Epileptic encephalopathy associated human *GABRB* mutations disrupt $GABA_A$ receptor function and results in Lennox-Gastaut syndrome in *Gabrb3*$^{+/D120N}$ knock-in mice

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CHAPTER I: INTRODUCTION

1.1 Epilepsy−Definition and epidemiology

Epilepsy is a neurological disease in patients with at least two unprovoked seizures that are more than 24 hours apart [1]. A seizure is a transient symptom due to abnormal, excessive and/or synchronous activity of neurons, while epilepsy is a predisposition to recurrent, unprovoked seizures. A familiar example of a seizure is a 'grand mal' or generalized tonic-clonic seizure (GTCS) that involves loss of consciousness, stiffening of arms and legs followed by their rapid jerking. Even though not all seizures are as detrimental as GTCSs, seizures such as absence seizures involving brief loss of consciousness also have a negative impact on the quality of life. One or more seizures induced by acute events such as fever, head injury, infection, stroke, brain tumor, medicine, etc. are not considered epilepsy. In 2014 the International League Against Epilepsy (ILAE) added a new definition of epilepsy as "one unprovoked seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years", for people at high risk of having two or more seizures such as those with trauma, stroke, or central nervous system (CNS) infections [1].

Epilepsy is one of the most common neurological diseases, affecting about 50 million people worldwide (WHO Factsheet, 2018) and 1 in 26 individuals in the United States [2]. The worldwide incidence of epilepsy is 67.7 per 100,000 individuals per year, and the lifetime prevalence is 7.6 per 1,000 persons [3, 4]. The first line and long-term treatments for the majority of patients are anti-epileptic drugs (AEDs). Over 20 AEDs currently are available [5], and seizures are well controlled for most patients by AEDs. However, about one third of epilepsy patients are unresponsive to multiple AEDs. These intractable patients bear greater morbidity and risk of mortality than individuals in the general population [6-10]. Some patients with drug

resistant seizures with two or more AEDs are candidates for resective epilepsy surgery or vagal nerve stimulation [11]. In addition the high fat and low carbohydrate ketogenic diet is also a treatment option for intractable patients [12].

Epilepsy has high health care cost resulting from disease assessment, treatment, rehabilitation, and hospitalization for seizures [13, 14]. In addition to managing symptoms and comorbidities, daily activities such as driving, attention in school, academic and employment productivity, and social interactions can be challenging for epilepsy patients and their families. This adds to indirect economic costs associated with epilepsy, especially when caring for children and elderly patients [15, 16]. In the United States alone, epilepsy has an estimated annual impact of $9.6 billion [17]. Thus, treatment and management of epilepsy are major public health concerns.

1.2 Classification of epilepsy syndromes

Epilepsy is a spectrum disorder with a range of seizure types that vary in their severity and causes. Thus, the impact of epilepsy differs for individuals, even among family members. This adds additional hurdles for accurate diagnosis and treatment. Often the diagnosis is based on family members' report or a self-report of the seizure semiology. Epilepsy syndromes have been classified to improve the accuracy of clinical diagnosis and to facilitate clinical epilepsy research. Classification of the epilepsies is based on two important aspects—(a) identifying the seizure type(s), and (b) determining their etiology. The initial classification schemes were primarily based on seizure semiology and electroencephalography (EEG). More recent classification schemes after 2010 have attempted to include etiology as an important aspect of classification based on scientific advances from many published studies and technological advances in clinical practice. Understanding the epilepsy etiology remains important in both

clinical practice and the laboratory since epilepsy etiology often drives seizure management, treatment options, and future therapies. However, determining epilepsy etiology remains challenging given that the brief, unpredictable, and pleomorphic seizures can occur due to either acquired or genetic causes.

In 2017 the ILAE revised the epilepsy classification with an emphasis on etiology due to tremendous progress in epilepsy genetics and neuroimaging. Currently epilepsy syndromes are grouped based on their onset as focal, generalized, or unknown [18-20]. Focal seizures originate in and remain localized to one hemisphere. They can be restricted to one brain region or widely distributed within one hemisphere. Focal seizures replaced the term 'partial' seizures. A generalized seizure rapidly engages both hemispheres, as reflected in the symptoms and EEG. For the "unknown" seizures the onset is uncertain, but motor and/or non-motor characteristics may be known. With further information, the unknown seizures can often be reclassified as focal or generalized.

Each of the three types of seizures, focal, generalized, and unknown, can be further classified. Focal seizures have been subclassified based on level of awareness during the focal seizures, with awareness used as a substitute for consciousness. If awareness is not lost during any portion of the seizure, it is classified as a focal seizure without impaired awareness. If awareness is lost for part or all of the seizure, it is classified as a focal seizure with impaired awareness. A caveat is that the patient may be aware during the seizure but may not remember the seizure due to transient epileptic amnesia, and thus accurate accounts of the seizure by observers are needed. Focal seizures are further classified into motor onset and nonmotor onset. Additionally, due to its common occurrence, "focal to bilateral tonic-clonic seizure" has been added as a special category, describing the spread of a seizure rather than a unique seizure type; it replaces the older term "secondarily GTCS".

For generalized seizures, awareness is not used for further classification since the majority of generalized seizures have associated impaired awareness, and thus, are categorized as motor and nonmotor (absence) seizures. Generalized seizures can have symmetrical or asymmetrical motor manifestations. In absence seizures, there is a sudden cessation of motor activity (behavioral arrest) and loss of awareness. Automatisms may be present, but they are less complex than those that occur with focal seizures with impaired awareness. Absence seizures have an abrupt onset and offset and primarily occur in children.

Unknown seizures can be classified further as motor, nonmotor, or unclassified. Unclassified indicates seizures that do not match the other categories or there is insufficient information to categorize them.

Epilepsy syndromes can be classified further based on etiologies. The current clinical care systems place an emphasis on determining seizure etiology if resources are available [21-25]. The recent ILAE classification identifies structural, genetic, infectious, metabolic, immune, and unknown etiologies [19], recognizing that patients can be grouped into more than one etiological category. Of note about 60-80% of all epilepsies are considered to have a genetic origin, and the genetic generalized epilepsies (GGEs) comprise most of the genetic epilepsies and one-third of all epilepsies [26]. This thesis explores the functional consequences of human genetic mutations identified in the pediatric population.

1.3 Genetic etiologies of epilepsy

The genetic basis of epilepsy was well recognized since epilepsy patients are more likely to have family members affected by epilepsy [27]. One of the early important findings that suggested a genetic component of epilepsy came from twin studies. In 1960 Dr. William Lennox (a renowned epileptologist, also well known for his contribution to the Lennox-Gastaut

syndrome) showed that monozygotic twins are more likely to share occurrence of generalized epilepsies and febrile seizures than expected by chance [28-30]. These results were later bolstered by studies that overcame confounds of environmental factors and demonstrated that monozygotic twins have a significantly higher coincidence of epilepsy than dizygotic twins [31-34].

Linkage analysis studies in the early 1990s revealed an association of chromosome locations 21q22 [35] and 20q13.2 [36] linked to progressive myoclonus epilepsy and autosomal dominant nocturnal frontal lobe epilepsy syndromes, respectively. These studies paved the way for isolating epilepsy-associated genes. In 1995 one of the first epilepsy gene mutations (missense) was identified in *CHRNA4*—encoding the α4 subunit of the nicotinic acetylcholine receptor—in members of a large Australian family with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [37]. This was immediately followed by discovery of mutations in *KCNQ2* [38], *KCNQ3* [39], *SCN1A* [40], *GABRG2* [41, 42], and *GABRA1* [43] in distinct families with members affected by generalized epilepsy syndromes. In addition, linkage analysis pointed to direct efforts at interesting loci such as chromosome 5q34 containing the gene cluster *GABRB2*[44, 45], *GABRA6* [46-49], *GABRA1*[43, 50-52], and *GABRG2* [41, 42]. All of these genes are currently recognized as epilepsy genes. However, co-segregation of mutated epilepsy genes (such as *GABRA2 and EFHC1*) was found to be limited to only a few families and was not broadly applicable to a significant portion of families with common epilepsy syndromes, even in studies with large sample size. The linked chromosomal loci could not be confirmed by independent studies or unequivocally mapped to susceptibility genes [53]. Nevertheless, identification of missense mutations following identification of important chromosomal loci marked the beginning of the 'channelopathy' era resulting in a wave of discovery of monogenic epilepsies associated with neuronal ion channel gene mutations. To date thousands of mutations have been reported in epilepsy patients [54] that include missense (the majority), nonsense, frame shift, insertion-deletion, promoter, splice-site, and intronic mutations (see section 15 and Table 1.1).

The genetic factors influencing epilepsy can be attributed primarily to inheritance of monogenic mutations including *de novo* mutations, polygenic or complex inheritance, with modifiers or susceptibility genes. Here I briefly discuss these topics and elaborate on the role of *de novo* mutations due to their prominence in EEs.

1. 4 Monogenic and polygenic epilepsies

Numerous autosomal dominant mutations have been identified in several families affected by epilepsy. However, the archetype familial cases with dominant mutations and epilepsy transmitted across generations account for a very small fraction (1-2%) of the epilepsy population [55]. Moreover, most familial epilepsies do not have Mendelian inheritance owing to incomplete penetrance, i.e. not all individuals with the mutation will have epilepsy. Furthermore, mutation carriers may have different expressivity, i.e. variable expression of symptoms including drug responses (Figure 1.2). Some authors consider variable expressivity a norm in human diseases owing to diverse genetic backgrounds [56]. The symptomatic differences (even among close family members) in the presence of the primary gene mutation attributed to epilepsy are thought to be due to variations in additional genes (genetic modifiers or susceptibility genes) or environmental factors [57, 58]. Indeed phenotypic differences among laboratory mice harboring the same genetic mutation but with different background strains support the involvement of genetic modifiers in variable expression of disease phenotypes [59-62]. For example, the epilepsy phenotype in a mouse model with the *Scn2a(Q54)* mutation in a congenic C57BL/6J background had delayed seizure onset, significantly reduced spontaneous seizures, and increased survival rates compared to mice with C57BL/6J × SJL/J mixed background [61], due to two potential modifiers [63].

Despite the caveats, studies of monogenic genetic generalized epilepsies have contributed enormously to understanding the molecular pathogenesis of epilepsy, several of which have been due to ion channel dysfunction. Moreover, it is easier to generate and study the effects of analogous single gene mutations in animal models of epilepsy compared to studying polygenic or complex epilepsies. Examples of monogenic epilepsies where mutation in a major gene explains the epilepsy phenotype include the generalized epilepsy syndromes: ADNFLE, benign familial neonatal seizures (BFNS), childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE), juvenile myoclonic epilepsy (JME), generalized epilepsy with febrile seizures plus (GEFS+), and GTCS and the focal epilepsies with *LGI1* [64-66], *DEPDC5* [67-69] (non-ion channel genes), and *GRIN2A* [70] mutations. Even though a certain gene can be repeatedly identified in an epilepsy syndrome, it can also lead to other epilepsies and other disorders, and vice versa. More than one gene, often several genes, can independently result in the same epilepsy syndrome. For example, the majority of Dravet syndrome patients have *SCN1A* mutations, although *SCN1A* mutations also result in hemiplegic migraine [71], GEFS+ [72], and other EEs [73, 74] . In addition, mutations in *GABRA1* [52], *GABRB3* [75], *GABRG2* [76], *SCN2A* [77], *SCN1B* [78], and *STXBP1* [50] have also be associated with Dravet syndrome.

On the other hand, polygenic or complex epilepsies arise from contributions of a few or many genes and are currently thought to account for the majority of epilepsy syndromes [79]. The dogma for polygenic epilepsies is that individuals have susceptibility gene variants that alone do not produce epilepsy, but certain numbers (additive effects) or combinations (modulatory effects) of susceptibility genes could result in hyperexcitable neuronal networks and lead to epilepsy in the given environmental conditions. This is also the foundation for genome wide association studies (GWAS) that aims to find commonly occurring susceptibility chromosomal loci that could increase the risk of developing diseases, but which by themselves have modest association with disease. Frequently, the associated chromosomal regions include more than

one gene. With next-generation sequencing technologies, GWAS studies seek disease association with single nucleotide polymorphisms or copy number variants at much higher resolution than traditional linkage studies. GWAS studies need large numbers (of the order of 1000s) of affected and unaffected individuals to capture several common variants (population frequency of 5-50%) of small effect size to be sufficiently powered. Not surprisingly, few GWAS studies exist in epilepsy, and the initial studies were not promising even with large sample sizes [80, 81]. Small family based linkage studies failed to replicate the genome-wide association in independent families [82-88]. This is not surprising given that millions of gene variants are documented in healthy and affected individuals in public databases (such as NCBI's Database of single nucleotide polymorphisms (dbSNP) [89], Human Gene Mutation Database (HGMD) [90], NHLBI Exome Variant Server, and others) that can potentially act as susceptibility genes [91]. However, the contribution of common polymorphisms to epilepsy risk is still not clear. Additionally, clear examples how these common polymorphisms with small effect can interact with each other or the environment to result in epilepsy phenotypes are still missing. Recent studies utilizing meta-analysis with larger sample sizes to be sufficiently powered had better outcomes. A study in a Chinese population with 1087 focal epilepsy patients (unknown and symptomatic causes) and 3444 controls identified a SNP in *CAMSAP1L1* on the 1q32.1 chromosomal region with genome-wide significance [92]. A large study by the EPICURE consortium in 379 multiplex European families (982 relatives) with genetic generalized epilepsy (GGE) found 5q34 as the susceptibility locus for a spectrum of familial GGE syndromes, while loci 2q34 and 13q31.3 specially affected susceptibility to myoclonic seizures or absence seizures, respectively [53]. Another meta study by the EPICURE consortium analyzed 3020 GGE patients and 3954 controls of European ancestry and identified significant genome-wide association of 2p16.1 and 17q21.32 loci to susceptibility of GGE syndromes, while loci 2q22.3

and 1q43 had syndrome-related association to CAE and JME, respectively [93]. These results have revived enthusiasm for GWAS in epilepsy.

A second and currently a popular approach of combing through genomic data is to look for rare single nucleotide variations (SNVs) (<5% population frequency), as opposed to the GWAS approach of identifying common variants (5-50% population frequency) with large effects on disease risk in large number of affected individuals. This approach has been made possible by next-generation sequencing technology and can directly identify rare causal variants. These rare (1-5% population frequency) or private (limited to probands and their families) mutations that are missed by GWAS are now well recognized as causes of several neurological and psychiatric diseases. Two such genetic alternations— *de novo* single nucleotide mutations and copy number variations (CNVs)—that contribute to epilepsy etiology are discussed below.

1.4.1 *De novo* single nucleotide mutations

De novo mutations are present in the carrier but absent in either parent, and arise due to erroneous DNA replication in germline cells (during meiosis) or somatic cells during early embryogenesis [94, 95]. Genetic variation via *de novo* mutations occurs spontaneously in somatic and germline cells throughout life and drives evolution. Both healthy and affected individuals have *de novo* mutations ranging from SNVs or single nucleotide polymorphisms (SNPs) to large CNVs (see below) to chromosomal rearrangements. An estimated SNV rate in humans is 1.0-1.8 × 10^{-8} per nucleotide per generation, or roughly 40-90 *de novo* mutations in each individual, of which 1 or 2 mutations are in the coding regions of the genome [95-99]. Additionally, the majority of the germline *de novo* mutations are of paternal origin and increase with age of the father [96, 99].

D*e novo* mutations occur naturally in all individuals both in their somatic and germline cells [100, 101]. However, germline *de novo* mutations that are lethal or result in severe diseases

(for example EEs) are not passed on to the next generation and thus do not undergo evolutionary selection. While the germline mutations that are tolerated (for example familial epilepsies) or promote survival (for example the *APP(A673T)* mutation that protects against Alzheimer's disease) are passed on to the next generation and become inherited mutations. Thus, *de novo* mutations are considered prime candidates for genetic diseases, especially sporadic diseases with unaffected parents [102]. Certainly an excess of missense *de novo* SNVs in the coding genes have been identified as a significant risk factor for several diseases including epilepsy [103-106], autism [107-114], schizophrenia [115-121], intellectual disability [122, 123], and developmental disorders [124].

De novo mutations in epilepsy were well recognized even before the advent of next generation sequencing (NGS) technologies [125]. Thousands of *SCN1A* mutations have been reported in 70-80% of Dravet syndrome patients [74, 126-128], more than 90% of which are *de novo* [74]. However, the homogenous genetic etiology of this severe childhood epileptic encephalopathy is an exception to the widely heterogeneous genetic etiologies of virtually all other epilepsy syndromes. The significance of *de novo* mutations in several epilepsy syndromes has been revealed only in last five years by large sequencing studies. These studies also explained why familial inheritance has been found only in a very small fraction of epilepsy patients (1-2%), the vast majority of which lack a family history of epilepsy. One of the first large scale (264 trios with affected probands and 610 control trios) exome sequencing studies was conducted by the Epi4K consortium in patients with two well-known epileptic encephalopathies, the Lennox-Gastaut syndrome and infantile spams. In 2013 this study reported that the probands had an excess of *de novo* mutations in 4,000 genes that are most intolerant to functional genetic variations. Mutations in nine genes were observed in ≥2 probands, and these mutations in *GABRB3, ALG13, CDKL 5, SCN1A, SCN2A,* and *STXBP1* showed strong statistical association with the two epileptic encephalopathies. *GABRB3*

mutations were previously reported only in the relatively milder childhood absence epilepsy syndrome [129], but after this and several following studies, *GABRB3* has been recognized as a risk factor for severe epilepsy syndromes [104, 130-133]. Today *de novo* mutations are well-recognized risk factors for epilepsy syndromes and many of them have been documented in epilepsy patients either in individual cases or in medium to large cohort studies [106]. The present challenge is to determine whether they lead to dysfunction that is sufficient to cause epilepsy. Nevertheless, these studies are anticipated to pave the way for future personalized therapies.

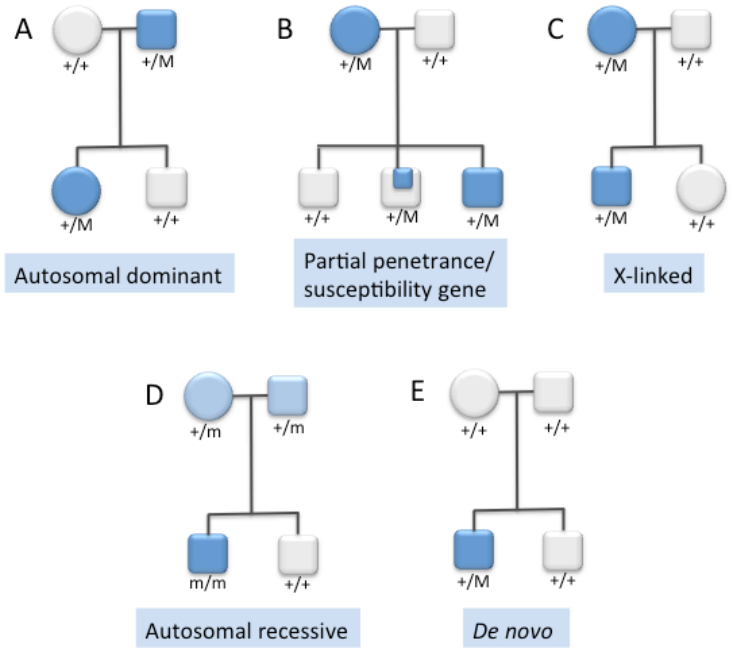


Figure 1.1 Common inheritance patterns seen in epilepsy.

(A) The autosomal dominant inherence is commonly observed pattern in monogenic epilepsies in which either parent can have a dominant mutation (M) and an epilepsy phenotype that is passed on to the off-spring. **(B)** Often the effect of the dominant mutation is not expressed in the same manner in all family members (partial penetrance). Family members with the same mutation can have varying degree of severity and epilepsy phenotypes, that can modified by other genes (susceptibility genes/modifiers). **(C)** The mutations on the X-chromosome gives rise to X-linked epilepsies in the offspring, male child is more susceptible; over 150 X-linked syndromes have been identified and several of them have seizures. When the parents are unaffected and the offspring can acquire epilepsy either when both parent harbor recessive mutations (m) which by themselves does not result in epilepsy **(D)** or when the mutation arises *de novo* in germline cells of either parent or during early embryonic stages **(E)**.

1.4.2 Copy number variations (CNVs)

CNVs are larger (>1 kilobase and up to a megabase) deletion, insertion, duplication or rearrangements in the genome [134-137]. As opposed to point, frame shift, small deletion and insertion mutations of few nucleotides, CNVs are large-scale alterations in the repetitive genomic regions that can span many genes but are smaller than structural variations in chromosomes. Unlike the familial mutations, CNVs could be inherited or appear *de novo* in the parent's germline cells or in somatic cells early in embryonic development.

Although, the presence of CNVs supports a polygenic etiology, and their role in complex diseases was speculated since their discovery, it is unclear how CNVs can lead to certain phenotypes. First, the majority of CNVs occur in all chromosomes [138] and only a few reoccur at the same location among different patients [139, 140]. Second, apparently healthy individuals can have CNVs (~12/individual) [141, 142] and are considered to be a major contributor of genomic variability among individuals [143]. It is estimated that there are about 100 genes that when completely deleted result in no apparent disease symptoms [143]. Third, CNVs can confer a selective evolutionary advantage such as disease resistance [144, 145].

Nonetheless, a significant role and high incidence of CNVs in epilepsy[139, 146] and other neurological conditions including intellectual disability (high penetrance), autism, schizophrenia, and attention-deficit hyperactivity disorder (ADHD) is now well-recognized [147-151]. Rare and non-recurrent (private) CNVs were identified in 5-15% of epileptic [152-155] and 4% of epileptic encephalopathy patients [156]. Additionally, chromosomal 'hot spots' for CNVs are associated with neurological diseases [152]. About 0.5-1 % of patients with genetic generalized epilepsies and focal epilepsies carry microdeletions of 1q21.1, 15q11.2, 15q13.3, 16p11.2, 16p13.11 or 17q12 chromosomal regions, but are rare in unaffected individuals [152, 153, 157-160]. The CNV frequency is even higher in patients with epilepsy and comorbidities such as intellectual

disability [161], developmental delay, or autism spectrum disorder (ASD) [162]. Since the function of most CNVs is unclear, several criteria must be considered to determine if they are benign or pathogenic. Certainly, CNV chromosomal location, size, and gene content seem critical; additionally recurrence of CNVs in patients is also an important indicator of pathogenicity. At present the overall pathogenicity of microdeletions is considered greater than those of duplication CNVs. Thus, CNVs could be risk factors or pathogenic for genetic generalized epilepsies, acting along with other genetic and environmental factors.

In summary, most of the patients without a clear structural/metabolic cause for epilepsy have a genetic origin of epilepsy. The genetic changes are inherited or appear *de novo*, and include single nucleotides, a few nucleotides, large DNA segments (CNVs), or structural chromosomal changes. Single gene mutations are monogenic causes of familial epilepsies and epileptic encephalopathies. Although, the majority of patients have a complex origin of epilepsy involving single or multiple genes; individual phenotypes depend on genetic background in addition to the gene mutation(s) [26, 57].

1.5. Complex genetic heritability of epilepsy revealed by whole exome sequencing

Whole exome sequencing is becoming a routine test to answer clinical and research questions, at least in developed countries [163], and works on the premise that human health and disease are influenced by mutations in the coding regions of the genome. The human coding genes are estimated to be 1% of the genome and predicted to account for 85% of the disease associated mutations [164-166], and the exome sequencing cost of ~$1000 has made its use attainable in research and clinical settings. This technique is especially useful for identifying *de novo* mutations associated with rare diseases, where the affected proband is presumed to have a

genetic epilepsy and the parents are unaffected by epilepsy. With whole exome sequencing, mutations are identified in virtually the entire coding region of the genome. Even then only a fraction of patients (~20-50%) [167-170] have genetic findings identified with whole exome sequencing. Only a fraction of the identified genetic changes can be concluded to cause epilepsy. For most mutations, either the gene lacks an association with epilepsy, the gene has unknown function, and/or the mutation is present only in a single individual. Thus sequencing studies account for etiology in only ~10-30% of epilepsy patients [106]. For example the Epi4K consortium identified 329 *de novo* mutations in 264 trios, of which 9 genes in 21 patients showed statistical significance to implicate them as causative [104], i.e. they explained the epilepsy phenotypes in only 8% (21/264) of patients. The current dogma in epilepsy is that the etiologies not explained by external agents such as trauma, stroke, infections, birth anoxia, etc have genetic origins, and thus the new classification replaced the term idiopathic with genetic. Thus, when genetic studies with good study design and methodology, large sample size, and good controls only identify genetic mutations in less than a quarter of the epilepsy patients, what explains the epilepsy phenotype in the rest (the majority?) of the patients with no known symptomatic or environmental contributions to etiology? I provide few plausible explanations from a point of view of a reader of these studies, one who has not participated in acquisition or analysis of sequencing data first hand. Two broad matters are discussed; the first involves the interpretation and the second explores expanding the search for etiology beyond the exome.

Typically, in large scale sequencing studies, 100s or 1000s of variants are identified in affected individuals that are absent in controls, and only a few affected individuals have mutations in the same gene. The probability of having multiple (≥2) mutations in the same gene in a patient cohort is calculated by taking into account the gene size, sequence specific mutation rate, and the number of patients enrolled. Additionally, computational predictions (using Polyphen-2, SIFT, nsSNP-Analyzer, and others) of the functional impact of the mutation aids in

the initial prioritizing of 100s or 1000s of potential disease-causing variations. However, lack of significant statistical association of the gene with a disease does not mean that the mutation has minimal disease risk. For example, of the 329 *de novo* mutations identified by the Epi4K consortium 35 (10.6%) were possibly damaging, 86 (26.1%) probably damaging, and 36 (10.9%) were either frameshift, insertion, deletion, splice donor site, splice acceptor site or nonsense mutations. Of these only 21 (~8%) mutations in 9 genes showed statistical association with infantile spasms and Lennox-Gastaut syndrome epilepsy syndromes (calculated from Supplementary Table 2) [104]. Among them the *GABRB1(F246S)* mutation was identified in a patient with infantile spasms and had a Polyphen-2 functional prediction to be probably damaging (score= 0.997, where 0 = benign and 1 = probably damaging); however, *GABRB1(F246S)* was not considered to be associated with infantile spasm as only one patient had a mutation in *GABRB1,* and this gene was previously not associated with epilepsy. Our functional studies however, showed that this mutation significantly altered γ aminobutyric acid type A ($GABA_A$) receptor current kinetics and reduced single channel current amplitudes (see Chapter 2) [171]. Later another *GABRB1(T287I)* mutation was identified in patient with early onset severe epilepsy and had clinical picture similar to the patients with *GABRB1(F246S)* mutation [172]. Similarly, DNM1 mutations were identified in two patients and were predicted to be probably damaging with Polyphen-2 but did not have the statistical significance to assign epilepsy risk. However, DNM1 mutations have been identified in epilepsy patients [173-176] and a mouse model with spontaneous DNM1 mutation has an epilepsy phenotype [177-179]. Thus, there may be many more genes that could be potentially pathogenic but are overlooked in sequencing studies due to lack of statistical significance, especially in the context of rare monogenic epilepsies.

Another possible explanation for identifying disease causing mutations in a small percentage of epileptic patients in large sequencing studies could be the involvement of non-exonic regions in the pathogenic processes. For example, the study by the Epi4K consortium

sequenced 264 trios and identified 329 *de novo* mutations in probands affected by infantile spasms or Lennox-Gastaut syndrome, 37.4% (123/329) of mutations were either synonymous or benign (calculated from Supplementary Table 2 [104]). It is possible that these patients had CNVs that were not detected, although at least some patients should have CNVs spanning the coding regions. Thus, non-coding regions may be involved. Whole genome sequencing will provide a more comprehensive role of genetic factors (in addition to exonic mutations) in the pathophysiology of epilepsy. Currently, whole exome sequencing takes precedence over whole genome sequencing as the human protein coding genes are best characterized and our current understanding of the non-coding genome is limited. However, whole genome sequencing studies in epilepsy are already in place and expected to increase the diagnostic rate and eventually replace whole exome sequencing as our understanding of the non-coding genome is enhanced [91, 180-182]

Additionally, current genetic studies also have full or partial sequence coverage in 3′ and 5′ untranslated regions (UTRs) and in introns. The significance of these regions is well recognized in the correct expression of genes and in human diseases [183-185]. However, the specific effects of mutations in the functional elements of UTRs or intronic regions are not always clear. Most studies are focused on the mutations in the coding region and few mutations are reported in non-coding regions. Further, the functional changes in non-coding regions are hard to predict compared to evaluating expression and functional changes of a mutated proteins.

1.6 Childhood epilepsies and associated comorbidities

Over 10.5 million children worldwide have epilepsy, accounting for about 25% of the epilepsy population, and epilepsy continues to be the most common long-term neurological condition for

this age group [186]. In the United States alone over 450,000 children from birth to 17 years old have active epilepsy [187]. Pediatric epilepsies are amongst the most catastrophic neurological disorders as the developing brain is susceptible to seizures, especially in the first two years of life [188-191]. Treating the young brain without interfering with the developmental processes presents unique treatment challenges in addition to managing drug resistant epilepsy in about 20-30% children [192]. Failure to treat pediatric epilepsy, however, increases the propensity to have subsequent seizures [193-196]. Children with epilepsy often exhibit social and learning deficits [197-199], are more likely to suffer from comorbidities such as attention-deficit/hyperactivity disorder (ADHD) [200, 201], learning difficulties [197-199], depression and anxiety [202, 203], and often are subject of stigma [204, 205]. Moreover individuals with epilepsy have 10 times the risk of a diagnosis of ASD [206, 207]. In addition children with epilepsy may suffer from developmental delays, emotional and behavioral difficulties, and migraine headaches [208]. Thus, the impact of epilepsy extends beyond management of seizures.

1.7 Epileptic encephalopathies

Epileptic encephalopathies (EEs) are catastrophic childhood epilepsy syndromes with severe pharmacoresistant seizures, varying levels of developmental delay and regression, cognitive impairment, and distinctive EEG patterns. The symptoms can worsen over time, and seizures and interictal epileptiform discharges in EEs are thought to contribute or worsen the cognitive and behavior impairment above and beyond that expected from the underlying pathology, especially in young children [209-211]. EEs account for a large fraction of early onset intractable childhood epilepsies, encompassing a variety of syndromes sometimes with overlapping and evolving symptoms. It should be recognized that the encephalopathic effects of intractable seizures may occur in any epilepsy syndrome, even in adults, but are commonly

observed in young children, and that the term EE is an evolving concept. Figure 1.2 lists EEs recognized by the International League Against Epilepsy (ILAE) in order of age of onset. Even with the well-studied epilepsy syndromes, accurate syndromic diagnosis is challenging due to range of severity of symptoms that occur in a relatively broad time frame and the patient may not experience all symptoms of a syndrome at once or throughout the course of the disease. Thus children with early onset catastrophic epilepsy syndromes are at increased risk for diagnostic delays and inadequate medical and mental health services [212]. Most EEs have neurodevelopmental delays as the primary feature rather than a secondary outcome to the excessive epileptiform activity. Thus, long-term care needed for seizure management and the associated co-morbidities poses a substantial economic burden on families and the health care system [14]. A significant challenge is to fulfill the unmet needs of this age group [213].

There is limited understanding of the pathological mechanisms involved in EEs; however, frequent seizures and epileptiform activity due to well recognized etiologies including genetic, structural brain malformations, infections, neuroinflammation, metabolic defects, are thought to hamper normal brain development and/or cause abnormal brain development. Until the last decade, etiologies beyond structural abnormalities (identified with neuroimaging) were rarely identified. In the past five years, however, there has been an explosion in the identification of genes associated with EEs using NGS technology that could explain 20-25 % of all early-onset catastrophic epilepsies previously regarded to have no identifiable cause [214, 215]. Mutated genes identified in EEs patients involve ion channels (majority), proteins involved in synaptic transmission and interneuron function, and neuro-inflammation. Thus, plausible mechanisms include altered cellular/network excitability, synaptic reorganization, and neuronal proliferation/migration defects. Although, how specifically these changes could result in age and syndrome specific symptoms remains an open question.

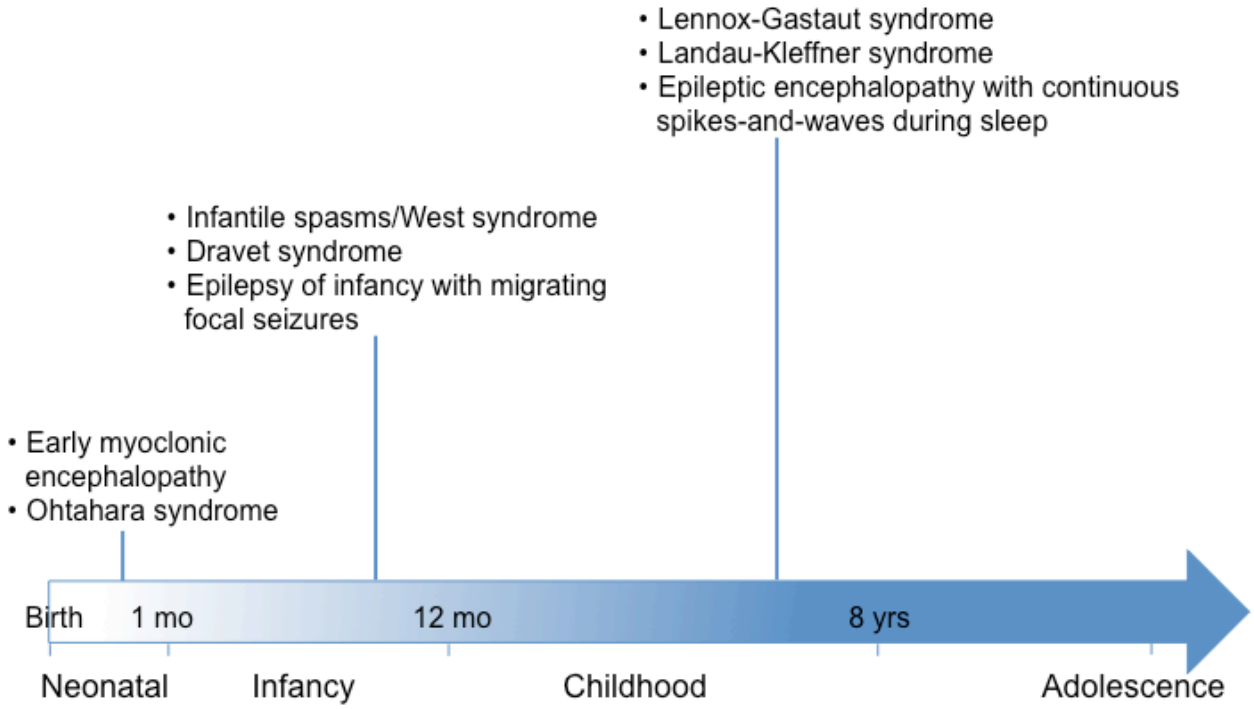


Figure 1.2 Age of onset of EEs.

EEs are a diverse group of severe epilepsies that have onset from a few hours after birth to late childhood.

Table 1.1 Commonly Occurring Seizures in Epileptic Encephalopathies.
Seizure types relevant for the descriptions and understanding of EEs are listed here.

Seizure	Typical manifestation
Absence	A sudden and brief (≤ 30s) interruption of awareness of the ongoing activity with unresponsiveness to external stimuli that appears as a blank stare. Appear as generalized SWDs of 3 Hz on EEG that are time locked with start and end of the seizure.
Atypical absence	A gradual and sustained (≥30s to minutes) loss of awareness of the ongoing activity with unresponsiveness to external stimuli that appears as a blank stare and often accompanied with automatisms. Appear as generalized SWDs of <2 Hz on EEG that are not always time locked with start and end of the seizure.
Atonic	A sudden loss or reduction of muscle tone that lasts for about 1–2 s and involves the head, trunk, jaw or limb muscles. Often atonic seizures result in falls due to loss of erect posture thus also known as drop seizures/attacks.
Clonic	Sustained (1-2 min), repetitive, and regular jerking of the body or body parts engaging same muscle group(s) affecting whole (generalized) or one side of the body (focal). Jerking movements are prolonged and cannot be controlled by restraining body parts.
GTCS	Seizure that begins with the tonic phase (sustained muscle contraction) and evolves to the clonic phase (repetitive jerking movements) lasting for 1-3 mins with loss of consciousness. The individual recovers slowly after the seizure and may feel confused, agitated, or depressed and may loose bladder control. A seizure lasting for >5 minutes is considered medical emergency.
Epileptic spasm	A sudden (~1s) flexion, extension or both of primarily the proximal and truncal muscles that is longer than a myoclonic seizure but shorted than a tonic seizure. Commonly occur in clusters. Infantile spasms is a well known example but also occurs in older age group.
Myoclonic	A sudden, brief (less than 100 ms), and involuntary contraction muscles(s) or muscle groups of variable topography (axial, proximal limb and distal).[30,35]
Myoclonic–atonic	Brief jerking of limbs or trunk muscles, followed by loss of muscle tone.
Tonic	A sustained muscle contraction lasting a few seconds to minutes. Neck, trunk, arms or entire body muscles are commonly involved. Can be accompanied by autonomic symptoms such as respiration, altered heart rate or loss of bladder control.

Next, I provide a brief overview of a few EEs that are the focus of this thesis and relevant to the discussion of the EEs, infantile spasms (IS) and Lennox-Gastaut syndrome (LGS). IS and LGS are described in detail in chapters II and III, respectively. The following section also highlights the diversity of EE phenotypes and emphasizes the need for syndrome-specific mouse models.

1.7.1 Neonatal EE

Ohtahara syndrome (OS) and Early Myoclonic Encephalopathy (EME) are two EEs with frequent seizures in the neonatal period and early infancy, and with a distinguishing feature of burst suppression on EEG during seizures. Burst suppression appears as brief high voltage activity alternating with background attenuation. Often seizures are intractable, and thus the prognosis is poor with severe to profound developmental delay.

OS, also known as early onset epileptic encephalopathy (EOEE), is rare but one of the most severe EEs. with seizure onset reported within 10 days of birth for most patients, ranging from hours after birth to three months of age [216-218]. The majority of patients have tonic spasms (muscle contractions lasting a few seconds and resembling those of West syndrome) with or without clustering; additionally focal seizures, myoclonic seizures, GTCS, and hemiconvulsions are also observed in some patients [219, 220]. A peculiar burst suppression pattern on EEG occurs both while awake and asleep and has the appearance of periodicity. Etiology is heterogeneous with structural brain abnormality being the most prominent [219-225], with congenital metabolic disorders, hypoxic-ischemic encephalopathy and gene mutations also being reported [226].

EME also has a very early seizure onset from few days after birth to three months of age, burst suppression on EEG, and intractable seizures with extremely poor prognosis [227-229].

Although unlike OS, the main seizure types in EME are fragmented myoclonic seizures, frequent focal seizures, and non-epileptic myoclonus; massive myoclonic seizures or tonic spasms are also observed less frequently. A myoclonic seizure involves a sudden, brief (less than 100 ms), involuntary contraction of muscles(s) or groups of muscles. Additionally, the burst suppression pattern on EEG is prominent in sleep. It has been reported that the majority of EME patients have a metabolic etiology and fewer have structural brain malformations [230].

There is disagreement [231-235] on whether OS and EME are the same syndromes or present as a continuum of syndromes with overlapping clinical presentations and etiologies. Both syndromes have early onset, burst suppression on EEG, pharmacoresistant seizures, and poor prognosis. Overlapping genetic etiologies [233] are now being reported and future studies will reveal if the prominence of structural etiologies in OS and metabolic etiologies in EME hold true. However, differences with respect to the major seizure types and occurrence of burst suppression during different vigilant states are well-documented in these two syndromes. Another key characteristic differentiating OS from EME is that patients with OS show age-dependent evolution of symptoms while those with EME do not. About 75% of OS patients progress to West syndrome after 3-4 months of age, and a further ~59% progress from West syndrome to Lennox-Gastaut syndrome after one year of age [234]. The transition of OS from one syndrome to another suggests overlapping pathophysiological mechanisms; additionally this could be regarded as a development specific response to brain insults irrespective of the heterogeneous etiologies [234]. EME displays no age-dependent evolution, and the symptoms remain stable for a long time or change to partial epilepsy or severe epilepsy with multiple independent spike foci. The lack of transition between OS and EME along with the evolution of symptoms with age in OS and relative consistency of EME symptoms with age reflect their pathophysiologic differences.

1.7.2 Infantile EE

Dravet syndrome, previously known as severe myoclonic epilepsy of infancy, is an EE with seizure onset from six months to a year. Beyond age two most patients develop refractory seizures accompanied by cognitive, behavioral, and motor (ataxia) impairment [236]. Typically, hemiclonic seizures or GTCS appear in the first year in otherwise healthy infants, which in some infants are be trigged by fever, hyperthermia (such as a warm bath) or vaccination. Beyond the first year, recurrent febrile and afebrile myoclonic, atypical absence, unilateral clonic, focal, and GTC seizures are observed. In some children focal or multifocal seizures are dominant and myoclonic seizures may not appear. EEG semiology is typically normal before seizure onset in the first year of life, following which generalized spike-wave and polyspike-wave discharges, and focal discharges are observed [237]. The head MRI is also normal at seizure onset or with non-specific findings for most patients [238-241] but may become abnormal with seizure progression [242, 243]. Thus, Dravet syndrome is considered a prototypical EE with cognitive, behavioral, and developmental regression after seizure onset. Furthermore, high mortality is observed in Dravet syndrome, especially during childhood frequently due to sudden unexpected death in epilepsy (SUDEP), status epilepticus, or accidental death due to injury or drowning [244, 245].

Additionally, Dravet syndrome alone accounts for a significant fraction of EEs with a population frequency of as high as 1 in 15,700 in the US [246] to 1: 40,000 in other parts of the world [126, 247-249]. The etiology of Dravet syndrome is uniquely homogeneous and well-studied compared to other EE syndromes. About 70-80% of Dravet syndrome patients have *SCN1A* mutation encoding Nav1.1 α1 subunit of the voltage-gated sodium channel, the majority of which are *de novo* missense mutations [126-128]. For the remaining ~30% of patients, other candidate genes including *GABRA1*, *GABRG2*, *SCN2A, SCN8A, SCN9A, HCN1, STXBP1, PCDH19* have been reported, but some of these patients may have borderline or Dravet

syndrome-like phenotypes [250]. Importantly not all patients with *SCN1A* mutations have Dravet syndrome. Over 1000 *SCN1A* mutations have been reported in epilepsy patients [74, 251]. Although the severity of symptoms and response to therapies would depend on the type and location of the *SCN1A* mutation in the channel [252-254] and the genetic background [57, 255], few common themes of mechanism of action have emerged from numerous *in vitro* studies and mouse models. Not surprisingly, mutations in the channel pore or those grossly altering the $Na_V1.1$ channels structure (eg. nonsense, frameshift, deletion, etc mutations) produced more severe epilepsy phenotypes with complete loss of channel function that were similar to that of haploinsufficiency [256]. As $Na_V1.1$ channels are excitatory channels, loss of function mutations should result in hypoexcitable rather than hyperexcitable neuronal networks. This conundrum was resolved using mouse models. Mice with loss of $Na_V1.1$ channels resulting from truncating the last exon of *Scn1a* (encoding S3 to S6 transmembrane segments of domain IV and the entire C-terminal tail) revealed reduced sodium currents in inhibitory GABAergic interneurons but not pyramidal cells, which could result in hyperexcitable networks and epilepsy [257, 258]. Loss of sodium currents from cerebellar Purkinje cells could explain ataxia in this mouse model similar to that seen in Dravet syndrome patients with loss-of-functions sodium channel mutations [259]. In agreement with this hypothesis, selective deletion of $Na_V1.1$ channels from whole brain interneurons but not from forebrain excitatory cells resulted in spontaneous epileptic seizures (more severe than global deletion of *Scn1a*), premature death, and ataxia [260]. In addition, loss of $Na_V1.1$ channels from parvalbumin positive (PV) interneurons was sufficient to replicate spontaneous epileptic seizures and ataxia in these mice [260, 261]. Further, inactivation of one *Scn1a* allele in PV interneurons but not in pyramidal cells produced spontaneous generalized seizures and increased susceptibility to heat and flurothyl induced seizures in mice [262]. Both pyramidal cells and GABAergic interneurons express $Na_V1.1$ channels. However, during development $Na_V1.1$ channels are predominantly expressed at the axon initial segment

of neocortical PV interneurons and on soma and axon initial segment of hippocampal PV interneurons, while their expression is extremely low on pyramidal cells [263]. Reduced interneuron excitability by loss of sodium currents is also replicated in mouse models with human Dravet syndrome mutations [264, 265], suggesting suppression of inhibitory activity to be a likely epileptic mechanism resulting from *SCN1A* mutations.

1.7.3 Childhood EE

Epileptic encephalopathy with continuous spike-and-wave during sleep (EECSWS) is a partly reversible childhood EE characterized by continuous spike-and-wave discharges during sleep (CSWS), variable seizure types, gradual but substantial decline in cognitive, motor, and psychiatric functions [266-268]. The disease progression involves first the appearance of seizures between 2 and 12 years of age (peak 4-5 years) in most but not all patients. Then CSWS appears 1-2 years after the incidence of first seizures concurrent with developmental decline. Lastly, remission of seizures and CSWS occurs usually between 2–7 years from the disease onset. The neuropsychological evaluation also improves but not to the level of unaffected children. Many patients with EECSWS have permanent and severe neuropsychological impairment despite the age-limited time course of epilepsy [269]. Nevertheless, the outcomes in this syndrome are better compared to EEs with progressively deteriorating symptoms.

Unlike other EEs, most (~80%), but not all, EECSWS patients have seizures at disease onset, and thus seizures prior to appearance of CSWS are not required for diagnosis [270-272]. The initial phase of EECSWS is free of CSWS; seizures if present at onset occur only occasionally, and most are nocturnal focal motor seizures or GTCSs. Occurrence of seizures increases in number and type as the CSWS and cognitive impairment appears. Some authors report up to 93% of affected children have multiple seizures per day [270, 271, 273-277]. Commonly

occurring seizures after appearance of CSWS include GTCSs, focal to bilateral tonic-clonic seizures (previously secondary generalized seizure), typical absence seizures, atypical absence seizures and atonic seizures, which may accompany falls, and focal seizures with or without impaired awareness (previously complex and simple partial seizures, respectively) [270, 271, 273]. Some patients have prenatal or perinatal abnormalities or a previous diagnosis of other epilepsy syndromes or related disorders [270].

The next phase of the disease appears 1-2 years after the seizure/disease onset marked by the characteristic CSWS on EEG, a necessary diagnostic criterion. CSWS are epileptic discharges that appear on EEG as diffuse and continuous spike-and-waves in non-rapid eye movement (Non-REM) sleep that can be focal, multifocal or generalized. The EEG may also show multi-focal spikes (associated with structural brain abnormalities) and/or bisynchronous generalized sharp discharges when awake or asleep. CSWS could occupy from 25% and to 90% of Non-REM sleep duration that significantly impacts the quality of sleep [278, 279]. The later stages of Non-REM sleep (deep/slow wave sleep) are considered to be important for cerebral restoration, recovery, memory and sleep consolidation, and particularly important for functions of the prefrontal cortex in humans [280-284]. This may explain the appearance of cognitive decline and behavioral symptoms along with CSWS in Non-REM sleep. The sudden appearance of aggression, hyperactivity, impulsivity, passivity, lack of interest in the surroundings and/or inflexibility takes over the lives of children and their caregivers and may overshadow the concordant cognitive decline [285].

In the remission phase, the seizures and CSWS begin to disappear just as they spontaneously appeared, simultaneously resolving behavioral issues and cognitive issues to variable degrees in each child. The extent of cognitive decline depends on the etiology. Most patients have structural brain abnormalities (70-85%), and other cases are presumed to have

genetic causes [278]. Structural abnormalities could be developmental or acquired and include, but are not limited to, polymicrogyria, hydrocephalus, schizencephaly, perinatal hypoxia-ischemia, and central cerebral atrophy. Patients with or without structural abnormalities have monogenic or polygenic etiologies that are inherited or appear *de novo*. Recently, mutations in several cell adhesion genes have been identified in EECSWS patients; several of these genes are also associated with ASD and Rolandic epilepsy [286]. The structural and genetic etiological groups have similar age of seizure onset and similar time elapsed between the first focal seizure and appearance of CSWS; however, patients with structural etiology have earlier onset of the focal seizures compared with patients with genetic or unknown etiologies [278]. Additionally, among the patients who respond to drugs, those with genetic etiologies have better cognitive outcomes than those with structural etiologies. Patients who had >75% seizure reduction or became seizure free with medication had significantly improved IQs and school performance [278].

Here I have used the term EECSWS as described by the ILAE [268], but some authors propose that CSWS is a common condition in a group of patients with EE and that drives symptoms depending upon the location of CSWS in brain [266, 287]. In the original description by Patry et al. in 1971, CSWS were described as subclinical electrical status epilepticus in children that is induced by sleep and terminated upon arousal [266]. As not all patients had overt motor seizures associated with CSWS, they were referred to as subclinical or electrical seizures. The syndrome was considered an encephalopathy because all children in that study and the subsequent studies had cognitive impairment (Dalla Bernardina et al., 1978; Kellermann, 1978; Laurette and Arfel, 1976). Since then the term electrical status epilepticus during sleep (ESES) became widely used. However, the term ESES was not considered accurate since without detectable motor seizures and EEG pattern alone is not considered equivalent to status epilepticus (as in GTCSs). Further, ESES only emphasizes the EEG pattern but not the

associated neuropsychological regression. Thus, some authors prefer to use the term ESES as "Encephalopathy with Status Epilepticus during Sleep", which reflects both the clinical findings and the EEG characteristics (Tassinari et al., 2000, 2005, 2009). Afterwards, the ILAE proposed the term CSWS as an epilepsy syndrome [209, 211]. Even today many authors use ESES and CSWS interchangeably. I point out these differences since now it is well recognized that CSWS (can be considered as reminiscent of atypical absence seizures present in LGS) occur in multiple EEs including Landau–Kleffner syndrome (LKS) [273, 288, 289], atypical benign epilepsy with centrotemporal spikes (BECTS) [290], and acquired opercular syndrome [291-294]. These disorders show a clinical spectrum with common features [269, 270, 273, 276, 279, 295, 296]. Along the same lines, LGS can be considered a variant in the CSWS spectrum [270, 297]. In LGS, however, atypical status epilepticus can occur when the child is awake and may progress to sleep. Age-related seizure remission does not occur, and the symptoms may worsen over time [298, 299]. Of particular note, LKS patients have CSWS that occur in parallel with language disturbances leading to aphasia. CSWS persist several months or years, and when CSWS begins to decrease or disappear, there is a concordant language recovery [277, 300]. Importantly epilepsy syndromes with CSWS and LGS may share the same reverberating thalamo-cortical circuit that generates oscillations during sleep (6-14 Hz) but switches to slow (1-4 Hz) oscillations in pathological states [301-306]. EE syndrome-specific mouse models will help understand their pathogenic mechanisms and the circuits involved.

1.8 GABA-mediated inhibitory neurotransmission

γ-aminobutyric acid (GABA) is the dominant inhibitory neurotransmitter in the CNS and acts by binding to $GABA_A$ receptors. Even though present in other tissues, GABA is present in high concentrations (mM) throughout brain and spinal cord, and occurs in about a third of the

synapses [307, 308]. GABAergic inhibitory interneurons are the most common cell types synthesizing GABA (10-30% of all neurons), although, GABA is also present in long-range projection neurons such as septo-hippocampal neurons [309], and in neurons with extended arborizations such Purkinje cells in the cerebellum [310] and nucleus reticularis thalami (nRT) neurons. GABA is also co-released with other neurotransmitters [311]. Glucose is the primary precursor for GABA synthesis; however, GABA can also be directly synthesized from glutamate, the most abundant excitatory neurotransmitter, by the enzyme L-glutamic acid decarboxylase (GAD) [312-314]. In fact, presence of GAD enzymes is used as a marker for inhibitory neurons. Following synthesis, the vesicular GABA transporter (VGAT) packages GABA into presynaptic vesicles [315] that then dock on the presynaptic terminals. Presynaptic vesicles fuse with the plasma membrane to release GABA either spontaneously at a constant low-level leak or in bulk after a depolarization event such an action potential to release a high concentration (~1-10 mM) of GABA into the synaptic cleft [316], where it diffuses rapidly due a steep concentration gradient and activates postsynaptic $GABA_A$ receptors. After synaptic release, GABA is rapidly cleared from the synaptic cleft via GABA transporters present on glial cells and presynaptic terminals [317]. After being transported into astrocytes, GABA is converted first to glutamate by GABA transaminase and then to glutamine by glutamine synthetase. Glutamine is then exported out in the extracellular space. Glutamine is taken up in the presynaptic terminal and converted back to glutamate and then to GABA that is packaged in synaptic vesicles for subsequent release [318].

1.9 Types of GABA receptors

GABAergic neurotransmission is mediated by two receptor types—$GABA_A$ and $GABA_B$. $GABA_A$ receptors are the principal inhibitory receptors abundantly expressed in the CNS that mediate the majority of the fast synaptic inhibition (few milliseconds duration) in adult CNS. In

addition, $GABA_A$ receptors mediate shunting and tonic inhibition, and thus, they are critical for maintaining inhibitory tone that prevents neuronal hyperexcitability. Pharmacological block of $GABA_A$ receptors quickly precipitates seizures in animals [319]. Similarly, several $GABA_A$ receptor mutations associated with GGE syndromes have been demonstrated to reduce $GABA_A$ receptor-mediated inhibition [320]. $GABA_A$ receptors are important targets for several AEDs as $GABA_A$ receptor dysfunction is one of the central mechanisms resulting in epilepsy,

The G-protein coupled $GABA_B$ receptors carry out the slow inhibition (seconds in duration) in neurons by activating outward rectifying voltage-gated potassium channels [321] or by inhibiting voltage-gated calcium channels [322]. Both $GABA_A$ and $GABA_B$ receptors are expressed pre- and post-synaptically where they reduce excitability of adult neurons.

1.10 Diversity of $GABA_A$ receptor subunits

$GABA_A$ receptors are ligand gated ion channels that assemble as psedosymmetrical heteropentamers composed of 2α, 2β, and 1z subunits, where z= γ, δ, ε, θ, π, or ρ subunit [323-326]. The subunits have a specific arrangement of z-β-α-β-α in counter clockwise direction as seen from the synaptic cleft [326-328]. The eight subunit families (α, β, γ, δ, ε, θ, π, and ρ) further have 19 subunit subtypes (α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-3}) [329], splice variants (β3v1 and β3v2, β2S and β2L, γ2S and γ2L), and alternatively edited mRNA transcripts (α3I and α3M) [330-334]. If $GABA_A$ receptors can assemble from any combination of 19 subunits (α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-3}, δ, ε, θ, π; excluding splice variants and alternately edited isoforms) with a 2α:2β:1z stoichiometry it would result in 6x3x6x3x10=3240 types of $GABA_A$ receptors. However, the biological diversity observed is much more limited [335, 336], suggesting restricted assembly. So far only 11 $GABA_A$ receptor isoforms have been identified *in vivo* (α1-4β2γ2, α4β2/3δ, α5βxγ2, α6βxγ2, α6β2/3δ,

and homomeric ρ receptors) and an additional eight isoforms are suspected to exist with high probability (α1β3γ2, α1βxδ, α5β3γ2, αxβ1γ, αxβx, α1α6βxγ, α1α6βxδ) [337]. Even then $GABA_A$ receptors subunits are immensely diverse, much more than the glutamate receptors [338]. This structural diversity allows functional diversity and fine-tuning of fast inhibitory currents. For example, the IPSCs from young mice are slow and long-lasting compared to the brief IPSCs from adult mice [339-341]. Further, the diversity of $GABA_A$ receptors increases with developmental maturation as evident by increased diversity of mRNA transcripts seen in single neurons [342-344]. Additional functional diversity arises due to differences in GABA potency; α6 subunit-containing $GABA_A$ receptors expressed especially in the extrasynaptic region have the highest GABA potency (0.17 μM), whereas the synaptic α2β3γ2 and α3β3γ2 receptors exhibit the lowest potency (12-13 μM) [345]. Expression of $GABA_A$ receptor subunits is highly regulated not only in certain brain regions but also within different compartments of a neuron. For example, the α6 subunits are preferentially expressed in cerebellar granule neurons and the low GABA affinity γ subunits are pre-dominantly synaptic while high affinity δ subunits are exclusively extrasynaptic [346, 347]. Also, α1 subunits are evenly distributed in synapses on the soma, dendrites, and axons whereas α2 subunits are primarily located at the axon initial segment (Klausberger et al., 2002; Nusser et al., 1996). Hence, the enormous diversity of $GABA_A$ receptor subunits offers wide-ranging functional flexibility.

In addition to the dominant 2α:2β:1x stoichiometry, others are also proposed in heterologous expression systems. For example, the θ and π subunits are most homologous to β and δ subunits, respectively, and have been reported to assemble as 2α:1β:1δ:1γ and 2α:1β:1γ:1π receptors [348, 349]. Similarly, the ε subunit with high homology to the γ subunit has been shown to assemble as 2α:1β:1γ:1ε receptors [350-353]. However, such receptor combinations have not been demonstrated in neurons.

1.11 Structural domains of $GABA_A$ receptor subunits

$GABA_A$ receptors are part of the Cys-loop family of receptors that include nicotinic acetylcholine (nAChR), serotonin (5-HT_3), and glycine receptors [354, 355]. The Cys-loop family of receptors contains a characteristic disulfide bond between N-terminal cysteine residues (the Cys-loop, hence the name of the family) and are arranged as pentamers with a central ion conducting pore. Moreover, each subunit shares a large β-sheet rich hydrophobic N-terminal domain, four hydrophilic α-helical transmembrane domains (M1-4), a large and flexible intracellular loop, and some subunits contain a very short extracellular C-terminal tail extending beyond the membrane in the extracellular side. The pore is formed by the M2 domains of the five subunits and ligand binding occurs at the interface of two pairs of α and β subunits in the extracellular N-terminal domains. The intracellular loop between M3 and M4 interacts with a large number of proteins and is involved in receptor trafficking, anchoring, phosphorylation, among important other functions [356]. The crystal structure of the heteropentameric $GABA_A$ receptor is not available but recently the high-resolution (3 A°) crystal structure of the human β3 homopentamer was reported [357]. This is by far the closest structure to heteropentameric $GABA_A$ receptors compared to the crystal structures of Acetylcholine binding protein (AChBP) [358], *Torpedo marmorata* nAChR [354, 359, 360] two bacterial analogues: *Erwinia chrysanthemi* ion channel (ELIC) [361] and GLIC [362], *Caenorhabditis elegans* glutamate-gated chloride channel α (GluCl) [363] that were used as templates for comparative structural modeling.

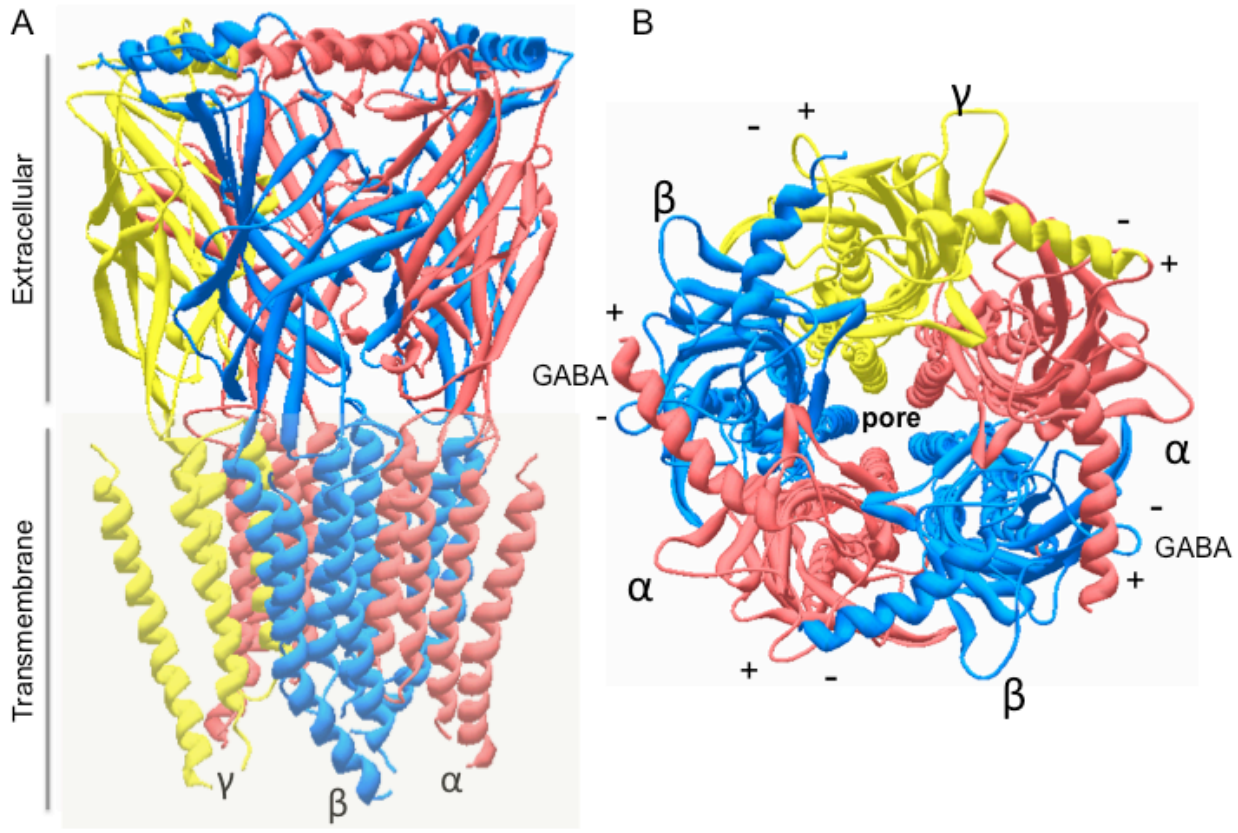


Figure 1.3 Three-dimensional structural model of GABAA receptor.

(A) Side view of $GABA_A$ receptor based on the crystal structure of the β3 pentamer [357] with 2α (red), 2β (blue), and 1γ (yellow) subunits. Each subunit has an extracellular N-terminal domain, four α-helical transmembrane domains, and the intracellular domain (not shown). **(B)** Top view of $GABA_A$ receptor as seen from the synaptic cleft. The five subunits assemble in a doughnut-like shape, the central cavity conducts Cl^- ions. For orientation purposes, each subunits is designated a principle (+) and a complementary (-) face. Full $GABA_A$ activation occurs when two GABA molecules bind at each of the β+/ α- interfaces.

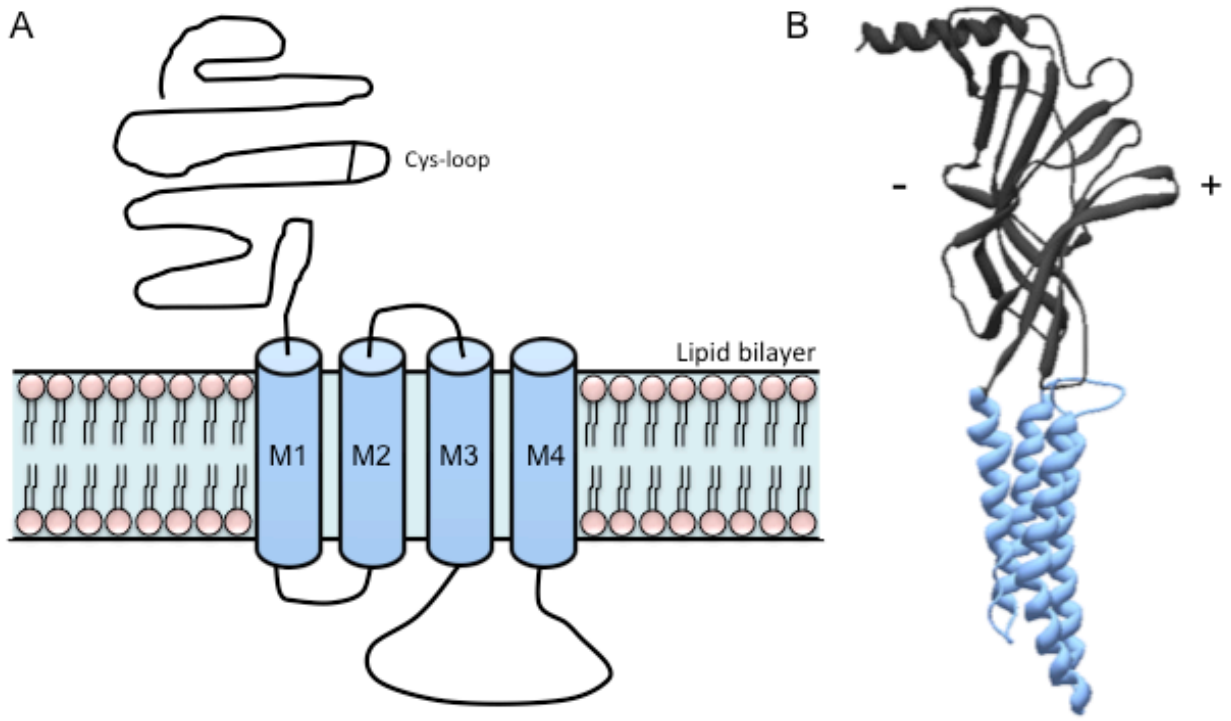


Figure 1.4 Structure of a single $GABA_A$ receptor subunit.
(A) Schematic showing extracellular domain, transmembrane domains (in the lipid bilayer), and intracellular domain of a single $GABA_A$ receptor subunit. The extracellular domain contains a highly conserved Cys loop (disulfide between two cysteine (Cys) residues). **(B)** Side view of $GABA_A$ receptor subunit. Within each subunit the extracellular N-terminal region begins with an α-helix followed by 10 β-sheets. The four α-helical transmembrane domains (M1-M4) remain embedded in the lipid bilayer, the M2 domains of each subunit comes together to form the channel pore. The structure of the intracellular domain is not known but thought to be a large flexible loop that binds to several intracellular components.

1.11.1 Extracellular domain

The large extracellular domains of $GABA_A$ receptor subunits assemble to give a doughnut-like appearance from the extracellular side (Figure 1.3B). Each subunit begins with an alpha helix that winds into two sets of β-strands that are connected by the highly conserved disulphide bond (Cys-loop). The rigid β-strands are connected by relatively flexible loops. For orientation purposes each subunit is designated to have a principal (+) side and a complementary (-) side. Full receptor activation occurs when two GABA molecules bind at two

of the β(+) and α(-) interfaces. GABA binding occurs in the pocket formed by the loops A, B, and C of the (+) side and the loops D, E, F, and G of the (-) side [357, 364]. The widely used antiepileptic benzodiazepines compounds (that are also sedative, anxiolytic, muscle relaxant) bind at the α(+) and γ(-) interface. The majority of the $GABA_A$ receptors with α1, α2, α3 or α5 and γ1-3 subunits are benzodiazepine sensitive and are synaptically located. While the α4, α6 or δ subunit-containing receptors are benzodiazepine insensitive and extrasynaptic. Additionally, the extracellular domains are critical for receptor assembly [365-368] and contain sites for binding of N-linked glycans that aid in surface expression [369] and function [370, 371] of $GABA_A$ receptors.

1.11.2 Transmembrane domains

The four helical domains (M1-4) following the extracellular domains of $GABA_A$ receptors remain embedded in the cell membrane (Figure 1.4). The M1 domain is important for channel function and required for actions of endogenous [372] and exogenous neuromodulators including barbiturates [373-380] and anesthetics [381, 382]. Five M2 domains assemble to form the lining of the channel pore that narrows towards that intracellular side. Thus the M2 domains determine ion selectivity [383], conductance, and gating [384-388]. As expected the critical pore lining M2 domains are highly conserved, within it the leucine residue at the 9′ position is highly conserved among the Cys-loop family members. The 9′ leucine was thought to line the narrowest part of the channel pore thus governing the closed channel state; not surprisingly mutation at this position has detrimental effects on channel functions [386, 389-393]. However, according to the recent crystal structure of the β3 homopentamer the 9′ leucine residues of M2 domain [357] are rotated out of the pore and their side chains are between the neighboring M2 helices. As much of the earlier $GABA_A$ receptor homology modeling was done using other Cys-loop channels [394], it is

interesting to note that the rotation at the 9′ leucine residues is not predicated in nAChR receptors during the open-to-closed M2 motion. Thus the closed state at least in the β3 homopentamer is primarily attributed to the unique conformation of the M2 helix [357]. The M3 domain strongly affects receptor desensitization [395] while the outermost M4 domain is thought to provide stability and can be substituted with an unrelated transmembrane domain in nAChRs, but substitution of the M1-3 domains produces non-functional channels [396]. Additionally, the M3 and M4 domains bind to multiple allosteric modulators such as intravenous anesthetics [397, 398].

1.11.3 Intracellular and extracellular loops

The flexible loops connecting the transmembrane domains also have crucial functions. The small intracellular loop between M1 and M2 domains extends beyond the pore formed by the M2 domain [383, 399, 400]. Recently the M1-M2 loop along with the end of M3 domain have been show to control $GABA_A$ and glycine receptor desensitization [395]. The extracellular loop between the M2 and M3 transmembrane domain transduces GABA binding to channel gating in the transmembrane domains by multiple polar interactions with the Cys-loop (β6-β7 loop) [357, 374, 401]. Importance of the M2-M3 loop mutations is further demonstrated by human mutations that reduce functional responses in GABA [402-404], glycine [405-407], and muscle ACh receptors [408]. This also demonstrates the conserved mechanisms that transduce ligand binding to channel gating in the Cys-loop family receptors. In patients with severe epilepsy syndromes, the *GABRA1(K306T)* [50], *GABRB3(Y302C)* [104, 171], *GABRB3(A305V)* [409], and *GABRG2(K328M)* [410, 411] mutations in the M2-M3 loop reduced $GABA_A$ receptor functions by either reducing peak GABA currents or altering current kinetics. The intracellular M3-M4 loop is the largest loop (>100 residues) in the Cys-loop family of receptors that provides the primary domain for interaction with the intracellular environment. The structure of this large intracellular loop is not

known; for channel crystallization purposes it is replaced with a small loop of <10 amino acid residues. This change is presumed to resemble the structure of the intact channel by and large. Within the cell, the M3-M4 loop is critical for $GABA_A$ receptor functions. Various protein interactions in the M3-M4 loop including those with scaffolding proteins (that govern receptor clustering, trafficking, sorting, expression, and endocytosis), protein kinases, and post-translation modification proteins govern channel function [412].

1.12 Modes of $GABA_A$ receptor-mediated inhibition: hyperpolarization, depolarization, shunting, and tonic inhibition

Binding of two GABA molecules opens $GABA_A$ receptors and results in Cl^- influx or efflux based on the local Cl^- concentration gradient inside and outside the cell and the local membrane potential. In adult neurons the intracellular Cl^- concentration is ~11 fold lower than the outside and has a reversal potential of approximately -65 mV, which is close to the resting membrane potential (~70 mV). Under these conditions $GABA_A$ receptor activation results in Cl^- influx and hyperpolarizes the cell [413]. In contrast, during development immature neurons have high intracellular Cl^- concentration $[Cl^-]$, and thus $GABA_A$ receptor activation results in Cl^- efflux that depolarizes the cell [414]. Since the GABAergic system develops before the glutaminergic system, GABA sets up the initial synaptic connectivity and acts as the major excitatory neurotransmitter during development that facilitates activity-dependent growth and synapse formation [415-418].

Developmental decline in $[Cl^-]$ is thought to drive the shift from depolarizing to hyperpolarizing actions of GABA. Immature neurons have elevated intracellular $[Cl^-]$ due to high expression of sodium potassium chloride co-transporters (NKCCs), sodium chloride co-

transporters (NCCCs), and sodium-independent anion exchangers (AE3). This leads to E_{Cl}^- that is more positive than the resting membrane potential, thus when $GABA_A$ receptors open in immature neurons Cl^- flows out of the cells to reach E_{Cl}^- and depolarizes the cells. Similarly, $GABA_A$ receptor activation in adult neural progenitor and immature neurons depolarizes them due to high intracellular $[Cl^-]$ [419, 420]. Of these NKCC1 levels have been shown to be important for regulation of intracellular $[Cl^-]$ and generation of hippocampal sharp waves in rat pups [421]. A large body of work on excitatory actions of GABA has been carried out *in vitro* conditions that provide ease of recording GABA currents and good control over internal and external Cl^- concentrations (that are challenging to measure and control precisely). It remains to be seen whether these conclusions remain valid in intact brains [422, 423]. As development progresses increased expression of potassium chloride co-transporters (KCCs), sodium dependent anion exchangers (NDAE), and chloride channel 2 (Clc2) that extrude Cl^- from neurons result in an E_{Cl}^- more negative than the membrane potential as the brain matures [424]. Thus, in mature adult neurons $GABA_A$ receptor activation produces Cl^- influx that hyperpolarizes neurons, thereby increasing the action potential threshold and minimizing the depolarization produced by glutamate or calcium receptors.

The Cl^- influx after activation of synaptic $GABA_A$ receptors does not always result in membrane hyperpolarization but can still reduce (or shunt) the likelihood of action potentials or membrane depolarization by increasing the conductance or decreasing membrane resistance, a phenomenon known as shunting inhibition. By Ohm's law V=IR, thus as the membrane resistance decreases (by channel opening) the same amount of current produces a smaller membrane depolarization. Additionally, since E_{Cl}^- is only slightly more positive than the resting membrane potential, it is uniquely suited for shunting inhibition, unlike the reversal potential for the excitatory channels that is typically much more positive than the resting membrane potential.

Thus, an excitatory input depolarizes cell membranes; however, an inhibitory input may not always hyperpolarize the cell membrane. Shunting inhibition is not just the difference in the membrane potential generated by EPSPs and IPSPs. Strength of shunting inhibition depends on the distance between the excitatory and inhibitory inputs, cell morphology, and location of GABAergic synapses. GABAergic inputs on proximal dendrites are particularly effective in shunting more distal excitatory inputs than those located more proximal to soma (Hao, 2009). Further, shunting inhibition persists only as long as the $GABA_A$ receptors are open, while hyperpolarizing IPSCs decay more slowly as governed by receptor kinetics and cable properties of neurons. Thus brief shunting inhibition has been proposed to determine the precise spike timing (millisecond range) of neurons that is essential for shaping network oscillations [425] and controls firing rate gain in neurons [426]. Moreover, shunting inhibition appears to be the main inhibitory mechanism in GABAergic hippocampal interneurons throughout development [427].

Tonic inhibition is a mode of long-lasting inhibition produced at the non-synaptic locations by a subpopulation of $GABA_A$ receptors, with δ subunits exclusively and α4 and α6 subunits predominantly expressed at extrasynaptic sites. The ε, π, and θ subunits are also expressed non-synaptically but are not widely expressed in all brain regions. Unlike the synaptic $GABA_A$ subunits, those mediating tonic inhibition are located in the vicinity (perisynaptic) or far (extrasynaptic) from GABAergic synapses. Thus, they are activated by GABA diffusion or spillover from synapses during repeated activation of presynaptic terminals during which transporters are unable clear GABA from the synaptic cleft [428-430]. Additionally, GABA has more recently been shown to be released by astrocytes (a major class of gila) [431-434] and neuronal dendrites [435, 436]. Further, this non-neuronal GABA has been shown to mediate tonic inhibition by non-synaptic $GABA_A$ receptors [434, 437-440]. Clearly much lower GABA concentrations (μM) [441] are available at non-synaptic sites (μM) than at synapses (mM), but they

are sufficient to activate high affinity non-synaptic $GABA_A$ receptors [442-444]. Additionally, δ subunit-containing $GABA_A$ receptors have reduced desensitization compared to those with γ subunits [445]. High affinity and reduced desensitization makes non-synaptic $GABA_A$ receptors effective for mediating tonic conductance. For example, tonic $GABA_A$ receptors in the hippocampus and cerebellum have been shown to conduct more charge than the high frequency phasic inhibition in the same cell [436, 446, 447]. Thus, tonic inhibition is critical for regulating cellular and network excitability, and not surprisingly, altered tonic inhibition is observed in disease states. SNPs and mutations in the δ subunit gene (*GABRD*) have been associated with GGEs [448, 449].

1.13 GABAergic signaling in neurodevelopment

Neurodevelopment is a highly orchestrated phenomenon with a plethora of coordinated events including actions of GABA and glutamate signaling. The $GABA_A$ neurotransmitter system is a key facilitator of embryonic neurodevelopment, demonstrated by the observation that cortical architecture is substantially altered when the $GABA_A$ agonist muscimol or antagonist bicuculline are applied to newborn rat parietal cortices [450]. The GABAergic system is one of the first to appear during development, even prior to the glutamatergic system [451-453]. However, maturation of the GABAergic system is more protracted than maturation of the glutamatergic system; mature EPSPs appear by P20-22 whereas mature adult-like IPSCs appear only by ~4 weeks after birth in rodents [454, 455]. GABAergic interneurons are the primary source of GABA—the primary inhibitory transmitter in the CNS. In the developing nervous system prior to synapse formation, GABA acts as neurotrophic factor that negatively regulates neurogenesis, promotes migration and differentiation of neurons, and synaptogenesis [452, 456, 457]. Additionally, ambient GABA is essential for maturation and integration of adult newborn neurons

[458], as well as for control of the number of newly born adult cells by inhibiting production of new neuroblasts [459-461]. In the adult brain each of the GABAergic interneuron types (≥20 in the cortex and hippocampus) fine-tune the output of microcircuits with their unique properties [462, 463]. Even though GABAergic interneurons in the adult mammalian cortex comprise only about 10-25% of all neurons, they are critical circuit components that regulate the activity and oscillatory behavior of excitatory pyramidal cell assemblies.

Murine interneurons start to appear in the ventral telencephalon on embryonic day (E) 9.5 and E12.5 in the medial and caudal ganglionic eminences, respectively [453, 464, 465]. Around the same time excitatory pyramidal cells start to emerge in the ventricular zone of the dorsal telencephalon at E11, then radially migrate to the cortical plate and form the six layered cerebral cortex by E17 [466-469]. Despite the earlier start, maturation of the GABAergic system is much more protracted. Interneurons that begin their tangential migration from the medial and caudal ganglionic eminences appear in the maturing dorsal telencephalon (that will become the cerebral cortex) where they begin the radial migration and integration with the glutamatergic cells. GABA immunoreactivity in the dorsal telencephalon, primarily in the cortical plate, sub-plate, marginal zone, and subventricular zone, can be seen from E14-19, but the pattern of GABA immunoreactivity as seen in adult brains appears much later postnatally from P16-21 [452, 470]. Additionally, GABAergic interneurons and synapses undergo pruning in the postnatal period. Interneuron numbers reach their maximum by P5 in mice, after which they undergo intrinsically determined cell death that reaches its maximum by P7. By P15 the interneuron cell death halts, and the P20 animals have adult interneuron levels; only 60% of the initial interneuron pool that reached the cortex survives [471]. The interneuron cell death is cell autonomous and independent of the competition for survival factors but synaptic transmission or cell–cell contact may play role in interneuron survival [472].

Similarly, the formation of GABAergic synapses can occur in the absence of GABA release primarily because cell-autonomous genetic programs regulate the physiological and morphological maturation of GABAergic interneurons and formation of GABAergic synapses [473, 474]. The initial foundation of GABAergic synapses is largely sensory input and activity independent, while their maturation is not. GABAergic synapses can be detected in the rat neocortex by E16 predominantly in the marginal zone and sub-plate [475], and glutamatergic synapses by E17 [476]. Functional GABAergic currents have been recorded only by E18 in the mouse neocortex. Thus even when $GABA_A$ receptors can be detected in neuronal stem cells and migrating neuroblasts, the formation of functional synapses with pre- and post-synaptic elements occurs much later in development. Once the synapses are formed their maturation is dependent on GABA release that itself depends on neuronal activity. When GABA release is dampened specifically from PV+ interneurons by knocking out the GABA synthesizing enzyme GAD67, perisomatic innervation by PV+ interneurons and their axonal branching are reduced [477]. On the contrary, complete blockade of GABA release from PV+ interneurons results in an excess of synapses and overgrowth of axons. These results indicate that synaptic GABA release regulates synapse elimination, as GABA is not needed for GABAergic synapse formation [478]. GABAergic circuits are refined by synapse pruning, a process that begins during neurodevelopment and continues in late adulthood [479, 480].

In addition to neurodevelopment, $GABA_A$ receptor activation is critical for adult neurogenesis involving proliferation, differentiation, migration, and appropriate synaptic integration of adult newborn neurons [481, 482]. Activation of $GABA_A$ receptors by ambient GABA, followed by depolarizing synaptic GABAergic inputs, and then embellishment of glutamatergic synapses in the juvenile and adult neuronal precursors is similar to the maturation pattern seen

during neurodevelopment [458, 483-489]. Thus, actions of GABA brought about by $GABA_A$ receptors are critical in shaping the developing nervous system.

1.14 $GABA_A$ receptor changes during development

Each of the 19 $GABA_A$ receptor and $GABA_{B1/2}$ receptor subunits are differentially expressed during embryonic and postnatal development. Prior to formation of GABAergic synapses, $GABA_A$ receptor activation serves autocrine and paracrine functions, following which GABA serves as an excitatory neurotransmitter [419, 490] in the immature nervous system, and then as the major inhibitory neurotransmitter in adults. The precise changes in the expression of each subunit are not well known as there are region specific, age specific, and species specific changes in expression patterns, and thus only some of the well-documented changes in the rodent brain are described here.

The α4, β1, and γ1 $GABA_A$ receptor subunits appear much earlier in rodent neurodevelopment and have been identified in neuronal stem cells [456, 491] and pre-migratory neuroblasts in the ventricular zone [492]. At this stage in rodent development, high levels of GABA promote proliferation of neuronal progenitors in the ventricular zone via $GABA_A$ receptor activation. As the neuroblasts approach their destination in the cortical plate, expression of α2, α3, β3, and γ2 subunits dominate while α4 subunit levels become undetectable [493], and $GABA_A$ receptor activation arrests their migration in rodents [452 494-496]. During the late embryonic stages, expression of α3, α5, β2/3, and γ2 subunits begins and peaks during initial postnatal stages. From this point, expression of β2/3 and γ2 subunits remains relatively constant, and they remain dominant subunits in the adult rodent cortex [494]. In the late embryonic stage, $GABA_A$ receptor activation results in phasic depolarization as early as E18 in the rodent neocortex and

hippocampus [475]. In the post-natal stages the dominant α3/α5 subunit-containing $GABA_A$ receptors of the early postnatal stages are replaced by predominantly α1 subunit-containing $GABA_A$ receptors [497-500] in most brain regions except in the thalamus. The α1 subunit from then becomes the dominant α subunit in most adult brain regions [494, 501-503]. This developmental change occurs concomitantly with replacement of slow-decaying IPSCs with adult-like fast decaying IPSCs [504], with increased sensitivity to zolpidem (α1 selective agonist) [505], benzodiazepines [504], and neurosteroids [506]. Consistently, mice lacking α1 subunits continue to have juvenile-like slow and long-lasting IPSCs [339-341]. Along with the α1 subunits, expression of α4, α6 (expressed only in the cerebellum), and δ subunits gradually increases during postnatal development [494]. The γ3 subunits are expressed only for about two weeks after birth [494] at a time when there is extensive differentiation and synaptogenesis but their role in this short post-natal period is not known. As ρ(1-3), ε, π, and θ subunits are not abundantly expressed throughout the brain, changes in their expression patterns are not well known. Next I will describe the changes in expression patterns of β3 and β1 subunits as effects of mutations in these subunits are the focus of this thesis.

1.14.1 Developmental expression of β3 subunits

Among β subunits, expression of β3 subunits dominates in the embryonic and early postnatal stages in most brain regions, while expression of β2 subunits is highest in the adult rodent cortex. Expression of β3 subunits begins at E12 in rodents, reaches its peak in the perinatal stage [494, 507] and plays a critical role in neuronal differentiation and migration. However, β3 subunit expression declines as the nervous system matures except in the hippocampus, olfactory bulb, cerebellum, and reticular thalamic nucleus (nRT), where they continue to be the predominant β subunit [494, 507, 508]. Additionally, β3 subunits have a

significantly higher GABA affinity than β1 or β2 subunits [509, 510], which may be well suited during the early neurodevelopmental period where low GABA concentrations exist due to maturating interneurons and GABAergic synapses. Furthermore, the β3 subunit occurs in two isoforms (1 and 2) that are developmentally regulated. The β3 subunits are encoded by the *GABRB3* gene located on chromosome 15q11.2-q12. *GABRB3* has nine coding exons [511] that are predicted to generate at least five mRNA transcripts (variants 1-5), though proteins from only variants 1 and 2 have been confirmed in the brain tissue. Variant 1 and 2 each encodes a full-length mature β3 subunit with 438 and 451 amino acids, respectively. Distinct transcriptional start sites in exon 1 and exon 1A produce mRNA transcripts variant 1 and variant 2, respectively, that encode identical β3 subunits apart from their signal peptide sequence and initial few amino acids of the mature peptide [512]. Interestingly, exon 1A is abundantly expressed in the fetal brain but not in the adult brain, while exon 1 is highly expressed in the adult brain. Although, in cortex and cerebellum, marginal expression of fetal variant 2 transcripts with exon 1A continues into adulthood [334, 512]. The β3 subunit isoform 4 is predicated to be 388 amino acids long (uniprot.org, NCBI Gene, but its presence in the CNS has not been experimentally verified. It could be speculated that the two alternative signal peptides provides temporal and tissue specific expression. The *GABRB3(N110D, D120N), GABRB3(E180G),* and *GABRB3(Y302C)* mutations described in this thesis are located in exons 4, 5 and 8, respectively. For this study, we used cDNA encoding human *GABRB3* variant 2 (NM_021912.4) that is abundant in fetal brain to express β3 subunits in heterologous HEK293T cells; in theory, β3 subunits produced from either variant 1 or 2 should not affect the properties of $GABA_A$ receptors, as the mature β3 subunits do not contain the signal peptide.

