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Identification and Evaluation of Novel Epilepsy-Associated Variants

By

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Identification and Evaluation of Novel Epilepsy-Associated Variants

By

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B.S., Clemson University, 2013

Advisor: Andrew Escayg, Ph.D.

An abstract of
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Abstract

Identification and Evaluation of Novel Epilepsy-Associated Variants

By Kameryn McCarty Butler

Epilepsy is a neurological disorder characterized by recurrent, unprovoked seizures caused by excessive neuronal synchrony and hyperexcitability. Although epilepsy can develop following insults such as head trauma, stroke, and infection, genetic factors are predicted to play a role in approximately 70-80% of epilepsy cases. To date, mutations in over 150 genes have been identified in different types of epilepsy; however, these mutations account for only a small fraction of the estimated genetic contribution to epilepsy. We collaborated with EGL Genetics, a CLIA-certified and CAP-accredited laboratory, to examine available sequence data from clinically-referred epilepsy patients. Upon referral, patient DNA samples were screened using the Epilepsy and Seizure Disorders (ESD) panel, a sequencing panel of 110 known epilepsy genes. The ESD panel is derived from a larger Mendeliome library of approximately 4,800 evidence-based disease genes, making this a valuable resource for the identification of putative disease-causing alleles as well as new disease associations. We determined the diagnostic yield of the ESD panel to be approximately 18% based on a review of panel results from 339 epilepsy patients, with most pathogenic variants affecting a small number of genes. From this analysis, we identified several factors that are likely to improve diagnostic yield. Additionally, we discovered five novel missense variants in the voltage-gated sodium channel gene *SCN8A*. Investigation of these variants revealed that both *de novo* and inherited *SCN8A* variants contribute to epilepsy, with inherited variants producing less severe forms of disease. Furthermore, we identified four regions of the sodium channel where disease variants cluster and discovered that an alternative coding exon of *SCN8A* was being overlooked by diagnostic laboratories. This dissertation also covers the examination of variants from the Mendeliome library from individuals with negative or inconclusive ESD panel results. Novel variants were identified in *GABRA5* and *GABRA2*, two genes not previously associated with disease, from individuals with severe early-onset epilepsy and developmental delay. Functional evaluation of these $GABA_A$ receptor pore mutations revealed potential gain- and loss-of-function changes. Overall, these results highlight the usefulness of utilizing clinical sequencing data for research-based epilepsy gene and variant discovery.

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To my husband Grant, my family, and my friends: Thank you for your unwavering support and encouragement, without which I would not have made it to this point.

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Tables and Figures

Abbreviations

AD	Autosomal Dominant
ADHD	Attention Deficit Hyperactivity Disorder
AED	Anti-Epileptic Drug
AR	Autosomal Recessive
ASD	Autism Spectrum Disorder
DD	Developmental Delay
EE	Epileptic Encephalopathy
EEG	Electroencephalogram
EGL	Emory Genetics Laboratory
EIEE	Early-Infantile Epileptic Encephalopathy
ESD	Epilepsy and Seizure Disorders Panel
ExAC	Exome Aggregation Consortium Database
GABA	γ-aminobutyric acid
GEFS+	Generalized Epilepsy with Febrile Seizures Plus
GERD	Gastroesophageal Reflux Disease
gnomAD	Genome Aggregation Database
GUS	Gene of Uncertain Significance
GWAS	Genome-Wide Association Study
HEK293T	Human Embryonic Kidney 293T
HGMD	Human Gene Mutation Database
ID	Intellectual Disability
LOF	Loss-of-Function
MAF	Minor Allele Frequency
MRI	Magnetic Resonance Imaging
NOS	Not Otherwise Specified
VUS	Variant of Uncertain Significance
WES	Whole-Exome Sequencing
WGS	Whole-Genome Sequencing
XLD	X-linked Dominant
XLR	X-linked Recessive

CHAPTER 1
EPILEPSY GENE AND VARIANT DISCOVERY

1.1 Overview

The overall goal of my graduate work was to further our understanding of the genetics of epilepsy through the identification and evaluation of novel genes and variants associated with epilepsy. This was accomplished using available sequencing data from epilepsy patients undergoing genetic testing at the Emory Genetics Laboratory (EGL). I evaluated hundreds of variants detected in known epilepsy genes as well as thousands of variants from genes not currently associated with epilepsy to identify those that could be contributing to the development of epilepsy.

In this introduction, I will first provide a general overview of epilepsy and the initial human epilepsy genes identified in order to help the reader understand the history of epilepsy genetics. This will be followed by a summary of the genes that have been identified in the last decade and the technologies used to detect variants in these genes, which is directly relevant to my research. I will also briefly discuss several approaches and concepts important for epilepsy gene discovery. Lastly, I will conclude with specific information on my experimental design and the advantages and limitations of using available clinical sequencing data.

1.2 General Overview of the Genetics of Epilepsy

Epilepsy refers to a group of neurological disorders that are characterized by recurrent, unprovoked seizures (ILAE, 1993). Collectively, epilepsy affects approximately 1% of the population and is the most common neurological disorder after migraine, stroke, and Alzheimer's disease. The risk for developing epilepsy is highest in young children and the elderly, and it can have a profound, long-term impact on quality

of life. Although epilepsy can develop following insults such as head trauma, stroke, and infection, the contribution of genetic factors as a primary cause of epilepsy has long been recognized from familial and twin studies (Berkovic et al., 1998; Helbig et al., 2008; Kjeldsen et al., 2003; Lennox, 1960; Poduri and Lowenstein, 2011; Vadlamudi et al., 2014). Currently, genetic factors are predicted to play a role in approximately 70-80% of epilepsy cases based on the observation that only 20-30% of epilepsy cases are acquired (Myers and Mefford, 2015).

1.2.1 Types of epilepsy

Although the classification criteria have changed over the years, epilepsy can be broken down into three broad categories: generalized epilepsies, focal epilepsies, and epileptic encephalopathies (Falco-Walter et al., 2017; Myers and Mefford, 2015). Genetic factors are known to contribute to all three categories. Generalized epilepsies, such as juvenile myoclonic epilepsy and childhood absence epilepsy, involve seizures that affect both hemispheres of the brain. In contrast, focal seizures originate from only one hemisphere or region of the brain. Examples of focal epilepsies include temporal lobe epilepsy and autosomal dominant nocturnal frontal lobe epilepsy. Epileptic encephalopathies (e.g., Dravet syndrome, West Syndrome) are characterized by severe, early-onset seizures that may be generalized or focal in nature. These seizures are generally refractory to treatment and are associated with developmental delay and regression as a result of ongoing epileptic activity. Unlike the other two categories, most epileptic encephalopathies have a poor prognosis.

In addition to the types of epilepsy described above, epilepsy can occur as the main symptom of a disease or it may occur as one symptom in the context of a broader disorder, such as tuberous sclerosis or fragile X syndrome.

1.2.2 The early genetics of epilepsy

The first epilepsy gene, an acetylcholine receptor encoded by *CHRNA4*, was identified in 1995 by linkage analysis in a large multiplex family with autosomal dominant nocturnal frontal lobe epilepsy (Steinlein et al., 1995). This discovery was followed by the identification of several other epilepsy genes encoding different types of ion channels and receptors, including sodium channels (*SCN1A*, *SCN1B*), potassium channels (*KCNQ2*, *KCNQ3*), and GABA-gated chloride channels (*GABRA1*, *GABRG2*) (Charlier et al., 1998; Cossette et al., 2002; Escayg et al., 2000; Singh et al., 1998; Wallace et al., 2001; Wallace et al., 1998). This led to the concept of epilepsy as a "channelopathy" (Berkovic and Scheffer, 1999). However, mutations were later identified in genes encoding non-ion channel proteins, such as *LGI1* and *ARX*, indicating that epilepsy could arise from more than just ion channel dysfunction (Kalachikov et al., 2002; Stromme et al., 2002).

1.2.3 Epilepsy genetics in the 21st century

Following a period of candidate gene screening and genome-wide association studies (GWASs), next-generation sequencing technologies allowed researchers to begin to identify pathogenic variants in an unbiased manner. This led to rapid discovery of new epilepsy genes, encoding ion channels, metabolic enzymes, transcriptional regulators,

synaptic proteins, and more. By 2013, variants in over one hundred genes had been implicated in epilepsy and seizure disorders (Michelucci et al., 2012; Nicita et al., 2012; Pal et al., 2010; Pandolfo, 2011; Poduri and Lowenstein, 2011). This number continues to grow as more genomes of individuals with epilepsy are sequenced in both research and diagnostic settings. **Table 1.1** lists 159 genes currently reported to be associated with epilepsy and seizures. While this list is not exhaustive, it does contain the genes most frequently reported for individuals with epilepsy.

Despite the progress that has been made in the last decade, a large percentage of epilepsy patients remain undiagnosed after genetic testing, suggesting that additional genetic factors that have yet to be identified are contributing to the development of epilepsy. These additional genetic variants are likely to be rare and only identified through the testing of larger numbers of individuals with epilepsy. My research utilized available sequencing data from >300 epilepsy patients, allowing me to identify novel and potentially rare genetic causes of epilepsy.

1.3 Genetic testing for epilepsy

Knowledge of the underlying genetic cause of disease can lead to a better understanding of the disease and improved treatments. Gene and variant discovery for epilepsy has been greatly accelerated by advances in the technologies used to detect genetic variants. These include array technologies to detect large chromosomal abnormalities and next-generation sequencing technologies to detect single nucleotide variants and microdeletions/duplications.

Table 1.1 Genes associated with Epilepsy and Seizures

ABAT	*CLN3*	*GAMT*	*LGI1*	*PNKP*	*SLC6A1*
ADSL	*CLN5*	*GATM*	*LIAS*	*POLG*	*SLC9A6*
ALDH5A1	*CLN6*	*GOSR2*	*MBD5*	*PPT1*	*SMARCA2*
ALDH7A1	*CLN8*	*GPHN*	*MCPH1*	*PRICKLE1*	*SMC1A*
ALG13	*CLTC*	*GRIN1*	*MDH2*	*PRICKLE2*	*SNAP25*
ANKRD11	*CNTNAP2*	*GRIN2A*	*MECP2*	*PRRT2*	*SPTAN1*
AP4B1	*CPA6*	*GRIN2B*	*MEF2C*	*PTEN*	*ST3GAL3*
ARHGEF9	*CSTB*	*GRIN2D*	*MFSD8*	*PURA*	*ST3GAL5*
ARX	*CTSD*	*HCN1*	*MTOR*	*QARS*	*STX1B*
ASPM	*CYP27A1*	*HNRNPU*	*NACC1*	*RELN*	*STXBP1*
ASXL1	*DCX*	*IQSEC2*	*NDE1*	*SCARB2*	*SYN1*
ATP1A2	*DEPDC5*	*KANSL1*	*NEDD4L*	*SCN1A*	*SYNGAP1*
ATP1A3	*DNM1*	*KCNA1*	*NHLRC1*	*SCN1B*	*SZT2*
ATP6AP2	*DNM1L*	*KCNA2*	*NPRL2*	*SCN2A*	*TBC1D24*
BCKDK	*DYRK1A*	*KCNB1*	*NPRL3*	*SCN3A*	*TCF4*
CACNA1A	*EEF1A2*	*KCNC1*	*NRXN1*	*SCN8A*	*TPP1*
CACNA2D2	*EHMT1*	*KCNH1*	*OPHN1*	*SCN9A*	*TSC1*
CACNB4	*EPM2A*	*KCNJ10*	*PACS1*	*SHANK3*	*TSC2*
CASK	*FLNA*	*KCNJ11*	*PAFAH1B1*	*SIK1*	*UBE3A*
CASR	*FOLR1*	*KCNMA1*	*PCDH19*	*SIX3*	*USP9X*
CDKL5	*FOXG1*	*KCNQ2*	*PHF6*	*SLC13A5*	*WDR45*
CENPJ	*GABBR2*	*KCNQ3*	*PIGA*	*SLC19A3*	*WDR62*
CHD2	*GABRA1*	*KCNT1*	*PIGC*	*SLC1A2*	*WWOX*
CHRNA2	*GABRB1*	*KCTD7*	*PIGT*	*SLC25A19*	*ZEB2*
CHRNA4	*GABRB2*	*KIAA2022*	*PIGQ*	*SLC25A22*	
CHRNB2	*GABRB3*	*KMT2A*	*PLCB1*	*SLC2A1*	
CLCN4	*GABRG2*	*KMT2D*	*PNPO*	*SLC35A2*	

Genes in red encode ion channels.

1.3.1 Chromosomal microarrays and copy number variations

Array comparative genomic hybridization (aCGH), sometimes referred to as chromosomal microarray (CMA), is a cytogenetic technique used to detect unbalanced chromosomal abnormalities, also known as copy number variations (CNVs). These include deletions, duplications, and insertions that would otherwise be missed by traditional karyotyping methods. Arrays can be designed to examine the whole genome, or they can be targeted to specific regions of the genome.

When this technology was applied to individuals with epilepsy, rare CNVs were identified in approximately 5-10% of cases, including individuals with focal epilepsy, generalized epilepsy, and epileptic encephalopathies (Heinzen et al., 2010; Mefford et al., 2010; Mefford et al., 2011). CMA has the highest diagnostic yield when applied to epilepsy patients with additional features, such as intellectual disability and/or dysmorphic facial features (Scheffer and Mefford, 2014). Several recurrent CNVs have been reported as risk factors for the development of epilepsy as well as other neurodevelopmental disorders, including autism spectrum disorder, intellectual disability, and schizophrenia. Deletions and duplications involving 1q21.1, 15q11.2, 15q13.3, 16p11.2, and 16p13.11 are amongst the most common recurrent CNVs identified in epilepsy patients (de Kovel et al., 2010; Heinzen et al., 2010; Mefford et al., 2010; Mullen et al., 2013).

CMA is currently recommended as a first-tier genetic test for individuals with unexplained epilepsy, although its yield is consistently lower than that of gene panels or whole-exome sequencing (discussed below). However, CMA will remain necessary to detect pathogenic CNVs until next-generation sequencing technologies can identify these types of variants more reliably.

1.3.2 Next-Generation Sequencing

Next-generation sequencing (NGS) is a set of high-throughput, massively-parallel sequencing techniques that have revolutionized the field of disease gene discovery. In addition to producing more sequencing reads than traditional Sanger sequencing, the cost of NGS continues to decrease as technologies improve. There are three major

applications of NGS for genetic diagnostics: targeted gene panels, whole-exome sequencing, and whole-genome sequencing.

1.3.2.1 Targeted Gene Panels

Targeted gene panels, also known as targeted resequencing, involve the simultaneous sequencing of a few to several hundred genes. On average, gene panels cost less than Sanger sequencing one to two large genes and are especially useful in cases where the patient's phenotype is not suggestive of a specific gene disorder. Lemke and colleagues reported the first diagnostic gene panel for epilepsy in 2012 (Lemke et al., 2012); however, several other laboratories also developed their own epilepsy gene panels around this time, including the panel used by EGL (discussed in more detail below).

The diagnostic yield of epilepsy gene panels has ranged from 8 to 48%, with the average yield being around 17% (**Table 1.2**). The yield of a panel greatly depends on the genes examined and the inclusion criteria for patients. Overall, the diagnostic yield of gene panels tends to be highest when applied to individuals with early-onset seizures (Moller et al., 2016; Parrini et al., 2017; Trump et al., 2016; Zhang et al., 2015).

1.3.2.2 Whole-exome sequencing

Whole-exome sequencing (WES) examines all the known coding regions of the human genome, approximately 22,000 genes. Due to the large number of variants identified by WES, it is most efficient when both parents of the proband are also screened, referred to as trio WES. Trio WES allows for the identification of *de novo* variants and for the phasing of recessive variants.

Table 1.2 Reported yields from studies using gene panels for epilepsy

Patient Inclusion Criteria (Reference)	No. Patients	Patients with finding	Diagnostic yield (%)	No. Genes included[a]
Severe unspecific seizure disorder (Lemke et al., 2012)	33	16	48	265
Epileptic encephalopathies (Carvill et al., 2013a)	500	52	10	65
EOEE (Kodera et al., 2013)	53	12	23	35
All referrals at epilepsy clinic (Wang et al., 2014)	28	6	21	38-53
Isolated or syndromic epilepsy (Della Mina et al., 2015)	19	9	47	67
Unexplained epilepsy and ID/DD (Zhang et al., 2015)	253	46	18	300
Early-onset epilepsy and severe developmental delay (Trump et al., 2016)	400	71	18	46
Epileptic encephalopathies and familial epilepsies (Moller et al., 2016)	216	49	23	46
Epileptic encephalopathies (de Kovel et al., 2016)	360	29	8	351
Medication-refractory epilepsy (Segal et al., 2016)	49	7	14	88-471
EOEE (Gokben et al., 2017)	30	12	40	16
Drug-resistant epilepsy in first years of life (Parrini et al., 2017)	349	71	20	30-95
Chinese epilepsy trios (Wang et al., 2017b)	63	15	24	412
Unspecified epilepsy (Bevilacqua et al., 2017)	305	46	15	377
All epilepsy referrals at EGL (Butler et al. 2017)	339	62	18	110
Total	**2997**	**503**	**17**	

[a]Ranges indicate studies that used multiple panels. Abbreviations: EOEE, Early-onset epileptic encephalopathy; ID, Intellectual disability; DD, Developmental delay; EGL, Emory Genetics Laboratory.

The use of WES for genetic testing in epilepsy is increasing, both in research and diagnostic settings; however, it is often used only after an individual has already had negative CMA and gene panel analysis. The Epi4k Consortium and the Epilepsy Phenome/Genome Project reported the first major WES study of 264 patients with epileptic encephalopathies (Lennox-Gastaut syndrome and infantile spasms) in 2013. They reported an 11% diagnostic yield; however, that is likely an underestimate as the authors only included positive findings from known epilepsy genes (Allen et al., 2013). More recently, Helbig and colleagues reported a diagnostic yield of 38% from 314 consecutive epilepsy patients undergoing diagnostic WES.

1.3.2.3 Whole-genome sequencing

Whole-genome sequencing (WGS) examines both the coding and noncoding regions of the genome. One advantage of WGS is that it produces even coverage over all genes as it does not require a target enrichment step like gene panels or WES. On the other hand, WGS is more computationally-intensive and less is known about the contribution of non-coding variants to disease. In addition, whole-genome sequencing using short read platforms, such as those manufactured by Illumina, work best in regions of the genome that are unique. Therefore, genomic regions with complex repetitive structures or simply repetitive sequences remain difficult to sequence to high quality. Similar to WES, WGS is most efficient when performed on trios.

To date, there has only been one large WGS study for epilepsy. Hamdan and colleagues examined 197 individuals with epileptic encephalopathy and pharmaco-resistant seizures using trio-based WGS. They reported a molecular diagnosis in 32% of

cases, including four *de novo* CNVs detected from the WGS data (Hamdan et al., 2017). Despite the advantage of CNV detection, the diagnostic yield of WGS is currently comparable to that WES.

1.3.3 *De novo* variants

'*De novo*' refers to variants that are detected in a proband but absent from the DNA of both biological parents. Such changes to the DNA sequence arise due to errors during DNA replication or repair and can range in size from a single nucleotide substitution to large CNVs. *De novo* variants can occur during gametogenesis, and therefore be present in the resulting offspring. Alternatively, *de novo* variants can occur post-zygotically in early embryonic development, sometimes leading to mosaicism in the proband.

The application of CMA testing and next-generation sequencing has highlighted the importance of *de novo* mutations in sporadic epilepsy cases, especially the epileptic encephalopathies (Allen et al., 2013; Carvill et al., 2013a; EuroEpinomics-R.E.S. et al., 2014; Mefford et al., 2011; Olson et al., 2014). While not all *de novo* variants cause disease, identification of a *de novo* variant in a known epilepsy gene is strong evidence for pathogenicity. Alternatively, patterns of *de novo* variants from epilepsy patients can be used to aid in disease gene discovery.

1.3.4 The problem of VUS (and GUS)

One of the major challenges that has arisen from the use of NGS technologies is the identification of variants of uncertain significance (VUS). VUS correspond to variants (in genes associated with disease) that lack sufficient evidence to be classified as either

pathogenic or benign. Their contribution to disease is unknown or uncertain, often leaving clinicians and families uncertain about how to proceed with further testing or treatments. In some cases, VUS can be reclassified by testing of parent samples to determine variant inheritance. In general, VUS may be reclassified over time as more individuals are sequenced (e.g., another patient is identified with the same variant or the variant is detected in multiple unaffected individuals). In other instances, VUS can only be reclassified after functional evaluation, which can be both costly and time-consuming.

For WES and WGS, there is also the complication of genes of uncertain significance (GUS), since these sequencing methods examine all genes in the genome. As the name implies, GUS are genes which have not yet been associated with the specific disease under investigation or whose disease association is questionable. Therefore, variants identified in these genes will be considered VUS and will require functional evaluation to support an association with disease. Alternatively, the identification of multiple rare, *de novo* variants from unrelated individuals with similar clinical presentations can statistically support the role of a novel gene in disease, although functional evidence is still valuable (Allen et al., 2013; EuroEpinomics-R.E.S. et al., 2014; Hamdan et al., 2017; Helbig et al., 2016).

1.3.5 Phenotype-first approach & Genetic heterogeneity

One common experimental design for gene discovery is to use a phenotype-first approach. This involves sequencing groups of individuals that have a very specific phenotype, such as Dravet syndrome, to see if they share a genetic basis. This strategy is also used for genome-wide association studies (GWAS) to identify common variants that

are associated with disease. The premise behind this approach is that by enriching your cohort for a specific phenotype, you will also enrich for variants in the genes that cause those phenotypes.

The Epi4K Consortium used this approach in their 2013 study in which they performed WES on 264 trios to identify *de novo* variants in individuals with Lennox-Gastaut syndrome (LGS) or infantile spasms (Allen et al., 2013). Unlike Dravet syndrome, where 85% of cases have a pathogenic variant in the *SCN1A* gene (Escayg and Goldin, 2010), the authors found that LGS and infantile spasms are genetically heterogeneous disorders. This means that variants in many different genes contribute to the development of these two types of epilepsy. Additionally, they identified variants in genes already associated with other neurodevelopmental conditions, expanding the phenotypic spectrum of those genes (Allen et al., 2013).

1.3.6 Genotype-first approach & Phenotypic heterogeneity

An alternative approach known as ‘genotype-first’ involves sequencing large groups of individuals with a broadly-defined disorder (epilepsy, autism, etc.) to first determine the genotype of each individual at many or all positions across the genome. Genes which exhibit an excess of variation in individuals with disease compared to healthy controls are identified as candidate disease genes. Individuals are then grouped based on the presence of variants at a common locus and the clinical presentation of individuals within groups are compared to search for patterns, such as common clinical features or disease severity. Using this approach, it is possible classify genetic subtypes

of disease as well as expand the phenotypic spectrum of genes to highlight the shared genetic etiology of neurodevelopmental disorders (Stessman et al., 2014).

Using this approach, Carvill and colleagues identified *de novo* variants in *CHD2* and *SYNGAP1* as novel causes of epileptic encephalopathy and were able to delineate common clinical features associated with these two genes (Carvill et al., 2013a). At the same time, the authors noted that variants in these genes were also associated with intellectual disability and autism, highlighting the phenotypic heterogeneity.

1.4 Using available sequence data for epilepsy gene discovery

Two of the biggest roadblocks for investigating the underlying genetic architecture of the epilepsies are patient recruitment and the cost of sequencing and analyzing large numbers of samples. Through our collaboration with the Emory Genetics Laboratory (known as EGL Genetics), we were uniquely positioned to overcome both of these hurdles.

1.4.1 EGL generates sequence data from hundreds of epilepsy patients

By curating the available scientific literature, EGL was able to develop a targeted sequencing library of approximately 4,800 evidence-based disease genes. Within this large ‘Mendeliome’ library is a smaller subset of 110 genes specifically associated with epilepsy, referred to as the ‘Epilepsy and Seizure Disorders’ (ESD) panel. EGL provides a diagnostic service whereby epilepsy patients are screened for pathogenic variants in the genes of the ESD panel; however, all genes of the Mendeliome are sequenced in the process, making this a valuable resource for identifying novel disease-causing alleles as

well as new epilepsy genes. EGL receives approximately 12 new epilepsy referrals per month and more than 500 epilepsy patients have been screened since the ESD panel was launched in 2013. The patient referrals are enriched for infants and children with severe epilepsy, often associated with additional clinical features (**Tables 1.3 and 1.4**).

Table 1.3 Demographic information for 275 consecutive individuals screened using the ESD panel

Demographic Information	
Average Age	7.53 yrs
Median Age	5.6 yrs
Maximum	74 yrs
Minimum	0.2 yrs
No. Females	139
No. Males	136
Total	275 cases

Table 1.4 Frequency of non-epileptic phenotypes reported for the 275 consecutive referrals

Phenotype Provided	% of cases
Developmental delay	31.2
Hypotonia	13.0
Epileptic encephalopathy	12.6
Movement disorders, ataxia	14.4
Intellectual disability	7.4
Autism	5.6
Developmental regression	4.7
Microcephaly	4.7
Family History	4.2

1.4.2 The Epilepsy and Seizure Disorders (ESD) Panel

The ESD panel was designed in 2012 to contain important genes associated with epilepsy. The ESD panel includes genes associated with nonsyndromic epilepsies (e.g., *ALDH7A1*, *KCNQ2*) and syndromic disorders (e.g., *TSC2*, *ZEB2*), as well as genes for metabolic and structural disorders that include epilepsy as a prominent clinical feature. The full list of the 110 genes analyzed by the ESD panel is provided in Chapter 2 (**Table 2.1**). The ESD panel is an excellent resource for identifying which genes are most frequently mutated in epilepsy. In Chapter 2, I will provide the results from a study in

which I determined the diagnostic yield from 339 consecutive individuals who were screened using the ESD panel.

The ESD panel is also useful for the identification of novel variants in the known epilepsy genes. Such information can improve our understanding of the types of variants likely to cause disease and enable genotype-phenotype correlations. In Chapter 3, I will present the results from a study in which I identified several novel variants in the *SCN8A* voltage-gated sodium channel.

1.4.3 The 'Mendeliome' sequencing library

The Mendeliome sequencing library contains approximately 4,800 genes that are associated with a broad range of human diseases based on available scientific literature and human disease databases such as the Human Gene Mutation Database (HGMD) and Online Mendelian Inheritance in Man (OMIM). Because it is time-consuming and costly to design disease-specific sequencing libraries, the use of the larger Mendeliome library allows EGL to maintain a single sequencing library for all their offered sequencing panels. Additionally, genes that are already present in the Mendeliome can easily be included in specific panels if disease relevance is established without having to update the sequencing library.

Nearly 70% of the genes in the Mendeliome library are expressed in the brain, and 20% have been previously associated with one or more neurodevelopmental disorders (e.g., autism spectrum disorder, intellectual disability, schizophrenia). Because the Mendeliome was curated to contain human disease genes, it is a rich resource for gene and variant discovery.

We used variant data from the Mendeliome library in a genotype-first approach to identify variants that could be contributing to epilepsy. I then attempted to compare clinical phenotypes of individuals with variants in the same genes. In Chapter 4, I will describe a study in which I identified missense variants in several subunits of the $GABA_A$ receptor family in epilepsy patients. The variants in *GABRA2* and *GABRB3* were identified from the examination of data from the Mendeliome library in individuals with negative or inconclusive ESD panel results. Additional findings from the Mendeliome library are presented in the Appendices.

1.4.4 Advantages of using available data from clinical testing

There are several obvious advantages of using available sequence data from genetic screening of epilepsy patients. Importantly, because epilepsy patients are clinically referred for the sequencing panel, we avoid the initial need for patient recruitment. Additionally, we avoid the costs associated with sample collection and sequencing. Furthermore, because the Mendeliome is curated to contain most of the known human disease genes, it is likely to be enriched for candidate epilepsy genes.

1.4.5 Limitations of using available data from clinical testing

On the other hand, there were several limitations to consider in using this available sequence data. First, some referrals do not include clinical information on the patient, meaning we might not initially know what type of epilepsy a patient has or if they have any additional clinical features, such as developmental delay or brain malformations, which could potentially aid in variant classification. Although we have

established a mechanism to recontact and enroll individuals with interesting variants, we lose many cases to follow-up, either because the referring facility does not inform the family about our study or because the family is not interested in participating in research. If we are unable to enroll an individual, then we will not be able to determine variant inheritance or gather additional information about the individual's clinical presentation. Second, we are limited to the genes present within the Mendeliome library. Although the Mendeliome has been designed to include all the known human disease genes, it still contains less than a quarter of the genes in the human genome. Similarly, the Mendeliome does not enable the identification of CNVs.

1.5 Project Aims

The overall goal of my dissertation research was to further our understanding of the genetics of epilepsy. This was accomplished by utilizing available sequence data from individuals with epilepsy who were screened by EGL, followed by bioinformatic and functional analysis of identified variants.

In Chapter 2, I report the diagnostic yield of the ESD panel from 339 consecutive epilepsy patients. Chapter 3 presents several novel *SCN8A* variants identified from gene panel testing. In Chapter 4, I provide evidence to support variants in *GABRA2* and *GABRA5* as novel contributors to severe, early-onset epilepsy. Finally, I provide a discussion of the future of epilepsy genetics in Chapter 5. The results of this study have the potential improve the interpretation and classification of future variants identified in epilepsy patients and may aid in the selection of genes that should be included in epilepsy gene panels.

CHAPTER 2
DIAGNOSTIC YIELD FROM 339 EPILEPSY PATIENTS SCREENED ON A CLINICAL GENE PANEL

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2.1 Summary

In this chapter, I describe the diagnostic yield from 339 consecutive epilepsy patients screened using the Epilepsy and Seizure Disorders (ESD) panel. I detected pathogenic variants in 18% of patients (62/339), with an additional 6% (21/339) of patients having potentially causative variants of uncertain significance that could be contributing to their epilepsy. Causative and potentially causative variants were identified in 30 of the 110 genes of the panel, with variants being most frequently identified in *SCN1A* (n = 15) and *KCNQ2* (n = 10). Other genes in which disease-causing variants were identified in multiple individuals included *CDKL5*, *SCN2A*, *SCN8A*, *SCN1B*, *STXBP1*, *TPP1*, *PCDH19*, *CACNA1A*, *GABRA1*, *GRIN2A*, *SLC2A1*, and *TSC2*. Sixteen additional genes had variants identified in single individuals.

Although the genes we identified most frequently were very similar to those reported by other panel studies, 54% of the variants described in this chapter were novel at the time of publication. Reporting of these variants should improve future variant interpretation, especially if these same variants are detected in other epilepsy patients.

Determining the diagnostic yield of the ESD panel was an important first step of my research project. Evaluating the variants identified from the ESD panel provided an opportunity for me to become very familiar with the known epilepsy genes, including gene-disease associations and inheritance patterns, as well as the databases and bioinformatic tools available to critically evaluate variants. The manuscript that resulted from this work contributed to our knowledge of epilepsy genetics by establishing the diagnostic yield from a relatively unselected cohort of epilepsy patients. This contrasts

with most of the prior panel reports that focused on patients with specific subtypes of epilepsy, such as early-onset epileptic encephalopathy (EOEE).

Finally, one of my biggest motivations for determining the yield from the ESD panel was to identify patients who did not have a pathogenic variant in a known epilepsy gene. Individuals with negative panel results were prime candidates for gene discovery as we could rule out variants in known genes as the cause of their epilepsy. Findings from the analysis of the negative cases is the focus of Chapter 4 and the Appendices.

The published manuscript that resulted from this work is reproduced below.

2.2 Introduction

Epilepsy comprises a group of disorders that are characterized by recurrent, unprovoked seizures that collectively affect about 1% of the population. Although epilepsy can develop following insults such as head trauma, stroke, and infection, it is now believed that genetic factors may contribute to 70-80% of epilepsy cases (Myers and Mefford, 2015).

Advances in next generation sequencing (NGS) have allowed for the identification of new causal genes in both familial and sporadic forms of epilepsy (Allen et al., 2013; Carvill et al., 2013a). To date, pathogenic variants in over one hundred different genes have been reported to cause epilepsy and seizures (Wang et al., 2017a). Many of these research findings have been used to develop clinical gene panels, which are available to physicians to aid in diagnosis. These panels can range in size from dozens to hundreds of genes and may be specific to epilepsy subtype, such as early-infantile epileptic encephalopathy, or more broadly encompass many known epilepsy genes.

In this study, we investigated the diagnostic yield of one such gene panel, known as the Epilepsy and Seizure Disorders (ESD) panel, in an unselected cohort of 339 clinically-referred patients. The ESD panel uses a targeted sequencing library of 110 genes that are associated with a spectrum of epilepsies, as well as genes associated with metabolic and structural disorders that include epilepsy as a prominent symptom. Identification of a causative variant can help families to better cope with the disorder, allow for genetic counseling to determine recurrence risk, and in some cases, can directly influence treatment options (Berkovic, 2015; Boerma et al., 2016; Pierson et al., 2014). Additionally, it can end the often long and expensive diagnostic odyssey that families experience when searching for answers.

2.3 Materials and Methods

Patients

Variant reports from 339 consecutive, clinically-referred patients screened between 2013 and 2016 were retrospectively reviewed for this study. All molecular diagnostic testing was performed at EGL Genetics, a Clinical Laboratory Improvement Amendments (CLIA) and College of American Pathologists (CAP) accredited laboratory. This study was approved by the institutional review board of Emory University.

Targeted gene panel testing

A custom-designed in-solution hybridization probe library (IDT or SureSelect, Agilent Technologies, Santa Clara, CA) was used to capture the coding exons of the 110 genes on the Epilepsy and Seizure Disorders (ESD) panel. Direct sequencing of the

amplified captured regions was performed using next-generation sequencing (2x100bp, paired-end reads) on an Illumina HiSeq 2500 (Illumina, San Diego, CA) in rapid run mode. The individual DNA sequence reads were aligned to the published human genome reference (hg19 build) and variants were called using NextGENe® (SoftGenetics, State College, PA). Further analysis was performed using the EGL bioinformatics pipeline, which annotates identified variants utilizing a variety of external and internal sources. Variants are called within the coding exons and +/- 10bp of flanking intronic sequence. Relevant regions of epilepsy genes not amenable to NGS were filled in using the Sanger sequencing method. **Table 2.1** contains a list of the 110 genes examined as part of the ESD panel.

Variant Evaluation

Variants were evaluated and classified by board certified clinical molecular geneticists using the ACMG guidelines for variant classification (Richards et al., 2015). Briefly, variants were classified as (1) pathogenic, (2) likely pathogenic, (3) of unknown significance, (4) likely benign, or (5) benign. For research purposes, we considered certain variants of unknown significance to be "potentially causative" if specific conditions were met, such as the variant was absent from the population, *in silico* analyses predicted the variant to be damaging, and/or the variant was located in a critical or evolutionarily conserved region of the protein (Richards et al., 2015). Classified variants were submitted to ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) as well as EGL Genetics' publicly-available database EmVClass (www.egl-eurofins.com/emvclass/emvclass.php).

Table 2.1. Genes present on the Epilepsy and Seizure Disorders panel

Disorder	Genes
Generalized epilepsies	*ADGRV1, ALDH5A1, ALDH7A1, ATP1A2, CACNA1A, CACNB4, CASR, EFHC1, GABRA1, GABRG2, KCNMA1, KCNQ2, KCNQ3, MBD5, PCDH19, PRRT2, SCN1A, SCN1B, SCN2A, SCN9A, SLC2A1, TBC1D24*
Focal epilepsies	*CHRNA2, CHRNA4, CHRNB2, CPA6, GRIN2A, KCNT1, LGI1, SCN3A*
Progressive myoclonic epilepsies	*CSTB, EPM2A, GOSR2, KCTD7, NHLRC1, PRICKLE1, PRICKLE2, SCARB2*
Early infantile epileptic encephalopathies	*ARHGEF2, ARX, CDKL5, FOXG1, GABRA1, HCN1, KCNQ2, KCNT1, MECP2, NRXN1, PCDH19, PLCB1, SCN1A, SCN1B, SCN2A, SCN8A, PNKP, SLC19A3, SLC25A22, SLC2A1, SPTAN1, STXBP1, ST3GAL3, ST3GAL5, TBC1D24*
Neuronal migration/ malformation disorders	*ADGRG1, ARX, DCX, EMX2, FLNA, NDE1, PAFAH1B1, RELN, SHH, SIX3, SRPX2*
Severe microcephaly	*ASPM, CASK, CENPJ, MCPH1, PNKP, SLC25A19, STIL, TSEN54, WDR62*
Epilepsy in X-linked intellectual disability	*ARHGEF2, ARX, ATP6AP2, CASK, CDKL5, DCX, OPHN1, PCDH19, PHF6, SLC9A6, SRPX2, SYN1*
Inborn errors of metabolism	*ABAT, ADSL, ALDH5A1, ALDH7A1, BCKDK, CYP27A1, FOLR1, GAMT, GATM, LIAS, MTHFR, PNKO, SLC19A3*
Mitochondrial disorders	*NDUFA1, POLG*
Neuronal ceroid lipofuscinosis	*CLN3, CLN5, CLN6, CLN8, CTSD, DNAJC5, MFSD8, PPT1, TPP1*
Syndromic disorders with epilepsy and others	*CNTNAP2, HCN4, KCNA1, KCNJ10, KCNJ11, MAGI2, MEF2C, TSC1, TSC2, TCF4, UBE3A, ZEB2*

2.4 Results

2.4.1 Patient demographics

Demographic information was available for approximately 80% of the 339 individuals. The age range of individuals referred for genetic testing was 2.5 months to 74 years; however, most of the referrals were children and more than half were below the

age of 5 years. There were equal numbers of males and females. The amount of clinical information that accompanied each referral varied widely, with nearly 22% of the referrals lacking any clinical information about the patient. For the referrals that did include clinical information, 75% of cases reported two or more phenotypes. Aside from seizures, developmental delay was the next most common phenotype reported, followed by hypotonia, epileptic encephalopathy, intellectual disability, autism, and developmental regression. Additional phenotypes frequently encountered included ataxia, microcephaly, and spasticity. A positive family history of seizures or other neurological disorders was noted for 4% of cases.

2.4.2 Yield from the Epilepsy and Seizure Disorders Panel

Pathogenic or likely pathogenic variants were identified in 62 (18%) of the 339 individuals screened (**Table 2.2**). Twenty-one additional patients (6%) had potentially causative variants. Pathogenic, likely pathogenic, and potentially causative variants were identified in 30 different genes, accounting for 27% of the 110 genes on the ESD panel. Approximately 75% of the variants were in genes associated with autosomal dominant inheritance, while 17% and 8% of the variants affected X-linked and autosomal recessive genes, respectively. Pathogenic, likely pathogenic, and potentially causative variants were most frequently identified in *SCN1A* (n = 15) and *KCNQ2* (n = 10). Other genes in which variants were identified in multiple individuals included *CDKL5* (n = 6), *SCN2A* (n = 6), *SCN8A* (n = 5), *SCN1B* (n = 4), *STXBP1* (n = 4), *TPP1* (n = 3), *PCDH19* (n = 3), *CACNA1A* (n = 3), *GABRA1* (n = 2), *GRIN2A* (n = 2), *SLC2A1* (n = 2), and *TSC2* (n = 2).