The β3 subunits continue to be highly expressed in regions involved in seizure generation such as cortex, thalamus, hippocampus, septum and basal forebrain; as well as in

the olfactory bulb, hypothalamus, epithalamus, and amygdala [494, 508, 513]. Also, the β3 subunit knockout mice develops seizures [514]. This indicates that β3 subunits have a critical role in normal brain function and that altered inhibition via β3 subunit-containing $GABA_A$ receptors during development or in the adult brain is sufficient to render brain circuits susceptible to seizures. Moreover, the thalamocortical circuits are essential to sensory processing, and thus it is not surprisingly that disruption of the GABAergic system in heterozygous *Gabrb3* mice elicits somatosensory deficits. *GABRB3* is also implicated in autism [515] in which somatosensory disturbances are common [516, 517].

1.14.2 Developmental expression of β1 subunit

The 449 amino acid long β1 subunit is encoded by *GABRB1* [518], which has 9 exons [511] similar to *GABRB3*. *GABRB1* is mapped to chromosome 4p12 in a cluster of $GABA_A$ receptor subunit genes encoding α4, α2, and γ1 subunits. In the rodents *in situ* hybridization starting on E14 revealed that β1 subunit mRNAs can be detected in the ventricular zone and the spinal cord [494], while the expression in the cortex, thalamus, and olfactory bulb begins after birth and undergoes significant down regulation in the early postnatal period. Each reaches adult levels after the first post-natal week [519]. Hippocampal β1 subunit mRNA levels are higher than cortex levels, but they peak in the postnatal period (~P12) and then decline to steady levels in adults [494, 519]. While in humans mRNA levels measured using microarray analysis with qPCR validation show that β1 subunit mRNAs decline after birth till 2-3 years of age and then remain at this level in adults [507]. However, unlike the β2 and β3 subunits, the contribution of β1 subunits to neurodevelopment and normal brain functions is not well understood.

Studies from the past decade highlight an important role of β1 subunits in the estrous cycle [520], sleep regulation [521], schizophrenia [522], and ataxia [523]. *GABRB1* polymorphisms have been shown to regulate alcohol dependence [524-527] and impair behavioral control predisposing individuals to addiction [528]. Recently, *GABRB1* mutations have been identified in two children with epileptic encephalopathy in two different studies [104, 172]. As *GABRB1* is identified in isolated cases, it lacks the statistical significance to be classified as an epilepsy gene. Although, our work [171] clearly demonstrates that mutant β1 subunits significantly disrupt GABA-evoked currents, suggesting that it could contribute to the epilepsy phenotypes (discussed in depth in Chapter 2). The presence of β1 subunits in circuits involved in seizure generation such as cortex, hippocampus, and thalamus during the early post-natal period and in adults [494, 507, 513, 519] also make their contribution to epilepsy plausible.

1.15 $GABA_A$ receptors: roles in epilepsy

As $GABA_A$ receptors are the dominant inhibitory receptors in the CNS, it is not surprising that mutations altering their functions can lead to epilepsy. Indeed, several mutations were identified in large families with epilepsy phenotypes (including CAE, JME, FS, GEFS+) beginning the early 2000s before NGS studies. These initial reports identified epilepsy-associated mutations in *GABRA1* [43], *GABRB3* [129, 529, 530], *GABRG2* [41, 42], *and GABRD* [531], establishing them as human epilepsy genes. Before NGS studies mutations in the remaining 15 of 19 $GABA_A$ receptor subunit genes were not identified in epilepsy patients, and a possible association of certain $GABA_A$ receptor genes with certain syndromes was pointed out. For example the association of *GABRB3* and *GABRA1* mutations with CAE or JME, respectively [43, 129]. Now, however, the continually expanding list of epilepsy-associated $GABA_A$ receptor subunit mutations includes

GABRA2 [532], *GABRA3* [533], *GABRB1* [172], and *GABRB2* [44, 45], which were previously not considered to be human epilepsy genes. It is only a matter of time before more epilepsy-associated $GABA_A$ mutations will emerge as additional risk factors for epilepsy. Currently $GABA_A$ receptor mutations have been identified in patients with different epilepsy syndromes including febrile seizures (FS), generalized epilepsy with febrile seizures plus (GEFS+), childhood absence epilepsy (CAE), juvenile myoclonic epilepsy (JME), and epileptic encephalopathies (EEs). Moreover, the contributions $GABA_A$ receptor gene mutations to diseases such as autism spectrum disorder and schizophrenia are also well recognized.

1.16 Pathophysiology of epilepsy-associated $GABA_A$ receptor mutations

The pathological impact of $GABA_A$ receptor mutations depends on their type (missense, nonsense, frameshift, insertion deletion, copy number variation (CNV)) and location (signal peptide, mature subunit, promoter region, intronic and 3′ and 5′ untranslated regions.). For missense mutations, the functional alteration varies even when different amino acid substitutions are observed at the same location [534]. Numerous mutations in $GABA_A$ receptor genes, especially missense mutations, have been documented so far [535] **(Table 1)**. The mechanisms of actions of few mutations are described here primarily to discuss the various ways, sometimes unanticipated, in which they disrupt $GABA_A$ receptor functions.

1.16.1 Missense mutations

The *GABRA1(A322D)* mutation was harbored by all individuals affected with autosomal dominant form of JME in a large French-Canadian family [43]. The symptoms included myoclonic seizures and GTCSs, and some family members also had absence seizures. This mutation

altered the non-polar amino acid alanine to an acidic polar aspartic acid residue in the M3 transmembrane helix of the $GABA_A$ receptor α1 subunit. When expressed in heterologous cells, the mutant α1*(A322D)* subunits misfolded and reduced incorporation of the M3 helix into the lipid bilayer [536]. The misfolded subunit was retained in the endoplasmic reticulum (ER), resulting in reduced total and surface levels [537]. This in turn led to significantly reduced GABA-evoked currents and GABA-sensitivity [538, 539]. In addition, when the mutant α1(A322D) subunits were expressed in neurons, they reduced miniature inhibitory postsynaptic current (mIPSC) amplitudes and altered receptor kinetics [540]. Thus, inhibition reduced by impaired subunit assembly and by dominant negative effects of this mutation could lead to hyperexcitable neuronal networks and in turn produce seizures.

1.16.2 Truncation mutations

The *GABRG2(Q390X)* mutation was identified in an Australian family with GEFS+ and Dravet syndrome [541]. It resulted in a truncated γ2 subunit that lacked the 78 C-terminal amino acids resulting in trapping and degradation of $GABA_A$ receptors in the ER and consequently severely reduced GABA-evoked currents [541, 542]. The mutant γ2(Q390X) subunits had dominant negative effects on GABA-evoked currents and surface expression of wild type receptors when α1, β2, γ2, and γ2(Q390X) subunits were co-expressed in heterologous cells and in cultured neurons [542]. Furthermore, a *Gabrg2*$^{+/Q390X}$ knock-in mouse model had spontaneous seizures, reduced mIPSC amplitudes, accumulation and aggregation of γ2(Q390X) subunits in neurons, and reduced partnering α and β subunits trafficking of heteropentameric $GABA_A$ receptors. Additionally, the accumulated γ2(Q390X) subunits promoted apoptosis and neuronal death, and in turn enhanced age related neurodegeneration [543]. Some of these adverse outcomes of the mutation were reduced by overexpression of wild type γ2 subunits in *Gabrg2*$^{+/Q390X}$ mice [544].

1.16.3 Frameshift mutations

The c.1329delC *GABRG2* frameshift mutation was identified in four family members affected with GEFS+ and in one unaffected family member [545]. The mutation is a deletion of the cytosine (C) nucleotide at the serine 443 codon resulting in loss of the natural stop codon and emergence of a new stop codon in the 3′ UTR, predicted to produce γ2(S443delC) subunits with an extended C-terminus that lacks 24 wild type amino acids and has an additional 50 cryptic amino acids. When the mutant γ2(S443delC) subunits were expressed in heterologous cell lines, they were retained in the ER and degraded, resulting in reduced total expression levels compared to the wild type γ2 subunits. Whole cell GABA-evoked currents from $GABA_A$ receptors containing α1, β2, and γ2(S443delC) subunits were only 30% of those with wild type subunits and had significantly increased desensitization and Zn^{2+} inhibition similar to those of α1 and β2 subunit-containing $GABA_A$ receptors. Thus, γ2(S443delC) subunits reduced inhibition by haploinsufficiency through loss of wild type γ2 subunits from the cell surface. Additionally, they also had dominant negative effects by increasing degradation of partnering αβ subunits and in turn reducing the surface expression of functional $GABA_A$ receptors.

1.16.4 Deletion-insertion mutations

The *GABRA1(K353delins18X)* mutation resulted from insertion of 25 nucleotides in intron 10 near the splice acceptor site of exon 11. Most eukaryotic mRNA contains introns that are spliced out to produce mature mRNA. During splicing the 5′ donor site first makes a lariat at the branch site, then the intron is spliced out at the 3′ acceptor site at the end of the intron. Common sequence length between the branch site and the acceptor site is 20-50 nucleotides.

Insertion of 25 nucleotides in intron 10 increased this distance to 63 nucleotides resulting in retention of intron 10 in the mRNA transcript and introduction of a premature translation termination codon (PTC). Although, not experimentally demonstrated, at least a fraction of the mutant mRNAs can likely be degraded by a cellular quality control mechanism known as nonsense-mediated mRNA decay (NMD) to prevent translation of truncated proteins. As mutant α1(K353delins18X) subunits have been identified using antibodies, some mRNAs do undergo translation and produce α1(K353delins18X*)* subunits with a lower molecular mass compared to wild type α1 subunits that are predicted to have 18 amino acids translated from intron 10 and deletion of the fourth transmembrane domain due to a PTC. When expressed in a heterologous cell line, the α1(K353delins18X*)* subunits were retained in the ER and not expressed on the cell surface. Not surprisingly GABA-evoked currents were absent from cells expressing the α1(K353delins18X), β, and γ subunits. The presence of the *GABRA1(K353delins18X)* mutation in three family members affected with GTCS [530] supports its role in epilepsy; however, one unaffected individual also harbored this mutation, suggesting significant effects of genetic background in expression of symptoms.

1.16.5 Intronic mutations

Exonic mutations are extensively studied while intronic mutations in $GABA_A$ receptor are less frequently reported and are difficult to study compared to point mutations. Non-coding regions such as introns and 3′ and 5′ untranslated regions can influence gene function and contribute to epilepsy pathogenesis. One such example is the *GABRG*2(IVS6+2T→G) mutation that altered the splice donor site sequence from GT to GG in intron 6, which was reported in an Australian family with CAE and FS [546]. When the mutation was expressed in HEK293T cells and in transgenic mice, the mRNAs with aberrant splice sites retained 53 base pairs of intron 6, had a

PTC in intron 7, and were partially subjected to degradation by NMD. This resulted in expression of a stable truncated protein with 217 wild type and 29 novel amino acids from intron 6 and frameshifted exon 7, but was not trafficked to the cell surface and was retained in the ER resulting in significantly reduced GABA-evoked currents resembling those of the αβ subunit-containing $GABA_A$ receptors. Thus, haploinsufficiency due to degradation of mutant mRNA and dominant negative effects of the mutation trapped the receptors in the ER, produced ER stress and reduced currents. Reduced inhibition resulting from these events was suggested to produce the epilepsy phenotype [547].

1.16.6 Promoter mutations

Single nucleotide polymorphisms (SNPs) in the *GABRB3* promoter regions were reported to significantly associate with CAE in a study by Urak and colleagues with 45 CAE patients and 75 controls [184]. To examine the effects of the promoter mutations an *in vitro* reporter gene assay was used, which compared the ability of the promoter regions of CAE patients and controls to drive protein expression. They found that the promoter region mutations of CAE patients significantly reduced transcriptional activity relative to that of the promoter regions of control individuals, resulting in reduced protein expression. Further *in silico* analysis predicted that the CAE-associated haplotype promoter impaired binding of a neuron specific transcriptional activator N-Oct-3, and an *in vitro* assay demonstrated reduced binding to nuclear proteins compared to the control promoter haplotype. Thus, the CAE-associated haplotype in principle could reduce β3 subunit expression and in turn reduce GABAergic inhibition during the critical developmental period and in adult CNS. However, the promoter haplotype (rs4906902) reported by Urak and colleagues failed to associate with CAE in two other studies with large (250 CAE patients, 559 controls) [548] and small (48 CAE patients, >500 controls) sample sizes

[549]. Recently, SNPs in *GABRB3* had been associated with schizophrenia [550, 551]. Non-coding genetic changes are expected to be identified more frequently as genomic sequencing becomes commonplace. Thus, the significance of non-coding sequences, which in principle can alter neuronal activity, is yet to be fully characterized.

Table 1.1 $GABA_A$ receptor mutations identified in patients with epilepsy, and in patients with intellectual disability or developmental delay with or without epilepsy.

Gene	Mutation	Diagnosis	Location	$GABA_A$ receptor functions			Reference
				Current amplitude	Current kinetics	Expression level	
GABRA1	T20I	GGE	N-terminus	-	-	-	Johannesen K, 2016
	V74I	GEFS+	N-terminus	-	-	-	Johannesen K, 2016
	S76R	DS-like/EE	N-terminus	Reduced	-	-	Johannesen K, 2016
	F104C	JME	N-terminus	-	-	-	Johannesen K, 2016
	R112Q	DS & IS	N-terminus	-	-	-	Carvill GL, 2014; Kodera H, 2016
	N115D	Mild DS	N-terminus	-	-	-	Johannesen K, 2016
	L146M	DS	N-terminus	-	-	-	Johannesen K, 2016
	R214H	DS/EE	N-terminus	Reduced	-	-	Johannesen K, 2016
	D192N	GGE	N-terminus	Reduced	Faster deactivation	Reduced surface and total α	Lachance-Touchette P, 2011; Chen X, et al., 2017
	G251S	DS	N-terminus	Reduced	-	-	Carvill GL, 2014
	P206L	OS, WS	M1	-	-	-	Kodera H, 2016
	M263T	WS	M1	-	-	-	Kodera H, 2016
	M2623I	WS	M1	-	-	-	Kodera H, 2016; Farnaes L, 2017

	V287L	EOEE	M2	-	-	-	Kodera H, 2016
	T289P	EIEE	M2	-	-	-	Johannesen K, 2016
	T289A	EIEE	M2	-	-	-	Johannesen K, 2016
	T292I	IS	M2	Reduced	-	-	Allen S, 2013; Chen X, et al., 2017
	A295D	JME	M3	-	-	-	
	K306T	DS, MAE-like	M2-M3 loop	Reduced	-	-	Carvill GL, 2014
	A322D	JME	M3	Reduced	Faster deactivation	Reduced surface and total	Cossette P, 2002; Gallagher MK, 2004
	S326fs238X	CAE	Splice site	Virtually no current	-	Reduced surface α1	Maljevic S, 2006
	K353delins18X	CAE	M1	Reduced	NA	Reduced surface and total	Lachance-Touchette P, 2011; Chen X, et al., 2017
	Y438C	ASD	M4	-	-	-	Heyne HO, 2018
GABRB1	F246S	IS	M1	Small reduction	Slow deactivation	Reduced surface	Allen S, 2013; Janve VS, 2016
	T287I	EE	M2	-	-	-	Lien E, 2016
GABRB2	L17S	ASD	Signal peptide	-	-	-	Heyne HO, 2018
	M79T	EE	N-terminus	-	-	-	Srivastava S, 2014; Hamdan FF, 2018
	D125N	DD, EE	N-terminus	-	-	-	Heyne HO, 2018; Hamdan FF, 2018
	Y183H	ID	N-terminus	-	-	-	Heyne HO, 2018
	Y244H	EE	N-terminus	-	-	-	Hamdan FF, 2017
	P252A	ID	M1	-	-	-	Heyne HO, 2018
	L277S	DD, EE	M2	-	-	-	Heyne HO, 2018; Hamdan FF, 2017
	V282A		M2	-	-	-	
	T284K	EE	M2	-	-	-	Hamdan FF, 2017
	T287P	DS	M2	Reduced	-	Reduced total and surface β2	Ishii A, 2017
	R293P	DD	M2-M3 loop				Hamdan FF, 2018

	Y301C	DD	M2-M3 loop	-	-	-	Heyne HO, 2018
	L303R	EOEE	M2-M3 loop	-	-	-	Hamdan FF, 2018
	L303N		M2-M3 loop	-	-	-	
	A304V	EE	M2-M3 loop	-	-	-	Hamdan FF, 2018
	V316I	EE	M3	-	-	-	Hamdan FF, 2018
GABRB3	-897T/C	CAE	Exon 1A promoter	-	-	-	Urak L, 2006
	P11S	CAE	Signal peptide	Reduced	-	Unaltered, hyperglycosylation	Tanaka M, 2008
	S15F	CAE	Signal peptide	Reduced	-	Unaltered, hyperglycosylation	Tanaka M, 2008
	G32R	CAE	N-terminus	Reduced	-	Increased total and surface	Tanaka M, 2008; Gurba KN, 2012
	V37G	GEFS+	N-terminus	-	-	-	Tanaka M, 2008
	N110D	IS	N-terminus	-	Faster deactivation	Increased total and surface β3	Allen S, 2013; Janve VS, 2016
	R111X	MAE	N-terminus				Moller RE, 2017
	D120N	LGS	N-terminus	Reduced	Slow rise	Unaltered	Allen S, 2013; Janve VS, 2016
	L127R	DD	N-terminus	-	-	-	Heyne HO, 2018
	T157M	GEFS+	N-terminus	-	-	-	Moller RE, 2017
	L170R	EOEE	N-terminus	Reduced	Altered	Reduced α,β,γ	Zhang Y, 2015; Hernandez CC,
	E180G	LGS	N-terminus	Reduced	Slow rise	Unaltered	Allen S, 2013; Janve VS, 2016
	Y182F	EE	N-terminus	-	-	-	Epi4K, 2016
	Y184H	MAE	N-terminus	-	-	-	Moller RE, 2017
	R232Q	DS	N-terminus	-	-	-	Le SV, 2017
	Q249K	EE	M1	-	-	-	Epi4K, 2016
	L256Q	WS/EE	M1	-	-	-	Moller RE, 2017
	T287I	EOEE	M2	-	-	-	Papandreou A, 2016
	T288N	EOEE	M2	Reduced	Altered	Reduced α,β,γ	Hernandez CC, 2017
	I306T	DD	M3				Heyne HO, 2018
	Y302C	LGS	M2-M3 loop	Reduced	Slow rise	Unaltered	Allen S, 2013,; Janve VS, 2016

	A305V	EOEE	M3-M4 loop	Reduced	Altered	Reduced α,β,γ	Zhang Y, 2015; Hernandez CC, 2017
	A305T	LGS	M3-M4 loop	-	-	-	Epi4K, 2016
	S420I	DD	M3-M4 loop	-	-	-	Heyne HO, 2018
	R429Q	GEFS+	M3-M4 loop	-	-	-	Epi-Phenome/Genome, 2013
	Y471C	DD	M4	-	-	-	Heyne HO, 2018
GABRG2	Q40X	DS	N-terminus	Reduced	Altered	γ subunits absent on	Huang X, 2012
	P59fsX12	FS	Frame shift/ N-terminus				Boillot M, 2015
	R82Q	CAE/FS	N-terminus				Wallace RH, 2001
	P83S	GGE	N-terminus			-	Lachance-Touchette P, 2011
	A106T	EE	N-terminus	Reduced	Altered	Reduced surface γ	Shen D, 2017
	I107T	EE	N-terminus	Reduced	Altered	Reduced surface γ	Shen D, 2017
	R136X	FS, GEFS+	N-terminus	Reduced	Altered	Reduced	Johnston AJ, 2014; Boillot M, 2015
	R177G	FS	N-terminus	-	-	-	Audenaert D, 2006
	IVS6+2T>G	CAE/FS	Splice site				Tian M, 2012
	M199V	CAE, GEFS+	N-terminus	-	-	-	Boillot M, 2015
	c.549-3T>G	RE	Splice site	-	-	-	Reinthaler EM, 2015
	G257R	RE	N-terminus	-	-	-	Reinthaler EM, 2015
	P282S	EE	M1	Reduced	Altered	Reduced surface γ	Shen D, 2017
GABRG2	P302L	DS	M2	Reduced	Altered	Slight reduction	Hernandez CC, 2017
	R323Q	MAE, RE, ID, EE	M2	Reduced	Altered	Reduced surface γ	Carvill GL, 2013; Hamdan FF, 2018; Reinthaler EM, 2015
	R323W	EE	M2	Reduced	Altered	Reduced surface γ	Shen D, 2017
	K328M	GEFS+	M2-M3 loop	Reduced	Altered	Surface γ virtually absent	Bulac S, 2001
	F343L	EE	M3	Reduced	Altered	Reduced surface γ	Shen D, 2017

	Q390X	GEFS+/ DS	M3-M4 loop	Reduced	-	Reduced total and surface β2	Harkin LA, 2002
	E402fsX3	FS/ TLE	Frame shift/ M3-M4 loop	-	-	-	Boillot M, 2015
	V402fs3	FS, TLE, GTCS	Frame shift/ M3-M4 loop	-	-	-	Boillot M, 2015
	W429X	DS, GEFS+	M3-M4 loop	Reduced	-	Reduced total and surface γ	Sun H, 2008; Kang JQ 2009
	S443delC	GEFS+	Frame shift/ M4	Reduced	-	Reduced total and surface γ	Tian M, 2013
	V462fsX33	FS	Frame shift/ M4	-	-	-	Boillot M, 2015

Abbreviations:
Epilepsy syndromes: **CAE** - child absence epilepsy, **DD** – Developmental delay, **DS** – Dravet syndrome, **EE** – Epileptic encephalopathy, **EOEE** – Early onset epileptic encephalopathy, **FS** – Febrile seizures, **GEFS+** – Generalized epilepsy with febrile seizures plus, **GTCS** – Generalized tonic-clonic seizures, **ID** – Intellectual disability, **JME** – Juvenile myoclonic epilepsy, **LGS** – Lennox-Gastaut syndrome, **MAE** – Myoclonic astatic epilepsy, **RE** – Rolandic epilepsy, **TLE** –Temporal lobe epilepsy, **WS** – West syndrome

Mutations:
Fs- Frameshift, X- Truncation, Del –Deletion, Delins- Deletion-insertion.

1.17 Rationale for experimental chapters

1.17.1 Increasing interest in EE syndromes

In the past decade, an increasing number of human diseases with complex genetic etiologies have been associated with *de novo* mutations. This has been a boon for genetic disease diagnosis and classification, especially for diseases that have sporadic presentations in which the genetic inheritance or environmental causes do not explain the disease phenotype. For the epilepsy field this had been a paradigm shift. Prior to identification of *de novo* mutations, most studies were focused on investigating the genetic etiologies in large multiplex families. This was a reasonable approach given that the mutation segregates well (but not 100%) with the affected individuals in a family. There was no theoretical framework, however, that explained the clear majority of epilepsy patients, including those with EEs, that had no family history or obvious environmental influences. In fact, the epilepsy in these patients was classified as idiopathic (unknown cause). In the past decade, genetic studies on large number of patients with unknown cause have become feasible due to next generation sequencing (NGS). Results from numerous NGS studies have shown that most patients with idiopathic epilepsies have a genetic origin of their epilepsy, which has resulted in a change in nomenclature for the epilepsy in these patients from idiopathic epilepsy to genetic generalized epilepsy. Currently, the number of epilepsies with unknown cause has been substantially reduced (Thomas and Berkovic, 2014). These advances have renewed interest in studying etiologies of epilepsy syndromes that could not be systematically studied in large patient populations, such as rare but severe EEs (Figure 1.6). The Epi4K consortium conducted an early study that examined large samples of EE patients and reported *GABRB3* mutations a genetic cause of IS and LGS. The mechanisms of action of these mutations were unknown, and previously the *GABRB3* mutations were associated with CAE, a relatively mild epilepsy syndrome. It seems plausible that *GABRB3*

mutations can lead to epilepsy. This could not be confirmed, however, in the absence of functional studies, and the extent of dysfunction could not be accurately predicted. Chapters II and III describe the functional impact of five *GABRB3* mutations reported by the Epi4K consortium in heterologous cells. Using electrophysiology, flow cytometry, and structural modeling, we showed that these mutations produced a clear $GABA_A$ receptor functional deficit and thus correlated with the severe epilepsy phenotype. Further, we show that mutation in *GABRB1*, a gene that was previously not associated with epilepsy and not reported by the Epi4K consortium as causative of EE, produced significant loss of receptor function, making it a likely candidate epilepsy gene.

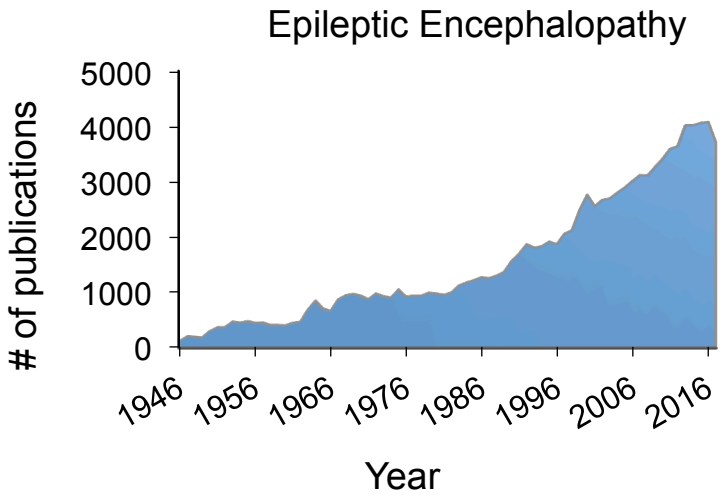


Figure 1.6 Several EEs were recognized as specific epilepsy syndromes during the mid1900s. In the past 20 years there has been a growing interest, especially after the ILEA recognized and postulated the term EE, which is reflected in the steady increase in the number of published studies as shown in the above graph. Search term 'epileptic encephalopathy' was used in Pubmed and results until 2017 are displayed here.

1.17.2 Establishing a model of LGS—a severe EE

Mouse models for most EEs do not exist, except for the mouse models of IS [552, 553] and the well-studied Dravet syndrome in which majority of patients have *SCN1A* mutations [263, 554]. Even though the genetic underpinnings of EEs (in addition to Dravet syndrome) are being discovered at an unprecedented rate, few mouse models of EE have been reported [555]. As EEs are heterogeneous syndromes both in terms of the clinical presentations and the genetic etiologies, models that replicate the salient features of specific EEs have been long awaited. Chapter III demonstrates that the human LGS *GABRB3(D120N)* mutation significantly reduced $GABA_A$ receptor function. Based on these results, our laboratory generated the *Gabrb3*$^{+/D120N}$ knock in mouse that harbors the mutation identified in a LGS patient. In Chapter IV examines the effects of the *GABRB3(D120N)* mutation in the LGS KI mouse model. The *Gabrb3*$^{+/D120N}$ mice display characteristic features of LGS: seizure onset in the first two weeks of life, characteristic seizure types, and sleep disturbances. These results demonstrate that *Gabrb3*$^{+/D120N}$ mice are a good model of LGS. Future studies will determine the mechanisms by which the *GABRB3(D120N)* mutation produces a LGS-like phenotype in these mice. Some of the possible ways to examine the mechanisms of actions are described in Chapter V.

REFERENCES

1. Fisher RS, Acevedo C, Arzimanoglou A, et al. ILAE official report: a practical clinical definition of epilepsy. Epilepsia. 2014 Apr;55(4):475-82.

2. England MJ, Liverman CT, Schultz AM, Strawbridge LM. Epilepsy across the spectrum: promoting health and understanding. A summary of the Institute of Medicine report. Epilepsy Behav. 2012 Oct;25(2):266-76.

3. Fiest KM, Sauro KM, Wiebe S, et al. Prevalence and incidence of epilepsy: A systematic review and meta-analysis of international studies. Neurology. 2017 Jan 17;88(3):296-303.

4. Bell GS, Neligan A, Sander JW. An unknown quantity--the worldwide prevalence of epilepsy. Epilepsia. 2014 Jul;55(7):958-62.

5. Schmidt D, Loscher W. Drug resistance in epilepsy: putative neurobiologic and clinical mechanisms. Epilepsia. 2005 Jun;46(6):858-77.

6. Tomson T, Walczak T, Sillanpaa M, Sander JW. Sudden unexpected death in epilepsy: a review of incidence and risk factors. Epilepsia. 2005;46 Suppl 11:54-61.

7. Massey CA, Sowers LP, Dlouhy BJ, Richerson GB. Mechanisms of sudden unexpected death in epilepsy: the pathway to prevention. Nat Rev Neurol. 2014 May;10(5):271-82.

8. Lhatoo S, Noebels J, Whittemore V, Research NCfS. Sudden unexpected death in epilepsy: Identifying risk and preventing mortality. Epilepsia. 2015 Nov;56(11):1700-6.

9. Forsgren L, Hauser WA, Olafsson E, Sander JW, Sillanpaa M, Tomson T. Mortality of epilepsy in developed countries: a review. Epilepsia. 2005;46 Suppl 11:18-27.

10. Nevalainen OP, Ansakorpi H, Auvinen A. Epilepsy-related clinical characteristics and mortality: a systematic review and meta-analysis. Neurology. 2015 Apr 28;84(17):1823-4.

11. Englot DJ. A modern epilepsy surgery treatment algorithm: Incorporating traditional and emerging technologies. Epilepsy Behav. 2018 Feb 1;80:68-74.

12. Neal EG, Chaffe H, Schwartz RH, et al. A randomized trial of classical and medium-chain triglyceride ketogenic diets in the treatment of childhood epilepsy. Epilepsia. 2009 May;50(5):1109-17.

13. Allers K, Essue BM, Hackett ML, et al. The economic impact of epilepsy: a systematic review. BMC Neurol. 2015 Nov 25;15:245.

14. Begley CE, Durgin TL. The direct cost of epilepsy in the United States: A systematic review of estimates. Epilepsia. 2015 Sep;56(9):1376-87.

15. Begley CE, Beghi E. The economic cost of epilepsy: a review of the literature. Epilepsia. 2002;43 Suppl 4:3-9.

16. Diederich F, Konig HH, Mietzner C, Brettschneider C. Costs of informal nursing care for patients with neurologic disorders: A systematic review. Neurology. 2018 Jan 2;90(1):28-34.

17. Yoon D, Frick KD, Carr DA, Austin JK. Economic impact of epilepsy in the United States. Epilepsia. 2009 Oct;50(10):2186-91.

18. Fisher RS, Cross JH, D'Souza C, et al. Instruction manual for the ILAE 2017 operational classification of seizure types. Epilepsia. 2017 Apr;58(4):531-42.

19. Scheffer IE, Berkovic S, Capovilla G, et al. ILAE classification of the epilepsies: Position paper of the ILAE Commission for Classification and Terminology. Epilepsia. 2017 Apr;58(4):512-21.

20. Fisher RS, Cross JH, French JA, et al. Operational classification of seizure types by the International League Against Epilepsy: Position Paper of the ILAE Commission for Classification and Terminology. Epilepsia. 2017 Apr;58(4):522-30.

21. Wilfert AB, Sulovari A, Turner TN, Coe BP, Eichler EE. Recurrent de novo mutations in neurodevelopmental disorders: properties and clinical implications. Genome Med. 2017 Nov 27;9(1):101.

22. Tacke M, Neubauer BA, Gerstl L, Roser T, Remi J, Borggraefe I. [Epilepsy-new diagnostic tools, old drugs? : Therapeutic consequences of epilepsy genetics]. Nervenarzt. 2017 Dec;88(12):1385-94.

23. Vollmar C, Noachtar S. [Importance of imaging diagnostics and EEG for differential diagnosis of epileptic seizures]. Nervenarzt. 2017 Oct;88(10):1119-25.

24. Sands TT, Choi H. Genetic Testing in Pediatric Epilepsy. Curr Neurol Neurosci Rep. 2017 May;17(5):45.

25. Mountz JM, Patterson CM, Tamber MS. Pediatric Epilepsy: Neurology, Functional Imaging, and Neurosurgery. Semin Nucl Med. 2017 Mar;47(2):170-87.

26. Thomas RH, Berkovic SF. The hidden genetics of epilepsy-a clinically important new paradigm. Nat Rev Neurol. 2014 May;10(5):283-92.

27. Annegers JF, Hauser WA, Anderson VE, Kurland LT. The risks of seizure disorders among relatives of patients with childhood onset epilepsy. Neurology. 1982 Feb;32(2):174-9.

28. Lennox WG. Epilepsy and related disorders: Little, Brown; 1960.

29. Vadlamudi L, Andermann E, Lombroso CT, et al. Epilepsy in twins: insights from unique historical data of William Lennox. Neurology. 2004 Apr 13;62(7):1127-33.

30. Lennox WG. The heredity of epilepsy as told by relatives and twins. J Am Med Assoc. 1951 Jun 9;146(6):529-36.

31. Berkovic SF, Howell RA, Hay DA, Hopper JL. Epilepsies in twins: genetics of the major epilepsy syndromes. Ann Neurol. 1998 Apr;43(4):435-45.

32. Kjeldsen MJ, Corey LA, Christensen K, Friis ML. Epileptic seizures and syndromes in twins: the importance of genetic factors. Epilepsy Research. 2003;55(1,Äì2):137-46.

33. Corey LA, Pellock JM, Kjeldsen MJ, Nakken KO. Importance of genetic factors in the occurrence of epilepsy syndrome type: a twin study. Epilepsy Res. 2011 Nov;97(1-2):103-11.

34. Vadlamudi L, Milne RL, Lawrence K, et al. Genetics of epilepsy: The testimony of twins in the molecular era. Neurology. 2014 Sep 16;83(12):1042-8.

35. Lehesjoki AE, Koskiniemi M, Sistonen P, et al. Localization of a gene for progressive myoclonus epilepsy to chromosome 21q22. Proc Natl Acad Sci U S A. 1991 May 01;88(9):3696-9.

36. Phillips HA, Scheffer IE, Berkovic SF, Hollway GE, Sutherland GR, Mulley JC. Localization of a gene for autosomal dominant nocturnal frontal lobe epilepsy to chromosome 20q 13.2. Nat Genet. 1995 May;10(1):117-8.

37. Steinlein OK, Mulley JC, Propping P, et al. A missense mutation in the neuronal nicotinic acetylcholine receptor alpha 4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy. Nat Genet. 1995 Oct;11(2):201-3.

38. Singh NA, Charlier C, Stauffer D, et al. A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. Nat Genet. 1998 Jan;18(1):25-9.

39. Charlier C, Singh NA, Ryan SG, et al. A pore mutation in a novel KQT-like potassium channel gene in an idiopathic epilepsy family. Nat Genet. 1998 Jan;18(1):53-5.

40. Escayg A, MacDonald BT, Meisler MH, et al. Mutations of SCN1A, encoding a neuronal sodium channel, in two families with GEFS+2. Nat Genet. 2000 Apr;24(4):343-5.

41. Wallace RH, Marini C, Petrou S, et al. Mutant GABA(A) receptor gamma2-subunit in childhood absence epilepsy and febrile seizures. Nat Genet. 2001 May;28(1):49-52.

42. Baulac S, Huberfeld G, Gourfinkel-An I, et al. First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. Nat Genet. 2001 May;28(1):46-8.

43. Cossette P, Liu L, Brisebois K, et al. Mutation of GABRA1 in an autosomal dominant form of juvenile myoclonic epilepsy. Nat Genet. 2002 Jun;31(2):184-9.

44. Ishii A, Kang JQ, Schornak CC, et al. A de novo missense mutation of GABRB2 causes early myoclonic encephalopathy. J Med Genet. 2017 Mar;54(3):202-11.

45. Srivastava S, Cohen J, Pevsner J, et al. A novel variant in GABRB2 associated with intellectual disability and epilepsy. Am J Med Genet A. 2014 Nov;164A(11):2914-21.

46. Hernandez CC, Gurba KN, Hu N, Macdonald RL. The GABRA6 mutation, R46W, associated with childhood absence epilepsy, alters 6beta22 and 6beta2 GABA(A) receptor channel gating and expression. J Physiol. 2011 Dec 1;589(Pt 23):5857-78.

47. Kumari R, Lakhan R, Kalita J, Garg RK, Misra UK, Mittal B. Potential role of GABAA receptor subunit; GABRA6, GABRB2 and GABRR2 gene polymorphisms in epilepsy susceptibility and pharmacotherapy in North Indian population. Clin Chim Acta. 2011 Jun 11;412(13-14):1244-8.

48. Prasad DK, Shaheen U, Satyanarayana U, Prabha TS, Jyothy A, Munshi A. Association of GABRA6 1519 T>C (rs3219151) and Synapsin II (rs37733634) gene polymorphisms with the development of idiopathic generalized epilepsy. Epilepsy research. 2014 Oct;108(8):1267-73.

49. Zeng Q, Yang X, Zhang J, et al. Genetic analysis of benign familial epilepsies in the first year of life in a Chinese cohort. J Hum Genet. 2018 Jan;63(1):9-18.

50. Carvill GL, Weckhuysen S, McMahon JM, et al. GABRA1 and STXBP1: novel genetic causes of Dravet syndrome. Neurology. 2014 Apr 8;82(14):1245-53.

51. Johannesen K, Marini C, Pfeffer S, et al. Phenotypic spectrum of GABRA1: From generalized epilepsies to severe epileptic encephalopathies. Neurology. 2016 Sep 13;87(11):1140-51.

52. Kodera H, Ohba C, Kato M, et al. De novo GABRA1 mutations in Ohtahara and West syndromes. Epilepsia. 2016 Apr;57(4):566-73.

53. Consortium E, Leu C, de Kovel CG, et al. Genome-wide linkage meta-analysis identifies susceptibility loci at 2q34 and 13q31.3 for genetic generalized epilepsies. Epilepsia. 2012 Feb;53(2):308-18.

54. Wang J, Lin ZJ, Liu L, et al. Epilepsy-associated genes. Seizure. 2017 Jan;44:11-20.

55. Weber YG, Lerche H. Genetic mechanisms in idiopathic epilepsies. Dev Med Child Neurol. 2008 Sep;50(9):648-54.

56. Nadeau JH. Modifier genes in mice and humans. Nat Rev Genet. 2001 Mar;2(3):165-74.

57. Meisler MH, O'Brien JE. Gene Interactions and Modifiers in Epilepsy. 2012.

58. Berkovic SF, Mulley JC, Scheffer IE, Petrou S. Human epilepsies: interaction of genetic and acquired factors. Trends Neurosci. 2006 Jul;29(7):391-7.

59. Kurtz BS, Lehman J, Garlick P, et al. Penetrance and expressivity of genes involved in the development of epilepsy in the genetically epilepsy-prone rat (GEPR). J Neurogenet. 2001;15(3-4):233-44.

60. Hawkins NA, Kearney JA. Confirmation of an epilepsy modifier locus on mouse chromosome 11 and candidate gene analysis by RNA-Seq. Genes Brain Behav. 2012 Jun;11(4):452-60.

61. Bergren SK, Chen S, Galecki A, Kearney JA. Genetic modifiers affecting severity of epilepsy caused by mutation of sodium channel Scn2a. Mamm Genome. 2005 Sep;16(9):683-90.

62. Frankel WN, Mahaffey CL, McGarr TC, Beyer BJ, Letts VA. Unraveling genetic modifiers in the gria4 mouse model of absence epilepsy. PLoS Genet. 2014 Jul;10(7):e1004454.

63. Bergren SK, Rutter ED, Kearney JA. Fine mapping of an epilepsy modifier gene on mouse Chromosome 19. Mamm Genome. 2009 Jun;20(6):359-66.

64. Kalachikov S, Evgrafov O, Ross B, et al. Mutations in LGI1 cause autosomal-dominant partial epilepsy with auditory features. Nat Genet. 2002 Mar;30(3):335-41.

65. Morante-Redolat JM, Gorostidi-Pagola A, Piquer-Sirerol S, et al. Mutations in the LGI1/Epitempin gene on 10q24 cause autosomal dominant lateral temporal epilepsy. Hum Mol Genet. 2002 May 1;11(9):1119-28.

66. Pizzuti A, Flex E, Di Bonaventura C, et al. Epilepsy with auditory features: a LGI1 gene mutation suggests a loss-of-function mechanism. Ann Neurol. 2003 Mar;53(3):396-9.

67. Dibbens LM, de Vries B, Donatello S, et al. Mutations in DEPDC5 cause familial focal epilepsy with variable foci. Nat Genet. 2013 May;45(5):546-51.

68. Ishida S, Picard F, Rudolf G, et al. Mutations of DEPDC5 cause autosomal dominant focal epilepsies. Nat Genet. 2013 May;45(5):552-5.

69. Scheffer IE, Heron SE, Regan BM, et al. Mutations in mammalian target of rapamycin regulator DEPDC5 cause focal epilepsy with brain malformations. Ann Neurol. 2014 May;75(5):782-7.

70. Lemke JR, Lal D, Reinthaler EM, et al. Mutations in GRIN2A cause idiopathic focal epilepsy with rolandic spikes. Nat Genet. 2013 Sep;45(9):1067-72.

71. Pelzer N, Haan J, Stam AH, et al. Clinical spectrum of hemiplegic migraine and chances of finding a pathogenic mutation. Neurology. 2018 Jan 17.

72. Gauthier AC, Manganas LN, Mattson RH. A novel inherited SCN1A mutation associated with GEFS+ in benign and encephalopathic epilepsy. J Clin Neurosci. 2017 Jun;40:82-4.

73. Sadleir LG, Mountier EI, Gill D, et al. Not all SCN1A epileptic encephalopathies are Dravet syndrome: Early profound Thr226Met phenotype. Neurology. 2017 Sep 5;89(10):1035-42.

74. Huang W, Liu M, Yan SF, Yan N. Structure-based assessment of disease-related mutations in human voltage-gated sodium channels. Protein Cell. 2017 Jun;8(6):401-38.

75. Le SV, Le PHT, Le TKV, Kieu Huynh TT, Hang Do TT. A mutation in GABRB3 associated with Dravet syndrome. Am J Med Genet A. 2017 Aug;173(8):2126-31.

76. Harkin LA, Bowser DN, Dibbens LM, et al. Truncation of the GABA(A)-receptor gamma2 subunit in a family with generalized epilepsy with febrile seizures plus. Am J Hum Genet. 2002 Feb;70(2):530-6.

77. Shi X, Yasumoto S, Kurahashi H, et al. Clinical spectrum of SCN2A mutations. Brain & development. 2012 Aug;34(7):541-5.

78. Ogiwara I, Nakayama T, Yamagata T, et al. A homozygous mutation of voltage-gated sodium channel beta(I) gene SCN1B in a patient with Dravet syndrome. Epilepsia. 2012 Dec;53(12):e200-3.

79. Dibbens LM, Heron SE, Mulley JC. A polygenic heterogeneity model for common epilepsies with complex genetics. Genes Brain Behav. 2007 Oct;6(7):593-7.

80. Kasperaviciute D, Catarino CB, Heinzen EL, et al. Common genetic variation and susceptibility to partial epilepsies: a genome-wide association study. Brain. 2010 Jul;133(Pt 7):2136-47.

81. Heinzen EL, Depondt C, Cavalleri GL, et al. Exome sequencing followed by large-scale genotyping fails to identify single rare variants of large effect in idiopathic generalized epilepsy. Am J Hum Genet. 2012 Aug 10;91(2):293-302.

82. Chioza BA, Aicardi J, Aschauer H, et al. Genome wide high density SNP-based linkage analysis of childhood absence epilepsy identifies a susceptibility locus on chromosome 3p23-p14. Epilepsy Res. 2009 Dec;87(2-3):247-55.

83. Durner M, Keddache MA, Tomasini L, et al. Genome scan of idiopathic generalized epilepsy: evidence for major susceptibility gene and modifying genes influencing the seizure type. Ann Neurol. 2001 Mar;49(3):328-35.

84. Hempelmann A, Taylor KP, Heils A, et al. Exploration of the genetic architecture of idiopathic generalized epilepsies. Epilepsia. 2006 Oct;47(10):1682-90.

85. Hempelmann A, Heils A, Sander T. Confirmatory evidence for an association of the connexin-36 gene with juvenile myoclonic epilepsy. Epilepsy Res. 2006 Oct;71(2-3):223-8.

86. Marini C, Scheffer IE, Crossland KM, et al. Genetic architecture of idiopathic generalized epilepsy: clinical genetic analysis of 55 multiplex families. Epilepsia. 2004 May;45(5):467-78.

87. Sander T, Schulz H, Saar K, et al. Genome search for susceptibility loci of common idiopathic generalised epilepsies. Hum Mol Genet. 2000 Jun 12;9(10):1465-72.

88. Zara F, Bianchi A, Avanzini G, et al. Mapping of genes predisposing to idiopathic generalized epilepsy. Hum Mol Genet. 1995 Jul;4(7):1201-7.

89. Sherry ST, Ward MH, Kholodov M, et al. dbSNP: the NCBI database of genetic variation. Nucleic Acids Res. 2001 Jan 1;29(1):308-11.

90. Stenson PD, Mort M, Ball EV, et al. The Human Gene Mutation Database: towards a comprehensive repository of inherited mutation data for medical research, genetic diagnosis and next-generation sequencing studies. Hum Genet. 2017 Jun;136(6):665-77.

91. Foo JN, Liu JJ, Tan EK. Whole-genome and whole-exome sequencing in neurological diseases. Nat Rev Neurol. 2012 Sep;8(9):508-17.

92. Guo Y, Baum LW, Sham PC, et al. Two-stage genome-wide association study identifies variants in CAMSAP1L1 as susceptibility loci for epilepsy in Chinese. Hum Mol Genet. 2012 Mar 1;21(5):1184-9.

93. Consortium E, Consortium EM, Steffens M, et al. Genome-wide association analysis of genetic generalized epilepsies implicates susceptibility loci at 1q43, 2p16.1, 2q22.3 and 17q21.32. Hum Mol Genet. 2012 Dec 15;21(24):5359-72.

94. Lynch M. Rate, molecular spectrum, and consequences of human mutation. Proc Natl Acad Sci U S A. 2010 Jan 19;107(3):961-8.

95. Roach JC, Glusman G, Smit AF, et al. Analysis of genetic inheritance in a family quartet by whole-genome sequencing. Science. 2010 Apr 30;328(5978):636-9.

96. Conrad DF, Keebler JE, DePristo MA, et al. Variation in genome-wide mutation rates within and between human families. Nat Genet. 2011 Jun 12;43(7):712-4.

97. Rahbari R, Wuster A, Lindsay SJ, et al. Timing, rates and spectra of human germline mutation. Nat Genet. 2016 Feb;48(2):126-33.

98. Goldmann JM, Wong WS, Pinelli M, et al. Parent-of-origin-specific signatures of de novo mutations. Nat Genet. 2016 Aug;48(8):935-9.

99. Francioli LC, Polak PP, Koren A, et al. Genome-wide patterns and properties of de novo mutations in humans. Nat Genet. 2015 Jul;47(7):822-6.

100. Kondrashov AS, Crow JF. A molecular approach to estimating the human deleterious mutation rate. Hum Mutat. 1993;2(3):229-34.

101. Crow JF. How much do we know about spontaneous human mutation rates? Environ Mol Mutagen. 1993;21(2):122-9.

102. Veltman JA, Brunner HG. De novo mutations in human genetic disease. Nat Rev Genet. 2012 Aug;13(8):565-75.

103. Epi Kc, Epilepsy Phenome/Genome P. Ultra-rare genetic variation in common epilepsies: a case-control sequencing study. Lancet Neurol. 2017 Feb;16(2):135-43.

104. Epi KC, Epilepsy Phenome/Genome P, Allen AS, et al. De novo mutations in epileptic encephalopathies. Nature. 2013 Sep 12;501(7466):217-21.

105. Epi KCEaekce, Epi KC. De Novo Mutations in SLC1A2 and CACNA1A Are Important Causes of Epileptic Encephalopathies. Am J Hum Genet. 2016 Aug 04;99(2):287-98.

106. Hamdan FF, Myers CT, Cossette P, et al. High Rate of Recurrent De Novo Mutations in Developmental and Epileptic Encephalopathies. Am J Hum Genet. 2017 Nov 2;101(5):664-85.

107. O'Roak BJ, Deriziotis P, Lee C, et al. Exome sequencing in sporadic autism spectrum disorders identifies severe de novo mutations. Nat Genet. 2011 Jun;43(6):585-9.

108. Sanders SJ, Murtha MT, Gupta AR, et al. De novo mutations revealed by whole-exome sequencing are strongly associated with autism. Nature. 2012 Apr 4;485(7397):237-41.

109. Neale BM, Kou Y, Liu L, et al. Patterns and rates of exonic de novo mutations in autism spectrum disorders. Nature. 2012 Apr 4;485(7397):242-5.

110. Iossifov I, Ronemus M, Levy D, et al. De novo gene disruptions in children on the autistic spectrum. Neuron. 2012 Apr 26;74(2):285-99.

111. O'Roak BJ, Vives L, Girirajan S, et al. Sporadic autism exomes reveal a highly interconnected protein network of de novo mutations. Nature. 2012 Apr 4;485(7397):246-50.

112. O'Roak BJ, Vives L, Fu W, et al. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science. 2012 Dec 21;338(6114):1619-22.

113. Michaelson JJ, Shi Y, Gujral M, et al. Whole-genome sequencing in autism identifies hot spots for de novo germline mutation. Cell. 2012 Dec 21;151(7):1431-42.

114. Yu TW, Chahrour MH, Coulter ME, et al. Using whole-exome sequencing to identify inherited causes of autism. Neuron. 2013 Jan 23;77(2):259-73.

115. Girard SL, Gauthier J, Noreau A, et al. Increased exonic de novo mutation rate in individuals with schizophrenia. Nat Genet. 2011 Jul 10;43(9):860-3.

116. Xu B, Ionita-Laza I, Roos JL, et al. De novo gene mutations highlight patterns of genetic and neural complexity in schizophrenia. Nat Genet. 2012 Dec;44(12):1365-9.

117. Fromer M, Pocklington AJ, Kavanagh DH, et al. De novo mutations in schizophrenia implicate synaptic networks. Nature. 2014 Feb 13;506(7487):179-84.

118. McCarthy SE, Gillis J, Kramer M, et al. De novo mutations in schizophrenia implicate chromatin remodeling and support a genetic overlap with autism and intellectual disability. Mol Psychiatry. 2014 Jun;19(6):652-8.

119. Guipponi M, Santoni FA, Setola V, et al. Exome sequencing in 53 sporadic cases of schizophrenia identifies 18 putative candidate genes. PLoS One. 2014;9(11):e112745.

120. Rees E, Kirov G, Walters JT, et al. Analysis of exome sequence in 604 trios for recessive genotypes in schizophrenia. Transl Psychiatry. 2015 Jul 21;5:e607.

121. Kranz TM, Harroch S, Manor O, et al. De novo mutations from sporadic schizophrenia cases highlight important signaling genes in an independent sample. Schizophr Res. 2015 Aug;166(1-3):119-24.

122. Huang K. De novo paradigm: the ultimate answer to the paradox in mental retardation? Clin Genet. 2011 May;79(5):427-8.

123. Vissers LE, de Ligt J, Gilissen C, et al. A de novo paradigm for mental retardation. Nat Genet. 2010 Dec;42(12):1109-12.

124. Deciphering Developmental Disorders S. Prevalence and architecture of de novo mutations in developmental disorders. Nature. 2017 Feb 23;542(7642):433-8.

125. Claes L, Del-Favero J, Ceulemans B, Lagae L, Van Broeckhoven C, De Jonghe P. De novo mutations in the sodium-channel gene SCN1A cause severe myoclonic epilepsy of infancy. Am J Hum Genet. 2001 Jun;68(6):1327-32.

126. Brunklaus A, Ellis R, Reavey E, Forbes GH, Zuberi SM. Prognostic, clinical and demographic features in SCN1A mutation-positive Dravet syndrome. Brain. 2012 Aug;135(Pt 8):2329-36.

127. Wallace RH, Hodgson BL, Grinton BE, et al. Sodium channel alpha1-subunit mutations in severe myoclonic epilepsy of infancy and infantile spasms. Neurology. 2003 Sep 23;61(6):765-9.

128. Meisler MH, Kearney JA. Sodium channel mutations in epilepsy and other neurological disorders. J Clin Invest. 2005 Aug;115(8):2010-7.

129. Tanaka M, Olsen RW, Medina MT, et al. Hyperglycosylation and reduced GABA currents of mutated GABRB3 polypeptide in remitting childhood absence epilepsy. Am J Hum Genet. 2008 Jun;82(6):1249-61.

130. Hernandez CC, Zhang Y, Hu N, et al. GABA A Receptor Coupling Junction and Pore GABRB3 Mutations are Linked to Early-Onset Epileptic Encephalopathy. Sci Rep. 2017 Nov 21;7(1):15903.

131. Le SV, Le PHT, Le TKV, Kieu Huynh TT, Hang Do TT. A mutation in GABRB3 associated with Dravet syndrome. Am J Med Genet A. 2017 Aug;173(8):2126-31.

132. Moller RS, Wuttke TV, Helbig I, et al. Mutations in GABRB3: From febrile seizures to epileptic encephalopathies. Neurology. 2017 Jan 04.

133. Papandreou A, McTague A, Trump N, et al. GABRB3 mutations: a new and emerging cause of early infantile epileptic encephalopathy. Dev Med Child Neurol. 2016 Apr;58(4):416-20.

134. Iafrate AJ, Feuk L, Rivera MN, et al. Detection of large-scale variation in the human genome. Nat Genet. 2004 Sep;36(9):949-51.

135. Sebat J, Lakshmi B, Troge J, et al. Large-scale copy number polymorphism in the human genome. Science. 2004 Jul 23;305(5683):525-8.

136. Freeman JL, Perry GH, Feuk L, et al. Copy number variation: new insights in genome diversity. Genome Res. 2006 Aug;16(8):949-61.

137. Redon R, Ishikawa S, Fitch KR, et al. Global variation in copy number in the human genome. Nature. 2006 Nov 23;444(7118):444-54.

138. Conrad DF, Pinto D, Redon R, et al. Origins and functional impact of copy number variation in the human genome. Nature. 2010 Apr 1;464(7289):704-12.

139. Olson H, Shen Y, Avallone J, et al. Copy number variation plays an important role in clinical epilepsy. Ann Neurol. 2014 Jun;75(6):943-58.

140. Wincent J, Kolbjer S, Martin D, et al. Copy number variations in children with brain malformations and refractory epilepsy. Am J Med Genet A. 2015 Mar;167A(3):512-23.

141. Feuk L, Carson AR, Scherer SW. Structural variation in the human genome. Nat Rev Genet. 2006 Feb;7(2):85-97.

142. Mills RE, Walter K, Stewart C, et al. Mapping copy number variation by population-scale genome sequencing. Nature. 2011 Feb 3;470(7332):59-65.

143. Zarrei M, MacDonald JR, Merico D, Scherer SW. A copy number variation map of the human genome. Nat Rev Genet. 2015 Mar;16(3):172-83.

144. Rees E, Kirov G, Sanders A, et al. Evidence that duplications of 22q11.2 protect against schizophrenia. Mol Psychiatry. 2014 Jan;19(1):37-40.

145. Gonzalez E, Kulkarni H, Bolivar H, et al. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science. 2005 Mar 4;307(5714):1434-40.

146. Fanciulli M, Pasini E, Malacrida S, et al. Copy number variations and susceptibility to lateral temporal epilepsy: a study of 21 pedigrees. Epilepsia. 2014 Oct;55(10):1651-8.

147. Zhang LN, Meng Z, He ZW, Li DF, Luo XY, Liang LY. [Clinical phenotypes and copy number variations in children with microdeletion and microduplication syndromes: an analysis of 50 cases]. Zhongguo Dang Dai Er Ke Za Zhi. 2016 Sep;18(9):840-5.

148. Nevado J, Mergener R, Palomares-Bralo M, et al. New microdeletion and microduplication syndromes: A comprehensive review. Genet Mol Biol. 2014 Mar;37(1 Suppl):210-9.

149. Weise A, Mrasek K, Klein E, et al. Microdeletion and microduplication syndromes. J Histochem Cytochem. 2012 May;60(5):346-58.

150. Vissers LE, Stankiewicz P. Microdeletion and microduplication syndromes. Methods Mol Biol. 2012;838:29-75.

151. Deak KL, Horn SR, Rehder CW. The evolving picture of microdeletion/microduplication syndromes in the age of microarray analysis: variable expressivity and genomic complexity. Clin Lab Med. 2011 Dec;31(4):543-64, viii.

152. Olson H, Shen Y, Avallone J, et al. Copy number variation plays an important role in clinical epilepsy. Ann Neurol. 2014 Jun;75(6):943-58.

153. Mefford HC, Muhle H, Ostertag P, et al. Genome-wide copy number variation in epilepsy: novel susceptibility loci in idiopathic generalized and focal epilepsies. PLoS Genet. 2010 May 20;6(5):e1000962.

154. Marini C, Mei D, Temudo T, et al. Idiopathic epilepsies with seizures precipitated by fever and SCN1A abnormalities. Epilepsia. 2007 Sep;48(9):1678-85.

155. Mulley JC, Nelson P, Guerrero S, et al. A new molecular mechanism for severe myoclonic epilepsy of infancy: exonic deletions in SCN1A. Neurology. 2006 Sep 26;67(6):1094-5.

156. Mefford HC, Yendle SC, Hsu C, et al. Rare copy number variants are an important cause of epileptic encephalopathies. Ann Neurol. 2011 Dec;70(6):974-85.

157. Helbig I, Mefford HC, Sharp AJ, et al. 15q13.3 microdeletions increase risk of idiopathic generalized epilepsy. Nat Genet. 2009 Feb;41(2):160-2.

158. de Kovel CG, Trucks H, Helbig I, et al. Recurrent microdeletions at 15q11.2 and 16p13.11 predispose to idiopathic generalized epilepsies. Brain. 2010 Jan;133(Pt 1):23-32.

159. Dibbens LM, Mullen S, Helbig I, et al. Familial and sporadic 15q13.3 microdeletions in idiopathic generalized epilepsy: precedent for disorders with complex inheritance. Hum Mol Genet. 2009 Oct 1;18(19):3626-31.

160. Heinzen EL, Radtke RA, Urban TJ, et al. Rare deletions at 16p13.11 predispose to a diverse spectrum of sporadic epilepsy syndromes. Am J Hum Genet. 2010 May 14;86(5):707-18.

161. Mullen SA, Carvill GL, Bellows S, et al. Copy number variants are frequent in genetic generalized epilepsy with intellectual disability. Neurology. 2013 Oct 22;81(17):1507-14.

162. Nicholl J, Waters W, Suwalski S, et al. Epilepsy with cognitive deficit and autism spectrum disorders: prospective diagnosis by array CGH. Am J Med Genet B Neuropsychiatr Genet. 2013 Jan;162B(1):24-35.

163. Thompson R, Drew CJ, Thomas RH. Next generation sequencing in the clinical domain: clinical advantages, practical, and ethical challenges. Adv Protein Chem Struct Biol. 2012;89:27-63.

164. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012 Sep 6;489(7414):57-74.

165. Altshuler D, Daly MJ, Lander ES. Genetic mapping in human disease. Science. 2008 Nov 7;322(5903):881-8.

166. Choi M, Scholl UI, Ji W, et al. Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. Proceedings of the National Academy of Sciences of the United States of America. 2009 Nov 10;106(45):19096-101.

167. Yang Y, Muzny DM, Xia F, et al. Molecular findings among patients referred for clinical whole-exome sequencing. JAMA. 2014 Nov 12;312(18):1870-9.

168. Taylor JC, Martin HC, Lise S, et al. Factors influencing success of clinical genome sequencing across a broad spectrum of disorders. Nat Genet. 2015 Jul;47(7):717-26.

169. Chong JX, Buckingham KJ, Jhangiani SN, et al. The Genetic Basis of Mendelian Phenotypes: Discoveries, Challenges, and Opportunities. Am J Hum Genet. 2015 Aug 6;97(2):199-215.

170. Shashi V, McConkie-Rosell A, Rosell B, et al. The utility of the traditional medical genetics diagnostic evaluation in the context of next-generation sequencing for undiagnosed genetic disorders. Genet Med. 2014 Feb;16(2):176-82.

171. Janve VS, Hernandez CC, Verdier KM, Hu N, Macdonald RL. Epileptic encephalopathy de novo GABRB mutations impair GABA receptor function. Ann Neurol. 2016 Mar 7.

172. Lien E, Vatevik AK, Ostern R, Haukanes BI, Houge G. A second patient with a De Novo GABRB1 mutation and epileptic encephalopathy. Ann Neurol. 2016 Aug;80(2):311-2.

173. Nakashima M, Kouga T, Lourenco CM, et al. De novo DNM1 mutations in two cases of epileptic encephalopathy. Epilepsia. 2016 Jan;57(1):e18-23.

174. Euro E-RESC, Epilepsy Phenome/Genome P, Epi KC. De novo mutations in synaptic transmission genes including DNM1 cause epileptic encephalopathies. Am J Hum Genet. 2014 Oct 02;95(4):360-70.

175. Dhindsa RS, Bradrick SS, Yao X, et al. Epileptic encephalopathy-causing mutations in DNM1 impair synaptic vesicle endocytosis. Neurol Genet. 2015 Jun;1(1):e4.

176. Deng XL, Yin F, Zhang CL, et al. [Dynamin-1-related infantile spasms: a case report and review of literature]. Zhonghua Er Ke Za Zhi. 2016 Nov 02;54(11):856-9.

177. Boumil RM, Letts VA, Roberts MC, et al. A missense mutation in a highly conserved alternate exon of dynamin-1 causes epilepsy in fitful mice. PLoS Genet. 2010 Aug 05;6(8).

178. Asinof SK, Sukoff Rizzo SJ, Buckley AR, et al. Independent Neuronal Origin of Seizures and Behavioral Comorbidities in an Animal Model of a Severe Childhood Genetic Epileptic Encephalopathy. PLoS Genet. 2015 Jun;11(6):e1005347.

179. Asinof S, Mahaffey C, Beyer B, Frankel WN, Boumil R. Dynamin 1 isoform roles in a mouse model of severe childhood epileptic encephalopathy. Neurobiol Dis. 2016 Nov;95:1-11.

180. Willig LK, Petrikin JE, Smith LD, et al. Whole-genome sequencing for identification of Mendelian disorders in critically ill infants: a retrospective analysis of diagnostic and clinical findings. Lancet Respir Med. 2015 May;3(5):377-87.

181. Martin HC, Kim GE, Pagnamenta AT, et al. Clinical whole-genome sequencing in severe early-onset epilepsy reveals new genes and improves molecular diagnosis. Hum Mol Genet. 2014 Jun 15;23(12):3200-11.

182. Dyment DA, Tetreault M, Beaulieu CL, et al. Whole-exome sequencing broadens the phenotypic spectrum of rare pediatric epilepsy: a retrospective study. Clin Genet. 2015 Jul;88(1):34-40.

183. Martin MS, Tang B, Ta N, Escayg A. Characterization of 5' untranslated regions of the voltage-gated sodium channels SCN1A, SCN2A, and SCN3A and identification of cis-conserved noncoding sequences. Genomics. 2007 Aug;90(2):225-35.

184. Urak L, Feucht M, Fathi N, Hornik K, Fuchs K. A GABRB3 promoter haplotype associated with childhood absence epilepsy impairs transcriptional activity. Hum Mol Genet. 2006 Aug 15;15(16):2533-41.

185. Li T, Kuang Y, Li B. The genetic variants in 3' untranslated region of voltage-gated sodium channel alpha 1 subunit gene affect the mRNA-microRNA interactions and associate with epilepsy. BMC Genet. 2016 Jul 29;17(1):111.

186. Guerrini R. Epilepsy in children. Lancet. 2006 Feb 11;367(9509):499-524.

187. Russ SA, Larson K, Halfon N. A national profile of childhood epilepsy and seizure disorder. Pediatrics. 2012 Feb;129(2):256-64.

188. Hauser WA, Annegers JF, Kurland LT. Incidence of epilepsy and unprovoked seizures in Rochester, Minnesota: 1935-1984. Epilepsia. 1993 May-Jun;34(3):453-68.

189. Chevrie JJ, Aicardi J. Convulsive disorders in the first year of life: persistence of epileptic seizures. Epilepsia. 1979 Dec;20(6):643-9.

190. Chevrie JJ, Aicardi J. Convulsive disorders in the first year of life: etiologic factors. Epilepsia. 1977 Dec;18(4):489-98.

191. Chevrie JJ, Aicardi J. Convulsive disorders in the first year of life: neurological and mental outcome and mortality. Epilepsia. 1978 Feb;19(1):67-74.

192. Oldham MS, Horn PS, Tsevat J, Standridge S. Costs and Clinical Outcomes of Epilepsy Surgery in Children With Drug-Resistant Epilepsy. Pediatr Neurol. 2015 Sep;53(3):216-20.

193. Stroink H, Brouwer OF, Arts WF, Geerts AT, Peters AC, van Donselaar CA. The first unprovoked, untreated seizure in childhood: a hospital based study of the accuracy of the diagnosis, rate of recurrence, and long term outcome after recurrence. Dutch study of epilepsy in childhood. J Neurol Neurosurg Psychiatry. 1998 May;64(5):595-600.

194. Camfield PR, Camfield CS, Dooley JM, Tibbles JA, Fung T, Garner B. Epilepsy after a first unprovoked seizure in childhood. Neurology. 1985 Nov;35(11):1657-60.

195. Annegers JF, Shirts SB, Hauser WA, Kurland LT. Risk of recurrence after an initial unprovoked seizure. Epilepsia. 1986 Jan-Feb;27(1):43-50.

196. Elwes RD, Chesterman P, Reynolds EH. Prognosis after a first untreated tonic-clonic seizure. Lancet. 1985 Oct 5;2(8458):752-3.

197. Pavlou E, Gkampeta A. Learning disorders in children with epilepsy. Childs Nerv Syst. 2011 Mar;27(3):373-9.

198. Beghi M, Cornaggia CM, Frigeni B, Beghi E. Learning disorders in epilepsy. Epilepsia. 2006;47 Suppl 2:14-8.

199. Sillanpaa M. Learning disability: occurrence and long-term consequences in childhood-onset epilepsy. Epilepsy Behav. 2004 Dec;5(6):937-44.

200. Besag F, Gobbi G, Caplan R, Sillanpaa M, Aldenkamp A, Dunn DW. Psychiatric and Behavioural Disorders in Children with Epilepsy (ILAE Task Force Report): Epilepsy and ADHD. Epileptic Disord. 2016 May 19.

201. Kaufmann R, Goldberg-Stern H, Shuper A. Attention-deficit disorders and epilepsy in childhood: incidence, causative relations and treatment possibilities. J Child Neurol. 2009 Jun;24(6):727-33.

202. Stevanovic D, Jancic J, Lakic A. The impact of depression and anxiety disorder symptoms on the health-related quality of life of children and adolescents with epilepsy. Epilepsia. 2011 Aug;52(8):e75-8.

203. Caplan R, Siddarth P, Gurbani S, Hanson R, Sankar R, Shields WD. Depression and anxiety disorders in pediatric epilepsy. Epilepsia. 2005 May;46(5):720-30.

204. Kanemura H, Sano F, Ohyama T, Sugita K, Aihara M. Correlation between perceived stigma and EEG paroxysmal abnormality in childhood epilepsy. Epilepsy Behav. 2015 Nov;52(Pt A):44-8.

205. Institute of Medicine Committee on the Public Health Dimensions of the E. 2012.

206. Sundelin HE, Larsson H, Lichtenstein P, et al. Autism and epilepsy: A population-based nationwide cohort study. Neurology. 2016 Jul 12;87(2):192-7.

207. El Achkar CM, Spence SJ. Clinical characteristics of children and young adults with co-occurring autism spectrum disorder and epilepsy. Epilepsy Behav. 2015 Jun;47:183-90.

208. Piccinelli P, Borgatti R, Nicoli F, et al. Relationship between migraine and epilepsy in pediatric age. Headache. 2006 Mar;46(3):413-21.

209. Berg AT, Berkovic SF, Brodie MJ, et al. Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia. 2010 Apr;51(4):676-85.

210. Engel J, Jr., International League Against E. A proposed diagnostic scheme for people with epileptic seizures and with epilepsy: report of the ILAE Task Force on Classification and Terminology. Epilepsia. 2001 Jun;42(6):796-803.

211. Engel J, Jr. Report of the ILAE classification core group. Epilepsia. 2006 Sep;47(9):1558-68.

212. Berg AT, Loddenkemper T, Baca CB. Diagnostic delays in children with early onset epilepsy: impact, reasons, and opportunities to improve care. Epilepsia. 2014 Jan;55(1):123-32.

213. Reilly C, Atkinson P, Das KB, et al. Neurobehavioral comorbidities in children with active epilepsy: a population-based study. Pediatrics. 2014 Jun;133(6):e1586-93.

214. Dunn P, Albury CL, Maksemous N, et al. Next Generation Sequencing Methods for Diagnosis of Epilepsy Syndromes. Front Genet. 2018;9:20.

215. Patel J, Mercimek-Mahmutoglu S. Epileptic Encephalopathy in Childhood: A Stepwise Approach for Identification of Underlying Genetic Causes. Indian J Pediatr. 2016 Oct;83(10):1164-74.

216. Ohtahara S, Ohtsuka Y, Yamatogi Y, Oka E. The early-infantile epileptic encephalopathy with suppression-burst: developmental aspects. Brain Dev. 1987;9(4):371-6.

217. Ohtsuka Y, Ogino T, Murakami N, Mimaki N, Kobayashi K, Ohtahara S. Developmental aspects of epilepsy with special reference to age-dependent epileptic encephalopathy. Jpn J Psychiatry Neurol. 1986 Sep;40(3):307-13.

218. Ohtahara S, Yamatogi Y. Epileptic encephalopathies in early infancy with suppression-burst. J Clin Neurophysiol. 2003 Nov-Dec;20(6):398-407.

219. Yamatogi Y, Ohtahara S. Early-infantile epileptic encephalopathy with suppression-bursts, Ohtahara syndrome; its overview referring to our 16 cases. Brain Dev. 2002 Jan;24(1):13-23.

220. Ohtsuka Y, Sato M, Sanada S, Yoshinaga H, Oka E. Suppression-burst patterns in intractable epilepsy with focal cortical dysplasia. Brain Dev. 2000 Mar;22(2):135-8.

221. Fusco L, Pachatz C, Di Capua M, Vigevano F. Video/EEG aspects of early-infantile epileptic encephalopathy with suppression-bursts (Ohtahara syndrome). Brain Dev. 2001 Nov;23(7):708-14.

222. Harding BN, Boyd SG. Intractable seizures from infancy can be associated with dentato-olivary dysplasia. J Neurol Sci. 1991 Aug;104(2):157-65.

223. Robain O, Dulac O. Early epileptic encephalopathy with suppression bursts and olivary-dentate dysplasia. Neuropediatrics. 1992 Jun;23(3):162-4.

224. Miller SP, Dilenge ME, Meagher-Villemure K, O'Gorman AM, Shevell MI. Infantile epileptic encephalopathy (Ohtahara syndrome) and migrational disorder. Pediatr Neurol. 1998 Jul;19(1):50-4.

225. Trinka E, Rauscher C, Nagler M, et al. A case of Ohtahara syndrome with olivary-dentate dysplasia and agenesis of mamillary bodies. Epilepsia. 2001 Jul;42(7):950-3.

226. Pavone P, Spalice A, Polizzi A, Parisi P, Ruggieri M. Ohtahara syndrome with emphasis on recent genetic discovery. Brain Dev. 2012 Jun;34(6):459-68.

227. Aicardi J, Goutieres F. [Neonatal myoclonic encephalopathy (author's transl)]. Rev Electroencephalogr Neurophysiol Clin. 1978 Jan-Mar;8(1):99-101.

228. Dalla Bernardina B, Dulac O, Fejerman N, et al. Early myoclonic epileptic encephalopathy (E.M.E.E.). Eur J Pediatr. 1983 Jun-Jul;140(3):248-52.

229. Dalla Bernardina B, Colamaria V, Capovilla G, Bondavalli S. Nosological classification of epilepsies in the first three years of life. Prog Clin Biol Res. 1983;124:165-83.

230. Djukic A, Lado FA, Shinnar S, Moshe SL. Are early myoclonic encephalopathy (EME) and the Ohtahara syndrome (EIEE) independent of each other? Epilepsy research. 2006 Aug;70 Suppl 1:S68-76.

231. Wang PJ, Lee WT, Hwu WL, Young C, Yau KI, Shen YZ. The controversy regarding diagnostic criteria for early myoclonic encephalopathy. Brain Dev. 1998 Oct;20(7):530-5.

232. Djukic A, Lado FA, Shinnar S, Moshe SL. Are early myoclonic encephalopathy (EME) and the Ohtahara syndrome (EIEE) independent of each other? Epilepsy Res. 2006 Aug;70 Suppl 1:S68-76.

233. Olson HE, Kelly M, LaCoursiere CM, et al. Genetics and genotype-phenotype correlations in early onset epileptic encephalopathy with burst suppression. Ann Neurol. 2017 Mar;81(3):419-29.

234. Ohtahara S, Yamatogi Y. Ohtahara syndrome: with special reference to its developmental aspects for differentiating from early myoclonic encephalopathy. Epilepsy Res. 2006 Aug;70 Suppl 1:S58-67.

235. Olson HE, Poduri A, Pearl PL. Genetic forms of epilepsies and other paroxysmal disorders. Semin Neurol. 2014 Jul;34(3):266-79.

236. Dravet C. Dravet syndrome history. Dev Med Child Neurol. 2011 Apr;53 Suppl 2:1-6.

237. Gaily E, Anttonen AK, Valanne L, et al. Dravet syndrome: new potential genetic modifiers, imaging abnormalities, and ictal findings. Epilepsia. 2013 Sep;54(9):1577-85.

238. Striano P, Mancardi MM, Biancheri R, et al. Brain MRI findings in severe myoclonic epilepsy in infancy and genotype-phenotype correlations. Epilepsia. 2007 Jun;48(6):1092-6.

239. Guerrini R, Striano P, Catarino C, Sisodiya SM. Neuroimaging and neuropathology of Dravet syndrome. Epilepsia. 2011 Apr;52 Suppl 2:30-4.

240. Catarino CB, Liu JY, Liagkouras I, et al. Dravet syndrome as epileptic encephalopathy: evidence from long-term course and neuropathology. Brain. 2011 Oct;134(Pt 10):2982-3010.

241. Akiyama M, Kobayashi K, Yoshinaga H, Ohtsuka Y. A long-term follow-up study of Dravet syndrome up to adulthood. Epilepsia. 2010 Jun;51(6):1043-52.

242. Siegler Z, Barsi P, Neuwirth M, et al. Hippocampal sclerosis in severe myoclonic epilepsy in infancy: a retrospective MRI study. Epilepsia. 2005 May;46(5):704-8.

243. Van Poppel K, Patay Z, Roberts D, et al. Mesial temporal sclerosis in a cohort of children with SCN1A gene mutation. J Child Neurol. 2012 Jul;27(7):893-7.

244. Sakauchi M, Oguni H, Kato I, et al. Mortality in Dravet syndrome: search for risk factors in Japanese patients. Epilepsia. 2011 Apr;52 Suppl 2:50-4.

245. Skluzacek JV, Watts KP, Parsy O, Wical B, Camfield P. Dravet syndrome and parent associations: the IDEA League experience with comorbid conditions, mortality, management, adaptation, and grief. Epilepsia. 2011 Apr;52 Suppl 2:95-101.

246. Wu YW, Sullivan J, McDaniel SS, et al. Incidence of Dravet Syndrome in a US Population. Pediatrics. 2015 Nov;136(5):e1310-5.

247. Hurst DL. Epidemiology of severe myoclonic epilepsy of infancy. Epilepsia. 1990 Jul-Aug;31(4):397-400.

248. Rosander C, Hallbook T. Dravet syndrome in Sweden: a population-based study. Dev Med Child Neurol. 2015 Mar 13.

249. Bayat A, Hjalgrim H, Moller RS. The incidence of SCN1A-related Dravet syndrome in Denmark is 1:22,000: a population-based study from 2004 to 2009. Epilepsia. 2015 Apr;56(4):e36-9.

250. Steel D, Symonds JD, Zuberi SM, Brunklaus A. Dravet syndrome and its mimics: Beyond SCN1A. Epilepsia. 2017 Nov;58(11):1807-16.

251. Parihar R, Ganesh S. The SCN1A gene variants and epileptic encephalopathies. J Hum Genet. 2013 Sep;58(9):573-80.

252. Catterall WA, Kalume F, Oakley JC. NaV1.1 channels and epilepsy. J Physiol. 2010 Jun 1;588(Pt 11):1849-59.

253. Ragsdale DS. How do mutant Nav1.1 sodium channels cause epilepsy? Brain Res Rev. 2008 Jun;58(1):149-59.

254. Escayg A, Goldin AL. Sodium channel SCN1A and epilepsy: mutations and mechanisms. Epilepsia. 2010 Sep;51(9):1650-8.

255. Miller AR, Hawkins NA, McCollom CE, Kearney JA. Mapping genetic modifiers of survival in a mouse model of Dravet syndrome. Genes Brain Behav. 2014 Feb;13(2):163-72.

256. Meng H, Xu HQ, Yu L, et al. The SCN1A mutation database: updating information and analysis of the relationships among genotype, functional alteration, and phenotype. Hum Mutat. 2015 Jun;36(6):573-80.

257. Yu FH, Mantegazza M, Westenbroek RE, et al. Reduced sodium current in GABAergic interneurons in a mouse model of severe myoclonic epilepsy in infancy. Nat Neurosci. 2006 Sep;9(9):1142-9.

258. Tai C, Abe Y, Westenbroek RE, Scheuer T, Catterall WA. Impaired excitability of somatostatin- and parvalbumin-expressing cortical interneurons in a mouse model of Dravet syndrome. Proc Natl Acad Sci U S A. 2014 Jul 29;111(30):E3139-48.

259. Kalume F, Yu FH, Westenbroek RE, Scheuer T, Catterall WA. Reduced sodium current in Purkinje neurons from Nav1.1 mutant mice: implications for ataxia in severe myoclonic epilepsy in infancy. J Neurosci. 2007 Oct 10;27(41):11065-74.

260. Ogiwara I, Iwasato T, Miyamoto H, et al. Nav1.1 haploinsufficiency in excitatory neurons ameliorates seizure-associated sudden death in a mouse model of Dravet syndrome. Hum Mol Genet. 2013 Dec 1;22(23):4784-804.

261. Cheah CS, Yu FH, Westenbroek RE, et al. Specific deletion of NaV1.1 sodium channels in inhibitory interneurons causes seizures and premature death in a mouse model of Dravet syndrome. Proc Natl Acad Sci U S A. 2012 Sep 4;109(36):14646-51.

262. Dutton SB, Makinson CD, Papale LA, et al. Preferential inactivation of Scn1a in parvalbumin interneurons increases seizure susceptibility. Neurobiol Dis. 2013 Jan;49:211-20.

263. Ogiwara I, Miyamoto H, Morita N, et al. Nav1.1 localizes to axons of parvalbumin-positive inhibitory interneurons: a circuit basis for epileptic seizures in mice carrying an Scn1a gene mutation. J Neurosci. 2007 May 30;27(22):5903-14.

264. Hedrich UB, Liautard C, Kirschenbaum D, et al. Impaired action potential initiation in GABAergic interneurons causes hyperexcitable networks in an epileptic mouse model carrying a human Na(V)1.1 mutation. J Neurosci. 2014 Nov 5;34(45):14874-89.

265. Martin MS, Dutt K, Papale LA, et al. Altered function of the SCN1A voltage-gated sodium channel leads to gamma-aminobutyric acid-ergic (GABAergic) interneuron abnormalities. J Biol Chem. 2010 Mar 26;285(13):9823-34.

266. Patry G, Lyagoubi S, Tassinari CA. Subclinical "electrical status epilepticus" induced by sleep in children. A clinical and electroencephalographic study of six cases. Arch Neurol. 1971 Mar;24(3):242-52.

267. Tassinari CA, Daniele O, Gambarelli F, Bureau-Paillas M, Robaglia L, Cicirata F. [Excessive 7-14-sec positive spikes during REM sleep in monozygotic non-epileptic twins with speech retardation (author's transl)]. Rev Electroencephalogr Neurophysiol Clin. 1977 Apr-Jun;7(2):192-3.

268. Proposal for revised classification of epilepsies and epileptic syndromes. Commission on Classification and Terminology of the International League Against Epilepsy. Epilepsia. 1989 Jul-Aug;30(4):389-99.

269. McVicar KA, Shinnar S. Landau-Kleffner syndrome, electrical status epilepticus in slow wave sleep, and language regression in children. Ment Retard Dev Disabil Res Rev. 2004;10(2):144-9.

270. Galanopoulou AS, Bojko A, Lado F, Moshe SL. The spectrum of neuropsychiatric abnormalities associated with electrical status epilepticus in sleep. Brain Dev. 2000 Aug;22(5):279-95.

271. Nieuwenhuis L, Nicolai J. The pathophysiological mechanisms of cognitive and behavioral disturbances in children with Landau-Kleffner syndrome or epilepsy with continuous spike-and-waves during slow-wave sleep. Seizure. 2006 Jun;15(4):249-58.

272. Scholtes FB, Hendriks MP, Renier WO. Cognitive deterioration and electrical status epilepticus during slow sleep. Epilepsy Behav. 2005 Mar;6(2):167-73.

273. Smith MC, Hoeppner TJ. Epileptic encephalopathy of late childhood: Landau-Kleffner syndrome and the syndrome of continuous spikes and waves during slow-wave sleep. J Clin Neurophysiol. 2003 Nov-Dec;20(6):462-72.

274. Buzatu M, Bulteau C, Altuzarra C, Dulac O, Van Bogaert P. Corticosteroids as treatment of epileptic syndromes with continuous spike-waves during slow-wave sleep. Epilepsia. 2009 Aug;50 Suppl 7:68-72.

275. Sanchez Fernandez I, Loddenkemper T, Peters JM, Kothare SV. Electrical status epilepticus in sleep: clinical presentation and pathophysiology. Pediatr Neurol. 2012 Dec;47(6):390-410.

276. Van Hirtum-Das M, Licht EA, Koh S, Wu JY, Shields WD, Sankar R. Children with ESES: variability in the syndrome. Epilepsy Res. 2006 Aug;70 Suppl 1:S248-58.

277. Scheltens-de Boer M. Guidelines for EEG in encephalopathy related to ESES/CSWS in children. Epilepsia. 2009 Aug;50 Suppl 7:13-7.

278. Caraballo RH, Veggiotti P, Kaltenmeier MC, et al. Encephalopathy with status epilepticus during sleep or continuous spikes and waves during slow sleep syndrome: a multicenter, long-term follow-up study of 117 patients. Epilepsy Res. 2013 Jul;105(1-2):164-73.

279. Tassinari CA, Cantalupo G, Rios-Pohl L, Giustina ED, Rubboli G. Encephalopathy with status epilepticus during slow sleep: "the Penelope syndrome". Epilepsia. 2009 Aug;50 Suppl 7:4-8.

280. Benington JH, Heller HC. Restoration of brain energy metabolism as the function of sleep. Prog Neurobiol. 1995 Mar;45(4):347-60.

281. Horne J. Human slow wave sleep: a review and appraisal of recent findings, with implications for sleep functions, and psychiatric illness. Experientia. 1992 Oct 15;48(10):941-54.

282. Sanchez-Vives MV, Mattia M. Slow wave activity as the default mode of the cerebral cortex. Arch Ital Biol. 2014 Jun-Sep;152(2-3):147-55.

283. Jerath R, Harden K, Crawford M, Barnes VA, Jensen M. Role of cardiorespiratory synchronization and sleep physiology: effects on membrane potential in the restorative functions of sleep. Sleep Med. 2014 Mar;15(3):279-88.

284. Halasz P, Bodizs R, Parrino L, Terzano M. Two features of sleep slow waves: homeostatic and reactive aspects--from long term to instant sleep homeostasis. Sleep Med. 2014 Oct;15(10):1184-95.

285. Seegmuller C, Deonna T, Dubois CM, et al. Long-term outcome after cognitive and behavioral regression in nonlesional epilepsy with continuous spike-waves during slow-wave sleep. Epilepsia. 2012 Jun;53(6):1067-76.

286. Lesca G, Rudolf G, Labalme A, et al. Epileptic encephalopathies of the Landau-Kleffner and continuous spike and waves during slow-wave sleep types: genomic dissection makes the link with autism. Epilepsia. 2012 Sep;53(9):1526-38.

287. Tassinari CA, Michelucci R, Forti A, et al. The electrical status epilepticus syndrome. Epilepsy Res Suppl. 1992;6:111-5.

288. Landau WM, Kleffner FR. Syndrome of acquired aphasia with convulsive disorder in children. Neurology. 1957 Aug;7(8):523-30.

289. Rossi PG, Parmeggiani A, Posar A, Scaduto MC, Chiodo S, Vatti G. Landau-Kleffner syndrome (LKS): long-term follow-up and links with electrical status epilepticus during sleep (ESES). Brain Dev. 1999 Mar;21(2):90-8.

290. Cianci V, Ferlazzo E, De Martino G, et al. Continuous spikes and waves during slow sleep in a child with karyotype 47, XYY. Epileptic Disord. 2014 Jun;16(2):223-6.

291. Pascual-Castroviejo I, Pascual-Pascual SI, Pena W, Talavera M. Status epilepticus-induced brain damage and opercular syndrome in childhood. Dev Med Child Neurol. 1999 Jun;41(6):420-3.

292. Prats JM, Garaizar C, Uterga JM, Urroz MJ. Operculum syndrome in childhood: a rare cause of persistent speech disturbance. Dev Med Child Neurol. 1992 Apr;34(4):359-64.

293. Shafrir Y, Prensky AL. Acquired epileptiform opercular syndrome: a second case report, review of the literature, and comparison to the Landau-Kleffner syndrome. Epilepsia. 1995 Oct;36(10):1050-7.

294. Desai SD, Patel D, Bharani S, Kharod N. Opercular syndrome: A case report and review. J Pediatr Neurosci. 2013 May;8(2):123-5.