Sixteen additional genes had variants identified in single individuals (**Fig. 2.1, Table 2.2**).

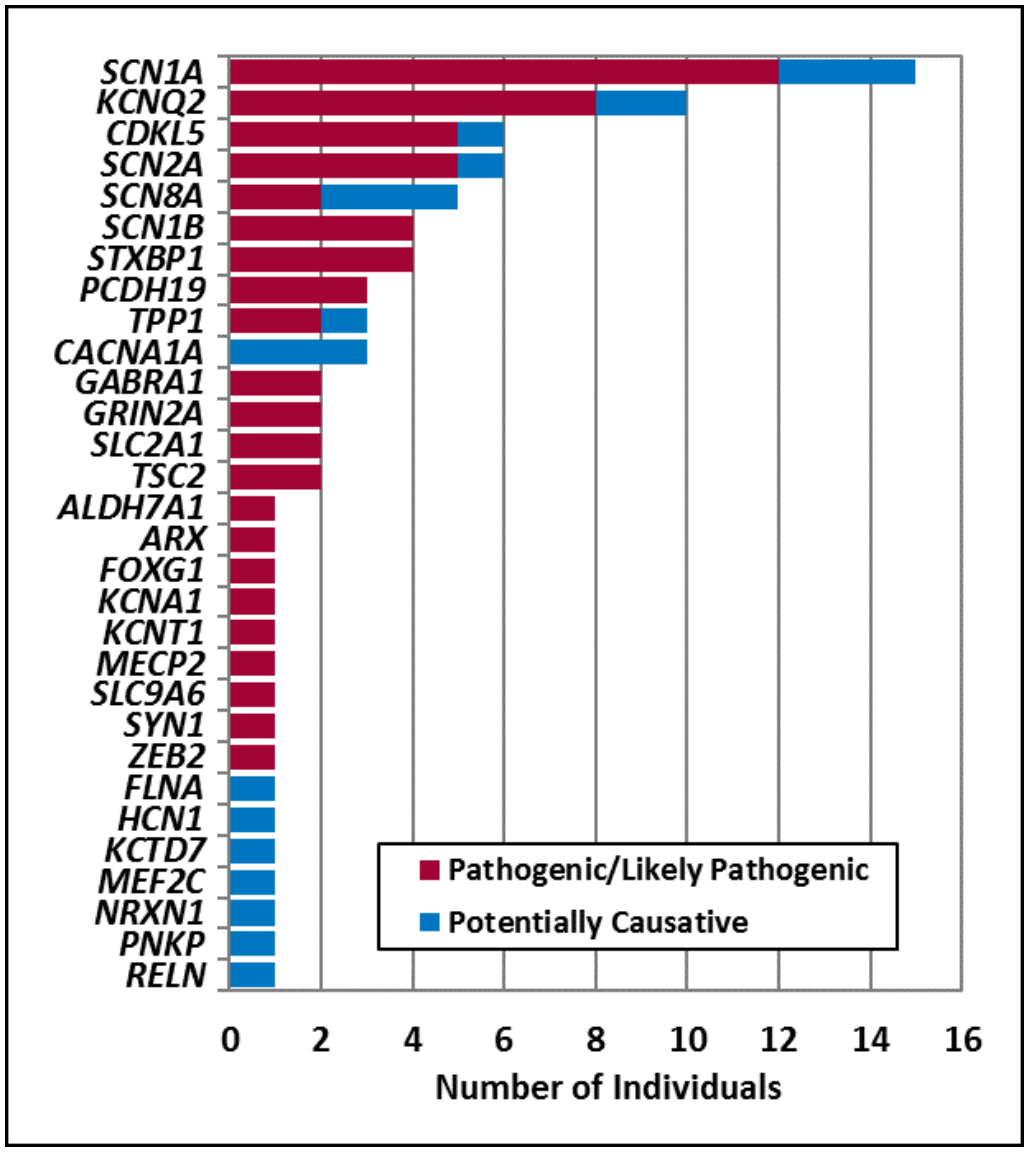

Figure 2.1. **Genes with pathogenic, likely pathogenic, and potentially causative variants detected in epilepsy patients.** Pathogenic/likely pathogenic variants (red) and potentially causative variants (blue) were identified in 30 genes of the ESD panel, most of which are associated with autosomal dominant epilepsy disorders.

Table 2.2. **Diagnostic yield from 339 epilepsy patients screened on the Epilepsy and Seizure Disorders panel.**

Pathogenic and Likely Pathogenic Variants					
Case	**Gene**	**Nucleotide**	**Protein**	**Classification**	**Phenotype**
1	*ALDH7A1*	c.1093+1G>A, c.1279G>C	p.E427Q	Pathogenic	Seizures, epileptic encephalopathy, macrocephaly, hypotonia, muscle weakness, and developmental delay
2	*ARX*	c.30C>A	p.C10*	Pathogenic	Infantile spasms, dysphagia, and poor weight gain
3	*CDKL5*	c.1891_1916delATAGGGCAAGGGATGGCAGCTAGAGC	p.I631Qfs*43	Pathogenic	Unspecified Epilepsy
4	*CDKL5*	c.1152C>G	p.Y384*	Pathogenic	Epileptic encephalopathy, myotonic and tonic-clonic seizures, and a course gyral pattern on brain imaging
5	*CDKL5*	c.1553delC	p.P518Hfs*5	Pathogenic	Seizures and spasticity
6	*CDKL5*	c.212delA	p.N71Tfs*5	Pathogenic	Infantile/epileptic spasms and hypotonia
7	*CDKL5*	c.1108_1109dupAA	p.N370Kfs*124	Pathogenic	NA
8	*FOXG1*	c.648_655delTTACTACC	p.Y217Rfs*235	Pathogenic	Unspecified Epilepsy
9	*GABRA1*	c.335G>A	p.R112Q	Likely Pathogenic	NA
10	*GABRA1*	c.640C>A	p.R214S	Likely pathogenic	Generalized convulsive epilepsy with intractable epilepsy
11	*GRIN2A*	c.2890delC	p.Q964Kfs*37	Pathogenic	Generalized seizures and speech disturbance
12	*GRIN2A*	c.3813G>A	p.W1271*	Pathogenic	NA
13	*KCNA1*	c.1222G>A	p.V408M	Likely Pathogenic	Seizures, developmental delay, and multiple joint contractures
14	*KCNQ2*	c.793G>A	p.A265T	Likely pathogenic	Neonatal seizure disorder
15	*KCNQ2*	c.637C>T	p.R213W	Pathogenic	Neonatal seizure disorder
16	*KCNQ2*	c.640C>T	p.R214W	Likely pathogenic	Autism, seizures, hydrocephaly, a brother

					with autism and epilepsy, and a father with a history of epilepsy in childhood
17	*KCNQ2*	c.1118+2T>C		Pathogenic	Infantile/epileptic spasms
18	*KCNQ2*	c.701C>T	p.T234I	Likely pathogenic	Seizures and developmental delay
19	*KCNQ2*	c.821C>T	p.T274M	Likely pathogenic	NA
20	*KCNQ2*	c.841G>A	p.G281R	Likely pathogenic	NA
21	*KCNQ2*	c.1088A>G	p.Y363C	Likely pathogenic	Neonatal seizure disorder and epileptic encephalopathy
22	*KCNT1*	c.2849G>A	p.R950Q	Likely Pathogenic	NA
23	*MECP2*	c.880C>T	p.R294*	Pathogenic	Delayed milestones and intractable epilepsy
24	*PCDH19*	c.814C>T	p.Q272*	Pathogenic	Seizure disorder
25	*PCDH19*	c.1091dupC	p.Y366Lfs*10	Pathogenic	Intellectual disability, autism, and seizures
26	*PCDH19*	c.1265_1266delCA	p.T422Nfs*23	Pathogenic	NA
27	*SCN1A*	c.2584C>T	p.R862*	Pathogenic	Psychomotor epilepsy, intellectual disability, phenotype consistent with Dravet syndrome
28	*SCN1A*	c.4907G>A	p.R1636Q	Likely pathogenic	Epileptic encephalopathy, myoclonic seizures, dystonia, and spasticity
29	*SCN1A*	c.602+1G>A		Pathogenic	Seizures and developmental delay
30	*SCN1A*	c.269T>C	p.F90S	Likely pathogenic	Prolonged seizures, possible myoclonus, and a clinical suspicion for Dravet syndrome
31	*SCN1A*	c.1264G>A	p.V422M	Likely pathogenic	Infantile spasms, focal seizures, hypotonia, bilateral polydactyly, lack of coordination, and grand mal status
32	*SCN1A*	c.1259C>A	p.A420D	Likely pathogenic	NA
33	*SCN1A*	c.302G>A	p.R101Q	Pathogenic	N/A
34	*SCN1A*	c.5389G>C	p.A1797P	Likely Pathogenic	Tonic-clonic and focal seizures
35	*SCN1A*	c.5348C>T	p.A1783V	Pathogenic	NA
36	*SCN1A*	c.4812delG	p.W1604*	Pathogenic	NA

37	*SCN1A*	c.5563C>T	p.P1855S	Likely Pathogenic	NA
38	*SCN1A*	c.1076A>G	p.N359S	Likely Pathogenic	NA
39	*SCN1B*	c.347delC	p.S116Wfs*31	Pathogenic	Hypotonia and seizures
40	*SCN1B*	c.653delG	p.S218Tfs*21	Likely Pathogenic	Developmental delay, seizures with an abnormal EEG, and high myopia
41	*SCN1B*	c.363C>G	p.C121W	Pathogenic	NA
42	*SCN1B*	c.363C>G	p.C121W	Pathogenic	NA
43	*SCN2A*	c.5387_5390dupAGAT	p.M1797Ifs*5	Pathogenic	Seizures and intellectual disability
44	*SCN2A*	c.5645G>A	p.R1882Q	Likely pathogenic	Lack of normal physiological development, autism spectrum disorder, and intractable epilepsy
45	*SCN2A*	c.2558G>A	p.R853Q	Pathogenic	Failure to thrive, developmental delay, speech delay, autism, seizures, dystonia, microcephaly, and gastroesophageal reflux disease
46	*SCN2A*	c.1178C>A	p.T393K	Likely Pathogenic	Seizures and developmental regression
47	*SCN2A*	c.2713A>G	p.K905E	Likely Pathogenic	Epileptic encephalopathy
48	*SCN8A*	c.3985A>G	p.N1329D	Likely pathogenic	Intractable epilepsy
49	*SCN8A*	c.2287A>G	p.I763V	Likely Pathogenic	Seizures and developmental delay
50	*SLC2A1*	c.997C>T	p.R333W	Pathogenic	Febrile seizures, ataxia, hypotonia, hypermobility, and a family history of seizures
51	*SLC2A1*	c.1006C>G	p.L336V	Likely Pathogenic	NA
52	*SLC9A6*	c.508-1G>A		Pathogenic	NA
53	*STXBP1*	c.1029+1G>T		Pathogenic	Seizures, developmental delay, and cognitive impairment.
54	*STXBP1*	c.364C>T	p.R122*	Pathogenic	Infantile/epileptic spasms and tonic seizures

55	*STXBP1*	c.548T>C	p.L183P	Likely Pathogenic	NA
56	*STXBP1*	c.704G>A	p.R235Q	Likely Pathogenic	Generalized, absence, and tonic-clonic seizures, infantile spasms, hypotonia, developmental delay, and cerebral palsy
57	*SYN1*	c.377G>A	p.W126*	Pathogenic	NA
58	*TPP1* [a]	c.509-1G>C		Pathogenic	NA
59	*TPP1*	c.509-1G>C, c.1016G>A	p.R339Q	Pathogenic	Seizures and speech delay
60	*TSC2*	c.4415delG	p.G1472Afs*4	Pathogenic	Seizure disorder and intellectual disability
61	*TSC2*	c.3598C>T	p.R1200W	Pathogenic	Infantile spasms and global developmental delay
62	*ZEB2*	c.1876G>T	p.G626*	Pathogenic	Developmental delay and psychomotor epilepsy
Potentially Causative Variants					
Case	**Gene**	**Nucleotide**	**Protein**	**Inheritance**	**Phenotype provided**
63	*CACNA1A*	c.5017C>T	p.R1673C	Unknown	Generalized convulsive epilepsy and hemiplegia
64	*CACNA1A*	c.4177G>A	p.V1393M	Unknown	Global developmental delay, seizures, and tremor
65	*CACNA1A*	c.4177G>A	p.V1393M	Unknown	NA
66	*CDKL5*	c.541G>A	p.E181K	Unknown	NA
67	*HCN1*	c.990G>C	p.W330C	Unknown	Epileptic encephalopathy, abnormal EEG, and other clinical features suggestive of Ohtahara syndrome
68	*FLNA*	c.4237G>A	p.E1413K	Unknown	Hypotonia, epilepsy, and abnormal MRI
69	*KCNQ2*	c.1627G>A	p.V543M	Inherited	Developmental delay, seizures, a ventricular septal defect, unilateral cryptorchidism, and macrocephaly
70	*KCNQ2*	c.1627G>A	p.V543M	Unknown	NA
71	*KCTD7*	c.190A>G, c.793G>A	p.T64A, p.G265R	Inherited from both parents	Status epilepticus, generalized, tonic-clonic, and myoclonic seizures

72	*MEF2C*	c.121T>C	p.C41R	Unknown	Generalized and myoclonic seizures, intellectual disability, hypotonia, spasticity, and muscle weakness
73	*NRXN1*	c.3619C>T	p.R1207*	Unknown	Autism, epilepsy, and developmental delay
74	*PNKP* [a]	c.1324G>A	p.G442S	Unknown	NA
75	*RELN*	c.1817C>T, c.2201T>A	p.T606I, p.V734D	Inherited from both parents	Cerebral palsy, abnormal EEG, developmental delay, and lack of coordination
76	*SCN1A*	c.638C>G	p.S213W	Unknown	Febrile and afebrile seizures and developmental delay
77	*SCN1A*	c.1703G>A	p.R568Q	Unknown	Seizures
78	*SCN1A*	c.2923G>C	p.V975L	Unknown	Seizures and developmental delay
79	*SCN2A*	c.4156T>G	p.C1386G	Unknown	Seizures, speech and developmental regression
80	*SCN8A*	c.491C>T	p.T164M	Inherited from affected mother	Focal seizures, developmental delay, failure to thrive, and a maternal family history of seizures
81	*SCN8A*	c.605T>A	p.I202N	Unknown	Seizures, developmental delay, dysmorphic features, pica, paroxysmal behavior with nonspecific encephalopathy on EEG, and a brother who also has seizures
82	*SCN8A*	c.1241A>T	p.Y414F	Unknown	NA
83	*TPP1* [a,ǂ]	c.523C>T	p.R175C	Unknown	Infantile spasms, learning disability, developmental delay, seizures, and hypotonia

NA, phenotype was not available

[a]Indicates a homozygous variant

[ǂ]This individual also had a novel homozygous missense variant in *SLC25A22*

2.5 Discussion

In this study, we determined the diagnostic yield of a clinical gene panel in an unselected cohort of 339 physician-referred epilepsy patients. This is one of the largest unselected epilepsy cohorts to be examined in this manner. We detected pathogenic or likely pathogenic variants in 18% of screened individuals, consistent with two recent studies that used gene panels to screen selected cohorts of individuals with severe, early-onset seizures, developmental delay, and intellectual disability (Trump et al., 2016; Zhang et al., 2015).

We also observed potentially causative variants in an additional 6% of screened cases (21 individuals). These variants occurred in many of the same genes as the pathogenic and likely pathogenic variants (**Fig. 2.1**), but were generally novel missense changes, which are more difficult to interpret clinically and will require additional functional evidence to be classified as pathogenic/likely pathogenic. Classification of these variants was often made more challenging due to a lack accompanying clinical information and parental samples to assist with the determination of variant inheritance.

The majority of the disease-causing variants identified in our study were detected in a small number of genes. *SCN1A* had the largest number of variants, followed by *KCNQ2,* together accounting for nearly 30% of the cases with a pathogenic, likely pathogenic, or potentially causative variant. Furthermore, multiple individuals had variants in *CDKL5*, *SCN2A*, *SCN8A*, *STXBP1,* and *PCDH19*, genes which are associated with epileptic encephalopathies, and in which pathogenic variants are consistently identified using gene panels and whole exome sequencing in different epilepsy studies (Allen et al., 2013; Carvill et al., 2013a; Trump et al., 2016). We also observed two or

more variants each in *CACNA1A*, *GABRA1*, *GRIN2A*, *SCN1B*, *SLC2A1*, *TPP1*, and *TSC2*. Pathogenic variants in these genes can cause severe seizures and epileptic encephalopathy, but each gene has also been associated with other clinical phenotypes, for instance movement disorders, migraine, and milder forms of epilepsy. Although we were limited in the amount of clinical information provided with each case, the identified genes would suggest that the individuals with pathogenic or potentially causative variants are most likely affected with severe forms of childhood epilepsy. This highlights the benefit of genetic testing in children with severe epilepsy.

Sixteen additional genes had variants that were seen in only one patient. This low frequency may be explained for some genes, such as *ZEB2* (Mowat-Wilson syndrome) and *MECP2* (Rett syndrome), which typically present with very distinctive phenotypes that may prompt gene-specific testing rather than broader screening on an epilepsy gene panel. In other instances, affected individuals may be less likely to be referred for an epilepsy-specific gene panel. For example, individuals with pathogenic variants in *FLNA* and *RELN*, which are associated with periventricular nodular heterotopia and lissencephaly, respectively, may be referred for testing on a brain malformations panel rather than an epilepsy panel. On the other hand, the individuals with variants in these genes that were referred for screening on an epilepsy-specific panel may represent cases with atypical presentation. For example, we identified the compound heterozygous *RELN* variants, Thr606Ile and Val734Asp, in an individual with epilepsy, cerebral palsy, and developmental delay, but without any significant magnetic resonance imaging (MRI) findings (**Table 2.2**). These individual cases emphasize the utility of referring individuals with unexplained epilepsy for a gene panel rather than testing single genes.

The epilepsy panel used in this study contained 110 genes curated for association with epilepsy and seizures (**Table 2.1**). Because of their known role in epilepsy, the majority of these genes are present on other commercially-available epilepsy panels. Therefore, it is likely that a similar diagnostic yield would be obtained if the cohort examined in this study was tested using another epilepsy panel, especially since most of the pathogenic variants were identified in a small number of genes (**Fig. 2.1**).

In summary, we identified 87 variants in 30 different genes that could explain disease, of which 54% were not previously reported. This study has the potential to aid future variant interpretation and confirms the utility of targeted gene panel analysis in individuals with epilepsy.

2.6 Acknowledgments

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CHAPTER 3
DE NOVO AND INHERITED *SCN8A* EPILEPSY MUTATIONS DETECTED BY GENE PANEL ANALYSIS

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3.1 Summary

During the analysis of the ESD panel data presented in Chapter 2, I identified five novel missense variants in the *SCN8A* voltage-gated sodium channel that were all classified as VUS. At the time, *SCN8A* was a relatively new epilepsy gene, making interpretation of these variants more difficult. We were intrigued by the frequency of rare variants identified in *SCN8A*, which was similar to better-known epilepsy genes such as *SCN2A* and *CDKL5*. This prompted us to seek parental samples and medical records to attempt to reclassify the variants, either as pathogenic or benign. Additionally, we were eager to investigate the clinical phenotypes of the patients, as little was known about the phenotypic spectrum of *SCN8A* at the time.

Of the five identified *SCN8A* variants, three were *de novo*, one was inherited from an affected parent, and one was inherited from an unaffected parent. Four of the individuals had epilepsy and developmental delay/intellectual disability. The remaining individual had a milder epilepsy presentation without cognitive impairment and had family history of mild epilepsy. Furthermore, taking advantage of the fact that the Mendeliome library is used for all panels at EGL, we also investigated the contribution of *SCN8A* to other neurodevelopmental disorders, including autism spectrum disorder, intellectual disability, and neuromuscular disorders. This analysis yielded an amino acid substitution at an evolutionarily conserved residue in a patient who was screened on the autism spectrum disorder panel, potentially suggesting a role for *SCN8A* in autism.

Finally, I examined the distribution of pathogenic *SCN8A* variants across the $Na_v1.6$ channel and identified four distinct clusters of epilepsy variants. These clusters were primarily located in regions of the channel that are important for the kinetics of

channel inactivation. One of the clusters occurred in a region of the sodium channel that is encoded by two developmentally-regulated alternative exons referred to as 5N and 5A.

One contribution of this study to the field of epilepsy genetics was the identification of mutation clustering in exon 5N, encoding part of the first repeat domain of *SCN8A*. More specifically, I recognized that diagnostic laboratories, including EGL, were not analyzing the 5A exon and were potentially missing variants in *SCN8A* that could contribute to disease. My hypothesis was later supported by a report from the Epilepsy Genetics Initiative in which three *de novo* variants in the 5A exon of *SCN8A* were identified in epilepsy patients who had undergone WES.

The manuscript that resulted from my work on *SCN8A* is reproduced below with some minor additions.

3.2 Introduction

Voltage-gated sodium channels (VGSCs) are important regulators of neuronal excitability. As a result, pathogenic variants in VGSCs are responsible for a number of pathophysiological conditions, particularly epilepsy. The VGSC *SCN8A* encodes the pore-forming α-subunit $Na_v1.6$, which is expressed in excitatory and inhibitory neurons throughout the central and peripheral nervous systems (Caldwell et al., 2000; Oliva et al., 2012).

VGSC α-subunits are made up of four homologous domains (DI-DIV), each containing six transmembrane segments (S1-S6). Mutations in the mouse homolog of *SCN8A* (*Scn8a*) were originally associated with motor disorders based on the phenotypes of more than ten different mouse lines with null or hypomorphic alleles of *Scn8a*. *Scn8a*

mouse mutants exhibit a range of phenotypes, including ataxia, tremor, dystonia, hind limb paralysis, and premature lethality with recessive inheritance (Meisler et al., 2001; O'Brien and Meisler, 2013). These mice do not exhibit spontaneous convulsive seizures, and furthermore, mice heterozygous for these mutant alleles were found to be more resistant to chemically and electrically induced seizures (Makinson et al., 2014; Martin et al., 2007). In 2006 the identification of a heterozygous, frameshift *SCN8A* variant in a patient exhibiting intellectual disability, cerebellar atrophy, and ataxia was consistent with the reported mouse models of *Scn8a* dysfunction (Trudeau et al., 2006).

The first *SCN8A* epilepsy mutation was identified in 2012 by whole-genome sequencing (WGS) in a patient with severe epileptic encephalopathy who exhibited early-onset seizures, autistic features, intellectual disability, ataxia, and sudden unexpected death in epilepsy (SUDEP) (Veeramah et al., 2012). The heterozygous missense variant, p.N1768D, was determined to be *de novo* in the patient. Since this initial discovery, there has been a sharp rise in the number of identified pathogenic *SCN8A* variants in patients with epilepsy, with over one hundred mutations reported to date (Meisler et al., 2016). Most of the *SCN8A* variants have been detected in individuals with early infantile epileptic encephalopathy (EIEE), similar to the initial patient. Furthermore, nearly all reported mutations are missense variants that were *de novo* or inherited from an unaffected parent who was found to be mosaic. Functional analysis of eight variants revealed gain-of-function effects as the predominant pathogenic mechanism, although two of the variants produced apparent loss-of-function effects *in vitro* (Blanchard et al., 2015; de Kovel et al., 2014; Estacion et al., 2014; Veeramah et al., 2012; Wagnon et al., 2016). In 2015, Wagnon et al. reported the generation of a mouse expressing the

p.N1768D mutation in the orthologous *Scn8a* gene. Unlike previous mouse lines, these mutants exhibit spontaneous seizures and premature lethality (Wagnon et al., 2015).

Recently, Gardella et al. reported three unrelated families carrying the same *SCN8A* missense variant, p.E1483K, in the inactivation gate of the channel (Gardella et al., 2016). Interestingly, all three families exhibited benign infantile seizures. This was the first report of *SCN8A*-associated epilepsy that showed a more benign seizure course without intellectual disability. The authors speculated that this variant in the inactivation gate might have a more modest effect on channel inactivation, thereby resulting in the milder phenotype seen in the three families.

While the application of whole-genome and -exome sequencing to severe epilepsy cases was essential to the discovery of the initial *SCN8A* pathogenic variants, many variants are now being identified via gene panel analysis (also known as targeted resequencing). One advantage of targeted gene panel analysis over whole-exome sequencing is that read coverage is increased across the genes of interest, thus reducing the possibility that clinically important variants in these genes are missed. More than 15 commercial epilepsy panels that include sequencing of *SCN8A* are currently available.

Here we report five new *SCN8A* epilepsy variants identified via gene panel analysis. We report several cases that have phenotypes consistent with previous reports for *SCN8A*-encephalopathy, as well as one case with an inherited *SCN8A* variant and a milder epilepsy presentation.

3.3 Materials and Methods

Patients

Sequence data from 275 patients screened by the Emory Genetics Laboratory (EGL) using the Epilepsy and Seizure Disorders (ESD) panel were reviewed for this study. Two additional patients with *SCN8A* variants had targeted gene panel testing performed by other diagnostic laboratories (GeneDx and Athena Diagnostics). All patients had epilepsy as one of the indications for genetic testing. Peripheral blood samples were obtained from family members to test variant inheritance after written consent was obtained. Clinical information for each patient was obtained from their corresponding clinicians. This study was approved by the Institutional Review Board of Emory University.

Targeted gene panel sequencing

Targeted gene panel analysis at EGL was performed as described in the Methods section of Chapter 2.

Sanger sequencing

All *SCN8A* variants were confirmed by Sanger sequencing. Parent and sibling samples were also Sanger sequenced to determine whether the *SCN8A* variant segregated with disease or arose *de novo*.

Deep amplicon sequencing

Sequencing of a 392-base pair amplicon encompassing the *SCN8A* c.2287A>G (p.I763V) variant from Patient 1 and his parents to approximately 400,000X coverage was performed by the Emory Integrated Genomics Core (EIGC).

3.4 Results

3.4.1 Identification of novel *SCN8A* epilepsy mutations

All patients had seizure onset within the first year of life and were referred for genetic testing. The identified *SCN8A* amino acid substitutions were distributed across the channel: one in the N-terminal domain (p.K101R), one in the DIIS1 (p.I763V), one in the linker between DIIIS4 and DIIIS5 (p.N1329D), one in DIIIS5 (p.L1332R), and one in the C-terminal domain (p.N1877S) (**Fig. 3.1A**). All five variants occur at evolutionarily conserved amino acid positions (**Fig. 3.1B**) and were predicted to be damaging to protein function by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) prediction algorithms. **Table 3.1** summarizes the clinical features of the patients.

3.4.2 Patient Descriptions

3.4.2.1 Patient 1: *SCN8A*, c.2287A>G, p.I763V

Patient 1 presented with seizures at four months of age and was recognized to have a subarachnoid hemorrhage. The hemorrhage was drained via a burr hole; however, he continued to have multiple generalized tonic-clonic seizures requiring over 20 hospitalizations, and at least one instance of status epilepticus. He is developmentally delayed, especially for speech and language. Electroencephalograms (EEGs) show a mild generalized slowing of the background with rare left frontal epileptiform discharges. Magnetic resonance imaging (MRI) showed areas of decreased linear signal as a result of the subarachnoid hemorrhage. Interestingly, the individual has a fraternal twin brother

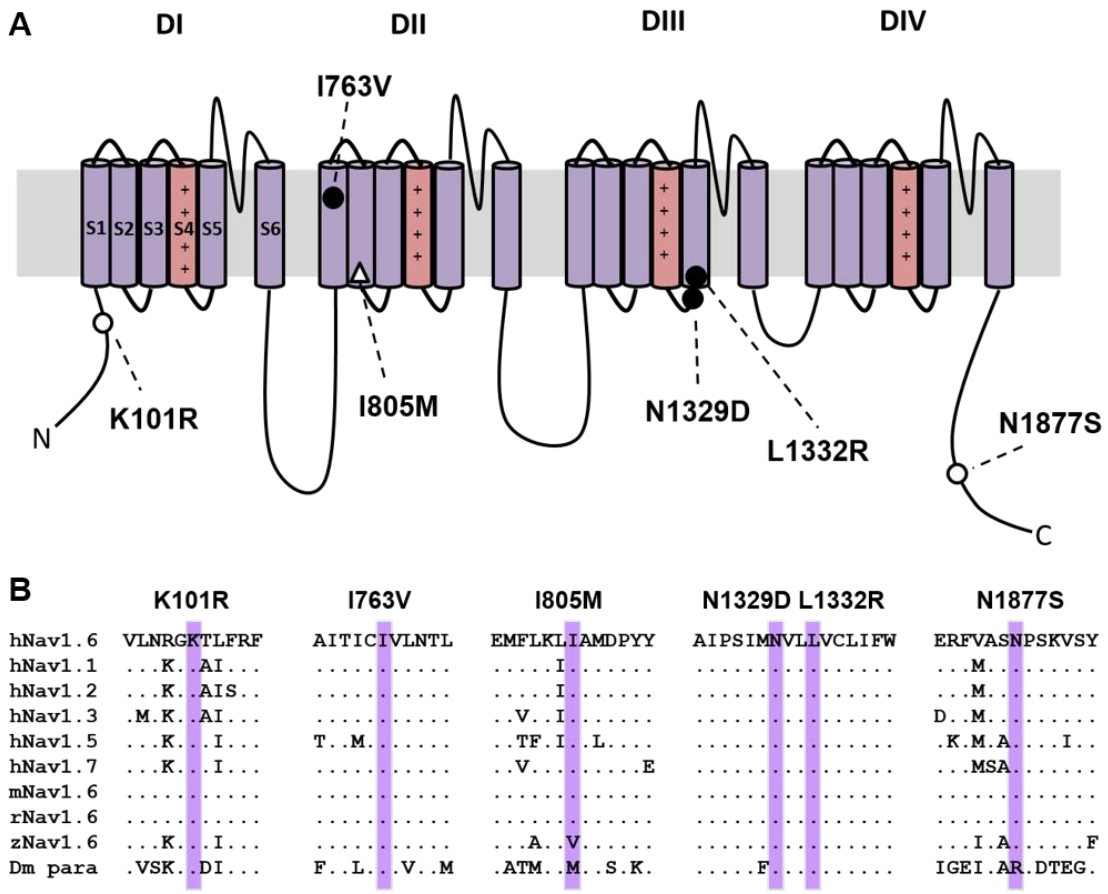

Figure 3.1 Location within the $Na_v1.6$ channel (A) and evolutionary conservation (B) of the identified *SCN8A* variants. (A) Location of the identified variants in the $Na_v1.6$ channel. Roman numerals correspond to the four repeat domains of the channel (DI-DIV). Cylinders represent the six transmembrane segments of each domain; red cylinders denote the voltage-sensing S4 domains. Filled circles denote *de novo* epilepsy variants. Open circles indicate inherited epilepsy variants. The triangle indicates a variant detected from an autism panel. (B) Protein alignment of the human, mouse, rat, zebrafish, and fruit fly VGSC α-subunits. $hNa_v1.6$: human, $mNa_v1.6$: mouse, $rNa_v1.6$: rat, $zNa_v1.6$: zebrafish, Dm para: *Drosophila melanogaster*.

Table 3.1 Clinical characteristics of epilepsy patients with *SCN8A* variants

Case	Patient 1	Patient 2	Patient 3
Sex	Male	Female	Male
Age	5 years	4 years	11 months
Nucleotide[a]	c.2287A>G	c.3985A>G	c.3995T>G
Protein	p.I763V	p.N1329D	p.L1332R
Inheritance	*de novo*	*de novo*	*de novo*
Variant Classification	Pathogenic	Pathogenic	Pathogenic
Family history	Affected fraternal twin brother	none	none
Age at onset	4 months	3 months	1 month
Diagnosis	Intractable epilepsy	Epileptic encephalopathy	Infantile spasms
Development	Delayed, especially speech and language	Global developmental delay	Developmental delay
EEG	Mild generalized slowing of background with rare left frontal epileptiform discharges	Diffuse cerebral dysfunction, slowing and disorganization of background, multifocal epileptiform discharges	Background disorganization and slowing, multiform epileptiform discharges
MRI	Linear areas of decreased signal from chronic subarachnoid hemorrhage	Mild cerebral volume loss	Left frontal lobe cortical dysplasia
Motor development	Mildly delayed	Wheelchair bound, minimal head control	Delayed
Other phenotypes	Obstructive sleep apnea, subdural hematoma, status epilepticus	Hypotonia, poor vision, nystagmus, feeding difficulties, GERD	Hypotonia, GERD, brachycephaly, nystagmus
Response to treatment	Refractory	Refractory	Refractory

Table 3.1 Continued

Case	Patient 4	Patient 5
Sex	Male	Female
Age	6 years	15 years
Nucleotide[a]	c.5630A>G	c.302A>G
Protein	p.N1877S	p.K101R
Inheritance	Inherited from affected father	Inherited from unaffected mother
Variant Classification	Likely Pathogenic	VUS
Family history	Affected brother and father	none
Age at onset	9 months	1 month
Diagnosis	Nocturnal Partial Complex seizures with secondary generalization	Seizures
Development	Normal	Developmental delay, intellectual disability, speech delay
EEG	Near continuous sleep enhanced spike and wave activity, generalized or shifting predominance between left and right anterior areas	Sharp spikes and waves in sleep
MRI	Normal	Inferior vermis hypoplasia
Motor development	Normal	---
Other phenotypes	---	Hypotonia, precocious puberty, short stature, facial dysmorphism
Response to treatment	Seizure-free for 3 years on LCM. Marked improvement on PHT	Seizure-free for several years on OXC

[a]Variants are annotated according to the reference transcript NM_014191.3.

Abbreviations: VUS, Variant of unknown significance; GERD, gastroesophageal reflux disease; EEG, electroencephalogram; MRI, magnetic resonance imaging; LCM, lacosamide; PHT, phenytoin; OXC, oxcarbazepine.

who had a cystic subdural hygroma and is similarly affected by seizures and developmental delays.

Genetic testing of Patient 1 at EGL uncovered heterozygous variants in three known epilepsy genes: *PRICKLE1* (c.2002T>A, p.S668T), *KCNQ3* (c.1720C>T, p.P574S), and *SCN8A* (c.2287A>G, p.I763V), as well as a deletion at 2q23.1 encompassing noncoding exon 5 of the *MBD5* gene (chr2: 149,057,023-149,166,288) (hg19). The *PRICKLE1* variant was not tested further since *PRICKLE1* is associated with autosomal recessive disease, and only one variant was detected in the patient. Parental testing detected the *MBD5* deletion in the unaffected mother and the *KCNQ3* variant in the unaffected father. The *KCNQ3* variant has also been observed 242 times in the ExAC database, indicating that it is likely to be a benign variant. In contrast, neither parent carried the *SCN8A* variant. The affected twin brother was also found to carry the *MBD5* deletion and the *SCN8A* missense variant. *SCN8A* p.I763V is located within the DIIS1 transmembrane domain near the previously published T767I epilepsy mutation (Estacion et al., 2014). The isoleucine residue is evolutionarily invariant in all members of the VGSC family (**Fig. 3.1**), and the change to valine is predicted to be damaging by PolyPhen-2 and SIFT.

Because the *SCN8A* variant was detected in both the patient and his brother but was absent in the parents, we attempted to determine whether one of the parents was mosaic. However, we could not detect the variant in DNA extracted from blood by deep amplicon sequencing in either parent (up to 400,000X coverage, data not shown). We conclude that mosaicism is likely to be limited to the germline of one of the parents. This variant has not been observed in the ExAC database but has been reported once as a

variant of unknown significance (VUS) from an epilepsy patient by GeneDx in ClinVar. Although we believe that the *SCN8A* variant is the causative mutation in these two brothers, we cannot rule out the possibility that the deletion of *MBD5* noncoding exon 5 may also contribute to the disease presentation in these individuals since heterozygous deletions of *MBD5* are associated with developmental delay, seizures, and language impairment due to haploinsufficiency. While deletions confined to the noncoding exons of *MBD5* (exons 1-5) have been reported in disease previously (Talkowski et al., 2011), none have been limited to exon 5 as in Patient 1, making it difficult to compare across cases. Exon 5 is the most proximal noncoding exon to the first coding exon (exon 6); however, this region is not evolutionary conserved, and the deletion was inherited from an unaffected parent.

3.4.2.2 Patient 2: *SCN8A*, c.3985A>G, p.N1329D

Patient 2 exhibited normal development until seizure onset at three months of age. She subsequently developed refractory epilepsy, global developmental delays, poor vision and nystagmus, and feeding difficulties and gastroesophageal reflux disease (GERD). EEG analysis showed diffuse cerebral dysfunction and slowing and disorganization of background with multifocal epileptiform discharges. MRI showed mild loss of cerebral volume. Previous genetic testing included a chromosomal microarray, an Infantile Epilepsy Panel, fragile X *FMR1* testing, and sequencing of *NPC1* and *NPC2*. *NPC2* sequencing revealed a heterozygous variant (c.352G>A, p.E118K), and the Infantile Epilepsy Panel uncovered a heterozygous variant of uncertain significance in *ALDH7A1* (c.235A>G, p.R79G). Since neither variant was thought to contribute to the patient's clinical phenotype, the ESD panel was ordered at EGL. This test not only

confirmed the presence of the *ALDH7A1* variant but also identified a heterozygous variant in *SCN8A* (c.3985A>G, p.N1329D) and a heterozygous intronic duplication in *CNTNAP2*. Parental testing revealed that the *SCN8A* variant was *de novo* in the patient, and parental identity was confirmed with microsatellite analysis. *ALDH7A1* and *CNTNAP2* were excluded from further analysis since these genes are associated with autosomal recessive disorders and were not consistent with the phenotype. *SCN8A* p.N1329D has not been seen before in disease or in the ExAC database. Furthermore, it is located in the intracellular linker between DIIIS4 and DIIIS5, in which several pathogenic variants have already been identified, including p.I1327V and p.L1331V. N1329 is evolutionarily invariant across species and the other VGSCs, and the change to a negatively-charged aspartic acid is predicted to be damaging.

3.4.2.3 Patient 3: *SCN8A*, c.3995T>G, p.L1332R

Patient 3 presented with abnormal involuntary movements at one month of age, and EEG analysis revealed hypsarrhythmia suggestive of infantile spasms. EEG at two months showed mild background slowing and disorganization with multifocal epileptiform discharges consistent with epileptic encephalopathy. He displays global developmental delays, hypotonia, nystagmus, and brachycephaly. MRI showed left frontal lobe cortical dysplasia. Chromosomal microarray analysis was normal. Epilepsy gene panel testing performed by GeneDx (Infantile Epilepsy panel) identified heterozygous variants in *SCN8A* (c.3995T>G, p.L1332R) and *KCNJ10* (c.1043G>A, p.R348H). Testing of the parents did not uncover the *SCN8A* variant, indicating that the variant arose *de novo* in the patient. The *KCNJ10* variant was not tested since it is associated with autosomal recessive disease and only one variant was detected. *SCN8A*

p.L1332R has not been seen previously in disease or in the ExAC database, but is directly adjacent to the reported pathogenic variant, p.L1331V, in the DIIIS5 transmembrane domain of the channel. The p.L1332R substitution affects an evolutionarily invariant residue and is predicted to be damaging by PolyPhen-2 and SIFT.