295. Tassinari CA, Rubboli G, Volpi L, et al. Encephalopathy with electrical status epilepticus during slow sleep or ESES syndrome including the acquired aphasia. Clin Neurophysiol. 2000 Sep;111 Suppl 2:S94-S102.

296. Nickels K, Wirrell E. Electrical status epilepticus in sleep. Semin Pediatr Neurol. 2008 Jun;15(2):50-60.

297. Yan Liu X, Wong V. Spectrum of epileptic syndromes with electrical status epilepticus during sleep in children. Pediatr Neurol. 2000 May;22(5):371-9.

298. Jayakar PB, Seshia SS. Electrical status epilepticus during slow-wave sleep: a review. Journal of clinical neurophysiology : official publication of the American Electroencephalographic Society. 1991 Jul;8(3):299-311.

299. Arzimanoglou A, French J, Blume WT, et al. Lennox-Gastaut syndrome: a consensus approach on diagnosis, assessment, management, and trial methodology. Lancet Neurol. 2009 Jan;8(1):82-93.

300. Deonna T, Roulet-Perez E. Early-onset acquired epileptic aphasia (Landau-Kleffner syndrome, LKS) and regressive autistic disorders with epileptic EEG abnormalities: the continuing debate. Brain Dev. 2010 Oct;32(9):746-52.

301. Huguenard JR, Prince DA. Intrathalamic rhythmicity studied in vitro: nominal T-current modulation causes robust antioscillatory effects. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1994 Sep;14(9):5485-502.

302. Sanchez-Vives MV, McCormick DA. Functional properties of perigeniculate inhibition of dorsal lateral geniculate nucleus thalamocortical neurons in vitro. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1997 Nov 15;17(22):8880-93.

303. Kim U, Sanchez-Vives MV, McCormick DA. Functional dynamics of GABAergic inhibition in the thalamus. Science. 1997 Oct 3;278(5335):130-4.

304. von Krosigk M, Bal T, McCormick DA. Cellular mechanisms of a synchronized oscillation in the thalamus. Science. 1993 Jul 16;261(5119):361-4.

305. Blumenfeld H, McCormick DA. Corticothalamic inputs control the pattern of activity generated in thalamocortical networks. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2000 Jul 1;20(13):5153-62.

306. Beenhakker MP, Huguenard JR. Neurons that fire together also conspire together: is normal sleep circuitry hijacked to generate epilepsy? Neuron. 2009 Jun 11;62(5):612-32.

307. Kuriyama K, Sisken B, Ito J, Simonsen DG, Haber B, Roberts E. The gamma-aminobutyric acid system in the developing chick embryo cerebellum. Brain research. 1968 Nov;11(2):412-30.

308. Roberts PJ. Gamma-aminobutyric acid homoexchange in sensory ganglia. Brain research. 1976 Aug 20;113(1):206-9.

309. Freund TF. GABAergic septohippocampal neurons contain parvalbumin. Brain research. 1989 Jan 30;478(2):375-81.

310. Kosaka T, Kosaka K, Nakayama T, Hunziker W, Heizmann CW. Axons and axon terminals of cerebellar Purkinje cells and basket cells have higher levels of parvalbumin immunoreactivity than somata and dendrites: quantitative analysis by immunogold labeling. Exp Brain Res. 1993;93(3):483-91.

311. Tritsch NX, Granger AJ, Sabatini BL. Mechanisms and functions of GABA co-release. Nat Rev Neurosci. 2016 Mar;17(3):139-45.

312. Bosma PT, Blazquez M, Collins MA, et al. Multiplicity of glutamic acid decarboxylases (GAD) in vertebrates: molecular phylogeny and evidence for a new GAD paralog. Mol Biol Evol. 1999 Mar;16(3):397-404.

313. Lernmark A. Glutamic acid decarboxylase--gene to antigen to disease. J Intern Med. 1996 Nov;240(5):259-77.

314. Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, Tobin AJ. Two genes encode distinct glutamate decarboxylases. Neuron. 1991 Jul;7(1):91-100.

315. Chaudhry FA, Reimer RJ, Bellocchio EE, et al. The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1998 Dec 1;18(23):9733-50.

316. Burger PM, Hell J, Mehl E, Krasel C, Lottspeich F, Jahn R. GABA and glycine in synaptic vesicles: storage and transport characteristics. Neuron. 1991 Aug;7(2):287-93.

317. Roth FC, Draguhn A. GABA metabolism and transport: effects on synaptic efficacy. Neural Plast. 2012;2012:805830.

318. Walls AB, Waagepetersen HS, Bak LK, Schousboe A, Sonnewald U. The glutamine-glutamate/GABA cycle: function, regional differences in glutamate and GABA production and effects of interference with GABA metabolism. Neurochem Res. 2015 Feb;40(2):402-9.

319. Kapur J, Macdonald RL. Rapid seizure-induced reduction of benzodiazepine and Zn2+ sensitivity of hippocampal dentate granule cell GABAA receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1997 Oct 1;17(19):7532-40.

320. Macdonald RL, Kang JQ, Gallagher MJ. Mutations in GABAA receptor subunits associated with genetic epilepsies. The Journal of physiology. 2010 Jun 1;588(Pt 11):1861-9.

321. Wagner PG, Dekin MS. GABAb receptors are coupled to a barium-insensitive outward rectifying potassium conductance in premotor respiratory neurons. Journal of neurophysiology. 1993 Jan;69(1):286-9.

322. Mintz IM, Bean BP. GABAB receptor inhibition of P-type Ca2+ channels in central neurons. Neuron. 1993 May;10(5):889-98.

323. Tretter V, Ehya N, Fuchs K, Sieghart W. Stoichiometry and assembly of a recombinant GABAA receptor subtype. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1997 Apr 15;17(8):2728-37.

324. Chang Y, Wang R, Barot S, Weiss DS. Stoichiometry of a recombinant GABAA receptor. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1996 Sep 1;16(17):5415-24.

325. Farrar SJ, Whiting PJ, Bonnert TP, McKernan RM. Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. J Biol Chem. 1999 Apr 9;274(15):10100-4.

326. Baumann SW, Baur R, Sigel E. Forced subunit assembly in alpha1beta2gamma2 GABAA receptors. Insight into the absolute arrangement. J Biol Chem. 2002 Nov 29;277(48):46020-5.

327. Sigel E, Baur R, Boulineau N, Minier F. Impact of subunit positioning on GABAA receptor function. Biochem Soc Trans. 2006 Nov;34(Pt 5):868-71.

328. Baur R, Minier F, Sigel E. A GABA(A) receptor of defined subunit composition and positioning: concatenation of five subunits. FEBS letters. 2006 Mar 6;580(6):1616-20.

329. Schofield PR, Darlison MG, Fujita N, et al. Sequence and functional expression of the GABA A receptor shows a ligand-gated receptor super-family. Nature. 1987 Jul 16-22;328(6127):221-7.

330. Dredge BK, Darnell RB. Nova regulates GABA(A) receptor gamma2 alternative splicing via a distal downstream UCAU-rich intronic splicing enhancer. Mol Cell Biol. 2003 Jul;23(13):4687-700.

331. Zhao C, Xu Z, Wang F, et al. Alternative-splicing in the exon-10 region of GABA(A) receptor beta(2) subunit gene: relationships between novel isoforms and psychotic disorders. PLoS One. 2009 Sep 18;4(9):e6977.

332. Ohlson J, Pedersen JS, Haussler D, Ohman M. Editing modifies the GABA(A) receptor subunit alpha3. Rna. 2007 May;13(5):698-703.

333. Whiting P, McKernan RM, Iversen LL. Another mechanism for creating diversity in gamma-aminobutyrate type A receptors: RNA splicing directs expression of two forms of gamma 2 phosphorylation site. Proceedings of the National Academy of Sciences of the United States of America. 1990 Dec;87(24):9966-70.

334. Kirkness EF, Fraser CM. A strong promoter element is located between alternative exons of a gene encoding the human gamma-aminobutyric acid-type A receptor beta 3 subunit (GABRB3). J Biol Chem. 1993 Feb 25;268(6):4420-8.

335. Sieghart W, Sperk G. Subunit composition, distribution and function of GABA(A) receptor subtypes. Curr Top Med Chem. 2002 Aug;2(8):795-816.

336. McKernan RM, Whiting PJ. Which GABAA-receptor subtypes really occur in the brain? Trends Neurosci. 1996 Apr;19(4):139-43.

337. Olsen RW, Sieghart W. GABA A receptors: subtypes provide diversity of function and pharmacology. Neuropharmacology. 2009 Jan;56(1):141-8.

338. Sigel E, Steinmann ME. Structure, function, and modulation of GABA(A) receptors. J Biol Chem. 2012 Nov 23;287(48):40224-31.

339. Bosman LW, Heinen K, Spijker S, Brussaard AB. Mice lacking the major adult GABAA receptor subtype have normal number of synapses, but retain juvenile IPSC kinetics until adulthood. J Neurophysiol. 2005 Jul;94(1):338-46.

340. Goldstein PA, Elsen FP, Ying SW, Ferguson C, Homanics GE, Harrison NL. Prolongation of hippocampal miniature inhibitory postsynaptic currents in mice lacking the GABA(A) receptor alpha1 subunit. Journal of neurophysiology. 2002 Dec;88(6):3208-17.

341. Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL, Homanics GE. GABA(A) receptor alpha1 subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2001 May 1;21(9):3009-16.

342. Pape JR, Skynner MJ, Sim JA, Herbison AE. Profiling gamma-aminobutyric acid (GABA(A)) receptor subunit mRNA expression in postnatal gonadotropin-releasing hormone (GnRH) neurons of the male mouse with single cell RT-PCR. Neuroendocrinology. 2001 Nov;74(5):300-8.

343. Brooks-Kayal AR, Shumate MD, Jin H, Rikhter TY, Kelly ME, Coulter DA. gamma-Aminobutyric acid(A) receptor subunit expression predicts functional changes in hippocampal dentate granule cells during postnatal development. Journal of neurochemistry. 2001 Jun;77(5):1266-78.

344. Daniel C, Ohman M. RNA editing and its impact on GABAA receptor function. Biochem Soc Trans. 2009 Dec;37(Pt 6):1399-403.

345. Mortensen M, Patel B, Smart TG. GABA Potency at GABA(A) Receptors Found in Synaptic and Extrasynaptic Zones. Front Cell Neurosci. 2011 Jan;6:1.

346. Nusser Z, Sieghart W, Somogyi P. Segregation of different GABAA receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1998 Mar 1;18(5):1693-703.

347. Jechlinger M, Pelz R, Tretter V, Klausberger T, Sieghart W. Subunit composition and quantitative importance of hetero-oligomeric receptors: GABAA receptors containing alpha6 subunits. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1998 Apr 1;18(7):2449-57.

348. Neelands TR, Macdonald RL. Incorporation of the pi subunit into functional gamma-aminobutyric Acid(A) receptors. Mol Pharmacol. 1999 Sep;56(3):598-610.

349. Bonnert TP, McKernan RM, Farrar S, et al. theta, a novel gamma-aminobutyric acid type A receptor subunit. Proceedings of the National Academy of Sciences of the United States of America. 1999 Aug 17;96(17):9891-6.

350. Neelands TR, Fisher JL, Bianchi M, Macdonald RL. Spontaneous and gamma-aminobutyric acid (GABA)-activated GABA(A) receptor channels formed by epsilon subunit-containing isoforms. Mol Pharmacol. 1999 Jan;55(1):168-78.

351. Davies PA, Kirkness EF, Hales TG. Evidence for the formation of functionally distinct alphabetagammaepsilon GABA(A) receptors. The Journal of physiology. 2001 Nov 15;537(Pt 1):101-13.

352. Wagner DA, Goldschen-Ohm MP, Hales TG, Jones MV. Kinetics and spontaneous open probability conferred by the epsilon subunit of the GABAA receptor. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2005 Nov 9;25(45):10462-8.

353. Jones BL, Henderson LP. Trafficking and potential assembly patterns of epsilon-containing GABAA receptors. Journal of neurochemistry. 2007 Nov;103(3):1258-71.

354. Unwin N. Nicotinic acetylcholine receptor at 9 A resolution. J Mol Biol. 1993 Feb 20;229(4):1101-24.

355. Connolly CN, Wafford KA. The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. Biochem Soc Trans. 2004 Jun;32(Pt3):529-34.

356. Vithlani M, Terunuma M, Moss SJ. The dynamic modulation of GABA(A) receptor trafficking and its role in regulating the plasticity of inhibitory synapses. Physiol Rev. 2011 Jul;91(3):1009-22.

357. Miller PS, Aricescu AR. Crystal structure of a human GABA receptor. Nature. 2014 Jun 8.

358. Brejc K, van Dijk WJ, Klaassen RV, et al. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature. 2001 May 17;411(6835):269-76.

359. Unwin N. Refined structure of the nicotinic acetylcholine receptor at 4A resolution. J Mol Biol. 2005 Mar 4;346(4):967-89.

360. Dellisanti CD, Yao Y, Stroud JC, Wang ZZ, Chen L. Crystal structure of the extracellular domain of nAChR alpha1 bound to alpha-bungarotoxin at 1.94 A resolution. Nat Neurosci. 2007 Aug;10(8):953-62.

361. Hilf RJ, Dutzler R. Structure of a potentially open state of a proton-activated pentameric ligand-gated ion channel. Nature. 2009 Jan 1;457(7225):115-8.

362. Bocquet N, Nury H, Baaden M, et al. X-ray structure of a pentameric ligand-gated ion channel in an apparently open conformation. Nature. 2009 Jan 1;457(7225):111-4.

363. Hibbs RE, Gouaux E. Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature. 2011 Jun 2;474(7349):54-60.

364. Ernst M, Brauchart D, Boresch S, Sieghart W. Comparative modeling of GABA(A) receptors: limits, insights, future developments. Neuroscience. 2003;119(4):933-43.

365. Enz R, Cutting GR. Identification of 70 amino acids important for GABA(C) receptor rho1 subunit assembly. Brain research. 1999 Nov 6;846(2):177-85.

366. Hackam AS, Wang TL, Guggino WB, Cutting GR. The N-terminal domain of human GABA receptor rho1 subunits contains signals for homooligomeric and heterooligomeric interaction. J Biol Chem. 1997 May 23;272(21):13750-7.

367. Hackam AS, Wang TL, Guggino WB, Cutting GR. Sequences in the amino termini of GABA rho and GABA(A) subunits specify their selective interaction in vitro. Journal of neurochemistry. 1998 Jan;70(1):40-6.

368. Klausberger T, Fuchs K, Mayer B, Ehya N, Sieghart W. GABA(A) receptor assembly. Identification and structure of gamma(2) sequences forming the intersubunit contacts with alpha(1) and beta(3) subunits. J Biol Chem. 2000 Mar 24;275(12):8921-8.

369. Connolly CN, Krishek BJ, McDonald BJ, Smart TG, Moss SJ. Assembly and cell surface expression of heteromeric and homomeric gamma-aminobutyric acid type A receptors. J Biol Chem. 1996 Jan 5;271(1):89-96.

370. Buller AL, Hastings GA, Kirkness EF, Fraser CM. Site-directed mutagenesis of N-linked glycosylation sites on the gamma-aminobutyric acid type A receptor alpha 1 subunit. Mol Pharmacol. 1994 Nov;46(5):858-65.

371. Lo WY, Lagrange AH, Hernandez CC, et al. Glycosylation of {beta}2 subunits regulates GABAA receptor biogenesis and channel gating. J Biol Chem. 2010 Oct 8;285(41):31348-61.

372. Hosie AM, Wilkins ME, da Silva HM, Smart TG. Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. Nature. 2006 Nov 23;444(7118):486-9.

373. Bianchi MT, Haas KF, Macdonald RL. Structural determinants of fast desensitization and desensitization-deactivation coupling in GABAa receptors. J Neurosci. 2001 Feb 15;21(4):1127-36.

374. Bianchi MT, Song L, Zhang H, Macdonald RL. Two different mechanisms of disinhibition produced by GABAA receptor mutations linked to epilepsy in humans. J Neurosci. 2002 Jul 1;22(13):5321-7.

375. Feng HJ, Macdonald RL. Barbiturates require the N terminus and first transmembrane domain of the delta subunit for enhancement of alpha1beta3delta GABAA receptor currents. J Biol Chem. 2010 Jul 30;285(31):23614-21.

376. Greenfield LJ, Jr., Zaman SH, Sutherland ML, et al. Mutation of the GABAA receptor M1 transmembrane proline increases GABA affinity and reduces barbiturate enhancement. Neuropharmacology. 2002 Mar;42(4):502-21.

377. Engblom AC, Carlson BX, Olsen RW, Schousboe A, Kristiansen U. Point mutation in the first transmembrane region of the beta 2 subunit of the gamma--aminobutyric acid type A receptor alters desensitization kinetics of gamma--aminobutyric acid- and anesthetic-induced channel gating. J Biol Chem. 2002 May 17;277(20):17438-47.

378. Jones-Davis DM, Song L, Gallagher MJ, Macdonald RL. Structural determinants of benzodiazepine allosteric regulation of GABA(A) receptor currents. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2005 Aug 31;25(35):8056-65.

379. Keramidas A, Kash TL, Harrison NL. The pre-M1 segment of the alpha1 subunit is a transduction element in the activation of the GABAA receptor. The Journal of physiology. 2006 Aug 15;575(Pt 1):11-22.

380. Akk G, Li P, Bracamontes J, Reichert DE, Covey DF, Steinbach JH. Mutations of the GABA-A receptor alpha1 subunit M1 domain reveal unexpected complexity for modulation by neuroactive steroids. Mol Pharmacol. 2008 Sep;74(3):614-27.

381. Hemmings HC, Jr., Akabas MH, Goldstein PA, Trudell JR, Orser BA, Harrison NL. Emerging molecular mechanisms of general anesthetic action. Trends Pharmacol Sci. 2005 Oct;26(10):503-10.

382. Chang CS, Olcese R, Olsen RW. A single M1 residue in the beta2 subunit alters channel gating of GABAA receptor in anesthetic modulation and direct activation. J Biol Chem. 2003 Oct 31;278(44):42821-8.

383. Jensen ML, Timmermann DB, Johansen TH, Schousboe A, Varming T, Ahring PK. The beta subunit determines the ion selectivity of the GABAA receptor. J Biol Chem. 2002 Nov 1;277(44):41438-47.

384. Giraudat J, Dennis M, Heidmann T, Chang JY, Changeux JP. Structure of the high-affinity binding site for noncompetitive blockers of the acetylcholine receptor: serine-262 of the delta subunit is labeled by [3H]chlorpromazine. Proceedings of the National Academy of Sciences of the United States of America. 1986 Apr;83(8):2719-23.

385. Akabas MH, Kaufmann C, Archdeacon P, Karlin A. Identification of acetylcholine receptor channel-lining residues in the entire M2 segment of the alpha subunit. Neuron. 1994 Oct;13(4):919-27.

386. Xu M, Akabas MH. Identification of channel-lining residues in the M2 membrane-spanning segment of the GABA(A) receptor alpha1 subunit. J Gen Physiol. 1996 Feb;107(2):195-205.

387. Serafini R, Bracamontes J, Steinbach JH. Structural domains of the human GABAA receptor 3 subunit involved in the actions of pentobarbital. The Journal of physiology. 2000 May 1;524 Pt 3:649-76.

388. Keramidas A, Moorhouse AJ, Schofield PR, Barry PH. Ligand-gated ion channels: mechanisms underlying ion selectivity. Prog Biophys Mol Biol. 2004 Oct;86(2):161-204.

389. Bianchi MT, Botzolakis EJ, Haas KF, Fisher JL, Macdonald RL. Microscopic kinetic determinants of macroscopic currents: insights from coupling and uncoupling of GABAA receptor desensitization and deactivation. J Physiol. 2007 Nov 1;584(Pt 3):769-87.

390. Bianchi MT, Macdonald RL. Mutation of the 9' leucine in the GABA(A) receptor gamma2L subunit produces an apparent decrease in desensitization by stabilizing open states without altering desensitized states. Neuropharmacology. 2001 Nov;41(6):737-44.

391. Chang Y, Weiss DS. Allosteric activation mechanism of the alpha 1 beta 2 gamma 2 gamma-aminobutyric acid type A receptor revealed by mutation of the conserved M2 leucine. Biophys J. 1999 Nov;77(5):2542-51.

392. Scheller M, Forman SA. Coupled and uncoupled gating and desensitization effects by pore domain mutations in GABA(A) receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2002 Oct 1;22(19):8411-21.

393. Cymes GD, Ni Y, Grosman C. Probing ion-channel pores one proton at a time. Nature. 2005 Dec 15;438(7070):975-80.

394. Unwin N, Fujiyoshi Y. Gating movement of acetylcholine receptor caught by plunge-freezing. J Mol Biol. 2012 Oct 5;422(5):617-34.

395. Gielen M, Thomas P, Smart TG. The desensitization gate of inhibitory Cys-loop receptors. Nat Commun. 2015 Apr 20;6:6829.

396. Tobimatsu T, Fujita Y, Fukuda K, et al. Effects of substitution of putative transmembrane segments on nicotinic acetylcholine receptor function. FEBS letters. 1987 Sep 28;222(1):56-62.

397. Schofield CM, Harrison NL. Transmembrane residues define the action of isoflurane at the GABAA receptor alpha-3 subunit. Brain research. 2005 Jan 25;1032(1-2):30-5.

398. Brannigan G, LeBard DN, Henin J, Eckenhoff RG, Klein ML. Multiple binding sites for the general anesthetic isoflurane identified in the nicotinic acetylcholine receptor transmembrane domain. Proceedings of the National Academy of Sciences of the United States of America. 2010 Aug 10;107(32):14122-7.

399. Filippova N, Wotring VE, Weiss DS. Evidence that the TM1-TM2 loop contributes to the rho1 GABA receptor pore. J Biol Chem. 2004 May 14;279(20):20906-14.

400. Wotring VE, Weiss DS. Charge scan reveals an extended region at the intracellular end of the GABA receptor pore that can influence ion selectivity. J Gen Physiol. 2008 Jan;131(1):87-97.

401. Kash TL, Jenkins A, Kelley JC, Trudell JR, Harrison NL. Coupling of agonist binding to channel gating in the GABA(A) receptor. Nature. 2003 Jan 16;421(6920):272-5.

402. O'Shea SM, Williams CA, Jenkins A. Inverse effects on gating and modulation caused by a mutation in the M2-M3 Linker of the GABA(A) receptor gamma subunit. Mol Pharmacol. 2009 Sep;76(3):641-51.

403. Davies M, Newell JG, Dunn SM. Mutagenesis of the GABA(A) receptor alpha1 subunit reveals a domain that affects sensitivity to GABA and benzodiazepine-site ligands. Journal of neurochemistry. 2001 Oct;79(1):55-62.

404. Bera AK, Chatav M, Akabas MH. GABA(A) receptor M2-M3 loop secondary structure and changes in accessibility during channel gating. J Biol Chem. 2002 Nov 8;277(45):43002-10.

405. Langosch D, Laube B, Rundstrom N, Schmieden V, Bormann J, Betz H. Decreased agonist affinity and chloride conductance of mutant glycine receptors associated with human hereditary hyperekplexia. Embo J. 1994 Sep 15;13(18):4223-8.

406. Lynch JW, Rajendra S, Pierce KD, Handford CA, Barry PH, Schofield PR. Identification of intracellular and extracellular domains mediating signal transduction in the inhibitory glycine receptor chloride channel. Embo J. 1997 Jan 2;16(1):110-20.

407. Harvey RJ, Topf M, Harvey K, Rees MI. The genetics of hyperekplexia: more than startle! Trends Genet. 2008 Sep;24(9):439-47.

408. Campos-Caro A, Sala S, Ballesta JJ, Vicente-Agullo F, Criado M, Sala F. A single residue in the M2-M3 loop is a major determinant of coupling between binding and gating in

neuronal nicotinic receptors. Proceedings of the National Academy of Sciences of the United States of America. 1996 Jun 11;93(12):6118-23.

409. Hernandez CC, Zhang Y, Hu N, et al. GABA A Receptor Coupling Junction and Pore GABRB3 Mutations are Linked to Early-Onset Epileptic Encephalopathy. Sci Rep. 2017 Nov 21;7(1):15903.

410. Macdonald RL, Bianchi MT, Feng H. Mutations linked to generalized epilepsy in humans reduce GABA(A) receptor current. Exp Neurol. 2003 Nov;184 Suppl 1:S58-67.

411. Baulac S, Huberfeld G, Gourfinkel-An I, et al. First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. Nat Genet. 2001 May;28(1):46-8.

412. Jacob TC, Moss SJ, Jurd R. GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. Nat Rev Neurosci. 2008 May;9(5):331-43.

413. Glickfeld LL, Roberts JD, Somogyi P, Scanziani M. Interneurons hyperpolarize pyramidal cells along their entire somatodendritic axis. Nat Neurosci. 2009 Jan;12(1):21-3.

414. Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL. Giant synaptic potentials in immature rat CA3 hippocampal neurones. The Journal of physiology. 1989 Sep;416:303-25.

415. Wang DD, Kriegstein AR, Ben-Ari Y. GABA regulates stem cell proliferation before nervous system formation. Epilepsy currents. 2008 Sep-Oct;8(5):137-9.

416. Wang DD, Kriegstein AR. GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2008 May 21;28(21):5547-58.

417. Young SZ, Taylor MM, Wu S, Ikeda-Matsuo Y, Kubera C, Bordey A. NKCC1 knockdown decreases neuron production through GABA(A)-regulated neural progenitor proliferation and delays dendrite development. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2012 Sep 26;32(39):13630-8.

418. Wester JC, McBain CJ. Interneurons Differentially Contribute to Spontaneous Network Activity in the Developing Hippocampus Dependent on Their Embryonic Lineage. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2016 Mar 2;36(9):2646-62.

419. Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Gaiarsa JL. GABAA, NMDA and AMPA receptors: a developmentally regulated 'menage a trois'. Trends Neurosci. 1997 Nov;20(11):523-9.

420. Ge S, Pradhan DA, Ming GL, Song H. GABA sets the tempo for activity-dependent adult neurogenesis. Trends Neurosci. 2007 Jan;30(1):1-8.

421. Sipila ST, Schuchmann S, Voipio J, Yamada J, Kaila K. The cation-chloride cotransporter NKCC1 promotes sharp waves in the neonatal rat hippocampus. The Journal of physiology. 2006 Jun 15;573(Pt 3):765-73.

422. Kirmse K, Kummer M, Kovalchuk Y, Witte OW, Garaschuk O, Holthoff K. GABA depolarizes immature neurons and inhibits network activity in the neonatal neocortex in vivo. Nat Commun. 2015 Jul 16;6:7750.

423. Ben-Ari Y. Commentary: GABA depolarizes immature neurons and inhibits network activity in the neonatal neocortex in vivo. Front Cell Neurosci. 2015;9:478.

424. Payne JA, Rivera C, Voipio J, Kaila K. Cation-chloride co-transporters in neuronal communication, development and trauma. Trends Neurosci. 2003 Apr;26(4):199-206.

425. Mann EO, Paulsen O. Role of GABAergic inhibition in hippocampal network oscillations. Trends Neurosci. 2007 Jul;30(7):343-9.

426. Prescott SA, De Koninck Y. Gain control of firing rate by shunting inhibition: roles of synaptic noise and dendritic saturation. Proceedings of the National Academy of Sciences of the United States of America. 2003 Feb 18;100(4):2076-81.

427. Banke TG, McBain CJ. GABAergic input onto CA3 hippocampal interneurons remains shunting throughout development. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006 Nov 8;26(45):11720-5.

428. Otis TS, Staley KJ, Mody I. Perpetual inhibitory activity in mammalian brain slices generated by spontaneous GABA release. Brain research. 1991 Apr 5;545(1-2):142-50.

429. Wei W, Zhang N, Peng Z, Houser CR, Mody I. Perisynaptic localization of delta subunit-containing GABA(A) receptors and their activation by GABA spillover in the mouse dentate gyrus. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2003 Nov 19;23(33):10650-61.

430. Rossi DJ, Hamann M. Spillover-mediated transmission at inhibitory synapses promoted by high affinity alpha6 subunit GABA(A) receptors and glomerular geometry. Neuron. 1998 Apr;20(4):783-95.

431. Barakat L, Bordey A. GAT-1 and reversible GABA transport in Bergmann glia in slices. Journal of neurophysiology. 2002 Sep;88(3):1407-19.

432. Kozlov AS, Angulo MC, Audinat E, Charpak S. Target cell-specific modulation of neuronal activity by astrocytes. Proceedings of the National Academy of Sciences of the United States of America. 2006 Jun 27;103(26):10058-63.

433. Jimenez-Gonzalez C, Pirttimaki T, Cope DW, Parri HR. Non-neuronal, slow GABA signalling in the ventrobasal thalamus targets delta-subunit-containing GABA(A) receptors. The European journal of neuroscience. 2011 Apr;33(8):1471-82.

434. Lee S, Yoon BE, Berglund K, et al. Channel-mediated tonic GABA release from glia. Science. 2010 Nov 5;330(6005):790-6.

435. Wall MJ, Usowicz MM. Development of action potential-dependent and independent spontaneous GABAA receptor-mediated currents in granule cells of postnatal rat cerebellum. The European journal of neuroscience. 1997 Mar;9(3):533-48.

436. Rossi DJ, Hamann M, Attwell D. Multiple modes of GABAergic inhibition of rat cerebellar granule cells. The Journal of physiology. 2003 Apr 1;548(Pt 1):97-110.

437. Heja L, Nyitrai G, Kekesi O, et al. Astrocytes convert network excitation to tonic inhibition of neurons. BMC Biol. 2012 Mar 15;10:26.

438. Wojtowicz AM, Dvorzhak A, Semtner M, Grantyn R. Reduced tonic inhibition in striatal output neurons from Huntington mice due to loss of astrocytic GABA release through GAT-3. Front Neural Circuits. 2013;7:188.

439. Yoon BE, Woo J, Chun YE, et al. Glial GABA, synthesized by monoamine oxidase B, mediates tonic inhibition. The Journal of physiology. 2014 Nov 15;592(22):4951-68.

440. Yoon BE, Woo J, Lee CJ. Astrocytes as GABA-ergic and GABA-ceptive cells. Neurochem Res. 2012 Nov;37(11):2474-9.

441. Tossman U, Jonsson G, Ungerstedt U. Regional distribution and extracellular levels of amino acids in rat central nervous system. Acta Physiol Scand. 1986 Aug;127(4):533-45.

442. Brown N, Kerby J, Bonnert TP, Whiting PJ, Wafford KA. Pharmacological characterization of a novel cell line expressing human alpha(4)beta(3)delta GABA(A) receptors. Br J Pharmacol. 2002 Aug;136(7):965-74.

443. Knoflach F, Benke D, Wang Y, et al. Pharmacological modulation of the diazepam-insensitive recombinant gamma-aminobutyric acidA receptors alpha 4 beta 2 gamma 2 and alpha 6 beta 2 gamma 2. Mol Pharmacol. 1996 Nov;50(5):1253-61.

444. Fisher JL, Macdonald RL. Single channel properties of recombinant GABAA receptors containing gamma 2 or delta subtypes expressed with alpha 1 and beta 3 subtypes in mouse L929 cells. The Journal of physiology. 1997 Dec 1;505 (Pt 2):283-97.

445. Saxena NC, Macdonald RL. Assembly of GABAA receptor subunits: role of the delta subunit. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1994 Nov;14(11 Pt 2):7077-86.

446. Nusser Z, Mody I. Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. Journal of neurophysiology. 2002 May;87(5):2624-8.

447. Semyanov A, Walker MC, Kullmann DM. GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. Nat Neurosci. 2003 May;6(5):484-90.

448. Dibbens LM, Feng HJ, Richards MC, et al. GABRD encoding a protein for extra- or peri-synaptic GABAA receptors is a susceptibility locus for generalized epilepsies. Human molecular genetics. 2004 Jul 1;13(13):1315-9.

449. Feng HJ, Kang JQ, Song L, Dibbens L, Mulley J, Macdonald RL. Delta subunit susceptibility variants E177A and R220H associated with complex epilepsy alter channel gating and surface expression of alpha4beta2delta GABAA receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006 Feb 1;26(5):1499-506.

450. Heck N, Kilb W, Reiprich P, et al. GABA-A receptors regulate neocortical neuronal migration in vitro and in vivo. Cereb Cortex. 2007 Jan;17(1):138-48.

451. Lauder JM, Han VK, Henderson P, Verdoorn T, Towle AC. Prenatal ontogeny of the GABAergic system in the rat brain: an immunocytochemical study. Neuroscience. 1986 Oct;19(2):465-93.

452. Haydar TF, Wang F, Schwartz ML, Rakic P. Differential modulation of proliferation in the neocortical ventricular and subventricular zones. J Neurosci. 2000 Aug 1;20(15):5764-74.

453. Miyoshi G, Fishell G. GABAergic interneuron lineages selectively sort into specific cortical layers during early postnatal development. Cereb Cortex. 2011 Apr;21(4):845-52.

454. Le Magueresse C, Monyer H. GABAergic interneurons shape the functional maturation of the cortex. Neuron. 2013 Feb 06;77(3):388-405.

455. Zhang ZW. Maturation of layer V pyramidal neurons in the rat prefrontal cortex: intrinsic properties and synaptic function. Journal of neurophysiology. 2004 Mar;91(3):1171-82.

456. LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR. GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron. 1995 Dec;15(6):1287-98.

457. Ben-Yaakov G, Golan H. Cell proliferation in response to GABA in postnatal hippocampal slice culture. Int J Dev Neurosci. 2003 May;21(3):153-7.

458. Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H. GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature. 2006 Feb 02;439(7076):589-93.

459. Liu X, Wang Q, Haydar TF, Bordey A. Nonsynaptic GABA signaling in postnatal subventricular zone controls proliferation of GFAP-expressing progenitors. Nat Neurosci. 2005 Sep;8(9):1179-87.

460. Stewart RR, Hoge GJ, Zigova T, Luskin MB. Neural progenitor cells of the neonatal rat anterior subventricular zone express functional GABA(A) receptors. J Neurobiol. 2002 Mar;50(4):305-22.

461. Wang DD, Krueger DD, Bordey A. GABA depolarizes neuronal progenitors of the postnatal subventricular zone via GABAA receptor activation. J Physiol. 2003 Aug 01;550(Pt 3):785-800.

462. Hu JS, Vogt D, Sandberg M, Rubenstein JL. Cortical interneuron development: a tale of time and space. Development. 2017 Nov 1;144(21):3867-78.

463. Pelkey KA, Chittajallu R, Craig MT, Tricoire L, Wester JC, McBain CJ. Hippocampal GABAergic Inhibitory Interneurons. Physiol Rev. 2017 Oct 1;97(4):1619-747.

464. Miyoshi G, Butt SJ, Takebayashi H, Fishell G. Physiologically distinct temporal cohorts of cortical interneurons arise from telencephalic Olig2-expressing precursors. J Neurosci. 2007 Jul 18;27(29):7786-98.

465. Miyoshi G, Hjerling-Leffler J, Karayannis T, et al. Genetic fate mapping reveals that the caudal ganglionic eminence produces a large and diverse population of superficial cortical interneurons. J Neurosci. 2010 Feb 03;30(5):1582-94.

466. Caviness VS, Jr., Takahashi T, Nowakowski RS. Numbers, time and neocortical neuronogenesis: a general developmental and evolutionary model. Trends Neurosci. 1995 Sep;18(9):379-83.

467. Takahashi T, Nowakowski RS, Caviness VS, Jr. The mathematics of neocortical neuronogenesis. Dev Neurosci. 1997;19(1):17-22.

468. Takahashi T, Nowakowski RS, Caviness VS, Jr. The leaving or Q fraction of the murine cerebral proliferative epithelium: a general model of neocortical neuronogenesis. J Neurosci. 1996 Oct 01;16(19):6183-96.

469. Caviness VS, Jr. Early events of neocortical assembly: experimental studies and human pathology. Int J Neurol. 1982;16-17:102-9.

470. Del Rio JA, Soriano E, Ferrer I. Development of GABA-immunoreactivity in the neocortex of the mouse. J Comp Neurol. 1992 Dec 22;326(4):501-26.

471. Southwell DG, Paredes MF, Galvao RP, et al. Intrinsically determined cell death of developing cortical interneurons. Nature. 2012 Nov 01;491(7422):109-13.

472. Nikoletopoulou V, Lickert H, Frade JM, et al. Neurotrophin receptors TrkA and TrkC cause neuronal death whereas TrkB does not. Nature. 2010 Sep 02;467(7311):59-63.

473. Oh WC, Smith KR. Activity-dependent development of GABAergic synapses. Brain research. 2018 Nov 12.

474. Huang ZJ. Activity-dependent development of inhibitory synapses and innervation pattern: role of GABA signalling and beyond. The Journal of physiology. 2009 May 1;587(Pt 9):1881-8.

475. Verhage M, Maia AS, Plomp JJ, et al. Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science. 2000 Feb 4;287(5454):864-9.

476. Konig N, Roch G, Marty R. The onset of synaptogenesis in rat temporal cortex. Anat Embryol (Berl). 1975 Nov 6;148(1):73-87.

477. Chattopadhyaya B, Di Cristo G, Wu CZ, et al. GAD67-mediated GABA synthesis and signaling regulate inhibitory synaptic innervation in the visual cortex. Neuron. 2007 Jun 21;54(6):889-903.

478. Wu X, Fu Y, Knott G, Lu J, Di Cristo G, Huang ZJ. GABA signaling promotes synapse elimination and axon pruning in developing cortical inhibitory interneurons. J Neurosci. 2012 Jan 04;32(1):331-43.

479. Chen Y. GABA-A receptor-dependent mechanisms prevent excessive spine elimination during postnatal maturation of the mouse cortex in vivo. FEBS Lett. 2014 Dec 20;588(24):4551-60.

480. Petanjek Z, Judas M, Simic G, et al. Extraordinary neoteny of synaptic spines in the human prefrontal cortex. Proc Natl Acad Sci U S A. 2011 Aug 9;108(32):13281-6.

481. Ben-Ari Y. Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci. 2002 Sep;3(9):728-39.

482. Marty A, Llano I. Excitatory effects of GABA in established brain networks. Trends Neurosci. 2005 Jun;28(6):284-9.

483. Tozuka Y, Fukuda S, Namba T, Seki T, Hisatsune T. GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron. 2005 Sep 15;47(6):803-15.

484. Song H, Kempermann G, Overstreet Wadiche L, Zhao C, Schinder AF, Bischofberger J. New neurons in the adult mammalian brain: synaptogenesis and functional integration. J Neurosci. 2005 Nov 09;25(45):10366-8.

485. Carleton A, Petreanu LT, Lansford R, Alvarez-Buylla A, Lledo PM. Becoming a new neuron in the adult olfactory bulb. Nat Neurosci. 2003 May;6(5):507-18.

486. Wang DD, Krueger DD, Bordey A. Biophysical properties and ionic signature of neuronal progenitors of the postnatal subventricular zone in situ. J Neurophysiol. 2003 Oct;90(4):2291-302.

487. Markwardt SJ, Wadiche JI, Overstreet-Wadiche LS. Input-specific GABAergic signaling to newborn neurons in adult dentate gyrus. J Neurosci. 2009 Dec 02;29(48):15063-72.

488. Overstreet Wadiche L, Bromberg DA, Bensen AL, Westbrook GL. GABAergic signaling to newborn neurons in dentate gyrus. J Neurophysiol. 2005 Dec;94(6):4528-32.

489. Wang LP, Kempermann G, Kettenmann H. A subpopulation of precursor cells in the mouse dentate gyrus receives synaptic GABAergic input. Mol Cell Neurosci. 2005 Jun;29(2):181-9.

490. Ben-Ari Y, Tseeb V, Raggozzino D, Khazipov R, Gaiarsa JL. gamma-Aminobutyric acid (GABA): a fast excitatory transmitter which may regulate the development of hippocampal neurones in early postnatal life. Prog Brain Res. 1994;102:261-73.

491. Owens DF, Boyce LH, Davis MB, Kriegstein AR. Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1996 Oct 15;16(20):6414-23.

492. Manent JB, Demarque M, Jorquera I, et al. A noncanonical release of GABA and glutamate modulates neuronal migration. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2005 May 11;25(19):4755-65.

493. Ma W, Barker JL. Complementary expressions of transcripts encoding GAD67 and GABAA receptor alpha 4, beta 1, and gamma 1 subunits in the proliferative zone of the embryonic rat central nervous system. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1995 Mar;15(3 Pt 2):2547-60.

494. Laurie DJ, Wisden W, Seeburg PH. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci. 1992 Nov;12(11):4151-72.

495. Ma W, Barker JL. Complementary expressions of transcripts encoding GAD67 and GABAA receptor alpha 4, beta 1, and gamma 1 subunits in the proliferative zone of the embryonic rat central nervous system. J Neurosci. 1995 Mar;15(3 Pt 2):2547-60.

496. Maric D, Maric I, Ma W, et al. Anatomical gradients in proliferation and differentiation of embryonic rat CNS accessed by buoyant density fractionation: alpha 3, beta 3 and gamma 2 GABAA receptor subunit co-expression by post-mitotic neocortical neurons correlates directly with cell buoyancy. Eur J Neurosci. 1997 Mar;9(3):507-22.

497. Bosman LW, Rosahl TW, Brussaard AB. Neonatal development of the rat visual cortex: synaptic function of GABAA receptor alpha subunits. J Physiol. 2002 Nov 15;545(Pt 1):169-81.

498. Liu Q, Wong-Riley MT. Developmental changes in the expression of GABAA receptor subunits alpha1, alpha2, and alpha3 in brain stem nuclei of rats. Brain Res. 2006 Jul 7;1098(1):129-38.

499. Okada M, Onodera K, Van Renterghem C, Sieghart W, Takahashi T. Functional correlation of GABA(A) receptor alpha subunits expression with the properties of IPSCs in the developing thalamus. J Neurosci. 2000 Mar 15;20(6):2202-8.

500. Duncan CE, Webster MJ, Rothmond DA, Bahn S, Elashoff M, Shannon Weickert C. Prefrontal GABA(A) receptor alpha-subunit expression in normal postnatal human development and schizophrenia. J Psychiatr Res. 2010 Jul;44(10):673-81.

501. Fritschy JM, Paysan J, Enna A, Mohler H. Switch in the expression of rat GABAA-receptor subtypes during postnatal development: an immunohistochemical study. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1994 Sep;14(9):5302-24.

502. Heinen K, Bosman LW, Spijker S, et al. GABAA receptor maturation in relation to eye opening in the rat visual cortex. Neuroscience. 2004;124(1):161-71.

503. Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience. 2000;101(4):815-50.

504. Rovira C, Ben-Ari Y. Developmental study of benzodiazepine effects on monosynaptic GABAA-mediated IPSPs of rat hippocampal neurons. Journal of neurophysiology. 1993 Sep;70(3):1076-85.

505. Mohler H, Benke D, Mertens S, Fritschy JM. GABAA-receptor subtypes differing in alpha-subunit composition display unique pharmacological properties. Adv Biochem Psychopharmacol. 1992;47:41-53.

506. Mtchedlishvili Z, Sun CS, Harrison MB, Kapur J. Increased neurosteroid sensitivity of hippocampal GABAA receptors during postnatal development. Neuroscience. 2003;118(3):655-66.

507. Fillman SG, Duncan CE, Webster MJ, Elashoff M, Weickert CS. Developmental co-regulation of the beta and gamma GABAA receptor subunits with distinct alpha subunits in the human dorsolateral prefrontal cortex. Int J Dev Neurosci. 2010 Oct;28(6):513-9.

508. Wisden W, Laurie DJ, Monyer H, Seeburg PH. The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci. 1992 Mar;12(3):1040-62.

509. Hadingham KL, Wingrove PB, Wafford KA, et al. Role of the beta subunit in determining the pharmacology of human gamma-aminobutyric acid type A receptors. Mol Pharmacol. 1993 Dec;44(6):1211-8.

510. Mortensen M, Patel B, Smart TG. GABA Potency at GABA(A) Receptors Found in Synaptic and Extrasynaptic Zones. Front Cell Neurosci. 2011 Jan;6:1.

511. Glatt K, Glatt H, Lalande M. Structure and organization of GABRB3 and GABRA5. Genomics. 1997 Apr 01;41(1):63-9.

512. Kirkness EF, Fraser CM. A strong promoter element is located between alternative exons of a gene encoding the human gamma-aminobutyric acid-type A receptor beta 3 subunit (GABRB3). J Biol Chem. 1993 Feb 25;268(6):4420-8.

513. Hortnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G. Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain. Neuroscience. 2013 Apr 16;236:345-72.

514. Homanics GE, DeLorey TM, Firestone LL, et al. Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. Proc Natl Acad Sci U S A. 1997 Apr 15;94(8):4143-8.

515. Noroozi R, Taheri M, Ghafouri-Fard S, et al. Meta-analysis of GABRB3 Gene Polymorphisms and Susceptibility to Autism Spectrum Disorder. J Mol Neurosci. 2018 Aug;65(4):432-7.

516. Puts NAJ, Wodka EL, Harris AD, et al. Reduced GABA and altered somatosensory function in children with autism spectrum disorder. Autism Res. 2017 Apr;10(4):608-19.

517. Russo N, Foxe JJ, Brandwein AB, Altschuler T, Gomes H, Molholm S. Multisensory processing in children with autism: high-density electrical mapping of auditory-somatosensory integration. Autism Res. 2010 Oct;3(5):253-67.

518. Kirkness EF, Kusiak JW, Fleming JT, et al. Isolation, characterization, and localization of human genomic DNA encoding the beta 1 subunit of the GABAA receptor (GABRB1). Genomics. 1991 Aug;10(4):985-95.

519. Garrett KM, Saito N, Duman RS, et al. Differential expression of gamma-aminobutyric acidA receptor subunits. Mol Pharmacol. 1990 May;37(5):652-7.

520. Griffiths JL, Lovick TA. GABAergic neurones in the rat periaqueductal grey matter express alpha4, beta1 and delta GABAA receptor subunits: plasticity of expression during the estrous cycle. Neuroscience. 2005;136(2):457-66.

521. Yanovsky Y, Schubring S, Fleischer W, et al. GABAA receptors involved in sleep and anaesthesia: beta1- versus beta3-containing assemblies. Pflugers Arch. 2012 Jan;463(1):187-99.

522. Mueller TM, Remedies CE, Haroutunian V, Meador-Woodruff JH. Abnormal subcellular localization of GABAA receptor subunits in schizophrenia brain. Transl Psychiatry. 2015 Aug 04;5:e612.

523. Gee KW, Tran MB, Hogenkamp DJ, et al. Limiting activity at beta1-subunit-containing GABAA receptor subtypes reduces ataxia. J Pharmacol Exp Ther. 2010 Mar;332(3):1040-53.

524. Parsian A, Zhang ZH. Human chromosomes 11p15 and 4p12 and alcohol dependence: possible association with the GABRB1 gene. Am J Med Genet. 1999 Oct 15;88(5):533-8.

525. Song J, Koller DL, Foroud T, et al. Association of GABA(A) receptors and alcohol dependence and the effects of genetic imprinting. Am J Med Genet B Neuropsychiatr Genet. 2003 Feb;117B(1):39-45.

526. Reck BH, Mukhopadhyay N, Tsai HJ, Weeks DE. Analysis of alcohol dependence phenotype in the COGA families using covariates to detect linkage. BMC Genet. 2005 Dec 30;6 Suppl 1:S143.

527. Anstee QM, Knapp S, Maguire EP, et al. Mutations in the Gabrb1 gene promote alcohol consumption through increased tonic inhibition. Nat Commun. 2013;4:2816.

528. Duka T, Nikolaou K, King SL, et al. GABRB1 Single Nucleotide Polymorphism Associated with Altered Brain Responses (but not Performance) during Measures of Impulsivity and Reward Sensitivity in Human Adolescents. Front Behav Neurosci. 2017;11:24.

529. Delahanty RJ, Kang JQ, Brune CW, et al. Maternal transmission of a rare GABRB3 signal peptide variant is associated with autism. Mol Psychiatry. 2011 Jan;16(1):86-96.

530. Lachance-Touchette P, Brown P, Meloche C, et al. Novel alpha1 and gamma2 GABAA receptor subunit mutations in families with idiopathic generalized epilepsy. Eur J Neurosci. 2011 Jul;34(2):237-49.

531. Dibbens LM, Feng HJ, Richards MC, et al. GABRD encoding a protein for extra- or peri-synaptic GABAA receptors is a susceptibility locus for generalized epilepsies. Hum Mol Genet. 2004 Jul 1;13(13):1315-9.

532. Orenstein N, Goldberg-Stern H, Straussberg R, et al. A de novo GABRA2 missense mutation in severe early-onset epileptic encephalopathy with a choreiform movement disorder. Eur J Paediatr Neurol. 2017 Dec 30.

533. Niturad CE, Lev D, Kalscheuer VM, et al. Rare GABRA3 variants are associated with epileptic seizures, encephalopathy and dysmorphic features. Brain. 2017 Nov 1;140(11):2879-94.

534. Shen D, Hernandez CC, Shen W, et al. De novo GABRG2 mutations associated with epileptic encephalopathies. Brain. 2017 Jan;140(Pt 1):49-67.

535. Macdonald RL, Kang JQ, Gallagher MJ. Mutations in GABAA receptor subunits associated with genetic epilepsies. J Physiol. 2010 Jun 1;588(Pt 11):1861-9.

536. Gallagher MJ, Ding L, Maheshwari A, Macdonald RL. The GABAA receptor alpha1 subunit epilepsy mutation A322D inhibits transmembrane helix formation and causes proteasomal degradation. Proc Natl Acad Sci U S A. 2007 Aug 7;104(32):12999-3004.

537. Gallagher MJ, Shen W, Song L, Macdonald RL. Endoplasmic reticulum retention and associated degradation of a GABAA receptor epilepsy mutation that inserts an aspartate in the M3 transmembrane segment of the alpha1 subunit. J Biol Chem. 2005 Nov 11;280(45):37995-8004.

538. Gallagher MJ, Song L, Arain F, Macdonald RL. The juvenile myoclonic epilepsy GABA(A) receptor alpha1 subunit mutation A322D produces asymmetrical, subunit position-dependent reduction of heterozygous receptor currents and alpha1 subunit protein expression. J Neurosci. 2004 Jun 16;24(24):5570-8.

539. Krampfl K, Maljevic S, Cossette P, et al. Molecular analysis of the A322D mutation in the GABA receptor alpha-subunit causing juvenile myoclonic epilepsy. Eur J Neurosci. 2005 Jul;22(1):10-20.

540. Ding L, Feng HJ, Macdonald RL, Botzolakis EJ, Hu N, Gallagher MJ. GABA(A) receptor alpha1 subunit mutation A322D associated with autosomal dominant juvenile myoclonic epilepsy reduces the expression and alters the composition of wild type GABA(A) receptors. J Biol Chem. 2010 Aug 20;285(34):26390-405.

541. Harkin LA, Bowser DN, Dibbens LM, et al. Truncation of the GABA(A)-receptor gamma2 subunit in a family with generalized epilepsy with febrile seizures plus. Am J Hum Genet. 2002 Feb;70(2):530-6.

542. Kang JQ, Shen W, Macdonald RL. The GABRG2 mutation, Q351X, associated with generalized epilepsy with febrile seizures plus, has both loss of function and dominant-negative suppression. J Neurosci. 2009 Mar 4;29(9):2845-56.

543. Kang JQ, Shen W, Zhou C, Xu D, Macdonald RL. The human epilepsy mutation GABRG2(Q390X) causes chronic subunit accumulation and neurodegeneration. Nat Neurosci. 2015 Jul;18(7):988-96.

544. Huang X, Zhou C, Tian M, et al. Overexpressing wild-type gamma2 subunits rescued the seizure phenotype in Gabrg2(+/Q390X) Dravet syndrome mice. Epilepsia. 2017 Aug;58(8):1451-61.

545. Tian M, Mei D, Freri E, et al. Impaired surface alphabetagamma GABA(A) receptor expression in familial epilepsy due to a GABRG2 frameshift mutation. Neurobiol Dis. 2013 Feb;50:135-41.

546. Kananura C, Haug K, Sander T, et al. A splice-site mutation in GABRG2 associated with childhood absence epilepsy and febrile convulsions. Arch Neurol. 2002 Jul;59(7):1137-41.

547. Tian M, Macdonald RL. The intronic GABRG2 mutation, IVS6+2T->G, associated with childhood absence epilepsy altered subunit mRNA intron splicing, activated nonsense-mediated decay, and produced a stable truncated gamma2 subunit. J Neurosci. 2012 Apr 25;32(17):5937-52.

548. Hempelmann A, Cobilanschi J, Heils A, et al. Lack of evidence of an allelic association of a functional GABRB3 exon 1a promoter polymorphism with idiopathic generalized epilepsy. Epilepsy research. 2007 Apr;74(1):28-32.

549. Tanaka M, Bailey JN, Bai D, Ishikawa-Brush Y, Delgado-Escueta AV, Olsen RW. Effects on promoter activity of common SNPs in 5' region of GABRB3 exon 1A. Epilepsia. 2012 Aug;53(8):1450-6.

550. Liu Y, Sun XF, Ding M, et al. The GABRB3 Polymorphism and its Association with Schizophrenia. J Mol Neurosci. 2018 Jan;64(1):75-9.

551. Sun XF, Ding M, Sun Y, et al. [Polymorphisms of rs4906902 and rs8179184 loci in the promoter of the GABRB3 gene and their relevance with schizophrenia]. Fa Yi Xue Za Zhi. 2012 Jun;28(3):195-7.

552. Dulla CG. Utilizing Animal Models of Infantile Spasms. Epilepsy Curr. 2018 Mar-Apr;18(2):107-12.

553. Galanopoulou AS, Moshe SL. Neonatal and Infantile Epilepsy: Acquired and Genetic Models. Cold Spring Harb Perspect Med. 2015 Dec 04;6(1):a022707.

554. Schutte SS, Schutte RJ, Barragan EV, O'Dowd DK. Model systems for studying cellular mechanisms of SCN1A-related epilepsy. J Neurophysiol. 2016 Apr;115(4):1755-66.

555. Maljevic S, Reid CA, Petrou S. Models for discovery of targeted therapy in genetic epileptic encephalopathies. J Neurochem. 2017 Oct;143(1):30-48.

CHAPTER II: Effects of Infantile spasms (IS)-associated *GABRB3(N110D)* and *GABRB1(F246S)* mutations on $GABA_A$ receptor ($GABA_AR$) function

2.1 IS: the most common seizures in infancy

IS are seizures manifested as sudden and brief (1-2 s) contractions (flexion or extension) of the arms, legs, head, neck, and trunk muscles, the most common being flexor spasms of the head and arms. Spasms are usually longer than myoclonic seizures, but not as prolonged as tonic seizures, and often occur in clusters. Some patients also experience a prolonged tonic contraction and/or arrest of activity following the spasm or in the absence of a spasm [1-4]. Most infants (~90%) experience more than one type of spasm [3, 5]. Spasms can be focal or generalized on an electroencephalogram (EEG) recording, but are almost always bilateral. Asymmetrical spasms due to greater flexion or extension on one side are also observed [2, 6]. For the vast majority of cases, IS abruptly begin in the first year of life with a peak incidence between 3-10 months of age and followed by developmental regression after seizure onset [7-12]. The initial spasms are subtle but rapidly become worse. The child may cry or become upset during the spasms and may experience a very brief loss of consciousness [13].

IS were first described by Dr. William James West in 1841 in a letter describing infantile seizures and developmental regression of his son. At four month of age his son experienced subtle forward head bobbing that increased in frequency and strength leading to clusters of spasms that occurred many times (50-60) per day [14]. At the time

the rare IS were known only to handful to experts. Today after 176 years, IS are the most frequently encountered infantile seizures with significant morbidity [15, 16], and they remain one of the most severe epilepsies of infancy due to poor neurodevelopmental outcomes [17]. However, the annual incidence is relatively low, 0.25 to 0.42 per 1000 live births, accounting for 13-45.5% of infantile epilepsies in population-based studies [15, 18-25], with a slight male predominance [7, 8, 10, 11, 26, 27].

2.1.1 Diagnosis

In addition to spasms, an important diagnostic consideration is hypsarrhythmia - a characteristic chaotic EEG pattern of high amplitude (>200 μV) slow waves with multiple independent spike foci [28-30]. Gibbs and Gibbs coined the term hypsarrhythmia for this EEG pattern in 1952 [31]. Hypsarrhythmia often fluctuates from one focus to another before becoming generalized, although it never becomes rhythmic or develops signature slow waves as seen in typical absence seizures. In most cases hypsarrhythmia appears as a continuous chaotic background activity during waking and sleep [29]. While IS patients may show other EEG abnormalities, hypsarrhythmia is virtually absent in other epilepsy syndromes. However, about a third of IS patients do not have hypsarrhythmia, hypsarrhythmia does not persist throughout the clinical course of the condition [32-36], and EEG patterns evolve [37-39]. Thus, according to some authors hypsarrhythmia is not required for diagnosis of IS [1], whereas hypsarrhythmia is prerequisite for the diagnosis of West syndrome - an early onset epileptic encephalopathy. A diagnosis of West syndrome is made when all three criteria are met:

IS, hypsarrhythmia, and cognitive decline [40-42]. Thus, EEG recording should be obtained as soon as a concern for IS arises and is required to confirm or rule out the diagnosis of IS or West syndrome. A sufficient recording duration to capture wakefulness, sleep, and awakening is recommended [43, 44]. Unlike hypsarrhythmia, spasms appear as distinct sharp wave complexes on the EEG and EMG that may be followed by voltage attenuation, which are more likely to occur during sleep-wake transitions and wakefulness than in sleep [3, 5, 33, 45, 46]. A 24-hour video-EEG has a high probability to capture both hypsarrhythmia and spasms and is recommended when facilities are available. Video-EEG is especially useful since hypsarrhythmia (and to a lesser extent spasms) shows numerous variations [32, 33, 47, 48]. In addition to the diagnostic value, early and aggressive treatment to control spasms and hypsarrhythmia has been shown to improve cognitive outcomes [25, 49-51].

Although not required for diagnosis, obtaining a brain MRI is an established practice to evaluate structural etiologies. In addition, genetic and metabolic screening are emerging practices and are becoming commonplace in developed countries. Although there is insufficient evidence that they improve immediate patient care, they are very likely to improve understanding of etiologies and future treatment options [52-56]. Occasionally, IS can be misdiagnosed as colic/gastroesophageal reflux or movement abnormalities in spastic infants [49], but the correct diagnosis is often made [7, 13] when the child is re-examined due to increased spasm frequency and developmental delays such as loss of head and trunk control or loss of babbling/verbalizations [57]. Moreover, during initial examinations it can be difficult to separate IS or West syndrome from other early

onset epilepsy syndromes such as early myoclonic encephalopathy, myoclonic epilepsy in infancy, Ohtahara syndrome, Dravet syndrome, epilepsy with myoclonic atonic (previously astatic) seizures/Doose syndrome, and partial seizures with secondary spasm-like manifestations. Synchronous video–EEG monitoring and presence of hypsarrhythmia can be especially useful for differential diagnosis [29, 33, 58].

2.1.2 Prognosis

Several factors are implicated to be significant for long-term prognosis of patients with IS and West syndrome. Those considered most important are: underlying etiology, presence or lack of per-existing seizures, developmental abnormalities, and time from the onset of seizures (to diagnosis and) to initiation of treatment [59-65]. In most patients spasms that primarily emerge in first year of life persist throughout the clinical time course of IS, but spontaneously resolve even in the absence of treatment in ~25% of patients at 12 months of age and in ~75% of patients by age of 3 or 4 [15, 33, 66-68]. However, there are a few accounts of persistent and drug resistant spasms that occur from infancy to teenage years [15, 68-70] or spasms reemerging in adulthood [71]. Late onset spasms that appear after one year of age are also noted [72, 73]. Similarly, as the brain develops the chaotic activity of hypsarrhythmia becomes less pronounced and is rare after ~5 years of age [74]. Several studies indicate viral infections as a precipitating factor for spontaneous remission [75-77].

Unfortunately, cessation of spasms and hypsarrhythmia with or without medication may not be the end of seizures for the majority of children with IS (~70%) as they develop other epilepsy syndromes. Only a small percentage (~7-30%) of IS patients show normal or mild developmental regression [4, 13, 15, 33, 78, 79]. Wide ranges of values are reported for the patients who spontaneously do or do not remit. The values here are from the most commonly cited articles and do not reflect all reported values. A close association exists between IS and Lennox Gastaut syndrome (LGS). About 20-50% of patients with IS evolve to LGS [25, 68, 80, 81], especially the drug resistant cases. Additionally, IS can evolve from other epilepsy syndromes. About 70% of patients with early infantile epileptic encephalopathy or Ohtahara syndrome progress to IS, some of which progress to LGS [8, 82-84]. A propensity to progress from Ohtahara syndrome to IS to LGS exists, even though patients can independently acquire them. It is not clear if they are etiologically related or what factors drive the progression in some patients but not others.

Furthermore IS and LGS are severe and often refractory epilepsies that are typically accompanied by comorbid cognitive and behavioral dysfunction including the risk of developing comorbid autism spectrum disorders [85-87]. About 15-33% of IS patients with have autism, and it could be as high as 70% in patients with tuberous sclerosis and IS [88]. Moreover, timely help to address impairments (sometimes subtle but clinically significant) in memory, speech, language, and social interactions, in addition to controlling spasms and hypsarrhythmia, improves functioning in social environments. Thus, overall most patients with IS have a poor prognosis.

2.2 What defines IS?

The definition and classification of IS remains debatable and confusing. Here I present the current views on the definition(s) of IS to give the readers a broader inclusion criteria when the term IS is used in the literature.

The term 'IS' is used to describe the seizure type or the epilepsy syndrome itself (also referred to as 'IS syndrome' in which patients experience other seizure types in addition to spasms and/or hypsarrhythmia). It is increasingly recognized that patients could present with just IS seizures or just hypsarrhythmia or a combination of both, in the presence or absence of developmental delays [1, 89] (Table 2.1). For example, patients who present with IS seizures and psychomotor delay, but not hypsarrhythmia, would be considered to have the epileptic encephalopathy IS without hypsarrhythmia (but not West syndrome). While, IS seizures are an obligatory feature of West syndrome, some authors consider West syndrome to be a subset of the IS syndrome [90]. The confusion arises when the terms IS and West syndrome are used interchangeably in the literature without providing the patient phenotypes [13, 91]. To further the confusion, spasms (that occur primarily in infancy) may continue beyond infancy or may begin after infancy (at 1-2 years of age, late onset spasms) [73, 92-99], and occur in other syndromes (such as Rett's syndrome [95], tuberous sclerosis [100-102], and patients with chromosomal abnormalities [103-108]. Thus some investigators, especially recently, use "epileptic spasms" rather than IS when the spasms persist beyond infancy [28, 41, 48, 71, 109, 110]. Despite these differences, the overall treatment and management of patients is identical.

Furthermore, IS is considered to be an epileptic encephalopathy. By definition epileptic encephalopathy implies that the seizures themselves contribute to cognitive and behavioral impairments (with a wide range of severity) beyond that expected from the underlying pathology (such as a structural brain damage) [41]. Thus, if a patient has IS seizures and/or hypsarrhythmia that spontaneously resolves during the first year of life and has mild to no cognitive or behavioral impairment, should this be considered an epileptic encephalopathy?

Lastly, the nuances concerning of the range of IS phenotypes may not make a big difference when these patients are treated similarly, and it is well appreciated that there is a heterogeneity of phenotypes even in well-defined epilepsy syndromes. Although, this makes a huge difference when new animal models are developed or drugs are tested to prevent spasms or hypsarrhythmia by targeting specific pathways that may or not overlap among these IS phenotypes. Conceptually it is important to know if these represent distinct syndromes or present as a continuum of disorders.

Table 2.1 IS phenotypes

Epileptic encephalopathy	Infantile/ epileptic spasms	Hypsarrhythmia	Developmental regression	Other seizure types at disease onset
IS	+	+/-	Mild to severe	-
IS syndrome	+	+/-	Mild to severe	+
West syndrome	+	+	Severe	-

The main argument in this chapter is that the *GABRB3(N110D)* and *GABRB1(F246S)* mutations disrupt $GABA_AR$ function in cultured HEK293T cells. The

Epi4K consortium described the patients with *GABRB3(N110D)* and *GABRB1(F246S)* mutations to have the epileptic encephalopathy IS [111]. I have used their terminology in the rest of the chapter, disregarding the fact that they may meet West syndrome criteria. Given the ambiguity concerning the definition of IS, I state the phenotypes of patients with these mutations (as described in their supplementary table 13 [111]). The patient with the *GABRB3(N110D)* mutation had classical spasms as the dominant seizure type beginning at 5 months of age and hypsarrhythmia that evolved from multifocal discharges, while information on developmental regression is lacking. The patient had a normal MRI and development prior to seizure onset. Myoclonic spells were the only other seizure type noted. The patient with the *GABRB1(F246S)* mutation had spasms at 35 months of age along with atypical absence, atonic, myoclonic seizures. Gross and fine motor delays and hypotonia observed prior to seizure onset turned into developmental regression after seizure onset at 35 months. Hypsarrhythmia with 2 Hz spike waves was recorded at 4 years, followed by a global developmental delay, low tone, ataxia, cortical visual impairment, and gastrostomy tube insertion to assist food intake at 4.5 years of age. MRI showed a thin corpus callosum but was otherwise normal. The patient with the *GABRB1(F246S)* mutation with IS, hypsarrhythmia, and developmental regression meets the criteria for West syndrome. Whereas the patient with the *GABRB3(N110D)* mutation had early onset classical spasms, and hypsarrhythmia, while his developmental trajectory is unknown.

2.3 Etiologies of IS

Etiology is an important predictor of treatment outcomes for children with IS [1, 90, 112]. Despite significant efforts directed to understand IS since the initial description in 1841, the etiology of IS remains uncertain in terms of the cellular, molecular, and electrophysiological mechanisms. As stated earlier, IS can result from a variety of causes including tuberous sclerosis, Down's syndrome, Ohtahara syndrome, prenatal and perinatal causes, hypoxia, chromosomal abnormalities, periventricular leukomalacia or hemorrhage, metabolic disorders, and trauma. However, only some patients with these antecedent origins develop spasms. Thus, instead of the true underlying molecular etiology and how that results in epilepsy, a diagnostic classification is commonly used. Typically, when the etiology is identified in a clinical setting for diagnostic purposes, the molecular mechanisms underlying the epilepsy syndrome are not precisely known. For example, if an IS patient is found to carry a mutation with known association to epilepsy, it is likely to be causative, and the diagnosis would be considered genetic. However, the protein/mRNA dysfunction brought about by the particular gene mutation may be unknown. According to the recent classification by the International League Against Epilepsy, the etiology can be classified as structural, genetic, infectious, metabolic, and immune, and unknown groups [113]. Thus, based on this broad classification scheme, etiology is known for the majority of the cases (~60%) in the developed world [114, 115], although the specific pathogenic mechanisms are unknown for most cases. A large-scale study found that 61% of patients had a proven

etiology, 33% of patients had no identified etiology, and 6% were not completely examined [114].

The majority of IS patients have structural abnormalities and often have poor developmental outcomes [68, 112, 114, 115]. A structural etiology refers to a brain abnormality visible on a neuroimaging scan, which in combination with the electroclinical assessment could be reasonably identified as the cause of the seizures. The structural etiologies may be acquired by hypoxic-ischemic encephalopathy, infection, stroke, trauma, or genetic abnormalities [113]. For example, if a patient with tuberous sclerosis complex (about 25% of IS patients have tuberous sclerosis [64, 87, 100, 116-120]) due to a genetic mutation has infantile spasms, the structural abnormalities associated with tuberous sclerosis likely cause the patient's epilepsy, although both structural and genetic etiologies could contribute. However, care must be taken when assigning all coincidental conditions as causes of IS. For instance, histidinemia, a relatively common and mild metabolic disorder that occurs in some patients with IS, has not been shown to play a causal role in development of IS.

In addition to structural abnormalities, the role of inborn errors of metabolism is well documented in IS patients. Epilepsy is considered to have a metabolic etiology when it results directly from a known or presumed metabolic disorder, and when seizures are a core symptom of the disorder [113]. Metabolic causes usually refer to a well-described defect of biochemical changes throughout the body, yet the newly discovered metabolic defects could have unknown biochemical pathways or identified gene mutation(s) [121, 122]. Although, several metabolic disorders are likely to have

genetic origins, others may be acquired. A few of the many well-known examples of IS with metabolic origins includes phenylketonuria, proprionic acidemia, nonketotic hyperglycinemia, methylmalonic acidemia, maple-syrup urine disease, pyridoxine-dependent seizures, and Menkes disease [123-131]. Metabolic disorders result in epilepsy by disrupting neuronal energetics, building up of toxins, altering synapses, or disrupting morphogenesis [132-134]. Metabolic screening is important for early diagnosis that allows use of specific treatments to reduce or prevent intellectual impairment.

A small percentage of IS patients have infectious or immune etiologies - two etiological groups in which the seizures are the primary symptom of the disorder as a direct outcome from a known infection or an immune disorder, respectively. The infectious etiology refers to epilepsy and not to seizures due to an acute infection such as encephalitis or meningitis. The human cytomegalovirus infection is the most common intrauterine viral infection, affecting the CNS in 95% of cases and leads to several epilepsy syndromes. However, several viral, bacterial, and parasitic infections, especially in the perinatal period, are attributed to several central nervous system (CNS) defects and symptomatic epilepsies, including IS [135-139]. As the infectious or immune etiologies have a variable prognosis and only account for a small portion of IS patients, they are not discussed in depth in this thesis.

Even today the underlying etiology of some IS patients cannot be determined, and they are classified under the unknown etiological group (previously cryptogenic or idiopathic). This group of patients are characterized by normal neurodevelopment before the time of clinical presentation of spasms, and at the time of diagnosis/treatment

are devoid of known underlying condition(s) that could be associated with IS. Since genetics has an important contribution in several epilepsy syndromes, these patients are referred for genetic testing in developed countries (frequently targeted sequencing, genomic hybridization, and/or whole exome sequencing) especially when clinical exams, MRI, and EEG do not reveal any etiologies [140]. An increasing number of genetic abnormalities are identified in IS patients who would have been otherwise classified as having unknown etiology, and they may account for the majority of unexplained cases. These IS patients are classified to have a genetic etiology (previously idiopathic also referred to as a presumed genetic defect), which refers to the concept that genetic mutation(s) or presumed genetic mutation(s) are directly responsible for seizures that are the core symptom of the disorder.

Our knowledge of the various ways in which genetic changes contribute to the pathogenesis of IS has broadened tremendously since the late 1990s. The genetic changes seen in IS patients include: *de novo* mutations, copy number variations (CNVs) [141, 142], X-linked inheritance (e.g. *ARX, CDKL5,* X-inactivation of STK9 [143]) [143-150], mutations leading to cortical malformations (e.g. *LIS1, DCX*) [151], and chromosomal abnormalities (trisomy, translocations, microdeletions, duplications, triplications, etc) [152-163]. Depending on the population examined, 7-25% (up to 50-60% also reported) of IS patients have a family history of epilepsy [15, 46, 80, 115, 164-166]. Even though a family history of epilepsy increases the risk (~3X) of developing IS when no known structural, metabolic or immune abnormalities, or infections are present (i.e. unknown etiology), some studies suggest that overall children with IS are less likely to have a family history

of epilepsy as compared to children with other epilepsy syndromes or febrile seizures [164]. This observation is supported by recent genetic studies that revealed *de novo* mutations (mostly autosomal) in numerous patients as important genetic contributors to IS [52-54, 167]. These findings clarified the puzzling absence of a family history when a genetic component was suspected and may underlie a large portion of the unexplained IS cases, even when family history of epilepsy is lacking [168, 169]. *GABRB1, GABRB3, GABRA1* are recent additions to the large and rapidly expanding list of genes in which *de novo* mutations have been found in IS patients. Collectively, these findings point to multiple genetic causes of IS.

Remarkable progress in uncovering genetic etiologies of IS, even at the levels of identifying ultra-rare deleterious variants [170] and coding variation among thousands of control subjects, is paving the way for translating these genetic findings into clinical practice. To impact patient care, however, knowledge of molecular mechanisms altered by the causative mutations is essential to alter current treatments or develop new ones. We have taken one of the first steps to this end by elucidating the actions of IS-associated *GABRB3(N110D)* and *GABRB1(F246S)* mutations identified by the Epi4K consortium in 2013 - one of the first large scale genetic sequencing studies that identified monogenic causes of IS and LGS [111] (Table 2.1). *In silico* methods (such as PolyPhen-2 or SIFT scores) are a good proxy to determine the effects of *de novo* mutations, although empirical studies to understand their functional consequences are critical as their impact is not always straight forward [171] and may not reveal unanticipated effects as described in chapters II and III.

Table 2.2. *De novo GABRB* mutations identified in patients with IS

Mutation	Nucleotide change	CCDS transcript ID	Location in β1/3 subunit	PolyPhen-2 score	Sex	Age at seizure onset	Initial seizure type
GABRB3 (N110D)	15:26866594 T→C	CCDS53920.1	N-terminal, β(-) interface	0.965	F	5 mo.	IS
GABRB1 (F246S)	4:47405630 T→C	CCDS3474.1	TM1	0.997	M	12 mo.	FDS

CCDS transcript ID = consensus coding sequence identifier of the GABRB3/1 genes, FDS=Focal dyscognitive seizures

2.4 Materials and methods

2.4.1 Complementary DNA (cDNA) Constructs

cDNAs encoding human $GABA_AR$ subunits α1 (NM_000806.5), β1 (NM_000812.3), β3 (NM_021912.4, variant 2), γ2L (NM_198904.2) and EGFP (LC008490.1) were each cloned into the pcDNA(3.1+) vector. Point mutations in the cDNA encoding β1 and γ2L subunits were introduced using the QuikChange Site-Directed Mutagenesis kit (Agilent). The hemagglutinin (HA) epitope tag (YPYDVPDYA) was inserted between amino acids 4 and 5 of the mature β1 and γ2L subunit proteins. The HA epitope tag insertion at this position in the γ2L subunit has minimal effects on the electrophysiological properties or surface levels of $GABA_ARs$. All constructs were sequenced prior to use at the Vanderbilt Technologies for Advanced Genomics core facility and verified against published sequences. The amino acids are numbered according to the immature peptide sequence.

2.1.2 Expression of recombinant $GABA_A$Rs

HEK293T cells (HEK 293T/17, ATCC® CRL-11268™) were cultured as monolayers at 37°C in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 100 IU/ml each of penicillin and streptomycin (Invitrogen).

For whole electrophysiology, cells were plated at a density of 4 x 10^5 in 60 mm culture dishes (Corning). cDNA transfections were done 24 hours after plating cells, and they were re-plated for recording using trypsin-EDTA digestion (Invitrogen) in 35 mm dishes 24 hours after transfection. For the homozygous expression condition, 0.6 µg cDNA of each α1, β1/3, and γ2L subunit and 0.1 µg of EGFP were transfected using X-tremeGENE9 DNA Transfection Reagent (Roche Diagnostics). For the heterozygous condition 0.6 µg α1 and γ2L, 0.3 µg wt β1/3, 0.3 µg mutant β1/3, and 0.1 µg EGFP of cDNAs were used. A ratio of 1.15 µl X-tremeGENE9:1 µg cDNA was used. EGFP and $GABA_A$R subunit cDNAs were co-transfected to identify transfected cells based on GFP fluorescence. For single channel recordings, HEK293T cells were plated at 4 x 10^4 in 35 mm culture dishes (Corning) and transfected after 24 hours with 0.3 µg cDNA of each α1, β1/3, and γ2L subunit and 0.05 µg cDNA of EGFP for the homozygous condition. Recordings were done 48 hours after transfection. The cDNA and XtremeGENE9 amounts were scaled up or down in proportion to the area of the culture dishes. Our unpublished observations show that cells lacking GFP expression do not produce GABA-evoked currents, indicating that GFP is a good proxy for α, β, and γ subunit expression. In addition when cells are transfected with wt α, β, and γ subunit-

encoding cDNAs, they produce typical αβγ subunit-like current responses and lack, for example, of αβ subunit-like currents. Although, αβ subunit-containing $GABA_AR$s could constitute a minor portion of all receptors and their presence cannot be ruled out, the majority of the receptors are likely to be αβγ subunit-containing.

For flow cytometry experiments, cells were plated at a density of 4-6 x 10^5 in 60 mm culture dish (Corning), and transfected with 0.6 µg cDNA for each α1, β1/3, and γ2L subunit 24 hours after plating using Polyethyleneimine (MW 40,000 KD, 24765, Polysciences Inc.). Cells were harvested using trypsin-EDTA (Invitrogen) 48 hours following transfection. For mock or single subunit expression, empty pcDNA3.1 vector was added to make the final cDNA transfection amount to be 1.8 µg.

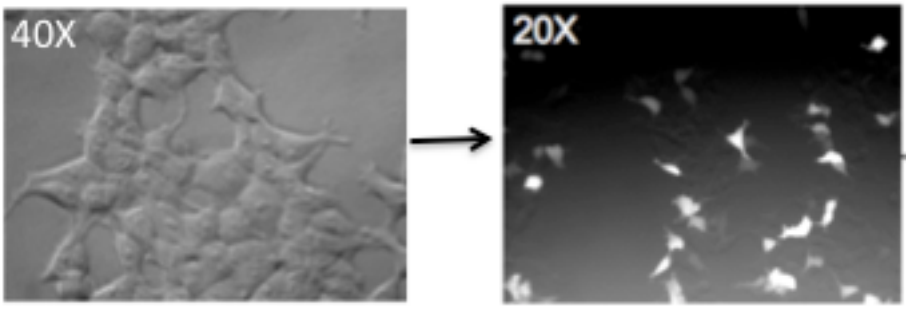


Figure 2.1 Expression of $GABA_AR$ subunits and selection marker GFP in HEK293T cells.

Bright filed image of non-transfected HEK293T cells (left) and fluorescent image (right) of HEK293T cells 48 hours after transfecting with cDNAs encoding GABAAR subunits and GFP. Cells expressing GFP were used for electrophysiological recordings. For flow cytometry analysis a threshold intensity of 103 arbitrary units of GFP fluorescence (488 nm) was used post-hoc to accept GFP-expressing cells.

2.4.3 Whole cell electrophysiology

Whole cell voltage clamp recordings were obtained at room temperature from lifted HEK293T cells bathed in an external solution composed of (in mM): 142 NaCl, 8 KCl, 10 D(+)-glucose, 10 HEPES, 6 MgCl2.6H2O, and 1 CaCl2 (pH 7.4, ~326 mOsm). Recording electrodes with series resistance of 1-2 MΩ were pulled from TW15OF-3 thin walled glass capillaries (World Precision Instruments, Inc.) using a P-2000 Quartz Micropipette Puller (Sutter Instruments), and the tip was polished using MF-830 Micro Forge (Narishige). The internal solution consisted of (in mM): 153 KCl, 10 HEPES, 5 EGTA 2 Mg-ATP, and 1 MgCl2.6H2O (pH 7.3, ~300 mOsm). The Cl- reversal potential was near 0 mV, and cells were voltage clamped at -20mV to get an inward GABA-evoked current. Whole-cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments) low-pass filtered at 2 kHz using the internal 4-Pole Bessel filter of the amplifier, digitized at 10KHz with Digidata 1322A (Axon Instruments) and stored for offline analysis. Drugs were gravity-fed to a four-barrel square glass tubing connected to a SF-77B Perfusion Fast-Step system (Warner Instruments Corporations). The 10-90 % rise times of open-tip liquid junction currents were 200-600 μs. 1 mM GABA was applied for 4s to study macroscopic current kinetics. Data were analyzed offline using Clampfit 9.0 (Axon Instruments). Data were expressed as mean ± standard error of the mean (SEM).

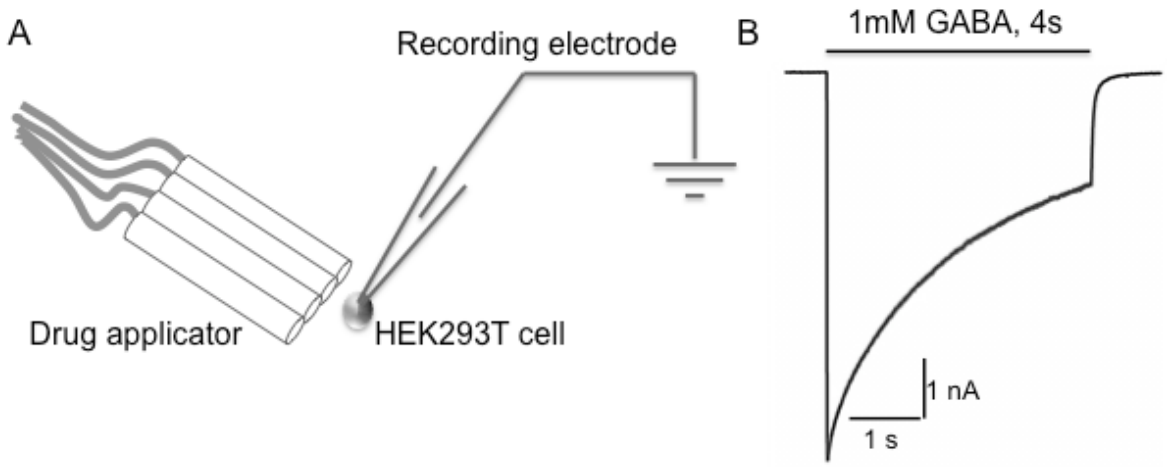


Figure 2.2 Schematic of single cell recording from lifted HEK293T cells expressing $GABA_A$ receptors.
(A) Rapid (<1 ms) application of GABA and Zn on lifted HEK293T cells was carried out using a glass barrel with multiple channels. GABA-evoked current were recorded by from an electrode inserted in a glass capillary. **(B)** Typical GABA-evoked current from cells expressing wild-type α, β, and γ $GABA_A$ receptor subunits with a profound desensitization during prolonged GABA application.

2.4.4 Single channel electrophysiology

$GABA_AR$ single channel currents were recorded in the cell-attached configuration as described previously [172, 173]. Briefly, single-channel currents were recorded from HEK293T cells bathed in external solution containing (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 glucose, and 10 HEPES (pH 7.4). Glass electrodes were pulled from thick-walled borosilicate capillary glass (World Precision Instruments) on a P-2000 Quartz Micropipette Puller (Sutter Instruments) and fire-polished to a resistance of 10–20 MΩ on an MF-830 Micro Forge (Narishige) before use. The electrode solution consisted of (in mM): 120 NaCl, 5 KCl, 10 MgCl2, 0.1 CaCl2, 10 glucose, and 10 HEPES (pH 7.4). The electrode potential was held at +80 mV. $GABA_AR$ spontaneous activity was recorded in absence of GABA and blocked by adding 100 μM picrotoxin

and 100 μM Zn^{+2}. GABA-evoked currents were recorder in presence of 1 mM GABA. Single channel currents were amplified and low-pass filtered at 2 kHz using an Axopatch 200B amplifier, digitized at 20 kHz using Digidata 1322A, and saved using pCLAMP 9 (Axon Instruments). Data were analyzed offline using TAC 4.2 and TACFit 4.2 (Bruxton Corporation) [172, 173]. Data were shown as the mean ± SEM. Statistical analysis was performed using GraphPad Prism (GraphPad Software 6.0). Statistical significance was taken as $p < 0.05$, using unpaired two-tailed Student's *t* test or one-way ANOVA as appropriate. GABA, Zn^{+2} and picrotoxin were obtained from Sigma.

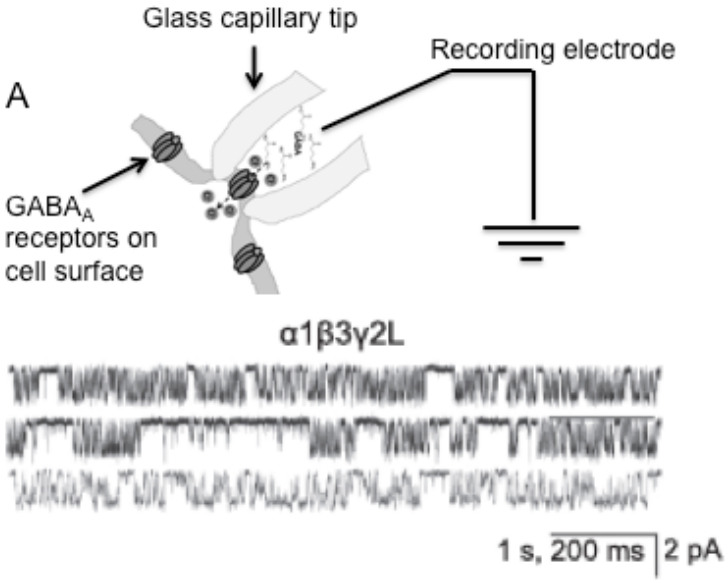


Figure 2.3 Schematic of single channel recording from HEK293T cells expressing GABA$_A$ receptors.

(A) A tight seal is made on the cell membrane of HEK293T cells expressing GABA$_A$ receptors such that one or few receptors are engulfed in the narrow tip of the glass capillary that contains saturating GABA concentration (1 mM). (B) Typical single channel currents from from cells expressing wild-type α, β, and γ GABA$_A$ receptor subunits. Each downward deflection is a channel opening.

2.4.5 Flow Cytometry

Flow cytometry protocols have been previously described in detail [174, 175]. Briefly, $GABA_AR$s were expressed as described above, and cells were harvested ~48 hours after transfection and immediately placed on ice in 4°C FACS buffer. Cell surface expression levels of α1, β3, $β1^{HA}$, $γ2L^{HA}$ subunits were determined using primary antibodies against human α1 subunits (N-terminal, clone BD24, Millipore; 2.5 g/ml), human β3 subunits (N-terminal, monoclonal, β2/3-PE, clone 62-3G1, Millipore; 2.5 g/ml), and the HA epitope tag (clone 16B12, Covance; 2.5 g/ml) respectively. Cells were washed 3 times using 4°C cold FACS buffer and fixed with 2% w/v paraformaldehyde. To determine the total cellular expression levels, cells were fixed for 15 min using BD Cytofix/CytopermTM fixation/permeabilization buffer (BD Biosciences) and washed 2 times using BD Perm/WashTM staining buffer (BD Biosciences) prior to staining. Following washes after primary antibody treatment cells were incubated with anti-mouse IgG1 secondary antibody conjugated to the Alexa647 fluorophore (Invitrogen) before additional washing and fixation.

Fluorescence intensity levels of cells were determined using a BD LSR II 3/5-laser flow cytometer (BD Biosciences). Data were analyzed offline using FlowJo 7.5.5 (Tree Star). For each condition, intensity levels of 50,000 cells were acquired. The non-viable cells were excluded using their forward and side-scatter properties. Net fluorescence intensity levels from cells expressing $GABA_AR$ subunits were calculated after subtracting the mean fluorescence intensities of the cells transfected with blank pcDNA(3.1+) vector. The relative fluorescence intensities for each condition were

calculated by normalizing the average intensity values to those obtained from cells expressing α1, β3/β1^{HA}, and γ2L^{HA} subunits (WT). One-way analysis of variance with Tukey's post-test was used to determine if the expression levels of different transfection conditions were significantly different from the WT condition. Data were expressed as mean ± SEM.

We have used β1 subunits for whole cell and single cell electrophysiology techniques, and β1 as well as β1^{HA} subunits for flowcytometry technique as none of the following β1 antibodies were found to be specific when tested in our laboratory. We used 1:500 of Millipore (AB9680), 1:500 of Neuromap (N95/55), and 1:300 of Novus (NB300-197) primary antibodies against human β1 subunits based on manufactures' instructions. Although, Millipore, (AB9680, 1:500) produced an expected sharp band (~55 kD) against rat β1 subunits.

2.4.6 Structural Modeling and Simulation

$GABA_AR$ subunit raw sequences in FASTA format were individually loaded into Swiss-PdbViewer 4.10 [176] for template searching against ExPDB database (ExPASy, http://www.expasy.org/). Then, the structure of the *C. elegans* glutamate-gated chloride channel (GluCl; PDB: 3RHW) [177] in the closed conformation was identified as the best template resulting in 33% and 36% sequence identity for γ2 and α1 subunits respectively. For β3 subunits, the human $GABA_AR$-β3 (PDB: 4COF) [178] crystal structure was used *per se* with no further modification. The initial sequence alignments between γ2 and α1 subunits and *C. elegans GluCl* subunits were generated with full-length

multiple alignments using ClustalW [179]. Sequence alignments were inspected manually to assure accuracy among structural domains solved from the template. Because the long cytoplasmic regions of the γ2 and α1 subunits were absent in the solved GluCl structure, it was excluded from the modeling, and separate alignments were generated for the TM4 domains. Then full-length multiple alignments were submitted for automated comparative protein modeling implemented in the program suite incorporated in SWISS-MODEL (http://swissmodel.expasy.org/SWISS-MODEL.html). Before energy minimization using GROMOS96 [180], resulting structural models of human γ2 and α1 subunits were inspected manually, their structural alignments confirmed and evaluated for proper h-bonds, presence of clashes and missing atoms using Molegro Molecular Viewer (www.clcbio.com). Then, pentameric $GABA_AR$ homology models were generated by combining α1, β3 and γ2 structural models in the stoichiometry 2β:2α:1γ with the subunit arrangement β-α-β-α-γ. Neighborhood structural conformational changes at the β3+/α1-, α1+/β3-, and γ2+/β3- interfaces of the $GABA_AR$ caused by a single mutated amino acid residue (*de novo* mutation) in the human β3 subunit were simulated using Rosetta 3.1 [181] implemented in the program suite incorporate in Rosetta Backrub (https://kortemmelab.ucsf.edu). Since Rosetta 3.1 does not allow Cysteine substitutions, $GABA_ARs$ carrying Cysteine mutations were exchanged by Alanine. Then, up to twenty of the best-scoring structures were generated at each time by choosing parameters recommended by the application for one mutation at the time for each correspondent subunit interface as follow: β3(D120N), β3(E18G), and β3(Y302A) within the β3+/α1- interface, and β3(N110D) and β3(F246S) at the α1+/β3- and γ2+/β3- interfaces. All single point mutations were incorporated the

β3 subunit since the full-length alignment between the β3 and β1 sequences displayed high similarity (91.2 %). Root mean square deviation (RMS) was calculated between the initial (wild-type) structures and superimposed simulated (mutated) structures. For each mutation, the RMS average over ten low energy structures was computed and conformational changes displayed among neighborhood structural domains. We prepared the figures using Chimera 1.7 [182] and Swiss-PdbViewer 4.1.0 [176].

2.4.7 Data analysis

Electrophysiology data were analyzed offline using Clampfit 10.3 software (Axon Instruments) and were expressed as mean ± standard error of the mean (SEM). One mM GABA was applied for 4 s to study macroscopic current kinetics. Peak currents and 10-90% rise time of currents were calculated using a built-in function in the Clampfit 10.3 software. The current desensitization time courses were fitted using the Levenberg-Marquardt least squares method with multiple component exponential functions of the form $\sum_{i=1}^{n} A_i \, e^{-t/\tau i} + C$, where n is the number of exponential components, A is the relative (fractional) amplitude of the component at time = 0, t is time, τ is the time constant, and C is a constant offset in the y direction. Fitting the decay of currents evoked with 4 s GABA applications resulted in three or four exponential components. The number of components were increased until additional components did not significantly improve the fit. The fit was determined by an F-test on the residuals. To simplify comparisons a weighted sum of desensitization time courses was used. The current deactivation following removal of GABA was fitted suing mono-

exponential equation (n = 1), % desensitization was calculated by subtracting the GABA current at the end of 4 s application from the peak GABA current, and then normalized to the peak GABA current. To determine the % Zn^{2+} inhibition of GABA currents, an extended wash for 40 s followed 4s GABA (1 mM) application and then 10 μM Zn^{2+} was applied for 10 s, followed by co-application of 1 mM GABA and 10 μM Zn^{2+} for 4 s. Peak currents from co-application of GABA and Zn^{2+} were subtracted from the peak GABA-evoked currents and then normalized to the peak GABA-evoked current. Holding current was calculated as the average of baseline current before GABA application. The outward Zn^{2+} current during the application of 10 μM Zn^{2+} for 10 s was calculated by subtracting the 6 s average baseline current before Zn^{2+} application from the average of current during the last 6 s of Zn^{2+} application.

2.5 RESULTS

2.5.1 *De novo GABRB3* and *GABRB1* mutations identified in patients with IS were located in conserved structural domains of $GABA_AR$ β subuni*ts.*

When protein sequences of $GABA_AR$ α,β, and γ subunits were aligned we found that the β3(N110) subunit residue was conserved in α1-6 and β1-3 subunits, while the β1(F246) subunit residue was conserved among all α, β, and γ subunits (Figure 2.2A). Next the mutations were mapped on the structural model of the $GABA_AR$. The β3(N110) subunit residue was in the N-terminal α2 helix at the β-/α+ interface, and the β1(F246) subunit residue was in the transmembrane domain 1 (TM1) of β1 subunit (Figure 2.2B).

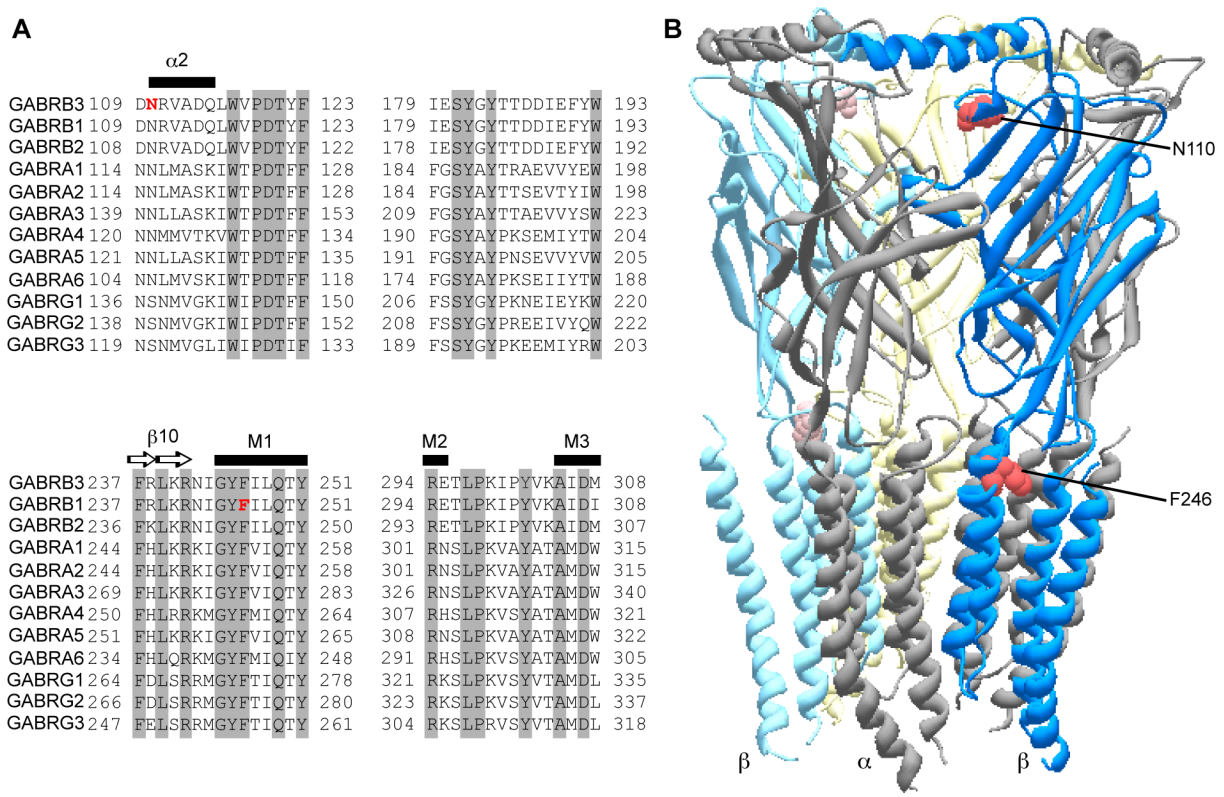


Figure 2.4 IS-associated *GABRB1/3* mutations alter conserved β1/3 subunit domains.

(A) Protein sequence alignment of $GABA_AR$ α (1-6), β (1-3), and γ (1-3) show that gene mutations alter conserved amino acids. Mutated amino residues are in red and residues conserved across α, β, and γ subunits in grey. Secondary structures are represented above the alignments as a helices (black bars) or β sheets (arrows). **(B)** Three-dimensional structural model of the $GABA_AR$ with amino acid residues altered by *GABRB3(N110D)* and *GABRB1(F246S)* mutations mapped on β3 subunits (shown in red). The β3 subunits are in blue, α1 subunits in gray, and γ2L subunit is in yellow.

2.5.2 IS-associated *de novo GABRB1/3* mutations had minimal effects on GABA-evoked current amplitudes.

Functional consequences of IS-associated mutations were determined by measuring whole cell GABA-evoked α1β1/3γ2L currents from lifted HEK293T cells following rapid application of 1 mM GABA. Cells were transfected with equimolar quantities of α1, β1/3 (wt or mutant), and γ2L subunit-expressing cDNAs in homozygous (hom) condition or with α1, 0.5β1/3 (wt), 0.5β1/3(mutant), and γ2L expressing cDNAs. GABA-evoked peak current amplitudes were unaltered for cells expressing β3(N110D) subunit-containing receptors in hom and het conditions, whereas GABA-evoked currents were significantly reduced (by 25%) from cells expressing β1(F246S) subunits in hom but not in the het condition (Figure 2.3, Table 2.2). This suggests that the loss of GABA-evoked currents is not the primary mechanism of action of IS-associated mutations.

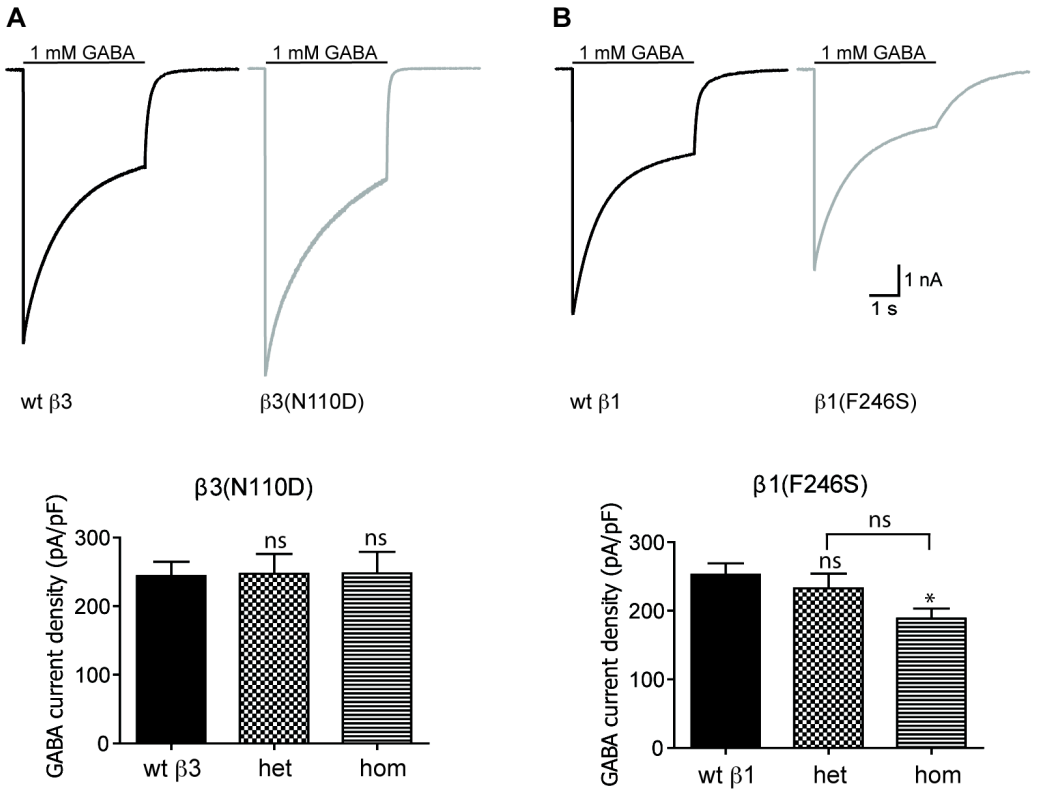


Figure 2.5 *De novo GABRB1/3* mutations found in IS patients produced either no change or small reduction of GABA-evoked currents.

(A) Representative GABA-evoked current traces obtained following rapid application of 1 mM GABA for 4 s to lifted HEK293T cells voltage clamped at -20 mV. The current traces from $GABA_AR$s containing mutant β1/3 subunits in the homozygous (hom) condition are compared to their respective wild-type (wt) current traces. **(B)** Bar graphs showing average peak current densities from cells expressing mutant β subunits in hom and heterozygous (het) conditions. Values are expressed as mean ± standard error of the mean (see Table 2.2 for details).

Values are expressed as mean ± standard error of the mean. One-way analysis of variance with Dunnett post-test was used to determine significance. */#Significant difference compared to the wt and het conditions, respectively. *p < 0.05, **/##p < 0.001, ***/###p < 0.0001. ns = not significant.

2.5.3 Predominant actions of IS-associated *de novo GABRB1/3* mutations were altered GABA-evoked current kinetics.

Next we examined the impact of IS-associated mutations on important current kinetic properties—activation, desensitization, and deactivation— that govern the shape and amplitude inhibitory post-synaptic currents (IPSCs) in neurons. We found that both the *GABRB3* and *GABRB1* mutations altered current kinetic properties but in opposite directions. The *GABRB3(N110D)* mutation significantly slowed current activation (longer 10–90% rise time) in the hom condition but not in the het condition (Figure 2.4A, left traces and bar graph, Table 2.2). In contrast, currents from β1(F246S) subunit-expressing cells had reduced rise times in the het condition but not in the hom condition (Figure 2.4A, right traces and bar graph). Furthermore, we calculated current deactivation by measuring current decay after termination of GABA application. The β3(N110D) subunit-containing receptors increased the current deactivation rate (reduced weighted deactivation rate constant) both in the hom and het conditions (Figure 2.4C left traces and bar graph, Table 2.2). Conversely, the β1(F246S) subunit-containing receptors decreased the deactivation rate (increased weighted deactivation rate constant) compared to currents from wt β1 containing receptors (Figure 2.4C right traces and bar graph, Table 2.2). In addition to 4s GABA application we did 10 ms rapid application of GABA pulses (a more accurate method to determine current rise and deactivation) and got similar results for current rise and deactivation (Figure 2.4E and F). Current desensitization was not affected by either of the *GABRB* mutations in the

hom or het expression conditions (see Table 2.2). Thus, the primary impact of IS-associated *GABRB* mutations was altered current kinetics.

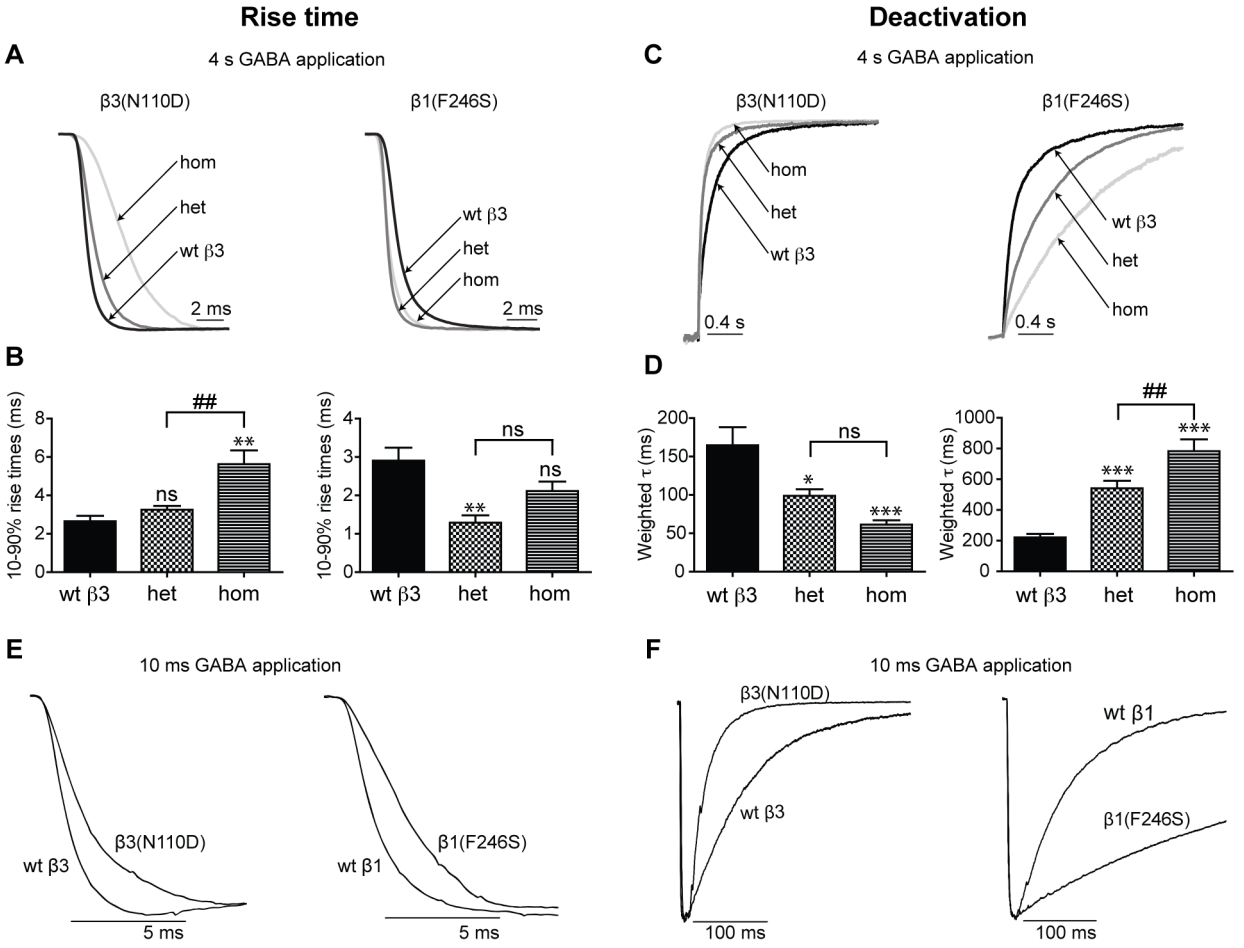


Figure 2.6 The *de novo GABRB1/3* mutations found in IS patients altered $GABA_AR$ current kinetic properties.
(A and C) Representative traces showing rise times of GABA-evoked currents produced by 4 second (A and C, top panel) application of 1mM GABA to wild-type (wt) receptors or receptors containing β3(N110D) or β1(F246S) subunits. **(B and D)** Bar graphs show average rise times from the cells expressing wt $GABA_ARs$ or receptors containing β3(N110D) or β1(F246S) subunits. **(E and F)** Representative current traces showing rise times and deactivation of current relaxation at the end of 10 millisecond GABA application (1 mM) to wt receptors or receptors containing the β3(N110D) or β1(F246S) subunits in the hom condition.

Values are expressed as mean ± standard error of the mean (see Table 2.2 for details). One-way analysis of variance with Dunnett post-test was used to determine significance. */# Significant difference compared to the wt and het conditions, respectively. *p < 0.05, **/##p < 0.001, ***p < 0.0001. ns = not significant.

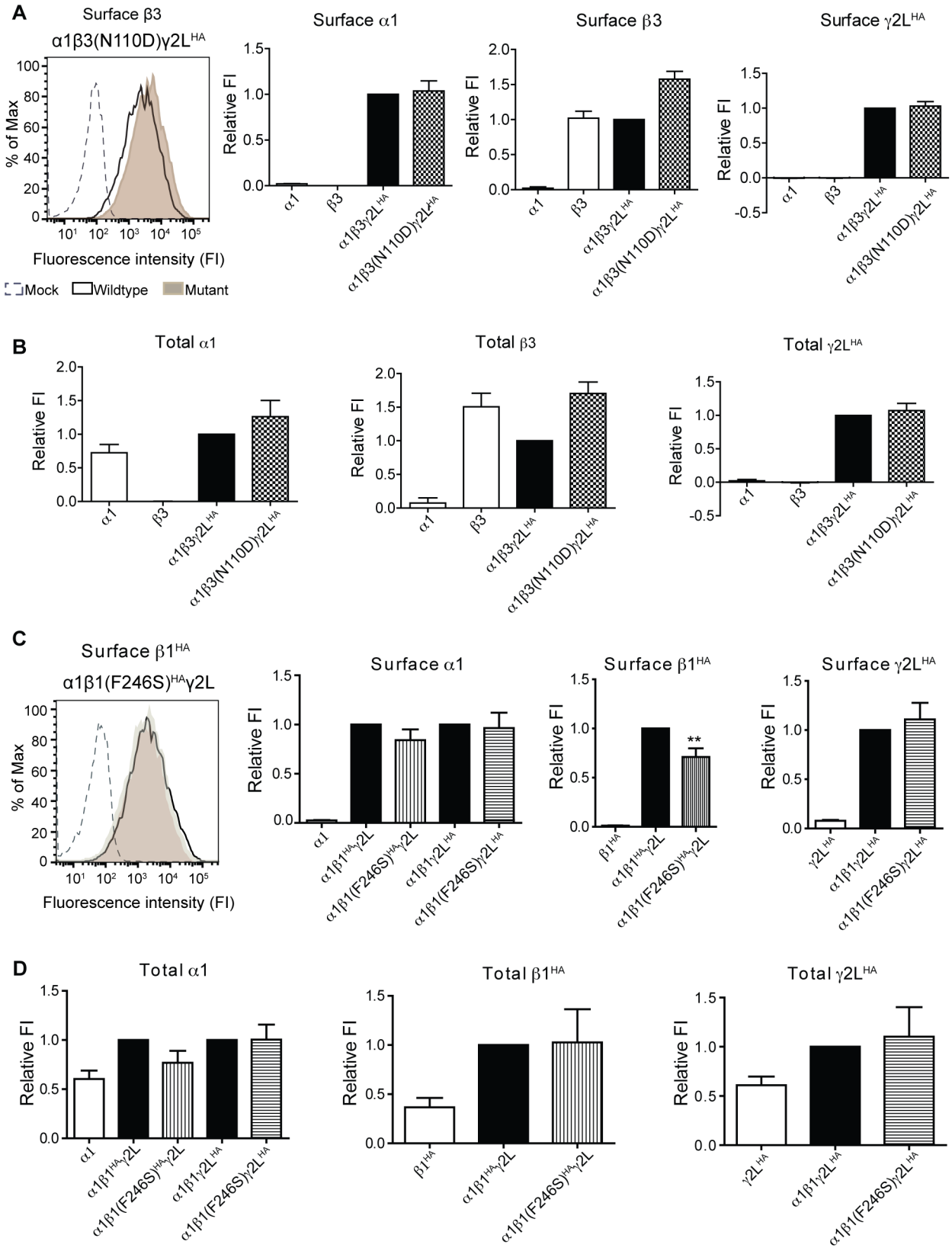
2.5.4 IS-associated *de novo GABRB* mutations did not reduce surface levels of α,β, or γ subunits.

As we observed a small reduction in the peak current amplitudes from cells expressing α1β1(F246S)γ2L subunits in hom condition (Figure 2.2B), we evaluated surface levels of $GABA_AR$ subunits using flow cytometry technique. Additionally, surface levels for the α1β3(N110D)γ2L hom condition were also examined. As none of the commercially available antibodies against the $GABA_AR$ β1 subunits were found to be specific when tested in our laboratory, we incorporated hemagglutinin (HA) epitope tag (YPYDVPDYA) between amino acids 4 and 5 of the mature β1 and β1(F246S) subunits. Moreover HA-tagged γ2L subunits were used due to better signal to noise ratio.

As expected we found that the neither the surface levels nor the total levels of α1, β3, and $\gamma2L^{HA}$ were reduced in the hom condition. However, surprisingly, the β3(N110D) subunit levels were significantly increased (surface levels=142.3 ± 8% and total levels = 170 ± 17% of wt condition), without increasing α1 or $\gamma2L^{HA}$ subunit levels (see Figure 2.5A and B, Table 2.2). An unusual property of wt β3 subunits is that when expressed alone (in absence of α1 or γ2 subunits) they are found on the cell surface suggesting they form homomeric β3 receptors[183, 184] (Figure 2.5. A, middle bar graph). These results indicate that the β3(N110D) subunits enhance the formation of surface

homomeric β3 receptors, although the significance of this finding is not clear as homomeric $GABA_A$Rs have not be described in neurons.

Furthermore, similar to a small decrease in the peak currents the β1(F246S)^{HA} subunit levels were slightly but significantly reduced compared to wt β1^{HA} levels, without any alteration in α1 or γ2L^{HA} levels (see Figure 2.5C). While the total expression levels of α1, β1^{HA}, and γ2L^{HA} were not changed (see Figure 2.5D). Reduced surface β1(F246S) subunit levels without a decrease in the total levels suggest that mutant subunits affected the assembly and/or trafficking of heteromeric $GABA_A$Rs but not the biogenesis of the mutant subunits. However, the presence of HA tag could affect the assembly and/or trafficking, especially when 2 tagged β1^{HA} subunits are present in the pentameric (β-α-β-α-γ) $GABA_A$R. In absence of β1 specific antibodies this issue remains unresolved.


A
Surface β3
α1β3(N110D)γ2L^HA
% of Max
Fluorescence intensity (FI)
Mock
Wildtype
Mutant
Surface α1
Surface β3
Surface γ2L^HA
Relative FI
α1
β3
α1β3γ2L^HA
α1β3(N110D)γ2L^HA
B
Total α1
Total β3
Total γ2L^HA
C
Surface β1^HA
α1β1(F246S)^HAγ2L
Surface α1
Surface β1^HA
Surface γ2L^HA
α1β1^HAγ2L
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α1β1(F246S)γ2L^HA
β1^HA
γ2L^HA
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D
Total α1
Total β1^HA
Total γ2L^HA

Figure 2.7 The *GABRB3(N110D)* mutation did not reduce surface and total levels of $GABA_AR$ subunits, while and *GABRB1(F246S)* mutation produced small reduction of $\beta1^{HA}$ surface levels.

Flow cytometry was used to determine surface **(A)** and total **(B)** levels of α1, $\beta1^{HA}$/ β3, and $\gamma2L^{HA}$ subunits in HEK293T cells. The *GABRB3(N110D)* mutation reduced surface or total levels of $GABA_AR$ subunits, while the *GABRB1(F246S)* mutation produced small but significant reduction of $\beta1^{HA}$ subunits. **(A and C left most panel)** Representative fluorescence intensity (FI) histograms showing the surface levels of $\beta1^{HA}$/ β3 subunits from cells expressing α1, mutant $\beta1^{HA}$/ β3, $\gamma2L^{HA}$ subunits in hom condition (shaded), α1, wt $\beta1^{HA}$/ β3, $\gamma2L^{HA}$ subunits (unfilled with solid black line), and empty vector (unfilled with dotted line). Normalized FI values of the Alexa 674 fluorophore are presented in bar graphs, with FI for each condition normalized to the FI of the wild-type (wt) condition (Relative FI). The **(A and C)** and total **(B and D)** panels respectively show the surface Relative FI levels of the α1, $\beta1^{HA}$/β3, and $\gamma2L^{HA}$ subunits. Cells expressing only α1, $\beta1^{HA}$/β3, or $\gamma2L^{HA}$ subunits were used as controls. Unlike the β3(N110D) subunit levels, the $\beta1(F246S)^{HA}$ subunit levels were decreased by 28.8% of the wt $\beta1^{HA}$ levels.

Values were expressed as mean ± standard error of the mean. One-way analysis of variance with Dunnett post-test was used to determine significance. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ compared to the wt condition.

2.5.5 IS-associated *GABRB* mutations impaired single channel gating properties of $GABA_AR$s.

Single channel currents are a direct readout of the channel gating properties. Thus, microscopic single channel currents of wt and mutant $\alpha1\beta1/3\gamma2L$ subunit-containing receptors were obtained from cell attached out-side-out patched in sustained presence of 1 mM GABA. In response to 1 mM GABA, wt $\alpha1\beta3\gamma2L$ and $\alpha1\beta1\gamma2L$ receptors opened into brief bursts and frequent prolonged (>500 milliseconds) clusters of bursts to a main conductance level of ~26 pS (Figs 2.6A, Tables 2.3) with channel open probabilities (Po) of 0.62 ± 0.05. and 0.48 ± 0.03, respectively. Open time distributions were best fitted by 3 weighted exponential functions (a_{o1}, a_{o2}, and a_{o3}) with 3 open time

constants (τ_{o1}, τ_{o2}, and τ_{o3}; Figs 2.6B and E, top left panels), suggesting channels open to at least 3 different states.

The *GABRB3(N110D)* mutation dramatically reduced channel Po (to 17.7% of wt) without changing the single channel opening frequency or mean open time (Figure 2.6, Table 2.3). Consistent with this, the open time constants and relative area under the curves were unaltered. The *GABRB3(N110D)* mutation decreased channel Po largely by reducing burst duration (to 70% of control), resulting from a shift to briefer bursts. The slow rise time and fast current deactivation of macroscopic currents from β3(N110D) subunit-containing receptors is accounted by reduced Po, number of openings per burst, and burst duration.

In contrast, the *GABRB1(F246S)* mutation did not alter channel Po in spite decreasing the opening frequency due to a gain of function resulting in increased mean open time (~3.75-fold) and burst duration (~3.2-fold) from prolonged openings. These changes are reflected as significant shifts in the distribution of the longest open states and may account for the prolonged macroscopic current deactivation. In addition, the *GABRB1(F246S)* mutation reduced single channel conductance level to ~21pS, which accounted for a small reduction of whole cell GABA-evoked currents in the α1β1(F246S)γ2L hom condition. Thus, *GABRB3(N110D)* and *GABRB1(F246S)* mutations have opposing effects on single channel properties similar to those seen with macroscopic current kinetics.

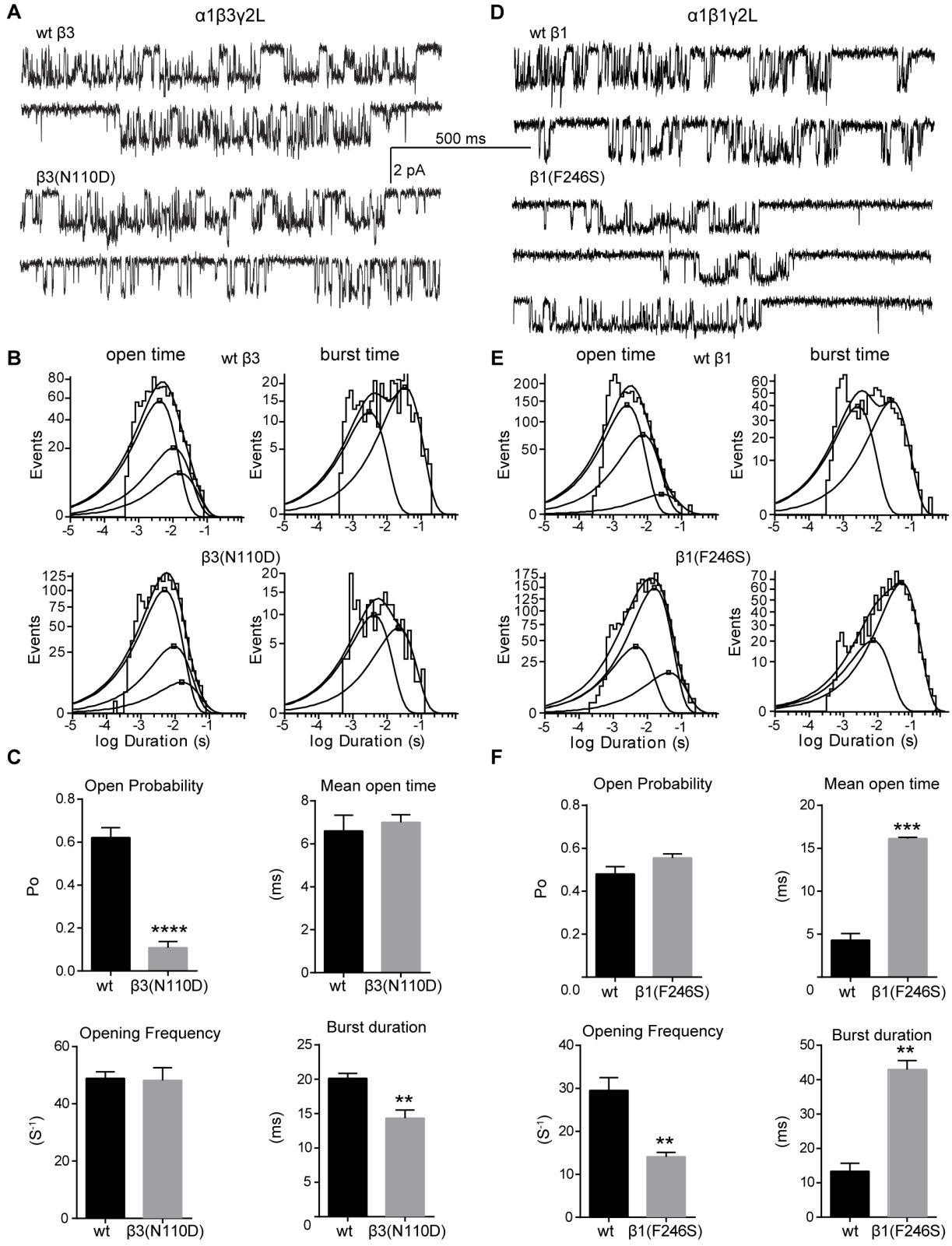
A
α1β3γ2L
wt β3
500 ms
2 pA
β3(N110D)
D
α1β1γ2L
wt β1
β1(F246S)
B
open time
wt β3
burst time
Events
β3(N110D)
log Duration (s)
E
open time
wt β1
burst time
β1(F246S)
C
Open Probability
Po
****
wt
β3(N110D)
Mean open time
(ms)
Opening Frequency
(S-1)
Burst duration
**
F
Open Probability
β1(F246S)
Mean open time
***
Opening Frequency
Burst duration

Figure 2.8 Single channel properties of $GABA_AR$s with wt and mutant β1/3 subunits.

Single channel currents were obtained using cell attached recordings with 1 mM GABA in the recording pipette. **(A and D)** Representative single channel current traces from cells expressing wild-type (wt) or mutant $GABA_AR$s (hom condition). **(B and E)** Mean open time (left panels) and burst duration (right panels) histograms for wt and mutant receptors were fitted best to 3 and 2 exponential functions, respectively. The average of open and burst duration histograms (sums of multiple exponential functions) are marked with a square. **(C and F)** Bar graphs summarize the effects of wt and LGS-associated *GABRB3* mutations on the kinetic properties of the receptors. *GABRB3(N110D)* and *GABRB1(F246S)* mutations had opposite effects on the single channel properties. The *GABRB3(N110D)* mutation reduced Po and burst duration of $GABA_AR$ channels. While the *GABRB1(F246S)* mutation reduced opening frequency and mean open time and burst duration.

Values represent mean ± standard error of the mean. Statistical differences were determined using one-way analysis of variance with Dunnett multiple comparisons test (see Table 2 for details). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Table 2.3. Single channel properties of the *de novo GABRB* mutations associated with IS.

	α1β3γ2L (n)		α1β1γ2L (n)	
	wt β3 (6)	β3(N110D) (5)	wt β1(6)	β1(F246S) (3)
Channel conductance (pS)	24.79 ± 1.62	23.66 ± 0.97	25.72 ± 1.09	22.15 ± 0.75*
Mean open time (ms)	6.60 ± 0.73	7.00 ± 0.36	4.30 ± 0.78	16.13 ± 0.14***
Opening frequency(S^{-1})	49 ± 2	48 ± 4	30 ± 3	14 ± 1**
Open probability (Po)	0.62 ± 0.05	0.11 ±0.03****	0.48 ±0.03	0.55 ±0.02
Open time constants:				
τ_{o1} (ms)	3.18 ± 0.28	3.98 ± 0.44	2.39 ± 0.14	4.49 ± 0.05***
τ_{o2} (ms)	9.77 ± 0.84	8.92 ± 1.17	8.33 ±1.01	16.6 ± 0.3**
τ_{o3} (ms)	20.8 ± 3.4	21.6 ± 5.1	17.4 ± 5.3	47.9 ± 3.5**
a_{o1} (%)	67 ± 1	66 ± 8	72 ± 6	19 ± 3***
a_{o2} (%)	28 ± 2	29 ± 8	25 ± 5	75 ± 4***
a_{o3} (%)	5 ± 2	5 ± 1	6 ± 2	6 ± 1
Burst duration (ms)	20.13 ± 0.72	14.34 ± 1.17**	13.33 ± 2.38	42.95 ± 2.61**
Openings/burst	3.12 ± 0.19	2.21 ± 0.16*	2.42 ± 0.11	2.49 ± 0.13
Burst time constants:				
τ_1(ms)	2.54 ± 0.20	4.85 ± 0.60*	2.42 ± 0.24	5.62 ± 1.04*
τ_1(ms)	35.4 ± 2.9	24.6 ± 3.4*	21.2 ± 3.1	52.0 ± 1.6***
a_1 (%)	37 ± 6	55 ± 3*	54 ± 3	20 ± 3***
a_2 (%)	63 ± 6	45 ± 3*	49 ± 2	80 ± 3***

Values represent mean ± S.E.M.*, **, *** and **** indicate $p < 0.05$, $p < 0.01$, $p<0.001$ and $p < 0.0001$ (unpaired t-test) statistically different from wt, respectively.

2.5.6 *De novo GABRB1(F246S)* mutation produced spontaneously gated $GABA_AR$s.

An unexpected finding from macroscopic current recordings was significantly increased holding currents (~5- fold) from α1β1(F246S)γ2L subunit-containing receptors in the hom condition (Figure 2.7A, Table 2.2). While the holding currents for receptors containing β3(N110D) subunits were significantly reduced in hom and het conditions (Table 2.2). As increased holding currents in general are indicators of poor cell health, and was also true for β1(F246S) subunit-containing receptors (our unpublished observations), we examined this further. Increased holding currents could be due to formation of homomeric or spontaneously gated β1(F246S) subunit-containing receptors, although, it is important to note that the wt β3 subunits themselves assemble to form homopentameric $GABA_AR$s [178]. To this end we used Zinc as it has been shown to block spontaneous $GABA_AR$ "leak" current that appears as a positive shift in the baseline current from cells expressing only β1 (or β3) subunits (subunits known to form homomeric $GABA_AR$s) [183-185]. Surprisingly, 10 to 15% of the holding current was blocked by 10μM Zinc in the hom condition (Figure 2.7B, Table 2.2). The holding current was blocked to a similar extent by 100μM zinc (data not shown). Additionally, there is small but significant decrease in the surface levels of β1(F246S) subunits in the hom condition (see Figure 2.5C). Taken together these results suggest that the β1(F246S) subunit results in spontaneously gated heteropentameric $GABA_AR$s (as previously reported [186]) rather than formation of homopentameric β1 subunit-containing receptors.

We further investigated the mechanisms by which the *de novo GABRB1(F246S)* mutation increased macroscopic holding currents at a single channel level. Analyzing spontaneous single channel currents in the absence of GABA revealed that the even wt β1 subunit-containing receptors opened spontaneously (Figure 2.7C). However, the spontaneous openings of wt α1β1γ2L receptors were very brief, with isolated low conductance openings, and with a Po that was ~68% of that observed in presence of 1 mM GABA. $GABA_A$Rs containing the mutant β1(F246S) subunits increased spontaneous Po by 4-fold without changing the single channel conductance (Figure 2.7C). *GABRB3(N110D)* mutation also produced spontaneous channel openings, although they were rare, brief, and with smaller conductance than wt receptors (1.1 ± 0.01pA and 0.001 ± 0.002pA, n = 3). The spontaneous activation of $GABA_A$Rs with mutant β3(N110D) and β1(F246S) subunits was blocked by picrotoxin (100 μM) in a fashion similar to that for receptors containing wt β1/3 subunits (data not shown). Thus, spontaneous activation of $GABA_A$Rs could be an additional pathogenic mechanism of action of the *GABRB1(F246S)* mutation.

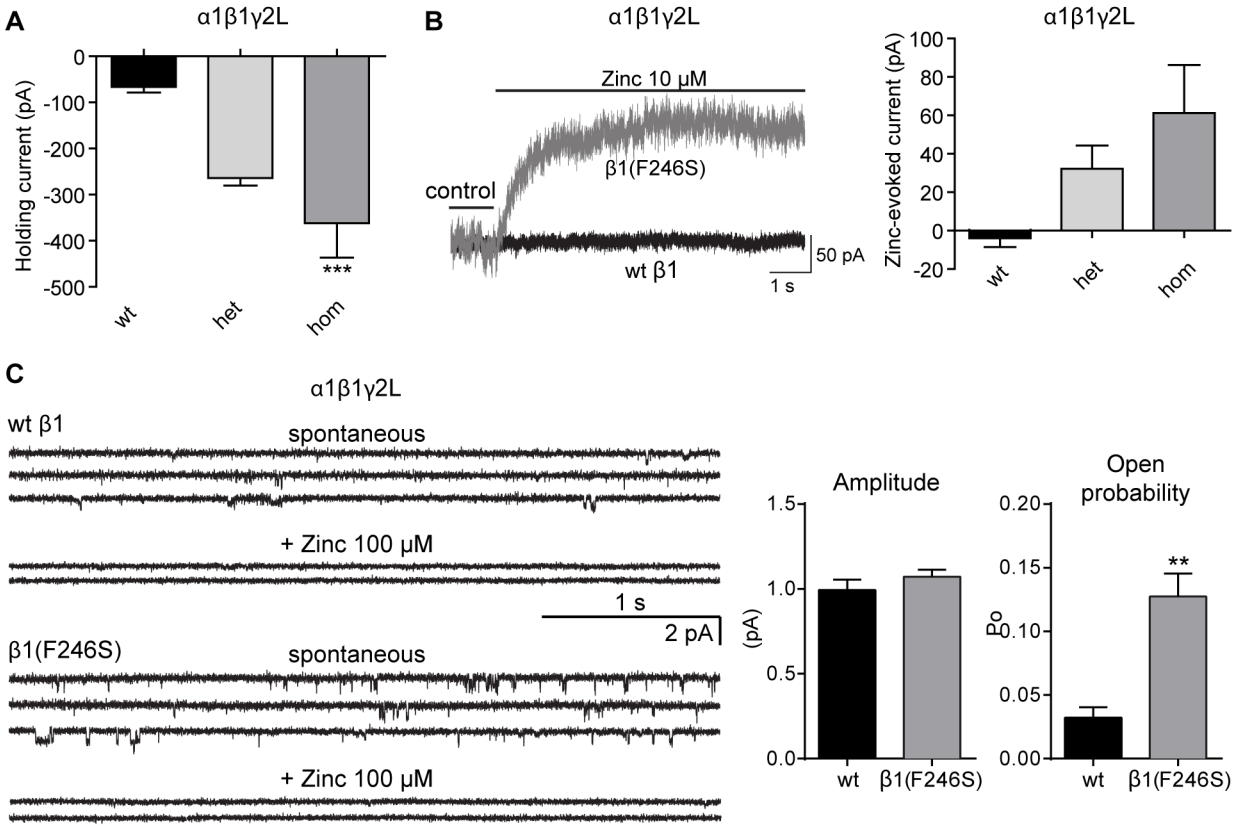

Figure 2.9 Mutant β1(F246S) subunits produced spontaneously gated $GABA_A$Rs leading to spontaneous macroscopic currents and single channel openings.

(A) Bar graph shows significantly higher holding currents were needed to clamp cells expressing β1(F246S) subunit-containing $GABA_A$Rs in het and hom conditions compared to cells with wt receptors. **(B, left panel)** Representative traces showing outward currents following Zn^{+2} application from cells with β1(F246S) (hom) subunit-containing $GABA_A$Rs. **(B, right panel)** Bar graph with average Zn^{+2}-evoked currents in wt, het, and hom conditions. **(C, left panel)** Representative single channel currents from cells with wt and β1(F246S) in hom condition. **(C, right panel)** Bar graphs showing single channel amplitude and Po of wt (black bars) and spontaneously activated mutant (gray bars) receptors. The β1(F246S) mutant subunits significantly increased the Po of low-conductance openings without altering single channel conductance.

Values represent mean ± standard error of the mean. One-way analysis of variance with Dunnett multiple comparisons test (A and B) or Two-way analysis of variance with Tukey multiple comparisons (C, right panel) test were used to determine statistical significance. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

2.5.7 *De novo GABRB1/3* mutations rearranged conserved structural domains related to $GABA_AR$ function.

The structural changes induced by the *GABRB* mutations were studied by creating the wt and mutant pentameric αβγ $GABA_AR$ simulations (see Figure 1B) using solved structures of both the *C. elegans* GluCl channel and the human $GABA_AR$ β3 subunit homopentamer as templates (see Materials and Methods for details). Rearrangements of the secondary structure and side chain conformation were computed as the root mean square (RMS) between wt and mutant receptors (Figure 2.8C). The D110 and F246 mutant residues are located at the β complimentary (-) interface, and thus the major structural changes induced by the IS-associated mutations occurred at two interfaces, between the principle (+) side of the γ and α subunits and the (-) side of the β subunit i.e. the γ+/β- and α+/β- interfaces (Figure 2.8B). These domains are in close contact with the structural assembly motifs [187, 188]. Conformational changes through neighborhood structural domains at the γ+/β-, and α+/β- interfaces were predicted for both IS-associated mutations. Perturbations in the secondary structure are presented as mutation associated alternative ribbon in rainbow when RMS > 0.03A°, and those in the side chain residues as plotted as box plots (Figure 2.9C). In addition to local effects (intrasubunit) confined to structural domains of the β subunit, and global effects (intersubunit) propagated to the nearest α and γ subunits via rearrangements of nearby residues and structural domains (Figure 2.9C).

The mutated β1(F246S) subunit had structural rearrangements largely restricted to the coupling zone domains and propagated to the TM domains at both the γ+/β- and

α+/β- interfaces (Figure 2.8C). The mutated β3(N110D) subunit acquired structural changes in the loops A, B, and α-β1 at the α+ and γ+ interfaces (Figure 2.8C). Remarkably, only at the γ+/β- interface, the β3(N110D) subunit predicted changes that were extended across the α-β1 loop of the γ+ subunit, a motif previously reported to impair receptor gating and βγ subunit interaction [171, 189, 190]. The N110D mutation lies in the inner β3 sheet on the opposite side of the ligand binding channel gating coupling interface, thus it seems unlikely to reduce the gating of the channel. However, $GABA_AR$ activation was decreased when glycine was inserted in the inner β4–β5 sheets at the β3(-) interface [191], in line with this, IS-associated mutations primarily affected the kinetic properties of the $GABA_ARs$. Both *de novo* GABRB mutations produced a wave of structural rearrangements that altered similar domains at the homologous γ+/β- and α+/β- interfaces required for receptor expression and function.

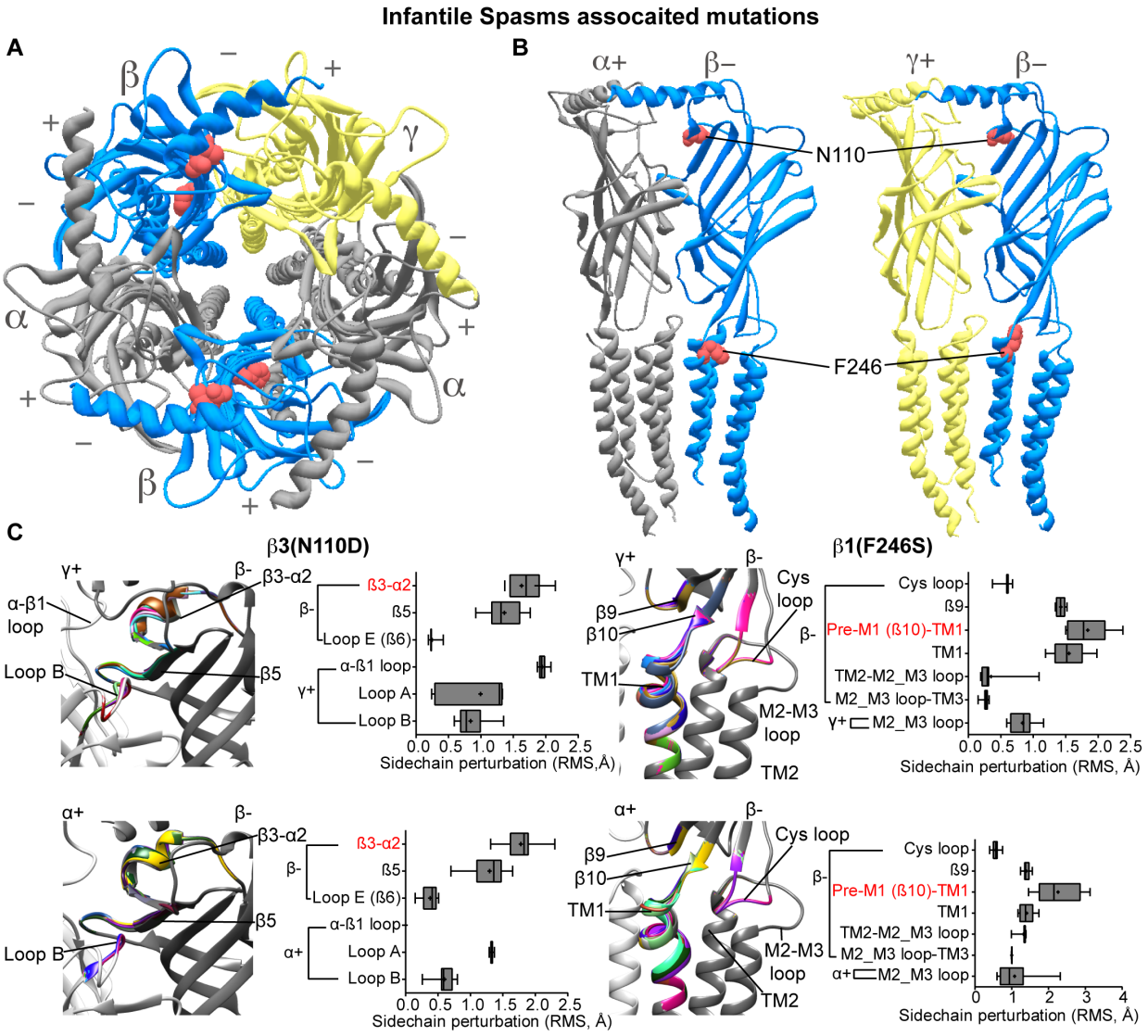

Figure 2.10 ***De novo GABRB1/3*** **mutations induced a wave of structural rearrangements in conserved structural domains important for $GABA_AR$ function.**

(A) Extracellular view of the N-terminal domains of a pentameric αβγ subunit-containing GABAAR structural model (as seen from the synaptic cleft) displaying LGS-associated (in red) GABRB1/3 mutations on β3 subunits (blue ribbons) subunits, α1 and γ2L subunits are represented as gray and yellow ribbons, respectively. The principal (+) and complementary (-) interfaces of each subunit are shown. The bottom panel lists the location of the mutations in their respective interfaces. **(B)** Zoomed in view of the β3+/α- subunit domains containing LGS-associated mutations (as seen almost parallel to the plasma membrane). **(C)** Enlarged view of the domains that had structural rearrangements caused by the IS-associated GABRB3(N110D) and GABRB1(F246S) mutations. The perturbations of the secondary structures that differ among the wt (in gray) and mutant (in rainbow) structures are indicated by solid black lines (left panels).

Box plots show perturbations (as root mean square deviation [RMS]) caused by the mutations in the side chain residues that are propagated through β sheets, loops, and TM helices (right panels). RMS values for up to 10 simulations are represented as interleaved box and whiskers plots (25–75% percentile, median, and minimum and maximum). The secondary structure containing the mutation is highlighted in red.

2.6 DISCUSSION

In this chapter I presented our analysis of the impaired function of $GABA_AR$s dysfunctions produced by the epilepsy-associated *GABRB*3*(N110D)* and *GABRB1(F246S)* mutations. The contribution of *GABRB*3 mutations to a relatively mild childhood absence epilepsy syndrome was reported in the past decade [174, 192], although association of *GABRB*3 mutations with severe epileptic encephalopathies was first provided only recently by the Epi4K consortium [111]. Several recent sequencing studies also report *GABRB*3 mutations in their epilepsy cohorts [111, 193-200]. On the contrary *GABRB*1 mutations are just beginning to appear in the epilepsy literature, even though their role in Schizophrenia, major depression, bipolar disorder [201-203] and increased alcohol consumption was suspected [186]. The Epi4K consortium was the first to report a *GABRB1* mutation (F246S) in an epileptic encephalopathy patient. However, as *GABRB1(F246S)* was found in a single patient, there was not enough statistical power to conclude that *GABRB1* mutations are causal for epileptic encephalopathies. Although, our functional study clearly demonstrated that the *GABRB*1*(F246S)* mutation disrupted $GABA_AR$ function, consistent with a contribution to the pathogenesis of epilepsy syndromes. In fact another report of the *GABRB1(T287I)* mutation in a patient with epileptic encephalopathy came out at the same time that our study was published

[204]. The patient with the *GABRB1(T287I)* mutation presented with clinical symptoms overlapping with the patient carrying the *GABRB*1*(F246S)* mutation [204]. These findings strongly suggested that *GABRB*1 mutations are causal for rare genetic epilepsies, and it would not be surprising to see additional epilepsy-associated *GABRB*1 mutations in the near future.

2.6.1 The IS-associated *de novo GABRB3* and *GABRB1* mutations had minor effects on $GABA_AR$ current amplitudes and surface levels.

IS is a catastrophic epilepsy syndrome and both patients with *GABRB*3*(N110D)* and *GABRB1(F246S)* mutations had severe epilepsy. Additionally, the PolyPhen-2 scores indicated that both of these mutations were probably damaging (Table 2.1). However, the major impact of IS-associated mutations were not loss of peak currents but they altered GABA-evoked current activation and deactivation times (Figure 2.4). The *GABRB1(F246S)* mutation reduced peak current amplitudes in the hom but not in the het condition, while the *GABRB3(N110D)* mutation did not reduce peak current amplitudes in het or hom conditions (Figure 2.3). As the patients were heterozygous for the mutations, it could be assumed that the current kinetic changes were pathogenic. This also highlights the value of functional studies, as the PolyPhen-2 scores were not sufficient to predict this outcome.

Consistent with minimal effects on peak GABA-evoked current amplitudes, both mutations had marginal effects on surface levels of α, β or γ subunits. The

GABRB3(N110D) mutation increased surface and total levels of $GABA_AR$ β3 but not of α1 or γ2L^{HA} subunits. This suggested that the *GABRB(N110D)* mutations favored the formation of surface β3(N110D) subunit homomers. As the total levels of β3(N110D) subunits were also increased, it could be speculated that these homomers were retained in the endoplasmic reticulum and might have impaired the oligomerization of β3(N110D) subunits with α1 and γ2L subunits. On the contrary the *GABRB1(F246S)* mutation produced a small but significant reduction of β1^{HA} surface levels but not those of α1 or γ2L^{HA} subunits. The total levels of α1, β1^{HA} or γ2L^{HA} subunits were also unaltered. It is unusual that only the surface levels of β1^{HA} subunits were reduced, as surface α1-γ2L^{HA} dimers do not assemble into functional pentamers. It can be speculated that the presence of two HA tags on each of the β1^{HA} subunits could hinder the formation of β1^{HA}-α1-β1^{HA}-α1-γ2L^{HA} subunit-containing $GABA_ARs$. Whereas, previous work from our laboratory demonstrated that the single HA tag had minimal effects on surface levels and electrophysiological properties of β-α-β-α-γ2L^{HA} subunit-containing $GABA_A$ receptors compared to β-α-β-α-γ2L subunit-containing $GABA_ARs$. The reduced surface β1^{HA} subunit levels could reduce the accessibility of anti-HA antibodies when two HA tags were present on β1 subunits rather than just one HA tag on the γ2L subunit. We used β1^{HA} subunits instead of β1 subunits since the antibodies against human β1 subunits were not specific (see methods). Thus the significance of reduced surface levels of β1^{HA} subunits but not of α1 or γ2L^{HA} subunits is not clear.

2.6.2 The predominant effect of IS-associated *de novo GABRB3* and *GABRB1* mutations were altered macroscopic $GABA_AR$ current kinetics and single channel properties.

The *GABRB*3*(N110D)* and *GABRB1(F246S)* mutations altered macroscopic current kinetics in opposite directions. The *GABRB3(N110D)* mutation significantly slowed current activation (longer 10–90% rise time) in the hom condition but not in the het condition (Figure 2.4A, left traces and bar graph, Table 2.2), but the *GABRB1(F246S)* mutation accelerated current activation (reduced rise times) in the het condition but not in the hom condition (Figure 2.4A, right traces and bar graph). The effects on rise times were inconsistent in hom and het condition for both mutations, and the significance of these findings is not clear. Brief application of GABA is a more precise technique for determining current rise and deactivation time courses. When GABA was applied for 10 ms in the hom condition, both mutations slowed current activation (increased rise times), but the effect was not significant for the *GABRB1(F246S)* mutation. Thus, the brief GABA application mimicked the effects seen with macroscopic currents.

On the other hand current deactivation was increased by the *GABRB3(N110D)* mutation (reduced weighted deactivation rate constant) and decreased (increased weighted deactivation rate constant) by the *GABRB1(F246S)* mutation in both the hom and het conditions (Figure 2.4C left traces and bar graph, Table 2.2). Both current rise times and deactivation rates determine the shape and peak of GABA-evoked currents. Thus slow rise rates and faster deactivation rates of GABA-evoked currents due to the *GABRB3(N110D)* mutation would result in brief and inhibitory post-synaptic currents

(IPSCs) and decreased charge transfer. In contrast, slow current deactivation due to the *GABRB1(F246S)* mutation would prolong the IPSC duration and increase the charge transfer during the first IPSC, but decrease charge transfer during subsequent IPSCs by reducing the number of unbound $GABA_A$Rs, especially during high-frequency neuronal firing. Counter intuitively, both faster and slower deactivation would result in the net loss of GABAergic inhibition, as previously reported for mutant γ2(K289M) and γ2(L313S/L9′S) subunit, respectively [205, 206].

The slower rise and faster deactivation rates of macroscopic currents due to the *GABRB3(N110D)* mutation results from reduced single channel open probability (Po) due to decreased burst duration and openings per burst (Figure 2.6 A-C, Table 2.3). Similarly, the prolonged current deactivation due to the *GABRB1(F246S)* mutation could be explained by significantly increased mean channel open time and burst duration. However, the Po is unaltered despite increased mean open time and burst duration likely due to reduced channel opening frequency (Figure 2.6 D-F, Table 2.3).

2.6.3 Mutant β1(F246S) subunits produced spontaneously gated $GABA_A$Rs

Additional pathogenic actions of the *GABRB1(F246S)* mutation are that it results in spontaneous $GABA_A$R opening that can be seen as drastically increased single channel openings in the absence of GABA and outward (positive) macroscopic currents in the presence of Zn. This also results in reduced HEK293T cell health and increased holding currents (Figure 2.7). Unlike the β3 subunit-containing $GABA_A$Rs those with β1

subunits have low Po in absence of GABA (Figure 2.7C); however, the mutant β1(F246S) subunits increased the channel Po by 4-fold without changing single channel conductance. These openings were almost completely blocked by Zn, which is also a blocker of homomeric β1 subunits [183-185], and the surface levels of β1(F246S) subunits were reduced. Taken together these finding suggesting that rather than altering the composition of $GABA_A$Rs, the β1(F246S) subunits resulted in spontaneously gated α1β1(F246S)γ2L channels. It could be speculated that the spontaneous channel openings could result in persistent tonic currents in neurons and reduce the population of $GABA_A$Rs in closed states that could respond to incoming synaptic inhibition. Although, the spontaneous currents in neurons have not been demonstrated, thus the consequence of these findings is unclear.

2.6.4 IS-associated *de novo GABRB1/3* mutations rearranged conserved structural domains related to $GABA_A$R function.

The *GABRB*3*(N110D)* and *GABRB1(F246S)* mutations located at the β- interface in the N-terminal domain of the β3 subunit and TM1 region of the β1 subunit, respectively, perturbed critical functional domains in the β subunits as well as in the neighboring α+/β- and γ+/β- interfaces (Figure 2.8). The *GABRB3(N110D)* mutation located in the inner β3 sheet and on the opposite side of the ligand binding channel gating coupling interface may not appear to affect gating, but previous studies also report reduced $GABA_A$R activation when glycine was inserted in the inner β4–β5 sheets at the β3- interface [191]. The effects of *GABRB1(F246S)* mutation were primarily restricted to the

coupling zone domains and propagated to the TM domains at both the γ+/β- and α+/β- interfaces. As these mutations are far from the GABA-binding site they did not have major effects on GABA-evoked current amplitudes but altered current kinetics. The changes predicted from the $GABA_AR$ structural model containing mutant β3(N110D) and β1(F246S) subunits support these observations.

2.6.5 How can IS-associated *GABRB3* and *GABRB1* mutations result in epilepsy syndromes?

Both β3 and β1 subunits are widely expressed in the developing and adult brain in seizure generation regions such as cortex, hippocampus, and thalamic reticular nucleus [207-209], where they mediate phasic and tonic inhibition. The β3 subunits are abundant during early development, whereas expression of β1 subunits begins after birth and undergoes down regulation until reaching stable levels in mature neurons [208, 210]. Even though the role of β1 subunits during development in not well understood, the ubiquitous expression of β1 subunits indicates their role in neurodevelopment after birth. Therefore, the loss of or altered depolarizing drive of mutant $GABA_ARs$ would hamper formation of appropriate neuronal circuits during critical periods of central nervous system development. Based on our results on GABA-evoked currents, it could be speculated that the *GABRB*3*(N110D)* mutation could have more prominent effects on phasic inhibition, whereas the *GABRB1(F246S)* mutation that resulted in spontaneous channel openings could affect both phasic and tonic inhibition.

2.7 REFERENCES

1. Lux AL, Osborne JP. A proposal for case definitions and outcome measures in studies of infantile spasms and West syndrome: consensus statement of the West Delphi group. Epilepsia. 2004 Nov;45(11):1416-28.

2. Fusco L, Vigevano F. Ictal clinical electroencephalographic findings of spasms in West syndrome. Epilepsia. 1993 Jul-Aug;34(4):671-8.

3. King DW, Dyken PR, Spinks IL, Jr., Murvin AJ. Infantile spasms: ictal phenomena. Pediatr Neurol. 1985 Jul-Aug;1(4):213-8.

4. Kossoff EH. Infantile spasms. Neurologist. 2010 Mar;16(2):69-75.

5. Kellaway P, Hrachovy RA, Frost JD, Jr., Zion T. Precise characterization and quantification of infantile spasms. Ann Neurol. 1979 Sep;6(3):214-8.

6. Gaily EK, Shewmon DA, Chugani HT, Curran JG. Asymmetric and asynchronous infantile spasms. Epilepsia. 1995 Sep;36(9):873-82.

7. Riikonen R. Epidemiological data of West syndrome in Finland. Brain Dev. 2001 Nov;23(7):539-41.

8. Cowan LD, Hudson LS. The epidemiology and natural history of infantile spasms. J Child Neurol. 1991 Oct;6(4):355-64.

9. Lux AL. Latest American and European updates on infantile spasms. Curr Neurol Neurosci Rep. 2013 Mar;13(3):334.

10. Sidenvall R, Eeg-Olofsson O. Epidemiology of infantile spasms in Sweden. Epilepsia. 1995 Jun;36(6):572-4.

11. Trevathan E, Murphy CC, Yeargin-Allsopp M. The descriptive epidemiology of infantile spasms among Atlanta children. Epilepsia. 1999 Jun;40(6):748-51.

12. Kurokawa T, Goya N, Fukuyama Y, Suzuki M, Seki T, Ohtahara S. West syndrome and Lennox-Gastaut syndrome: a survey of natural history. Pediatrics. 1980 Jan;65(1):81-8.

13. Pellock JM, Hrachovy R, Shinnar S, et al. Infantile spasms: a U.S. consensus report. Epilepsia. 2010 Oct;51(10):2175-89.

14. West WJ. ON A PECULIAR FORM OF INFANTILE CONVULSIONS. The Lancet. 1841;35(911):724-5.

15. Jeavons PM, Bower BD, Dimitrakoudi M. Long-term prognosis of 150 cases of "West syndrome". Epilepsia. 1973 Jun;14(2):153-64.

16. Wilmshurst JM, Gaillard WD, Vinayan KP, et al. Summary of recommendations for the management of infantile seizures: Task Force Report for the ILAE Commission of Pediatrics. Epilepsia. 2015 Aug;56(8):1185-97.

17. Widjaja E, Go C, McCoy B, Snead OC. Neurodevelopmental outcome of infantile spasms: A systematic review and meta-analysis. Epilepsy Res. 2015 Jan;109:155-62.

18. Verity CM, Ross EM, Golding J. Epilepsy in the first 10 years of life: findings of the child health and education study. BMJ. 1992 Oct 10;305(6858):857-61.

19. Freitag CM, May TW, Pfafflin M, Konig S, Rating D. Incidence of epilepsies and epileptic syndromes in children and adolescents: a population-based prospective study in Germany. Epilepsia. 2001 Aug;42(8):979-85.

20. Dura-Trave T, Yoldi-Petri ME, Gallinas-Victoriano F. Incidence of epilepsies and epileptic syndromes among children in Navarre, Spain: 2002 through 2005. J Child Neurol. 2008 Aug;23(8):878-82.

21. Eltze CM, Chong WK, Cox T, et al. A population-based study of newly diagnosed epilepsy in infants. Epilepsia. 2013 Mar;54(3):437-45.

22. Masri A, Badran E, Hamamy H, Assaf A, Al-Qudah AA. Etiologies, outcomes, and risk factors for epilepsy in infants: a case-control study. Clin Neurol Neurosurg. 2008 Apr;110(4):352-6.

23. Matsumoto A, Watanabe K, Sugiura M, Negoro T, Takaesu E, Iwase K. Long-term prognosis of convulsive disorders in the first year of life: mental and physical development and seizure persistence. Epilepsia. 1983 Jun;24(3):321-9.

24. Riikonen R, Donner M. Incidence and aetiology of infantile spasms from 1960 to 1976: a population study in Finland. Dev Med Child Neurol. 1979 Jun;21(3):333-43.

25. Rantala H, Putkonen T. Occurrence, outcome, and prognostic factors of infantile spasms and Lennox-Gastaut syndrome. Epilepsia. 1999 Mar;40(3):286-9.

26. Luthvigsson P, Olafsson E, Sigurthardottir S, Hauser WA. Epidemiologic features of infantile spasms in Iceland. Epilepsia. 1994 Jul-Aug;35(4):802-5.

27. Brna PM, Gordon KE, Dooley JM, Wood EP. The epidemiology of infantile spasms. Can J Neurol Sci. 2001 Nov;28(4):309-12.

28. Noachtar S, Binnie C, Ebersole J, Mauguiere F, Sakamoto A, Westmoreland B. A glossary of terms most commonly used by clinical electroencephalographers and proposal for the report form for the EEG findings. The International Federation of Clinical Neurophysiology. Electroencephalogr Clin Neurophysiol Suppl. 1999;52:21-41.

29. Gibbs EL, Fleming MM, Gibbs FA. Diagnosis and prognosis of hypsarhythmia and infantile spasms. Pediatrics. 1954 Jan;13(1):66-73.

30. Lennox WG, Davis JP. Clinical correlates of the fast and the slow spike-wave electroencephalogram. Pediatrics. 1950 Apr;5(4):626-44.

31. Gibbs FA GE. Atlas of electroencephalography.

. 1952;2:24–30.

32. Hrachovy RA, Frost JD, Jr., Kellaway P. Hypsarrhythmia: variations on the theme. Epilepsia. 1984 Jun;25(3):317-25.

33. Hrachovy RA, Frost JD, Jr. Infantile epileptic encephalopathy with hypsarrhythmia (infantile spasms/West syndrome). J Clin Neurophysiol. 2003 Nov-Dec;20(6):408-25.

34. Hussain SA, Shinnar S, Kwong G, et al. Treatment of infantile spasms with very high dose prednisolone before high dose adrenocorticotropic hormone. Epilepsia. 2014 Jan;55(1):103-7.

35. Caraballo RH, Ruggieri V, Gonzalez G, et al. Infantile spams without hypsarrhythmia: a study of 16 cases. Seizure. 2011 Apr;20(3):197-202.

36. Caraballo RH, Fejerman N, Bernardina BD, et al. Epileptic spasms in clusters without hypsarrhythmia in infancy. Epileptic Disord. 2003 Jun;5(2):109-13.

37. Altunel A, Sever A, Altunel EO. Hypsarrhythmia paroxysm index: A tool for early prediction of infantile spasms. Epilepsy Res. 2015 Mar;111:54-60.

38. Philippi H, Wohlrab G, Bettendorf U, et al. Electroencephalographic evolution of hypsarrhythmia: toward an early treatment option. Epilepsia. 2008 Nov;49(11):1859-64.

39. Watanabe K, Iwase K, Hara K. The evolution of EEG features in infantile spasms: a prospective study. Dev Med Child Neurol. 1973 Oct;15(5):584-96.

40. ILAE CoCaTot. Proposal for revised classification of epilepsies and epileptic syndromes. Commission on Classification and Terminology of the International League Against Epilepsy. Epilepsia. 1989 Jul-Aug;30(4):389-99.

41. Berg AT, Berkovic SF, Brodie MJ, et al. Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia. 2010 Apr;51(4):676-85.

42. ILAE CoCaTot. Workshop on Infantile Spasms. Epilepsia. 1992;33(1):195-.

43. Wilmshurst JM, Ibekwe RC, O'Callaghan FJ. Epileptic spasms - 175 years on: Trying to teach an old dog new tricks. Seizure. 2017 Jan;44:81-6.

44. Korff CM, Nordli DR, Jr. The clinical-electrographic expression of infantile seizures. Epilepsy Res. 2006 Aug;70 Suppl 1:S116-31.

45. Baird HW, 3rd. Convulsions in infancy and childhood. Conn Med. 1959 Mar;23(3):149-51.

46. Druckman R, Chao D. Massive spasms in infancy and childhood. Epilepsia. 1955 Nov;4:61-72.

47. Hussain SA, Kwong G, Millichap JJ, et al. Hypsarrhythmia assessment exhibits poor interrater reliability: a threat to clinical trial validity. Epilepsia. 2015 Jan;56(1):77-81.

48. Lee YJ, Berg AT, Nordli DR, Jr. Clinical spectrum of epileptic spasms in children. Brain Dev. 2015 Jan;37(1):37-48.

49. Auvin Sp, Hartman AL, Desnous Ba, et al. Diagnosis delay in West syndrome: misdiagnosis and consequences. European Journal of Pediatrics. 2012 2012//;171(11):1695-701.

50. Riikonen R. Long-term otucome of West syndrome: a study of adults with a history of infantile spasms. Epilepsia. 1996 Apr;37(4):367-72.

51. Primec ZR, Stare J, Neubauer D. The risk of lower mental outcome in infantile spasms increases after three weeks of hypsarrhythmia duration. Epilepsia. 2006 Dec;47(12):2202-5.

52. Gonsales MC, Montenegro MA, Soler CV, Coan AC, Guerreiro MM, Lopes-Cendes I. Recent developments in the genetics of childhood epileptic encephalopathies: impact in clinical practice. Arq Neuropsiquiatr. 2015 Nov;73(11):946-58.

53. Orsini A, Zara F, Striano P. Recent advances in epilepsy genetics. Neurosci Lett. 2017 May 10.

54. Sands TT, Choi H. Genetic Testing in Pediatric Epilepsy. Curr Neurol Neurosci Rep. 2017 May;17(5):45.

55. Noebels J. Precision physiology and rescue of brain ion channel disorders. J Gen Physiol. 2017 May 01;149(5):533-46.

56. Thomas RH, Berkovic SF. The hidden genetics of epilepsy-a clinically important new paradigm. Nat Rev Neurol. 2014 May;10(5):283-92.

57. Donat JF, Wright FS. Clinical imitators of infantile spasms. J Child Neurol. 1992 Oct;7(4):395-9.

58. Donat JF, Wright FS. Simultaneous infantile spasms and partial seizures. J Child Neurol. 1991 Jul;6(3):246-50.

59. Appleton RE. West syndrome: long-term prognosis and social aspects. Brain Dev. 2001 Nov;23(7):688-91.

60. Caplan R, Siddarth P, Mathern G, et al. Developmental outcome with and without successful intervention. Int Rev Neurobiol. 2002;49:269-84.

61. Montenegro MA, Eck K, Jacob S, et al. Long-term outcome of symptomatic infantile spasms established by video-electroencephalography (EEG) monitoring. J Child Neurol. 2008 Nov;23(11):1288-92.

62. Guggenheim MA, Frost JD, Jr., Hrachovy RA. Time interval from a brain insult to the onset of infantile spasms. Pediatr Neurol. 2008 Jan;38(1):34-7.

63. Matsumoto A, Watanabe K, Negoro T, et al. Prognostic factors of infantile spasms from the etiological viewpoint. Brain Dev. 1981;3(4):361-4.

64. Goh S, Kwiatkowski DJ, Dorer DJ, Thiele EA. Infantile spasms and intellectual outcomes in children with tuberous sclerosis complex. Neurology. 2005 Jul 26;65(2):235-8.

65. Sillanpaa M, Schmidt D. Early seizure frequency and aetiology predict long-term medical outcome in childhood-onset epilepsy. Brain. 2009 Apr;132(Pt 4):989-98.

66. Hrachovy RA, Glaze DG, Frost JD, Jr. A retrospective study of spontaneous remission and long-term outcome in patients with infantile spasms. Epilepsia. 1991 Mar-Apr;32(2):212-4.

67. Bachman DS. Spontaneous remission of infantile spasms with hypsarhythmia. Arch Neurol. 1981 Dec;38(12):785.

68. Riikonen R. A long-term follow-up study of 214 children with the syndrome of infantile spasms. Neuropediatrics. 1982 Feb;13(1):14-23.

69. Talwar D, Baldwin MA, Hutzler R, Griesemer DA. Epileptic spasms in older children: persistence beyond infancy. Epilepsia. 1995 Feb;36(2):151-5.

70. de Menezes MA, Rho JM. Clinical and electrographic features of epileptic spasms persisting beyond the second year of life. Epilepsia. 2002 Jun;43(6):623-30.

71. Camfield P, Camfield C, Lortie A, Darwish H. Infantile spasms in remission may reemerge as intractable epileptic spasms. Epilepsia. 2003 Dec;44(12):1592-5.

72. Eisermann MM, Ville D, Soufflet C, et al. Cryptogenic late-onset epileptic spasms: an overlooked syndrome of early childhood? Epilepsia. 2006 Jun;47(6):1035-42.

73. Nordli DR, Jr., Korff CM, Goldstein J, Koh S, Laux L, Kelley KR. Cryptogenic late-onset epileptic spasms or late infantile epileptogenic encephalopathy? Epilepsia. 2007 Jan;48(1):206-8.

74. Trojaborg W, Plum P. Treatment of "hypsarrhythmia" with ACTH. Acta Paediatr. 1960 Sep;49:572-82.

75. Hattori H. Spontaneous remission of spasms in West syndrome--implications of viral infection. Brain Dev. 2001 Nov;23(7):705-7.

76. Iwasaki T, Nonoda Y, Ishii M. Remission associated with human herpesvirus infection in west syndrome. J Child Neurol. 2006 Oct;21(10):886-90.

77. Yamamoto H, Yamano T, Niijima S, Kohyama J, Yamanouchi H. Spontaneous improvement of intractable epileptic seizures following acute viral infections. Brain Dev. 2004 Sep;26(6):377-9.

78. Riikonen R. Long-term outcome of patients with West syndrome. Brain Dev. 2001 Nov;23(7):683-7.

79. Iwatani Y, Kagitani-Shimono K, Tominaga K, et al. Long-term developmental outcome in patients with West syndrome after epilepsy surgery. Brain Dev. 2012 Oct;34(9):731-8.

80. Lombroso CT. A prospective study of infantile spasms: clinical and therapeutic correlations. Epilepsia. 1983 Apr;24(2):135-58.

81. Yamatogi Y, Ohtahara S. Age-dependent epileptic encephalopathy: a longitudinal study. Folia Psychiatr Neurol Jpn. 1981;35(3):321-32.

82. Chakova L. On a rare form of epilepsy in infants--Ohtahara syndrome. Folia Med (Plovdiv). 1996;38(2):69-73.

83. Clarke M, Gill J, Noronha M, McKinlay I. Early infantile epileptic encephalopathy with suppression burst: Ohtahara syndrome. Dev Med Child Neurol. 1987 Aug;29(4):520-8.

84. Ohtahara S, Ohtsuka Y, Yamatogi Y. The West syndrome: developmental aspects. Acta Paediatr Jpn. 1987 Feb;29(1):61-9.

85. Paciorkowski AR, Thio LL, Dobyns WB. Genetic and biologic classification of infantile spasms. Pediatr Neurol. 2011 Dec;45(6):355-67.

86. Besag F, Gobbi G, Aldenkamp A, Caplan R, Dunn DW, Sillanpaa M. Psychiatric and Behavioural Disorders in Children with Epilepsy (ILAE Task Force Report): Behavioural and psychiatric disorders associated with childhood epilepsy syndromes. Epileptic Disord. 2016 May 16.

87. Curatolo P, Cusmai R. Autism and infantile spasms in children with tuberous sclerosis. Dev Med Child Neurol. 1987 Aug;29(4):551.

88. Askalan R, Mackay M, Brian J, et al. Prospective preliminary analysis of the development of autism and epilepsy in children with infantile spasms. J Child Neurol. 2003 Mar;18(3):165-70.

89. Pavone P, Striano P, Falsaperla R, Pavone L, Ruggieri M. Infantile spasms syndrome, West syndrome and related phenotypes: what we know in 2013. Brain Dev. 2014 Oct;36(9):739-51.

90. Pavone P, Striano P, Falsaperla R, Pavone L, Ruggieri M. Infantile spasms syndrome, West syndrome and related phenotypes: What we know in 2013. Brain Dev. 2013 Nov 19.

91. Wheless JW, Gibson PA, Rosbeck KL, et al. Infantile spasms (West syndrome): update and resources for pediatricians and providers to share with parents. BMC Pediatr. 2012 Jul 25;12:108.

92. Metsahonkala L, Gaily E, Valanne L, Blomstedt G. Etiology and Long-Term Outcomes of Late-Onset Infantile Spasms. Neuropediatrics. 2015 Aug;46(4):269-76.

93. Ronzano N, Valvo G, Ferrari AR, Guerrini R, Sicca F. Late-onset epileptic spasms: clinical evidence and outcome in 34 patients. J Child Neurol. 2015 Feb;30(2):153-9.

94. Fukui M, Shimakawa S, Kuki I, et al. Effect of adrenocorticotropic hormone therapy for epileptic spasms developing after the age of 1 year. Seizure. 2014 Aug;23(7):521-6.

95. Caumes R, Boespflug-Tanguy O, Villeneuve N, et al. Late onset epileptic spasms is frequent in MECP2 gene duplication: electroclinical features and long-term follow-up of 8 epilepsy patients. Eur J Paediatr Neurol. 2014 Jul;18(4):475-81.

96. Ishikawa N, Kobayashi Y, Fujii Y, Tajima G, Kobayashi M. Ictal electroencephalography and electromyography features in symptomatic infantile epileptic encephalopathy with late-onset spasms. Neuropediatrics. 2014 Feb;45(1):36-41.

97. Ricard-Mousnier B, Dorfmuller G, Fohlen M, et al. Late-onset epileptic spasms may be cured by focal cortical resective surgery. Epileptic Disord. 2012 Sep;14(3):313-20.

98. Auvin S, Lamblin MD, Pandit F, Vallee L, Bouvet-Mourcia A. Infantile epileptic encephalopathy with late-onset spasms: report of 19 patients. Epilepsia. 2010 Jul;51(7):1290-6.

99. Caraballo RH, Fortini S, Reyes G, Carpio Ruiz A, Sanchez Fuentes SV, Ramos B. Epileptic spasms in clusters and associated syndromes other than West syndrome: A study of 48 patients. Epilepsy Res. 2016 Jul;123:29-35.

100. Capal JK, Bernardino-Cuesta B, Horn PS, et al. Influence of seizures on early development in tuberous sclerosis complex. Epilepsy Behav. 2017 May;70(Pt A):245-52.

101. Overwater IE, Verhaar BJ, Lingsma HF, et al. Interdependence of clinical factors predicting cognition in children with tuberous sclerosis complex. J Neurol. 2017 Jan;264(1):161-7.

102. Kannan L, Vogrin S, Bailey C, Maixner W, Harvey AS. Centre of epileptogenic tubers generate and propagate seizures in tuberous sclerosis. Brain. 2016 Oct;139(Pt 10):2653-67.

103. Conant KD, Finucane B, Cleary N, et al. A survey of seizures and current treatments in 15q duplication syndrome. Epilepsia. 2014 Mar;55(3):396-402.

104. Battaglia A. The inv dup (15) or idic (15) syndrome (Tetrasomy 15q). Orphanet J Rare Dis. 2008 Nov 19;3:30.

105. Bingham PM, Spinner NB, Sovinsky L, Zackai EH, Chance PF. Infantile spasms associated with proximal duplication of chromosome 15q. Pediatr Neurol. 1996 Sep;15(2):163-5.

106. Datta A, Picker J, Rotenberg A. Trisomy 8 mosaicism and favorable outcome after treatment of infantile spasms: case report. J Child Neurol. 2010 Oct;25(10):1275-7.

107. Kumada T, Maihara T, Higuchi Y, Nishida Y, Taniguchi Y, Fujii T. Epilepsy in children with trisomy 18. Am J Med Genet A. 2013 Apr;161A(4):696-701.

108. Yamanouchi H, Imataka G, Nakagawa E, et al. An analysis of epilepsy with chromosomal abnormalities. Brain Dev. 2005 Aug;27(5):370-7.

109. Blume WT, Luders HO, Mizrahi E, Tassinari C, van Emde Boas W, Engel J, Jr. Glossary of descriptive terminology for ictal semiology: report of the ILAE task force on classification and terminology. Epilepsia. 2001 Sep;42(9):1212-8.

110. Goldstein J, Slomski J. Epileptic spasms: a variety of etiologies and associated syndromes. J Child Neurol. 2008 Apr;23(4):407-14.

111. Epi KC, Epilepsy Phenome/Genome P, Allen AS, et al. De novo mutations in epileptic encephalopathies. Nature. 2013 Sep 12;501(7466):217-21.

112. Lux AL, Osborne JP. The influence of etiology upon ictal semiology, treatment decisions and long-term outcomes in infantile spasms and West syndrome. Epilepsy Res. 2006 Aug;70 Suppl 1:S77-86.

113. Scheffer IE, Berkovic S, Capovilla G, et al. ILAE classification of the epilepsies: Position paper of the ILAE Commission for Classification and Terminology. Epilepsia. 2017 Apr;58(4):512-21.

114. Osborne JP, Lux AL, Edwards SW, et al. The underlying etiology of infantile spasms (West syndrome): information from the United Kingdom Infantile Spasms Study (UKISS) on contemporary causes and their classification. Epilepsia. 2010 Oct;51(10):2168-74.

115. Wirrell EC, Shellhaas RA, Joshi C, et al. How should children with West syndrome be efficiently and accurately investigated? Results from the National Infantile Spasms Consortium. Epilepsia. 2015 Apr;56(4):617-25.

116. Curatolo P, Seri S, Verdecchia M, Bombardieri R. Infantile spasms in tuberous sclerosis complex. Brain Dev. 2001 Nov;23(7):502-7.

117. Ess KC. Treatment of infantile spasms in tuberous sclerosis complex: dismal outcomes but future hope? Nat Clin Pract Neurol. 2009 Feb;5(2):72-3.