3.4.2.4 Patient 4: *SCN8A*, c.5630A>G, p.N1877S

Patient 4 presented with seizures at nine months of age, which included staring spells during wakefulness and later included generalized tonic-clonic seizures during sleep. He was started on oxcarbazepine, which controlled his father's seizures, but developed a severe allergic reaction. Levetiracetam was ineffective. Seizures were fully controlled for three years on lacosamide but then recurred. He continued to have 1-2 seizures during sleep per month despite treatment. Initial EEG was normal. EEG at age six years revealed very active, sleep enhanced spike and wave activity, which was generalized or shifting predominance between left and right anterior areas. The individual performs reasonably well in school but is somewhat behind his twin sister. He has had marked improvement, but not complete control, on phenytoin.

The individual has a family history of epilepsy. His father (**Fig. 3.2**, I:1) started having seizures at six months of age and continued to have frequent seizures despite treatment with phenobarbital until age six, at which point his seizures improved. At age 13, treatment was switched to carbamazepine, from which point he continued to have seizures about twice a year. Seizures stopped in his late teens, but he has had seizures on several occasions when he tried to stop taking his medication. All EEGs and MRIs were reported to be normal, and the father has normal cognition. Patient 4 also has a younger brother (**Fig. 3.2,** II:3) who presented with seizures at 7 months of age. The younger

brother's EEG was normal, and his seizures are fully controlled on oxcarbazepine. An epilepsy gene panel performed by Athena Diagnostics (Epilepsy Advanced Sequencing Evaluation) identified a number of heterozygous single nucleotide variants in the younger brother, including *SCN8A* (c.5630A>G, p.N1877S), *GRIN2A* (c.4353A>T, p.R1451S), *TSC1* (c.1631G>A, p.G544E), *ATP2A2* (c.1912A>G, p.I638V), and *LBR* (c.899A>G, p.Y300C). We performed familial segregation analysis for the *SCN8A*, *GRIN2A*, *ATP2A2*, and *TSC1* variants (*LBR* was excluded since seizures have only been seen in homozygous patients and only one variant was identified from panel testing). The *SCN8A* variant was present in all three affected family members, but was absent from the unaffected mother (I:2) and the patient's unaffected twin (II:1). The *GRIN2A* variant was present in the father (I:1) and younger brother (II:3) but absent from Patient 4 (II:2). The *ATP2A2* variant was present in the unaffected mother (I:2), unaffected sister (II:1), and the younger brother (II:3). Finally, the *TSC1* variant was detected in the mother and both her sons. This pattern of inheritance lends support to the *SCN8A* variant being causative, although we cannot rule out the possibility that the *GRIN2A* variant carried by the father and the younger brother may act as a modifier, decreasing the severity of their epilepsy and the EEG abnormalities.

The *SCN8A* p.N1877S variant resides in the C-terminal domain of the channel, proximal to the mutational hotspot p.R1872, at which three different amino acid substitutions have been identified in ten unrelated epilepsy patients (Wagnon et al., 2016). N1877S is predicted to be damaging by PolyPhen-2 and SIFT. *SCN8A* p.N1877S has not been observed in the ExAC database but has been reported twice in ClinVar (once as a VUS and once as a likely pathogenic *de novo* variant) and has recently been

reported in a father and son with early-onset epilepsy without cognitive impairment (Anand et al., 2016). This additional instance of parental transmission and milder epilepsy presentation associated with the same *SCN8A* variant again lends support to the prediction that this is a pathogenic variant.

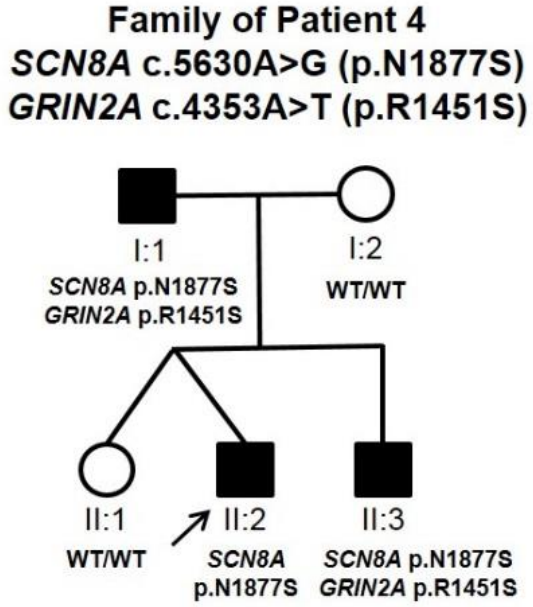

Figure 3.2 Pedigree of Patient 4 showing the inheritance pattern of the *SCN8A* and *GRIN2A* variants. Filled symbols indicate affected individuals. The arrow denotes Patient 4.

3.4.2.5 Patient 5: *SCN8A*, c.302A>G, p.K101R

Seizure onset in Patient 5 began in the neonatal period, and seizures occurred approximately once a year between ages one and four. At age seven, she began to have more frequent seizures, which were controlled on oxcarbazepine. Staring spells were also reported. The individual's history is significant for developmental delay, aphasia,

hypotonia, short stature, and moderate to severe cognitive impairment. Patient 5 also exhibits craniofacial abnormalities, including brachycephaly with a broad, tall forehead, bilateral epicanthal folds, flattening of the nasal bridge, low hairline, very full lips, and a high arched palate. EEG revealed sharp waves and spikes in sleep; however, this activity was brief and did not correlate with any clinical events. MRI showed the stable appearance of an inferior vermian hypoplasia. Prior genetic testing included two microarrays to detect unbalanced chromosomal abnormalities, *UBE3A* sequence and deletion/duplication analysis, and *SNRPN* methylation analysis, all of which were normal. Testing on EGL's ESD panel uncovered a heterozygous variant in the N-terminal domain of *SCN8A* (c.302A>G, p.K101R). K101 is evolutionarily invariant, and the identified substitution is predicted to be damaging by PolyPhen-2 and SIFT. Parental testing revealed that the variant was inherited from her unaffected mother. The p.K101R variant was also reported once in the ExAC database. The presence of this variant in an unaffected parent raises two possibilities: the variant might not be the cause of disease in Patient 5, or this variant may exhibit reduced penetrance in the mother.

Further sequence analysis revealed the presence of a known pathogenic variant in the *PACS1* gene (c.607C>T, p.R203W) in Patient 5. *PACS1* p.R203W has been reported previously as a *de novo* variant from whole-exome sequencing in three unrelated male patients with intellectual disability and similar facial dysmorphisms (Gadzicki et al., 2015; Helbig et al., 2016). Two of these patients had seizures in the neonatal period similar to Patient 5. Parental testing did not detect the *PACS1* variant in the mother; the father was unavailable for testing. *PACS1* p.R203W has also been reported once in the ExAC database.

3.4.3 *SCN8A* variation in other neurodevelopmental disorders

In addition to seizures, individuals with *SCN8A* encephalopathy often experience other neurodevelopmental abnormalities, including intellectual disability, autism, and movement disorders such as ataxia and dystonia (Larsen et al., 2015). To investigate whether variation in *SCN8A* contributes to these other neurodevelopmental disorders with or without seizures, we examined variants compiled from gene panel testing at EGL for autism spectrum disorder, intellectual disability, and neuromuscular disorders. A total of 107 *SCN8A* variants were detected from 91 patients (52 autism referrals, 26 intellectual disability referrals, and 13 neuromuscular referrals). Seventy-two variants (67%) were located in intronic sequences, none of which affected consensus splice sites. Twenty-three variants (22%) were synonymous substitutions and were not predicted to affect the protein sequence or splicing. Twelve patients (13%) were found to have missense substitutions that altered the amino acid sequence of the protein. The polymorphisms c.3076C>T (p.R1026C) and c.2098A>T (p.I700L) were identified in six individuals and one individual, respectively (**Table 3.2**). Of the remaining five missense variants, only one (p.I805M) altered an evolutionarily conserved residue and was predicted to be damaging by *in silico* analysis (Adzhubei et al., 2010). I805M is located within the DIIS2 transmembrane domain (**Fig. 3.1**), and substitution of this amino acid has not been observed in ExAC or in any other database. This variant was identified from an autism spectrum disorder referral; however, we do not have information on the clinical presentation of the patient. Three of the other four rare missense variants (p.I68V, p.G1050S, p.R1960Q) have been reported before in the ClinVar database. The N-

Table 3.2 *SCN8A* variants identified from panel testing for other neuro-developmental disorders

Variant[a]	Protein	No. Obs.	Location in Channel	Evolutionarily Conserved	ExAC[b]	Gene Panel
c.202A>G	p.I68V	1	NT	No	0	NM
c.2098A>T	p.I700L	1	CL1	No	315	ID
c.2415A>G	p.I805M	1	DIIS2	Yes	0	ASD
c.3076C>T	p.R1026C	6	CL2	No	1418	ASD, ID
c.3097C>G	p.P1033A	1	CL2	No	0	ASD
c.3148G>A	p.G1050S	1	CL2	No	10	ASD
c.5879G>A	p.R1960Q	1	CT	No	3	ASD

[a]Variants are annotated according to the reference transcript NM_014191.3.

[b]Number of times observed in the ExAC database out of approximately 121,400 alleles

Abbreviations: ASD, Autism spectrum disorder; ID, Intellectual disability; NM, Neuromuscular; NT, N-terminus; CL1, Cytoplasmic loop 1; CL2, Cytoplasmic loop 2; CT, C-terminus.

terminal variant (p.I68V) was reported as a likely benign variant by the Genetics Services Laboratory at University of Chicago. The C-terminal variant (p.R1960Q) was reported as a VUS from an infant-epilepsy panel by GeneDx and has been seen three times in the ExAC database. The p.G1050S variant was reported previously as a VUS from an epilepsy panel by GeneDx, once as a likely pathogenic *de novo* variant in a patient with hemiplegic cerebral palsy and intellectual disability, and ten times in the ExAC database (McMichael et al., 2015). At this time, it is unclear whether these rare variants contribute to disease or represent benign variants. *SCN8A* may therefore contribute to other neurodevelopmental disorders, and additional screening for *SCN8A* mutations should be

conducted to explore the full spectrum of phenotypes associated with *SCN8A* dysfunction.

3.4.4 Mutation clusters within the $Na_v1.6$ channel

When the locations of all the reported *SCN8A* epilepsy variants are overlaid on the $Na_v1.6$ channel, several patterns emerge (**Fig. 3.3**). Similar to reports for the other VGSCs, many variants reside within the transmembrane domains of the channel, especially the voltage-sensing S4 segment of each repeat domain; however, several intriguing clusters of variants are observed outside of the transmembrane domains. One of these clusters involves the extracellular linker between DIS3 and DIS4 (**Fig. 3.3**, **cluster A**). Interestingly, this region of the channel is encoded by two mutually exclusive, alternatively spliced exon 5s (5N and 5A). The usage of these exons is developmentally regulated, with preferential usage of exon 5N early in development and exon 5A during adulthood. Inclusion of each exon has been reported to differentially alter the excitability of the encoded channel (Fletcher et al., 2011; Gazina et al., 2010; Raymond et al., 2004). To date, all the pathogenic variants identified in this region are located in exon 5N. Another observed cluster of variants is located in the 19-amino acid linker between the DIIIS4 and DIIIS5 domains (**cluster B**). Clustering of pathogenic variants in this region has also been observed for the $Na_v1.2$ channel (Howell et al., 2015). This linker region is reported to participate in channel inactivation through stabilizing interactions with the nearby inactivation gate (Smith and Goldin, 1997). Consistent with this, we also see a cluster of pathogenic *SCN8A* variants within the inactivation gate, which is located between DIIIS6 and DIVS1 (**cluster C**). Finally, there appears to be a cluster of pathogenic variants in the C-terminus, including the recurrently mutated position at

p.R1872 (**cluster D**). Previous electrophysiological analysis of substitutions at R1872 revealed a delay in the inactivation of the mutant channels, which is predicted to increase channel activity (Wagnon et al., 2016).

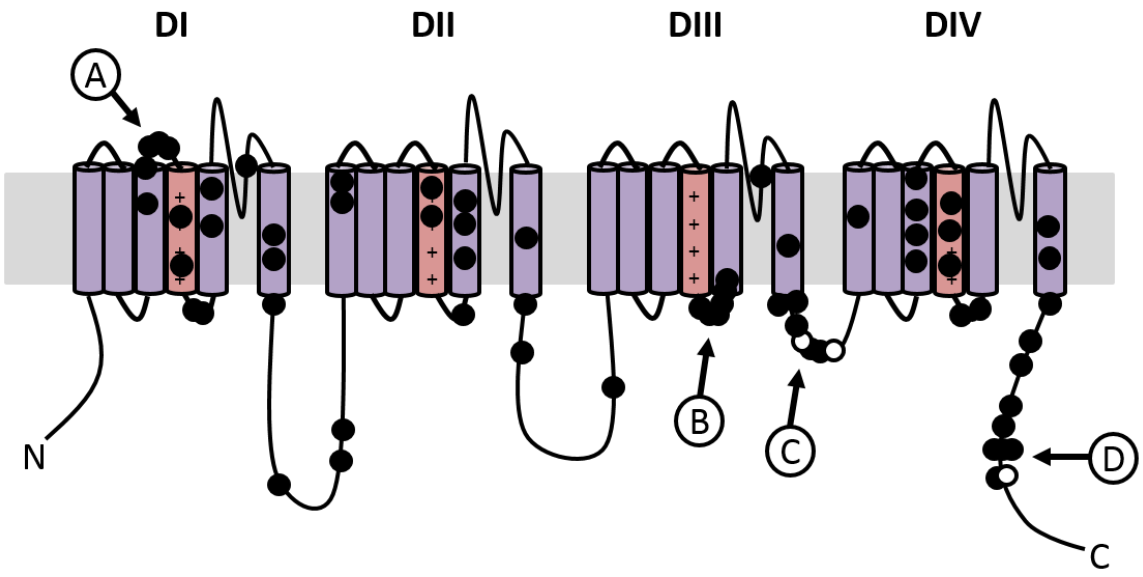

Figure 3.3 Distribution of pathogenic epilepsy variants across the $Na_v1.6$ α-subunit. The filled circles denote the location of pathogenic epilepsy variants reported to be *de novo*. The open circles denote inherited variants. The four mutation clusters are indicated by lettered circles with arrows.

3.5 Discussion

The list of *SCN8A* epilepsy variants has been rapidly growing since the first report in 2012, due in part to the wider availability of genetic testing that includes the *SCN8A* gene. Pathogenic *SCN8A* variants appear to account for approximately 1% of patients with epileptic encephalopathies, with over a hundred pathogenic variants identified in *SCN8A* to date (Meisler et al., 2016). Similar to previous observations, we estimate that

variants in *SCN8A* are responsible for disease in approximately 1% of the cohort of 275 epilepsy patients screened by the Emory Genetics Laboratory, although this cohort is not limited to the epileptic encephalopathies. Pathogenic *SCN8A* variants are predominantly *de novo* or inherited from an unaffected parent found to be mosaic. This was true for three of the variants reported in this study (Patients 1-3). Although most pathogenic *SCN8A* variants are found in individuals with early infantile epileptic encephalopathy (EIEE), an inherited missense variant (p.E1483K) was recently identified in three unrelated families with benign infantile spasms and paroxysmal dyskinesia (Gardella et al., 2016).

In a recent report, Anand and colleagues described a heterozygous *SCN8A* variant, p.N1877S, in an affected father and son that experienced seizure onset at four and five months of age, respectively, and presented with focal and generalized tonic-clonic seizures. However, neither individual exhibited cognitive impairment. The son's EEG showed background slowing and disorganization with active focal epileptiform discharges, but his seizures were controlled on carbamazepine (Anand et al., 2016). Here we report an additional family segregating the *SCN8A* p.N1877S variant. Similar to the family presented by Anand et al, the family described here appears to have a milder epilepsy presentation without cognitive impairment and shows response to treatment with sodium channel blockers such as oxcarbazepine and phenytoin. In contrast to these two families, *SCN8A* p.N1877S has also been observed *de novo* in at least two other epilepsy patients with developmental delay and intellectual disability. Anand and colleagues speculated that additional modifying genetic variants may explain the reduced disease severity in their two cases, although no candidates were revealed by the gene panel

analysis (Anand et al., 2016). In the family presented here, variants in additional genes were uncovered from the gene panel analysis. This included the NMDA receptor gene, *GRIN2A,* which is associated with a broad spectrum of epilepsy and speech disorders (Carvill et al., 2013b). The C-terminal *GRIN2A* p.R1451S variant is predicted to be tolerated by *in silico* analysis and was observed 12 times in the ExAC database. We found that the two family members with p.R1451S (**Fig. 3.2**, I:1 and II:3) have milder epilepsy presentations and normal EEGs compared to the proband with only the *SCN8A* variant (II:2). Further studies are needed to determine the functional effects of the *SCN8A* p.N1877S variant, as well as whether this *GRIN2A* variant is capable of modifying those effects.

Our last case (Patient 5) highlights the challenges associated with variant interpretation from gene panel analysis. The *SCN8A* p.K101R variant was the only reported finding from the 110 genes of the ESD panel analysis. This very rare variant (seen in only 1/120,220 alleles in ExAC) was predicted to be damaging by MutationTaster and SIFT (and possibly damaging by PolyPhen-2), and the phenotype of the patient overlaps with what has been reported previously for *SCN8A*-associated epilepsy. Parental testing can be critical for distinguishing pathogenic variants from rare benign familial variants. In this case, the *SCN8A* variant was inherited from the unaffected mother, suggesting that p.K101R may represent a rare benign variant. The identification of a second variant predicted to be pathogenic (*PACS1* p.R203W) also raises the possibility that the *SCN8A* p.K101R variant may not be pathogenic in this individual. Functional analysis will be necessary to determine whether the p.K101R variant alters the electrophysiological properties of the $Na_v1.6$ channel.

The VGSC genes *SCN1A* and *SCN2A*, encoding the $Na_v1.1$ and 1.2 sodium channels, respectively, are associated with a spectrum of epileptic phenotypes, including severe sporadic EIEE and milder familial epilepsies. Mutations in *SCN1A* were first identified in families with genetic epilepsy with febrile seizures plus (GEFS+) and later recognized as a cause of the catastrophic EIEE Dravet syndrome (Claes et al., 2001; Escayg et al., 2000). Similarly, mutations in *SCN2A* were originally associated with benign familial neonatal-infantile seizures (BFNIS) and GEFS+, but were later identified in patients with EIEEs, such as Ohtahara syndrome and Dravet syndrome (Berkovic et al., 2004; Nakamura et al., 2013). Consistent with these observations, *SCN8A* mutations also appear to underlie a spectrum of epilepsy phenotypes.

A small number of *SCN8A* variants have also been found in individuals with intellectual disability, ataxia, and cerebral palsy, without seizures (McMichael et al., 2015; Rauch et al., 2012; Trudeau et al., 2006). Here we identified an *SCN8A* missense variant at an evolutionarily conserved amino acid position from a patient with autism. Although we do not know the inheritance of the variant or whether this individual also experienced seizures, autism spectrum disorder is a common comorbidity in patients with pathogenic *SCN8A* variants (Larsen et al., 2015). Additionally, pathogenic *SCN8A* variants were recently detected from diagnostic whole-exome sequencing in patients with neurodevelopmental disorders without seizures (Helbig et al., 2016). This is similar to findings for *SCN1A* and *SCN2A*, both of which are implicated in autism with and without seizures (Li et al., 2015; O'Roak et al., 2011; Sanders et al., 2012). Consequently, it may be beneficial to add *SCN8A* to gene panels designed for intellectual disability, autism,

and movement disorders, since these disorders share underlying genetic pathways with epilepsy (Li et al., 2015).