118. Riikonen R, Simell O. Tuberous sclerosis and infantile spasms. Dev Med Child Neurol. 1990 Mar;32(3):203-9.

119. Fukushima K, Inoue Y, Fujiwara T, Yagi K. Long-term follow-up study of West syndrome associated with tuberous sclerosis. Brain Dev. 2001 Nov;23(7):698-704.

120. Webb DW, Fryer AE, Osborne JP. Morbidity associated with tuberous sclerosis: a population study. Dev Med Child Neurol. 1996 Feb;38(2):146-55.

121. Kumaran A, Kar S, Kapoor RR, Hussain K. The clinical problem of hyperinsulinemic hypoglycemia and resultant infantile spasms. Pediatrics. 2010 Nov;126(5):e1231-6.

122. Shah NS, Mitchell WG, Boles RG. Mitochondrial disorders: a potentially under-recognized etiology of infantile spasms. J Child Neurol. 2002 May;17(5):369-72.

123. Zhongshu Z, Weiming Y, Yukio F, Cheng LZ, Zhixing W. Clinical analysis of West syndrome associated with phenylketonuria. Brain Dev. 2001 Nov;23(7):552-7.

124. Gkampeta A, Pavlou E. Infantile spasms (West syndrome) in children with inborn errors of metabolism: a review of the literature. J Child Neurol. 2012 Oct;27(10):1295-301.

125. Rossi S, Daniele I, Bastrenta P, Mastrangelo M, Lista G. Early myoclonic encephalopathy and nonketotic hyperglycinemia. Pediatr Neurol. 2009 Nov;41(5):371-4.

126. Tekgul H, Serdaroglu G, Karapinar B, et al. Vigabatrin caused rapidly progressive deterioration in two cases with early myoclonic encephalopathy associated with nonketotic hyperglycinemia. J Child Neurol. 2006 Jan;21(1):82-4.

127. Sfaello I, Castelnau P, Blanc N, Ogier H, Evrard P, Arzimanoglou A. Infantile spasms and Menkes disease. Epileptic Disord. 2000 Dec;2(4):227-30.

128. Bahi-Buisson N, Kaminska A, Nabbout R, et al. Epilepsy in Menkes disease: analysis of clinical stages. Epilepsia. 2006 Feb;47(2):380-6.

129. Fois A. Infantile spasms: review of the literature and personal experience. Ital J Pediatr. 2010 Feb 08;36:15.

130. Basura GJ, Hagland SP, Wiltse AM, Gospe SM, Jr. Clinical features and the management of pyridoxine-dependent and pyridoxine-responsive seizures: review of 63 North American cases submitted to a patient registry. Eur J Pediatr. 2009 Jun;168(6):697-704.

131. Bennett CL, Chen Y, Hahn S, Glass IA, Gospe SM, Jr. Prevalence of ALDH7A1 mutations in 18 North American pyridoxine-dependent seizure (PDS) patients. Epilepsia. 2009 May;50(5):1167-75.

132. Pascual JM, Campistol J, Gil-Nagel A. Epilepsy in inherited metabolic disorders. Neurologist. 2008 Nov;14(6 Suppl 1):S2-S14.

133. Glushakov AV, Glushakova O, Varshney M, et al. Long-term changes in glutamatergic synaptic transmission in phenylketonuria. Brain. 2005 Feb;128(Pt 2):300-7.

134. Dobyns WB. Agenesis of the corpus callosum and gyral malformations are frequent manifestations of nonketotic hyperglycinemia. Neurology. 1989 Jun;39(6):817-20.

135. Nieto-Barrera M. [Clinical, neuro-radiological and prognostic aspects of post-encephalitic catastrophic epilepsies]. Rev Neurol. 2002 Sep;35 Suppl 1:S30-8.

136. Gripper LB, Welburn SC. The causal relationship between neurocysticercosis infection and the development of epilepsy - a systematic review. Infect Dis Poverty. 2017 Apr 05;6(1):31.

137. Ayaz E, Turkoglu SA, Orallar H. Toxoplasma gondii and Epilepsy. Turkiye Parazitol Derg. 2016 Jun;40(2):90-6.

138. Lo Presti A, Aguirre DT, De Andres P, Daoud L, Fortes J, Muniz J. Cerebral sparganosis: case report and review of the European cases. Acta Neurochir (Wien). 2015 Sep;157(8):1339-43; discussion 43.

139. Vezzani A, Fujinami RS, White HS, et al. Infections, inflammation and epilepsy. Acta Neuropathol. 2016 Feb;131(2):211-34.

140. Ottman R, Hirose S, Jain S, et al. Genetic testing in the epilepsies--report of the ILAE Genetics Commission. Epilepsia. 2010 Apr;51(4):655-70.

141. Mefford HC, Yendle SC, Hsu C, et al. Rare copy number variants are an important cause of epileptic encephalopathies. Ann Neurol. 2011 Dec;70(6):974-85.

142. Paciorkowski AR, Thio LL, Rosenfeld JA, et al. Copy number variants and infantile spasms: evidence for abnormalities in ventral forebrain development and pathways of synaptic function. Eur J Hum Genet. 2011 Dec;19(12):1238-45.

143. Kalscheuer VM, Tao J, Donnelly A, et al. Disruption of the serine/threonine kinase 9 gene causes severe X-linked infantile spasms and mental retardation. Am J Hum Genet. 2003 Jun;72(6):1401-11.

144. Claes S, Devriendt K, Lagae L, et al. The X-linked infantile spasms syndrome (MIM 308350) maps to Xp11.4-Xpter in two pedigrees. Ann Neurol. 1997 Sep;42(3):360-4.

145. Bruyere H, Lewis S, Wood S, MacLeod PJ, Langlois S. Confirmation of linkage in X-linked infantile spasms (West syndrome) and refinement of the disease locus to Xp21.3-Xp22.1. Clin Genet. 1999 Mar;55(3):173-81.

146. Moey C, Topper S, Karn M, et al. Reinitiation of mRNA translation in a patient with X-linked infantile spasms with a protein-truncating variant in ARX. Eur J Hum Genet. 2016 May;24(5):681-9.

147. Morleo M, Iaconis D, Chitayat D, et al. Disruption of the IQSEC2 transcript in a female with X;autosome translocation t(X;20)(p11.2;q11.2) and a phenotype resembling X-linked infantile spasms (ISSX) syndrome. Mol Med Rep. 2008 Jan-Feb;1(1):33-9.

148. Scala E, Ariani F, Mari F, et al. CDKL5/STK9 is mutated in Rett syndrome variant with infantile spasms. J Med Genet. 2005 Feb;42(2):103-7.

149. Hjalmgrim H, Hansen LK, Ousager LB, Moller RS. [Early-onset epileptic encephalopathy caused by CDKL5 mutation]. Ugeskr Laeger. 2014 Dec 15;176(25A).

150. Zhang Q, Li J, Zhao Y, Bao X, Wei L, Wang J. Gene mutation analysis of 175 Chinese patients with early-onset epileptic encephalopathy. Clin Genet. 2017 May;91(5):717-24.

151. Guerrini R, Dobyns WB. Malformations of cortical development: clinical features and genetic causes. Lancet Neurol. 2014 Jul;13(7):710-26.

152. Gerard-Blanluet M, Romana S, Munier C, et al. Classical West "syndrome" phenotype with a subtelomeric 4p trisomy. Am J Med Genet A. 2004 Oct 15;130A(3):299-302.

153. Akabori S, Takano T, Fujito H, Takeuchi Y. West syndrome in a patient with balanced translocation t(X;18)(p22;p11.2). Pediatr Neurol. 2007 Jul;37(1):64-6.

154. Vandeweyer G, Van der Aa N, Ceulemans B, van Bon BW, Rooms L, Kooy RF. A de novo balanced t(2;6)(p15;p22.3) in a patient with West Syndrome disrupts a lnc-RNA. Epilepsy Res. 2012 May;99(3):346-9.

155. Lacaze E, Gruchy N, Penniello-Valette MJ, et al. De novo 15q13.3 microdeletion with cryptogenic West syndrome. Am J Med Genet A. 2013 Oct;161A(10):2582-7.

156. Allen NM, Conroy J, Shahwan A, et al. Excellent outcome with de novo 15q13.3 microdeletion causing infantile spasms--a further patient. Am J Med Genet A. 2014 Jul;164A(7):1863-6.

157. Striano P, Paravidino R, Sicca F, et al. West syndrome associated with 14q12 duplications harboring FOXG1. Neurology. 2011 May 03;76(18):1600-2.

158. Hackmann K, Stadler A, Schallner J, et al. Severe intellectual disability, West syndrome, Dandy-Walker malformation, and syndactyly in a patient with partial tetrasomy 17q25.3. Am J Med Genet A. 2013 Dec;161A(12):3144-9.

159. Riikonen RS, Wallden T, Kokkonen H. Infantile spasms and 15q11.2q13.1 chromosome duplication in two successive generations. Eur J Paediatr Neurol. 2016 Jan;20(1):164-7.

160. Tapp S, Anderson T, Visootsak J. Neurodevelopmental outcomes in children with Down syndrome and infantile spasms. J Pediatr Neurol. 2015 Jun;13(2):74-7.

161. Stafstrom CE, Konkol RJ. Infantile spasms in children with Down syndrome. Dev Med Child Neurol. 1994 Jul;36(7):576-85.

162. Lujic L, Bosnjak VM, Delin S, Duranovic V, Krakar G. Infantile spasms in children with Down syndrome. Coll Antropol. 2011 Jan;35 Suppl 1:213-8.

163. Goldberg-Stern H, Strawsburg RH, Patterson B, et al. Seizure frequency and characteristics in children with Down syndrome. Brain Dev. 2001 Oct;23(6):375-8.

164. Rantala H, Shields WD, Christenson PD, et al. Risk factors of infantile spasms compared with other seizures in children under 2 years of age. Epilepsia. 1996 Apr;37(4):362-6.

165. Millichap JG, Bickford RG, Klass DW, Backus RE. Infantile spasms, hypsarhythmia, and mental retardation. A study of etiologic factors in 61 patients. Epilepsia. 1962 Jun;3:188-97.

166. Karvelas G, Lortie A, Scantlebury MH, Duy PT, Cossette P, Carmant L. A retrospective study on aetiology based outcome of infantile spasms. Seizure. 2009 Apr;18(3):197-201.

167. Noebels J. Pathway-driven discovery of epilepsy genes. Nat Neurosci. 2015 Mar;18(3):344-50.

168. Hino-Fukuyo N, Kikuchi A, Arai-Ichinoi N, et al. Genomic analysis identifies candidate pathogenic variants in 9 of 18 patients with unexplained West syndrome. Hum Genet. 2015 Jun;134(6):649-58.

169. Mercimek-Mahmutoglu S, Patel J, Cordeiro D, et al. Diagnostic yield of genetic testing in epileptic encephalopathy in childhood. Epilepsia. 2015 May;56(5):707-16.

170. Epi Kc, Epilepsy Phenome/Genome P. Ultra-rare genetic variation in common epilepsies: a case-control sequencing study. Lancet Neurol. 2017 Feb;16(2):135-43.

171. Klassen T, Davis C, Goldman A, et al. Exome sequencing of ion channel genes reveals complex profiles confounding personal risk assessment in epilepsy. Cell. 2011 Jun 24;145(7):1036-48.

172. Hernandez CC, Gurba KN, Hu N, Macdonald RL. The GABRA6 mutation, R46W, associated with childhood absence epilepsy, alters 6beta22 and 6beta2 GABA(A) receptor channel gating and expression. J Physiol. 2011 Dec 1;589(Pt 23):5857-78.

173. Tang X, Hernandez CC, Macdonald RL. Modulation of spontaneous and GABA-evoked tonic alpha4beta3delta and alpha4beta3gamma2L GABAA receptor currents by protein kinase A. J Neurophysiol. 2010 Feb;103(2):1007-19.

174. Gurba KN, Hernandez CC, Hu N, Macdonald RL. GABRB3 mutation, G32R, associated with childhood absence epilepsy alters alpha1beta3gamma2L gamma-aminobutyric acid type A (GABAA) receptor expression and channel gating. J Biol Chem. 2012 Apr 6;287(15):12083-97.

175. Lo WY, Botzolakis EJ, Tang X, Macdonald RL. A conserved Cys-loop receptor aspartate residue in the M3-M4 cytoplasmic loop is required for GABAA receptor assembly. J Biol Chem. 2008 Oct 31;283(44):29740-52.

176. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res. 2003 Jul 1;31(13):3381-5.

177. Hibbs RE, Gouaux E. Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature. 2011 Jun 2;474(7349):54-60.

178. Miller PS, Aricescu AR. Crystal structure of a human GABA receptor. Nature. 2014 Jun 8.

179. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994 Nov 11;22(22):4673-80.

180. Lukas D. Schuler XDaWFvG. An improved GROMOS96 force field for aliphatic hydrocarbons in the condensed phase. Journal of Computational Chemistry. 2001 2001;22(11):14.

181. Smith CA, Kortemme T. Backrub-like backbone simulation recapitulates natural protein conformational variability and improves mutant side-chain prediction. J Mol Biol. 2008 Jul 18;380(4):742-56.

182. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem. 2004 Oct;25(13):1605-12.

183. Dunne EL, Hosie AM, Wooltorton JR, et al. An N-terminal histidine regulates Zn(2+) inhibition on the murine GABA(A) receptor beta3 subunit. Br J Pharmacol. 2002 Sep;137(1):29-38.

184. Wooltorton JR, McDonald BJ, Moss SJ, Smart TG. Identification of a Zn2+ binding site on the murine GABAA receptor complex: dependence on the second transmembrane domain of beta subunits. J Physiol. 1997 Dec 15;505 (Pt 3):633-40.

185. Krishek BJ, Moss SJ, Smart TG. Homomeric beta 1 gamma-aminobutyric acid A receptor-ion channels: evaluation of pharmacological and physiological properties. Mol Pharmacol. 1996 Mar;49(3):494-504.

186. Anstee QM, Knapp S, Maguire EP, et al. Mutations in the Gabrb1 gene promote alcohol consumption through increased tonic inhibition. Nat Commun. 2013;4:2816.

187. Klausberger T, Fuchs K, Mayer B, Ehya N, Sieghart W. GABA(A) receptor assembly. Identification and structure of gamma(2) sequences forming the intersubunit contacts with alpha(1) and beta(3) subunits. J Biol Chem. 2000 Mar 24;275(12):8921-8.

188. Sarto I, Wabnegger L, Dogl E, Sieghart W. Homologous sites of GABA(A) receptor alpha(1), beta(3) and gamma(2) subunits are important for assembly. Neuropharmacology. 2002 Sep;43(4):482-91.

189. Lo WY, Lagrange AH, Hernandez CC, Gurba KN, Macdonald RL. Co-expression of gamma2 subunits hinders processing of N-linked glycans attached to the N104 glycosylation sites of GABAA receptor beta2 subunits. Neurochem Res. 2014 Jun;39(6):1088-103.

190. Lo WY, Lagrange AH, Hernandez CC, et al. Glycosylation of {beta}2 subunits regulates GABAA receptor biogenesis and channel gating. J Biol Chem. 2010 Oct 8;285(41):31348-61.

191. Venkatachalan SP, Czajkowski C. Structural link between gamma-aminobutyric acid type A (GABAA) receptor agonist binding site and inner beta-sheet governs

channel activation and allosteric drug modulation. J Biol Chem. 2012 Feb 24;287(9):6714-24.

192. Tanaka M, Olsen RW, Medina MT, et al. Hyperglycosylation and reduced GABA currents of mutated GABRB3 polypeptide in remitting childhood absence epilepsy. Am J Hum Genet. 2008 Jun;82(6):1249-61.

193. Le SV, Le PHT, Le TKV, Kieu Huynh TT, Hang Do TT. A mutation in GABRB3 associated with Dravet syndrome. Am J Med Genet A. 2017 May 24.

194. Hernandez CC, Kong W, Hu N, et al. Altered Channel Conductance States and Gating of GABAA Receptors by a Pore Mutation Linked to Dravet Syndrome. eNeuro. 2017 Jan-Feb;4(1).

195. Euro E-RESC, Epilepsy Phenome/Genome P, Epi KC. De novo mutations in synaptic transmission genes including DNM1 cause epileptic encephalopathies. Am J Hum Genet. 2014 Oct 02;95(4):360-70.

196. Hamdan FF, Srour M, Capo-Chichi JM, et al. De novo mutations in moderate or severe intellectual disability. PLoS Genet. 2014 Oct;10(10):e1004772.

197. Papandreou A, McTague A, Trump N, et al. GABRB3 mutations: a new and emerging cause of early infantile epileptic encephalopathy. Dev Med Child Neurol. 2016 Apr;58(4):416-20.

198. Wolff M, Johannesen KM, Hedrich UB, et al. Genetic and phenotypic heterogeneity suggest therapeutic implications in SCN2A-related disorders. Brain. 2017 Mar 04.

199. Zerem A, Haginoya K, Lev D, et al. The molecular and phenotypic spectrum of IQSEC2-related epilepsy. Epilepsia. 2016 Nov;57(11):1858-69.

200. Zhang Y, Wang Y, Zhou Y, Zhang L, Yu L, Zhou S. Therapeutic effects of the ketogenic diet in children with Lennox-Gastaut syndrome. Epilepsy Res. 2016 Dec;128:176-80.

201. Mueller TM, Remedies CE, Haroutunian V, Meador-Woodruff JH. Abnormal subcellular localization of GABAA receptor subunits in schizophrenia brain. Transl Psychiatry. 2015 Aug 04;5:e612.

202. Bristow GC, Bostrom JA, Haroutunian V, Sodhi MS. Sex differences in GABAergic gene expression occur in the anterior cingulate cortex in schizophrenia. Schizophr Res. 2015 Sep;167(1-3):57-63.

203. Fatemi SH, Folsom TD, Rooney RJ, Thuras PD. Expression of GABAA alpha2-, beta1- and epsilon-receptors are altered significantly in the lateral cerebellum of subjects with schizophrenia, major depression and bipolar disorder. Transl Psychiatry. 2013 Sep 10;3:e303.

204. Lien E, Vatevik AK, Ostern R, Haukanes BI, Houge G. A second patient with a De Novo GABRB1 mutation and epileptic encephalopathy. Ann Neurol. 2016 Aug;80(2):311-2.

205. Bianchi MT, Macdonald RL. Mutation of the 9' leucine in the GABA(A) receptor gamma2L subunit produces an apparent decrease in desensitization by stabilizing open states without altering desensitized states. Neuropharmacology. 2001 Nov;41(6):737-44.

206. Bianchi MT, Song L, Zhang H, Macdonald RL. Two different mechanisms of disinhibition produced by GABAA receptor mutations linked to epilepsy in humans. J Neurosci. 2002 Jul 1;22(13):5321-7.

207. Hortnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G. Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain. Neuroscience. 2013 Apr 16;236:345-72.

208. Laurie DJ, Wisden W, Seeburg PH. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci. 1992 Nov;12(11):4151-72.

209. Wisden W, Laurie DJ, Monyer H, Seeburg PH. The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci. 1992 Mar;12(3):1040-62.

210. Fillman SG, Duncan CE, Webster MJ, Elashoff M, Weickert CS. Developmental co-regulation of the beta and gamma GABAA receptor subunits with distinct alpha subunits in the human dorsolateral prefrontal cortex. Int J Dev Neurosci. 2010 Oct;28(6):513-9.

CHAPTER III: Effects of Lennox-Gastaut syndrome-associated GABRB3 (D120N, E180G, Y302C) mutations on $GABA_A$ receptor function

3.1 Lennox-Gastaut syndrome (LGS): A rare but catastrophic childhood epilepsy

The Lennox-Gastaut syndrome is a severe childhood epilepsy syndrome with multiple seizure types, characteristic EEG patterns, and a typical age of onset between 1-8 years. LGS remains to be one of the most difficult to manage epilepsies with nearly all patients experiencing intractable seizures, profound cognitive decline, and behavioral deficits, features that rarely remit [1-3]. Anti-epileptic drugs (AEDs), surgical interventions, and dietary modifications provide inadequate relief from frequent seizures and associated comorbidities. A high seizure frequency is common, and most patients receive polytherapy with multiple AEDs [3-6]. Cognitive impairment progresses after onset of seizures in 75-90% of patients at the time of diagnosis, and within five years of onset serious impairment is apparent [3, 7]. Cognitive impairment is more severe in children with early onset of seizures or when LGS evolves from other epilepsy syndromes such as West syndrome/infantile spams (in ~20-50% cases) [8-13]. Though, cases with late onset (>8 years) have much better developmental and cognitive outcomes prior to seizure onset [6, 8, 14-19].

LGS is a rare epilepsy syndrome with an annual incidence of ~2/100,000 births [8, 20], a prevalence of 1-10% of all childhood epilepsies [21-25], and with a slight male predominance [17, 20, 21, 23]. The higher prevalence rates (>5%) could be skewed by misdiagnosing all patients with multiple seizure types and cognitive impairment to be

affect by LGS. For example diagnosing patients with Doose syndrome/myoclonic–astatic epilepsy as LGS [26] or from cases such as infantile spasms with high prevalence rates (~25%) that progress to LGS. While a lower prevalence rate (<5%) could result from adhering to stringent diagnostic criteria. Despite the wide range of reported prevalence rates, a prevalence rate of ~4-6% is generally agreed upon. In the recent classification by the International League Against Epilepsy (ILAE), LGS is defined as an epileptic encephalopathy-epilepsy in which epileptic activity (seizures and interictal discharges) contributes to cognitive decline beyond that expected from the underlying pathology [27, 28]. With more stringent diagnostic criteria, growing awareness, and increasing number of studies on LGS, future studies could provide much better estimates of prevalence. Even though LGS is a rare disease, patients with this devastating disorder require a substantial amount of clinical resources. Further lifelong seizures and associated comorbidities have a tremendously negative impact on patients and caregivers.

3.2 Discovery of LGS

The first systematic report of what would be called the Lennox-Gastaut syndrome (LGS) was published by Frederic A. Gibbs, Erna L. Gibbs, and William G. Lennox in 1939 [29]. They described it as “petit mal variant” (now called atypical absence seizures) since the SWDs frequency was slower (<2.5 Hz) than that observed during petit mal (generalized absence) seizures (~3 Hz). They also noted that (1) atypical absence seizures were common in post-central and occipital areas, while absence seizures were most common

in the frontal and the pre-central areas. (2) atypical absence seizures were insensitive to blood glucose and CO_2 levels, unlike the absence seizures that are decreased by high and increased by low blood sugar levels. However, this study was largely unrecognized, probably because electrographic features were primarily used to distinguish the two absence seizure types. A large body of work followed this seminal observation. In 1943 Lennox and colleagues demonstrated that patients with slow SWDs (< 2.5 Hz) were clinically distinct from the patients with typical SWDs (~3 Hz), and had higher likelihood of developing intractable seizure and cognitive impairment. Additionally, they showed that unaffected controls and epileptic patients can indeed be parsed based on electrographic SWD frequencies [30]. Patients with a clinical diagnosis of absence seizures had the highest coincidence (in 77% of patients) of observing typical electrographic seizures (3 Hz) during routine EEGs compared to patients with complex partial seizures or GTCS [30]. Later they described a working definition of absence seizures, noted early age of onset of absence seizures, provided clinical utility of EEG measures, and tested several drugs (including caffeine) on patients with atypical absence seizures and found that the unmarketed drug Tridione was the most effective [31, 32]. In 1950 Lennox and Davis carried out an extensive study involving 200 patients with atypical and 200 with typical absence seizures [33]. In this seminal paper they described the clinical features that are to date considered the hallmark features of LGS. They also reported that patients with atypical absence seizures had greater variety of seizures and EEG abnormalities, lesser impact of family history, and multiple seizure types. Additionally, they observed that in striking contrast to children with typical absence seizures, those with atypical absence seizures had diminished response to

Tridione and presented with learning and intellectual disabilites. In depth findings from the above studies were published in "Epilepsy and Related Disorders" [34], which reemphasized that patients with atypical absence seizures are clinically different from those with typical absence seizures, and associated the clinical findings with the electrographic features of the patients.

Interest in LGS was revived in 1965 when Charlotte Dravet reported findings from 50 children with slow SWDs in her medical doctoral thesis under the supervision of Henri Gastaut [35]. Later these findings were extended to a total of 100 patients [36]. They confirmed the EEG findings, multiple seizure phenotypes (refractory to medication), and cognitive deficits reported by Lennox. In addition they extended the clinical findings and reported that the most common age on onset of LGS was 1-6 years, West syndrome could progress to LGS, the interictal EEG in patients with LGS was abnormal. They also introduced the terms ‘epileptic encephalopathy with slow spike-waves’ or ‘Lennox Syndrome’ to describe the syndrome. Later Lennox’s daughter proposed to expand the term to Lennox-Gastaut syndrome in the XIV European meeting on EEG Information [37, 38]. The term Lennox-Gastaut syndrome was first used by Niedermeyer in 1969 [39], and studies by Chevrie, Aicardi, Penry, and Markand furthered the field and refined the EEG with simultaneous video-EEG monitoring [40-44].

3.3 LGS: current definition

The current clinical diagnostic criteria of LGS have changed a little over the past 67 years the since Lennox and Davis' description in 1950. Even though the precise definition of LGS may vary, currently most authors agree that the presence of following triad of features is diagnostic [19, 27, 28].

1. Multiple seizure types including tonic and atypical absence seizures as the most frequent seizures, drop attacks (resulting from tonic and atonic seizures), myoclonic seizures, generalized tonic-clonic seizures, and focal seizures. Seizure onset is 1-8 years (peak 3-5 years, rarely in adolescence). However, LGS can evolve from West syndrome/infantile spams, which has a much earlier seizure onset (typically between 3 and 8 months). Seizures are refractory to AED treatment for most patients.
2. EEG abnormalities include slow SWDs (<2.5 Hz) in wakefulness and sleep, and fast rhythms (10-12 Hz) during sleep that may be associated with tonic seizures.
3. Profound cognitive decline and developmental delay. Although, patients with late onset (> 8 years) may have better cognitive outcomes [19, 45].

However, patients may not display all diagnostic and clinical features, especially at the onset of LGS, and a definitive diagnosis may take several years. A detailed description of the clinical manifestations and EEG signatures of the common seizure types in LGS patients are described in the next chapter.

3.4 Etiology of LGS

Comprehending the etiologies of LGS is important for treatment, counseling, and research purposes. Being a rare disorder, the clinical and research community has only started to scratch the surface of the underlying etiologies of LGS. The causes of LGS are extremely heterogeneous, although the majority of the LGS patients' etiology could be broadly categorized either as structural (previously symptomatic, 60-80%) or as genetic/presumed genetic or unknown (previously idiopathic or cryptogenic, 20-40%) [6, 46]. Rarely patients present with infectious, metabolic, or immune etiologies [19, 47-50]. A structural etiology refers to the concept that structural abnormalities (such as those acquired from trauma, stroke, hypoxic–ischemic injury, infection, or cortical malformation due to a genetic mutation) markedly increases the risk of epilepsy [51]. For LGS patients, neuroimaging and EEG are required to ascertain whether a structural abnormality is the cause of the seizures. According to the recent ILAE classification, the structural etiology takes precedence over genetic classification when the structural malformations underlie the epilepsy, although, both etiological terms could be used [51].

Genetic etiology implies that the epilepsy results directly from a known or presumed genetic mutation [51]. The causative mutation could be monogenic (inherited or *de novo*) or polygenic with or without contribution of environmental factors and/or susceptibility genes. In addition to missense or small deletion/insertion mutations, copy number variants (>1 kb DNA deletions or duplications) are emerging as important genetic contributors to epilepsy [52, 53]. A genetic etiology for LGS was long suspected for the unknown (or cryptogenic) cases, but only recently the genetic etiology has been

confirmed. Factors that make establishing the genetic etiology of LGS difficult include: (1) rarity of the disorder, (2) sporadic occurrence with limited family history of epilepsy (family history of epilepsy for LGS patients is same as those for non-epileptic patients, 4-10 %) [2], and (3) lack of next generation sequencing for patients to identify small genetic changes prior to the last decade .

Even though complex inheritance was suspected for LGS, whole exome sequencing studies in the current decade indicate a prominent role for *de novo* mutations in LGS etiology that also explain the occurrence of sporadic cases. There has been an exponential increase in the number of genes associated with LGS in the past five years, with epilepsy-associated genes discovered on a monthly basis. *De novo* missense mutations in ***GABRB3* and *ALG13*** were reported as causative for LGS [54] by the Epi4K consortium in 2013. Since then a number of genes have been reported either in isolated cases or designated as causative in cohort studies. A few in the fast growing list of genes include *CACNA1A* [54], *CDKL5* [54], *CHD2* [54, 55], *DNM1* [54, 56-58], *FLNA* [54], *FOXG1* [59], *GABRA1* [54], *GABRG2* [60], *GRIN1* [54], *GRIN2A*, *GRIN2B* [54], *HDAC3*, *HDAC4* [54], *HNRNPU* [54], *IQSEC2* [54, 61], *MTOR* [54], *NEDD4L* [54], *SCN1A* [54], *SCN2A* [54, 62], *SCN8A* [54], *STXBP1,* and *SYNGAP1*. Thus, previously classified idiopathic and cryptogenic LGS cases can very well be classified as genetic. However, until genetic testing becomes commonplace in majority of epilepsy clinics, the causative genes for most LGS cases will remain unknown.

Unknown etiology implies that the cause of epilepsy is not yet understood based on the clinical picture, but basic diagnosis could be made from electroclinical semiology

such as generalized or focal epilepsy [51]. Currently the percentage of patients with truly unknown diagnosis in the developed countries has been decreasing due to increasing access to specialized neurology clinics, EEG monitoring, neuroimaging, and genetic testing. The degree to which the cause could be ascertained depends on the availability and accessibility to health care resources for patient evaluation. The hope is that the etiology for more unknown cases would emerge as diagnostic resources and understanding of LGS continues to increase.

3.5 *GABRB3* mutations as cause of LGS

After decades of pursuit, the genetic contribution to LGS became apparent when the Epi4K/EPGP consortium identified 329 *de novo* mutations in 264 LGS and IS patients by whole exome sequencing of trios (proband and their unaffected parents) [54]. These mutations were absent in 610 control trios. Among the 329 genes, four mutations in *GABRB3* were implicated as monogenic causes of LGS and IS. Additionally, two or more probands had *de novo* mutations in eight other genes (*ALG13,* n=2; *CDKL5*, n=3; *DNM1*, n=2; *HDAC4*, n=2; *SCN1A*, n=7; *SCN2A*, n=2; *SCN8A*, n=2; and *STXBP1*, n=5). Patients also had significant excess of *de novo* mutations in the ~4000 genes intolerant to functional genetic variation and enriched in genes previously associated with autism and regulated by fragile X protein [54]. The promising results from this study has revived interest in LGS in recent times, and a number of sequencing studies in EE patients have followed. Since then several novel *de novo* mutations have been identified and over 20 have been reported in *GABRB3* [54, 57, 63-68].

Although, the four *GABRB3* mutations were implicated to be causative in LGS, their impact on $GABA_A$ functions were unknown, further *in silico* functional prediction scores (Table 2.1) offer no clues to the mechanisms of actions of these mutations. Thus, we determined the effects of the *de novo GABRB3* mutations on $GABA_A$ receptor function and biogenesis *in vitro* using the HEK 293T cell expression system. In this chapter I describe the actions of three *GABRB3(D120N, E180G, Y302C)* mutations identified in LGS patients.

Table 3.1. *De novo GABRB3* mutations identified in patients with Lennox-Gastaut syndrome.

Mutation	Nucleotide change	CCDS transcript ID	Location in β3 subunit	PolyPhen-2 score	Sex	Age at seizure onset	Initial seizure type
GABRB3 (D120N)	15:26866564 C→T	CCDS539 20.1	GABA binding pocket, loop A	1	M	10 mo.	IS
GABRB3 (E180G)	15:26828484 T→C	CCDS539 21.1	GABA binding pocket, loop B	0.974	M	10 mo.	LGS
GABRB3 (Y302C)	15:26806254 T→C	CCDS100 19.1	M2-M3 loop	0.938	F	10 mo.	FDS, staring spells

CCDS transcript ID = consensus coding sequence identifier of the GABRB3 gene,

FDS=Focal dyscognitive seizures

3.5 MATERIALS AND METHODS

3.5.1 Complementary DNA (cDNA) Constructs

cDNAs encoding human $GABA_AR$ subunits α1 (NM_000806.5), β1 (NM_000812.3), β3 (NM_021912.4, variant 2), γ2L (NM_198904.2) and EGFP (LC008490.1) were each cloned into the pcDNA(3.1+) vector. Point mutations in the cDNA encoding β1 and β3 subunits were introduced using the QuikChange Site-Directed Mutagenesis kit (Agilent). The hemagglutinin (HA) epitope tag (YPYDVPDYA) was inserted between amino acids 4 and 5 of the mature γ2L subunit protein. The HA epitope tag insertion at this position in the γ2L subunit has minimal effects on the electrophysiological properties or surface levels of $GABA_AR$s. All constructs were sequenced prior to use at the Vanderbilt Technologies for Advanced Genomics core facility and verified against published sequences. The amino acids are numbered according to the immature peptide sequence.

3.5.2 Expression of recombinant $GABA_A$ Receptors

HEK293T cells (HEK 293T/17, ATCC® CRL-11268™) were cultured as monolayers at 37°C in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 IU/ml each of penicillin and streptomycin (Invitrogen). For whole electrophysiology, cells were plated at a density of 4×10^5 in 60 mm culture dishes (Corning). cDNA transfections were done 24 hours after plating cells, and they were re-plated for recording using trypsin-EDTA digestion (Invitrogen) in 35 mm dishes 24 hours after transfection. For the homozygous expression condition, 0.6 μg cDNA of

each α1, β3, and γ2L subunit and 0.1 μg of EGFP were transfected using X-tremeGENE9 DNA Transfection Reagent (Roche Diagnostics). For the heterozygous condition 0.6 μg α1 and γ2L, 0.3 μg wt β1/3, 0.3 μg mutant β1/3, and 0.1 μg EGFP of cDNAs were used. A ratio of 1.15 μl X-tremeGENE9:1 μg cDNA was used. EGFP and $GABA_AR$ subunit cDNAs were co-transfected to identify transfected cells based on GFP fluorescence. For single channel recordings, HEK293T cells were plated at 4 x 10^4 in 35 mm culture dishes (Corning) and transfected after 24 hours with 0.3 μg cDNA of each α1, β3, and γ2L subunit and 0.05 μg cDNA of EGFP for the homozygous condition. Recordings were done 48 hours after transfection. The cDNA and XtremeGENE9 amounts were scaled up or down in proportion to the area of the culture dishes. Our unpublished observations show that cells lacking GFP expression do not produce GABA-evoked currents, indicating that GFP is a good proxy for α, β, and γ subunit expression. In addition when cells are transfected with wt α, β, and γ subunit-encoding cDNAs, they produce typical $\alpha\beta\gamma$ subunit-like current responses and lack, for example, of $\alpha\beta$ subunit-like currents. Although, $\alpha\beta$ subunit-containing $GABA_A$ receptors could constitute a minor portion of all receptors and their presence cannot be ruled out, the majority of the receptors are likely to be $\alpha\beta\gamma$ subunit-containing.

For flow cytometry experiments, cells were plated at a density of 4-6 x 10^5 in 60 mm culture dish (Corning), and transfected with 0.6 μg cDNA for each α1, β3, and γ2L subunit 24 hours after plating using Polyethyleneimine (MW 40,000 KD, 24765, Polysciences Inc.). Cells were harvested using trypsin-EDTA (Invitrogen) 48 hours

following transfection. For mock or single subunit expression, empty pcDNA3.1 vector was added to make the final cDNA transfection amount to be 1.8 µg.

3.5.3 Whole cell electrophysiology

Whole cell voltage clamp recordings were obtained at room temperature from lifted HEK293T cells bathed in an external solution composed of (in mM): 142 NaCl, 8 KCl, 10 D(+)-glucose, 10 HEPES, 6 MgCl2.6H2O, and 1 CaCl2 (pH 7.4, ~326 mOsm). Recording electrodes with series resistance of 1-2 MΩ were pulled from TW15OF-3 thin walled glass capillaries (World Precision Instruments, Inc.) using a P-2000 Quartz Micropipette Puller (Sutter Instruments), and the tip was polished using MF-830 Micro Forge (Narishige). The internal solution consisted of (in mM): 153 KCl, 10 HEPES, 5 EGTA 2 Mg-ATP, and 1 MgCl2.6H2O (pH 7.3, ~300 mOsm). The Cl- reversal potential was near 0 mV, and cells were voltage clamped at -20mV to get an inward GABA-evoked current. Whole-cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments) low-pass filtered at 2 kHz using the internal 4-Pole Bessel filter of the amplifier, digitized at 10KHz with Digidata 1322A (Axon Instruments) and stored for offline analysis. Drugs were gravity-fed to a four-barrel square glass tubing connected to a SF-77B Perfusion Fast-Step system (Warner Instruments Corporations). The 10-90 % rise times of open-tip liquid junction currents were 200-600 µs. 1 mM GABA was applied for 4s to study macroscopic current kinetics. Data were analyzed offline using Clampfit 9.0 (Axon Instruments). Data were expressed as mean ± standard error of the mean (SEM).

3.5.4 Single channel electrophysiology

$GABA_A$ receptor single channel currents were recorded in the cell-attached configuration as described previously [69, 70]. Briefly, single-channel currents were recorded from HEK293T cells bathed in external solution containing (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 glucose, and 10 HEPES (pH 7.4). Glass electrodes were pulled from thick-walled borosilicate capillary glass (World Precision Instruments) on a P-2000 Quartz Micropipette Puller (Sutter Instruments) and fire-polished to a resistance of 10–20 MΩ on an MF-830 Micro Forge (Narishige) before use. The electrode solution consisted of (in mM): 120 NaCl, 5 KCl, 10 MgCl2, 0.1 CaCl2, 10 glucose, and 10 HEPES (pH 7.4). The electrode potential was held at +80 mV. $GABA_A$ receptor spontaneous activity was recorded in absence of GABA and blocked by adding 100 μM picrotoxin and 100 μM Zn^{+2}. GABA-evoked currents were recorder in presence of 1 mM GABA. Single channel currents were amplified and low-pass filtered at 2 kHz using an Axopatch 200B amplifier, digitized at 20 kHz using Digidata 1322A, and saved using pCLAMP 9 (Axon Instruments). Data were analyzed offline using TAC 4.2 and TACFit 4.2 (Bruxton Corporation) [69, 70]. Data were shown as the mean ± SEM. Statistical analysis was performed using GraphPad Prism (GraphPad Software 6.0). Statistical significance was taken as $p < 0.05$, using unpaired two-tailed Student's *t* test or one-way ANOVA as appropriate. GABA, Zn^{+2} and picrotoxin were obtained from Sigma.

3.5.5 Flow Cytometry

Flow cytometry protocols have been previously described in detail [71, 72]. Briefly, $GABA_A$ receptors were expressed as described above, and cells were harvested ~48 hours after transfection and immediately placed on ice in 4°C FACS buffer. Cell surface expression levels of α1, β3, $\gamma2L^{HA}$ subunits were determined using primary antibodies against human α1 subunits (N-terminal, clone BD24, Millipore; 2.5 g/ml), human β3 subunits (N-terminal, monoclonal, β2/3-PE, clone 62-3G1, Millipore; 2.5 g/ml), and the HA epitope tag (clone 16B12, Covance; 2.5 g/ml) respectively. Cells were washed 3 times using 4°C cold FACS buffer and fixed with 2% w/v paraformaldehyde. To determine the total cellular expression levels, cells were fixed for 15 min using BD Cytofix/CytopermTM fixation/permeabilization buffer (BD Biosciences) and washed 2 times using BD Perm/WashTM staining buffer (BD Biosciences) prior to staining. Following washes after primary antibody treatment cells were incubated with anti-mouse IgG1 secondary antibody conjugated to the Alexa647 fluorophore (Invitrogen) before additional washing and fixation.

Fluorescence intensity levels of cells were determined using a BD LSR II 3/5-laser flow cytometer (BD Biosciences). Data were analyzed offline using FlowJo 7.5.5 (Tree Star). For each condition, intensity levels of 50,000 cells were acquired. The non-viable cells were excluded using their forward and side-scatter properties. Net fluorescence intensity levels from cells expressing $GABA_A$ receptor subunits were calculated after subtracting the mean fluorescence intensities of the cells transfected with blank pcDNA(3.1+) vector. The relative fluorescence intensities for each condition

were calculated by normalizing the average intensity values to those obtained from cells expressing α1, β3 and γ2L^{HA} subunits (WT). One-way analysis of variance with Tukey's post-test was used to determine if the expression levels of different transfection conditions were significantly different from the WT condition. Data were expressed as mean ± SEM.

3.5.6 Structural Modeling and Simulation

$GABA_A$ receptor subunit raw sequences in FASTA format were individually loaded into Swiss-PdbViewer 4.10 [73] for template searching against ExPDB database (ExPASy, http://www.expasy.org/). Then, the structure of the *C. elegans* glutamate-gated chloride channel (GluCl; PDB: 3RHW) [74] in the closed conformation was identified as the best template resulting in 33% and 36% sequence identity for γ2 and α1 subunits respectively. For β3 subunits, the human $GABA_AR$-β3 (PDB: 4COF) [75] crystal structure was used *per se* with no further modification. The initial sequence alignments between γ2 and α1 subunits and *C. elegans GluCl* subunits were generated with full-length multiple alignments using ClustalW [76]. Sequence alignments were inspected manually to assure accuracy among structural domains solved from the template. Because the long cytoplasmic regions of the γ2 and α1 subunits were absent in the solved GluCl structure, it was excluded from the modeling, and separate alignments were generated for the TM4 domains. Then full-length multiple alignments were submitted for automated comparative protein modeling implemented in the program suite incorporated in SWISS-MODEL (http://swissmodel.expasy.org/SWISS-MODEL.html).

Before energy minimization using GROMOS96 [77], resulting structural models of human γ2 and α1 subunits were inspected manually, their structural alignments confirmed and evaluated for proper h-bonds, presence of clashes and missing atoms using Molegro Molecular Viewer (www.clcbio.com). Then, pentameric $GABA_A$ receptor homology models were generated by combining α1, β3 and γ2 structural models in the stoichiometry 2β:2α:1γ with the subunit arrangement β-α-β-α-γ. Neighborhood structural conformational changes at the β3+α1-, α1+β3-, and γ2+β3- interfaces of the $GABA_A$ receptor caused by a single mutated amino acid residue (*de novo* mutation) in the human β3 subunit were simulated using Rosetta 3.1 [78] implemented in the program suite incorporate in Rosetta Backrub (https://kortemmelab.ucsf.edu). Since Rosetta 3.1 does not allow Cysteine substitutions, $GABA_A$ receptors carrying Cysteine mutations were exchanged by Alanine. Then, up to twenty of the best-scoring structures were generated at each time by choosing parameters recommended by the application for one mutation at the time for each correspondent subunit interface as follow: β3(D120N), β3(E18G), and β3(Y302A) within the β3+α1- interface, and β3(N110D) and β3(F246S) at the α1+β3- and γ2+β3- interfaces. All single point mutations were incorporated the β3 subunit since the full-length alignment between the β3 and β1 sequences displayed high similarity (91.2 %). Root mean square deviation (RMS) was calculated between the initial (wild-type) structures and superimposed simulated (mutated) structures. For each mutation, the RMS average over ten low energy structures was computed and conformational changes displayed among neighborhood structural domains. We prepared the figures using Chimera 1.7 [79].

3.5.6 Data analysis

Electrophysiology data were analyzed offline using Clampfit 10.3 software (Axon Instruments) and were expressed as mean ± standard error of the mean (SEM). One mM GABA was applied for 4 s to study macroscopic current kinetics. Peak currents and 10-90% rise time of currents were calculated using a built-in function in the Clampfit 10.3 software. The current desensitization time courses were fitted using the Levenberg-Marquardt least squares method with multiple component exponential functions of the form $\sum_{i=1}^{n} A_i\, e^{-t/\tau i} + C$, where n is the number of exponential components, A is the relative (fractional) amplitude of the component at time = 0, t is time, τ is the time constant, and C is a constant offset in the y direction. Fitting the decay of currents evoked with 4 s GABA applications resulted in three or four exponential components. The number of components were increased until additional components did not significantly improve the fit. The fit was determined by an F-test on the residuals. To simplify comparisons a weighted sum of desensitization time courses was used. The current deactivation following removal of GABA was fitted suing mono-exponential equation (n=1), % desensitization was calculated by subtracting the GABA current at the end of 4 s application from the peak GABA current, and then normalized to the peak GABA current. To determine the % Zn^{2+} inhibition of GABA currents, an extended wash for 40 s followed 4s GABA (1 mM) application and then 10 μM Zn^{2+} was applied for 10 s, followed by co-application of 1 mM GABA and 10 μM Zn^{2+} for 4 s. Peak currents from co-application of GABA and Zn^{2+} were subtracted from the peak GABA-evoked currents and then normalized to the peak GABA-evoked current. Holding current was calculated as the average of baseline current before GABA

application. The outward Zn^{2+} current during the application of 10 μM Zn^{2+} for 10 s was calculated by subtracting the 6 s average baseline current before Zn^{2+} application from the average of current during the last 6 s of Zn^{2+} application.

3.6 RESULTS

3.6.1 LGS-associated de novo *GABRB3* mutations were located in conserved structural domains of $GABA_A$ receptor β subunits.

We found that all three LGS-associated mutations were located in conserved domains of β3 subunits that are critical for $GABA_A$ receptor functions. By aligning protein sequences encoded by *GABR* genes we found that D120 and Y302 were invariant residues across all $GABA_A$ receptor subunits, while E180 was invariant among β subunits (Figure 3A). Mutated residues are part of major structural domains (Figure 3B; mutated residues are shown in red) such as loop A (D120), loop B (β7 sheet, E180), and the M2–M3 loop (Y302), which are involved in the ligand binding channel gating-coupling mechanisms [75, 80-84]

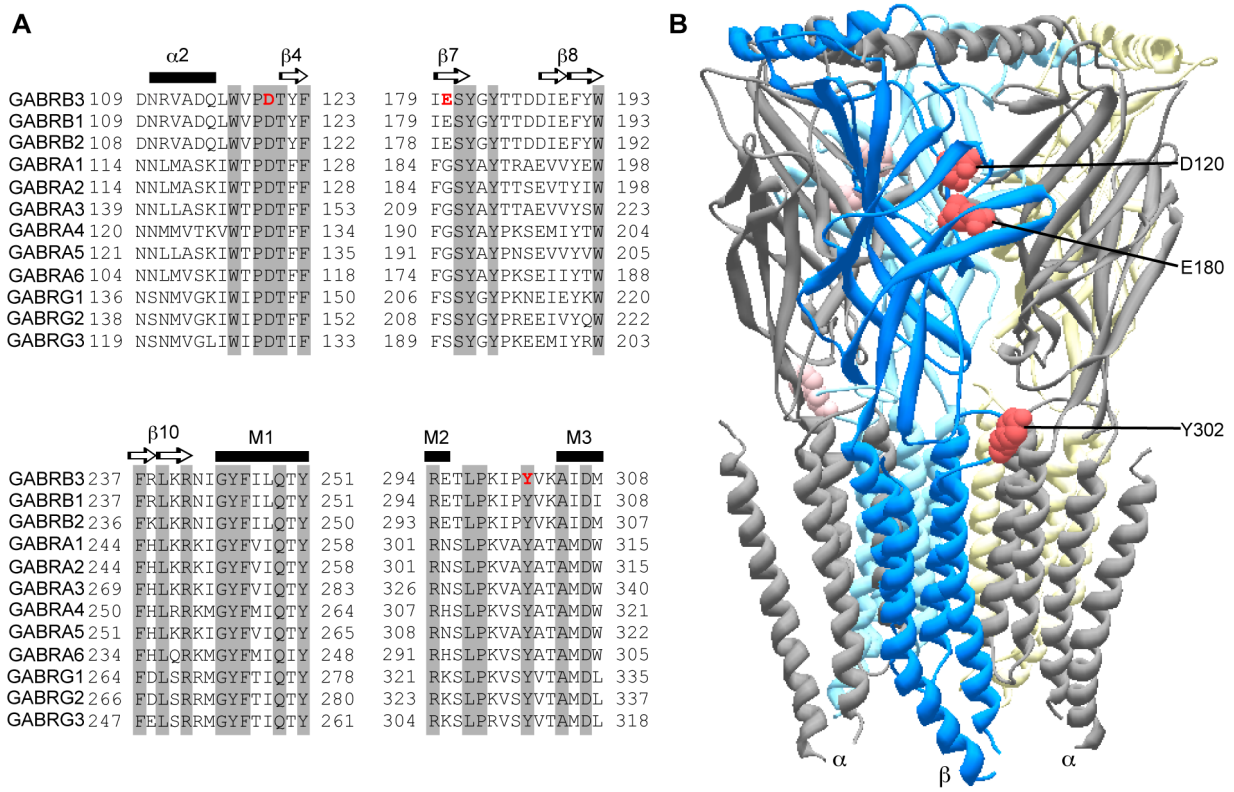

Figure 3.1 LGS-associated *GABRB3* mutations alter conserved β3 subunit domains.

(A) Protein sequence alignment of $GABA_A$ receptor α (1-6), β (1-3), and γ (1-3) show that gene mutations alter conserved amino acids. Mutated amino residues are in red and residues conserved across α, β, and γ subunits in grey. Secondary structures are represented above the alignments as a helices (black bars) or β sheets (arrows). **(B)** Three-dimensional structural model of the $GABA_A$ receptor was made with amino acid residues altered by *GABRB3* mutations shown in red. The β subunits are in blue, α subunits in gray, and γ subunit in yellow.

3.6.2 LGS-associated *de novo GABRB3* mutations reduced GABA-evoked currents.

To determine the consequences of LGS-associated *GABRB3* mutations on $GABA_A$ receptor function, we measured macroscopic GABA-evoked current amplitudes

recorded with the whole cell configuration following rapid application of 1 mM GABA. In the homozygous (hom) condition (cells expressing α1, β3 (mutant), and γ2L subunits) GABA-evoked peak amplitudes were significantly reduced compared to the wild type (wt) condition (cells expressing α1, β3 (wt), and γ2L subunits). The peak current densities from cells expressing β3(D120N), β3(E180G), and β3(Y302C) subunit-containing $GABA_A$ receptors were reduced to ~24%, 1%, and 5% of the wt condition, respectively (Figure 3.3, Table 3.2). Since the LGS patients were heterozygous (het) for the *de novo GABRB3* mutations, we also examined the impact of these mutations in the *in vitro* het expression condition (cells expressing α1, β3 (wt), β3 (mutant), and γ2L subunits). Peak current amplitudes were also significantly reduced in the het condition to a lesser extent than the hom condition (het β3(D120N) = 64%, het β3(E180G) = 61%, and het β3(Y302C) = 44% of wt current densities; see Figure 3.3, Table 3.2).

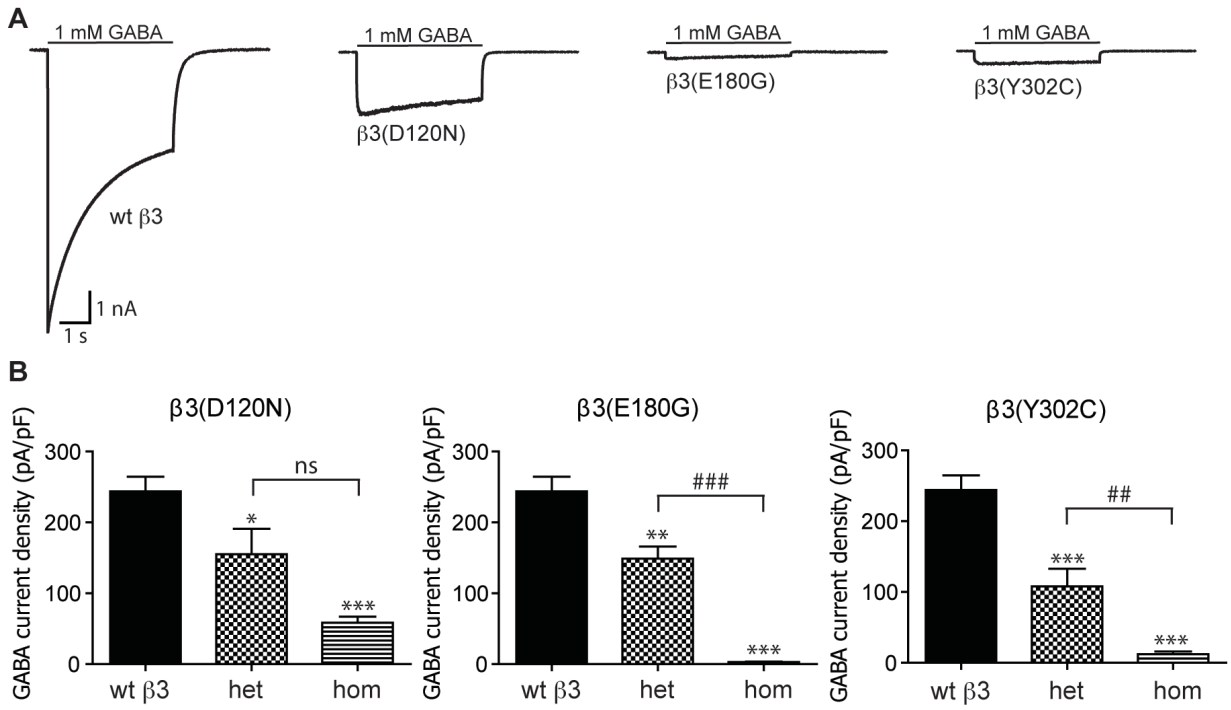

Figure 3.2 The *de novo GABRB3* mutations found in LGS patients produced substantial loss of GABA-evoked currents.

(A) Representative GABA-evoked current traces obtained following rapid application of 1 mM GABA for 4 s to lifted HEK293T cells voltage clamped at -20 mV. The current traces from $GABA_A$ receptors containing mutant β3 subunits in the homozygous (hom) condition are compared to their respective wild-type (wt) current traces. **(B)** Bar graphs showing average peak current densities from cells expressing mutant β subunits in hom and heterozygous (het) conditions. Values are expressed as mean ± standard error of the mean (see Table 2.2 for details).

Values are expressed as mean ± standard error of the mean. One-way analysis of variance with Dunnett post-test was used to determine significance. */#Significant difference compared to the wt and het conditions, respectively. *p < 0.05, **/##p < 0.001, ***/###p < 0.0001. ns = not significant.

3.6.3 LGS-associated *de novo GABRB3* mutations altered GABA-evoked current kinetics.

We examined macroscopic properties such activation, desensitization, and deactivation of GABA-evoked currents. When these important properties are altered, they affect shape and amplitude of the inhibitory post-synaptic currents (IPSCs) in neurons. All three β3 subunit mutations significantly slowed current activation (longer 10–90% rise time) in the hom condition but not in the het condition (Figure 3.4A, Table 3.2). Current desensitization during GABA application was unaltered in hom and het conditions by β3(D120N) and β3(Y302C) mutant subunits, while the β3(E180G) subunits produced strong current desensitization only in the hom condition (Figure 3.4 B and C, Table 3.2). Further, β3(D120N) and β3(Y302C) mutant subunits increased current deactivation (reduced weighted deactivation rate constant) after GABA application was terminated. The β3(E180G) subunits resulted in an unusual positive overshoot of the currents from the baseline after removal of GABA. This prevented fitting exponential functions in a meaningful way to determine deactivation rate constants (resulting in negative time constants). In the het condition, the β3(E180G) subunits did not affect current deactivation. Although, the current kinetic changes due to β3(D120N, E180G, Y302C) subunits were hard to interpret as the GABA-currents were profoundly reduced (76.1–98.9% of wt) and seemed to be less relevant than the substantial loss of peak currents.

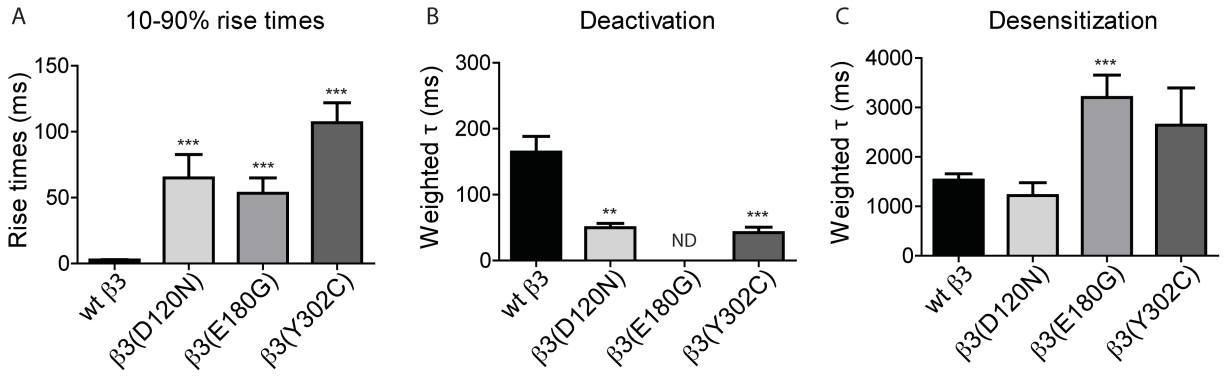

Figure 3.3 The *de novo* GABRB3 mutations alter GABA-evoked whole cell current kinetic properties.

Bar graphs show average rise times **(A)**, deactivation τ **(B),** and desensitization τ **(C)** of whole cell GABA-evoked currents in wt and hom conditions (see Table 2.3 for het results). All three *GABRB3* mutations considerably increased rise times **(A)** and reduced deactivation time constants **(B),** although for a large portion of cells the deactivation could not be meaningfully extracted for the β3(E180G) hom condition. **(C)** Deactivation of GABA-evoked currents was seen only for the β3(E180G) hom condition.

Values are expressed as mean ± standard error of the mean. One-way analysis of variance with Dunnett post-test was used to determine significance. */#Significant difference compared to the wt and het conditions, respectively. *p < 0.05, **/##p < 0.001, ***/###p < 0.0001. ND= not determined.

Table 3.2 Effects LGS-associated *GABRB3* mutations on whole cell GABA- evoked currents and expession levels of $GABA_A$ receptor subunits.

	α1β3γ2L (n)			
	wt β3 (8-16)	β3(D120N) (5-8)	β3(E180G) (5-21)	β3(Y302C) (8-10)
Peak GABA current density (pA/pF), homozygous	244 ± 21	58.3 ± 8.8***	2.8 ± 0.7***	12.4 ± 3.7***
Peak GABA current density (pA/pF), heterozygous	-	155 ± 36*	149 ± 17**###	108 ± 25***##
10-90% rise time (ms), homozygous	2.6 ± 0.3	64.9 ± 17.8***	53.3 ± 11.7***	107 ± 15***
10-90% rise time (ms), heterozygous	-	4.4 ± 1.2###	3.1 ± 0.4###	5.9 ± 1.2###
Desensitization τ (ms), homozygous	1530 ± 128	1218 ± 262	3202 ± 452***	2640 ± 755.4
Desensitization τ (ms), heterozygous	-	1559 ± 320.1	2024 ± 285.1	1701 ± 424
Deactivation τ (ms), homozygous	165 ± 24	49.9 ± 6.5**	ND	42.5 ± 8.1***
Deactivation τ (ms), heterozygous	-	78.6 ± 11.9*	93.2 ± 15.4	70.6 ± 9.5***
Holding current (pA), homozygous	-85.8 ± 12	-35.3 ± 17.3	-466 ± 87***	-10.8 ± 4.7***
Holding current (pA), heterozygous	-	-31.7 ± 10.4*	-209 ± 69	-25.8 ± 8.2***
Outward Zn^{2+} current (pA), homozygous	+7.2 ± 2.3	+0.9 ± 0.6	+47.5 ± 13.8**	-0.9 ± 1.3*
Outward Zn^{2+} current (pA), heterozygous	-	+3.6 ± 1.6	+15.8 ± 3.9#	+3.9 ± 1.3
% Zn^{2+} inhibition, homozygous	13.2 ± 2.1	13.6 ± 1.3	33.7 ± 8**	-9.9 ± 6.0***

% Zn^{2+} inhibition, heterozygous	-	15.0 ± 1.4	12.2 ± 2.7$^{\#\#}$	10.8 ± 2.0$^{\#\#\#}$
		(12/5)	(12/5)	(15/8)
Surface/Total a1, homozygous	-	0.93 ± 0.04/ 1.00 ± 0.14	1.01 ± 0.13/ 1.10 ± 0.13	1.08 ± 0.09/ 1.30 ± 0.15
Surface/Total $b1^{HA}$/3, homozygous	-	0.98 ± 0.05/ 1.10 ± 0.11	1.25 ± 0.08/ 1.26 ± 0.17	1.059 ± 0.06/ 1.73 ± 0.21**
Surface/Total $g2L^{HA}$, homozygous	-	0.98 ± 0.07/ 0.97 ± 0.17	1.01± 0.16/ 1.33 ± 0.45	1.04 ± 0.07/ 1.15 ± 0.14

Values represent mean ± S.E.M.*, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p<0.001$ statistically different from wt homozygous, and $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ indicate $p < 0.05$, $p < 0.01$, and $p<0.001$ statistically different between *de novo GABRB* mutations in homozygous and heterozygous conditions (one-way ANOVA with Dunnett's multiple comparisons test or unpaired t-test). ND=non-determinate. Surface and total expression levels were normalized against the wt condition.

3.6.4 LGS-associated *de novo GABRB3* mutations did not reduce surface levels of α, β or γ subunits.

To determine if the loss of GABA-evoked currents resulted from loss of surface $GABA_A$ receptors, we examined surface levels of α1, β3 or $\gamma2L^{HA}$ subunits using flow cytometry. We found that none of the LGS-associated mutations reduced total or surface levels of α1, β3 or $\gamma2L^{HA}$ subunits in the hom condition (Figure 3.5 A,B), suggesting that none of the mutations affected biogenesis or trafficking of $GABA_A$ receptors. As we saw greater impact of the mutations on GABA-evoked currents in hom than the het condition, but did not see loss of surface receptors in hom condition we did not extend the flow cytometry experiments to the het condition. Surprisingly, in the hom condition surface levels of

β3(E180G) subunits were 24.9 ± 0.08% higher (but not significantly higher) than the wt β3 subunit levels. The majority of the $GABA_A$ receptor subunits are not expressed on the cell surface without partnering subunits, although wt β3 subunits are found on cell surface in absence of α and γ subunits, at levels similar to those of β3 subunits in the wt condition. When β3(E180G) subunits were expressed alone, they had a 37.3% higher surface expression level compared to wt β3 subunits expressed alone. These data suggest that the β3(E180G) subunits favor the formation of homomeric β3 receptors or $GABA_A$ receptors with subunit stoichiometry distinct from wt receptors.

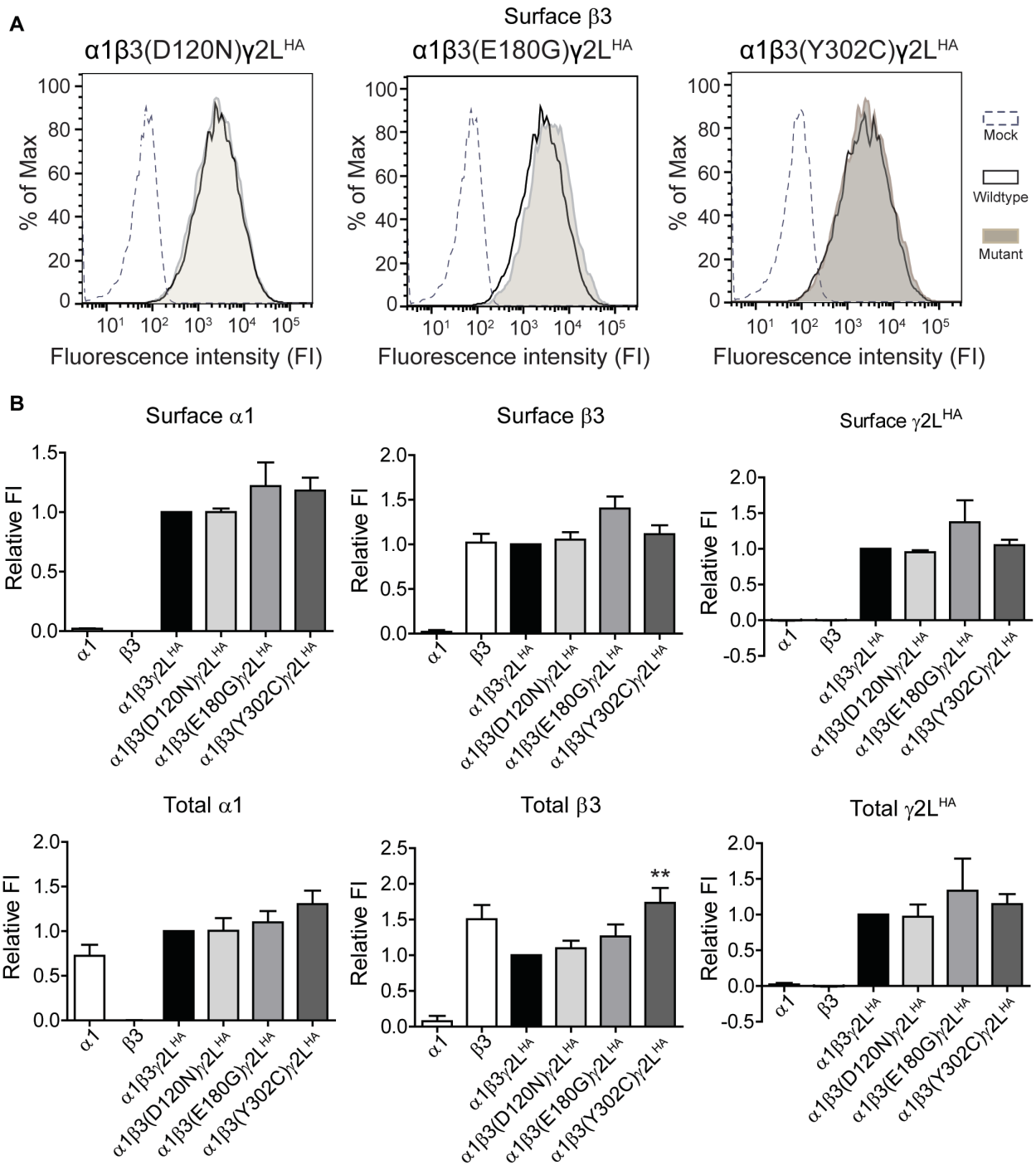
A
Surface β3
α1β3(D120N)γ2L^HA
α1β3(E180G)γ2L^HA
α1β3(Y302C)γ2L^HA
% of Max
Fluorescence intensity (FI)
Mock
Wildtype
Mutant
B
Surface α1
Surface β3
Surface γ2L^HA
Total α1
Total β3
Total γ2L^HA
Relative FI
α1
β3
α1β3γ2L^HA
α1β3(D120N)γ2L^HA
α1β3(E180G)γ2L^HA
α1β3(Y302C)γ2L^HA
**

Figure 3.4 The GABRB3 mutations did not reduce surface and total levels of GABAA receptor subunits.
Flow cytometry was used to determine surface **(A)** and total **(B)** levels of α1, β3, and γ2L^{HA} subunits in HEK293T cells. None of the *GABRB3* mutations reduced surface or total levels of $GABA_A$ receptor subunits. **(A)** Representative fluorescence intensity (FI) histograms showing the surface levels of β3 subunits from cells expressing α1, mutant β3, γ2L^{HA} subunits in hom condition (shaded), α1, wt β3, γ2L^{HA} subunits (unfilled with solid black line), and empty vector (unfilled with dotted line). **(B)** Normalized FI values of the Alexa 674 fluorophore are presented in bar graphs, with FI for each condition normalized to the FI of the wild-type (wt) condition (Relative FI). The top two panels and the bottom panel respectively show the surface and total Relative FI levels of the α1, β3, and γ2L^{HA} subunits. Cells expressing only α1, β3, or γ2L^{HA} subunits were used as controls. Unlike the β3(D120N) and β(Y302C) subunits, the β3(E180G) subunit levels were increased by 25% of the wt β3 levels, although they were not significantly different. Additionally, the β3(Y302C) total levels were significantly higher than the wt β3 levels by 73%.

Values were expressed as mean ± standard error of the mean. One-way analysis of variance with Dunnett post-test was used to determine significance. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ compared to the wt condition.

3.6.5 LGS-associated *GABRB3* mutations reduced GABA-activated currents by reducing GABA potency or efficacy.

Results from Figure 3.3 and Figure 3.4 reveal that LGS-associated mutations reduced GABA-evoked current amplitudes, increased/slowed rise times, and speeded deactivation. Similar changes are seen in when sub-saturating concentrations of GABA (<1 mM) is applied to wt $GABA_A$ receptors [85, 86]. Since the β3(D120N, E180G, and Y302C) mutant subunits were located in loop A and loop B (β7 sheet) of the GABA binding pocket and M2–M3 loop (involved in the ligand binding channel gating coupling mechanism), respectively (see Figure 3.2B and Figure 3.9, mutated residues are in red), it is possible that these mutations disrupted GABA binding and/or coupling of GABA binding to channel gating, resulting in reduced GABA potency and/or efficacy.

To determine if this could be the case, we measured current responses to a supersaturating concentration of 10 mM GABA. We found that with 10 mM GABA, β3(D120N) subunit-containing receptors produced current amplitudes similar to those containing wt receptors with 1 mM GABA (Figure 3.6A, B left graph). The current response increased to 83.6 ± 7.3% of wt current with 10 mM GABA compared to 23.9 ± 3.6% of the wt current with 1 mM GABA. This result suggested that the β3(D120N) subunits reduce GABA potency. In contrast, currents from β3(E180G) and β3(Y302C) containing $GABA_A$ receptors produced minimal increase with 10 mM GABA (1.2 ± 0.3% and 5.1 ± 1.5% of wt in 10 mM GABA, respectively), suggesting a major reduction in GABA efficacy (Figure 3.6A B left graph).

Furthermore, the current rise times for β3(D120N) subunit-containing receptors with 10 mM GABA were similar to those of wt receptors with 1 mM GABA. While for the β3(E180G) and β3(Y302C) subunit-containing $GABA_A$ receptors the rise times were much slower even with 10 mM GABA (Figure 3.6B B right graph). Although, the rise times were ~14-fold, ~3-fold, and ~3-fold faster with 10 mM GABA from cells containing β3(D120N), β3(E180G) and β3(Y302C) subunit-containing $GABA_A$ receptors, respectively, compared to wt receptors with 1 mM GABA. These findings further support that β3(D120N) subunits reduced GABA potency while β3(E180G) and β3(Y302C) subunits reduced GABA efficacy.

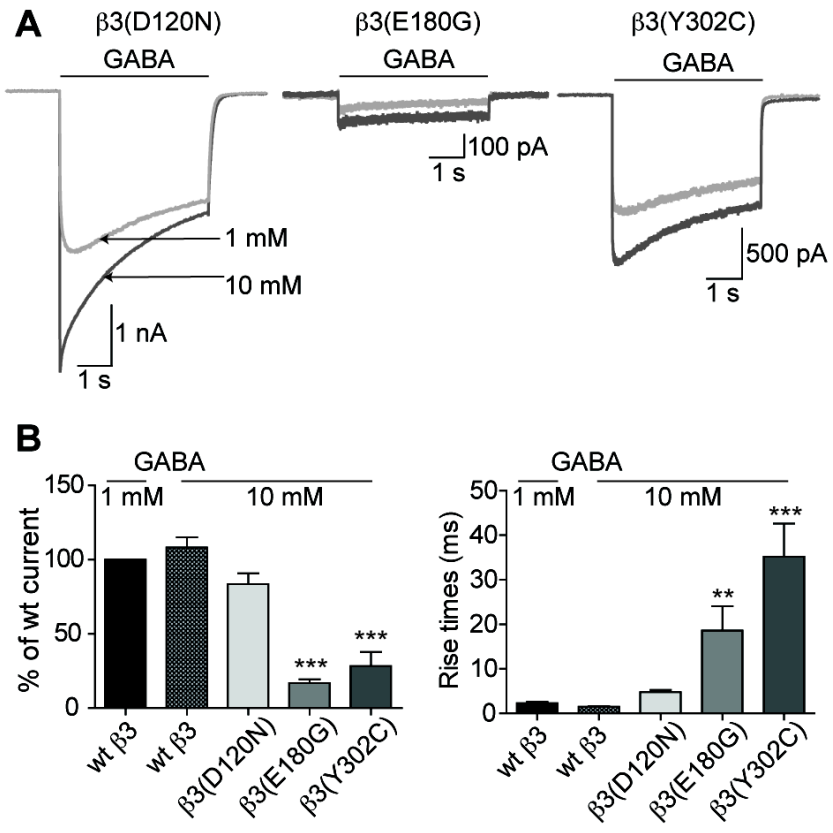

Figure 3.5 The mutant β3 subunits reduced GABA potency or efficacy.
To test if high concentrations of GABA can restore current form mutant receptors to wt levels 10 mM GABA was applied. **(A)** Representative whole cell current responses following 1 mM GABA (light gray) and 10 mM (dark gray) application from cells expressing wild-type (wt) or mutant receptors in hom condition. **(B, left)** Bar graph show average peak current responses to 10 mM GABA application as a percentage of wt response to 1mM GABA. Average currents from β3(D120N), β3(E180G), and β3(Y302C) subunit-containing receptors with 10 mM GABA were 83.6 ± 7.3%, 16.9 ± 2.5%, and 28.3 ± 9.6% of the wt currents, respectively, with 1mM GABA. **(B, right)** Bar graph show the average rise times of GABA-evoked currents to 1- and 10mM GABA application from cells with wt or mutant subunits in hom condition. Rise times for β3(D120N), β3(E180G), and β3(Y302C) subunit-containing receptors were 4.8 ± 0.5 ms, 18.6 ± 5.5 ms, and 35.2 ± 7.4 ms, respectively. These results indicate that the *GABRB3(D120N)* mutation reduced GABA potency while the *GABRB3(E180G, Y302C)* mutations reduced GABA efficacy.

Values were expressed as mean ± standard error of the mean. One-way analysis of variance with Dunnett post-test was used to determine significance. **p < 0.001, ***p < 0.0001 compared to the wt condition with 1mM GABA application.

3.6.6 LGS-associated mutations impaired the single channel gating properties of $GABA_A$ receptors.

Single channel recordings provide a direct measurement of currents and kinetic behavior of the ion channels. These microscopic current measurements may explain the macroscopic properties of GABA-evoked currents containing mutant subunits, such as reduced peak currents and altered current kinetics. Therefore, we recorded single channel currents in the continuous presence of 1 mM GABA in a cell attached out-side-out configuration. Wt $\alpha 1\beta 3\gamma 2L$ receptors opened in brief bursts and recurrent prolonged (>500 ms) clusters of bursts with a main conductance level of ~26 pS and an open channel probability (Po) of 0.62 ± 0.05 (Figure 3.7, Table 2.3). Open time distributions were fitted best with three weighted exponential functions (a_{o1}, a_{o2}, and a_{o3}) suggesting openings to at least three different open states with open time constants τ_{o1}, τ_{o2}, and τ_{o3} (Figure 3.7 B).

All three *GABRB3(D120N, E180G, and Y302C)* mutations that reduced peak current amplitudes also significantly reduced single channel Po compared to wt receptors (Figure 3.7C, Table 3.3). While the *GABRB3(Y320C)* mutation reduced single channel conductance (~21 pS), the *GABRB3(D120N, E180G)* mutations did not (Table 3.3). Additionally, *GABRB3(D120N, Y302C)* mutations also reduced opening frequency without altering the single channel mean open time (Figure 3.7C, Table 3.3). Concordantly, the three open time distributions were minimally affected for $\beta 3$(D120N) and $\beta 3$(Y302C) subunit-containing receptors (Figure 3.7B, Table 3.3). On the other hand the $\beta 3$(E180G) subunit containing-receptors did not alter single channel opening

frequency but reduced single channel mean open time, resulting from reduced open time constants and a substantial increase (88 ± 5% in the relative proportion [a_{o1}]) of occurrence of the shortest open state (τ_{o1}). In addition to channels gating properties, the bursts were affected by the LGS-associated mutations (Figure 3.7A-C, Table 3.3). All three mutations increased the duration and frequency of short bursts, and decreased the duration and frequency of long bursts (Figure 3.7B and C, Table 3.3).

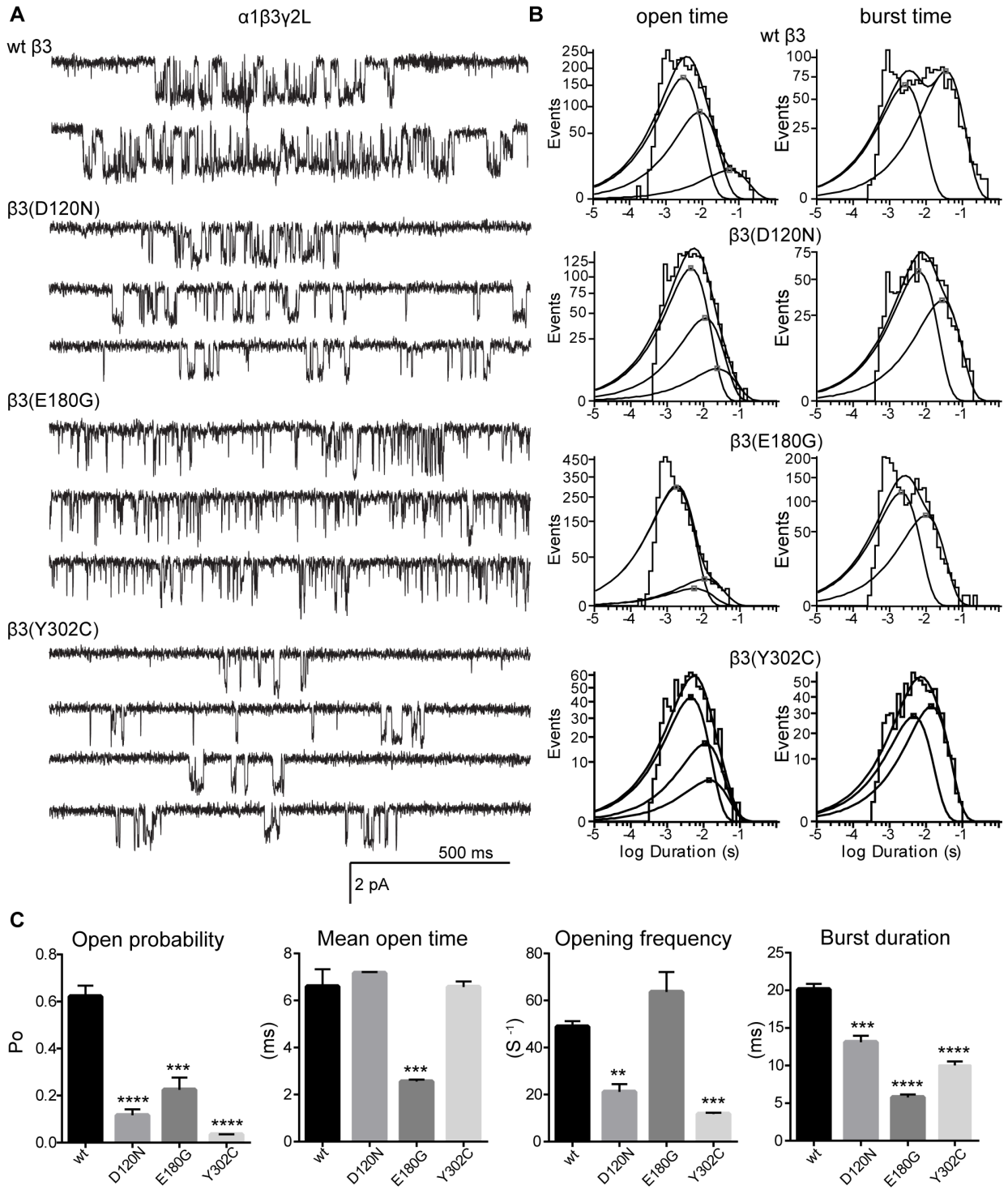
A
α1β3γ2L
wt β3
β3(D120N)
β3(E180G)
β3(Y302C)
500 ms
2 pA
B
open time
burst time
wt β3
β3(D120N)
β3(E180G)
β3(Y302C)
Events
log Duration (s)
C
Open probability
Po
Mean open time
(ms)
Opening frequency
(S-1)
Burst duration
(ms)
wt
D120N
E180G
Y302C

Figure 3.6 Single channel properties of GABAA receptors with wt and mutant β3 subunits.

Single channel currents were obtained using cell attached recordings with 1 mM GABA in the recording pipette. **(A)** Representative single channel current traces from cells expressing wild-type (wt) or mutant $GABA_A$ receptors (hom condition). **(B)** Mean open time (left panels) and burst duration (right panels) histograms for wt and mutant receptors were fitted best to 3 and 2 exponential functions, respectively. The average of open and burst duration histograms (sums of multiple exponential functions) are marked with a square. **(C)** Bar graphs summarize the effects of wt and LGS-associated *GABRB3* mutations on the kinetic properties of the receptors. All three mutations reduced Po and burst duration of $GABA_A$ receptor channels. The *GABRB3(E180G)* mutation significantly increased brief channel openings reflected as reduced mean open time and increased channel opening frequency.

Values represent mean $\pm$ standard error of the mean. Statistical differences were determined using one-way analysis of variance with Dunnett multiple comparisons test (see Table 2 for details). **p < 0.01, ***p <0.001, ****p < 0.0001.

3.6.7 The mutant β3(E180G) subunit produced spontaneously gated $GABA_A$ receptor channels.

One unexpected finding from recording macroscopic currents was that the cells expressing α1β3(E180G)γ2L receptors required higher holding currents (5.4-fold and 2.4-fold in hom and het conditions, respectively; Figure 3.8A, Table 3.2) to keep cells clamped at -20 mV compared to those expressing wt receptors. This suggests spontaneous channel openings in the hom and the het condition. Additionally, the cells expressing β3(E180G) were unhealthy (had significantly increased holding currents to keep cells at -20 mV) compared to those expressing wt β3, β3(D120N) or β3(Y302C) subunits. We knew from flowcytometry experiments that in the hom condition the β3(E180G) subunits had higher cell surface expression levels (by 24.9 ± 0.08%), but not significantly different from wt (Figure 3.5B middle graph, Table 2.3), while the α1 and

γ2L surface levels were unaltered. Furthermore, co-application of Zn and GABA is used as a proxy to determine the composition of surface $GABA_A$ receptors. $GABA_A$ receptors with αβ or just β subunits have significantly higher inhibition of peak GABA currents in presence of Zn^{+2} compared those with αβγ subunits. Similarly, peak currents from cell expressing α1β3(E180G)γ2L subunits in hom condition have significantly higher Zn^{+2} (10 μM) inhibition of GABA currents compared to those expressing α1β3γ2L subunits (Table 3.3). This further indicates that β3(E180G) subunits change the composition of surface $GABA_A$ receptors. While the surface levels of α and γ subunits did not change, it is likely that the increased holding currents and increased Zn^{+2} inhibition could be due to formation of homomeric β3(E180G) receptors. Zn^{+2} application has also been shown to block spontaneous $GABA_A$ receptor "leak" current leading to a positive shift in the baseline current from cell expressing only β3 subunits (known to form homomeric $GABA_A$ receptors with spontaneous single channel openings) [87-89]. With 10 μM Zn^{+2} about 10% and 7% of the holding current was blocked in the hom and het conditions, respectively (Figure 3.8, Table 3.3). These results suggest that β3(E180G) form Zn^{+2} sensitive homomeric receptors.

Indeed when single channel currents were recorded from cells expressing α1β3(E180G)γ2L receptors in the hom condition in the absence of GABA, spontaneous single channel openings were observed (Figure 3.8C). Although, it should be noted that even cells expressing wt α1β3γ2L receptors also displayed spontaneous channel opening but with conductance levels of ~12.5 pS and ~21 pS (as opposed to ~26 pS with 1 mM GABA) with the same Po (Figure 3.8C). The spontaneous α1β3γ2L

openings occurred as frequent isolated single channel openings and brief bursts, unlike the prolonged bursts with 1 mM GABA. In contrast the α1β3(E180G)γ2L receptors had significantly higher spontaneous single channel openings, more brief bursts and prolonged clusters of bursts (>1 s). These receptors also opened to two conductance levels that resembled the GABA-evoked openings from wt receptors. The increased burst openings were a result of ~3-fold increase in Po of the low-conductance openings (Figure 3.8C, D). The spontaneous openings from wt and β3(E180G) subunit-containing receptors were inhibited by 100 μM Zn^{+2}, consistent with the block of macroscopic baseline currents with Zn^{+2} (Figure 3.8C).

The receptors containing β3(D120N) and β3(Y302C) subunits also displayed spontaneous channel openings but with smaller conductance than the wt receptors. The β3(D120N) subunit-containing $GABA_A$ receptors had low-conductance (1.2 ± 0.12 pA, n = 4 and 0.08 ± 0.01 pA, n = 4) spontaneous openings with Po similar to wt receptors, while receptors with β3(Y302C) subunits had spontaneous openings that were rare, brief, and low-conductance (1.0 ± 0.18 pA, n = 3 and 0.001 ± 0.003 pA, n = 3). The spontaneous openings of $GABA_A$ receptors with either β3(D120N), β3(E180G) or β3(Y302C) mutant subunits were blocked by picrotoxin (100 μM) in a manner similar to that for wt receptors. Overall, all the *de novo* mutations affected spontaneous activation of $GABA_A$ receptors.

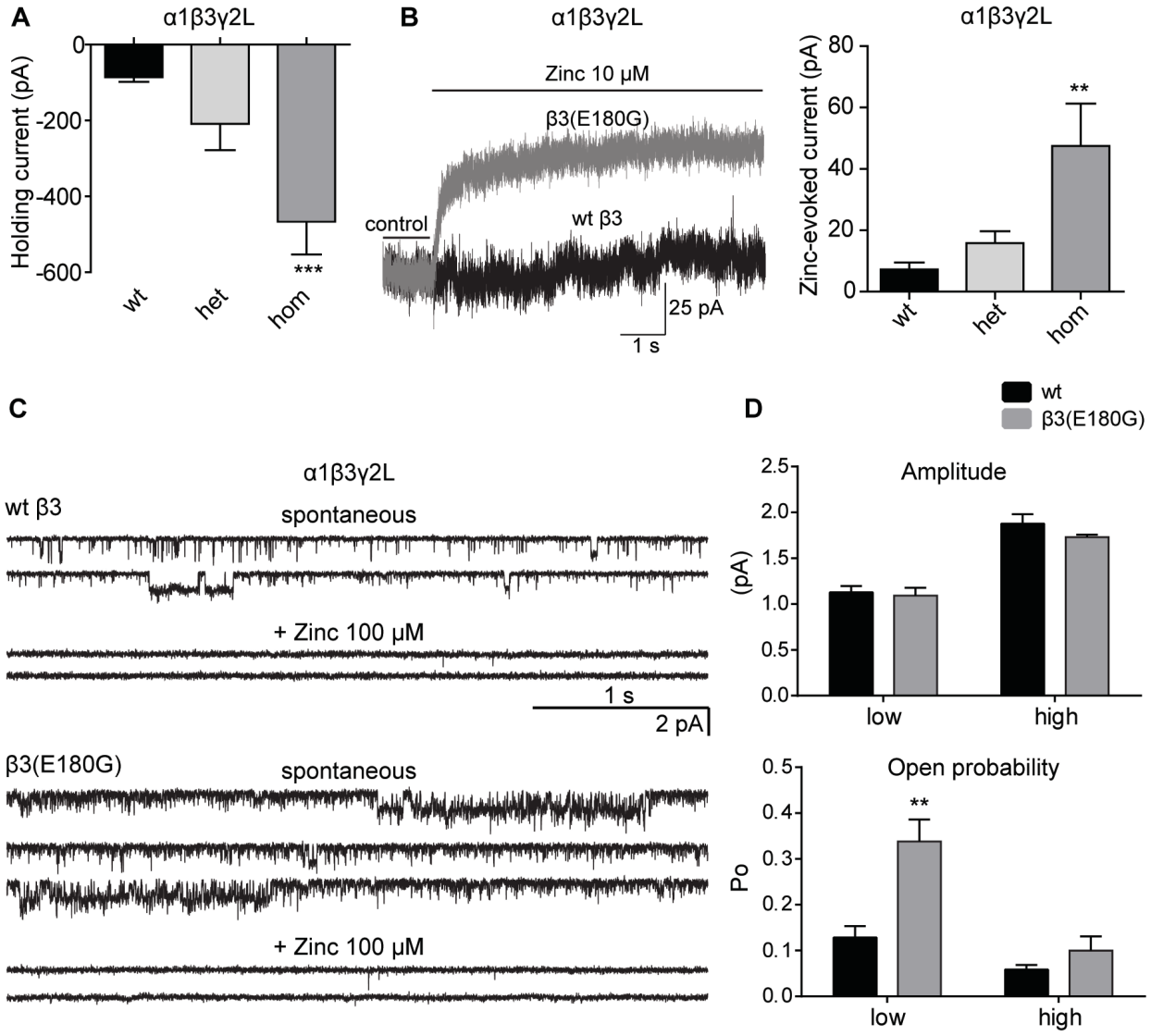

Figure 3.7 Mutant β3(E180G) subunits produced spontaneously gated $GABA_A$ receptors leading to spontaneous macroscopic currents and single channel openings. **(A)** Bar graph shows significantly higher holding currents were needed to clamp cells expressing β3(E180G) subunit-containing $GABA_A$ receptors in het and hom conditions compared to cells with wt receptors. (B, left) Representative traces showing outward currents following Zn^{+2} application from cells with wt β3 and β3(E180G) (hom) subunit-containing $GABA_A$ receptors. **(B, right)** Bar graph with average Zn^{+2}-evoked currents in wt, het, and hom conditions. **(C)** Representative single channel currents from cells with wt and β3(E180G) hom condition. **(D)** Bar graphs showing single channel amplitude and Po of wt (black bars) and spontaneously activated mutant (gray bars) receptors. For wt β3 subunit-containing receptors, the low- and high-conductance openings were 2.5 pS (1.1 ± 0.07pA, n = 7) and 21 pS (1.8 ± 0.10 pA, n = 4) with Po of 0.13 ± 0.02 (n = 7) and 0.06 ± 0.01 (n = 4), respectively. The β3(E180G) mutant subunits significantly

increased the Po of low-conductance openings (0.34 ± 0.05, 1.1 ± 0.08pA, n = 10), without altering high-conductance openings (0.10 ± 0.03, 1.7 ± 0.03 pA, n = 4, p > 0.05).

Values represent mean ± standard error of the mean. One-way analysis of variance with Dunnett multiple comparisons test (A and B) or Two-way analysis of variance with Tukey multiple comparisons (D) test were used to determine statistical significance. **p < 0.01, ***p <0.001, ****p < 0.0001.

Table 3.3 Single channel properties of $GABA_A$ receptors with the *de novo GABRB3* mutations associated with Lennox-Gastaut Syndrome.

	α1β3γ2L (n)			
	wt β3 (6)	β3(D120N) (5)	β3(E180G) (3)	β3(Y302C) (3)
Channel conductance (pS)	24.79 ± 1.62	21.77 ± 2.18	22.55 ± 2.39	18.88 ± 2.47**
Mean open time (ms)	6.60 ± 0.73	7.17 ± 0.04	2.56 ± 0.08***	6.58 ± 0.23
Opening frequency (S^{-1})	49 ± 2	21 ± 3**	64 ± 8	12 ± 1***
Open probability (Po)	0.62 ± 0.05	0.12 ± 0.03****	0.23 ± 0.05***	0.035 ± 0.001****
Open time constants:				
τ_{o1} (ms)	3.18 ± 0.28	4.37 ±0.11*	1.90 ± 0.06*	3.75 ±0.26
τ_{o2} (ms)	9.77 ± 0.84	9.94 ± 0.84	4.97 ± 1.55**	9.99 ± 0.29
τ_{o3} (ms)	20.8 ± 3.4	21.1 ± 1.6	9.98 ± 1.92	16.4 ± 3.1
a_{o1} (%)	67 ± 1	59 ± 8	88 ± 5*	63 ± 2
a_{o2} (%)	28 ± 2	37 ± 8	5 ± 2*	30 ± 5
a_{o3} (%)	5 ± 2	5 ± 0.3	7 ± 3	8 ± 4
Burst duration (ms)	20.13 ± 0.72	13.11 ± 0.86***	5.78 ± 0.38****	10.04 ± 0.52****
Openings/burst	3.12 ± 0.19	1.64 ± 0.09****	1.65 ± 0.06****	1.40 ± 0.02****
Burst time constants:				
τ_1(ms)	2.54 ± 0.20	6.67 ± 0.36***	2.28 ±0.13	4.40 ± 0.49**
τ_1(ms)	35.4 ± 2.9	23.5 ± 3.7*	10.7 ± 0.8***	14.2 ± 0.1***
a_1 (%)	37 ± 6	61 ± 4*	59 ± 8*	43 ± 4
a_2 (%)	63 ± 6	39 ± 4*	41 ± 8*	57 ±4

Values represent mean ± S.E.M.*, **, *** and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ statistically different from wt, respectively. One-way ANOVA with Dunnett's multiple comparisons test was used.

3.6.8 Mutant β3 subunits rearrange conserved structural domains critical for $GABA_A$ receptor function.

The above results and PolyPhen-2 scores (Table 3.1) indicate that all three LGS-associated mutations disrupt $GABA_A$ receptor functions. To gain insights into the structural changes produced by *GABRB3(D120N, E180G, and Y302C)* mutations, we generated homology models for wt and mutant pentameric $GABA_A$ receptors with a stoichiometry of 2β:2α:1γ and the anti-clock wise arrangement of β-α-β-α-γ when viewed from the synaptic cleft. The rearrangement of the subunits' secondary structure and side chains were quantified as the root mean square (RMS) difference between the wt and mutant structures when superimposed (see methods for details).

The LGS-associated mutations were located in the principal (+) side of the β3 subunit, thus the predominant structural changes were in the (+) side of β3 subunits and adjacent to the complimentary (-) side of α subunits (β+/α- interface) that outline the important ligand binding pockets (Figure 3.8A). Thus, perturbations in the secondary structures (presented as mutation-associated alternative ribbon in rainbow when RMS >0.03A°) and side chain residues (box-plots) were measured at the β+/α- interfaces. The mutant β3(D120N) and β3(E180G) subunits mainly induced structural perturbations in loops A, B and C of the GABA binding pocket (Figure 3.8B), which are critical for GABA binding, while the Y302C mutation in the M2-M3 loop disrupted the Cys loop, β1-β2 loop, and the M2-M3 loop, which are part of the ligand binding-channel gating coupling zone (Figure 3.8B). Therefore, not surprisingly the predominant effect of LGS-

associated mutations was reduced GABA-evoked currents by decreasing responses to GABA. LGS-associated mutations caused both local (intra-subunit) perturbations confined to structural domains of the subunit, and global (inter-subunit) changes propagated to the nearest subunit via rearrangements to the neighboring residues and structural domains (Figure 3.8B, box plots).

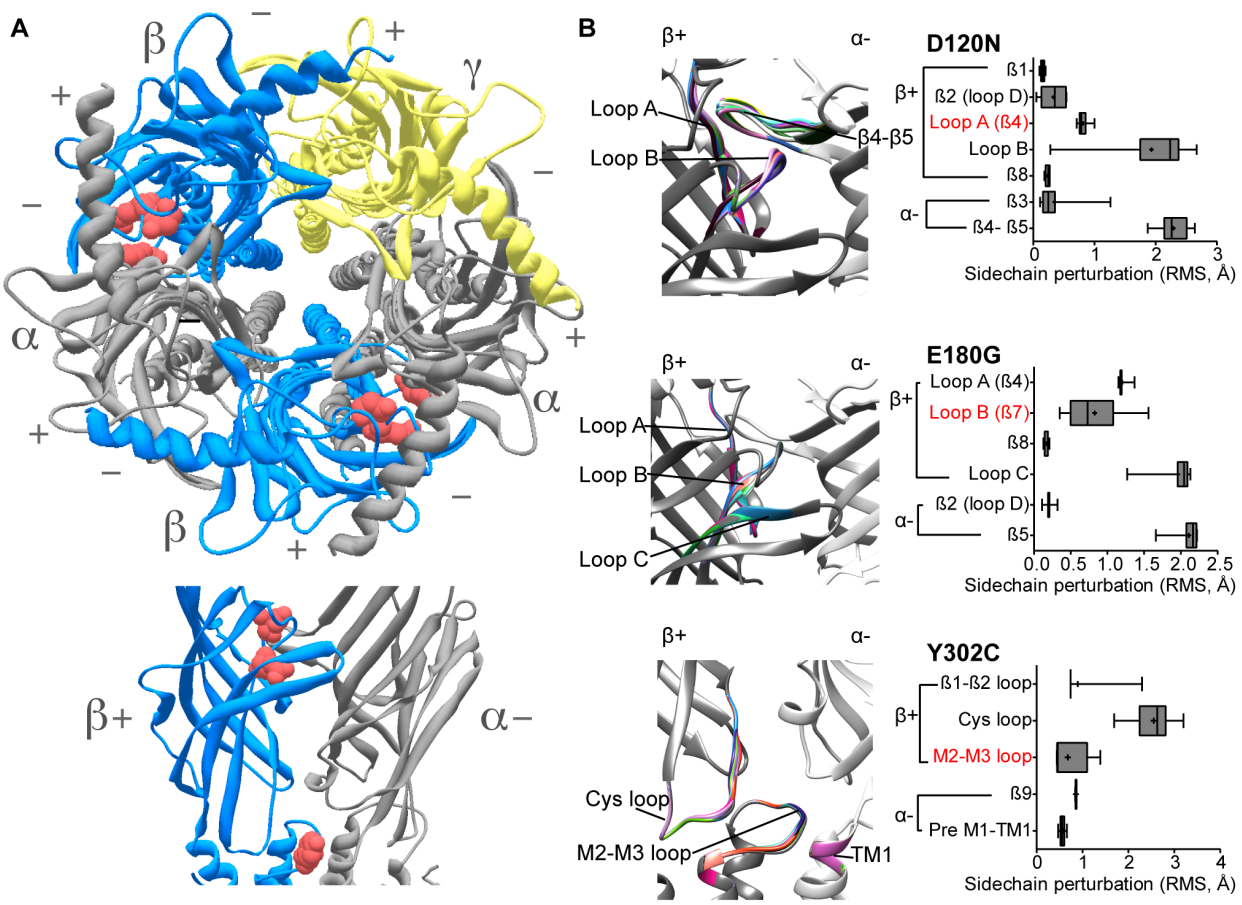

FIGURE 3.8 *De novo GABRB3* mutations induced a wave of structural rearrangements in conserved structural domains important for GABAA receptor function. **(A, top)** Extracellular view of the N-terminal domains of a pentameric αβγ subunit-containing $GABA_A$ receptor structural model (as seen from the synaptic cleft) displaying LGS-associated (in red) *GABRB3* mutations on β3 subunits (blue ribbons) subunits, α1 and γ2L subunits are represented as gray and yellow ribbons, respectively. The principal (+) and complementary (-) interfaces of each subunit are shown. The bottom panel lists the location of the mutations in their respective interfaces. **(A, bottom)** Zoomed in view of the β3+/α- subunit domains containing LGS-associated mutations (as seen almost parallel to the plasma membrane). **(B)** Enlarged view of the domains that had structural rearrangements caused by the LGS-associated *GABRB3(D120N, E180G, Y302C)* mutations. The perturbations of the secondary structures that differ among the wt (in gray) and mutant (in rainbow) structures are indicated by solid black lines (left panels). Box plots show perturbations (as root mean square deviation [RMS]) caused by the mutations in the side chain residues that are propagated through β sheets, loops, and TM helices (right panels). RMS values for up to 10 simulations are represented as interleaved box and whiskers plots (25–75% percentile, median, and minimum and maximum). The secondary structure containing the mutation is highlighted in red.

3.9 DISCUSSION

The Epi4K consortium provided the first evidence of the contribution of *GABRB*3 in EEs and several recent sequencing studies reached the same conclusion [52, 55, 61-66]. Although, the functional consequences of *GABRB3(D120N, E180G, Y302C)* mutations reported by the Epi4K consortium were not know, and this remains true for most mutations identified by large-scale bioinformatics studies. We systematically examined the impact of LGS-associated mutations and found that the mutations disrupt crucial functional domains of $GABA_A$ receptor resulting in result in severe loss of functions, supporting their contribution to EEs.

3.9.1 The principle effects of the LGS-associated mutations are loss of GABA-evoked currents.

The most consequential actions of LGS-associated mutations were significant reduction of whole cell GABA-evoked currents in both hom and het conditions (Figure 3.3). As opposed to several previously reported $GABA_A$ receptor mutations [90], loss of GABA-evoked currents induced by LGS-associated mutations were not due to loss of surface $GABA_A$ receptors (Figure 3.5). Rather the *GABRB3(D120N)* mutation reduced GABA potency while the *GABRB3(E180G, Y302C)* mutations dramatically reduced GABA efficacy (Figure 3.6). In addition all three mutations slowed activation and accelerated deactivation of GABA currents (Figure 3.4). Furthermore, the reduction of GABA-evoked currents due to LGS-associated mutations was much higher than the small

decrease in currents due to previously reported *GABRB(* P11S, S15F and G32R) mutations, which are associated with the less severe absence epilepsy syndrome [71, 91].

The loss of macroscopic currents could be explained by the substantial reduction of Po, openings per burst, and burst duration of single channels (Figure 3.7). Additionally, the *GABRB3(D120N, Y302C)* mutations reduced single channel opening frequency. The *GABRB3(E180G)* mutation produced brief and frequent channel opening and thus did decrease the channel opening frequency. At a neuronal level the mutant subunits could reduce channel openings at both synaptic and extra-synaptic sites, resulting in smaller IPSCs with slower rise and faster decay rates leading to reduced GABAergic inhibition.

3.9.2 Spontaneous currents due to the *GABRB3(E180G)* mutation produced additional $GABA_A$ receptor dysfunction.

In addition to loss of GABA-evoked currents, the *GABRB3(E180G)* mutation produced spontaneous macroscopic and single channel currents that could be blocked by Zn^{+2} application. Spontaneous currents resulted in significantly higher holding currents to clamp cells (Figure 3.8) and reduced overall cell health (unpublished observation). Furthermore, the spontaneous low amplitude channel openings were significantly higher in $GABA_A$ receptors with mutant β3(E180G) subunits than those containing wt β3 subunits. Moreover, there was a small but non-significant increase in β3(E180G) subunits on the cell surface, likely due to formation of β3(E180G) subunit homomers.

These abnormal properties of β3(E180G) subunits could add to the pathogenicity of the mutation beyond the loss of GABA-evoked currents. Although the presence of homomeric $GABA_A$ receptors and spontaneous $GABA_A$ channel opening have not been demonstrated in neurons, a few speculated effects of β3(E180G) subunits could be increased energy spent in maintaining Cl^- concentration gradients, disrupted GABA dependent migration and maturation of interneurons during development, and reduced IPSC amplitudes resulting from reduced drive for Cl^- ions across neuronal membranes.

3.9.3 LGS-associated mutations are predicted to disrupt the GABA binding and channel coupling domains.

When the structural changes induced by the LGS-associated mutations were modeled using the homology models based on crystal structures of the *C. elegans* GluCl and the human $GABA_A$ receptor β3 homopentamer, we saw a structure-dysfunction correlation. The LGS-associated *GABRB3(D120N, E180G, Y302C)* mutations located in crucial GABA binding and channel gating domains at the β+/α- interface produced a substantial loss of currents (reduced to ~24, ~1, ~5% of wt currents, respectively). In comparison mutations located in the signal peptide—*GABRB3(P11S, S15F)* [91] and at the γ+/β- interface—*GABRB3(G32R)* [71] and *GABRG2(R82Q,P83S)* [92] produced smaller current loss (reduced to ~42, ~48, ~50-62, ~34, and ~12% of wt currents, respectively). Similarly, the IS-associated *GABRB3(N110D)* and *GABRB1(F246S)* mutations located at the α+/β- and γ+/β- interfaces either do not alter GABA-evoked current amplitudes or

reduced it to ~75% of the wt currents, respectively (see Chapter II). Further, the *GABRA1(D219N)* mutation also located at the β+/α- interface reduced currents to 30% of the wt currents [93]. Additionally, recent whole exome sequencing studies associated *GABRA1(R112Q, G251S)* [94], *GABRB2(M79T)*[95] mutations located at the β+/α- interface with severe developmental disorders such as Dravet syndrome and intellectual disability. These findings are in line with the assumption that the mutations at the β+/α- interface perturbing the domains crucial for ligand binding and channel coupling are more disruptive of $GABA_A$ receptor functions than those at the α+/β- and γ+/β- interfaces. This might explain, at least in part, how different *GABRB3* mutations with varying extent of channel dysfunction could contribute to pathological outcomes of both mild (childhood absence epilepsy) and severe (LGS, IS) epilepsy syndromes.

3.10 How can *GABRB3* mutations result in epilepsy syndromes?

The β3 subunits are widely expressed and abundant in developing and adult mammalian brain where they mediate tonic and phasic GABAergic input. During development, the majority of $GABA_A$ receptors contain β3 subunits, as opposed to β2 subunits in adult brains [96-99]. Moreover the developing and adult mammalian brains ubiquitously express β3 subunits in regions involved with seizure generation/maintenance such as cortex, thalamus, hippocampus, septum and basal forebrain, as well as in other brain regions—olfactory bulb, hypothalamus, epithalamus, and amygdala [96, 97, 99, 100]. Thus it not hard to imagine that reduced GABAergic input

during the critical early stages of development (when GABA is an excitatory neurotransmitter [101]) could lead formation of abnormal and potentially hyperexcitable circuits resulting in seizures. Similarly reduced GABAergic inhibition in adult circuits could generate seizures. Additionally, the β3 subunit knockout mice displays seizures, developmental (some display cleft palate, runted growth, and reduced life span) and behavioral (hyperactive, mothers neglect off springs) deficits, and most mice have neonatal mortality [102], suggesting a critical role of these subunits for neurodevelopment and adult brain functions.

Seizure generation due to disrupted $GABA_A$ functions by mutated β3 subunits seems quite plausible given the widespread expression and critical neurodevelopmental role of β3 subunits, although, the extent of contribution of a particular mutation to the epilepsy phenotype is not straightforward in patients with diverse genetic backgrounds. For example, the *GABRB3(Y302C)* mutation results in both the severe LGS (onset at 17 months and frequent seizures) and the milder focal epilepsy (onset at 7 months and rare seizures) [68]. Nevertheless when an identical *GABRB3(Y302C)* mutation is identified in three epilepsy patients (one reported by the Epi4K consortium [54] and two by Moller et al. [68]), it is likely to have significant contribution to the epilepsy syndromes. Moreover, with more than 20 *GABRB3* epilepsy-associated mutations identified so far, a contribution to epilepsy is very likely [54, 57, 63-68]. The findings from chapters II and III provide strong *in vitro* evidence of the contribution of LGS- and IS-associated *GABARB3* mutations to EEs. In the next chapter I examine the impact of the *GABARB3(D120N)* mutation in a mouse model.

3.11 REFERENCES

1. Arzimanoglou A, French J, Blume WT, et al. Lennox-Gastaut syndrome: a consensus approach on diagnosis, assessment, management, and trial methodology. Lancet Neurol. 2009 Jan;8(1):82-93.

2. Dulac O, N'Guyen T. The Lennox-Gastaut syndrome. Epilepsia. 1993;34 Suppl 7:S7-17.

3. Hancock EC, Cross HH. Treatment of Lennox-Gastaut syndrome. Cochrane Database Syst Rev. 2009 Jul 08(3):CD003277.

4. van Rijckevorsel K. Treatment of Lennox-Gastaut syndrome: overview and recent findings. Neuropsychiatr Dis Treat. 2008 Dec;4(6):1001-19.

5. Hancock EC, Cross JH. Treatment of Lennox-Gastaut syndrome. Cochrane Database Syst Rev. 2013 Feb 28(2):CD003277.

6. Kim HJ, Kim HD, Lee JS, Heo K, Kim DS, Kang HC. Long-term prognosis of patients with Lennox--Gastaut syndrome in recent decades. Epilepsy Res. 2015 Feb;110:10-9.

7. Oguni H, Hayashi K, Osawa M. Long-term prognosis of Lennox-Gastaut syndrome. Epilepsia. 1996;37 Suppl 3:44-7.

8. Rantala H, Putkonen T. Occurrence, outcome, and prognostic factors of infantile spasms and Lennox-Gastaut syndrome. Epilepsia. 1999 Mar;40(3):286-9.

9. Kotagal P. Multifocal independent Spike syndrome: relationship to hypsarrhythmia and the slow spike-wave (Lennox-Gastaut) syndrome. Clin Electroencephalogr. 1995 Jan;26(1):23-9.

10. Watanabe K, Iwase K, Hara K. The evolution of EEG features in infantile spasms: a prospective study. Dev Med Child Neurol. 1973 Oct;15(5):584-96.

11. Koo B, Hwang PA, Logan WJ. Infantile spasms: outcome and prognostic factors of cryptogenic and symptomatic groups. Neurology. 1993 Nov;43(11):2322-7.

12. Trevathan E, Murphy CC, Yeargin-Allsopp M. The descriptive epidemiology of infantile spasms among Atlanta children. Epilepsia. 1999 Jun;40(6):748-51.

13. Lombroso CT. A prospective study of infantile spasms: clinical and therapeutic correlations. Epilepsia. 1983 Apr;24(2):135-58.

14. Orrico A, Zollino M, Galli L, Buoni S, Marangi G, Sorrentino V. Late-onset Lennox-Gastaut syndrome in a patient with 15q11.2-q13.1 duplication. Am J Med Genet A. 2009 May;149A(5):1033-5.

15. Ferlazzo E, Adjien CK, Guerrini R, et al. Lennox-Gastaut syndrome with late-onset and prominent reflex seizures in trisomy 21 patients. Epilepsia. 2009 Jun;50(6):1587-95.

16. Shyu H-Y, Lin J-H, Chen C, Kwan S-Y, Yiu C-H. An atypical case of Lennox-Gastaut syndrome not associated with mental retardation: A nosological issue. Seizure. 2011;20(10):820-3.

17. Widdess-Walsh P, Dlugos D, Fahlstrom R, et al. Lennox-Gastaut syndrome of unknown cause: phenotypic characteristics of patients in the Epilepsy Phenome/Genome Project. Epilepsia. 2013 Nov;54(11):1898-904.

18. Park KM, Hur YJ, Kim SE. Brainstem dysfunction in patients with late-onset Lennox-Gastaut syndrome: Voxel-based morphometry and tract-based spatial statistics study. Ann Indian Acad Neurol. 2016 Oct-Dec;19(4):518-22.

19. Goldsmith IL, Zupanc ML, Buchhalter JR. Long-term seizure outcome in 74 patients with Lennox-Gastaut syndrome: effects of incorporating MRI head imaging in defining the cryptogenic subgroup. Epilepsia. 2000 Apr;41(4):395-9.

20. Heiskala H. Community-based study of Lennox-Gastaut syndrome. Epilepsia. 1997 May;38(5):526-31.

21. Trevathan E, Murphy CC, Yeargin-Allsopp M. Prevalence and descriptive epidemiology of Lennox-Gastaut syndrome among Atlanta children. Epilepsia. 1997 Dec;38(12):1283-8.

22. Camfield PR. Definition and natural history of Lennox-Gastaut syndrome. Epilepsia. 2011 Aug;52 Suppl 5:3-9.

23. Sidenvall R, Forsgren L, Heijbel J. Prevalence and characteristics of epilepsy in children in northern Sweden. Seizure. 1996 Jun;5(2):139-46.

24. Hauser WA. The prevalence and incidence of convulsive disorders in children. Epilepsia. 1994;35 Suppl 2:S1-6.

25. Steffenburg U, Hagberg G, Kyllerman M. Characteristics of seizures in a population-based series of mentally retarded children with active epilepsy. Epilepsia. 1996 Sep;37(9):850-6.

26. Camfield P, Camfield C. Long-term prognosis for symptomatic (secondarily) generalized epilepsies: a population-based study. Epilepsia. 2007 Jun;48(6):1128-32.

27. Epilepsy ILA. Proposal for revised classification of epilepsies and epileptic syndromes. Commission on Classification and Terminology of the International League Against Epilepsy. Epilepsia. 1989 Jul-Aug;30(4):389-99.

28. Berg AT, Berkovic SF, Brodie MJ, et al. Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia. 2010 Apr;51(4):676-85.

29. Gibbs FA, Gibbs EL, WG L. Influence of the blood sugar level on the wave and spike formation in Petit Mal epilepsy. Archives of Neurology and Psychiatry. 1939;41(6):1111-6.

30. Gibbs FA, Gibbs EL, Lennox WG. Electroencephalographic classification of epileptic patients and control subjects. Archives of Neurology & Psychiatry. 1943;50(2):111-28.

31. Lennox WG. The petit mal epilepsies; their treatment with tridione. J Am Med Assoc. 1945 Dec 15;129:1069-74.

32. Lennox WG. Tridione in the treatment of epilepsy. J Am Med Assoc. 1947 May 10;134(2):138-43.

33. Lennox WG, Davis JP. Clinical correlates of the fast and the slow spike-wave electroencephalogram. Pediatrics. 1950 Apr;5(4):626-44.

34. Lennox WG. Epilepsy and related disorders: Little, Brown; 1960.

35. Dravet C. Encéphalopathie épileptique de l'enfant avec pointe-onde lente diffuse (petit mal variant). PhD thesis, University of Marseilles. 1965.

36. Gastraut H, Roger J, Soulayrol R, et al. Childhood epileptic encephalopathy with diffuse slow spike-waves (otherwise known as "petit mal variant") or Lennox syndrome. Epilepsia. 1966 Jun;7(2):139-79.

37. Gastaut H. The Lennox-Gastaut syndrome: comments on the syndrome's terminology and nosological position amongst the secondary generalized epilepsies of childhood. Electroencephalogr Clin Neurophysiol Suppl. 1982(35):71-84.

38. Crumrine PK. Management of seizures in Lennox-Gastaut syndrome. Paediatr Drugs. 2011 Apr 01;13(2):107-18.

39. Niedermeyer E. The Lennox-Gastaut syndrome: a severe type of childhood epilepsy. Dtsch Z Nervenheilkd. 1969;195(4):263-82.

40. Chevrie JJ, Aicardi J. Childhood epileptic encephalopathy with slow spike-wave. A statistical study of 80 cases. Epilepsia. 1972 Apr;13(2):259-71.

41. Aicardi J. The problem of the Lennox syndrome. Dev Med Child Neurol. 1973 Feb;15(1):77-80.

42. Penry JK, Porter RJ, Dreifuss RE. Simultaneous recording of absence seizures with video tape and electroencephalography. A study of 374 seizures in 48 patients. Brain. 1975 Sep;98(3):427-40.

43. Markand ON. Slow spike-wave activity in EEG and associated clinical features: often called 'Lennox' or "Lennox-Gastaut' syndrome. Neurology. 1977 Aug;27(8):746-57.

44. Chevrie JJ, Aicardi J. Convulsive disorders in the first year of life: persistence of epileptic seizures. Epilepsia. 1979 Dec;20(6):643-9.

45. Aicardi J, Levy Gomes A. Clinical and electroencephalographic symptomatology of the 'genuine' Lennox-Gastaut syndrome and its differentiation from other forms of epilepsy of early childhood. Epilepsy Res Suppl. 1992;6:185-93.

46. Pati S, Deep A, Troester MM, Kossoff EH, Ng YT. Lennox-Gastaut syndrome symptomatic to hypothalamic hamartoma: evolution and long-term outcome following surgery. Pediatr Neurol. 2013 Jul;49(1):25-30.

47. Smeraldi E, Scorza Smeraldi R, Cazzullo CL, Guareschi Cazzullo A, Fabio G, Canger R. Immunogenetics of the Lennox-Gastaut syndrome: frequency of HL-A antigens and haplotypes in patients and first-degree relatives. Epilepsia. 1975 Dec;16(5):699-703.

48. Ong MS, Kohane IS, Cai T, Gorman MP, Mandl KD. Population-level evidence for an autoimmune etiology of epilepsy. JAMA Neurol. 2014 May;71(5):569-74.

49. Camfield PR, Bahi-Buisson N, Trinka E. Transition issues for children with diffuse cortical malformations, multifocal postnatal lesions, (infectious and traumatic) and Lennox-Gastaut and similar syndromes. Epilepsia. 2014 Aug;55 Suppl 3:24-8.

50. Agapejev S, Padula NA, Morales NM, Lima MM. [Neurocysticercosis and Lennox-Gastaut syndrome: case report]. Arq Neuropsiquiatr. 2000 Jun;58(2B):538-47.

51. Scheffer IE, Berkovic S, Capovilla G, et al. ILAE classification of the epilepsies: Position paper of the ILAE Commission for Classification and Terminology. Epilepsia. 2017 Apr;58(4):512-21.

52. Mefford HC. CNVs in Epilepsy. Curr Genet Med Rep. 2014;2:162-7.

53. Epilepsy Phenome/Genome Project Epi KC. Copy number variant analysis from exome data in 349 patients with epileptic encephalopathy. Ann Neurol. 2015 Aug;78(2):323-8.

54. Epi KC, Epilepsy Phenome/Genome P, Allen AS, et al. De novo mutations in epileptic encephalopathies. Nature. 2013 Sep 12;501(7466):217-21.

55. Lund C, Brodtkorb E, Oye AM, Rosby O, Selmer KK. CHD2 mutations in Lennox-Gastaut syndrome. Epilepsy Behav. 2014 Apr;33:18-21.

56. Nakashima M, Kouga T, Lourenco CM, et al. De novo DNM1 mutations in two cases of epileptic encephalopathy. Epilepsia. 2016 Jan;57(1):e18-23.

57. Euro E-RESC, Epilepsy Phenome/Genome P, Epi KC. De novo mutations in synaptic transmission genes including DNM1 cause epileptic encephalopathies. Am J Hum Genet. 2014 Oct 02;95(4):360-70.

58. Deng XL, Yin F, Zhang CL, et al. [Dynamin-1-related infantile spasms: a case report and review of literature]. Zhonghua Er Ke Za Zhi. 2016 Nov 02;54(11):856-9.

59. Terrone G, Bienvenu T, Germanaud D, et al. A case of Lennox-Gastaut syndrome in a patient with FOXG1-related disorder. Epilepsia. 2014 Nov;55(11):e116-9.

60. Shen D, Hernandez CC, Shen W, et al. De novo GABRG2 mutations associated with epileptic encephalopathies. Brain. 2017 Jan;140(Pt 1):49-67.

61. Zerem A, Haginoya K, Lev D, et al. The molecular and phenotypic spectrum of IQSEC2-related epilepsy. Epilepsia. 2016 Nov;57(11):1858-69.

62. Wolff M, Johannesen KM, Hedrich UB, et al. Genetic and phenotypic heterogeneity suggest therapeutic implications in SCN2A-related disorders. Brain. 2017 Mar 04.

63. Hamdan FF, Srour M, Capo-Chichi JM, et al. De novo mutations in moderate or severe intellectual disability. PLoS Genet. 2014 Oct;10(10):e1004772.

64. Zhang Y, Kong W, Gao Y, et al. Gene Mutation Analysis in 253 Chinese Children with Unexplained Epilepsy and Intellectual/Developmental Disabilities. PLoS One. 2015;10(11):e0141782.

65. Epi KCEaekce, Epi KC. De Novo Mutations in SLC1A2 and CACNA1A Are Important Causes of Epileptic Encephalopathies. Am J Hum Genet. 2016 Aug 04;99(2):287-98.

66. Papandreou A, McTague A, Trump N, et al. GABRB3 mutations: a new and emerging cause of early infantile epileptic encephalopathy. Dev Med Child Neurol. 2016 Apr;58(4):416-20.

67. Trump N, McTague A, Brittain H, et al. Improving diagnosis and broadening the phenotypes in early-onset seizure and severe developmental delay disorders through gene panel analysis. J Med Genet. 2016 May;53(5):310-7.

68. Moller RS, Wuttke TV, Helbig I, et al. Mutations in GABRB3: From febrile seizures to epileptic encephalopathies. Neurology. 2017 Jan 04.

69. Hernandez CC, Gurba KN, Hu N, Macdonald RL. The GABRA6 mutation, R46W, associated with childhood absence epilepsy, alters 6beta22 and 6beta2 GABA(A) receptor channel gating and expression. J Physiol. 2011 Dec 1;589(Pt 23):5857-78.

70. Tang X, Hernandez CC, Macdonald RL. Modulation of spontaneous and GABA-evoked tonic alpha4beta3delta and alpha4beta3gamma2L GABAA receptor currents by protein kinase A. J Neurophysiol. 2010 Feb;103(2):1007-19.

71. Gurba KN, Hernandez CC, Hu N, Macdonald RL. GABRB3 mutation, G32R, associated with childhood absence epilepsy alters alpha1beta3gamma2L gamma-aminobutyric acid type A (GABAA) receptor expression and channel gating. J Biol Chem. 2012 Apr 6;287(15):12083-97.

72. Lo WY, Botzolakis EJ, Tang X, Macdonald RL. A conserved Cys-loop receptor aspartate residue in the M3-M4 cytoplasmic loop is required for GABAA receptor assembly. J Biol Chem. 2008 Oct 31;283(44):29740-52.

73. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res. 2003 Jul 1;31(13):3381-5.

74. Hibbs RE, Gouaux E. Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature. 2011 Jun 2;474(7349):54-60.

75. Miller PS, Aricescu AR. Crystal structure of a human GABA receptor. Nature. 2014 Jun 8.

76. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994 Nov 11;22(22):4673-80.

77. Lukas D. Schuler XDaWFvG. An improved GROMOS96 force field for aliphatic hydrocarbons in the condensed phase. Journal of Computational Chemistry. 2001 2001;22(11):14.

78. Smith CA, Kortemme T. Backrub-like backbone simulation recapitulates natural protein conformational variability and improves mutant side-chain prediction. J Mol Biol. 2008 Jul 18;380(4):742-56.

79. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem. 2004 Oct;25(13):1605-12.

80. Althoff T, Hibbs RE, Banerjee S, Gouaux E. X-ray structures of GluCl in apo states reveal a gating mechanism of Cys-loop receptors. Nature. 2014 Aug 21;512(7514):333-7.

81. Klausberger T, Fuchs K, Mayer B, Ehya N, Sieghart W. GABA(A) receptor assembly. Identification and structure of gamma(2) sequences forming the intersubunit contacts with alpha(1) and beta(3) subunits. J Biol Chem. 2000 Mar 24;275(12):8921-8.

82. Sarto I, Wabnegger L, Dogl E, Sieghart W. Homologous sites of GABA(A) receptor alpha(1), beta(3) and gamma(2) subunits are important for assembly. Neuropharmacology. 2002 Sep;43(4):482-91.

83. Venkatachalan SP, Czajkowski C. A conserved salt bridge critical for GABA(A) receptor function and loop C dynamics. Proc Natl Acad Sci U S A. 2008 Sep 09;105(36):13604-9.

84. Wagner DA, Czajkowski C, Jones MV. An arginine involved in GABA binding and unbinding but not gating of the GABA(A) receptor. J Neurosci. 2004 Mar 17;24(11):2733-41.

85. Feng HJ, Macdonald RL. Multiple actions of propofol on alphabetagamma and alphabetadelta GABAA receptors. Mol Pharmacol. 2004 Dec;66(6):1517-24.

86. Macdonald RL, Rogers CJ, Twyman RE. Kinetic properties of the GABAA receptor main conductance state of mouse spinal cord neurones in culture. J Physiol. 1989 Mar;410:479-99.

87. Angelotti TP, Macdonald RL. Assembly of GABAA receptor subunits: alpha 1 beta 1 and alpha 1 beta 1 gamma 2S subunits produce unique ion channels with dissimilar single-channel properties. J Neurosci. 1993 Apr;13(4):1429-40.

88. Dunne EL, Hosie AM, Wooltorton JR, et al. An N-terminal histidine regulates Zn(2+) inhibition on the murine GABA(A) receptor beta3 subunit. Br J Pharmacol. 2002 Sep;137(1):29-38.

89. Wooltorton JR, McDonald BJ, Moss SJ, Smart TG. Identification of a Zn2+ binding site on the murine GABAA receptor complex: dependence on the second transmembrane domain of beta subunits. J Physiol. 1997 Dec 15;505 (Pt 3):633-40.

90. Macdonald RL, Kang JQ, Gallagher MJ. Mutations in GABAA receptor subunits associated with genetic epilepsies. J Physiol. 2010 Jun 01;588(Pt 11):1861-9.

91. Tanaka M, Olsen RW, Medina MT, et al. Hyperglycosylation and reduced GABA currents of mutated GABRB3 polypeptide in remitting childhood absence epilepsy. Am J Hum Genet. 2008 Jun;82(6):1249-61.

92. Huang X, Hernandez CC, Hu N, Macdonald RL. Three epilepsy-associated GABRG2 missense mutations at the gamma+/beta- interface disrupt GABAA receptor assembly and trafficking by similar mechanisms but to different extents. Neurobiol Dis. 2014 Aug;68:167-79.

93. Lachance-Touchette P, Brown P, Meloche C, et al. Novel alpha1 and gamma2 GABAA receptor subunit mutations in families with idiopathic generalized epilepsy. Eur J Neurosci. 2011 Jul;34(2):237-49.

94. Carvill GL, Weckhuysen S, McMahon JM, et al. GABRA1 and STXBP1: novel genetic causes of Dravet syndrome. Neurology. 2014 Apr 8;82(14):1245-53.

95. Srivastava S, Cohen J, Pevsner J, et al. A novel variant in GABRB2 associated with intellectual disability and epilepsy. Am J Med Genet A. 2014 Nov;164A(11):2914-21.

96. Wisden W, Laurie DJ, Monyer H, Seeburg PH. The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci. 1992 Mar;12(3):1040-62.

97. Laurie DJ, Wisden W, Seeburg PH. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci. 1992 Nov;12(11):4151-72.

98. Fillman SG, Duncan CE, Webster MJ, Elashoff M, Weickert CS. Developmental co-regulation of the beta and gamma GABAA receptor subunits with distinct alpha subunits in the human dorsolateral prefrontal cortex. Int J Dev Neurosci. 2010 Oct;28(6):513-9.

99. Zhang JH, Sato M, Tohyama M. Different postnatal development profiles of neurons containing distinct GABAA receptor beta subunit mRNAs (beta 1, beta 2, and beta 3) in the rat forebrain. J Comp Neurol. 1991 Jun 22;308(4):586-613.

100. Hortnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G. Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain. Neuroscience. 2013 Apr 16;236:345-72.

101. Ben-Ari Y. Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci. 2002 Sep;3(9):728-39.

102. Homanics GE, DeLorey TM, Firestone LL, et al. Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. Proc Natl Acad Sci U S A. 1997 Apr 15;94(8):4143-8.

CHAPTER IV: Mice harboring the LGS-associated *GABRB3(D120N)* mutation (*Gabrb3*$^{+/D120N}$ mice) have spontaneous seizures and EEG abnormalities

In this chapter I expand the *in vitro* findings from Chapter III to an *in vivo* knock in (KI) mouse model harboring the *GABRB3(D120N)* mutation (the heterozygous (het) *Gabrb3*$^{+/D120N}$ KI mouse). Our *in vitro* studies revealed that the *GABRB3(D120N)* mutation disrupted $GABA_A$ receptor function by perturbing the GABA blinding pocket, resulting in reduced macroscopic GABA-evoked currents and GABA potency, as well as reduced single channel Po, opening frequency, and burst duration. Spontaneous seizures in the *Gabrb3*$^{+/D120N}$ mice further support the contribution of the *GABRB3(D120N)* mutation to LGS. Here I primarily discuss the epilepsy and electroencephalograph (EEG) findings from *Gabrb3*$^{+/D120N}$ mice.

4.1 Mouse models of epilepsy

Epilepsy is caused by a complex interplay of different brain regions that recruit millions of neurons. Mouse models are used extensively to understand the underpinnings of epilepsy due to their brain structures having similarities to human brain structures, their ease of breeding and the ability to manipulate their well-characterized genomes. Moreover, the similarities in disease pathogenesis in humans and mice make genetically engineered mice attractive disease models. Likewise the EEG, etiology,

pathology, behavioral manifestations, and responses to antiepileptic drugs in mouse models can be compared to those observed in humans.

Numerous mouse models of spontaneous and induced seizures have been developed that have expanded our understanding of epileptogenesis. However, few mouse models of epileptic encephalopathies (EEs) are available. EEs encompass a broad range of catastrophic childhood epilepsy syndromes with an early onset and pharmacoresistant seizures for most patients. Not surprisingly patients have diverse seizure types, ages of onset, EEG activity, developmental outcomes, comorbidities, and responses to medication. Grouping these rare, but severe, childhood epilepsies has helped raise awareness of these disorders and has resulted in joint cohort studies to examine their underlying genetic etiology [1-4]. At the same time, pre-clinical studies need specific animal models that recapitulate the hallmark clinical features of each EE syndrome to probe the underlying molecular pathogenesis and to explore potential treatment options.

Etiologies of EEs include structural brain malformations, acquired brain insults, inborn errors of metabolism, and genetic defects. Recent studies using next generation sequencing technologies suggest important contribution of spontaneous *de novo* mutations in EEs [5, 6]. *De novo* mutations are rare genetic variations in the parent's germ line cells or a somatic mutation at very early stages of embryonic development. As *de novo* mutations are not subjected to evolutionary selection, they are generally more deleterious than inherited mutations [7, 8]. Following these genetic findings, substantial progress has been made to understand the pathophysiology of EEs and has

led to development of mouse models harboring human mutations. Genetically modified KI models, in which a normal nucleotide is replaced with a mutant nucleotide that codes for a different amino acid residue and causes epilepsy in humans, are among the most useful models to study the basis for epilepsy. It is hoped that the animal models with the human mutations provide close matches to human epilepsies and thus could recapitulate the clinical symptoms, EEG findings, and behavioral deficits seen in patients. In addition they provide the means to understand the circuits and molecular pathways that the human mutation(s) could perturb in specific epilepsy syndromes and tests which drugs could be most effective during different phases of the disease. They are valuable for the much-anticipated 'precision medicine' era of drug discovery. Even though it is not possible to have a mouse model for each human epilepsy-associated mutation, the diversity of epilepsy syndromes demands models that can recapitulate specific epilepsy phenotypes. Furthermore some investigators argue that genetic mouse models could also aid in development of novel therapeutics for epilepsy and non-epilepsy disorders, and uncover unforeseen molecular targets and pathways [9]. Patients with EEs progressively deteriorate, with seizures thought to contribute to or worsen the underlying brain malfunction. EE mouse models would help test the hypothesis that blocking seizures would prevent the cognitive and behavioral decline.

4.1.1. Mouse models of EEs

Here I discuss the most relevant findings from animal models of EEs in the context of my work concerning seizure semiology from our mouse model of LGS. Detailed

descriptions of the features of LGS are described in Chapter III; in the following sections, I will reiterate clinical concepts pertinent to understand the seizure types and the EEG findings from mouse models.

Lack of genetic mouse models of Lennox-Gastaut syndrome

LGS patients present heterogeneous symptoms. The majority of patients experience a triad of symptoms that include multiple seizure types (prevalent types are atypical absence, tonic, atonic, myoclonic and generalized tonic-clonic (GTC) seizures), moderate to severe cognitive dysfunction, and pharmacoresistant seizures that persist into adulthood. EEG tracings show characteristic slow spike discharges (SWD) of ≤ 2.5 Hz and generalized paroxysmal fast activity of 10-20 Hz. Currently, mouse models exhibiting the triad features of LGS have not been reported, and to date there are no animal models carrying LGS-associated human mutations. Although, mouse models with induced seizures that resemble atypical absence seizures and mice with genetic mutations that have multiple seizure types have contributed to our understanding of some molecules and circuits involved in LGS. Two of these models are described below. However, the lack of appropriate animal models has severely hampered understanding of the pathophysiology of LGS and development of potential treatments specifically for LGS.

4.1.2 Rodent models of atypical absence seizures

One of the diagnostic criteria of LGS is atypical absence seizures that appear as high voltage, bilaterally synchronized, and irregular SWDs on EEG [10-12]. Atypical absences seizures are in stark contrast with typical absence seizures; these differences are important to note even in non-clinical settings to distinguish them in animal models. Although both seizure types are observed in children with LGS, atypical absence seizures are rare but associated with severe cognitive and developmental impairment and poor response to medication [13-16]. In contrast, typical absence seizures are associated with minimal or no cognitive impairment [17] and are well controlled in most patients with ethosuximide, valproic acid or lamotrigine [18-22]. Atypical absence seizures emerge gradually and end abruptly, and patients have fragmented consciousness with subtle movements or automatisms. Typical absence seizures start and end abruptly with complete loss of consciousness and motor and behavioral arrest. Another prominent difference is that while SWDs associated with typical absence seizures are brief (<15 s) [23] and have a characteristic high amplitude 3 Hz SWD pattern on EEG, atypical absence seizures are prolonged (15 s to several minutes) and have SWDs with frequency of <2.5 Hz during the ictal phase [10, 24, 25]. SWDs of atypical absence seizures can vary among patients. They may attenuate with paroxysmal fast activity (10-13 Hz) during the ictal phase or synchronous SWDs may build up after fast activity of increasing amplitude [15]. Additionally, atypical absence seizures are insensitive to blood glucose levels while absence seizures are exacerbated by low blood glucose levels [26-30].

As atypical absence seizures are only seen in rare disorders such as LGS and myoclonic–astatic epilepsy (Doose syndrome), they are not well studied in animal models. A model of absence seizures was generated by acute administration of the cholesterol synthesis inhibitor AY-9944 to rats during early development (post-natal day (P)2-P50 every 6th day). Administration of the drug produced recurrent SWDs of 2-15 s duration at relative constant rate throughout the life of the animal that were reduced by the clinically used anti-epileptic drugs ethosuximide, diazepam, and clonazepam [31]. Snead and colleagues examined the AY-9944 treated rat model (P2-P33 every 6th day) in detail using synchronized video-EEG recordings and proposed it to be a model of atypical absence seizures [32] rather than typical absence seizures. Later they introduced a two-hit model in which the pregnant rats were treated with methylazoxymethanol (MAM) followed by postnatal administration of AY-9944. MAM is an antimitotic agent that results in brain dysgenesis and produces absence seizures that are refractory to ethosuximide, CGP 35348, and diazepam, resembling drug resistant atypical absence seizures [33]. The behavioral arrest and SWDs in AY-9944 treated mice did not perfectly align in time. There was a gradual onset of the behavioral arrest after the SWDs began, while the offset could outlast the end of the SWDs. Similarly, patients show varying degree of awareness during atypical absence seizures. Furthermore, movement was seen in mice during SWDs as is seen in patients [34] with atypical absence seizures. This is in sharp contrast to typical absence seizures with abrupt onset and offset of the behavioral arrest that aligns well with the start and stop of SWDs seen both in patients

[35] and in the rodent models [36-38]. Additionally, AY-9944 treated rats had SWDs associated with myoclonic jerks during sleep [32], similar to those seen in LGS patients, while SWDs during sleep in rat models of typical absence seizures have not been described. Another feature that distinguishes the AY-9944 treated rat and mouse models from typical absence seizures is that atypical absence seizures involve the hippocampus [32, 39] along with thalamocortical circuits, whereas typical absence seizures are limited to thalamocortical circuits [40 41]. Hippocampal involvement during atypical absence seizures in patients is plausible but not definitely established [42-45]. The AY-9944 rat model also recapitulates the higher propensity of absence seizures in females as seen in patients [46-49]. The duration of individual SWDs during atypical absence seizures is longer while the frequency of the waveform is shorter than those seen in typical absence seizures [50], although duration of individual SWDs is not commonly discussed in the literature. The AY-9944 treated rodents are also reported to have longer SWDs with a waveform frequency of 4-6 Hz, which is less than that observed mouse models of typical absence seizures (7-11 Hz) [51-56].

In spite of the fact that the AY-9944 treated rats reproduce the atypical absence seizure phenotype in a consistent manner, they do not recapitulate other features of LGS. First, no cognitive or behavioral disturbances, one of the primary features of EEs, have been reported in these mice. Second, LGS patients often have multiple seizure types. Atypical absence seizures are the predominant seizure in the AY-9944 treated mice, and they have myoclonic jerks in during sleep. This differs from having multiple seizure types during the wake states. Additionally, LGS patients have chaotic baseline

EEG activity [12, 24, 57, 58] that is not seen in the AY-9944 treated mice. Third, LGS is progressively debilitating for most patients. Apart from increased SWD duration from P21-P33 after AY-9944 treatment, seizure severity in terms of number of absence and myoclonic seizures has not been reported for adult or aged mice. Finally, EE patients with a genetic contribution are likely to have a different developmental trajectory prior to birth and seizure onset that cannot be replicated with postnatal treatment of AY-9944.

4.1.3 *DNM1* associated EE

Recently unique *de novo* mutations in the gene encoding dynamin 1 (DNM1) have been suggested to underlie LGS or IS in five patients [3] and were identified in two more patients with seizures and developmental disorders [59]. Dynamin 1 is a neuron specific large multimeric GTPase critical for activity-dependent membrane recycling, including fission of synaptic vesicle during endocytosis [60-63] and is important for synapse formation and maintenance [64]. Three mutations identified in patients that progressed from IS to LGS inhibited endocytosis in a dominant negative manner in HeLa and COS-7 cells [65], suggesting dysfunctional synaptic vesicle trafficking as a putative mechanism for DNM1 associated EEs.

Furthermore, in 2010 a C57BL/6J inbred mouse strain with a spontaneous novel mutation (fitful) in the DNM1 gene were reported to have seizures in hom and het [66]. The hom mice had ataxia and tonic-clonic seizures (at P14-P16) leading to mortality in two to three weeks of birth, while the het mice developed generalized tonic-clonic

seizures (GTCSs) and partial seizures from two to three months of age. A thorough analysis of the seizure types and background activity on EEG was not examined in the het mice and the fitful mice were initially suggested to be a model of genetic generalized (idiopathic) epilepsy [66]. At the molecular level mutant dynamin 1 protein in fitful mice decreased endocytosis in a dominant negative manner [66], similar to a mutation found in an LGS patient [65] and to the mutants generated to elucidate dynamin 1 function [62]. Although, the fitful mutation has not been reported in humans, fitful mice are valuable for study of the role of Dynamin 1/synaptic vesicle trafficking in the pathogenesis of EEs. Future studies are needed to carefully determine if fitful mice have EEG abnormalities and cognitive and behavioral deficits as in LGS patients.

4.2 Concerns with rodent models

As with all models, rodent models of human diseases are not without caveats, despite their widespread use. For mouse models of pediatric epilepsies the problem is twofold. First, needless to say, human and rodent maturation is quite different. The well documented difference in the developmental rates of rodent and human brains prevent exact age equivalent comparisons between these species [67]. Second, as children are not just miniature adults, results from models of epilepsies developed in adult mice would be less informative of the pathophysiology in young mice. In essence the goal with models of pediatric epilepsy is to capture the insults of early life that produce epilepsy. Unfortunately, difficulty in handling and performing experimental procedures on mice of pre-weaning age poses significant limitation to the use of these animals.

Next, I provide a brief history and basic principles of electroencephalography as this is the primary technique used in the clinic to determine the seizure types and in my work with *Gabrb3*$^{+/D120N}$ mice.

4.3 Electroencephalography is the most common method used to document and diagnose seizures and epilepsy.

In 1875 Richard Caton, a physiologist in Liverpool, was among the first to study spontaneous electrical activity from rabbit and monkey brains [68, 69]. Another pioneer of EEG was a Polish physiologist Adolf Beck who recorded electrical potentials evoked by peripheral sensory stimuli in multiple brain regions of different animal species. He discovered that the electrical oscillations due to brain activity stopped after sensory stimulation, i.e. desynchronization, now a well-documented phenomenon [70]. Investigating the brain's electrical activity non-invasively in humans became widespread after the groundbreaking work of the German physiologist Hans Berger in 1929 [71]. It is noteworthy that EEG recordings by early investigators were also technical feats as they recorded the microvolt changes on the mammalian skull that are 10,000 times smaller than the current AA batteries. The present EEG instruments are at least 100 times more sensitive. To date EEG remains one of the fundamental tools for basic and clinical epilepsy research.

The potential of EEG to study epileptiform activity in humans was demonstrated in 1934 by Frederic A. Gibbs in collaboration with Hallowell Davis. They demonstrated the 3 Hz SWDs seen in two patients with absence epilepsy (earlier known as petit mal epilepsy) [10, 72]. Their subsequent study (on 12 children) suggested the association of absence seizures with the 3 Hz SWDs. Today the 3 Hz SWD discharge is considered the hallmark of absence seizures and is used in routine clinical diagnosis. Later Gibbs and William G. Lennox delineated the EEG characteristics of complex partial seizures and GTCSs [11]. These findings revolutionized clinical epileptology and became the foundation for localizing seizures and brain regions for lobectomies as a treatment for drug resistant seizures. Over the last eight decades EEG has become the mainstay for diagnosis and management of seizure disorders. A few examples of the current applications of EEG include differentiating focal from generalized seizures, identifying syndrome specific EEG patterns, identifying epileptogenic regions in candidates for epilepsy surgery, differential diagnosis of paroxysmal events, monitoring effectiveness of drug and surgical treatments, and monitoring convulsive and non-convulsive seizures. The non-invasive nature, relative convenience and ease of acquiring patient data, and high temporal resolution make EEG the most useful diagnostic tool for epilepsy.

4.3.1 Physiological basis of the EEG signal

Human EEG recordings are most widely used to identify focal epileptic cortical regions and generalized epileptiform discharges. The technique involves placement of multiple

electrodes on standard landmarks of the skull for spatial resolution and provides high temporal resolution in the millisecond time scale. Thus EEG provides a “real time” and direct measurement of changing brain activities in normal and disease states. Here I will discuss the most commonly used scalp EEG recordings in humans, their physiological basis, and how they differ from the EEG recordings obtained from mice.

The principal source of the EEG signal is the synchronized synaptic activity of populations of cortical neurons. Synaptic excitation and inhibition of neurons is produced by transmembrane currents. This time varying current generates transmembrane potentials in neurons. With EEG the potential difference between two different brain regions is measured using scalp electrodes. The two primary sources of transmembrane neuronal currents are fast action potentials and slow excitatory and inhibitory post-synaptic potentials. The fast action potentials occur on a low millisecond time scale and are generated by voltage gated sodium and potassium channels. They produce a sudden change of the transmembrane potential from negative to positive inside neurons. The slower synaptic potentials occur on a multiple millisecond time scale and are produced by several neurotransmitters acting on ion channels or G-protein coupled receptors, the two most prominent being excitatory post synaptic potentials (EPSPs) and inhibitory post synaptic potentials (IPSPs). Broadly speaking, an EPSP generates a transmembrane current by opening cation channels that produce an net inward flow of positive ions (Na^+ and K^+), and an IPSP generates current by opening anion channels that produce an inward flow of negative ions (Cl^-) or outward

flow of positive ions (K^+) through ion selective channels that span the neuronal membrane.

Let us consider a scenario in which an action potential or EPSP originating at the cell soma results in a net positive voltage at the soma of a cortical neuron and thus a net negative potential (relative to the soma and the extracellular matrix) along the apical dendrites. In this state of charge separation, the neuron acts as an electrical dipole, where the positively charged soma would be the current 'source' and the negatively charged dendrites would be the current 'sink'. If we put an imaginary high sensitivity electrode that could measure the tiniest potential differences on the skull, it would experience a sudden drop in voltage. Alternatively, if an IPSC is generated at the soma, the soma would be negative with respect to the dendrites, which would produce a sudden rise in the voltage at the skull electrode. In addition, the deflection of the scalp electrode would depend upon where the input arrives along the length of the neuron. For example, if a cortical neuron receives EPSPs on the apical dendrites, the extracellular matrix becomes negative relative to the dendrites, although the final outcomes resemble the scenario of receiving an IPSC at the soma. Another important aspect to consider is that not all neurons are perpendicular to the skull as the invagination of the cortex produces gyri and sulci. Thus neurons whose processes are parallel to the skull would produce both positive and negative deflections, and if the electrode were equidistant from the both the poles, it would measure a net neutral potential. In reality, of course, electrodes can only measure activity from thousands of cortical neurons immediately underneath the skull. In addition to the number, neurons

must be stacked parallel to each other (so that the dipoles add up and do not cancel each other out).

4.3.2. EEG signals reach from the brain to the scalp electrode via volume and capacitive conduction.

The charge separation generated by neuronal dipoles in the cortex is ultimately propagated to the scalp electrode through the neighboring tissue via volume conduction i.e. groups of ions of the same charge repel each other and of the opposite charges attract each other, and capacitive conduction i.e. charge built up across insulating tissue layers. Volume conduction propagates the electrical signal within the brain and comes in play immediately after the synaptic activity on neurons resulting in charge separation within the neuron and the surrounding extracellular medium by movement of charged ions until they encounter an insulating material such as cell membranes, myelin, meningeal membranes, bone, skin, etc. At the edges of these insulators, charged particles accumulated by volume conduction on one side will attract opposite charges on the other side. As the closely situated meninges, skull, and skin stack in parallel layers, the charge built up in the brain spreads across the meninges and then to the next layers until it reaches the electrode by capacitive conductance. Within these insulating layers, the charges are mobile and opposite charges separate on the two layers, similar in arrangement to parallel capacitors Once the electrical signal reaches the skin surface, it encounters the poorly conducting air, thus high conducting gel (to allow maximum voltage signal to go through the circuit) is applied between the skin and

the recording electrode. The gel makes another capacitive layer, although some volume conduction occurs between the gel and the electrode. Modern day electrodes are connected to an amplifier and digitized, and the signal is transmitted to a computer where it can be seen and analyzed in real time.

Each of the multiple tissue layers adds noise to the EEG signal before it reaches the electrode. In addition, the EEG signal amplitude (microvolts) drops exponentially with distance from the source. Thus, it needs local amplification to maximize the signal to noise ratio (SNR). The common sources of electrical noise (60 Hz) include electrical wiring of the building, video equipment and their electrical source, lights, computer monitors, etc [73]. Often the remedy is to use high impedance local amplifiers so as to allow minimum current to pass through the amplifier, or a minimum voltage drop across the amplifier so that maximum voltage difference between the electrode and amplifier could be measured. Another common remedy is to shield the signal in a Faraday cage, although for experimental EEG recording this may not be always feasible. Furthermore, noise from subjects due to breathing, blinking, heartbeat, other movements cannot be eliminated while acquiring data and are often removed by sophisticated post processing analysis methods. These movement artifacts are less interfering in the rodent EEG recordings with the tethered systems. It is also noteworthy to mention that the net voltage contributions from neurons, glia, extracellular matrix, and properties of the brain tissue, and the skull (attenuate higher frequencies, ≥30 Hz) shape the EEG signal.

4.3.3 What does the EEG measure?

The EEG signal is the average activity of millions of neurons underneath the electrode, with distortions from capacitive currents of the lipid membranes, glial cells, blood vessels, meninges, skull, and scalp [74]. The majority of the EEG signal originates from the superficial layers of cortex; contributions from deep cortical layers are substantially reduced, and those from sub-cortical regions are negligible. Highly synchronized activity of a large number of neurons produces high amplitude signals on EEG such as seen in sleep and seizures, while desynchronized activity that appears as small amplitude signals is commonly observed in active awake states. The ensemble of neuronal activity appears as oscillations of varying frequencies on EEG depending on the brain state and the brain region.

4.4 Oscillations of the normal adult brain

Following Hans Berger's original description and naming of alpha [71] and beta [75] waves, the International Federation of Societies for Electroencephalography and Clinical Neurophysiology adapted the nomenclature to classify different oscillations on EEG as delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), beta (12–30 Hz), and gamma (30-100 Hz) [76].

Neuronal network oscillations are considered the basic means of communication among different neural networks and have been implied to be involved in a plethora of critical functions including learning and memory [77], cognition [78], perception [79],

movement control [80], and sleep [81]. Abnormal oscillatory activity is seen in several diseases including epilepsy [82, 83], schizophrenia [96, 97, 84], autism [85-87], Alzheimer's disease [88-90], Parkinson's disease [91-93], and sleep disorders [94, 95]. To some degree, specific oscillations have been associated with certain cognitive functions. This does not indicate that the brain is operating only in a frequency range for a certain process. Rather it indicates that a dominant amplitude of the EEG signal for specific frequencies is seen in certain brain regions during a cognitive specific process. For example, alpha oscillations of 8-12 Hz are prominently seen (as spindles) in posterior brain regions when a person is relaxed, and the eyes are closed. The power of alpha oscillations decreases when the eyes are opened and replaced by faster oscillations of smaller amplitude. By and large an overall reduction of alpha power is associated with increased demands of attention and alertness [78]. Recent work suggests that alpha oscillations also occur in the activated cortex but are very different from the classical alpha rhythm (Klimesch, 2000 and Başar, 2004).[96-98].

In contrast, waking produces low amplitude beta oscillations (12–30 Hz) in the fronto-centro-temporal regions [99]. Beta oscillations are considered a measure of cortical arousal. Low amplitude beta oscillations are primarily observed in the sensorimotor cortex ('Rolandic' areas) during motor tasks with movements and motor imagery; and high amplitude beta is seen during postural maintenance [100]. Often the amplitude of beta oscillations is enhanced in the pre-central regions with drowsiness. Frontal or generalized high amplitude beta oscillations are prominent after intake of benzodiazepines or barbiturates [101, 102]. Additionally, enhanced background beta in the

waking states is correlated with disease states, and this “excess beta” is considered abnormal [103].

Low amplitude gamma oscillations (30-100 Hz, although some authors define gamma oscillations from 30–>80 Hz) are typically observed in awake states spontaneously or during active cognitive states [104-106], as well as in sleep states [107-110]. Gamma oscillations often arise first in response to a sensory stimulus, and the subsequent gamma reflects cognitive processing [111]. Gamma oscillations have been the prime focus of a large number of studies due to their association with cognitive functions [112-114], while altered gamma oscillations are implicated in a number of disease states [95, 115-117]. Gamma oscillations were missing from earlier studies using analog EEGs and became visible with digital broad-band EEG recordings. They are easily detected with invasive intracranial recordings or depth recordings from the cortex and from brain slices [118-120]. During generalized absence seizures, a sudden and pronounced increase in cortical gamma coherence time-locked to the onset of the SWDs and a gradual reduction in gamma power and coherence prior to seizure termination have been observed recently using scalp EEG recordings [121]. Moreover, intracranial recordings reveal heightened gamma and high frequency oscillations (HFOs). HFOs are generally 100-600 Hz (further sub-divided as ripple > 80–200 Hz, fast ripple > 200–600, and sigma ≥ 600 Hz) in the neocortical and mesiotemporal epileptic zone during normal appearing background before seizure onset, and thus, have been suggested to be possible biomarkers for epileptic tissue [122-128]. HFOs are typically observed with intracranial electrodes implanted in patients with medically

refractory focal epilepsies for localizing epileptic tissue for surgical removal. Recent studies have identified HFOs from scalp EEG [129-136] and magnetoencephalography (MEG) recordings [137-142], expanding their applications to pediatric and generalized epilepsies.

The slow delta (0.5–4 Hz) and theta (4–8 Hz) oscillations are prominent in drowsiness and sleep, but are rare or non-existent in normal awake EEG recordings [143-146]. In general, slow oscillations travel much further and connect different brain regions, and thus are associated with more global brain events such as sleep and epilepsy. Fast oscillations such as beta, gamma and HFOs, however, are restricted to local brain circuits [147]. Delta oscillations are increased in epileptic discharges particularly during SWDs [148, 149].

Oscillations are generally studied within different frequency bands, which serve distinct and overlapping cognitive and sensory functions; however, cross-frequency coupling across different oscillations is well known. For example theta-gamma coupling is observed in hippocampal place cells when rats explore their environment and are enhanced when they remember the spatial information correctly[150, 151]. Different oscillatory frequency bands may carry different aspect of complex information and may improve information transfer and storage opportunities across different neuronal assemblies [152, 153].

Below I will discuss relevant changes in oscillatory frequency and power of EEG signals during ictal and inter-ictal events in LGS patients. Some seizure types produce

characteristic oscillatory changes in patients that are used as identifying features for disease classification.

4.5 EEG abnormalities associated with the most common seizure types in LGS

Atypical absence, tonic, and atonic seizures (drop attacks) are the most common seizure types in LGS, and myoclonic seizures and GTCSs are less common in LGS. Atypical absence seizures and interictal periods are seen as prominent high amplitude slow SWDs of <2.5 Hz on EEG. The particular details of SWDs, however, vary widely among patients (such as in waveform, frequency, amplitude, duration) and even for the same patient during the same study session [24]. The average frequency of SWDs ranges from 1.5-2 Hz, but some rapid SWDs of 3 or 4 Hz are also seen. The SWDs last for 1-2 s or up to few minutes. During long events, multiple SWDs appear (continuous or in bursts) that are often interrupted by low amplitude activity accompanied by disappearance of SWDs (discontinuous). Most commonly seen patterns are biphasic or triphasic sharp waves, polyspikes and slow waves. The SWDs can be generalized, synchronized and symmetrical across both hemispheres; alternatively, they can be asymmetric with spike foci (such as temporal focus) or lateralized, which is usually associated with a focal neurological deficit. The focal spikes can evolve to diffuse discharges and later synchronize bilaterally [24, 25, 50, 154]. The states of relaxation and drowsiness promote occurrence of SWDs, while stimuli such as calling the patient's name, pain, or eye opening terminates or decreases the occurrence of SWDs. Similarly, sleep profoundly increases SWDs and could occupy ~50% (almost

continuous) of the time in drowsiness and sleep [155]. SWDs in sleep could be hypsarrhythmic or fragmented by electrodecrement ('flattening of signal amplitude'), resulting in appearance of pseudo-periodic activity [24, 50]. In addition, SWDs can transition to polyspike-waves with an increase in amplitude and frequency [24]. Additionally, it is important to note that the typical 3 Hz SWDs are not uncommon in LGS patients and can be seen during sleep, especially in patients with multiple spike patterns on EEG. However, 3 Hz SWDs occur occasionally and constitute only a minor portion of the SWDs on EEG [156].

Tonic seizures are the hallmark seizures of LGS and are reported in over 50-70% of LGS patients [15, 156, 157]. They are considered a prerequisite for LGS diagnosis; however, they may not be seen at the onset of LGS [158], and thus may delay the diagnosis. However, tonic seizures were not a requirement in the 1989 ILAE criteria for LGS diagnosis and some authors still hold that view [158-160]. In a typical tonic seizure, the neck and trunk flex, the arms are raised in semiflexed or extended position, the legs extend, the facial muscles contract, and the eyes deviate upwards. Additionally, tonic seizures terminating with a clonic jerk or loss of tone have been described [24]. Tonic seizures have notable features on EEG including desynchronization and/or paroxysmal fast rhythmic activity. Commonly reported ictal patterns are: (a) desynchronization (flattening of EEG signal) during the entire tonic seizure [156, 161, 50], (b) rapid rhythmic activity of 15-25 Hz that typically has low amplitude and progressively assumes high amplitude [15, 24], (c) desynchronization followed by rapid rhythmic activity [156, 50, 162], (d) high amplitude rhythmic activity at 10-15 Hz [15, 24]. Rarely, tonic seizures may be

followed by automatisms. On an electromyogram (EMG) recordings, the intensity of muscle contraction peaks within 1-2 s of the onset of a tonic seizure and remains constant throughout the seizure. Similar to atypical absence seizures, the occurrence of tonic seizures is altered by the state of vigilance, with sleep enhancing their occurrence particularly in first few hours of non-REM sleep [163]. In sleep, tonic seizures may present with features as in diurnal seizures or may have diminished features such as autonomic symptoms (bradypnea, tachycardia), or could be subclinical with slow-spikes or polyspikes or rhythmic SWDs of ~10 Hz on EEG.

Atonic seizures are the third most common seizure types in LGS and are reported to occur in 30-66% of patients [156, 164, 165]. They involve a sudden loss of muscle tone resulting in falls due to loss of posture. The loss of muscle tone is seen as a reduction in the amplitude of the EMG signal. The fall could involve just the head (head drop) or the entire body. Consciousness is partly or completely impaired for the brief period of the atonic seizure, and post ictal confusion is seen in some patients [166]. In addition, the abrupt falls may follow a myoclonic jerk [167] producing a myoclonic-atonic (myoclonic-astatic; Doose syndrome) seizure. Since the myoclonus lasts for ≤1s, it is likely to be missed, especially in the presence of a massive fall. Studies that monitored muscle activity with EMG along with EEG have revealed co-existence of global myoclonic seizures with atonic seizures [168]. On EEG, pure atonic seizures as well as myoclonic-atonic seizures appear as brief generalized voltage attenuation (<1s) followed by polyspike and wave or rhythmic spike and wave (1-3 Hz) discharges or as mixture of fast and slow wave discharges with infrequent SWD patterns [169] [170]. It is

important to have both EEG and EMG monitoring to identify atonic seizures, as falls could be due to brief tonic seizures or massive bilateral myoclonic seizures [171] [24] [172]. Even though atonic seizures are less common than tonic seizures, injuries due to the abrupt falls during atonic seizures are a prime health concern.

Other common co-existing seizure types in LGS patients include myoclonic seizures and GTCSs. Myoclonic seizures are manifested as sudden and brief jerks of a muscle or group of muscles. They are seen in ~18% of LGS patients [154]. Similar to tonic seizures, most myoclonic seizures are associated with electrodecrement of EEG activity that lasts for 1-2 s and occurs concurrently with or just following the brief jerk. However, some myoclonic seizures may not result in electrodecrement on EEG, while some traces with electrodecrement may not be associated with myoclonic seizures [50]. However, distinctions between spams (massive myoclonic jerks), myoclonic jerks, and tonic seizures ending with a brief clonic jerk are not always clear, especially in the absence of EEG and EMG monitoring [173] [159]. Parsing these sudden movements is even more difficult in mice.

GTCSs are less frequent and present in a fraction of LGS patients (15-45%) [24, 25, 154, 174], although some studies also report much higher incidence in a cohort but lower frequency of GTCSs in individuals [50]. GTCSs are quite variable in duration and progression [175-177,178], but are generally ≥ 5 minutes long [179] and appear on EEG as high voltage recurrent polyspike and waves in the earlier stages of seizures followed by a brief low voltage fast activity (20-40 Hz). Next in the tonic phase rhythmic alpha activity (10-12 Hz) is seen that progressively increases in amplitude for 8-10 s. Thereafter,

myoclonic jerks may emerge when the frequency decreases from to 7-8 Hz to 4-5 Hz. This is followed by the clonic phase with slow activity of 1-2 Hz. At the end of the seizure the voltage suddenly drops (EEG attenuation) followed by delta activity that gradually increases in amplitude and frequency. After a variable period of time, the EEG eventually returns to baseline [180, 181]. As these seizures are remarkable and hard to miss, EEG is not necessary, per se, to monitor them, but does provide information about the progression and features such as lateralization, waveform frequency, and duration of GTCSs.

The usefulness of EEG studies in clinical and research settings cannot be understated. However, it should be noted that there is not always a one-to-one correlation between EEG findings and ictal clinical signs; additionally, the characteristic EEG patterns described above may evolve with age [182-190].

4.6 Interictal EEG abnormalities in LGS patients

In addition to ictal activity, interictal activity is considered to be strongly associated with cognitive decline and neurodevelopmental regression [191-193] and is supported by the observation that patients successfully treated with AEDs or surgery to reduce or eliminate interictal activity regain cognitive functions [194, 195]. In addition to the slow ictal EEG abnormalities, background activity for the majority of LGS patients has been reported to be moderately or severely slower during awake states than expected for that age [50, 61, 154, 166]. Similar to atypical absence seizures, awake Interictal activity may

show extended or almost continuous sequence of slow spike waves (1.5-2.5 Hz) and bursts of generalized paroxysmal fast activity [164, 196, 197]. LGS patients with brain lesions may show frequent focal slow wave or spike anomalies [198]. Furthermore, interictal activity is enhanced by sleep, especially in stage 2 and slow wave sleep and to a lesser extent in rapid eye movement (REM) sleep [163, 199]. A striking similarity is seen in the interictal discharges and tonic seizures in patients with and without brain lesions, especially in sleep; both appear as high-amplitude polyspikes on EEG [196, 198]. Thus treatments should be considered to curb interictal activity in addition to controlling seizures, which may significantly improve cognitive and developmental trajectories of LGS patients.

4.7 Differences in mouse and human EEG

Seizures in mouse models of epilepsy are commonly monitored using EEG techniques and are the primary focus of this chapter. Although the basic principles of EEG remain the same, rodent and human EEGs differ in a number of ways. The most obvious differences include much smaller mouse brain size and lissencephalic cortex (lack of gyri and sulci). The commonly used human EEG electrodes are ~1 mm in diameter; in comparison, the distance between lambda and bregma sutures, where the head mount is placed, is about 4 mm [200]. Thus, the majority of EEG devices for rodents do not have the array of electrodes that are commonplace in the clinic, resulting in decreased spatial resolution. However, recent studies have used microelectrode arrays in adult mice [201-203], but synchronization between the electrodes due to volume conduction has also

been reported [204]. Microelectrode arrays currently are not widely used. Most rodent EEGs involve fewer electrodes and a relatively invasive method of implanting subdural electrodes that are typically metal wires or micro-screws secured on the skull. The advantage is fewer artifacts, with movement artifacts or electrical noise being most common in rodent EEGs. Awake human EEG recordings suffer from common artifacts from scalp muscles, eye blinking, swallowing, saccades, and spontaneous change of gaze [205]. The placement of subdural metal electrodes in mice is less precise than the standardized International 10–20 system of scalp electrode placement in humans. Despite these drawbacks rodent EEG, especially with synchronous video monitoring, provides valuable information about ictal and interictal patterns.

4.8 *Gabrb3*$^{+/D120N}$ mice as a model for LGS

To determine whether the *GABRB3(D120N)* mutation identified in a LGS patient could result in epilepsy, we generated the het *Gabrb3*$^{+/D120N}$ knock-in (KI) mouse. The *Gabrb3*$^{+/D120N}$ mouse harbors the *GABRB3(D120N)* mutation on one allele at a location equivalent to that of the human mutation and leads to production of mutant β3(D120N) subunits; the 2nd *Gabrb3* allele produces wt β3 subunits. To our knowledge this is the first mouse model harboring a gene mutation from an LGS patient. In the following sections I describe the EEG findings from this mouse model, a critical step to ascertain spontaneous seizures and test whether this mouse model recapitulates the LGS syndrome. Furthermore, $GABA_A$ receptor mutations have been recently identified in LGS patients, and thus this model is likely to provide new insights into the pathological

mechanisms of LGS. Additionally, lack of appropriate animal models had severely hampered understanding the pathophysiology of LGS. If the *Gabrb3*$^{+/D120N}$ mouse recapitulates LGS symptoms, this will be the first genetic model of LGS harboring a human mutation. Understanding disease mechanisms at the molecular, cellular, circuit, and whole brain level could potentially provide a platform for testing antiepileptic drugs that specifically target seizures in LGS patients. We hope that this work would significantly contribute to the epilepsy field.

Table 4.1. Phenotype LGS patient with *GABRB3(D120N)* mutation.

Mutations	Age at seizure onset	Development	Initial seizure types	Additional seizure types	EEG features	Other features
GABRB3 (D120N) *RAPGEF6* (in 3′ UTR)	10 M	Mild delay prior to seizure onset, no regression	Infantile spasms	Atypical absence, atonic, myoclonic, GTCS	Sharp spike-wake complexes of 2 Hz	ADHD, Mood lability with impulsive behavior, adaptive score <20

Modified from Table 13 of Allen AS, et al., 2013 [1].

4.9 MATERIALS AND METHODS

4.9.1 Generation and housing of *Gabrb3*$^{+/D120N}$ mice

Dr. Shimian Qu from our laboratory generated the het *Gabrb3*$^{+/D120N}$ KI mouse using standard protocols on congenic C57BL/6N background (Primogenix, Inc) in the Vanderbilt Transgenic Mouse/Embryonic Stem Cell Shared Resource facility. Briefly, a

G358A mutation in exon 4 of *Gabrb3* was introduced by site-directed mutagenesis in the targeting vector to generate the D120N substitution (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies). The targeting vector containing the *Gabrb3(D120N)* and positive and negative selection markers was electroporated into B6N embryonic stem (ES) cells. Targeted ES cells, identified by long range PCR and confirmed by restriction enzyme digestions and sequencing, were injected into the C57BL/6N albino blastocyst and transferred into pseudo-pregnant female mice. Five chimeric mice (chimera rate was about 90%) were produced from two independent ES cell clones. Chimeric mice with confirmed germ-line transmission were backcrossed with C57BL/6J mice and bred further to obtain $Gabrb3^{+/D120N}$ mice on a C57BL/6J background. Breeding, care, and all experiments were conducted in accordance with Vanderbilt Animal Care and Use Committee (IACUC) guidelines.

4.9.2 Behavioral video monitoring of young mice

All animals were cared for and used in accordance with the policies of Vanderbilt University's IACUC and to National Institutes of Health Guide for Care and Use of Laboratory Mice. $Gabrb3^{+/D120N}$ females and C57Bl/6 males were used for breeding, as breeding using $Gabrb3^{+/D120N}$ males was not successful. Pups were marked on the tail to track them, and I was blinded to the genotype during the behavioral assessment. Pups from post-natal day P1-6 were observed in their home cage with their parents to reduce separation-induced stress and rejection by their mothers. Later P6-P11 and P12-18 old pups were separated from the home cage for 2-5 and 5-10 minutes,

respectively, and observed individually in small plastic boxes for general behaviors and spontaneous seizures. Mothers immediately accepted the pups when they were reintroduced in the cage on all days from P6-P18, and the behavioral observations did not hamper their development. As some pups from *Gabrb3*$^{+/D120N}$ female and C57Bl/6 male parents were small in size at P21, all litters had extended weaning to ~P28 and were genotyped after weaning.

4.9.3 EEG-EMG head mount implantation

To record seizures, EEG-EMG head mounts (Pinnacle Technology, #8201; Figure 4.1) were surgically implanted on the skulls of *Gabrb3*$^{+/D120N}$ and wild type C57Bl/6 mice between the bregma and lambdoid sutures (Figure 4.2). The size of our current EEG-EMG head mount system (Pinnacle Technology, #8201) allows us to implant them on animals ≥60 days of age or ≥25 g in weight, and thus all animals were adults when the recordings were acquired. The data presented in this thesis are from 5-6.5 month old mice. For the surgery, each mouse was weighed and then anesthetized using 2-4% isoflurane in an induction chamber. After the animal was unconscious, it was placed on a paper towel and the scalp hair was carefully cut using scissors, making sure not to cut the whiskers. The mouse was then placed in a stereotactic apparatus, and its head was secured using ear bars. Five mg/kg Ketofen (an anti-inflammatory and analgesic agent) was subcutaneously administered with a 1 ml syringe by lifting back the skin. Eye ointment was applied to keep the eyes moist. The skin over the skull was disinfected using an iodine solution. A small incision was made in the skin and pulled apart to

expose the skull. To improve adherence of the head mount on the skull, clear the dura matter, and better expose coronal and lambdoid sutures, 30% hydrogen peroxide was applied using a cotton swab. The skull was immediately wiped using a clean and dry cotton swab. The two EMG leads of the head mount were slightly bent downward and cyanoacrylate glue was applied at the bottom of the head mount. The EMG wires were inserted in the nuchal muscles by gently pulling the skin at the back of the head mount. Immediately the headmount was placed on the skull between the bregma and lambda landmarks, and held in place on the skull until the glue dried. Small holes were made through the head mounts by rotating and gently pushing a 23 gauge needle. The head mount was then secured using 0.10" screws (#8209). The screws provide electrical contact between the brain surface and the head mount. After all four screws were secured dental cement was applied on the screw heads, around the edge of the head mount, and under and over the skin. If the skin was not entirely covered under the dental cement it was carefully stitched together around the head mount. To ensure electrical continuity between the head mount to the pre-amplifier, the holes of 6 pin head mount surface connectors were cleaned with an extra needle ensuring they were free of dental cement. Mice were then removed from the stereotactic apparatus and allowed to recover on a heating pad. Then 500 μl of warmed saline was administered intraperitoneally to rehydrate the animal. After the mouse started moving, it was placed back in the housing cage. Mice were monitored for at least three days post surgery for signs of pain, infection, or other distress and were injected with 5 mg/kg of Ketofen for pain relief. The genotypes were blinded for data acquisition and analysis.

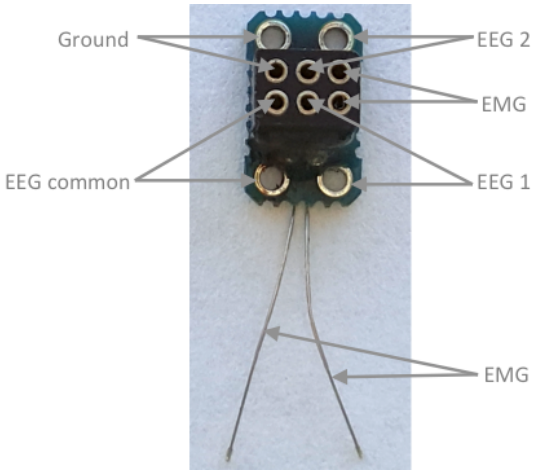

Figure 4.1: Head-mount that was used for EEG-EMG recordings.
The head-mount allows acquisition of data from 2 EEG and 1 EMG channels at 400 Hz.

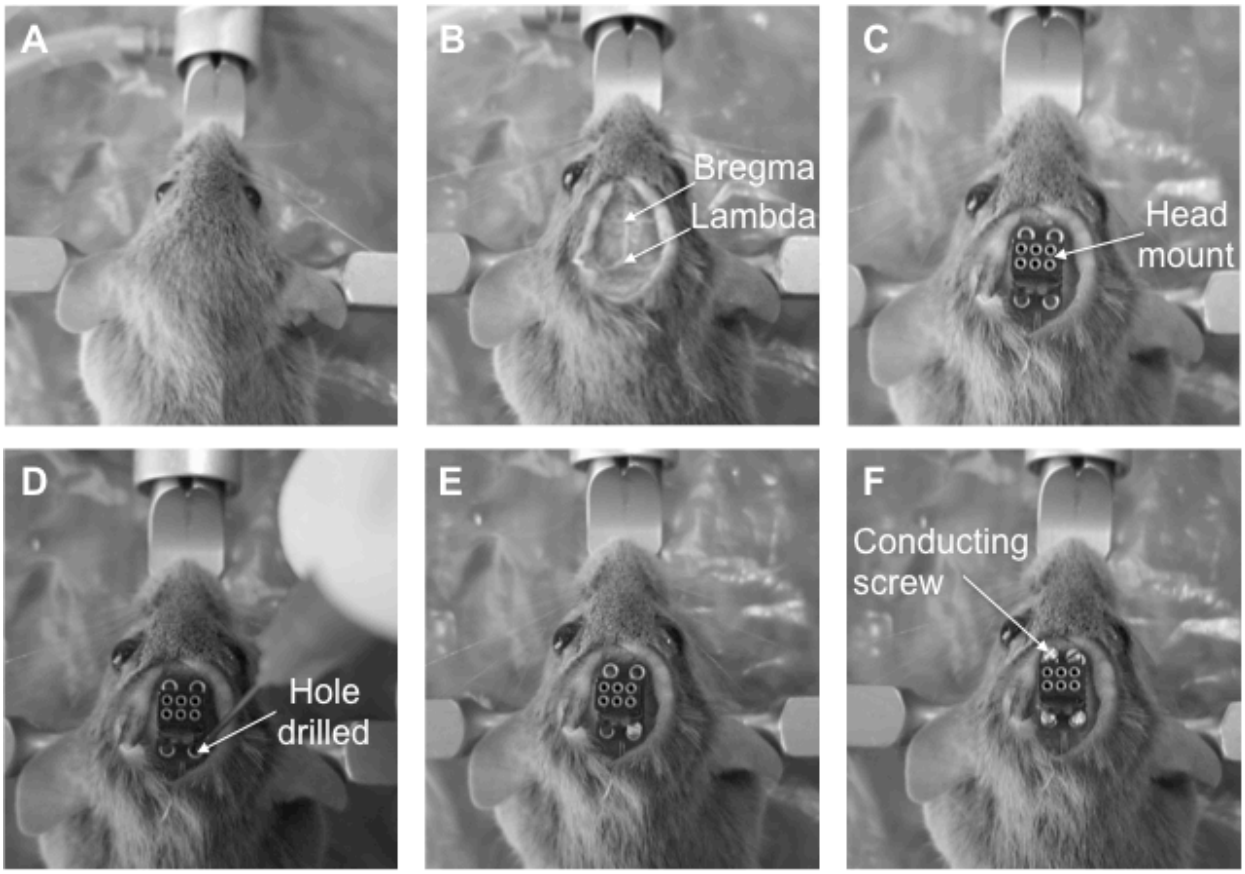

Figure 4.2: Surgical procedure for implanting head-mount to acquire EEG-EMG recordings.
(A) Mouse was anesthetized, and the head was secured on the stereotactic apparatus such that the head lays flat. **(B)** An incision was made to expose bregma and lambda sutures. **(C)** The sutures were used as guides to place and secure the head-mount using super-glue. **(D)** After the head-mount was secured with glue, holes were drilled in the skull using a 23 gauge needle. **(E and F)** The head-mount was further secured using conducting screws in each of the four screw slots. The screws provided electrical contact with the brain.