To date, five epilepsy variants have been identified in the region of *SCN8A* encoded by the two alternate exon 5s (5N and 5A). All five of these amino acid substitutions are located within exon 5N (Allen et al., 2013; de Kovel et al., 2014; Larsen et al., 2015; Mercimek-Mahmutoglu et al., 2015; Ohba et al., 2014). Studies in mice and non-human primates (*Macaca fascicularis*) have shown that the 5N exon, referred to as the neonatal isoform, is expressed early in development and is gradually replaced by isoforms containing the 5A exon (adult isoform) as development progresses (Gazina et al., 2010; Raymond et al., 2004). Little is currently known about the developmental expression pattern of these two exons in humans, although Raymond et al. reported that the 5N exon was observed at higher levels in fetal brain, whereas the 5A exon was incorporated more frequently in transcripts from adult brain (Raymond et al., 2004). The 5N and 5A exons are both 92 nucleotides in length (encoding 30 amino acids) and are separated by a short 155 bp intron. The exons differ at 19 nucleotide positions but only two amino acid positions (p.207I>V and p.212N>D) (Raymond et al., 2004). The usage of these two alternatively spliced exons, encoding a portion of the first domain, is evolutionarily conserved across most of the VGSC α-subunits, including all of the human VGSCs expressed in the CNS. Several studies have examined the functional differences between the two corresponding alternatively spliced isoforms in $Na_v1.1$ and $Na_v1.2$. Electrophysiological studies revealed that alternative splicing of exon 5 modifies the inactivation kinetics of the sodium channel, such that isoforms including the 5N exon inactivate more rapidly compared to those with the 5A exon (Fletcher et al., 2011; Xu et

al., 2007). Additionally, the "neonatal" $Na_v1.2$ isoform appears to reduce neuronal excitability in mice (Gazina et al., 2015). Taken together, we hypothesize that mutations in the 5N exon of *SCN8A* could cause disease by altering the inactivation kinetics of the $Na_v1.6$ channels expressed in early development, leading to hyperexcitability in neurons expressing those channels.

Until recently, there were no reports of pathogenic variants in the 5A exon. The lack of pathogenic variants in 5A could reflect differences in the electrophysiological properties of the different channel isoforms, such that substitutions in the adult isoform are less detrimental than substitutions in the neonatal isoform. However, when the two exons are examined in the ExAC database, four missense changes are seen in the 5N exon, and only one missense change for the 5A exon. This high level of conservation would suggest both exons are important for proper channel function. Alternatively, the lack of 5A mutations could be the result of the use of sequencing libraries that do not target both *SCN8A* exons. The current reference transcript for *SCN8A* (NM_014191.3), used by most laboratories for mutation identification, includes only the 5N exon. As a result, variants in the 5A exon might be missed. Indeed, the Epilepsy Genetics Initiative, a program created to periodically reanalyze the exomes and genomes of epilepsy patients, identified three pathogenic variants in the 5A exon of *SCN8A* that were initially missed during exome analysis (Epilepsy Genetics, 2017). All three individuals carrying the 5A variants presented with early-onset epileptic encephalopathy, consistent with variants identified elsewhere in the *SCN8A* gene. Prior to this discovery, it was unclear whether variants in the 5A exon of *SCN8A* could also cause disease and, if so, whether disease severity would be similar to that reported for pathogenic variants in other regions of the

channel. Interestingly, pathogenic variants have been observed in both alternate exons of *SCN2A* (denoted as exons 6N and 6A) (Kodera et al., 2013; Nakamura et al., 2013). In these cases, the disease severity did not appear to correlate with the affected exon.

The other three observed clusters of *SCN8A* variants affect regions of the sodium channel known to have roles in channel inactivation. Two of these clusters, the inactivation gate and the proximal portion of the C-terminus, were noted previously as being enriched for pathogenic variants (Wagnon and Meisler, 2015). The third cluster in the DIIIS4-S5 linker region (**Fig. 3.3, cluster B**) has not been reported before. This linker is approximately 19 amino acids in length, and five of these positions are reported to be mutated in epilepsy. Two additional epilepsy variants are found directly adjacent to this linker in the DIIIS5 transmembrane domain. This highly conserved linker is known to interact with the inactivation gate by serving as a docking site to stabilize the gating particle in VGSCs. Specifically, the alanine residue at position 1319 of the DIIIS4-S5 linker is known to interact with the hydrophobic isoleucine-phenylalanine-methionine-threonine (IFMT) amino acid sequence in the inactivation gate (Goldin, 2003; Smith and Goldin, 1997). Two recent studies characterized the biophysical consequences of two pathogenic variants located in the DIIIS4-S5 linker, I1327V and L1331V. As predicted, both variants impair channel inactivation, resulting in channels that are slower to transition from the open state to the inactive state (Barker et al., 2016; Patel et al., 2016). Additionally, amino acid substitutions have been identified at p.A1319 in the DIIIS4-S5 linker, as well as at the isoleucine (p.I1479), phenylalanine (p.F1480), and methionine (p.M1481) residues in the inactivation gate in epilepsy patients. A total of seven unique epilepsy variants have been observed within the *SCN8A* inactivation gate, including the

recurrent p.E1483K inherited missense variant. Variants that alter the structure of the inactivation gate also have the potential to alter channel inactivation, producing hyperexcitable channels.

The C-terminal domain also plays a role in modulating fast inactivation of VGSCs. Specifically, the proximal half of the C-terminus contains six α-helices that are predicted to interact with the inactivation gate, as well as with calmodulin, a calcium-responsive protein known to modulate channel inactivation (Cormier et al., 2002; O'Brien and Meisler, 2013). The cluster of seven pathogenic *SCN8A* variants observed in the C-terminus primarily affect the fifth α-helix, which contains multiple residues known to be important for inactivation kinetics (Nguyen and Goldin, 2010). Wagnon et al. showed that substitutions at the positively charged p.R1872 residue within the fifth α-helix destabilizes interactions with the inactivation gate, producing hyperexcitable channels (Wagnon et al., 2016). One can hypothesize that other variants in this region of the C-terminus may have similar effects on the inactivation kinetics of the channel, including the inherited variant p.N1877S reported in this study. Alternatively, substitutions in this region of the C-terminus may disrupt interactions with proteins that normally regulate channel activity or trafficking (O'Brien and Meisler, 2013).

In summary, we report several novel epilepsy variants in *SCN8A* identified by gene panel analysis. As genetic testing technologies continue to improve and become more integrated into routine clinical care, we will undoubtedly find other pathogenic *SCN8A* variants in patients with epilepsy and other neurodevelopmental disorders. These additional patients will provide insight into the full phenotypic spectrum of *SCN8A* dysfunction and improve our ability to interpret new variants uncovered by diagnostic

testing. Efforts are currently underway to launch an *SCN8A* variant registry (http://www.scn8a.net/), which will allow families, clinicians, and researchers to come together to advance our understanding of *SCN8A* and epilepsy.

3.6 Acknowledgments

We would like to thank the families for their participation. We are grateful for the assistance of Cynthia Freehauf in the collection of clinical data. This study was supported by funding from Children's Healthcare of Atlanta to A.E., the Emory University Research Council to A.E. and J.J.A., and a training grant appointment (5T32GM008490-23) to K.M.B. Deep amplicon sequencing was performed by the Emory Integrated Genomics Core (EIGC), which is subsidized by the Emory University School of Medicine and is one of the Emory Integrated Core Facilities.

CHAPTER 4
DE NOVO VARIANTS IN *GABRA2* AND *GABRA5* ALTER RECEPTOR FUNCTION AND CONTRIBUTE TO EARLY-ONSET EPILEPSY

In revision for submission to *Brain*:

Butler, K.M.*, Moody, O.*, Schuler, E., Coryell, J., Alexander, J.J., Jenkins, A., and Escayg, A. *De novo* variants in *GABRA2* and *GABRA5* alter receptor function and contribute to early-onset epilepsy.

*Co-first authors

4.1 Summary

I initially identified a *de novo* variant in *GABRA5* from trio-WGS of a German patient with severe, unexplained epilepsy who was referred to our lab for genetic testing. This discovery prompted me to query the Mendeliome data for additional variants in *GABRA5*. Unfortunately, *GABRA5* was not included in the Mendeliome library; however, there were nine other $GABA_A$ receptor genes present in the library. From this, I detected two variants in *GABRA2* and *GABRB3* that stood out as plausible disease variants and warranted functional evaluation.

My contribution to the paper that forms the basis of this chapter included the initial identification of the $GABA_A$ receptor variants (**Table 4.1**), the determination of variant inheritance for *GABRA2* and *GABRA5*, and the coordinated collection of clinical phenotype information (**Table 4.2**). I created the protein alignment and receptor structure in **Fig. 4.1A-B** and the lollipop plots in **Fig. 4.2**. Additionally, I performed the site-directed mutagenesis to introduce the *GABRA2* and *GABRB3* variants into the corresponding cDNA plasmids and performed the protein extraction and Western blotting for the biotinylation assays (**Fig. 4.3E-F** and **Fig. 4.4D-E**).

This study identified two new genes (*GABRA2* and *GABRA5*) as causes of severe, early-onset epilepsy. Although, these genes represent rare causes of epilepsy, it is likely that additional variants will be identified now that these genes have been associated with disease. The full manuscript, of which I am co-first author, is reproduced below.

4.2 Introduction

GABA is the major inhibitory neurotransmitter in the brain. $GABA_A$ receptors are heteropentameric, ligand-gated anion channels that are activated by GABA to mediate both phasic synaptic transmission and tonic extrasynaptic inhibition in the brain. Functional $GABA_A$ receptors are typically formed from three of 19 different gene products encoded by *GABRA1-GABRA6* (α1-6), *GABRB1-GABRB3* (β1-3), *GABRG1-GABRG3* (γ1-3), *GABRR1-GABRR3* (ρ1-3), *GABRD* (δ), *GABRE* (ε), *GABRP* (π), and *GABRQ* (θ), with most synaptic receptors consisting of two α, two β, and one γ subunit (Olsen and Sieghart, 2009).

Considering the role of $GABA_A$ receptors in neuronal inhibition, the genes encoding these receptors (*GABRs*) are of obvious interest in disorders of altered neuronal excitability such as epilepsy. Pathogenic variants in these genes have been identified in patients with various types of epilepsy. *GABRA1* is associated with hereditary generalized epilepsies, like juvenile myoclonic epilepsy, as well as sporadic epileptic encephalopathies, such as Dravet and Ohtahara syndromes (Carvill et al., 2014; Cossette et al., 2002; Kodera et al., 2016). *GABRG2* mutations were found in patients with GEFS+, absence epilepsy, and epileptic encephalopathies (Baulac et al., 2001; Kang and Macdonald, 2016; Shen et al., 2017; Wallace et al., 2001). Mutations in *GABRB3* have recently emerged as a cause of febrile seizures, GEFS+, focal epilepsy, myoclonic-atonic epilepsy, and early-onset epileptic encephalopathy (Allen et al., 2013; Janve et al., 2016; Moller et al., 2017). There have also been a few of reports of epilepsy-associated variants in *GABRA3, GABRB1*, *GABRB2,* and *GABRD* (Ishii et al., 2017; Janve et al., 2016; Niturad et al., 2017; Okamoto et al., 2015; Srivastava et al., 2014). A single *de novo*

missense variant was recently reported in *GABRA2* in an individual with epileptic encephalopathy; however, the functional consequence of the variant was not investigated (Orenstein et al., 2018). Additionally, Hernandez and colleagues recently identified variants in several *GABRs* that may modify epilepsy susceptibility (Hernandez et al., 2016).

Here, we provide the first functional evidence that *de novo* variants in the *GABRA2* and *GABRA5* genes, encoding the α_2 and α_5 $GABA_A$ receptor subunits, respectively, contribute to early-onset epilepsy and developmental delay. We also present functional characterization of a recurrent missense variant identified in *GABRB3*.

4.3 Methods and Materials

Next-Generation Sequencing and Sanger Confirmation

Whole-genome sequencing was performed on DNA extracted from whole blood from Patient 1 and his parents at 30X coverage using the Illumina HiSeq X Ten platform (Illumina, San Diego, CA) with 150-bp paired-end reads. Reads were mapped to the human reference genome using PEMapper, and variants were called using PECaller (Johnston et al., 2017). Variants were annotated using SeqAnt 2.0 (Shetty et al., 2010). *De novo* variants identified in the proband were confirmed with Sanger sequencing.

Targeted resequencing of DNA samples from 279 epilepsy patients was performed using a custom in-solution hybridization probe library (IDT or SureSelect, Agilent Technologies, Santa Clara, CA) to capture the coding exons of approximately 4,800 genes associated with human disease, as described in Chapter 2. The following *GABRs* were present in the sequencing library: *GABRA1, GABRA2, GABRA6, GABRB2,*

GABRB3, GABRG1, GABRG2, GABRD, and *GABRR2*. Variants were filtered for call quality and frequency in the genome aggregation database (gnomAD). Peripheral blood or saliva samples were obtained from family members to test variant inheritance after written consent was obtained. The *GABRA2* variant (c.875C>A, p.T292K) was determined to be *de novo* by Sanger sequencing of Patient 2 and her parents. Parental samples were unavailable for Patient 3. This study was approved by the Institutional Review Boards of Emory University and Heidelberg University Hospital.

$GABA_A$ Receptor cDNAs and Mutagenesis

The human $GABA_A$ receptor α_1 (NM_000806), α_2 (NM_000807), α_5 (NM_000810), β_3 (NM_000814), γ_{2s} (NM_000816), and rat β_2 (NM_012957) subunit cDNAs were each cloned into the pcDNA3.1+ expression vector containing a cytomegalovirus promoter. The rat β_2 cDNA (NM_012957) was humanized with a single N323S substitution to match the human β_2 peptide sequence (NP_000804). Site-directed mutagenesis (QuikChange Lightening, Agilent Technologies) was performed to introduce the following variants: α_2(T292K), α_5(V294L), and β_3(P301L) into their respective clones. Numbering of variants was based on the full-length protein sequences, which include the signal peptides. Sanger sequencing was used to confirm each variant and to ensure the absence of unwanted substitutions.

Cell Culture and Transfection

Human embryonic kidney 293T (HEK293T) cells (CRL-3216, ATCC®) were maintained at 37°C in 5% CO_2 in Eagle's Minimum Essential Medium supplemented with 5% fetal bovine serum, 40 μM *L*-glutamine, 100 U/ml penicillin, and 0.1 mM streptomycin. Cells were grown on poly-D-lysine-coated glass coverslips (No.2, VWR)

and transfected with X-tremeGENE™ (Roche Diagnostics) with the $GABA_A$ receptor cDNAs at a 1:1:1 ratio to express $\alpha_x\beta_x\gamma_{2s}$ receptors (0.5 μg per cDNA) and 0.5 μg green fluorescent protein (GFP). GFP was used as an expression marker for transfection efficiency. Patch-clamp experiments were performed on cells at 24-72 hrs post-transfection. All experiments were performed at 22°C using cells from at least two transfections and across multiple days to control for cell health and transfection efficiency. All reagents were purchased from Sigma unless otherwise noted.

Whole-Cell Patch-Clamp Recordings

Whole-cell patch-clamp recording was performed on HEK293T cells expressing $\alpha_x\beta_x\gamma_{2s}$ $GABA_A$ receptors and GFP, similar to methods previously described (Williams et al., 2010). Patch pipettes were fabricated from thin-walled borosilicate glass (TW150F-4, World Precision Instruments, Inc.) using a horizontal puller (P-97, Sutter Instruments, Inc.) to give a resistance of 2-8 MΩ when filled with intracellular solution (120 mM KCl, 2 mM $MgCl_2$, 10 mM EGTA, and 10 mM HEPES, adjusted to pH 7.2 with NaOH, 315 mOsm). Extracellular solution contained 161 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, and 6 mM D-glucose, adjusted to pH 7.4 with NaOH (320-330 mOsm). A rapid solution changer (RSC-160, BioLogics Science Instruments) connected to a 10-channel infusion pump (KD Scientific Inc) was used to deliver GABA and picrotoxin solutions. The rapid solution changer was controlled by protocols written in pClamp 9 (Molecular Devices, LLC). Whole-cell currents were recorded at -60 mV, filtered at 100 Hz, and sampled at 200 Hz with a MultiClamp 700B amplifier and DigiData 1322A (Molecular Devices, LLC) digitizer.

GABA concentration-response assays were performed by exposing each whole-cell patch to increasing concentrations of GABA (0.3, 1, 3, 10, 30, 100, 300, and 1000 μM) for 2 seconds, with an 8-second washout between concentrations. Recordings were baseline corrected and analyzed in MATLAB (MathWorks, Inc.). Peak currents (*I*) were measured from GABA exposures and fitted using least-of-squares nonlinear regression analysis based on the Hill equation: $I = I_{max} * [A]^{nH} / (EC_{50}^{nH} + [A]^{nH})$, where *I* is current peak amplitude, I_{max} is maximum current amplitude, EC_{50} is the GABA concentration producing the half-maximal response, *A* is agonist concentration, and *nH* is the Hill coefficient. GABA concentration-response assays were individually fitted to the Hill equation for each whole-cell recording. The maximum peak current, EC_{50}, and Hill coefficient were estimated based on averaged values for each mutant receptor and are shown as mean ± standard error of the mean (SEM). The EC_{50}, also known as apparent-affinity, is a compound measure of the binding affinity and gating efficacy of GABA for the receptor (Colquhoun, 1998).

The degree of baseline leak current for cells expressing α_2- and α_2(T292K)-containing receptors was calculated using whole-cell recordings from GABA concentration-response assays. The first 41 points (0.2 sec) of whole-cell baseline current in extracellular solution was averaged for each patch to give a measurement of baseline leak. This was performed for all eight concentrations in the GABA concentration assay, and the values averaged for each cell.

Desensitization was measured for α_5- and α_5(V294L)-containing receptors from the whole-cell recordings of GABA concentration-response assays. Whole-cell analysis of desensitization was performed using previously described methods (Moody et al.,

2017). Briefly, desensitization was measured from 2-second GABA exposures as follows: $(I_{peak} - I_{end})/I_{peak}*100$, where I_{peak} was the amplitude of the total peak current response and I_{end} was the amplitude of the peak current response at the end of the agonist exposure (at 2 sec). For each cell, desensitization was measured for the eight GABA concentration responses. The log-linear concentration-desensitization relationship was fitted by linear regression. The slope of this function describes the extent of desensitization as GABA concentration increased.

Picrotoxin Assay

Picrotoxin (Sigma) was dissolved in 0.1% DMSO and diluted in extracellular solutions to final concentrations of 1, 10, and 100 μM. Picrotoxin solutions were applied in increasing concentrations to patched cells for 3 seconds, with an 8-second washout between each concentration. Recordings were baseline corrected and peak current amplitude was measured at each picrotoxin concentration.

Biotinylation Assay and Western Blotting

Cell surface biotinylation was performed on transfected HEK293T cells as previously described (Thompson et al., 2012). Total and surface fractions were separated by SDS-PAGE (4-15% gel) and transferred to a PVDF membrane (Bio-Rad). Membranes were blocked with 5% nonfat milk, then incubated with anti-α_2 (1:500; 822-GA2CL Phosphosolutions), anti-α_5 (1:500; N415/24 NeuroMab), and Na^+/K^+-ATPase (1:10,000; ab76020 abcam) primary antibodies. Membranes were stripped and incubated with monoclonal mouse anti-β-actin (1:2000; A00702 GenScript) primary antibody to verify that the biotinylating reagent did not cross the cell membrane. Signal intensities were

quantified using Image Lab™ software (Bio-Rad). $GABA_A$ receptor expression levels were normalized to Na^+/K^+-ATPase.

Structural Modelling and Lollipop Diagrams

A three-dimensional model of the assembled $GABA_A$ receptor was generated using PyMOL software and is based on the crystal structure of the human $GABA_A$ β_3 homopentamer presented by Miller and colleagues (PDB: 4COF) (Miller and Aricescu, 2014).

Lollipop diagrams were generated using the freely available Lollipops software (Jay and Brouwer, 2016). After downloading the source code, the software was run directly from the command line interface using the following UniProt accession numbers to draw the protein domains: P31644 (*GABRA5*), P47869 (*GABRA2*), and P28472 (*GABRB3*). Synonymous and nonsynonymous variants were downloaded from the gnomAD Browser for each gene. The resulting diagrams for the synonymous and nonsynonymous variants were merged into a single image for easier visualization.

Statistical Analysis

Individual parameters from the whole-cell patch-clamp recordings and Western blot experiments were compared using unpaired two-way *t*-tests ($\alpha = 0.05$). A two-way unpaired *t*-test ($\alpha = 0.05$) with Welch's correction was used to evaluate group differences in baseline leak current. A linear regression analysis was used to evaluate differences in receptor desensitization as GABA concentration increased. For the picrotoxin assay, a two-way repeated measures ANOVA ($\alpha = 0.05$) with a Sidak *post-hoc* test for multiple comparisons was performed to compare levels of picrotoxin block across groups. Statistical analyses were carried out using Prism 7.0 (GraphPad Software, Inc.). All data

are presented as mean ± SEM. Statistical differences are indicated in the figures and tables using the following symbols: ***p* ≤ 0.05, ****p* ≤ 0.01, *****p* ≤ 0.001, ******p* < 0.0001.

4.4 Results

4.4.1 $GABA_A$ Receptor Variants Detected from Individuals with Epilepsy

Using trio-based whole-genome sequencing, we identified the novel *de novo* *GABRA5* variant c.880G>C (p.V294L) in a proband (Patient 1) with severe epilepsy and developmental delay. To identify additional disease-associated variants in *GABRs,* we next examined available sequence data from 279 clinically referred epilepsy patients screened at EGL Genetics using a targeted sequencing panel of approximately 4,800 genes. Most of the patients referred for genetic testing were children (average age 7 years) with severe pediatric-onset epilepsy. Nine *GABRs* were included in the sequencing panel: *GABRA1, GABRA2, GABRA6, GABRB2, GABRB3, GABRG1, GABRG2, GABRD,* and *GABRR2*. *GABRA5* was not included in the sequencing panel. Variants identified in these nine genes were filtered to remove those seen at a frequency greater than 1% in the gnomAD database (**Table 4.1**). There were 19 unique heterozygous missense variants identified from 20 individuals after filtering. Three of the 19 variants were absent from the gnomAD database, which includes whole-exome and genome data from approximately 138,600 individuals and excludes individuals with severe pediatric diseases. Although the frameshifting *GABRR2* variant c.57_67delCCTCACAGATG was absent from gnomAD, at least 31 other loss-of-function (LOF) *GABRR2* variants are listed in the database, suggesting that *GABRR2* is tolerant of heterozygous LOF variation.

We selected the remaining two variants, *GABRA2* c.875C>A (p.T292K) and *GABRB3* c.902C>T (p.P301L) for further investigation (**Table 4.1, Fig. 4.1**). The clinical features of the individuals carrying the α_5(V294L), α_2(T292K), and β_3(P301L) variants are summarized in **Table 4.2**.

4.4.2 *GABRA5* c.880G>C (p.V294L)

Patient 1 is the second child of unrelated parents with no family history of epilepsy. At four months of age, he developed seizures during sleep consisting of myoclonic and tonic seizures, oral automatisms, coughing, tonic-clonic generalized seizures, and migrating partial seizures. Seizure frequency increased from one seizure per week to clusters of up to 100 seizures/day within six months. Cognitive and motor development slowed severely at the time of seizure onset. The patient developed secondary microcephaly. At the age of 24 months, Patient 1 shows muscular hypotonia, tetraspasticity, and autistic behavior. EEG analysis revealed epileptiform discharges, predominantly within the temporal and posterior parts of the brain. MRI showed hypomyelination. Seizures were unresponsive to treatment with phenobarbital, pyridoxine, folinic acid, pyridoxal phosphate, valproate, lacosamide, clonazepam, levetiracetam (alone), and levetiracetam and topiramate in combination; however, the patient became seizure-free at 14 months of age on a combination of zonisamide, levetiracetam, and oxcarbazepine.

Previous metabolic analyses and gene panel testing for epileptic encephalopathies (CeGAT Tubingen) were negative. Through whole-genome sequencing (WGS) of Patient 1 and his parents, we identified the *de novo GABRA5* c.880G>C (p.V294L) variant.

Table 4.1 *GABR* variants identified from screening of 279 individuals with epilepsy

Gene	Refseq	Nucleotide	Protein	Domain	gnomAD[a]	Polyphen-2/SIFT[b]
GABRA1	NM_000806.5	c.85C>T	p.P29S	NT	10	B/T
GABRA2	**NM_000807.2**	**c.875C>A**	**p.T292K**	**M2**	**0**	**PrD/D**
GABRA2	NM_000807.2	c.1131T>A	p.N377K	CL	7	B/T
GABRA6	NM_000811.2	c.178A>G	p.T60A	NT	30	PrD/D
GABRA6	NM_000811.2	c.251G>A	p.R84H	NT	17	PrD/T
GABRA6	NM_000811.2	c.915G>A	p.W305*	M3	3	-
GABRA6	NM_000811.2	c.1142T>C	p.I381T	CL	36	B/T
GABRB2	NM_021911.2	c.1118G>A	p.R373Q	CL	17	B/T
GABRB3	**NM_000814.**5	**c.902C>T**	**p.P301L**	**M2-M3**	**0**	**PrD/D**
GABRD	NM_000815.4	c.1166G>C	p.G389A	CL	5	B/T
GABRG1	NM_173536.3	c.835A>G	p.I279V	M1	4	B/T
GABRG2	NM_000816.3	c.691G>A	p.D231N	NT	4	PrD/D
GABRR2	NM_002043.3	c.57_67delCCTCACAGATG	p.L20Pfs*44	SP	0	-
GABRR2	NM_002043.3	c.376G>A	p.D126N	NT	219	PrD/D
GABRR2	NM_002043.3	c.539T>G	p.M180R	NT	95	PrD/D
GABRR2	NM_002043.3	c.860G>A	p.R287H	M1-M2	138	PoD/T
GABRR2	NM_002043.3	c.961G>A	p.V321I	M2-M3	176	B/T
GABRR2	NM_002043.3	c.1130A>G	p.Q377R	CL	951	B/T
GABRR2	NM_002043.3	c.1298G>A	p.R433H	CL	975	B/T

[a]Number of times the allele was observed in the gnomAD database out of approximately 277,200 alleles. [b]Polyphen-2 and SIFT prediction algorithms only score missense variants. Bolded rows highlight variants that were functionally evaluated. PrD, Probably Damaging; PoD, Possibly Damaging; B, Benign; D, Deleterious; T, Tolerated; NT = N-terminal; SP = signal peptide; CL = cytoplasmic loop between M3 and M4 transmembrane domains; M1-M2 = linker between M1 and M2 transmembrane domains; M2-M3 = linker between M2 and M3 transmembrane domains.

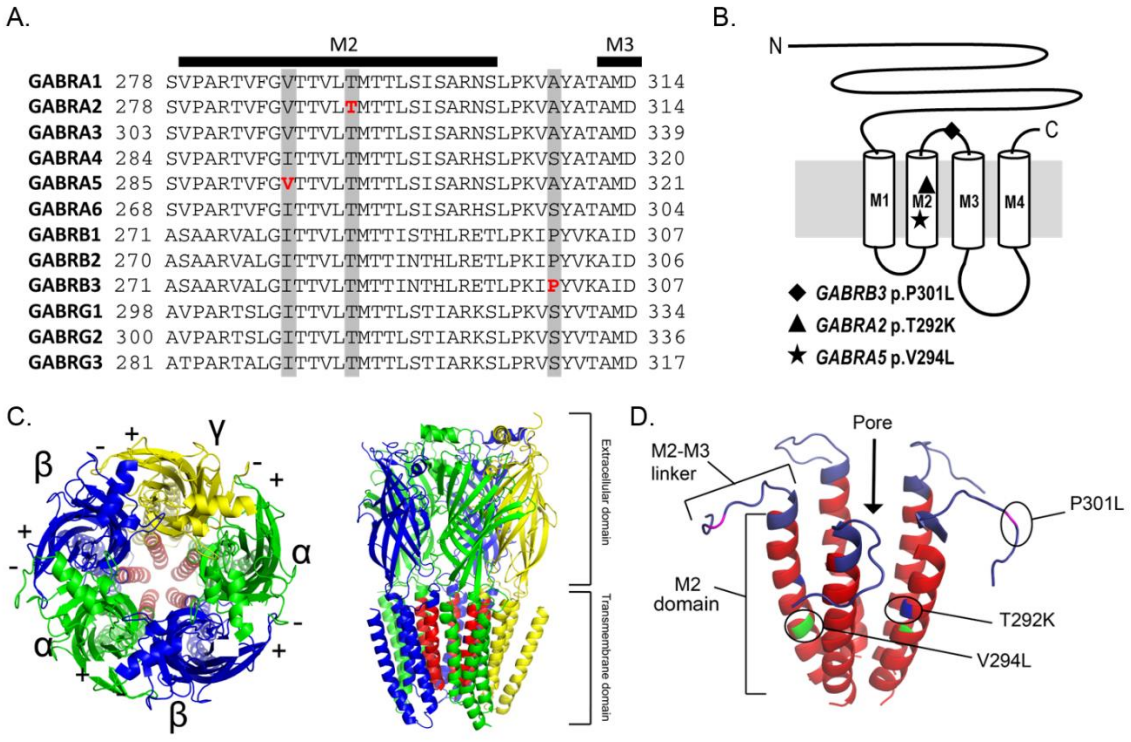

Figure 4.1 Location of epilepsy variants in the GABA$_A$ receptor subunits. (A) Alignment of human α, β, and γ subunits. The location of the α_5(V294L), α_2(T292K), and β_3(P301L) variants are highlighted in grey and the specific amino acids affected are bolded in red. Secondary structures (M2 and M3 transmembrane domains) are also shown above the alignment. The following protein sequences were used to make the alignment: *GABRA1*, NP_000797; *GABRA2*, NP_000798; *GABRA3*, NP_000799; *GABRA4*, NP_000800; *GABRA5*, NP_000801; *GABRA6*, NP_000802; *GABRB1*, NP_000803; *GABRB2*, NP_000804; *GABRB3*, NP_000805; *GABRG1*, NP_775807; *GABRG2*, NP_000807; *GABRG3*, NP_150092. (B) Schematic representation of a single GABA$_A$ receptor subunit with the approximate locations of the three missense variants shown. (C) Top-down and side view of an assembled GABA$_A$ receptor containing two α (green), two β (blue), and one γ (yellow) subunit. The M2 domain of each subunit has been colored red to show the location of the channel pore. (D) Three-dimensional representation of the five M2 domains of the assembled GABA$_A$ receptor pore with the three patient variants indicated.

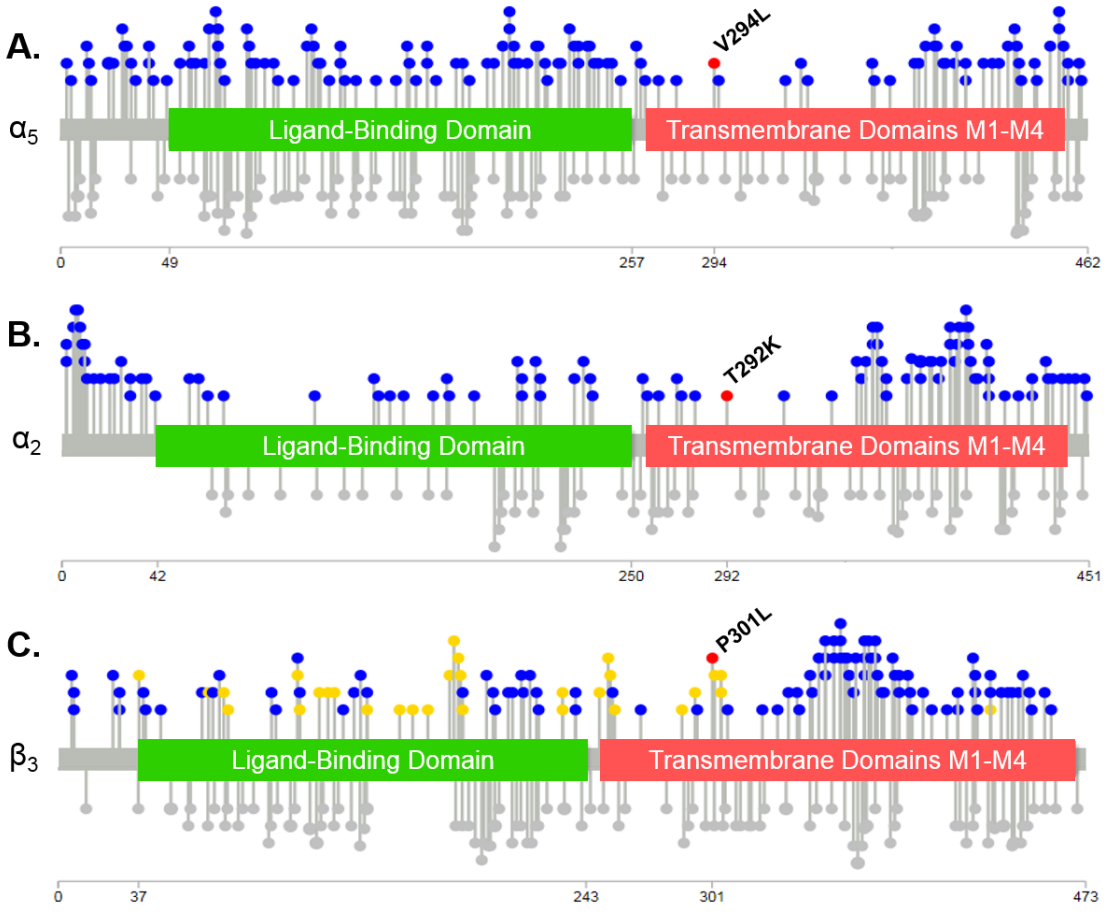

Figure 4.2 Distribution of variants from the gnomAD database across the three GABA$_A$ receptor proteins. In each lollipop diagram, the ligand binding domain is shown in green, the transmembrane domain, which includes all four transmembrane segments, is shown in red, and the unstructured regions are shown in gray. The numbers beneath each figure refer to the amino acid numberings. Missense (blue) and synonymous (gray) variants observed in the gnomAD database are shown. Red lollipops represent the candidate disease variants identified in this study. (A) Lollipop diagram for α_5 (encoded by *GABRA5*) showing the location of the p.V294L variant in the transmembrane region of the receptor. Protein domain annotation is based on UniProt accession P31644. (B) Lollipop diagram for the α_2 receptor showing the location of the p.T292K variant. Based on UniProt accession P47869. (C) Lollipop diagram of the β_3 receptor showing the location of p.P301L (red) as well as the locations of previously reported pathogenic *GABRB3* variants (gold). Based on UniProt accession P28472.

Table 4.2 Genetic and clinical details of epilepsy patients with $GABA_A$ receptor variants

	Patient 1	Patient 2	Patient 3
Age, sex	2 years, male	11 years, female	6 years, male
Detection method	WGS	Gene Panel	Gene Panel
Genomic position[a]	Chr15:27188364	Chr4:46264127	Chr15:26806257
Gene	*GABRA5*	*GABRA2*	*GABRB3*
Nucleotide	c.880G>C (NM_000810.3)	c.875C>A (NM_000807.2)	c.902C>T (NM_000814.5)
Protein	p.V294L	p.T292K	p.P301L
Inheritance	*De novo*	*De novo*	Unknown
Seizures	Myoclonic, tonic, tonic-clonic seizures, oral automatisms, migrating partial seizures	Clustered partial seizures, tonic, tonic-clonic, myoclonic seizures, epileptic spasms	Intractable seizures
Onset	4 months	6 weeks	na
Development	Secondary microcephaly, delayed milestones, nonverbal	Developmentally delayed, microcephaly, nonverbal, profound intellectual disability	Developmentally delayed
Motor development	Truncal hypotonia, tetraspasticity	Central hypotonia, spasticity, nonambulatory, cerebral palsy	na
EEG	Epileptiform discharges predominantly located within temporal and posterior parts of the brain	Slow and disorganized background, multifocal epileptiform discharges	na
MRI	Hypomyelination	Hypomyelination	na
Other	Autistic features	Cortical visual impairment	Behavioral/psychiatric abnormality

[a]Genomic positions are relative to the GRCh37/hg19 human genome assembly. na: not available; WGS: whole-genome sequencing.

Another *de novo* variant in the *MIA2* gene (c.1001_1004delACAA) was also detected but was considered unlikely to contribute to the patient's phenotype since multiple LOF variants in this gene were observed in the gnomAD database. No other candidate variants were identified by WGS. The *GABRA5* p.V294L variant is located in the pore-forming M2 transmembrane domain of the receptor and the affected valine is conserved across the benzodiazepine-sensitive alpha subunits (α1-3, α5, **Fig. 4.1**). The V294L substitution was absent from the gnomAD database and occurs in a region of the receptor where very few missense variants are seen in population controls (**Fig. 4.2A**). Finally, *GABRA5* p.V294L was predicted to be damaging by PolyPhen-2 and SIFT algorithms.