4.9.5 Video-EEG-EMG monitoring

After seven days of recovery, video-EEG-EMG data were collected from freely moving mice for 24 hours using Pinnacle's Sirenia® Acquisition Software. First, the head mounts on the mice were connected to a pre-amplifier (#8202-SE), and then the mice

were transferred to the recording cage. Using the low-torque 6-pin commutator wire (#8204), the pre-amplifier was connected to the 3-channel (2 EEG channels and 1 EMG channel) data acquisition/conditioning system (#8206-SE, Model 4100) mounted on top of the cage. The acquisition rate for the EEG-EMG channels was 400 Hz. The pre-amplifier filtered (1 Hz high pass) and amplified the signal (gain 100X), and additional filtering occurred at the data acquisition/conditioning system. Synchronized video-EEG recordings were obtained by using an infrared camera at 15 frames/second. The digitized video and EEG-EMG signals were visualized and recorded on a computer. See Figure 4.3.

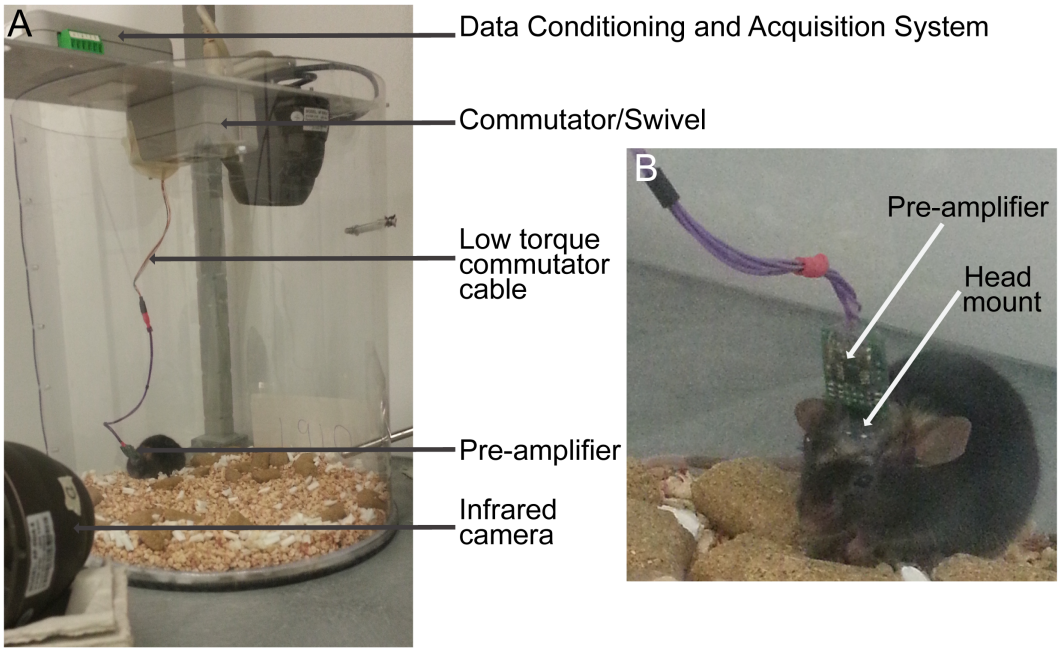

Figure 4.3 Synchronous video-EEGs.
(A) Components of the acquisition system that was used for 24-hour video-EEG recordings in a cylindrical plexiglass cage with food and water. **(B)** Enlarged image shows the pre-amplifier attached (right before the recordings) to the head-mount secured on the skull of the mouse.

4.9.6 Data analysis

Video-EEG-EMG data were analyzed offline with Sirenia® Seizure software using a widely used approach of visual inspection. Unlike the common sampling of only ~5 minutes/hour, the entire 24-hour recording was analyzed. Seizures were marked on the EEG-EMG recordings if they exceeded the baseline by 2X (Figure 4.4), and a behavioral feature was seen on the video (such as behavioral arrest, sudden pauses, repetitive jerking, rearing, rearing and falling, automatisms (aberrant grooming, circling), circadian behaviors (sleep) or other ictal behaviors) [206, 207]. The tonic seizures did not have strikingly increased EEG amplitude compared to the baseline, and thus, tonic seizures were scored positive irrespective of the change in the baseline. Additionally, SWDs in *Gabrb3*$^{+/D120N}$ mice observed during moving, grooming, chewing, etc were not scored. Seizure annotations were exported as a text file and number of seizures, seizure duration, sleep duration, light-dark effects, gender differences, etc were calculated in Microsoft Excel. Power spectra from the unfiltered EEG channels were generated using EDF browser.

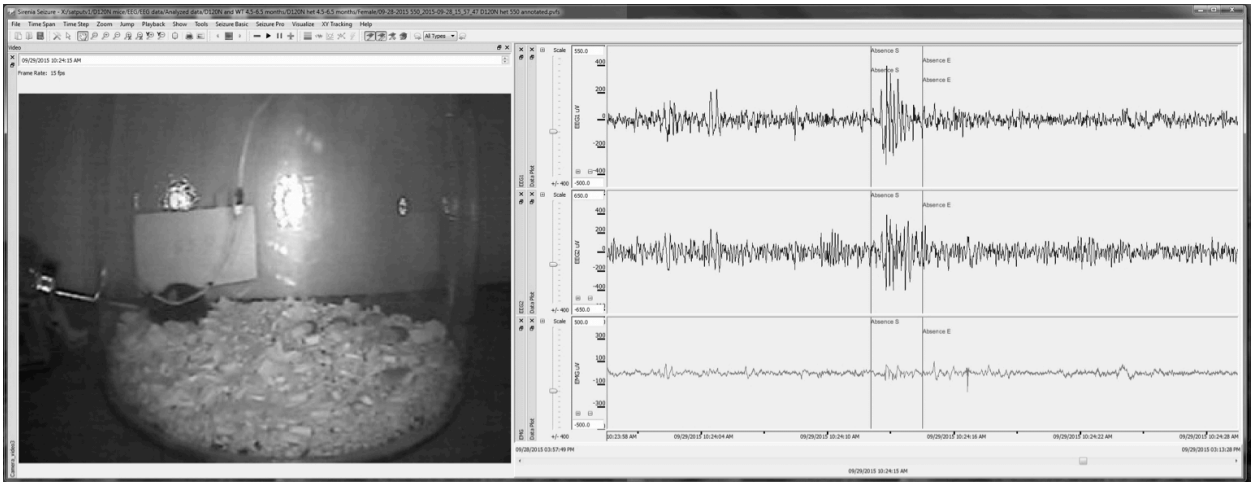

Figure 4.4 Video-EEG-EMG data analysis method.
Snap shot from Sirenia® Seizure software shows the seizure identification and scoring scheme. Seizures seen on both the EEG channels (top two traces) were correlated with the video record, and each seizure was manually scored.

4.9 RESULTS

4.9.1. Young *Gabrb3*$^{+/D120N}$ mice have spasm-like motor seizures.

As LGS is a childhood EE that can sometimes evolve from other EEs, especially infantile spasms (as seen in the patient with the *GABRB3(D120N)* mutation, Table 4.1), I observed *Gabrb3*$^{+/D120N}$ mice and their wild type (wt) littermates after birth for seizures and other abnormalities. The pups from *Gabrb3*$^{+/D120N}$ female x C57Bl/6 male parents were observed in their home cage with their parents until they were post-natal day 6 (P6), as separating young pups from their mother even for few minutes could drop their body temperature, and it is stressful for the pups and mother. From P6-P11 each pup was separated from the home cage for 2-5 minutes and observed for general appearance, skin pigmentation, ear openings, righting reflexes, and eye opening. From

P12-P18 the observations were 5-10 minutes long and focused on motor spasms, grooming, gait, head drops, staring, activity level, and how well the mouse explored its new environment. Mothers immediately accepted the pups when they were reintroduced in the cage on all days from P6-P18. Apart from the minimal stress that the pups and the mother may have experienced, the observations otherwise appeared to have no impact on their overall development. I was blinded to the genotype during the observations.

Observations in the home cage from P1-P6 were not very informative as the pups were clustered in the nest with the mother even when the mother was displaced for few minutes. No major differences were observed between wt and mutant *Gabrb3*$^{+/D120N}$ pups. From P6-11 observations only revealed a slight lag in development of skin pigmentation and smaller size for the *Gabrb3*$^{+/D120N}$ pups compared to the wt pups. Also the *Gabrb3*$^{+/D120N}$ pups had difficulty in righting when they were on their backs at P6 but eventually they caught up by P8. Only 3 litters were observed from P1-P11 as this time period did not reveal seizures or major differences in the *Gabrb3*$^{+/D120N}$ pups.

In contrast, distinct infantile spasm-like seizures, rapid and brief extension followed by flexion of the fore limbs, hind limbs, trunk, and the tail, that stretches the entire body, were seen in *Gabrb3*$^{+/D120N}$ pups between P14-P17 with a significantly higher likelihood compared to wt pups (Figure 4.5 A, B). A small fraction of wt pups were scored positive for spasm-like seizures, although, in the five litters (wt = 16 and *Gabrb3*$^{+/D120N}$ = 19) I observed, none of the *Gabrb3*$^{+/D120N}$ pups lacked spasm-like

seizures between P14-P18. The spasm-like seizures occurred in clusters and were accompanied by behavioral arrest and staring during the events. Typically the pups would sit at the edge of the cage (in between exploring or grooming) as if anticipating the event and would have a fixed gaze. The spasm-like seizures were distinct from sudden motor/myoclonic twitches or startles seen during grooming. Further, the *Gabrb3*$^{+/D120N}$ pups also displayed brief trunk extension and flexion before or after the spasm-like seizures, but these events were not scored.

Additionally, when the pups were lifted by their tail, they clasped their fore and hind limbs at once or sequentially, this behavior was absent or rarely seen in wt pups (Figure 4.5 C). .Also, the *Gabrb3*$^{+/D120N}$ pups weighed significantly less on P21 compared to the wt pups (wt = 10.9 ± 0.3 g, n = 10; *Gabrb3*$^{+/D120N}$ = 8.4 ± 0.4 g, n = 11; p = 0.0001; 3 litters; Figure 4.5 D) but this difference disappeared after weaning when they were put on a solid diet.

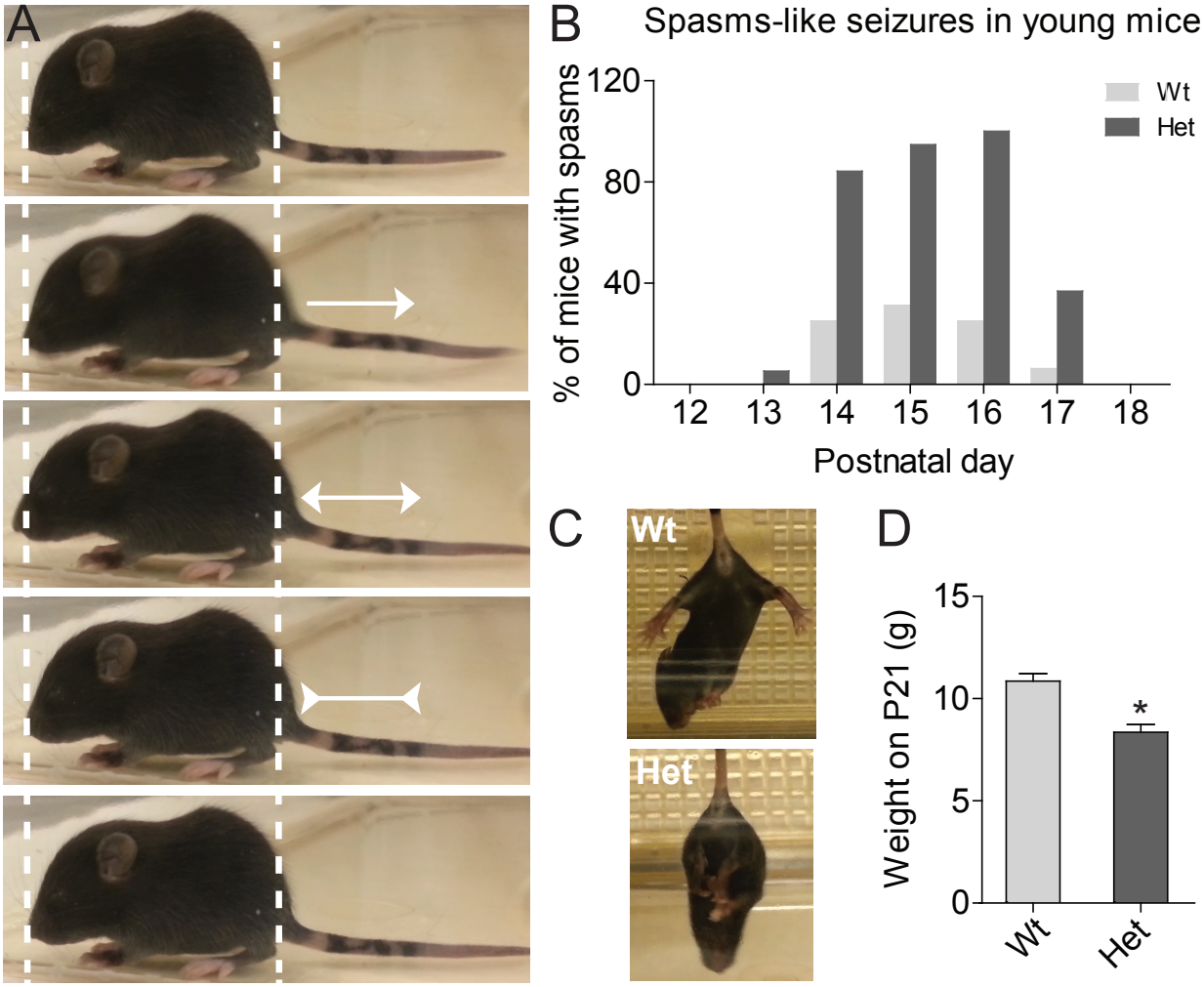

Figure 4.5 Young *Gabrb3*$^{+/D120N}$ mice had spontaneous spasm-like seizures.
(A) Typical spasm-like seizure in a P15 *Gabrb3*$^{+/D120N}$ mouse. The spontaneous spasm-like seizure involves behavioral arrest followed by clusters of brief extension of fore limbs, hind limbs, trunk, and the tail, followed by their flexion. **(B)** Spasm-like seizures appeared typically at P13 and disappeared by P18 in wt and *Gabrb3*$^{+/D120N}$ pups, but occurred more frequently in *Gabrb3*$^{+/D120N}$ pups. **(C)** Abnormal fore and hind limb clenching seen in *Gabrb3*$^{+/D120N}$ pups (here both wt and mutant mice were P15). **(D)** *Gabrb3*$^{+/D120N}$ pups had reduced body weight compared to wt littermates prior to weaning.

4.9.2. Adult *Gabrb3*$^{+/D120N}$ mice had multiple types of spontaneous seizures.

To determine whether *Gabrb3*$^{+/D120N}$ mice had spontaneous electrographic seizures, we performed prolonged 24-hour video-EEG-EMG recordings from chronically implanted *Gabrb3*$^{+/D120N}$ and wt mice. Our recording system can only be implanted on adult mice (≥25 g), thus we could not determine the age of onset of these seizures. Spontaneous seizures were observed in mice from 3.5 months of age. The data presented here are from 5-6.5 month old mice (wt n = 4, *Gabrb3*$^{+/D120N}$ n = 7).

Multiple types of spontaneous seizures were seen in *Gabrb3*$^{+/D120N}$ mice including typical absence, atypical absence, myoclonic, tonic, and generalized tonic-clonic (GTC) seizures (Figure 4.6). Absence seizures and myoclonic jerks were the predominant seizure types in *Gabrb3*$^{+/D120N}$ mice, while tonic seizures and GTCSs were rare. Typical absence and atypical absence seizures involved behavioral arrest (also seen as reduced EMG activity) with a staring gaze ranging from a few seconds to minutes and accompanied by a conspicuous SWDs on EEG (Figure 4.7A, right traces). The myoclonic jerks were brief (~0.5 s) and sudden muscle contraction involving the entire body, seen as extension followed by flexion of the body and had a prominent sharp spike on EEG and EMG channels. The majority of myoclonic jerks (94.6%) occurred during the light period (inactive time for mice). GTCSs began with tonic stiffening and extension of the limbs, followed by rapid clonic movements of the limbs, and then proceeded to strong tonic-clonic activity with abrupt jumps and Straub tail; the seizures ended with behavioral arrest. Although, GTCSs were seen multiple times during routine handling and captured on video in the housing facility, they were rarely captured during

the 24-hour EEG-EMG recordings. The tonic phase had high amplitude and high frequency on EEG, while the clonic and tonic-clonic phases had high amplitude and lower frequency than the tonic phase. This was followed by an electrodecrement of EEG amplitude (Figure 4.6C). Tonic seizures were infrequent and involved a sudden contraction of the limbs and sometime stiffening of tail that was associated with either no change in EEG amplitude or with low-amplitude high frequency activity, as previously described [208] (Figure 4.6D) that lasted a few seconds. Although, the number of tonic seizures in 24 hours described here are likely an underestimate as tonic seizures did not have a characteristic EEG pattern in *Gabrb3*$^{+/D120N}$ mice, they were are hard to establish especially when then the mice were not moving immediately prior to the seizure. Moreover, the male mice had more absence seizures, (males = 544.33 ± 128.78, females = 364.50 ± 100.93) and myoclonic jerks (males = 206.33 ± 130.32, females = 18 ± 10.46); although these differences were not statistically different amongst 4 female and 3 male *Gabrb3*$^{+/D120N}$ mice. The tonic seizures were significantly more frequent in males (6.66 ± 0.45, n = 3) than in females (2 ± 0.61, n = 4). The EEG data from mice ~3-4 month old (current data set had ~5-6.5 month old mice) were not extensively analyzed; brief observations indicated that the seizure severity increased with age and was certainly true for the increased occurrence of GTCSs.

Furthermore, the *Gabrb3*$^{+/D120N}$ mice showed other abnormal behaviors such as increased rearings in 24 hours compared to the wt mice (wt = 260.5 ± 17.32, n = 4; *Gabrb3*$^{+/D120N}$ = 596.57 ± 218.13, n = 7; $p > 0.5$). Some *Gabrb3*$^{+/D120N}$ mice were hyperactive and showed repetitive running interrupted by rearings around the edges of

the cage up to several hours at a time. The *Gabrb3*$^{+/D120N}$ mice were hyper-sensitive to touch during routine handling.

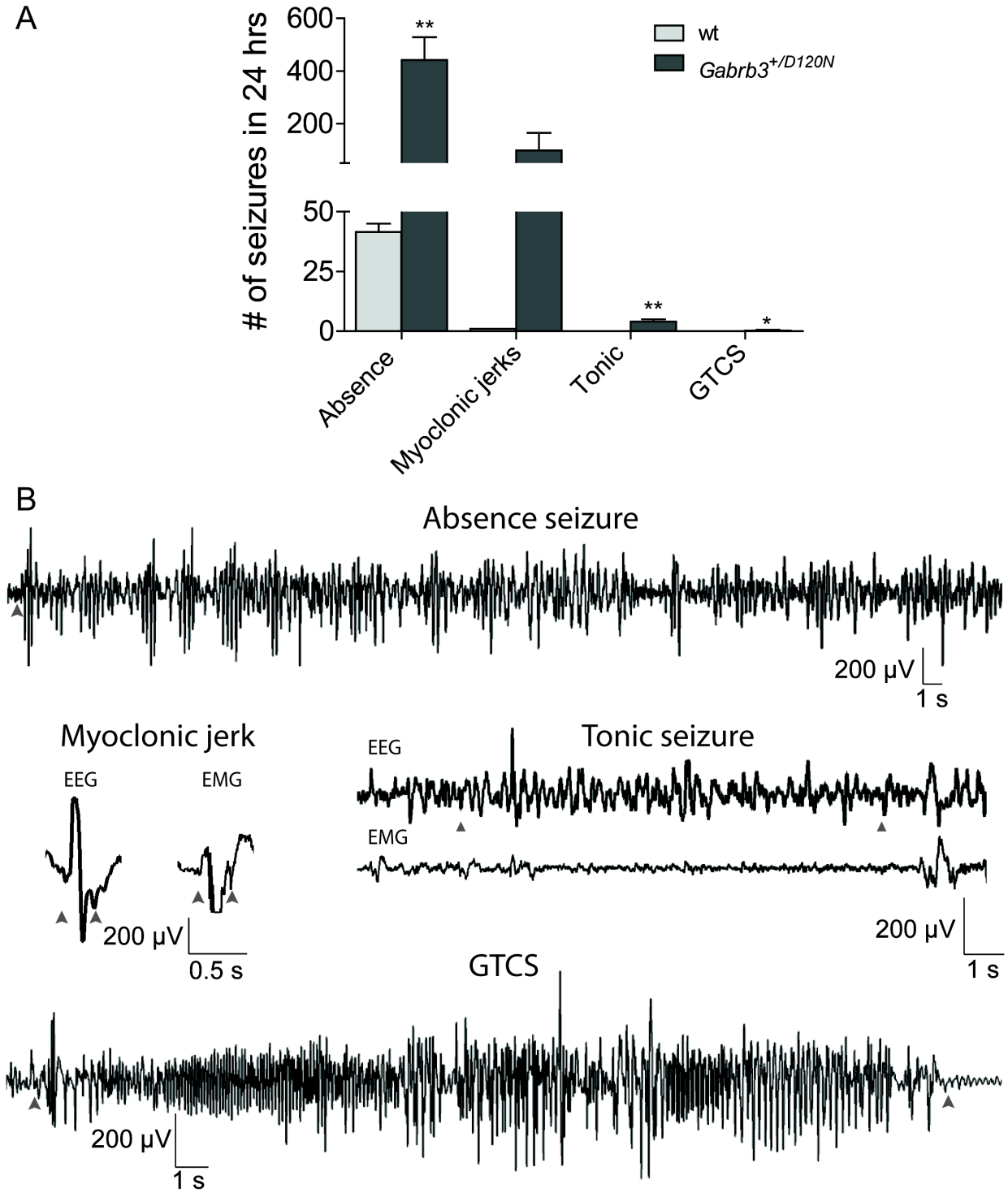

Figure 4.6 EEG features of spontaneous seizures in adult *Gabrb3*$^{+/D120N}$ mice.
(A) Bar graph showing the average number seizures in wt and *Gabrb3*$^{+/D120N}$ mice in a 24-hour period. Absence seizures and myoclonic jerks were the two dominant seizure types in *Gabrb3*$^{+/D120N}$ mice. **(B)** Representative EEG traces from 4 different seizure types. The arrowheads indicate the beginning and end of the seizure. Note that absence seizures start with a typical SWDs pattern and transition to a more chaotic and slow-spike-wave like pattern. Only a segment of the absence seizure is depicted here so the trace does not have an end point. Myoclonic jerks were noted as brief (~500 μs) and prominent spikes in both EEG and EMG channels. Tonic seizures did not stand out from the baseline, showed a slight increase in amplitude but were high frequency events compared to the baseline. GTCS were striking with the largest increase in EEG amplitude. The tonic phase had slightly lower amplitude but higher waveform frequency compared to the clonic phase, which was followed by electrodecrement.

4.9.3. Atypical absence seizures were the predominant seizure type in adult *Gabrb3*$^{+/D120N}$ mice

The *Gabrb3*$^{+/D120N}$ mice had over 400 absence seizures on average in 24 hours, about 10 times of that observed in wt mice. Thus, I examined this seizure type further. The wt C57Bl/6 mice had typical brief (9.9 ± 0.1 s) and infrequent absence seizures with high amplitude 6-8 Hz SWDs (Figure 4.7 A, B left panels), as previously reported in other rodent models of absence seizures [51, 209-214] and other control background strains [215, 216]. Whereas the *Gabrb3*$^{+/D120N}$ mice had both typical absence and atypical absence seizures with an average duration of 20 ± 10.2 s (Figure 4.7 A, B left panels). The typical absence seizures had a characteristic spindle-like SWD pattern of 4-5 Hz. While the majority of the atypical absence seizures began with typical SWDs and progressed to distinctive high amplitude SWDs of 2-3 Hz that were less organized and lacked the characteristic spindle-like appearance. Given the large number of absence seizures in *Gabrb3*$^{+/D120N}$ mice, it is not surprising that they spent a significantly larger amount of

time in absence seizures compared to the wt mice (Figure 4.7 C, wt = 7.43 ± 1.05 s, n = 4 mice; *Gabrb3*$^{+/D120N}$ = 150.5 ± 36.83 s, n = 7 mice). However, the absence seizures with durations ≥30s accounted for ~70% of the time spent in absence seizures even though they comprised only ~25% of all absence seizures in *Gabrb3*$^{+/D120N}$ mice. Occasionally the prolonged atypical absence seizures (>30 s) with slow disorganized SWDs progressed to states of drowsiness and sleep. Moreover, the behavioral arrest seen during atypical absence seizures was not always time-locked with the onset of SWDs on EEG and was occasionally associated with behaviors such as head nods, eyelid myoclonus or whisker twitches. Movements and automatisms were also reported in patients during atypical absence seizures. Additionally, the majority of the typical absence and atypical absence seizures occurred during the dark period (active period for mice) with a peak occurrence right after the light to dark period transition (Figure 4.7 D).

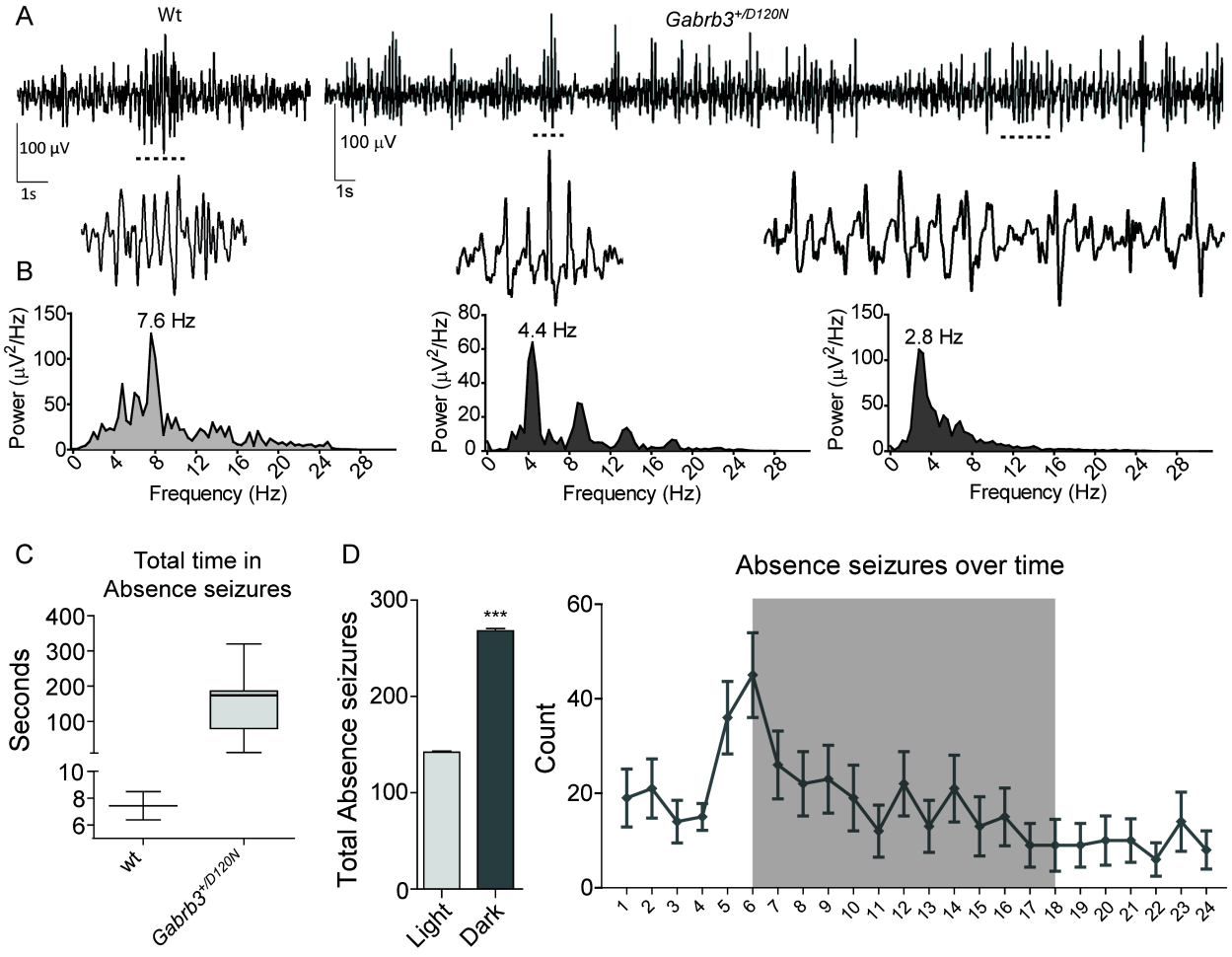

Figure 4.7 *Gabrb3*$^{+/D120N}$ mice had spontaneous typical and atypical absence seizures.

(A) Representative traces showing absence seizures in wt (left) and *Gabrb3*$^{+/D120N}$ (right) mice. Wt mice had brief SWDs time-locked with behavioral arrest while the *Gabrb3*$^{+/D120N}$ mice had both brief and prolonged absence seizures. Prolonged absence seizures were not always time-locked with the behavioral onset and/or had brief movements during the seizure. Apart from the duration**,** the waveform frequencies in *Gabrb3*$^{+/D120N}$ mice were lower in both brief (with typical SWDs) and prolonged absence seizures (with typical SWDs and chaotic slow-spike-waves) **(B)**, middle and right graphs) compared to the wt mice (B, left graph). **(C)** Due to the prolonged duration of absence seizures**,** the *Gabrb3*$^{+/D120N}$ mice spent a large amount of time in absence seizures (wt = 7.43 ± 1.05 s, *Gabrb3*$^{+/D120N}$ = 150.5 ± 36.83 s), although these durations were not statistically different. **(D)** The majority of absence seizures in *Gabrb3*$^{+/D120N}$ mice occurred during the dark (active) period (left graph) with a peak incidence right at the light-dark period transition (right graph, dark period in grey).

4.9.4. Adult *Gabrb3*$^{+/D120N}$ mice had seizures during sleep.

Total sleep duration in *Gabrb3*$^{+/D120N}$ mice was similar to wt controls (wt = 12.49 ± 0.01 hours, *Gabrb3*$^{+/D120N}$ = 10.81 ± 2.6 hours, p = 0.45). Although, unlike the wt mice the *Gabrb3*$^{+/D120N}$ mice had several absence-like SWDs and polyspikes in non-rapid eye movement (NREM) and REM sleep (Figure 4.8). Absence-like SWDs were prominent at the beginning of NREM sleep and during REM sleep, while polyspikes appeared as NREM sleep progressed. Sleep disturbances and presence of SWDs and polyspikes are also reported in LGS patients [163] and are thought to contribute to cognitive deficits [217, 218].

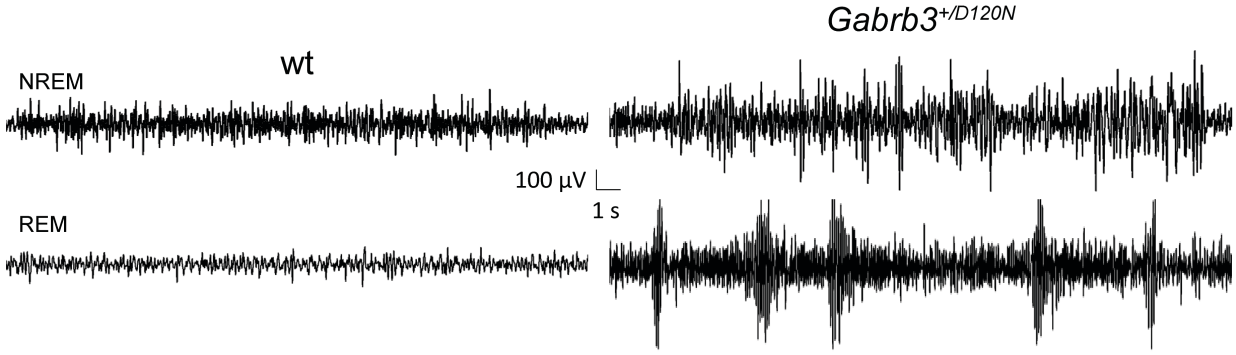

Figure 4.8 Adult *Gabrb3*$^{+/D120N}$ mice have spontaneous seizures in sleep.
Representative traces from NREM and REM sleep in wt (left) and *Gabrb3*$^{+/D120N}$ mice (right). Prominent SWDs in both sleep stages were observed in all *Gabrb3*$^{+/D120N}$ mice and were absent in wt mice. The SWDs resembled those seen during absence seizures.

4.10 DISCUSSION

The *Gabrb3*$^{+/D120N}$ mice recapitulate the salient features of LGS, namely spontaneous atypical absence seizures, myoclonic jerks, GTCS, and tonic seizures. Importantly, the

Gabrb3$^{+/D120N}$ mice show early seizure onset and progression of seizure severity. Additionally, the mice also exhibit sleep disturbances and behavioral abnormalities, comorbidities seen in LGS patients (Table 4.2). Thus, we believe that *Gabrb3*$^{+/D120N}$ mice present a valuable model to study the pathophysiological mechanisms of LGS and efficacy of AEDs used for its treatment.

Table 4.2. Phenotypes LGS patients and *Gabrb3*$^{+/D120N}$ mice.

	LGS patients	LGS patient with *GABRB3(D120N)* mutation	*Gabrb3*$^{+/D120N}$ mice
Seizure onset	1-8 years, earlier if progresses from other epilepsies	10 M, initial seizure infantile spasms	Mild delay prior to seizure onset, no regression
Seizure types	Atypical absence, atonic, tonic, myoclonic, GTCSs	Atypical absence, myoclonic, GTCSs	Atypical absence, myoclonic, tonic, GTCSs
EEG features	<3 Hz SWDs, slow background, 10 Hz fast rhythm in sleep	Bursts of 2 Hz sharp-spike-wave complexes	2-3 Hz slow SWDs often prolonged to minutes, high background EEG
Behavioural abnormalities	Cognitive impairment, behavioral difficulties, sleep disturbances	ADHD, impulsivity, adaptive score <20, sleeping difficulties	Hyperactive, hyper responsive to touch, seizures during sleep

Although postnatal ages of humans and mice cannot be directly compared, the P13-P20 rats are roughly comparable to human infancy and early childhood, a period of susceptibility to infantile spasms and LGS in children [219-222]. The P21 rats are considered analogous to 5-10 year old humans [219, 220]. Mice have slightly earlier maturation compared to rats. The P14-P17 *Gabrb3*$^{+/D120N}$ mice had spontaneous spasm-like seizures, extended staring, and reduced pre-weaning weight compared to wt

mice. Further, when the young *Gabrb3*$^{+/D120N}$ mice were suspended by the tail, they showed abnormal fore and hind limb clenching, which is considered an indicator of neurological abnormality. Seizure onset in LGS patients is 1-8 years or earlier when LGS progresses from other syndromes such as infantile spasms [24, 156, 164]. Seizure onset in young P14-P17 *Gabrb3*$^{+/D120N}$ mice was in the age range analogous to seizure onset in LGS patients.

Atypical absence, tonic, myoclonic, and atonic seizures are the most common seizures in LGS patients. For the *Gabrb3*$^{+/D120N}$ mice, the predominant seizure types were atypical absence and myoclonic seizures. Tonic seizures are one of the main seizure types pf LGS and observed in majority of LGS patients while they are awake and asleep. While tonic seizures were not the predominant seizure type in *Gabrb3*$^{+/D120N}$ mice, they occurred on average four times in 24 hours and showed male predominance. As tonic seizures are hard to establish, especially when the mice are asleep and not facing the camera, they could be slightly underestimated in this study. Atonic seizures or drop attacks result in sudden loss of muscle tone resulting in head or whole body falls leading to injuries in LGS patients. Very few atonic-like seizures were observed during the 24 hour video-EEG recordings in *Gabrb3*$^{+/D120N}$ mice, but they were not scored as the mice did not fall on their sides and atonic seizure in mice are not well characterized. Additionally, most seizure types had increased incidence in male *Gabrb3*$^{+/D120N}$ mice, but were not significantly higher compared to female mice. Even though not all seizure types seen in LGS patients were present in *Gabrb3*$^{+/D120N}$ mice,

they captured most of the characteristic LGS seizure types and thus should be a useful model of LGS.

Typical sleep disturbances documented in LGS patients include augmentation of slow spike waves, generalized polyspikes, and generalized paroxysmal fast activity in NREM sleep and a reduction of REM sleep [24, 156, 199, 223-225]. Disruption of sleep features with large numbers of awakenings and altered sleep stages was also reported in LGS patients [163]. The *Gabrb3*$^{+/D120N}$ mice also exhibited significant sleep disturbances including SWDs in NREM and REM sleep, myoclonic jerks, and reduced REM sleep. Qualitatively, the waveform and frequency of SWDs in NREM sleep resembled those of atypical absence seizures (4-5 Hz) seen in *Gabrb3*$^{+/D120N}$ mice, while SWDs of REM sleep resembled typical absence seizures with characteristic spindle-like appearance with frequencies of 4-5 Hz and 7-8 Hz.

To date very few mouse models mimicking the LGS phenotype exist. The *Gabrb3*$^{+/D120N}$ mouse is the first mouse model harboring a human mutation associated with LGS. A mouse model of atypical absence seizures was developed by administering AY-9944, a cholesterol biosynthesis inhibitor in young rats, which resulted in recurrent SWDs at 5-6 Hz (slower than SWDs of 7-8 Hz seen in typical absence seizures in rodents) that lasted several seconds. The SWDs persisted in sleep and were accompanied by myoclonic jerks [32]. However, other seizure types seen in LGS patients were lacking, and the pathological mechanisms during the pre-natal period and early post-natal period preceding seizure onset at P21 cannot be achieved. Importantly, absence seizures in *Gabrb3*$^{+/D120N}$ mice and the AY-9944 mice were qualitatively

different. The *Gabrb3*$^{+/D120N}$ mice had both typical SWDs of 4-6 Hz and less organized slow-spike-waves of 2-3 Hz. Similarly, LGS patients are reported to have both typical (3 Hz) and less organized atypical (1-2.5 Hz) absence seizures [24]. Additionally, cognitive and behavioral deficits have not been reported in AY-9944 treated rats, although decreased hippocampal long-term potentiation has been suggested [32]. The AY-9944 model showed a higher propensity for absence seizures in females, while the male *Gabrb3*$^{+/D120N}$ mice show higher propensity for absence, myoclonic, and tonic seizures. Male predominance was reported for LGS patients [158, 165], but it is not clear if male LGS patients had increased seizure severity compared to female LGS patients.

Recently, *DNM1* was associated with LGS [3, 226], and the 'fitful' mouse harboring a spontaneous novel mutation in *DNM1* was initially proposed as a model of genetic generalized (idiopathic) epilepsy [66] and later of EE [227, 228]. Both homozygous (hom) and het fitful mice had spontaneous seizures. Young hom (P14-P16) mice had ataxia and tonic-clonic seizures resulting in mortality during the pre-weaning period, while the hets developed GTCSs and partial seizures from two to three months of age [66]. The fitful mice exhibited ataxia and reduced dendritic arbors and branching of cerebellar Purkinje cells. Ataxia and cerebellar dysfunctions were not typically observed in LGS patients but were reported in other EEs [229]. However, the characteristic LGS seizure types (atypical absence, tonic, atonic, and myoclonic seizures), cognitive and behavioral abnormalities, and sleep disturbances have not been reported in the fitful mice. Future studies are needed to carefully assess whether the fitful mice display predominant LGS seizure types and associated comorbidities.

Lastly, hom *Gabrb3*$^{-/-}$ mice have been proposed as a model for Angelman syndrome, a severe neurodevelopmental disorder associated with epilepsy in the majority of cases [230-233]. A large proportion of Angelman syndrome cases result from deletion of the maternally imprinted chromosomal 15q11-13 region containing *UBE3A*, *GABRB3*, *GABRA5*, and *GABRG3* [231, 234-237]. In neurons, the chromosomal 15q11-13 segment is maternally imprinted, i.e. exclusively expressed by the maternal copy while expression of the paternal chromosomal segment is suppressed. As about 20% of patients with Angelman syndrome are non-deletion cases and those with *UBE3A* mutations have milder phenotypes with few or nonexistence seizures compared to the chromosomal 15q11-13 deletion cases, Olsen and colleagues suggested that *GABRB3* could be an Angelman syndrome candidate gene. However, the current consensus is that Angelman syndrome results from loss of function of *UBE3A*, and Angelman syndrome cases can arise either due to the deletion or mutations of *UBE3A*, paternal uniparental disomy or genomic imprinting defects [233, 235, 238, 239], all coinciding with loss of function of the maternal copy of *UBE3A*.

As *UBE3A* is the principal gene involved in the Angelman syndrome pathology, the hom *Gabrb3*$^{-/-}$ *KO* mice mimics the pathology of a minute fraction of patients with just the loss of *GABRB3*, and the het *Gabr3*$^{+/-}$ *KO* mice could represent an extremely rare genetic condition. Nevertheless, the hom *Gabrb3*$^{-/-}$ and het *Gabr3b*$^{+/-}$ *KO* mice displayed several characteristics that have clinical correlates to human EE phenotypes including spontaneous seizures (absence, myoclonic jerks, and GTCS), runted pre-weaning growth, hyperactivity, and increased severity of seizures with age. The

Gabrb3$^{-/-}$ mice had a high neonatal mortality (~90%) within 24 hours of birth that was attributed to feeding difficulties resulting from cleft palate and reduced life span of 18 weeks [240]. Cleft palate was also described in Angelman syndrome patients [241]. The phenotype of het KO mice was milder than that of hom KO mice. Ethosuximide is a commonly used AED to treat absence seizures in humans. When administered to *Gabrb3*$^{-/-}$ mice, ethosuximide reduced absence and myoclonic seizures, and abolished slow background and interictal EEG activity. In contrast, the AED carbamazepine and the $GABA_B$ receptor agonist baclofen triggered seizures and aggravated EEG abnormalities. The $GABA_B$ receptor antagonist CGP 35348 suppressed atypical absence seizures in the AY-9944 model but was ineffective in reducing seizures or normalizing EEG activity in *Gabrb3*$^{-/-}$ mice [32, 242].

As the same gene is disrupted in *Gabrb3*$^{-/-}$ and *Gabr3*$^{+/D120N}$ mice, the two mice are likely to share some features. In addition to the presence of absence seizures, myoclonic jerks, and GTCS, both of these mice have runted growth until weaning, some mice are hyperactive (run in circles), both mice are hyper responsive to human touch - both mice jump when they are approached to be held, both mice clench paws when held by their tail, and the *Gabrb3*$^{-/-}$ and *Gabrb3*$^{+/D120N}$ mothers fail to nurture their offspring irrespective of the littermate genotype. Additionally, GABA-evoked currents from cultured sensory dorsal root ganglion (DRG) cells of hom and het *Gabrb3* KO mice were decreased by ~80% and ~25%, respectively. Similarly, mutant β3(D120N) subunits significantly reduced GABA-evoked currents to ~63% and ~24% of wt currents, respectively, in hom and het conditions *in vitro* [243]. Based on these results and the

presence of spontaneous seizures, loss of GABAergic inhibition would be expected in *Gabrb3*$^{+/D120N}$ mice. While the seizures manifested in both strains differ; *Gabrb3*$^{-/-}$ mice had absence, myoclonic, and GTCS seizures, whereas the predominant phenotype of adult *Gabrb3*$^{+/D120N}$ mice was atypical absence and myoclonic seizures with occasional tonic seizures and GTCS. Since the precise numbers of seizures in hom *Gabrb3*$^{-/-}$ and het *Gabrb3*$^{+/-}$ KO mice are not known, seizure severity cannot be directly compared with *Gabrb3*$^{+/D120N}$ mice. Still based on the phenotypes of the three mice described here, it appears that the order of severity could be *Gabrb3*$^{-/-}$ > *Gabrb3*$^{+/D120N}$ > *Gabrb3*$^{+/-}$ KO mice.

In conclusion, the EEG findings presented here unambiguously establish the spontaneous seizure types in *Gabrb3*$^{+/D120N}$ mice. Further, our data show that *Gabrb3*$^{+/D120N}$ mice display spontaneous seizures and other features relevant to clinical symptoms in LGS patients, and thus are a valuable model to study pathogenesis of LGS and genetic EEs. Spontaneous seizures in *Gabrb3*$^{+/D120N}$ mice are in agreement with *in vitro* findings that mutant β3(D120N) subunits in hom and het conditions reduce GABA-evoked inhibitory currents in HEK293T cells [243]. The β3 subunits are expressed in regions that are important for generation of sezirues including cortex, thalamus, and the nRT. Thus, reduced post-synaptic inhibition in these regions resulting from β3(D120N) subunits can generate hyperexcitable networks that in turn can produce generalized seizures and epilepsy. These EEG studies lays the groundwork for future studies to determine the age of seizure onset of electrographic seizures, molecular, cellular, and circuits level changes that promote seizures, degree of loss of GABAergic

inhibition, efficacy of AEDs, most effective treatment window, among other important questions.

4.11 REFERENCES

1. Epi KC, Epilepsy Phenome/Genome P, Allen AS, et al. De novo mutations in epileptic encephalopathies. Nature. 2013 Sep 12;501(7466):217-21.

2. de Kovel CG, Brilstra EH, van Kempen MJ, et al. Targeted sequencing of 351 candidate genes for epileptic encephalopathy in a large cohort of patients. Mol Genet Genomic Med. 2016 Sep;4(5):568-80.

3. Euro E-RESC, Epilepsy Phenome/Genome P, Epi KC. De novo mutations in synaptic transmission genes including DNM1 cause epileptic encephalopathies. Am J Hum Genet. 2014 Oct 02;95(4):360-70.

4. Carvill GL, Heavin SB, Yendle SC, et al. Targeted resequencing in epileptic encephalopathies identifies de novo mutations in CHD2 and SYNGAP1. Nat Genet. 2013 Jul;45(7):825-30.

5. Thomas RH, Berkovic SF. The hidden genetics of epilepsy-a clinically important new paradigm. Nat Rev Neurol. 2014 May;10(5):283-92.

6. Noebels J. Pathway-driven discovery of epilepsy genes. Nat Neurosci. 2015 Mar;18(3):344-50.

7. Veltman JA, Brunner HG. De novo mutations in human genetic disease. Nat Rev Genet. 2012 Aug;13(8):565-75.

8. Helbig KL, Farwell Hagman KD, Shinde DN, et al. Diagnostic exome sequencing provides a molecular diagnosis for a significant proportion of patients with epilepsy. Genet Med. 2016 Sep;18(9):898-905.

9. Rogawski MA. Molecular targets versus models for new antiepileptic drug discovery. Epilepsy Res. 2006 Jan;68(1):22-8.

10. Gibbs FA, Davis H, WG L. The electro-encephalogram in epilepsy and in conditions of impaired consciousness. Arch Neurol Psychiatry. 1935;34 (6):1133-48.

11. Gibbs FA, Gibbs EL, WG L. Epilepsy: a paroxysmal cerebral dysrhythmia. Brain. 1937;60(4):377-88.

12. Camfield PR. Definition and natural history of Lennox-Gastaut syndrome. Epilepsia. 2011 Aug;52 Suppl 5:3-9.

13. Velazquez JL, Huo JZ, Dominguez LG, Leshchenko Y, Snead OC, 3rd. Typical versus atypical absence seizures: network mechanisms of the spread of paroxysms. Epilepsia. 2007 Aug;48(8):1585-93.

14. Carmant L, Kramer U, Holmes GL, Mikati MA, Riviello JJ, Helmers SL. Differential diagnosis of staring spells in children: a video-EEG study. Pediatr Neurol. 1996 Apr;14(3):199-202.

15. Yaqub BA. Electroclinical seizures in Lennox-Gastaut syndrome. Epilepsia. 1993 Jan-Feb;34(1):120-7.

16. van Rijckevorsel K. Treatment of Lennox-Gastaut syndrome: overview and recent findings. Neuropsychiatr Dis Treat. 2008 Dec;4(6):1001-19.

17. Farwell JR, Dodrill CB, Batzel LW. Neuropsychological abilities of children with epilepsy. Epilepsia. 1985 Sep-Oct;26(5):395-400.

18. Tenney JR, Jain SV. Absence Epilepsy: Older vs Newer AEDs. Curr Treat Options Neurol. 2014 May;16(5):290.

19. DM IJ, van Veenendaal TM, Debeij-van Hall MH, et al. The Cognitive Profile of Ethosuximide in Children. Paediatr Drugs. 2016 Oct;18(5):379-85.

20. Glauser TA, Cnaan A, Shinnar S, et al. Ethosuximide, valproic acid, and lamotrigine in childhood absence epilepsy: initial monotherapy outcomes at 12 months. Epilepsia. 2013 Jan;54(1):141-55.

21. Hwang H, Kim H, Kim SH, et al. Long-term effectiveness of ethosuximide, valproic acid, and lamotrigine in childhood absence epilepsy. Brain Dev. 2012 May;34(5):344-8.

22. Glauser TA, Cnaan A, Shinnar S, et al. Ethosuximide, valproic acid, and lamotrigine in childhood absence epilepsy. N Engl J Med. 2010 Mar 4;362(9):790-9.

23. Penry JK, Porter RJ, Dreifuss RE. Simultaneous recording of absence seizures with video tape and electroencephalography. A study of 374 seizures in 48 patients. Brain. 1975 Sep;98(3):427-40.

24. Gastraut H, Roger J, Soulayrol R, et al. Childhood epileptic encephalopathy with diffuse slow spike-waves (otherwise known as "petit mal variant") or Lennox syndrome. Epilepsia. 1966 Jun;7(2):139-79.

25. Lennox WG, Davis JP. Clinical correlates of the fast and the slow spike-wave electroencephalogram. Pediatrics. 1950 Apr;5(4):626-44.

26. Gibbs FA, Gibbs EL, WG L. Influence of the blood sugar level on the wave and spike formation in Petit Mal epilepsy. Archives of Neurology and Psychiatry. 1939;41(6):1111-6.

27. Verrotti A, D'Egidio C, Agostinelli S, Gobbi G. Glut1 deficiency: when to suspect and how to diagnose? Eur J Paediatr Neurol. 2012 Jan;16(1):3-9.

28. Nakano K, Kobayashi K, Okano Y, Aso K, Ohtsuka Y. Intractable absence seizures in hyperinsulinism-hyperammonemia syndrome. Pediatr Neurol. 2012 Aug;47(2):119-22.

29. Pearson TS, Akman C, Hinton VJ, Engelstad K, De Vivo DC. Phenotypic spectrum of glucose transporter type 1 deficiency syndrome (Glut1 DS). Curr Neurol Neurosci Rep. 2013 Apr;13(4):342.

30. Ochs RF, Gloor P, Tyler JL, et al. Effect of generalized spike-and-wave discharge on glucose metabolism measured by positron emission tomography. Ann Neurol. 1987 May;21(5):458-64.

31. Smith KA, Bierkamper GG. Paradoxical role of GABA in a chronic model of petit mal (absence)-like epilepsy in the rat. Eur J Pharmacol. 1990 Jan 25;176(1):45-55.

32. Cortez MA, McKerlie C, Snead OC, 3rd. A model of atypical absence seizures: EEG, pharmacology, and developmental characterization. Neurology. 2001 Feb 13;56(3):341-9.

33. Serbanescu I, Cortez MA, McKerlie C, Snead OC, 3rd. Refractory atypical absence seizures in rat: a two hit model. Epilepsy Res. 2004 Nov;62(1):53-63.

34. Bare MA, Glauser TA, Strawsburg RH. Need for electroencephalogram video confirmation of atypical absence seizures in children with Lennox-Gastaut syndrome. J Child Neurol. 1998 Oct;13(10):498-500.

35. Crunelli V, Leresche N. Childhood absence epilepsy: genes, channels, neurons and networks. Nat Rev Neurosci. 2002 May;3(5):371-82.

36. Vergnes M, Marescaux C, Depaulis A, Micheletti G, Warter JM. Spontaneous spike and wave discharges in thalamus and cortex in a rat model of genetic petit mal-like seizures. Exp Neurol. 1987 Apr;96(1):127-36.

37. Vergnes M, Marescaux C, Micheletti G, et al. Spontaneous paroxysmal electroclinical patterns in rat: a model of generalized non-convulsive epilepsy. Neurosci Lett. 1982 Nov 16;33(1):97-101.

38. van Luijtelaar EL, Coenen AM. Two types of electrocortical paroxysms in an inbred strain of rats. Neurosci Lett. 1986 Oct 20;70(3):393-7.

39. Jung S, Jeong Y, Jeon D. Epileptic activity during early postnatal life in the AY-9944 model of atypical absence epilepsy. Cell Calcium. 2015 May;57(5-6):376-84.

40. Blumenfeld H. Cellular and network mechanisms of spike-wave seizures. Epilepsia. 2005;46 Suppl 9:21-33.

41. Avoli M. A brief history on the oscillating roles of thalamus and cortex in absence seizures. Epilepsia. 2012 May;53(5):779-89.

42. Kawashima T, Adachi T, Tokunaga Y, et al. Immunohistochemical analysis in a case of idiopathic Lennox-Gastaut syndrome. Clin Neuropathol. 1999 Nov-Dec;18(6):286-92.

43. Velasco M, Velasco F, Velasco AL. Centromedian-thalamic and hippocampal electrical stimulation for the control of intractable epileptic seizures. J Clin Neurophysiol. 2001 Nov;18(6):495-513.

44. Pedersen M, Curwood EK, Archer JS, Abbott DF, Jackson GD. Brain regions with abnormal network properties in severe epilepsy of Lennox-Gastaut phenotype: Multivariate analysis of task-free fMRI. Epilepsia. 2015 Nov;56(11):1767-73.

45. Camfield PR, Gibson PA, Douglass LM. Strategies for transitioning to adult care for youth with Lennox-Gastaut syndrome and related disorders. Epilepsia. 2011 Aug;52 Suppl 5:21-7.

46. Persad V, Ting Wong CG, Cortez MA, Wang YT, Snead OC, 3rd. Hormonal regulation of atypical absence seizures. Ann Neurol. 2004 Mar;55(3):353-61.

47. Seneviratne U, Cook M, D'Souza W. Epileptiform K-Complexes and Sleep Spindles: An Underreported Phenomenon in Genetic Generalized Epilepsy. J Clin Neurophysiol. 2016 Apr;33(2):156-61.

48. Christensen J, Kjeldsen MJ, Andersen H, Friis ML, Sidenius P. Gender differences in epilepsy. Epilepsia. 2005 Jun;46(6):956-60.

49. Miskov S. Gender differences in epilepsy. Acta Neuropsychiatr. 2009 Jun;21 Suppl 2:41-4.

50. Markand ON. Slow spike-wave activity in EEG and associated clinical features: often called 'Lennox' or "Lennox-Gastaut' syndrome. Neurology. 1977 Aug;27(8):746-57.

51. Marescaux C, Vergnes M, Depaulis A. Genetic absence epilepsy in rats from Strasbourg--a review. J Neural Transm Suppl. 1992;35:37-69.

52. Coenen AM, Drinkenburg WH, Inoue M, van Luijtelaar EL. Genetic models of absence epilepsy, with emphasis on the WAG/Rij strain of rats. Epilepsy Res. 1992 Jul;12(2):75-86.

53. Depaulis A, David O, Charpier S. The genetic absence epilepsy rat from Strasbourg as a model to decipher the neuronal and network mechanisms of generalized idiopathic epilepsies. J Neurosci Methods. 2016 Feb 15;260:159-74.

54. Powell KL, Tang H, Ng C, et al. Seizure expression, behavior, and brain morphology differences in colonies of Genetic Absence Epilepsy Rats from Strasbourg. Epilepsia. 2014 Dec;55(12):1959-68.

55. van Luijtelaar G, Sitnikova E. Global and focal aspects of absence epilepsy: the contribution of genetic models. Neurosci Biobehav Rev. 2006;30(7):983-1003.

56. Robinson PF, Gilmore SA. Spontaneous generalized spike-wave discharges in the electrocorticograms of albino rats. Brain Res. 1980 Nov 17;201(2):452-8.

57. ILAE CoCaTot. Proposal for revised classification of epilepsies and epileptic syndromes. Commission on Classification and Terminology of the International League Against Epilepsy. Epilepsia. 1989 Jul-Aug;30(4):389-99.

58. Zupanc ML. Clinical evaluation and diagnosis of severe epilepsy syndromes of early childhood. J Child Neurol. 2009 Aug;24(8 Suppl):6S-14S.

59. Deciphering Developmental Disorders S. Large-scale discovery of novel genetic causes of developmental disorders. Nature. 2015 Mar 12;519(7542):223-8.

60. van der Bliek AM, Redelmeier TE, Damke H, Tisdale EJ, Meyerowitz EM, Schmid SL. Mutations in human dynamin block an intermediate stage in coated vesicle formation. J Cell Biol. 1993 Aug;122(3):553-63.

61. Ferguson SM, De Camilli P. Dynamin, a membrane-remodelling GTPase. Nat Rev Mol Cell Biol. 2012 Jan 11;13(2):75-88.

62. Marks B, Stowell MH, Vallis Y, et al. GTPase activity of dynamin and resulting conformation change are essential for endocytosis. Nature. 2001 Mar 08;410(6825):231-5.

63. Ferguson SM, Brasnjo G, Hayashi M, et al. A selective activity-dependent requirement for dynamin 1 in synaptic vesicle endocytosis. Science. 2007 Apr 27;316(5824):570-4.

64. Fan F, Funk L, Lou X. Dynamin 1- and 3-Mediated Endocytosis Is Essential for the Development of a Large Central Synapse In Vivo. J Neurosci. 2016 Jun 01;36(22):6097-115.

65. Dhindsa RS, Bradrick SS, Yao X, et al. Epileptic encephalopathy-causing mutations in DNM1 impair synaptic vesicle endocytosis. Neurol Genet. 2015 Jun;1(1):e4.

66. Boumil RM, Letts VA, Roberts MC, et al. A missense mutation in a highly conserved alternate exon of dynamin-1 causes epilepsy in fitful mice. PLoS Genet. 2010 Aug 05;6(8).

67. Dutta S, Sengupta P. Men and mice: Relating their ages. Life Sci. 2016 May 1;152:244-8.

68. Caton R. The electric currents of the brain. British Medical Journal. 1875(2):278–.

69. Caton R. Interim report on investigations of the electric currents of the brain. . British Medical Journal. 1877(1):62–5.

70. Beck A. O pobudliwości rónych miejsc tego samego nerwu. [On the excitability of various parts of the same nerve]. . Rozprawy Akademii Umiejtnoci, Wydzia Matematyczno-Przyrodniczy. 1888(15):165–95.

71. Berger H. Über das Elektrenkephalogramm des Menschen. [On the electroencephalogram of humans]. Archiv für Psychiatrie und Nervenkrankheiten. 1929(87):527–70.

72. Gibbs FA, H D. Changes in the human electroencephalogram associated with loss of consciousness. 1935;113:49-50.

73. Ferree TC, Luu P, Russell GS, Tucker DM. Scalp electrode impedance, infection risk, and EEG data quality. Clin Neurophysiol. 2001 Mar;112(3):536-44.

74. Nunez PL. Toward a quantitative description of large-scale neocortical dynamic function and EEG. Behav Brain Sci. 2000 Jun;23(3):371-98; discussion 99-437.

75. Berger H. Ueber das Elekroenkephalogramm des Menschen. Journal of Psychological Neurology. 1930;40:160-79.

76. International Federation of Societies for Electroencephalography and Clinical Neurophysiology. Electroencephalogr Clin Neurophysiol. 1974 Nov;37(5):521.

77. Sauseng P, Griesmayr B, Freunberger R, Klimesch W. Control mechanisms in working memory: a possible function of EEG theta oscillations. Neurosci Biobehav Rev. 2010 Jun;34(7):1015-22.

78. Klimesch W. EEG alpha and theta oscillations reflect cognitive and memory performance: a review and analysis. Brain Res Brain Res Rev. 1999 Apr;29(2-3):169-95.

79. Rodriguez E, George N, Lachaux JP, Martinerie J, Renault B, Varela FJ. Perception's shadow: long-distance synchronization of human brain activity. Nature. 1999 Feb 04;397(6718):430-3.

80. Sanes JN, Donoghue JP. Oscillations in local field potentials of the primate motor cortex during voluntary movement. Proc Natl Acad Sci U S A. 1993 May 15;90(10):4470-4.

81. Bazhenov M, Timofeev I, Steriade M, Sejnowski TJ. Model of thalamocortical slow-wave sleep oscillations and transitions to activated States. J Neurosci. 2002 Oct 01;22(19):8691-704.

82. Britton JW, Frey LC, Hopp JL, et al. 2016 Abnormal Findings in Adults, Children, and Infants.

83. Staba RJ, Bragin A. High-frequency oscillations and other electrophysiological biomarkers of epilepsy: underlying mechanisms. Biomark Med. 2011 Oct;5(5):545-56.

84. Gonzalez-Burgos G, Cho RY, Lewis DA. Alterations in cortical network oscillations and parvalbumin neurons in schizophrenia. Biol Psychiatry. 2015 Jun 15;77(12):1031-40.

85. Thompson L, Thompson M, Reid A. Functional neuroanatomy and the rationale for using EEG biofeedback for clients with Asperger's syndrome. Appl Psychophysiol Biofeedback. 2010 Mar;35(1):39-61.

86. David N, Schneider TR, Peiker I, Al-Jawahiri R, Engel AK, Milne E. Variability of cortical oscillation patterns: A possible endophenotype in autism spectrum disorders? Neurosci Biobehav Rev. 2016 Dec;71:590-600.

87. Sinclair D, Oranje B, Razak KA, Siegel SJ, Schmid S. Sensory processing in autism spectrum disorders and Fragile X syndrome-From the clinic to animal models. Neurosci Biobehav Rev. 2017 May;76(Pt B):235-53.

88. Yener GG, Basar E. Biomarkers in Alzheimer's disease with a special emphasis on event-related oscillatory responses. Suppl Clin Neurophysiol. 2013;62:237-73.

89. Basar E, Basar-Eroglu C, Guntekin B, Yener GG. Brain's alpha, beta, gamma, delta, and theta oscillations in neuropsychiatric diseases: proposal for biomarker strategies. Suppl Clin Neurophysiol. 2013;62:19-54.

90. Klein AS, Donoso JR, Kempter R, Schmitz D, Beed P. Early Cortical Changes in Gamma Oscillations in Alzheimer's Disease. Front Syst Neurosci. 2016;10:83.

91. Brown P. Oscillatory nature of human basal ganglia activity: relationship to the pathophysiology of Parkinson's disease. Mov Disord. 2003 Apr;18(4):357-63.

92. Kuhn AA, Kupsch A, Schneider GH, Brown P. Reduction in subthalamic 8-35 Hz oscillatory activity correlates with clinical improvement in Parkinson's disease. Eur J Neurosci. 2006 Apr;23(7):1956-60.

93. Brown P, Oliviero A, Mazzone P, Insola A, Tonali P, Di Lazzaro V. Dopamine dependency of oscillations between subthalamic nucleus and pallidum in Parkinson's disease. J Neurosci. 2001 Feb 01;21(3):1033-8.

94. Basar E. Brain oscillations in neuropsychiatric disease. Dialogues Clin Neurosci. 2013 Sep;15(3):291-300.

95. Yener GG, Basar E. Brain oscillations as biomarkers in neuropsychiatric disorders: following an interactive panel discussion and synopsis. Suppl Clin Neurophysiol. 2013;62:343-63.

96. Herrmann CS, Senkowski D, Rottger S. Phase-locking and amplitude modulations of EEG alpha: Two measures reflect different cognitive processes in a working memory task. Exp Psychol. 2004;51(4):311-8.

97. Reuter BM, Linke DB. [Are cognitive processes related to the EEG alpha phase? An experimental study using P300]. EEG EMG Z Elektroenzephalogr Elektromyogr Verwandte Geb. 1987 Jun;18(2):58-60.

98. Ray WJ, Cole HW. EEG alpha activity reflects attentional demands, and beta activity reflects emotional and cognitive processes. Science. 1985 May 10;228(4700):750-2.

99. Jasper HH, Andrews HL. Electro-encephalography: Iii. normal differentiation of occipital and precentral regions in man. Archives of Neurology & Psychiatry. 1938;39(1):96-115.

100. MacKay WA, Mendonca AJ. Field potential oscillatory bursts in parietal cortex before and during reach. Brain Res. 1995 Dec 18;704(2):167-74.

101. Domino EF, French J, Pohorecki R, Galus CF, Pandit SK. Further observations on the effects of subhypnotic doses of midazolam in normal volunteers. Psychopharmacol Bull. 1989;25(3):460-5.

102. Feshchenko VA, Veselis RA, Reinsel RA. Comparison of the EEG effects of midazolam, thiopental, and propofol: the role of underlying oscillatory systems. Neuropsychobiology. 1997;35(4):211-20.

103. Wu JY, Koh S, Sankar R, Mathern GW. Paroxysmal fast activity: an interictal scalp EEG marker of epileptogenesis in children. Epilepsy Res. 2008 Nov;82(1):99-106.

104. Tallon-Baudry C, Bertrand O, Delpuech C, Pernier J. Stimulus specificity of phase-locked and non-phase-locked 40 Hz visual responses in human. J Neurosci. 1996 Jul 01;16(13):4240-9.

105. Lachaux JP, George N, Tallon-Baudry C, et al. The many faces of the gamma band response to complex visual stimuli. Neuroimage. 2005 Apr 01;25(2):491-501.

106. Jensen O, Kaiser J, Lachaux JP. Human gamma-frequency oscillations associated with attention and memory. Trends Neurosci. 2007 Jul;30(7):317-24.

107. Valderrama M, Crepon B, Botella-Soler V, et al. Human gamma oscillations during slow wave sleep. PLoS One. 2012;7(4):e33477.

108. Dalal SS, Hamame CM, Eichenlaub JB, Jerbi K. Intrinsic coupling between gamma oscillations, neuronal discharges, and slow cortical oscillations during human slow-wave sleep. J Neurosci. 2010 Oct 27;30(43):14285-7.

109. Cantero JL, Atienza M, Madsen JR, Stickgold R. Gamma EEG dynamics in neocortex and hippocampus during human wakefulness and sleep. Neuroimage. 2004 Jul;22(3):1271-80.

110. Llinas R, Ribary U. Coherent 40-Hz oscillation characterizes dream state in humans. Proc Natl Acad Sci U S A. 1993 Mar 01;90(5):2078-81.

111. Basar-Eroglu C, Brand A, Hildebrandt H, Karolina Kedzior K, Mathes B, Schmiedt C. Working memory related gamma oscillations in schizophrenia patients. Int J Psychophysiol. 2007 Apr;64(1):39-45.

112. Fries P. Neuronal gamma-band synchronization as a fundamental process in cortical computation. Annu Rev Neurosci. 2009;32:209-24.

113. Fries P. A mechanism for cognitive dynamics: neuronal communication through neuronal coherence. Trends Cogn Sci. 2005 Oct;9(10):474-80.

114. Varela F, Lachaux JP, Rodriguez E, Martinerie J. The brainweb: phase synchronization and large-scale integration. Nat Rev Neurosci. 2001 Apr;2(4):229-39.

115. Lewis DA. Inhibitory neurons in human cortical circuits: substrate for cognitive dysfunction in schizophrenia. Curr Opin Neurobiol. 2014 Jun;26:22-6.

116. Gonzalez-Burgos G, Hashimoto T, Lewis DA. Alterations of cortical GABA neurons and network oscillations in schizophrenia. Curr Psychiatry Rep. 2010 Aug;12(4):335-44.

117. Herrmann CS, Demiralp T. Human EEG gamma oscillations in neuropsychiatric disorders. Clin Neurophysiol. 2005 Dec;116(12):2719-33.

118. Bartos M, Vida I, Jonas P. Synaptic mechanisms of synchronized gamma oscillations in inhibitory interneuron networks. Nat Rev Neurosci. 2007 Jan;8(1):45-56.

119. Buzsaki G, Wang XJ. Mechanisms of gamma oscillations. Annu Rev Neurosci. 2012;35:203-25.

120. Whittington MA, Doheny HC, Traub RD, LeBeau FE, Buhl EH. Differential expression of synaptic and nonsynaptic mechanisms underlying stimulus-induced gamma oscillations in vitro. J Neurosci. 2001 Mar 01;21(5):1727-38.

121. Benedek K, Berenyi A, Gombkoto P, Piilgaard H, Lauritzen M. Neocortical gamma oscillations in idiopathic generalized epilepsy. Epilepsia. 2016 May;57(5):796-804.

122. Andrade-Valenca LP, Dubeau F, Mari F, Zelmann R, Gotman J. Interictal scalp fast oscillations as a marker of the seizure onset zone. Neurology. 2011 Aug 09;77(6):524-31.

123. Bragin A, Engel J, Jr., Wilson CL, Fried I, Buzsaki G. High-frequency oscillations in human brain. Hippocampus. 1999;9(2):137-42.

124. Bragin A, Engel J, Jr., Wilson CL, Fried I, Mathern GW. Hippocampal and entorhinal cortex high-frequency oscillations (100--500 Hz) in human epileptic brain and in kainic acid--treated rats with chronic seizures. Epilepsia. 1999 Feb;40(2):127-37.

125. Fisher RS, Webber WR, Lesser RP, Arroyo S, Uematsu S. High-frequency EEG activity at the start of seizures. J Clin Neurophysiol. 1992 Jul;9(3):441-8.

126. Jirsch JD, Urrestarazu E, LeVan P, Olivier A, Dubeau F, Gotman J. High-frequency oscillations during human focal seizures. Brain. 2006 Jun;129(Pt 6):1593-608.

127. Medvedev AV, Murro AM, Meador KJ. Abnormal interictal gamma activity may manifest a seizure onset zone in temporal lobe epilepsy. Int J Neural Syst. 2011 Apr;21(2):103-14.

128. Zijlmans M, Jiruska P, Zelmann R, Leijten FS, Jefferys JG, Gotman J. High-frequency oscillations as a new biomarker in epilepsy. Ann Neurol. 2012 Feb;71(2):169-78.

129. Pizzo F, Frauscher B, Ferrari-Marinho T, Amiri M, Dubeau F, Gotman J. Detectability of Fast Ripples (>250 Hz) on the Scalp EEG: A Proof-of-Principle Study with Subdermal Electrodes. Brain Topogr. 2016 May;29(3):358-67.

130. Chaitanya G, Sinha S, Narayanan M, Satishchandra P. Scalp high frequency oscillations (HFOs) in absence epilepsy: An independent component analysis (ICA) based approach. Epilepsy Res. 2015 Sep;115:133-40.

131. Irahara K, Nakagawa E, Honda R, et al. High gamma activity of 60-70 Hz in the area surrounding a cortical tuber in an infant with tuberous sclerosis. Ital J Pediatr. 2012 May 03;38:15.

132. Chu CJ, Chan A, Song D, Staley KJ, Stufflebeam SM, Kramer MA. A semi-automated method for rapid detection of ripple events on interictal voltage discharges in the scalp electroencephalogram. J Neurosci Methods. 2017 Feb 01;277:46-55.

133. Kobayashi K, Akiyama T, Oka M, Endoh F, Yoshinaga H. A storm of fast (40-150Hz) oscillations during hypsarrhythmia in West syndrome. Ann Neurol. 2015 Jan;77(1):58-67.

134. Fahoum F, Melani F, Andrade-Valenca L, Dubeau F, Gotman J. Epileptic scalp ripples are associated with corticothalamic BOLD changes. Epilepsia. 2014 Oct;55(10):1611-9.

135. Besio WG, Martinez-Juarez IE, Makeyev O, et al. High-Frequency Oscillations Recorded on the Scalp of Patients With Epilepsy Using Tripolar Concentric Ring Electrodes. IEEE J Transl Eng Health Med. 2014;2:2000111.

136. Melani F, Zelmann R, Dubeau F, Gotman J. Occurrence of scalp-fast oscillations among patients with different spiking rate and their role as epileptogenicity marker. Epilepsy Res. 2013 Oct;106(3):345-56.

137. Papadelis C, Tamilia E, Stufflebeam S, et al. Interictal High Frequency Oscillations Detected with Simultaneous Magnetoencephalography and Electroencephalography as Biomarker of Pediatric Epilepsy. J Vis Exp. 2016 Dec 06(118).

138. Nissen IA, van Klink NE, Zijlmans M, Stam CJ, Hillebrand A. Brain areas with epileptic high frequency oscillations are functionally isolated in MEG virtual electrode networks. Clin Neurophysiol. 2016 Jul;127(7):2581-91.

139. Tang L, Xiang J, Huang S, et al. Neuromagnetic high-frequency oscillations correlate with seizure severity in absence epilepsy. Clin Neurophysiol. 2016 Feb;127(2):1120-9.

140. Xiang J, Tenney JR, Korman AM, et al. Quantification of Interictal Neuromagnetic Activity in Absence Epilepsy with Accumulated Source Imaging. Brain Topogr. 2015 Nov;28(6):904-14.

141. Miao A, Xiang J, Tang L, et al. Using ictal high-frequency oscillations (80-500Hz) to localize seizure onset zones in childhood absence epilepsy: a MEG study. Neurosci Lett. 2014 Apr 30;566:21-6.

142. Jacobs J, Staba R, Asano E, et al. High-frequency oscillations (HFOs) in clinical epilepsy. Prog Neurobiol. 2012 Sep;98(3):302-15.

143. Dijk DJ. EEG slow waves and sleep spindles: windows on the sleeping brain. Behav Brain Res. 1995 Jul-Aug;69(1-2):109-16.

144. Steriade M, Amzica F. Sleep oscillations developing into seizures in corticothalamic systems. Epilepsia. 2003;44 Suppl 12:9-20.

145. Steriade M, Amzica F. Slow sleep oscillation, rhythmic K-complexes, and their paroxysmal developments. J Sleep Res. 1998;7 Suppl 1:30-5.

146. Sanchez-Vives MV, Mattia M. Slow wave activity as the default mode of the cerebral cortex. Arch Ital Biol. 2014 Jun-Sep;152(2-3):147-55.

147. von Stein A, Sarnthein J. Different frequencies for different scales of cortical integration: from local gamma to long range alpha/theta synchronization. Int J Psychophysiol. 2000 Dec 01;38(3):301-13.

148. Halasz P, Terzano MG, Parrino L. Spike-wave discharge and the microstructure of sleep-wake continuum in idiopathic generalised epilepsy. Neurophysiol Clin. 2002 Jan;32(1):38-53.

149. van Luijtelaar G, Hramov A, Sitnikova E, Koronovskii A. Spike-wave discharges in WAG/Rij rats are preceded by delta and theta precursor activity in cortex and thalamus. Clin Neurophysiol. 2011 Apr;122(4):687-95.

150. Buzsaki G, Leung LW, Vanderwolf CH. Cellular bases of hippocampal EEG in the behaving rat. Brain Res. 1983 Oct;287(2):139-71.

151. Tort AB, Komorowski RW, Manns JR, Kopell NJ, Eichenbaum H. Theta-gamma coupling increases during the learning of item-context associations. Proc Natl Acad Sci U S A. 2009 Dec 08;106(49):20942-7.

152. Moran LV, Hong LE. High vs low frequency neural oscillations in schizophrenia. Schizophr Bull. 2011 Jul;37(4):659-63.

153. Buzsaki G, Draguhn A. Neuronal oscillations in cortical networks. Science. 2004 Jun 25;304(5679):1926-9.

154. Chevrie JJ, Aicardi J. Childhood epileptic encephalopathy with slow spike-wave. A statistical study of 80 cases. Epilepsia. 1972 Apr;13(2):259-71.

155. Papini M, Pasquinelli A, Armellini M, Orlandi D. Alertness and incidence of seizures in patients with Lennox-Gastaut syndrome. Epilepsia. 1984 Apr;25(2):161-7.

156. Niedermeyer E. The Lennox-Gastaut syndrome: a severe type of childhood epilepsy. Dtsch Z Nervenheilkd. 1969;195(4):263-82.

157. Gastaut H, Roger J, Ouahchi S, Timsit M, Broughton R. An electro-clinical study of generalized epileptic seizures of tonic expression. Epilepsia. 1963 Mar;4:15-44.

158. Widdess-Walsh P, Dlugos D, Fahlstrom R, et al. Lennox-Gastaut syndrome of unknown cause: phenotypic characteristics of patients in the Epilepsy Phenome/Genome Project. Epilepsia. 2013 Nov;54(11):1898-904.

159. Aicardi J. The problem of the Lennox syndrome. Dev Med Child Neurol. 1973 Feb;15(1):77-80.

160. Aicardi J, Levy Gomes A. Clinical and electroencephalographic symptomatology of the 'genuine' Lennox-Gastaut syndrome and its differentiation from other forms of epilepsy of early childhood. Epilepsy Res Suppl. 1992;6:185-93.

161. Kobayashi K, Inoue T, Watanabe Y, et al. Spectral analysis of EEG gamma rhythms associated with tonic seizures in Lennox-Gastaut syndrome. Epilepsy Res. 2009 Sep;86(1):15-22.

162. Intusoma U, Abbott DF, Masterton RA, et al. Tonic seizures of Lennox-Gastaut syndrome: periictal single-photon emission computed tomography suggests a corticopontine network. Epilepsia. 2013 Dec;54(12):2151-7.

163. Sforza E, Mahdi R, Roche F, Maeder M, Foletti G. Nocturnal interictal epileptic discharges in adult Lennox-Gastaut syndrome: the effect of sleep stage and time of night. Epileptic Disord. 2016 Mar;18(1):44-50.

164. Arzimanoglou A, French J, Blume WT, et al. Lennox-Gastaut syndrome: a consensus approach on diagnosis, assessment, management, and trial methodology. Lancet Neurol. 2009 Jan;8(1):82-93.

165. Beaumanoir A. The Lennox-Gastaut syndrome: a personal study. Electroencephalogr Clin Neurophysiol Suppl. 1982(35):85-99.

166. Bauer G, Aichner F, Saltuari L. Epilepsies with diffuse slow spikes and waves of late onset. Eur Neurol. 1983;22(5):344-50.

167. Patry G, Lyagoubi S, Tassinari CA. Subclinical "electrical status epilepticus" induced by sleep in children. A clinical and electroencephalographic study of six cases. Arch Neurol. 1971 Mar;24(3):242-52.

168. Oguni H, Uehara T, Imai K, Osawa M. Atonic epileptic drop attacks associated with generalized spike-and-slow wave complexes: video-polygraphic study in two patients. Epilepsia. 1997 Jul;38(7):813-8.

169. Gastaut H. Clinical and electroencephalographical classification of epileptic seizures. Epilepsia. 1970 Mar;11(1):102-13.

170. Hrachovy RA, Frost JD, Jr. The EEG in selected generalized seizures. J Clin Neurophysiol. 2006 Aug;23(4):312-32.

171. Egli M, Mothersill I, O'Kane M, O'Kane F. The axial spasm--the predominant type of drop seizure in patients with secondary generalized epilepsy. Epilepsia. 1985 Sep-Oct;26(5):401-15.

172. Ikeno T, Shigematsu H, Miyakoshi M, Ohba A, Yagi K, Seino M. An analytic study of epileptic falls. Epilepsia. 1985 Nov-Dec;26(6):612-21.

173. Dulac O, N'Guyen T. The Lennox-Gastaut syndrome. Epilepsia. 1993;34 Suppl 7:S7-17.

174. Ferlazzo E, Adjien CK, Guerrini R, et al. Lennox-Gastaut syndrome with late-onset and prominent reflex seizures in trisomy 21 patients. Epilepsia. 2009 Jun;50(6):1587-95.

175. Dobesberger J, Ristic AJ, Walser G, et al. Duration of focal complex, secondarily generalized tonic-clonic, and primarily generalized tonic-clonic seizures--A video-EEG analysis. Epilepsy Behav. 2015 Aug;49:111-7.

176. Pan SP, Wang F, Zhang Y, Wang J. The electroclinical-semiology of generalized tonic-clonic seizures among different epilepsies. Eur Rev Med Pharmacol Sci. 2015 Nov;19(22):4249-53.

177. Theodore WH, Porter RJ, Albert P, et al. The secondarily generalized tonic-clonic seizure: a videotape analysis. Neurology. 1994 Aug;44(8):1403-7.

178. Pan S, Wang F, Wang J, Li X, Liu X. Factors influencing the duration of generalized tonic-clonic seizure. Seizure. 2016 Jan;34:44-7.

179. Lowenstein DH, Bleck T, Macdonald RL. It's time to revise the definition of status epilepticus. Epilepsia. 1999 Jan;40(1):120-2.

180. Gursahani R, Gupta N. The adolescent or adult with generalized tonic-clonic seizures. Ann Indian Acad Neurol. 2012 Apr;15(2):81-8.

181. Niedermeyer E, Lopes da Silva F. Electroencephalography: Basic Principles, Clinical Applications, and Related Fields. 5th ed: Lippincott Williams & Wilkins; 2005.

182. Hirfanoglu T, Serdaroglu A, Capraz I, Bilir E, Arhan EP, Aydin K. Comparison of ILAE 2010 and semiological seizure classification in children with epilepsy. Epilepsy Res. 2016 Nov 21;129:41-50.

183. Alan S, Yalnizoglu D, Turanli G, et al. Semiological seizure classification of epileptic seizures in children admitted to video-EEG monitoring unit. Turk J Pediatr. 2015 Jul-Aug;57(4):317-23.

184. Dikmen PY, Unlusoy Acar Z, Gurses C. Clinical events in psychogenic non-epileptic seizures based on semiological seizure classification. Neurol Res. 2013 Dec;35(10):1070-5.

185. Hirfanoglu T, Serdaroglu A, Cansu A, Bilir E, Gucuyener K. Semiological seizure classification: before and after video-EEG monitoring of seizures. Pediatr Neurol. 2007 Apr;36(4):231-5.

186. Lin JH, Kwan SY, Wu D, Su MS, Yiu CH. [Another seizure classification--Semiological Seizure Classification]. Acta Neurol Taiwan. 2004 Sep;13(3):136-48.

187. Kim KJ, Lee R, Chae JH, Hwang YS. Application of semiological seizure classification to epileptic seizures in children. Seizure. 2002 Jul;11(5):281-4.

188. Bautista JF, Luders HO. Semiological seizure classification: relevance to pediatric epilepsy. Epileptic Disord. 2000 Mar;2(1):65-72; discussion 3.

189. Tich SN, Pereon Y. Semiological seizure classification. Epilepsia. 1999 Apr;40(4):531.

190. Luders H, Acharya J, Baumgartner C, et al. Semiological seizure classification. Epilepsia. 1998 Sep;39(9):1006-13.

191. McBride MC, Laroia N, Guillet R. Electrographic seizures in neonates correlate with poor neurodevelopmental outcome. Neurology. 2000 Aug 22;55(4):506-13.

192. Holmes GL, Lenck-Santini PP. Role of interictal epileptiform abnormalities in cognitive impairment. Epilepsy Behav. 2006 May;8(3):504-15.

193. Aarts JH, Binnie CD, Smit AM, Wilkins AJ. Selective cognitive impairment during focal and generalized epileptiform EEG activity. Brain. 1984 Mar;107 (Pt 1):293-308.

194. Matsuzaka T, Baba H, Matsuo A, et al. Developmental assessment-based surgical intervention for intractable epilepsies in infants and young children. Epilepsia. 2001;42 Suppl 6:9-12.

195. Asarnow RF, LoPresti C, Guthrie D, et al. Developmental outcomes in children receiving resection surgery for medically intractable infantile spasms. Dev Med Child Neurol. 1997 Jul;39(7):430-40.

196. Archer JS, Warren AE, Stagnitti MR, Masterton RA, Abbott DF, Jackson GD. Lennox-Gastaut syndrome and phenotype: secondary network epilepsies. Epilepsia. 2014 Aug;55(8):1245-54.

197. Barreira AA, Lison MP. [Interictal electroencephalographic paroxysms in Lennox-Gastaut syndrome of late onset]. Arq Neuropsiquiatr. 1984 Mar;42(1):1-8.

198. Kaminska A, Oguni H. Chapter 67 - Lennox‚ÄìGastaut syndrome and epilepsy with myoclonic‚Äìastatic seizures. In: Olivier Dulac ML, Harvey BS, editors. Handbook of Clinical Neurology: Elsevier; 2013. p. 641-52.

199. Eisensehr I, Parrino L, Noachtar S, Smerieri A, Terzano MG. Sleep in Lennox Gastaut Syndrome: the role oft the cyclic alternating pattern (CAP) in the gate control of clinical seizures and generalized polyspikes. Epilepsy Research. 2001 2001/09/01/;46(3):241-50.

200. Kawakami M, Yamamura K. Cranial bone morphometric study among mouse strains. BMC Evol Biol. 2008;8:73.

201. Choi JH, Koch KP, Poppendieck W, Lee M, Shin HS. High resolution electroencephalography in freely moving mice. J Neurophysiol. 2010 Sep;104(3):1825-34.

202. Park AH, Lee SH, Lee C, et al. Optogenetic Mapping of Functional Connectivity in Freely Moving Mice via Insertable Wrapping Electrode Array Beneath the Skull. ACS Nano. 2016 Feb 23;10(2):2791-802.

203. Shulyzki R, Abdelhalim K, Bagheri A, et al. 320-channel active probe for high-resolution neuromonitoring and responsive neurostimulation. IEEE Trans Biomed Circuits Syst. 2015 Feb;9(1):34-49.

204. Stienen PJ, Venzi M, Poppendieck W, Hoffmann KP, Aberg E. Precaution for volume conduction in rodent cortical electroencephalography using high-density polyimide-based microelectrode arrays on the skull. J Neurophysiol. 2016 Apr;115(4):1970-7.

205. Britton JW, Frey LC, Hopp JL, et al. The Normal EEG. . In: St. Louis EK, LC F, editors. Electroencephalography (EEG): An Introductory Text and Atlas of Normal and Abnormal Findings in Adults, Children, and Infants American Epilepsy Society; 2016.

206. Michalakis M, Holsinger D, Ikeda-Douglas C, et al. Development of spontaneous seizures over extended electrical kindling. I. Electrographic, behavioral, and transfer kindling correlates. Brain Res. 1998 May 18;793(1-2):197-211.

207. Singh NA, Otto JF, Dahle EJ, et al. Mouse models of human KCNQ2 and KCNQ3 mutations for benign familial neonatal convulsions show seizures and neuronal plasticity without synaptic reorganization. J Physiol. 2008 Jul 15;586(14):3405-23.

208. Gohma H, Kuramoto T, Matalon R, et al. Absence-like and tonic seizures in aspartoacylase/attractin double-mutant mice. Exp Anim. 2007 Apr;56(2):161-5.

209. Cox GA, Lutz CM, Yang CL, et al. Sodium/hydrogen exchanger gene defect in slow-wave epilepsy mutant mice. Cell. 1997 Oct 3;91(1):139-48.

210. Fletcher CF, Lutz CM, O'Sullivan TN, et al. Absence epilepsy in tottering mutant mice is associated with calcium channel defects. Cell. 1996 Nov 15;87(4):607-17.

211. Letts VA, Felix R, Biddlecome GH, et al. The mouse stargazer gene encodes a neuronal Ca2+-channel gamma subunit. Nat Genet. 1998 Aug;19(4):340-7.

212. Barclay J, Balaguero N, Mione M, et al. Ducky mouse phenotype of epilepsy and ataxia is associated with mutations in the Cacna2d2 gene and decreased calcium channel current in cerebellar Purkinje cells. J Neurosci. 2001 Aug 15;21(16):6095-104.

213. Coenen AM, Van Luijtelaar EL. Genetic animal models for absence epilepsy: a review of the WAG/Rij strain of rats. Behav Genet. 2003 Nov;33(6):635-55.

214. Burgess DL, Jones JM, Meisler MH, Noebels JL. Mutation of the Ca2+ channel beta subunit gene Cchb4 is associated with ataxia and seizures in the lethargic (lh) mouse. Cell. 1997 Feb 7;88(3):385-92.

215. Letts VA, Beyer BJ, Frankel WN. Hidden in plain sight: spike-wave discharges in mouse inbred strains. Genes Brain Behav. 2014 Jul;13(6):519-26.

216. Strohl KP, Gallaugher L, Lynn A, et al. Sleep-related epilepsy in the A/J mouse. Sleep. 2007 Feb;30(2):169-76.

217. Tassinari CA, Cantalupo G, Rios-Pohl L, Giustina ED, Rubboli G. Encephalopathy with status epilepticus during slow sleep: "the Penelope syndrome". Epilepsia. 2009 Aug;50 Suppl 7:4-8.

218. Hoffmann-Riem M, Diener W, Benninger C, et al. Nonconvulsive status epilepticus--a possible cause of mental retardation in patients with Lennox-Gastaut syndrome. Neuropediatrics. 2000 Aug;31(4):169-74.

219. Dobbing J, Sands J. Comparative aspects of the brain growth spurt. Early Hum Dev. 1979 Mar;3(1):79-83.

220. Gottlieb A, Keydar I, Epstein HT. Rodent brain growth stages: an analytical review. Biol Neonate. 1977;32(3-4):166-76.

221. Clancy B, Finlay BL, Darlington RB, Anand KJ. Extrapolating brain development from experimental species to humans. Neurotoxicology. 2007 Sep;28(5):931-7.

222. Dobbing J, Sands J. Quantitative growth and development of human brain. Arch Dis Child. 1973 Oct;48(10):757-67.

223. Markand ON. Lennox-Gastaut syndrome (childhood epileptic encephalopathy). J Clin Neurophysiol. 2003 Nov-Dec;20(6):426-41.

224. Blume WT, David RB, Gomez MR. Generalized sharp and slow wave complexes. Associated clinical features and long-term follow-up. Brain. 1973 Jun;96(2):289-306.

225. Amir N, Shalev RS, Steinberg A. Sleep patterns in the Lennox-Gastaut syndrome. Neurology. 1986 Sep;36(9):1224-6.

226. Nakashima M, Kouga T, Lourenco CM, et al. De novo DNM1 mutations in two cases of epileptic encephalopathy. Epilepsia. 2016 Jan;57(1):e18-23.

227. Asinof SK, Sukoff Rizzo SJ, Buckley AR, et al. Independent Neuronal Origin of Seizures and Behavioral Comorbidities in an Animal Model of a Severe Childhood Genetic Epileptic Encephalopathy. PLoS Genet. 2015 Jun;11(6):e1005347.

228. Asinof S, Mahaffey C, Beyer B, Frankel WN, Boumil R. Dynamin 1 isoform roles in a mouse model of severe childhood epileptic encephalopathy. Neurobiol Dis. 2016 Nov;95:1-11.

229. Noh GJ, Jane Tavyev Asher Y, Graham JM, Jr. Clinical review of genetic epileptic encephalopathies. Eur J Med Genet. 2012 May;55(5):281-98.

230. Fiumara A, Pittala A, Cocuzza M, Sorge G. Epilepsy in patients with Angelman syndrome. Ital J Pediatr. 2010 Apr 16;36:31.

231. Valente KD, Koiffmann CP, Fridman C, et al. Epilepsy in patients with angelman syndrome caused by deletion of the chromosome 15q11-13. Arch Neurol. 2006 Jan;63(1):122-8.

232. Galvan-Manso M, Campistol J, Conill J, Sanmarti FX. Analysis of the characteristics of epilepsy in 37 patients with the molecular diagnosis of Angelman syndrome. Epileptic Disord. 2005 Mar;7(1):19-25.

233. Buiting K, Williams C, Horsthemke B. Angelman syndrome - insights into a rare neurogenetic disorder. Nat Rev Neurol. 2016 Oct;12(10):584-93.

234. Knoll JH, Nicholls RD, Magenis RE, Graham JM, Jr., Lalande M, Latt SA. Angelman and Prader-Willi syndromes share a common chromosome 15 deletion but differ in parental origin of the deletion. Am J Med Genet. 1989 Feb;32(2):285-90.

235. Jiang Y, Lev-Lehman E, Bressler J, Tsai TF, Beaudet AL. Genetics of Angelman syndrome. Am J Hum Genet. 1999 Jul;65(1):1-6.

236. Kuwano A, Mutirangura A, Dittrich B, et al. Molecular dissection of the Prader-Willi/Angelman syndrome region (15q11-13) by YAC cloning and FISH analysis. Hum Mol Genet. 1992 Sep;1(6):417-25.

237. Christian SL, Robinson WP, Huang B, et al. Molecular characterization of two proximal deletion breakpoint regions in both Prader-Willi and Angelman syndrome patients. Am J Hum Genet. 1995 Jul;57(1):40-8.

238. Malcolm S, Clayton-Smith J, Nichols M, et al. Uniparental paternal disomy in Angelman's syndrome. Lancet. 1991 Mar 23;337(8743):694-7.

239. Buiting K, Saitoh S, Gross S, et al. Inherited microdeletions in the Angelman and Prader-Willi syndromes define an imprinting centre on human chromosome 15. Nat Genet. 1995 Apr;9(4):395-400.

240. Homanics GE, DeLorey TM, Firestone LL, et al. Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. Proc Natl Acad Sci U S A. 1997 Apr 15;94(8):4143-8.

241. Tsai CH, Taylor M, Siegel-Bartelt J. Early clinical features of Angelman Syndrome in infants with chromosomal deletion of 15g11-q13. Genet Med. 2000;2(1):89-.

242. DeLorey TM, Handforth A, Anagnostaras SG, et al. Mice lacking the beta3 subunit of the GABAA receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome. J Neurosci. 1998 Oct 15;18(20):8505-14.

243. Janve VS, Hernandez CC, Verdier KM, Hu N, Macdonald RL. Epileptic encephalopathy de novo GABRB mutations impair GABA receptor function. Ann Neurol. 2016 Mar 7.

CHAPTER V: Conclusions and Future Directions

5.1 Summary of experimental chapters

Epilepsy is one of the most common neurological conditions in pediatric and adult populations with tremendous social and economic cost [1,2]. The pediatric population is at a greater risk for severe brain damage as the seizures may interfere with normal brain development. This is especially true for EEs in which the ongoing seizures are thought to result in progressive cognitive and behavioral impairment [3–5]. In this dissertation, I investigated the influence of five EE-associated *GABRB* mutations on $GABA_A$ receptor functions in a heterologous cell line and a knock-in mouse model (*Gabrb3*$^{+/D120N}$) that harbors one of the EE-associated mutations.

In **Chapters II and III** I describe the effects of five mutations identified in a large-scale study that investigated the genetic contributions of EE in two well defined syndromes- infantile spasms (IS) and Lennox-Gastaut syndrome (LGS) [6]. Four of the five mutations (*GABRB3(N110D, D120N, E108G, Y302C)*) were identified in the gene encoding the β3 subunit of $GABA_A$ receptors (*GABRB3*) that was previously associated with childhood absence epilepsy, a milder form of epilepsy. While a F246S mutation in *GABRB1* was not previously associated with epilepsy. Our functional studies clearly demonstrate that all five mutations resulted in a clear loss of $GABA_A$ receptor function (Table 5.1). This work was published in the Annals of Neurology [7].

In **Chapter IV** I examined the EEGs of *Gabrb3*$^{+/D120N}$ mice that harbor the LGS-associated D120N mutation and observed that *Gabrb3*$^{+/D120N}$ mice have myoclonic jerks that peak at the beginning of the 2nd post-natal week and disappear after about 5 days, which is followed by seizure progression. The adult *Gabrb3*$^{+/D120N}$ mice display many features of LGS, namely spontaneous atypical absence seizures (~400/day), myoclonic seizures, GTCS, and tonic seizures. Additionally, the *Gabrb3*$^{+/D120N}$ mice have sleep disturbances and behavioral abnormalities, comorbidities that are common in LGS patients. Thus, we believe these mice will be useful for examining the pathophysiological mechanisms of LGS and for testing the efficacy of AEDs. This work has been submitted for peer review.

Corroboration of *in vitro* findings in a mouse model, though not always possible, remains an important and interesting scientific endeavor. Several findings from the *in vitro* studies of the mutant β3(D120N) subunits presented in Chapter III were also found in the *Gabrb3*$^{+/D120N}$ mice. Here I briefly describe these findings that were determined by several members of our laboratory.

First, mIPSC amplitudes were significantly reduced in layer V/VI pyramidal neurons of the somatosensory cortex of *Gabrb3*$^{+/D120N}$ mice. The frequency of mIPSCs was unaltered indicating postsynaptic loss of $GABA_A$ receptor currents as the primary contributor of reduced inhibition, which can generate hyperexcitable networks that in turn lead to seizures and epilepsy. These results are concordant with the significant reduction (63.5% of wt) of whole cell currents from HEK293T cells transfected in the heterozygous condition (α1, β3, β3(D120N) and γ2 subunits). Second, there was no

significant reduction in expression of $GABA_A$ receptor α1, β3, and γ2 subunits in cortex, hippocampus, thalamus, and cerebellum of *Gabrb3*$^{+/D120N}$ mice, additionally indicating that mutant β3(D120N) subunits do not interfere with production, assembly, trafficking, or surface expression of $GABA_A$ receptors. Furthermore, postsynaptic expression of $GABA_A$ receptors (in synaptosomes) was unaltered. Thus, surface expression of both wt and mutant receptors have equal likelihoods, and loss of GABA-evoked currents from the mutant receptors reduced GABAergic inhibition.

5.2 Scope of future directions

Now that we have demonstrated that the *Gabrb3*$^{+/D120N}$ mice are a useful model of LGS pathogenesis, the next phase of research awaits exploration of interesting and important questions, some of which are discussed below.

5.2.1 What is the age of seizure onset and developmental progression of seizures?

In Chapter IV I presented the data from visual observations of P6-P18 pups from *Gabrb3*$^{+/D120N}$ mice and wt mice. The peak incidence of spasm-like seizures in *Gabrb3*$^{+/D120N}$ pups was from P14-P17. This is comparable to age of peak seizure onset (3-5 years) in children with LGS (range 1-8 years) [8–11]. Another suspected seizure type at this age was absence-like seizures; some pups were staring and unresponsive to loud noises for short durations. However, absence seizures cannot be determined with certainty solely by visual observations in mice especially in this age

range; seizures can be missed due to their brief duration or a pause in movement could be just a resting period. Furthermore, absence seizures can progress to other seizure types such as myoclonic and tonic seizures in children [12]. Thus, without EEG/ECoG monitoring it is hard to determine whether absence-like seizures preceded the motor spasm-like seizures and could not be noticed during the brief observations made in young mice (P6-P18) or followed the spasm-like seizures.

The developmental progression of seizures varies in LGS. The core seizure types in LGS include tonic, atonic, and atypical absence seizures (additional types are myoclonic seizures and GTCS). However, these seizures and the characteristic EEG pattern of 1-2.5 Hz slow SWDs may not appear at disease onset. Further, the developmental regression and cognitive impairment also varies. The variability in clinical presentation makes the diagnosis difficult and often late when the patient has already progressed well into the disease. Additionally, the seizure types that first appear depend on the maturation stage of the brain and whether or not LGS evolved from other epilepsy syndromes. For example, about 70-80% of children with LGS have a preceding diagnosis of other epilepsy syndromes [13–15], about 30-40% of which evolve from West syndrome [16,17,18 19]. When LGS develops with no prior recognizable cause, it begins commonly with episodes of atonic (drop attacks) seizures and atypical absence seizures that are usually followed by tonic seizures (typically nocturnal) and other seizure types [20]. Given the wide range of seizure types and the age of seizure onset in LGS patients, it is hard to predict the seizure types that may follow/precede the motor spasms seen in P14-P17 *Gabrb3*$^{+/D120N}$ mice. Additionally, since typically developing

rodent pups often have sudden abrupt movements that can be mistaken for spasm-like seizures, EEG analysis is required to ascertain the presence and the age of onset of seizures in young mice. Hence, at least intermittent EEG monitoring is required from one week of age (roughly correspond to human infants) to adulthood (≥8 weeks).

The challenge, however, is that the skull in mouse pups is paper thin and small; it cannot support implantation of the widely used headmounts for adult mouse EEG recordings. Special flexible silicone electrodes or miniaturized EEG radio telemetry transponders that can mount on the skull without the need of the conducting screws have been in recent use for this purpose [12,21,22] but were cost prohibitive for our experiments (+$20K for an initial set up). As an alternative, I custom made intra-cortical electrodes penetrating all layers of the cortex from thin bare silver wires soldered to thin gold pins and recorded the EEG signal using the human EEG acquisition system (Figure 1). This basic setup is useful to record SWDs during quiet rest from pups as young as P13 (total of 6 pups were successfully recorded from P13-P16 but were from different genetic background than *Gabrb3*$^{+/D120N}$ mice). Surprisingly, when the pups were reintroduced to their home cage after surgery, they were immediately accepted by their dam, were active and interacted with other pups in the cage, and they remarkably continued to nurse for up to two days even with the implanted electrodes (not monitored further). Recordings were obtained only for an hour shortly after surgery as prolonged recordings could dehydrate pups. This setup has two major drawbacks - electrical noise and poor video resolution. Even subtle movements produce noise and thus are not ideal for prolonged (~4 hours) recording from pups. Moreover, the camera cannot

be set up close to the pups, and the resolution of video recording is poor. The ECoG and video are time stamped but are tedious to watch simultaneously. Even with these drawbacks, this set-up can be used to get glimpses of seizures in young pups at minimal cost (~$20 of supplies for ~8-10 pups), which can be supplemented later with more sophisticated probes. Based on my experience with the *Gabrb3*$^{+/D120N}$ mice, monitoring mice on P10, P14, P20, P30, and +P60 is likely to capture the progression of major seizure types. Although these recordings will require large number of animals and tremendous effort analyzing the data, they will answer important questions: (1) what is the age of seizure onset, (2) what types of seizures occur at onset and how do they progress, and (3) what are baseline ECoGs prior to and following seizure onset as little is known about EEG patterns in LGS patients before the diagnosis of epilepsy.

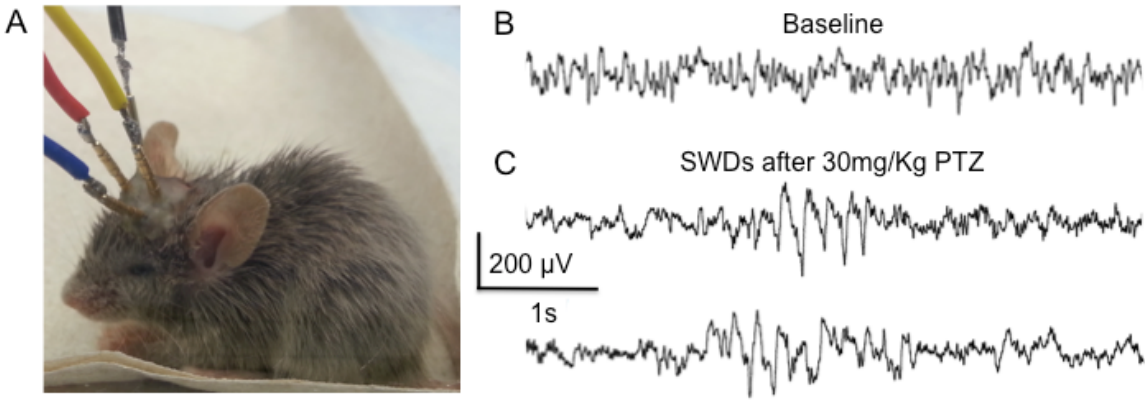

Figure 5.1: Synchronized video and intra-cortical recording from a wild type P15 pup.

(A) Image shows a custom made intra-cortical recording setup on a P15 pup. Thin sliver wire electrodes (penetrating all layers of the cortex) were soldered on gold connector pins and secured using dental cement on the skull. The gold pins on the head were connected to another set of gold pins that were soldered onto cables connecting the amplifier. Stable baseline recording during quite rest **(B)** and spike-and-wave discharges (SWDs) (C) after intraperitoneal injection of 30 mg/Kg of the convulsant drug pentylenetetrazol (PTZ) that blocks $GABA_A$ receptors.

5.2.2 Could treatment in the early postnatal period prevent or reduce seizures in adult *Gabrb3*$^{+/D120N}$ mice?

Developmental and cognitive outcomes for most LGS patients are poor once the disease progresses (often diagnosis comes well into the disease). Thus, studies evaluating the effects of the early treatment of LGS are rare, especially when LGS does not progress from other epilepsy syndromes [20]. As there are no animal models of LGS, the effectiveness of early intervention remains an open question. A fraction (~40%) of children with the severe epileptic encephalopathy, infantile spasms (IS), who received early treatment with existing therapies prevented progression of spasms later in life and had better developmental outcomes [23,24]. Similarly, mouse models of IS show that early treatment prior to and during early stages of seizure onset reduced seizure frequency in adults [25]. Although, IS and LGS have quite different ages of onset and seizure types, it is valuable to determine whether the promising results of early interventions prior to or shortly after seizure onset also hold true in *Gabrb3*$^{+/D120N}$ mice. Since children are not given antiseizure medication until at least one seizure is observed, the ideal treatment regime for *Gabrb3*$^{+/D120N}$ pups should begin at or shortly after seizure onset. However, in this pre-clinical model we can begin treatment shortly after birth that can also aid in determining if there is a critical treatment window. One of the caveats here is that the LGS patients have pharmacoresistant seizures and are on multiple anti-epileptic drugs [26]. Thus, one of the expected outcomes is that early treatment of *Gabrb3*$^{+/D120N}$ pups may not reduce seizures in pups and/or adult mice. Nonetheless, these experiments are worth pursuing.

5.2.3 Does reducing the slow background activity rescue cognitive deficits?

If seizures can be rescued in *Gabrb3*$^{+/D120N}$ pups, another important question can be asked – can early treatment reduce or eliminate cognitive decline? Cognitive and psychiatric comorbidities are well documented in almost all forms of epilepsy [27,28,29]. This is especially true for EEs, including LGS, where seizures and interictal activity are considered the primary drivers of cognitive decline that begins shortly after seizure onset [30,31,32]. Implied in this notion is that reducing or preventing seizures should rescue the cognitive decline. Indeed, children with late onset LGS (>8 years) have much better developmental and cognitive outcomes prior to seizure onset [33,34]. Additionally, LGS patients that show reduction in seizure frequency after surgical interventions also show cognitive improvements [35,36,37,38,39–41,42], but these benefits become less prominent as the time interval between seizure onset and surgery increases [43,44], a trend found in other EEs [45–48]. Consequently, there appears to be a critical window during which the developing brain is both susceptible to developing seizures and to recovering from seizures. Treatment during this window may offer the most benefit for reversing cognitive and development decline. Cognitive abilities in adult *Gabrb3*$^{+/D120N}$ mice that show seizure control can be tested using commonly used methods including Barnes maze and novel object recognition tests.

5.2.4 Which brain regions should be prioritized to examine neuronal networks engaged in generalized seizures of *Gabrb3*$^{+/D120N}$ mice?

The *Gabrb3*$^{+/D120N}$ mice can assist in developing significant insights into the underlying neural networks that are recruited and possibly altered in LGS as mouse models of this syndrome have not been reported. Being a rare disorder, only a handful of human studies have examined the brain regions activated during resting states and seizures in LGS patients. Further, these studies often have low sample size (often ≤15-20), which in part may be due to difficulty in recruiting children who frequently have cognitive disabilities, thus limiting the ability to conduct studies in MRI scanners and/or with scalp EEGs. Even though LGS patients display a range of etiologies with and without structural brain abnormalities, remarkably patients display fairly uniform types of generalized seizures. Thus, common neuronal networks are likely recruited during LGS seizures, while the origin of abnormal neural activity may differ depending on the etiology. Although there is considerable debate about the cortical versus thalamic origins of generalized seizures, the following discussion explores identifying key brain regions involved in LGS pathology that can serve as starting points for pre-clinical studies in animal models and does not focus on the seizure initiating region(s) per se.

Cortex: Cortical involvement in generalized seizures in LGS patients is evident from EEG recordings. Studies from the 1960s and 70s showed prominent SSWs in the frontal and frontocentral areas, and in some patients in the occipital regions [14,49]. Recent functional neuroimaging studies reveal that both cortical and subcortical regions

are active during generalized seizures in LGS patients. Positron emission tomography (PET) imaging studies measure glucose uptake as an indirect marker of neuronal activity. PET studies in LGS patients show focal (that correlates with the structural brain abnormalities) or diffuse glucose hypometabolism [50–54], glucose hypermetabolism [50,55] or normal [56] glucose metabolism predominantly in the frontal and temporal lobes, and infrequently in parietal lobes [56]. Simultaneous EEG and functional magnetic

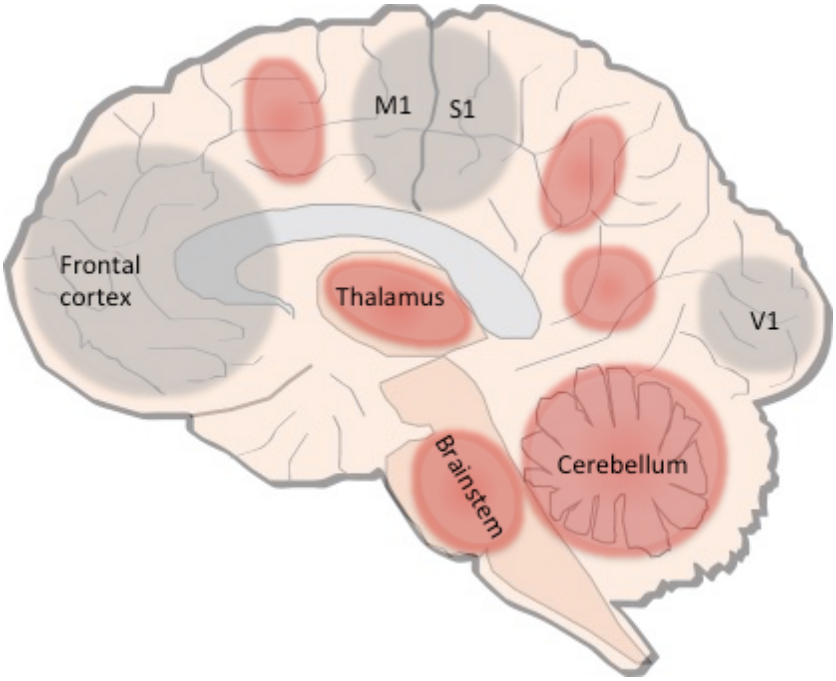

Figure 5.2: Schematic showing brain regions engaged in seizures and interictal epileptiform discharges in LGS patients.
Based on PET and EEG-fMRI studies, the red bubbles show regions that are activated during seizures including motor, somatosensory, and visual association cortices, thalamus, brainstem, and the cerebellum. The grey bubbles highlight regions that show hypoactivity, and include prefrontal cortex, and primary motor (M1), somatosensory (S1), and visual (V1) association cortices.

resonance imaging (fMRI) in LGS patients allows visualization of brain regions active during seizures and interictal events by measuring changes in blood oxygen level dependent (BOLD) response. Both PET [39,57,58] and EEG-fMRI [48] studies in LGS patients show abnormal hypoactivity to be largely confined to association cortices in the frontal, temporal, and parietal lobes, typically sparing the primary sensory areas (primary visual and motor cortices), and hyperactivity in the thalamus in the resting state and during seizures. In addition, Archer and colleagues found that interictal slow-spike-waves (1-2.5 Hz) and generalized paroxysmal fast activity (10-15 Hz) reduced BOLD signal in the primary cortical regions and increased signal in the thalamus, while the paroxysmal fast activity increased BOLD signal in the association cortices, thalamus, brainstem, and basal ganglia, irrespective of the presence or absence of underlying epileptogenic lesions [59,60]. The authors further proposed that the epileptic seizures in LGS patients arise from amplification of neuronal activity due to unusual co-activation of the two inversely related networks with opposing cognitive functions, the attention/executive-control and default mode networks. These findings corroborate numerous previous findings that normal brain functions and generalized seizures both engage thalamocortical circuits [32]. Thus, cortical structure, function, and networks should be further explored in *Gabrb3*$^{+/D120N}$ mice to understand LGS pathogenesis and to potentially rescue seizures.

Thalamus: Some LGS patients have seizure remission or reduction after surgical resection of cortical lesions [37]. However, in some LGS patients epileptic discharges did not initiate from cortical lesions [60], and their surgical removal did not

reduce generalized seizures [61]. Thus, it was concluded that at least for these patients, lesions interact with the underlying epileptic networks and aid in generation of seizures, but by themselves do not trigger seizures. In support of this, recent studies with simultaneous EEG-functional magnetic resonance imaging (fMRI) studies have identified thalamus and brainstem as two key sub-cortical regions that are activated during seizures in LGS patients irrespective of the underlying etiology [59,60,62,63]. Involvement of thalamus is not surprising since the thalamocortical system has been shown previously to be involved in other generalized epilepsies including absence epilepsy and GTC seizures [39,57,58]. Reciprocal interactions between thalamus and cortex are critical in the generation of spike-wave discharges characteristic of generalized absence seizures and have been extensively studied [64–67]. However, there are no known genetic models that have spontaneous atypical absence seizures, characteristic seizures in LGS patients and the most frequent seizures in *Gabrb3*$^{+/D120N}$ mice. Of particular interest is the centromedian thalamic nucleus [63] that projects to cortical layers I (extensively) and III of the prefrontal and motor cortices and to nRT, basal ganglia, and hypothalamus [68,69]. Patients with strokes in this region perform poorly on cognitive tests [68], and its stimulation in patients with refractory epilepsies including LGS has been an effective treatment strategy, especially for treating atypical absence seizures [70,71,72]. Interestingly, Velasco and colleagues noted that in humans, atypical absence seizures do not originate in the centromedian nucleus, but rather they propagate through it [70,73–75,76]. They suggested that atypical absence seizures originate in the reticular thalamocortical systems, most likely in the upper mesencephalon. Additionally, a recent study showed enhanced connectivity between mediodorsal and

ventrolateral thalamic nuclei in LGS patients [63]. The mediodorsal nucleus is a higher order thalamic nucleus (receives major input from cortical layers V and VI, but does not receive sensory information) that extensively projects to prefrontal cortex and has roles in working memory [68,77]. The ventrolateral thalamic nucleus projects to the motor cortex and receives input from pallidum, cerebellum and red nucleus. The role of this nucleus in seizure propagation is unclear.

In the context of epilepsy, the ventral posterior nucleus that receives the somatosensory input has been studied extensively. However, roles of the centromedian, mediodorsal, and ventrolateral nuclei in seizure generation have been reported in a handful of studies. These three nuclei can synchronize cortical activity and imaging studies of LGS patients show their relevance during seizures and interictal events. Thus, the potential role of these thalamic nuclei in *Gabrb3*$^{+/D120N}$ mice can be further explored to gain insights about the pathways involved in seizure generation and propagation.

Brainstem: The brainstem has been repeatedly shown in animal models and human studies to be necessary and sufficient for tonic seizures [78–81]. For example stimulation of the pons produces tonic-like seizures in animals [82]. Furthermore, tonic seizures were seen in a hydranencephalic patient [74], were not prevented by corpus callosotomy [74], and were less responsive to resection of epileptogenic cortical regions [74].

Tonic seizures are seen in the vast majority of LGS patients and used as a

diagnostic criterion. Further, increased BOLD signal in the brainstem during tonic seizures was seen in LGS patients and in the majority of IS/West syndrome patients (some of which frequently progress to LGS), regardless of the underlying etiology, medications used or other confounds [83,84]. Often brainstem and thalamus were both activated in LGS patients, so it is not clear which of these structure influences the other. Whether cortical hyperexcitability activates the thalamus that in turn engages the brainstem or whether the brainstem controls the thalamic activation via the reticular thalamic nucleus (nRT) and in turn induces cortical hyperexcitability [74]. Nonetheless the brainstem seems to be important in LGS pathogenesis and at least cursory examination should be performed given that adult *Gabrb3*$^{+/D120N}$ mice rarely have tonic seizures.

5.2.5 Potential ways to examine cortical, thalamic, and brainstem dysfunction in *Gabrb3*$^{+/D120N}$ mice.

Observations from human studies can be applied in a number of ways to study of LGS pathophysiology in *Gabrb3*$^{+/D120N}$ mice. Three approaches are described here.

1. Cortical microstructure/organization: Brains of *Gabrb3*$^{+/D120N}$ mice look grossly normal in structure. However, fine morphological changes in cortical layers and other brain regions are easy to miss just by visual observation. In a study with 27 LGS patients who under went resective brain surgery, only high-resolution MRI revealed cerebral lesions in 85.2% patients [40]. Similarly, cortical abnormalities of the order of

100s of micrometers have been observed in mouse models after staining with layer specific markers [85,86] Cortical layers can be examined first using NeuN antibodies that label all mature neurons [87], followed by a few of the layer specific markers including RELN (neuropil of layer I), CUX1 (layers II-IV), BCL11B/CTIP2 (layers V and VI), FOXP2 (labels layer VI), CTGF (Layer VIb) [88,89]. The cortex should be evaluated more carefully looking for fine morphological changes that could only be visualized using cortical layer specific markers, as seen the autism model. Additionally, special attention should be paid to regions that have dominant expression of β3 subunits including nRT, hippocampus, cerebellum, and the olfactory bulb [90–93].

2. Interneurons: Changes in the number and/or localization of interneurons is a common finding in a number of neurological diseases and is especially relevant to epilepsy. Thus, the distribution and number of Interneurons should be determined. Among the numerous interneuronal types, a good starting point can be to determine changes in parvalbumin (PV), somatostatin (SOM), and 5-HT3 receptor-containing interneurons that have been well studied. In the context of $GABA_A$ receptor β3 subunits, the PV interneurons are of special interest as a recent study showed that knocking out β3 subunits from hippocampal CA1 pyramidal cells impaired inhibitory synaptic currents from PV-, but not from SOM-, containing interneurons [77]. Furthermore, the β3 subunits are among the first to be expressed during development. Loss of functional $GABA_A$ receptors during early critical developmental periods could affect migration and integration of interneurons in cortical circuits. If interneuron defects are observed in *Gabrb3*$^{+/D120N}$ mice, the mice can be crossed with reporter mice

expressing fluorescent proteins in specific interneuron pollutions (PV, SOM, etc) [77] to facilitate identification of specific interneuron populations for functional studies.

3. Circuit mapping: The two dominant seizure types in adult *Gabrb3*$^{+/D120N}$ mice are atypical absence seizures and myoclonic seizures. In humans, deep brain stimulation of the centromedian nucleus is used as a target for treatment of refractory seizures, and it has been reported to be especially effective for treating atypical absence seizures [70,71]. To understand key circuits involved in these generalized seizures, a commonly used approach of injecting recombinant viral vectors (such as AAV) expressing photoexcitable proteins (such as channel rhodopsin, halorhodopsin) and fluorescent reporter proteins can be utilized. One approach could be to stereotactically inject channelrhodopsin and GFP expressing AAV in the centromedian nucleus and use depth light probes for optogenetic thalamic stimulation.

Even though, myoclonic seizures are the most common seizures in juvenile myoclonic epilepsy (JME), brain regions involved in myoclonic seizures are not know [94]. A few studies concluded that there were structural and functional abnormalities in the frontal lobes, but this is not been a consistent finding in JME patients, especially without neuropsychological deficits [95,96]. Thus, at this point, EEG using multielectrode array [97,98] that covers large portions of the cortical surface can guide us to brain regions that have prominent activity during myoclonic seizures in adult *Gabrb3*$^{+/D120N}$ mice.

Mediodorsal and centromedian thalamic nuclei: Since stimulation of the centromedian thalamic nuclei is used as a therapeutic strategy to control atypical

absence seizures, electrical or optogenetic stimulation of this region can be used to determine if such effects can be reproduced in *Gabrb3*$^{+/D120N}$ mice, and whether the blocking of atypical absence seizures improves cognition. Further, unlike other thalamic nuclei that project to cortical layer IV, the centromedian thalamus projects widely to cortical layers I and III of prefrontal and motor cortices, and basal ganglia. Thus, if atypical absence seizures are indeed rescued by stimulating the centromedian thalamus, viral injections can be performed to express channelrhodopsin and fluorescent proteins to trace and stimulate its postsynaptic targets, and determine if excitatory synaptic inputs are altered. This in an understudied region in epilepsy and reveals previously unknown pathways involved with generation of atypical absence seizures.

The mediodorsal nucleus is a higher order thalamic nucleus that receives cortical information, has roles in learning and memory, and shows enhanced activation in LGS patients. Thus, it can be determined directly whether learning and memory deficits in *Gabrb3*$^{+/D120N}$ mice can be reduced by repetitive electrical or optogenetic stimulation of this region, and can be assessed using the Barnes maze test before and after stimulation. If there are learning and memory improvements, synaptic and circuit defects in the thalamocortical circuit could be further explored by measuring the excitatory and inhibitory postsynaptic currents in the cortex and the mediodorsal nucleus. One major hindrance for these experiments would be the difficulty to accurately target the small and adjacent thalamic nuclei in *Gabrb3*$^{+/D120N}$ mice.

4. Brainstem: Since the adult *Gabrb3*$^{+/D120N}$ mice rarely have tonic seizures, it is not

clear whether brainstem dysfunction is present in this region that express β3 subunits. Of particular interest is the raphe nucleus in the brainstem that contains serotonergic neurons. Serotonin metabolism defects have been reported in IS/West syndrome patients, however, both increased and decreased levels of serotonin have been reported [99–101], and serotonin depletion is used to induce IS in rodent models [102]. Thus, serotonin levels from brain homogenates can be determined in P14-P17 *Gabrb3*$^{+/D120N}$ pups using HPLC [103]. Further, brainstem activation has been reported in the majority of IS/West syndrome patients (that frequently progress to LGS) [83,84,76]. The dorsal and medial raphe nucleus projects extensively to prefrontal cortex, and medial prefrontal cortex in turn projects back onto GABAergic neurons of the raphe nucleus. While the caudal raphe nucleus projects to other brainstem nuclei. Thus, this circuit can be examined for structural and functional defects using recombinant viral vectors and optogenetic tools [104,105]. The raphe nucleus can be a starting point to determine whether the serotonergic system or other brainstem nuclei are involved in generation of spasm-like seizures in P14-P17 *Gabrb3*$^{+/D120N}$ pups.

5. Hippocampus: Involvement of the temporal cortex has been described in a few studies of LGS patients, but definitive activation of the hippocampus in atypical absence seizures or interictal epileptiform discharges has not been reported. This is likely not due to the inability to detect hippocampal activation; the detection limit of both structural and functional imaging studies are well above the size of the hippocampus as demonstrated by the ability to detect activation of sub-cortical structures of comparable size during seizures and ability to identify cortical regions for

surgical resection. In animal studies only Snead and colleagues have reported involvement of the hippocampus in the AY-9944 treated rat model of atypical absence seizures. As LGS patients show severe learning and memory deficits, the hippocampus could be engaged during atypical absence seizures. Furthermore, the hippocampus highly expresses β3 subunits, and thus, IPSCs in hippocampal pyramidal cells and interneurons should be explored further.

Next, I discuss advances and barriers in translating molecular genetic findings, as well some ways by which these can be overcome.

5.3 Advances in epilepsy: role of genetic testing

Establishing an epilepsy diagnosis is a complex, time consuming, and expensive endeavor, especially for patients with neurodevelopmental, motor, and cognitive delays. A full work up often includes ordering MRI, EEG, metabolic and biochemical tests, biopsies, and genetic tests. Prior to modern DNA sequencing technologies, genetic tests in the 1960-70s could identify large genomic changes such as chromosomal abnormalities via karyotyping (for example Down syndrome due to trisomy of chromosome 21[106]) and in the 1990s micro chromosomal deletions, duplications, inversion, and translocations could be identified via fluorescence in situ hybridization (FISH)[107]. This was followed by an era of Sanger sequencing that lasted for about 30 years [108], which could determine single nucleotide changes but was limited to sequencing single or few candidate genes, and is still used to verify mutations identified

by sequencing studies. Even though slow and cumbersome, Sanger sequencing resulted in identification of several epilepsy gene mutations. In 1995 a mutation in *CHRNA4* that encodes the alpha 4 subunit of the nicotinic acetylcholine receptor, was identified as the first epilepsy gene in a large family with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [72]. This was followed by identification of numerous single gene mutations in ion channel and non-ion channel encoding genes in multiplex families including those encoding $GABA_A$ receptor subunits in 2001 [109,110]. The next approaches were to identify causative variants that impart susceptibility to complex diseases using genome wide association studies (GWAS), but these were generally unsuccessful in epilepsy [111,112].

The unparalleled success in the past decade has been due to advent of next generation sequencing (NGS) technologies, which allows parallel sequencing of millions of gene fragments from the entire exome or genome as opposed to the traditional Sanger sequencing of single gene fragments [113]. This transformed the pace of discovery from a few genetic findings in isolated patients/families to a virtual explosion of genetic findings from hundreds of patients. The NGS platform became viable in research and clinical settings as they allowed sequencing of hundreds of patient genomes in a timely and cost effective manner. Sequencing the first human genome (~3 billion base pairs) took 10 years [114,115] and about $500 million to $1 billion [116], while today a single patient genome can be sequenced in 3-4 weeks for about $300 to $1000. Thus, using NGS technology new epilepsy (and most human disease) associated gene mutations are being discovered on a weekly basis and are anticipated to grow

exponentially. Several hundred genes have been implicated to cause epilepsy as the core disease, and additionally hundreds of genes are associated with neurological diseases that have seizures as a symptom [117]. Equal contributors to this revolution are the advances in computational methods that allow interpretation of the sequencing data such as calling variants from the patient genomes and comparing them to genomes of hundreds of unaffected individuals [118]. One of the most important outcomes of these technological advances in epilepsy has been the discovery that *de novo* mutations account for a substantial portion of non-inherited severe epilepsies and developmental disorders. Additionally, monogenic epilepsies documented prior to NGS were of rare occurrence (1-2% of GGEs); currently they comprise a significant proportion of GGEs.

Today, numerous genetic tests are accessible to patients that utilize the power of high-throughput NGS that include gene panels, whole exome sequencing (WES), or whole genome sequencing (WGS) to determine if the observed epilepsy phenotype results from gene mutations. Among these, gene panels are used to screen genes with known roles in epilepsy, previous association with epilepsy, and/or are relevant to the patient phenotype. Gene panels are relatively less expensive and typically have fast (3-4 week) turnover time with high sequencing depth. WES and WGS are most recent genetic diagnostic tests that allow examination of genetic changes at the level of single nucleotides in all human genes and offer an unbiased approach to determine which gene(s) can result in the epilepsy phenotype. WES/WGS has been successful in identifying epilepsy-causing mutations in patients with no prior family history, absence of abnormalities in imaging and biochemical tests, and in patients with multiple phenotypic

features that overlap with other neurological disorders. WGS is likely to capture all variations in genomic and non-exomic regions of mitochondrial DNA. However, these tests are more expensive than a gene panel and take about 8-12 weeks to complete. There has been a considerable debate regarding the utility of WGS as a stand-alone or first-choice diagnostic tests as the interpretation of identified variant(s) can be uncertain, and currently WES is cheaper and faster than WGS [119,120]. However, recently a study examined this concern by carrying out WGS in 105 patients from whom previous gene panel results resulted either in identification of causative gene(s) or did not identify any associated gene(s) [121]. The authors found that the diagnostic yield of WGS was significantly higher (41%) compared to combined yield of gene panels and WES (24%), and that WGS reduced the need for multiple genetic tests without increasing the testing cost. Thus, they provided a systematic comparison of gene panels and WEG/WGS and argue towards offering WGS as stand-alone and first-choice diagnostic tests. A number of recent reports favor this view as WGS offers over all time and cost-savings (reduces need of multiple sequential genetic tests), and a more complete view of genetic changes [122–124]. Furthermore, the cost and speed of NGS techniques is constantly improving; thus WGS is going to be mainstream genetic test in the coming years.

5.4 Translating genetic findings to epilepsy treatments: paths towards precision medicine.

We are not in the era of precision medicine yet, and therapies for most diseases offer sub-optimal control of symptoms, do not alter disease outcome and have adverse

effects. However, given the tremendous success in understanding genetic contribution of diseases and few successful treatment outcomes, we are at the cusp of the precision medicine era. Precision medicine generally means identifying treatments that meet needs of groups of patients based on their genetics, environment, lifestyle, etc. It is thought that precision medicine would eventually move us towards personalized medicine i.e. treatments specifically designed towards an individual. One of the first reports of epilepsy precision medicine includes repurposing quinidine, a drug used for the treatment of cardiac arrhythmias, for the treatment of epilepsy in patients with *KCNT* gain of function mutations identified vis WES [125–127]. In the first report the *KCNT1(R428Q)* mutation was identified in a patient with migrating partial seizures of infancy (MPSI), which typically has drug resistant seizures, poor developmental outcomes, and early mortality [126]. This identical mutation had been previously reported in three patients with MPSI [128]. *In vitro* examination showed that quinidine reversibly blocked mutant *KCNT1* potassium channels and partially restored potassium currents to wild-type levels [129]. Quinidine treatment in the MPSI patient showed marked improvements with seizures occurring only during periods of intercurrent illness and improved developmental outcomes [126], while nine traditional AEDs were ineffective. Following this remarkable recovery, quinidine treatment has been tried on two other patients with drug resistant severe childhood epilepsies and gain of function *KCNT1* mutations [127]. One of the patients had the *KCNT1(K629N)* mutation and epilepsy of infancy with migrating focal seizures (EIMFS), and the second patient had the *KCNT1(Y796H)* mutation and nocturnal seizures. Following quinidine treatment the patient with EIMFS showed 80% reduction in seizure frequency (8 AEDs and ketogenic

diet previously failed), while the patient with nocturnal seizures did not improve (12 AEDs and ketogenic diet previously failed). The reasons for this discrepancy are not clear. More perplexingly is the fact that the *in vitro* results are opposite to the clinical results. The *KCNT1(K629N)* mutation increased potassium currents more prominently than the *KCNT1(Y796H)* mutation, and concordantly quinidine only marginally restored currents from *KCNT1(K629N)* to wild type levels compared to *KCNT1(Y796H)* channels [127]. While we do not know the expression levels of *KCNT1* channels in both patients, they underwent WES and only *KCNT1* mutations were identified. The authors offer few reasons for the varying results including the plasma levels of quinidine and drug interactions, but limited data prohibit any conclusions. These mixed results urge more targeted therapies for KCNT1 epilepsies and cautions against early use of quinidine; nonetheless, if other treatments fail, trying quinidine seems worthwhile. Another successful report demonstrating utility of precision medicine in epilepsy is the use of memantine, a FDA-approved use-dependent NMDA receptor channel blocker, to treat a patient with EE and a gain-of-function *GRIN2A(L812M)* mutation. Prolonged memantine treatment significantly reduced seizure frequency in this child [130].

The discovery of monogenic epilepsies with highly penetrant and deleterious *de novo* mutations and few positive results has further fueled the optimism of treating individuals based on their genetic diagnosis. The futuristic proposal in this view also includes pharmacogenetics, i.e. examining drug response based genetic findings, specifically how a particular variant would alter drug responses, and pharmacogenomics i.e. how all gene variants on a given genetic background responds to drugs. However,

this would require precise understanding of mechanisms of drug action and how a specific genetic variation on a given genetic background would respond to it. Next, I will discuss a few barriers to reaching precision medicine objectives and some ways to overcome them.

5.5 Road blocks to precision medicine

Precision medicine therapies require a detailed picture of genetic epilepsy pathogenesis. Although, not necessary for all patients, understanding the contribution of mutated genes/proteins at the cellular and neuronal network levels holds the most promise for the targeted treatments. Primary factors that hinder clinical implementation of precision medicine approaches even after a definitive genetic diagnosis is achieved are discussed below. It should be also noted that with our current understanding of the mechanisms of epilepsy pathogenesis, precision medicine therapies cannot be applied to all genetic findings.

5.5.1 Data interpretation bottleneck: lack of actionable knowledge despite a wealth of information.

We live in times of unprecedented growth in genetic findings from patients and unaffected individuals. Hundreds of epilepsy mutations have been identified by NGS and new mutations are being discovered each day. Collectively this appears to be a flood of seemingly unrelated genetic information. The biggest impediment in epilepsy

genetics is interpretation and clustering of results from numerous studies in meaningful ways (by gene, function, disease, etc) [130]. A clinical diagnosis that identifies a gene mutation often has no actionable treatment modifications. In practice, information from clinical diagnosis, genetic analysis, functional validation, and results from model systems are determined by diverse individuals that often never communicate with each other or are aware of the results at each level. For precision medicine to be useful in everyday clinical practice, a centralized information hub that allows easy access to query genetic and functional data in different ways is essential. This massive and expensive bioinformatics endeavor would necessitate international multicenter collaborations. Online Mendelian Inheritance in Man (OMIM, https://www.omim.org/) is a public database that clusters published literature on a particular gene, its function, associated diseases, mutations, functional studies, and much more. This site is primarily designed for physicians, researchers, and experts who seek information pertaining to genetic disorders. Vanderbilt University's 'My Cancer Genome' (https://www.mycancergenome.org/) is an excellent example of a hub that provides current information on cancer mutations, their therapeutic implications, and associated clinical trials and is user-friendly for experts and non-experts. Such efforts are paving the way forward for precision medicine to be mainstream.

5.5.2 Functional validation

The rate of discovery of epilepsy genes has the pace of a cheetah, while that of understanding its functional impact is that of a snail. This is because the current

bioinformatics software functional predications are quite reliable in determining whether or not the protein function will be disrupted, but not how the protein function will change (gain or loss of function, and to what degree, unexpected results of the mutations, shift in molecular interactions, etc). This functional discovery bottleneck has tremendously decelerated our ability to measure the pathological impact of gene mutations, their contribution to disease phenotypes, and translation of these findings to clinical practice.

Several factors hinder translation of genetic findings to useful therapies. For example, numerous epilepsy mutations result in loss of function. Although, not always true, in general loss of function or haploinsufficiency of the native protein is hard to correct compared to gain of function. Developing subunit specific potentiators/activators is extremely difficult compared to a global blocker of gain-of-function mutant proteins and their subtypes. Further, non-ion channel epilepsy mutations are hard to incorporate in precision medicine and are not targets of traditional epilepsy therapies. *CDKL5, LGI1, PCDH19, STXBP1,TCF4*, etc are few in the expanding list of non-ion channel epilepsy associated genes [130]. An example of a non-ion channel gene targeted by precision medicine treatment includes that of Everolimus (inhibitor of mammalian target of rapamycin (mTOR)) in tuberous sclerosis [131] but with limited success; further examples may appear at a slow pace. In addition, not all mutations are in protein coding genes, and effects of mutations in non-coding RNA, introns, intron splice sites, and 3'/5' untranslated regions or CNVs are often hard or not feasible to study. Functional studies based on WGS may provide ways to test the

effects of mutations in non-protein coding genes and other genomic elements that constitute approximately 99% of the human genome.

Several approaches are currently being implemented or pursued to increase the pace of functional analysis of mutant proteins. High-throughput screening of compounds in heterologous cell lines and neurons expressing mutant proteins is most promising for ion channel mutations [132–134]. However, for outcomes to be reproducible, at least some of the results need to be confirmed by manual patch clamp, which is considered a gold standard technique. Besides, screening several hundreds of known drugs on multiple mutant proteins (http://www.drugbank.ca/stats) can increase the combination of drug-mutant protein testing by several fold, making the experimental screening exhaustive or in some instances unfeasible. Computational drug design and virtual screening may reduce burden of exhaustive screening efforts and novel drug target validation [135–137]. The lack of complexity of interconnected neurons and the inability to test off-target side effects limit the utility of these reductive cell culture based assays beyond the initial screening step. In contrast, rodent models of acquired and genetic epilepsies are low throughput but retain high complexity. Of course, rodent models for each mutation cannot be generated and screening neurotherapeutics in them is several hundred-fold slower. Also, off-target effects in animal models are often measured by simple metrics and behavioral readouts that may fail to discern the underlying deficits vs off-target effects. Further, since rodents are meticulously inbred to get rid of inter-animal variation, they cannot recapitulate differences in drug responses due to genetic variations in the same strain. Even with these caveats,

rodent models have tremendously advanced understanding the pathophysiology of epilepsies and still play a vital part in neurotherapeutic research.

Newly developed drug high throughput screening tools include patient-derived organoids [138] and induced pluripotent stem cells (iPSCs) [139,140,141], and zebrafish models of genetic epilepsy [142]. These model systems retain some complexity of the nervous system and provide testing off-target effects and toxicity at a pace that is more conducive with the current pace of epilepsy genetic data generation. Additionally, they can be used for precision medicine. However, they have their own caveats but can complement conventional rodent models with each providing insights where the other has limitations. Together, these models can deliver precision medicine therapies at a faster pace compared to traditional approaches.

5.5.3 Complexity of epilepsy genetics: lack of genotype-phenotype correlations.

The complexity of genetic architecture of epilepsy, though well recognized, has been exemplified by NGS studies. Efforts to develop precision medicine for epilepsy have been limited by the complexity of the underlying genotype–phenotype correlations. Prior to genetic findings from NGS, it was well known that the phenotypic expression and severity of symptoms even for monogenic epilepsies among family members depends on the dominant gene mutation(s) as well as the genetic background, modifier genes, life style, and environmental factors. For the nervous system this also depends on cell-cell interactions and network-level integration, especially during

development. Now, with numerous gene mutations identified in epilepsy patients by NGS, especially those without a family history, highlight that (1) mutations in the same gene can result in a spectrum of epilepsy syndromes (or even different neurodevelopmental diseases), and (2) that a particular epilepsy syndrome can occur by mutations in different genes [143,144].

Prior to the WES era *GABRB3* mutations were identified in families with relatively mild epilepsy, CAE. *GABRG2* and *GABRA1* mutations were associated with severe epilepsy syndromes, and *GABRB2* mutations were not reported. As *GABRB2* encodes the dominant β2 subunits of $GABA_A$ receptors that are ubiquitously expressed in the nervous system, it was not clear whether β2 subunits could compensate for dysfunctions resulting from *GABRB3* mutations. However, findings from the Epi4K consortium clearly demonstrated that *GABRB3* mutations can be associated with severe EE syndromes. Now several publications report the *GABRB3* mutations in a spectrum of epilepsy syndromes including IS/West syndromes, LGS, DS, myoclonic-astatic epilepsy, FS, GEFS+, and other EEs [6,145–147]. Similarly, the phenotypic spectrum of *GABRA1* [148,149,150] and *GABRG2* [151–153] has also expanded. Currently, other than the influence of modifier and susceptibility genes (genetic background), we do not have good models that explain how different mutations in the same gene result in such vastly different epilepsy syndromes. For example, in our laboratory we have generated $Gabrb3^{+/P11S}$, $Gabrb3^{+/N110D,}$ and $Gabrb3^{+/D120N}$ mice, which harbor mutations identified in patients with CAE, IS, and LGS. All three KI mice are on C57Bl/6 background with vastly different seizure semiologies and behavioral

comorbidities. Differences in $GABA_A$ receptor dysfunctions seen in *in vitro* and brain slices do not sufficiently explain such degree of phenotypic differences, and is quite perplexing. On the other hand, Dravet syndrome (DS) is an exemplar of monogenic epilepsies in which ~70-80% cases have a mutation in *SCN1A,* the majority of which are *de novo* missense mutations. Recently, *GABRA1* mutations have been reported in DS patients, and its genetic causes continuously expand with mutations in identified in *GABRG2*, *GABRA1, SCN2A, SCN8A, SCN9A, HCN1, STXBP1, PCDH19, HCN1, KCNA2,* and others. This genetic complexity in epilepsy makes it difficult to predict responses of precision medicine treatments.

Furthermore, we are just beginning to understand the natural variations in healthy individuals. Human genetic backgrounds are extremely diverse and more than 10 million SNPs have been identified in human genomes, some of which are nonsynonymous SNPs and predicted to be deleterious [138]. Thus, further research would reveal what regulates fitness vs disease susceptibility.

5.6 CONCLUSIONS

In conclusion, work presented in this thesis is a continuation of the efforts to understand the effects of genetic mutations in human epilepsies. Specifically, my research provided empirical evidence that the five *GABRB* mutations identified in EE significantly disrupted $GABA_A$ receptor function, thus confirming their role in EE pathology. Further, I examined the impact of one these mutations, *GABRB3(D120N)*, in a KI mouse model and found early seizure onset and adult seizure semiologies similar to that of LGS patients. In the

absence of genetic models of LGS, this is a significant advance in the field and a promising starting point to examine the complex role human epilepsy mutations play in epileptogenesis during and after development. We hope this, and other EE models, also serve as drug screening modalities for pharmacoresistant epilepsies, especially those that can be targeted during childhood.

5.7 REFERENCES

1. Begley, C. E. & Durgin, T. L. The direct cost of epilepsy in the United States: A systematic review of estimates. *Epilepsia* **56**, 1376–87 (2015).
2. Allers, K. *et al.* The economic impact of epilepsy: a systematic review. *BMC Neurol.* **15**, 245 (2015).
3. Engel, J. Report of the ILAE Classification Core Group. *Epilepsia* **47**, 1558–1568 (2006).
4. Engel, J. & International League Against Epilepsy (ILAE). A proposed diagnostic scheme for people with epileptic seizures and with epilepsy: report of the ILAE Task Force on Classification and Terminology. *Epilepsia* **42**, 796–803 (2001).
5. Berg, A. T. *et al.* Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. *Epilepsia* **51**, 676–85 (2010).
6. Allen, A. S. *et al.* De novo mutations in epileptic encephalopathies. *Nature* **501**, 217–221 (2013).
7. Janve, V. S., Hernandez, C. C., Verdier, K. M., Hu, N. & Macdonald, R. L. Epileptic encephalopathy de novo GABRB mutations impair γ-aminobutyric acid type A receptor function. *Ann. Neurol.* **79**, 806–825 (2016).
8. Arzimanoglou, A. *et al.* Lennox-Gastaut syndrome: a consensus approach on diagnosis, assessment, management, and trial methodology. *Lancet Neurol.* **8**, 82–93 (2009).
9. Markand, O. N. Slow spike-wave activity in EEG and associated clinical features: often called ‘Lennox’ or "Lennox-Gastaut’ syndrome. *Neurology* **27**, 746–57 (1977).
10. LENNOX, W. G. & DAVIS, J. P. CLINICAL CORRELATES OF THE FAST AND THE SLOW SPIKE-WAVE ELECTROENCEPHALOGRAM. *Pediatrics* **5**, (1950).
11. GASTAUT, H. *et al.* Childhood Epileptic Encephalopathy with Diffuse Slow Spike-Waves (otherwise known as “Petit Mal Variant”) or Lennox Syndrome. *Epilepsia* **7**, 139–179 (2010).
12. Gataullina, S. *et al.* Epilepsy in young *Tsc1* $^{+/-}$ mice exhibits age-dependent expression that mimics that of human tuberous sclerosis complex. *Epilepsia* **57**, 648–659 (2016).
13. Chevrie, J. J. & Aicardi, J. Childhood epileptic encephalopathy with slow spike-wave. A statistical study of 80 cases. *Epilepsia* **13**, 259–71 (1972).
14. Markand, O. N. Slow spike-wave activity in EEG and associated clinical features: often called ‘Lennox’ or "Lennox-Gastaut’ syndrome. *Neurology* **27**, 746–57 (1977).

15. Heiskala, H. Community-based study of Lennox-Gastaut syndrome. *Epilepsia* **38**, 526–31 (1997).

16. Rantala, H. & Putkonen, T. Occurrence, outcome, and prognostic factors of infantile spasms and Lennox-Gastaut syndrome. *Epilepsia* **40**, 286–9 (1999).

17. Ohtahara, S., Yamatogi, Y. & Ohtsuka, Y. Prognosis of the Lennox syndrome-long-term clinical and electroencephalographic follow-up study, especially with special reference to relationship with the West syndrome. *Folia Psychiatr. Neurol. Jpn.* **30**, 275–87 (1976).

18. Trevathan, E., Murphy, C. C. & Yeargin-Allsopp, M. The descriptive epidemiology of infantile spasms among Atlanta children. *Epilepsia* **40**, 748–51 (1999).

19. You, S. J., Kim, H. D. & Kang, H.-C. Factors Influencing the Evolution of West Syndrome to Lennox-Gastaut Syndrome. *Pediatr. Neurol.* **41**, 111–113 (2009).

20. Resnick, T. & Sheth, R. D. Early Diagnosis and Treatment of Lennox-Gastaut Syndrome. doi:10.1177/0883073817714394

21. Chemaly, N., Nehlig, A., Chiron, C. & Nabbout, R. Electrocorticographic telemetric recording in unrestrained mouse pups. *J. Neurosci. Methods* **305**, 17–27 (2018).

22. Pirone, A. *et al.* APC conditional knock-out mouse is a model of infantile spasms with elevated neuronal β-catenin levels, neonatal spasms, and chronic seizures. *Neurobiol. Dis.* **98**, 149–157 (2017).

23. SOREL, L. & DUSAUCY-BAULOYE, A. [Findings in 21 cases of Gibbs' hypsarrhythmia; spectacular effectiveness of ACTH]. *Acta Neurol. Psychiatr. Belg.* **58**, 130–41 (1958).

24. Riikonen, R. A Long-term Follow-up Study of 214 Children with the Syndrome of Infantile Spasms. *Neuropediatrics* **13**, 14–23 (1982).

25. Olivetti, P. R., Maheshwari, A. & Noebels, J. L. Neonatal estradiol stimulation prevents epilepsy in arx model of X-linked infantile spasms syndrome. *Sci. Transl. Med.* **6**, 220ra12-220ra12 (2014).

26. Parisi, P. *et al.* Epileptic encephalopathy of infancy and childhood: electro-clinical pictures and recent understandings. *Curr. Neuropharmacol.* **8**, 409–21 (2010).

27. Boro, A. & Haut, S. Medical comorbidities in the treatment of epilepsy. *Epilepsy Behav.* **4 Suppl 2**, S2-12 (2003).

28. Berg, A. T., Caplan, R. & Hesdorffer, D. C. Psychiatric and neurodevelopmental disorders in childhood-onset epilepsy. *Epilepsy Behav.* **20**, 550–5 (2011).

29. Jalava, M. & Sillanpää, M. Concurrent illnesses in adults with childhood-onset epilepsy: a population-based 35-year follow-up study. *Epilepsia* **37**, 1155–63 (1996).

30. Sforza, E., Mahdi, R., Roche, F., Maeder, M. & Foletti, G. Nocturnal interictal

epileptic discharges in adult Lennox-Gastaut syndrome: the effect of sleep stage and time of night. *Epileptic Disord.* **18**, 44–50 (2016).

31. Parisi, P. *et al.* 'Epileptic encephalopathy' of infancy and childhood: electro-clinical pictures and recent understandings. *Curr. Neuropharmacol.* **8**, 409–21 (2010).

32. Aldenkamp, A. P. Effect of seizures and epileptiform discharges on cognitive function. *Epilepsia* **38 Suppl 1**, S52-5 (1997).

33. Aicardi, J. & Levy Gomes, A. Clinical and electroencephalographic symptomatology of the 'genuine' Lennox-Gastaut syndrome and its differentiation from other forms of epilepsy of early childhood. *Epilepsy Res. Suppl.* **6**, 185–93 (1992).

34. Goldsmith, I. L., Zupanc, M. L. & Buchhalter, J. R. Long-term seizure outcome in 74 patients with Lennox-Gastaut syndrome: effects of incorporating MRI head imaging in defining the cryptogenic subgroup. *Epilepsia* **41**, 395–9 (2000).

35. Freeman, J. L. *et al.* Generalized epilepsy in hypothalamic hamartoma: evolution and postoperative resolution. *Neurology* **60**, 762–7 (2003).

36. Quarato, P. P., Di Gennaro, G., Manfredi, M. & Esposito, V. Atypical Lennox-Gastaut syndrome successfully treated with removal of a parietal dysembryoplastic tumour. *Seizure* **11**, 325–9 (2002).

37. Angelini, L., Broggi, G., Riva, D. & Lazzaro Solero, C. A case of Lennox-Gastaut syndrome successfully treated by removal of a parietotemporal astrocytoma. *Epilepsia* **20**, 665–9 (1979).

38. Liu, S. *et al.* Pediatric intractable epilepsy syndromes: reason for early surgical intervention. *Brain Dev.* **29**, 69–78 (2007).

39. You, S. J., Lee, J.-K. & Ko, T.-S. Epilepsy surgery in a patient with Lennox-Gastaut syndrome and cortical dysplasia. *Brain Dev.* **29**, 167–70 (2007).

40. Lee, Y. J. *et al.* Resective Pediatric Epilepsy Surgery in Lennox-Gastaut Syndrome. *Pediatrics* **125**, e58–e66 (2010).

41. Lee, Y.-J. *et al.* Outcomes of epilepsy surgery in childhood-onset epileptic encephalopathy. *Brain Dev.* **36**, 496–504 (2014).

42. Lee, Y. J. *et al.* Resective Pediatric Epilepsy Surgery in Lennox-Gastaut Syndrome. *Pediatrics* **125**, e58–e66 (2010).

43. Liu, S.-Y. *et al.* Surgical treatment of patients with Lennox-Gastaut syndrome phenotype. *ScientificWorldJournal.* **2012**, 614263 (2012).

44. Bladin, P. F. Adult Lennox Gastaut syndrome: patients with large focal structural lesions. *Clin. Exp. Neurol.* **21**, 105–14 (1985).

45. Loddenkemper, T. *et al.* Developmental Outcome After Epilepsy Surgery in Infancy. *Pediatrics* **119**, 930–935 (2007).

46. Freitag, H. & Tuxhorn, I. Cognitive function in preschool children after epilepsy surgery: rationale for early intervention. *Epilepsia* **46**, 561–7 (2005).

47. Bourgeois, M. *et al.* Surgery of epilepsy associated with focal lesions in childhood. *J. Neurosurg.* **90**, 833–842 (1999).

48. Asarnow, R. F. *et al.* Developmental outcomes in children receiving resection surgery for medically intractable infantile spasms. *Dev. Med. Child Neurol.* **39**, 430–40 (1997).

49. GIBBS, F. A., GIBBS, E. L. & LENNOX, W. G. INFLUENCE OF THE BLOOD SUGAR LEVEL ON THE WAVE AND SPIKE FORMATION IN PETIT MAL EPILEPSY. *Arch. Neurol. Psychiatry* **41**, 1111 (1939).

50. Theodore, W. H. *et al.* Cerebral glucose metabolism in the Lennox-Gastaut syndrome. *Ann. Neurol.* **21**, 14–21 (1987).

51. Yanai, K. *et al.* Cerebral glucose utilization in pediatric neurological disorders determined by positron emission tomography. *Eur. J. Nucl. Med.* **13**, 292–6 (1987).

52. Iinuma, K. *et al.* Cerebral glucose metabolism in five patients with Lennox-Gastaut syndrome. *Pediatr. Neurol.* **3**, 12–8

53. Gur, R. C. *et al.* Positron emission tomography in two cases of childhood epileptic encephalopathy (Lennox-Gastaut syndrome). *Neurology* **32**, 1191–4 (1982).

54. Miyauchi, T., Nomura, Y., Ohno, S., Kishimoto, H. & Matsushita, M. Positron emission tomography in three cases of Lennox-Gastaut syndrome. *Jpn. J. Psychiatry Neurol.* **42**, 795–804 (1988).

55. Parker, A. P. *et al.* Neuroimaging and spectroscopy in children with epileptic encephalopathies. *Arch. Dis. Child.* **79**, 39–43 (1998).

56. Ferrie, C. D. *et al.* Focal abnormalities detected by 18FDG PET in epileptic encephalopathies. *Arch. Dis. Child.* **75**, 102–7 (1996).

57. Lee, Y. J. *et al.* Resective Pediatric Epilepsy Surgery in Lennox-Gastaut Syndrome. *Pediatrics* **125**, e58–e66 (2010).

58. Lee, Y.-J. *et al.* Outcomes of epilepsy surgery in childhood-onset epileptic encephalopathy. *Brain Dev.* **36**, 496–504 (2014).

59. Pillay, N. *et al.* Networks underlying paroxysmal fast activity and slow spike and wave in Lennox-Gastaut syndrome. *Neurology* **81**, 665–673 (2013).

60. Archer, J. S. *et al.* Lennox-Gastaut syndrome and phenotype: Secondary network epilepsies. *Epilepsia* **55**, 1245–1254 (2014).

61. Gupta, A. *et al.* Pediatric Epilepsy Surgery in Focal Lesions and Generalized Electroencephalogram Abnormalities. *Pediatr. Neurol.* **37**, 8–15 (2007).

62. Park, K., Hur, Y. & Kim, S. Brainstem dysfunction in patients with late-onset

Lennox-Gastaut syndrome: Voxel-based morphometry and tract-based spatial statistics study. *Ann. Indian Acad. Neurol.* **19**, 518 (2016).

63. Siniatchkin, M., Coropceanu, D., Moeller, F., Boor, R. & Stephani, U. EEG-fMRI reveals activation of brainstem and thalamus in patients with Lennox-Gastaut syndrome. *Epilepsia* **52**, 766–774 (2011).

64. Maheshwari, A. & Noebels, J. L. Monogenic models of absence epilepsy. in *Progress in brain research* **213**, 223–252 (2014).

65. Fogerson, P. M. & Huguenard, J. R. Tapping the Brakes: Cellular and Synaptic Mechanisms that Regulate Thalamic Oscillations. *Neuron* **92**, 687–704 (2016).

66. Walker, M. C. & Kullmann, D. M. *Tonic GABAA Receptor-Mediated Signaling in Epilepsy*. *Jasper's Basic Mechanisms of the Epilepsies* (2012).

67. Pearce, P. S. *et al.* Spike–wave discharges in adult Sprague–Dawley rats and their implications for animal models of temporal lobe epilepsy. *Epilepsy Behav.* **32**, 121–131 (2014).

68. Saalmann, Y. B. Intralaminar and medial thalamic influence on cortical synchrony, information transmission and cognition. *Front. Syst. Neurosci.* **8**, 83 (2014).

69. Royce, G. J. & Mourey, R. J. Efferent connections of the centromedian and parafascicular thalamic nuclei: An autoradiographic investigation in the cat. *J. Comp. Neurol.* **235**, 277–300 (1985).

70. Velasco, F. *et al.* Deep brain stimulation for treatment of the epilepsies: the centromedian thalamic target. *Acta Neurochir. Suppl.* **97**, 337–42 (2007).

71. Son, B. *et al.* Clinical Outcome of Patients with Deep Brain Stimulation of the Centromedian Thalamic Nucleus for Refractory Epilepsy and Location of the Active Contacts. *Stereotact. Funct. Neurosurg.* **94**, 187–197 (2016).

72. Steinlein, O. K. *et al.* A missense mutation in the neuronal nicotinic acetylcholine receptor α4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy. *Nat. Genet.* **11**, 201–203 (1995).

73. Velasco, F., Velasco, M., Jimenez, F., Velasco, A. L. & Marquez, I. Stimulation of the Central Median Thalamic Nucleus for Epilepsy. *Stereotact. Funct. Neurosurg.* **77**, 228–232 (2001).

74. Velasco, M., Velasco, F., Gardea, G., Gordillo, F. & Diaz de León, A. E. Polygraphic characterization of the sleep-epilepsy patterns in a hydranencephalic child with severe generalized seizures of the Lennox-Gastaut syndrome. *Arch. Med. Res.* **28**, 297–302 (1997).

75. Velasco, M. *et al.* REVIEW ARTICLE Acute and Chronic Electrical Stimulation of the Centromedian Thalamic Nucleus: Modulation of Reticulo-Cortical Systems and Predictor Factors for Generalized Seizure Control. *Arch. Med. Res.* **31**, 304–315 (2000).

76. Velasco, A. L. *et al.* Neuromodulation of the Centromedian Thalamic Nuclei in the Treatment of Generalized Seizures and the Improvement of the Quality of Life in Patients with Lennox–Gastaut Syndrome. *Epilepsia* **47**, 1203–1212 (2006).

77. Parnaudeau, S. *et al.* Inhibition of Mediodorsal Thalamus Disrupts Thalamofrontal Connectivity and Cognition. *Neuron* **77**, 1151–1162 (2013).

78. Kreindler, A., Zuckermann, E., Steriade, M. & Chimion, D. ELECTROCLINICAL FEATURES OF CONVULSIONS INDUCED BY STIMULATION OF BRAIN STEM. *J. Neurophysiol.* **21**, 430–436 (1958).

79. Blumenfeld, H. *et al.* Cortical and subcortical networks in human secondarily generalized tonic–clonic seizures. *Brain* **132**, 999–1012 (2009).

80. DeSalvo, M. N. *et al.* Focal BOLD fMRI changes in bicuculline-induced tonic–clonic seizures in the rat. *Neuroimage* **50**, 902–909 (2010).

81. Applegate, C. D., Samoriski, G. M. & Burchfiel, J. L. Evidence for the interaction of brainstem systems mediating seizure expression in kindling and electroconvulsive shock seizure models. *Epilepsy Res.* **10**, 142–7

82. Faingold, C. L. *The Role of the Brain Stem in Generalized Epileptic Seizures t*. *Metabolic Brain Disease* **2**, (1987).

83. Siniatchkin, M. *et al.* Different Neuronal Networks Are Associated with Spikes and Slow Activity in Hypsarrhythmia. *Epilepsia* **0**, 070816162212002–??? (2007).

84. Intusoma, U. *et al.* Tonic seizures of Lennox-Gastaut syndrome: Periictal single-photon emission computed tomography suggests a corticopontine network. *Epilepsia* **54**, 2151–2157 (2013).

85. Choi, G. B. *et al.* The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science (80-.).* **351**, 933–939 (2016).

86. Kim, S. *et al.* Maternal gut bacteria promote neurodevelopmental abnormalities in mouse offspring. *Nature* **549**, 528–532 (2017).

87. Gusel'nikova, V. V & Korzhevskiy, D. E. NeuN As a Neuronal Nuclear Antigen and Neuron Differentiation Marker. *Acta Naturae* **7**, 42–7 (2015).

88. Brunjes, P. C. & Osterberg, S. K. Developmental Markers Expressed in Neocortical Layers Are Differentially Exhibited in Olfactory Cortex. *PLoS One* **10**, e0138541 (2015).

89. Hevner, R. F. Layer-Specific Markers as Probes for Neuron Type Identity in Human Neocortex and Malformations of Cortical Development. *J. Neuropathol. Exp. Neurol.* **66**, 101–109 (2007).

90. Laurie, D. J., Seeburg, P. H. & Wisden, W. The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. *J. Neurosci.* **12**, 1063–76 (1992).

91. Fritschy, J.-M. & Mohler, H. GABAA-receptor heterogeneity in the adult rat brain: Differential regional and cellular distribution of seven major subunits. *J. Comp. Neurol.* **359**, 154–194 (1995).

92. Laurie, D. J., Wisden, W. & Seeburg, P. H. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J. Neurosci.* **12**, 4151–72 (1992).

93. Wisden, W., Laurie, D. J., Monyer, H. & Seeburg, P. H. *The Distribution of 13 GABA, Receptor Subunit mRNAs in the Rat Brain. I. Telencephalon, Diencephalon, Mesencephalon*. *The Journal of Neuroscience* **2**, (1992).

94. Meador, K. J. Brain function and anatomy in juvenile myoclonic epilepsy. *Epilepsy Curr.* **10**, 13–4 (2010).

95. de Araújo Filho, G. M. *et al.* Personality traits related to juvenile myoclonic epilepsy: MRI reveals prefrontal abnormalities through a voxel-based morphometry study. *Epilepsy Behav.* **15**, 202–207 (2009).

96. de Araújo Filho, G. M. *et al.* Are personality traits of juvenile myoclonic epilepsy related to frontal lobe dysfunctions? A proton MRS study. *Epilepsia* **50**, 1201–1209 (2009).

97. Choi, J. H., Koch, K. P., Poppendieck, W., Lee, M. & Shin, H.-S. High Resolution Electroencephalography in Freely Moving Mice. *J. Neurophysiol.* **104**, 1825–1834 (2010).

98. Lee, M., Kim, D., Shin, H.-S., Sung, H.-G. & Choi, J. H. High-density EEG recordings of the freely moving mice using polyimide-based microelectrode. *J. Vis. Exp.* (2011). doi:10.3791/2562

99. Silverstein, F. & Johnston, M. V. Cerebrospinal fluid monoamine metabolites in patients with infantile spasms. *Neurology* **34**, 102–5 (1984).

100. Airaksinen, E., Tuomisto, L. & Riikonen, R. The concentrations of GABA, 5-HIAA and HVA in the cerebrospinal fluid of children with infantile spasms and the effects of ACTH treatment. *Brain Dev.* **14**, 386–90 (1992).

101. Coleman, M. Infantile spasms associated with 5-hydroxytryptophan administration in patients with Down's syndrome. *Neurology* **21**, 911–9 (1971).

102. Scantlebury, M. H. *et al.* A model of symptomatic infantile spasms syndrome. *Neurobiol. Dis.* **37**, 604–12 (2010).

103. Bonnin, A. *et al.* A transient placental source of serotonin for the fetal forebrain. *Nature* **472**, 347–350 (2011).

104. Pollak Dorocic, I. *et al.* A Whole-Brain Atlas of Inputs to Serotonergic Neurons of the Dorsal and Median Raphe Nuclei. *Neuron* **83**, 663–678 (2014).

105. Challis, C. & Berton, O. Top-Down Control of Serotonin Systems by the Prefrontal Cortex: A Path toward Restored Socioemotional Function in Depression. *ACS*

Chem. Neurosci. **6**, 1040–54 (2015).

106. Ellis, H. John Langdon Down: Down's syndrome. *J. Perioper. Pract.* **23**, 296–297 (2013).

107. Korenberg, J. R., Yang-Feng, T., Schreck, R. & Chen, X. N. Using fluorescence in situ hybridization (FISH) in genome mapping. *Trends Biotechnol.* **10**, 27–32 (1999).

108. Sanger, F., Nicklen, S. & Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* (1977). doi:10.1073/pnas.74.12.5463

109. Baulac, S. *et al.* First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. *Nat. Genet.* **28**, 46–48 (2001).

110. Wallace, R. H. *et al.* Mutant GABA(A) receptor gamma2-subunit in childhood absence epilepsy and febrile seizures. *Nat. Genet.* **28**, 49–52 (2001).

111. Poduri, A. Meta-Analysis Revives Genome-Wide Association Studies in Epilepsy. *Epilepsy Curr.* **15**, 122–3 (2015).

112. International League Against Epilepsy Consortium on Complex Epilepsies. Electronic address: epilepsy-austin@unimelb.edu.au. Genetic determinants of common epilepsies: a meta-analysis of genome-wide association studies. *Lancet. Neurol.* **13**, 893–903 (2014).

113. Koboldt, D. C., Steinberg, K. M., Larson, D. E., Wilson, R. K. & Mardis, E. R. XThe next-generation sequencing revolution and its impact on genomics. *Cell* **155**, 27–38 (2013).

114. Lander, E. S. *et al.* Initial sequencing and analysis of the human genome. *Nature* (2001). doi:10.1038/35057062

115. Lander, E. S. *et al.* Initial sequencing and analysis of the human genome. *Nature* (2001). doi:10.1038/35057062

116. The Cost of Sequencing a Human Genome - National Human Genome Research Institute (NHGRI). Available at: https://www.genome.gov/27565109/the-cost-of-sequencing-a-human-genome/. (Accessed: 29th September 2018)

117. Devinsky, O. *et al.* Epilepsy. *Nat. Rev. Dis. Prim.* **4**, 18024 (2018).

118. Medvedev, P., Stanciu, M. & Brudno, M. Computational methods for discovering structural variation with next-generation sequencing. *Nat. Methods* **6**, S13–S20 (2009).

119. Tinhofer, I. *et al.* Next-generation sequencing: hype and hope for development of personalized radiation therapy? *Radiat. Oncol.* **10**, 183 (2015).

120. Schrijver, I. & Galli, S. J. Between hype and hope: whole-genome sequencing in clinical medicine. *Per. Med.* **9**, 243–246 (2012).

121. Lionel, A. C. *et al.* Improved diagnostic yield compared with targeted gene

sequencing panels suggests a role for whole-genome sequencing as a first-tier genetic test. *Genet. Med.* **20**, 435–443 (2018).

122. Urban, T. J. Whole-genome sequencing in pharmacogenetics. *Pharmacogenomics* **14**, 345–348 (2013).

123. Ellingford, J. M. *et al.* Whole Genome Sequencing Increases Molecular Diagnostic Yield Compared with Current Diagnostic Testing for Inherited Retinal Disease. *Ophthalmology* **123**, 1143–50 (2016).

124. Ostrander, B. E. P. *et al.* Whole-genome analysis for effective clinical diagnosis and gene discovery in early infantile epileptic encephalopathy. *npj Genomic Med.* **3**, 22 (2018).

125. Ambrosino, P. *et al.* De novo gain-of-function variants in *KCNT2* as a novel cause of developmental and epileptic encephalopathy. *Ann. Neurol.* **83**, 1198–1204 (2018).

126. Bearden, D. *et al.* Targeted treatment of migrating partial seizures of infancy with quinidine. *Ann. Neurol.* **76**, 457–461 (2014).

127. Mikati, M. A. *et al.* Quinidine in the treatment of KCNT1-positive epilepsies. *Ann. Neurol.* **78**, 995–999 (2015).

128. Barcia, G. *et al.* De novo gain-of-function KCNT1 channel mutations cause malignant migrating partial seizures of infancy. *Nat. Genet.* **44**, 1255–1259 (2012).

129. Milligan, C. J. *et al. KCNT1* gain of function in 2 epilepsy phenotypes is reversed by quinidine. *Ann. Neurol.* **75**, 581–590 (2014).

130. Pierson, T. M. *et al.* GRIN2A mutation and early-onset epileptic encephalopathy: personalized therapy with memantine. *Ann. Clin. Transl. Neurol.* **1**, 190–198 (2014).

131. Capal, J. K. & Franz, D. N. Profile of everolimus in the treatment of tuberous sclerosis complex: an evidence-based review of its place in therapy. *Neuropsychiatr. Dis. Treat.* **12**, 2165–72 (2016).

132. Stett, A., Burkhardt, C., Weber, U., Van Stiphout, P. & Knott, T. CYTOCENTERING : A Novel Technique Enabling Automated Cell-by-Cell Patch Clamping with the C YTO P ATCH ^{TM} Chip. *Recept. Channels* **9**, 59–66 (2003).

133. Natarajan, A., Molnar, P., Sieverdes, K., Jamshidi, A. & Hickman, J. J. Microelectrode array recordings of cardiac action potentials as a high throughput method to evaluate pesticide toxicity. *Toxicol. Vitr.* **20**, 375–381 (2006).

134. Wang, X. & Li, M. Automated Electrophysiology: High Throughput of Art. *Assay Drug Dev. Technol.* **1**, 695–708 (2003).

135. Kitchen, D. B., Decornez, H., Furr, J. R. & Bajorath, J. Docking and scoring in virtual screening for drug discovery: methods and applications. *Nat. Rev. Drug*

Discov. **3**, 935–949 (2004).

136. Sliwoski, G., Kothiwale, S., Meiler, J. & Lowe, E. W. Computational methods in drug discovery. *Pharmacol. Rev.* **66**, 334–95 (2014).

137. Jorgensen, W. L. The many roles of computation in drug discovery. *Science* **303**, 1813–8 (2004).

138. Boehnke, K. *et al.* Assay Establishment and Validation of a High-Throughput Screening Platform for Three-Dimensional Patient-Derived Colon Cancer Organoid Cultures. *J. Biomol. Screen.* **21**, 931–941 (2016).

139. Del Álamo, J. C. *et al.* High throughput physiological screening of iPSC-derived cardiomyocytes for drug development. *Biochim. Biophys. Acta* **1863**, 1717–27 (2016).

140. Kiskinis, E. *et al.* All-Optical Electrophysiology for High-Throughput Functional Characterization of a Human iPSC-Derived Motor Neuron Model of ALS. *Stem Cell Reports* **10**, 1991–2004 (2018).

141. Heilker, R., Traub, S., Reinhardt, P., Schö Ler, H. R. & Sterneckert, J. iPS cell derived neuronal cells for drug discovery. doi:10.1016/j.tips.2014.07.003

142. Eimon, P. M. *et al.* Brain activity patterns in high-throughput electrophysiology screen predict both drug efficacies and side effects. *Nat. Commun.* **9**, 219 (2018).

143. Zhu, X., Need, A. C., Petrovski, S. & Goldstein, D. B. One gene, many neuropsychiatric disorders: lessons from Mendelian diseases. *Nat. Neurosci.* **17**, 773–81 (2014).

144. Trump, N. *et al.* Improving diagnosis and broadening the phenotypes in early-onset seizure and severe developmental delay disorders through gene panel analysis. *J. Med. Genet.* **53**, 310–317 (2016).

145. Hamdan, F. F. *et al.* High Rate of Recurrent De Novo Mutations in Developmental and Epileptic Encephalopathies. *Am. J. Hum. Genet.* **101**, 664–685 (2017).

146. Møller, R. S. *et al.* Mutations in GABRB3: From febrile seizures to epileptic encephalopathies. *Neurology* **88**, 483–492 (2017).

147. Papandreou, A. *et al.* GABRB3 mutations: a new and emerging cause of early infantile epileptic encephalopathy. *Dev. Med. Child Neurol.* **58**, 416–20 (2016).

148. Kodera, H. *et al.* De novo *GABRA1* mutations in Ohtahara and West syndromes. *Epilepsia* **57**, 566–573 (2016).

149. Johannesen, K. *et al.* Phenotypic spectrum of *GABRA1*. *Neurology* **87**, 1140–1151 (2016).

150. Carvill, G. L. *et al.* GABRA1 and STXBP1: Novel genetic causes of Dravet syndrome. *Neurology* **82**, 1245–1253 (2014).

151. Zou, F. *et al.* Expanding the phenotypic spectrum of *GABRG2* variants: a

recurrent *GABRG2* missense variant associated with a severe phenotype. *J. Neurogenet.* **31**, 30–36 (2017).

152. Kang, J.-Q. & Macdonald, R. L. Molecular Pathogenic Basis for *GABRG2* Mutations Associated With a Spectrum of Epilepsy Syndromes, From Generalized Absence Epilepsy to Dravet Syndrome. *JAMA Neurol.* **73**, 1009 (2016).

153. Shen, D. *et al. De novo GABRG2* mutations associated with epileptic encephalopathies. *Brain* **140**, 49–67 (2017).