4.4.3 α_5(V294L)$\beta_2\gamma_{2s}$ Receptors are More Sensitive to GABA but Exhibit Increased Desensitization

When the α_5(V294L) variant was coexpressed with β_2 and γ_{2s} subunits in HEK293T cells, the GABA concentration-response relationship was altered relative to channels expressing wild-type α_5. First, the maximum GABA-evoked current produced by mutant receptors was significantly lower than that of wild-type (WT) receptors (WT α_5: -4165 ± 314 pA, n = 18 cells; α_5(V294L): -2717 ± 324 pA, n = 22 cells, p = 0.0024, **Fig. 4.3A-B**, **Table 4.3**). Second, there was a leftward shift in the GABA concentration-response curve relative to wild-type $\alpha_5\beta_2\gamma_{2s}$ receptors (**Fig. 4.3C**). This shift is exemplified by the EC_{50} of α_5(V294L)$\beta_2\gamma_{2s}$ receptors being approximately one tenth that of wild-type receptors (WT α_5: 2.041 ± 0.314 μM; α_5(V294L): 0.238 ± 0.028 μM, p = 0.0024). Third, the Hill coefficient, a measure of the cooperativity of GABA binding, was significantly higher for mutant receptors (WT α_5: 1.120 ± 0.061; α_5(V294L): 1.562 ±

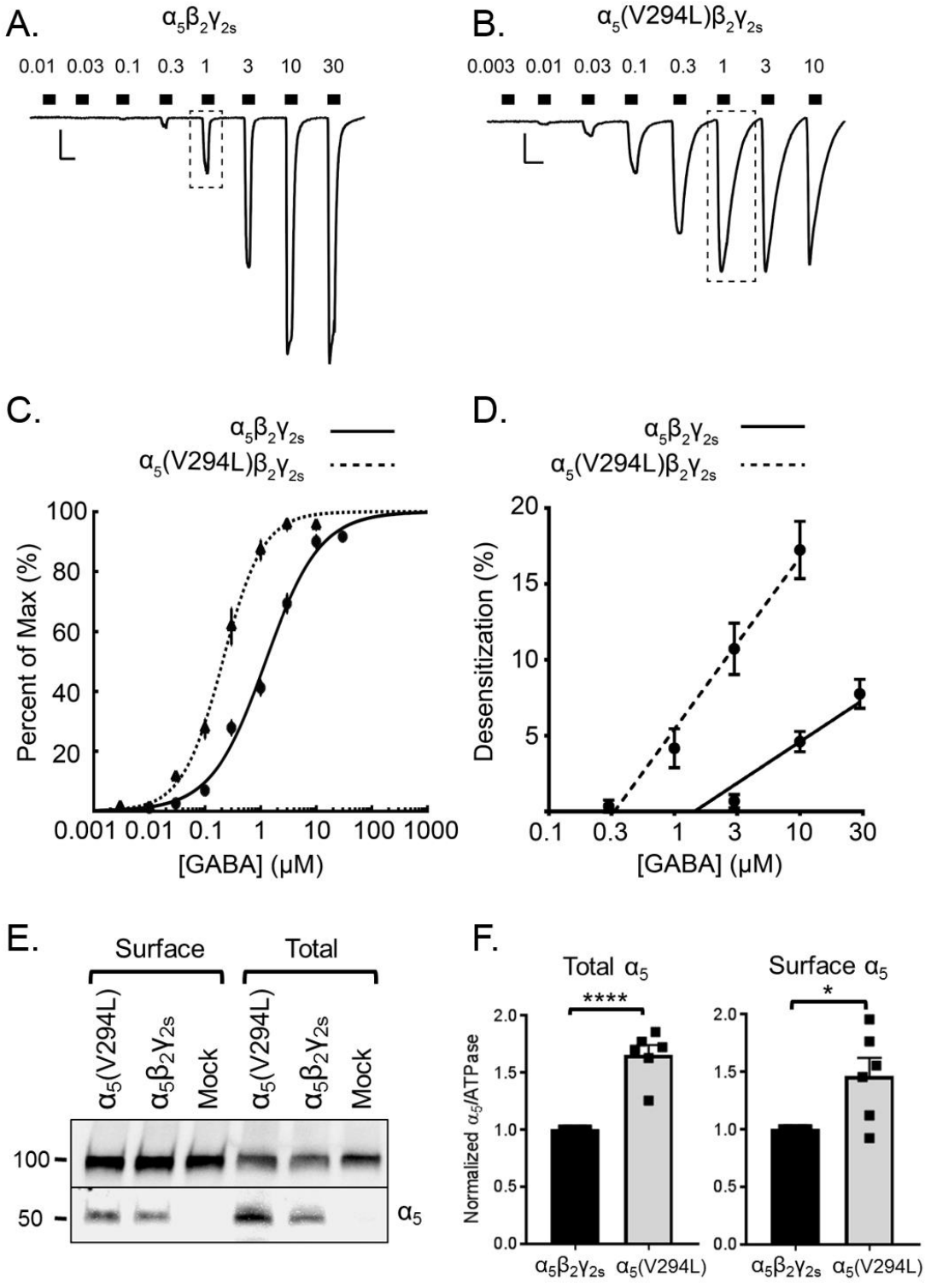

Figure 4.3 Increased GABA apparent-affinity and increased desensitization of α_5(V294L)$\beta_2\gamma_{2s}$ receptors. (A-B) Example traces of GABA concentration-response assays for (A) $\alpha_5\beta_2\gamma_{2s}$ receptors (0.01-30 μM) and (B) α_5(V294L)$\beta_2\gamma_{2s}$ receptors (0.003-10 μM)

expressed in HEK293T cells. Scale bars: horizontal = 5sec, vertical = 500pA. Dotted boxes highlight the 1 μM GABA response, which is larger and more desensitized in mutant receptors. (C) GABA concentration-response curves for $\alpha_5\beta_2\gamma_{2s}$ (solid line, n = 18 cells) and α_5(V294L)$\beta_2\gamma_{2s}$ (dotted line, n = 22 cells) receptors. Points are mean ± SEM and error bars are not shown where bars are smaller than points. The drawn line is a representative fit based on the average GABA concentration responses. (D) Average desensitization of peak currents from $\alpha_5\beta_2\gamma_{2s}$ (solid line) and α_5(V294L)$\beta_2\gamma_{2s}$ receptors (dotted line). Linear regressions to calculate desensitization are: Y = 5.508X + 0.909 ($\alpha_5\beta_2\gamma_{2s}$) and Y = 9.584X – 6.277 (α_5(V294L)), where Y is the percent of desensitization, and X is the log[GABA] in micromolar. Points represent mean ± SEM. (E) Total and cell surface protein lysates were analyzed by SDS-PAGE and blotted by anti-α_5 and anti-ATPase antibodies. Experiments were performed in triplicate on protein from two separate transfections. A representative Western blot is shown. (F) Band intensities of α_5 protein were normalized to the ATPase signal. Bars represent mean ± SEM. An unpaired *t*-test was used to determine significance. ***** $p \leq 0.05$, ******** $p < 0.0001$.

0.071, $p < 0.0001$). These changes are consistent with mutant α_5(V294L)$\beta_2\gamma_{2s}$ receptors having increased GABA apparent-affinity, causing them to near maximal activation around 1 μM GABA instead of 10 μM GABA like wild-type receptors (**Fig. 4.43A-C**).

One possible explanation for the reduced maximum GABA-evoked currents observed for α_5(V294L)$\beta_2\gamma_{2s}$ receptors is increased receptor desensitization. Since the mutant receptors are more sensitive to GABA, they are more likely to desensitize at lower GABA concentrations. To quantify this, we measured the degree of desensitization occurring over the 2-second GABA exposure during whole-cell GABA concentration-response assays. In whole-cell recordings, desensitization was seen as a decrease in current amplitude in the continued presence of GABA. The relationship between GABA concentration and the degree of desensitization could be described by the lines: Y = 5.508X + 0.909 (WT) and Y = 9.584X – 6.277 (α_5(V294L)), where Y is the percent of desensitization, and X is the log[GABA] concentration in micromolar (**Fig. 4.3D**). The results show that mutant receptors desensitized more than wild-type receptors as GABA

concentration increased ($F_{1,7}$ = 15.03, p = 0.0061). The α_5(V294L) variant was not found to reduce the total or cell surface protein expression. In fact, total and cell surface levels of α_5(V294L) were increased compared to wild-type α_5 (**Fig. 3E-F**). Increased expression of mutant receptors has been reported previously and could indicate the formation of homomeric receptors or altered subunit stoichiometry compared to wild-type receptors (Janve et al., 2016). Overall, our results show that α_5(V294L)$\beta_2\gamma_{2s}$ receptors are more sensitive to GABA but are also more likely to desensitize, thereby reducing the receptor's capacity to pass inhibitory chloride current.

4.4.4 *GABRA2* c.875C>A (p.T292K)

Patient 2 is the second child of unrelated healthy parents with no family history of epilepsy. Seizures began at six weeks of age and included clustered focal seizures and infantile spasms. Monthly clustered focal seizures were accompanied by eye dilation, alternating laughing and crying, breath holding, and behavioral arrest. The patient has also experienced tonic, tonic-clonic, and myoclonic seizures. EEG analysis at two years of age showed slow and disorganized background with multifocal epileptiform discharges. MRI performed at age nine showed hypomyelination. Now, at age 11, Patient 2 exhibits microcephaly, cerebral palsy with severe central hypotonia and asymmetric lower extremity spasticity, and cortical visual impairment. She is nonambulatory and nonverbal and has profound intellectual disability.

Her seizures failed to be controlled by treatment with oxcarbazepine, levetiracetam, phenobarbital, lamotrigine, topiramate, vigabatrin, adrenocorticotropic hormone, pyridoxal phosphate, vitamin E, ketogenic diet, or vagal nerve stimulation. She

is currently treated with a combination of valproic acid, phenobarbital, and clobazam but still experiences seizures.

Patient 2 was previously screened using a 110-gene epilepsy panel; however, the results were inconclusive. Using the larger next-generation sequencing library containing 4,800 genes, we identified the novel *GABRA2* c.875C>A (p.T292K) variant. Sanger sequencing of the parents confirmed that *GABRA2* p.T292K was *de novo* in Patient 2. Thr292 is located in the M2 domain and is conserved across all human α, β, and γ $GABA_A$ subunits (**Fig. 4.1A-B**). T292K is predicted to be damaging by Polyphen-2 and SIFT programs (**Table 4.1**). It is absent from the gnomAD database and there are few missense variants reported in close proximity to this position (**Fig. 4.2B**).

4.4.5 α_2(T292K)$\beta_2\gamma_{2s}$ Receptors are Tonically Open and Unresponsive to GABA

When the α_2(T292K) variant was coexpressed with β_2 and γ_{2s} subunits, it produced dysfunctional $GABA_A$ receptors that did not generate GABA-evoked whole-cell currents within the GABA concentration range of 0.3-1000 μM. This range normally evokes up to several nanoamps of current, as seen with wild-type $\alpha_2\beta_2\gamma_{2s}$ receptors (**Fig. 4.4A, Table 4.3**). For α_2(T292K)$\beta_2\gamma_{2s}$ receptors, the average current responses to 300, 1000, and 3000 μM GABA were: -22.32 ± 7.11 pA, -23.57 ± 76.27 pA, and -7.33 ± 1.82 pA, respectively (n = 9 cells). As a result, no Hill parameters could be estimated from the mutant data.

We noted that the basal leak current of cells expressing α_2(T292K)$\beta_2\gamma_{2s}$ receptors was twice as large as those expressing wild-type receptors ($t(24.45) = 3.37$, $p < 0.05$). Basal leak current refers to the baseline current that passes into cells in the absence of GABA, in part due to spontaneous channel openings. This observation, in combination

with the location of the variant in the pore-forming M2 domain of the receptor, led us to hypothesize that the mutant channels might be trapped in an open state. We used the $GABA_A$ receptor antagonist picrotoxin, a known open-channel blocker, to test this hypothesis. Mutant α_2(T292K)$\beta_2\gamma_{2s}$ receptors showed increased suppression of the basal leak current when exposed to increasing concentrations of picrotoxin in the absence of GABA (**Fig. 4.4B**). This suppression is due to picrotoxin blocking the tonic $GABA_A$ receptor-mediated leak current and is reflected in the membrane current moving closer to zero during picrotoxin exposure. Mutant receptors had significantly larger suppression of leak current in the presence of picrotoxin relative to wild-type receptors at both 10 μM ($p = 0.0017$) and 100 μM ($p < 0.0001$) concentrations (**Fig. 4.4B-C**). Given that $GABA_A$ receptors are generally closed in the absence of GABA, the observation of a leak current that could be blocked by picrotoxin indicates that the mutant α_2(T292K)$\beta_2\gamma_{2s}$ channel is likely trapped in an open state.

Based on the magnitude of leak current suppressed by picrotoxin, we hypothesized that the α_2(T292K) variant may also reduce expression of α_2-containing receptors. To test this, we performed a cell-surface biotinylation assay and measured total and cell surface expression of wild-type and α_2(T292K)-containing receptors (**Fig. 4.4D**). There was a significant reduction in the total amount of mutant receptor (~60% of WT levels, $p < 0.0001$) and a further reduction in the amount of mutant protein at the cell surface (~27% of WT levels, $p < 0.0001$, **Fig. 4.4E**). These results confirm that mutant α_2(T292K)$\beta_2\gamma_{2s}$ receptors are expressed at the cell surface, albeit at lower levels compared to wild-type receptors.

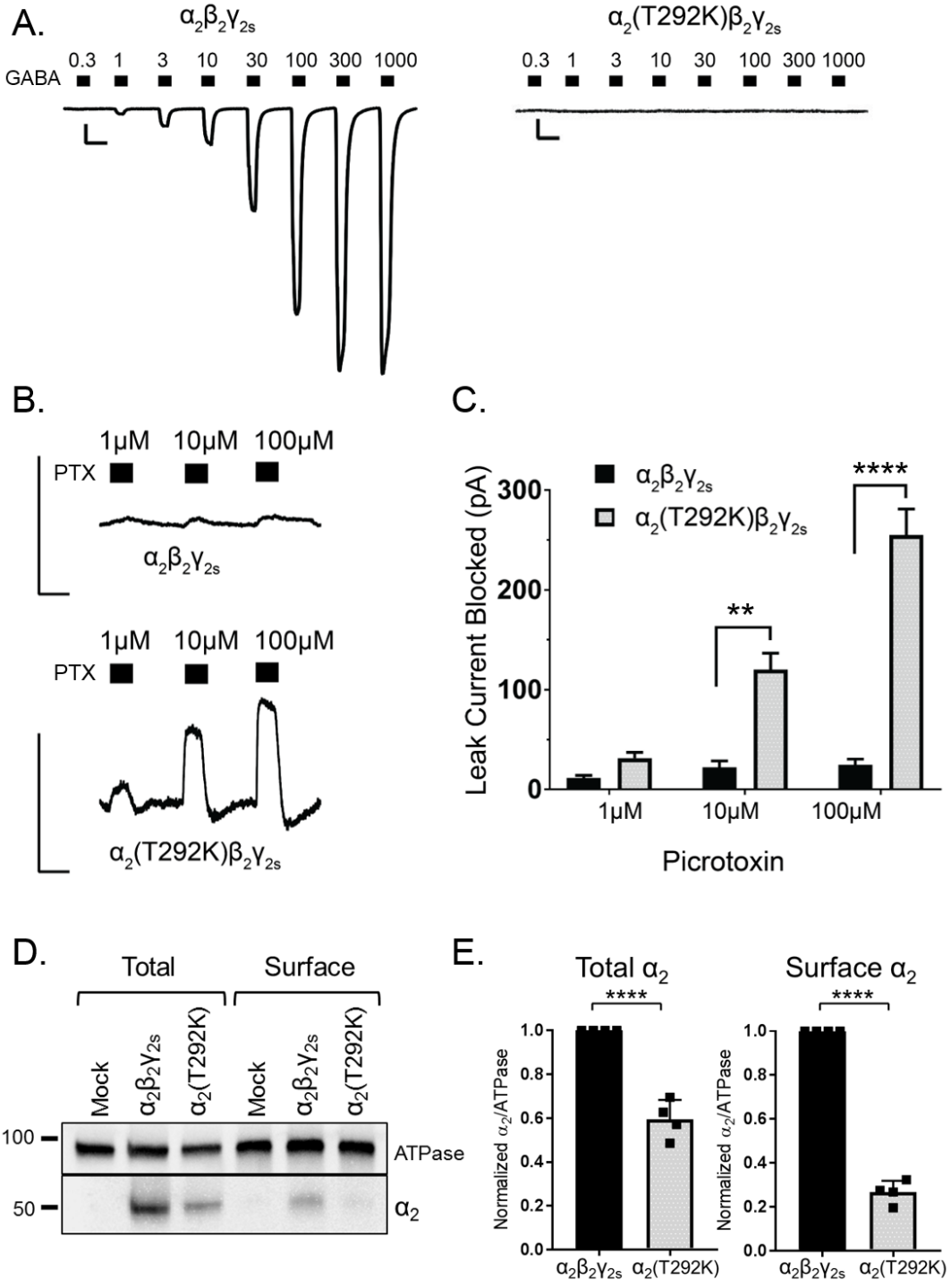

Figure 4.4 α_2(T292K)$\beta_2\gamma_{2s}$ receptors are predominantly open and produce leak current that can be blocked by picrotoxin. (A) Example leak-subtracted trace of GABA concentration-response assays (0.3-1000 μM) for $\alpha_2\beta_2\gamma_{2s}$ and α_2(T292K)$\beta_2\gamma_{2s}$ receptors expressed in HEK293T cells. Traces are aligned for easier visualization, although the

mutant example starts at a greater baseline leak current. Measured with whole-cell patch-clamp recording. Scale bars: horizontal = 5sec, vertical = 500pA. (B) Picrotoxin (PTX) blocked tonic leak current of α_2(T292K)$\beta_2\gamma_{2s}$ receptors in the absence of GABA, while wild-type $\alpha_2\beta_2\gamma_{2s}$ receptors showed little block. (WT α_2: n = 4 cells; α_2(T292K): n = 10 cells). Scale bars: horizontal = 5sec, vertical = 300pA. (C) Quantification of the leak current (pA) suppressed by picrotoxin. Picrotoxin block was significantly larger for mutant receptors at concentrations 10 μM ($p = 0.0017$) and 100 μM ($p < 0.0001$) (two-way repeated-measures ANOVA, Sidak post-hoc test). Bars represent mean ± SEM. **$p \leq 0.01$, ****$p < 0.0001$, respectively. (D) Total and cell surface protein lysates were blotted by anti-α_2 and anti-ATPase antibodies. Experiments were performed in duplicate on protein from two separate transfections. A representative Western blot is shown. (E) Band intensities of α_2 protein were normalized to the ATPase signal. Bars represent mean ± SEM. An unpaired *t*-test was used to determine significance. ****$p < 0.0001$.

4.4.6 *GABRB3* c.902C>T (p.P301L)

Patient 3 is a six-year-old male with intractable seizures, developmental delay, and an unspecified psychiatric abnormality. He was referred for genetic testing at age five, but a detailed clinical history and parental samples were unavailable.

Patient 3 was previously screened using a 110-gene epilepsy panel, but the results were inconclusive. We identified the *GABRB3* c.902C>T (p.P301L) variant using the larger sequencing library. Pro301 is located in the extracellular loop between the M2 and M3 transmembrane domains and is conserved across the human beta subunits (**Fig. 4.1A-B**). *GABRB3* p.P301L is absent from gnomAD and is predicted to be damaging by PolyPhen-2 and SIFT programs (**Table 4.1**). Additionally, it is proximal to several reported pathogenic *GABRB3* variants (**Fig. 4.2C**). Recently, Møller and colleagues reported the same *GABRB3* variant as *de novo* in an individual with focal epilepsy, supporting the pathogenicity of this variant (Moller et al., 2017).

4.4.7 $\alpha_1\beta_3$(P301L)γ_{2s} Receptors are Less Sensitive to GABA and Produce Less GABA-Evoked Current

The maximum GABA-evoked current produced by $\alpha_1\beta_3$(P301L)γ_{2s} receptors was significantly lower than for wild-type receptors (WT β_3: -1742.0 ± 157.1 pA; β_3(P301L): -540.2 ± 43.0 pA, $p < 0.0001$, **Fig. 4.5A**, **Table 4.3**). Additionally, expression of $\alpha_1\beta_3$(P301L)γ_{2s} receptors shifted the GABA concentration-response curve rightwards relative to wild-type receptors (**Fig. 4.5B**), resulting in a GABA EC_{50} that was significantly higher for mutant receptors than for wild-type receptors (WT β_3: 120 ± 14.37 μM, n = 21 cells; β_3(P301L): 298.10 ± 16.51 μM, n = 20 cells, $p < 0.0001$). These results suggest that β_3(P301L)-containing receptors have a reduced capacity for passing current and are less likely to be activated to the same degree as wild-type $\alpha_1\beta_3\gamma_{2s}$ receptors in response to GABAergic synaptic events.

Table 4.3. **Electrophysiological recordings from whole-cell patch-clamping of wild-type and mutant $GABA_A$ receptors**

Gene	Genotype	Maximum Current (pA)	Hill Coefficient	EC_{50} (μM)	N
GABRA5	WT	-4165 ± 314	1.120 ± 0.061	2.041 ± 0.314	18
	V294L	-2717 ± 324**	1.562 ± 0.071****	0.238 ± 0.028**	22
GABRA2	WT	-3340 ± 392	1.344 ± 0.069	5.97 ± 1.16	9
	T292K	-	-	-	9
GABRB3	WT	-1742 ± 157.1	1.235 ± 0.046	120.0 ± 14.4	21
	P301L	-540.2 ± 43.02****	1.474 ± 0.050***	298.1 ± 16.5****	20

Lack of GABA-evoked currents prevented Hill fit of α_2(T292K)$\beta_2\gamma_{2s}$ receptor data. Mean ± SEM values from N number of cells. WT; wild-type. **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$, unpaired two-way *t*-test.

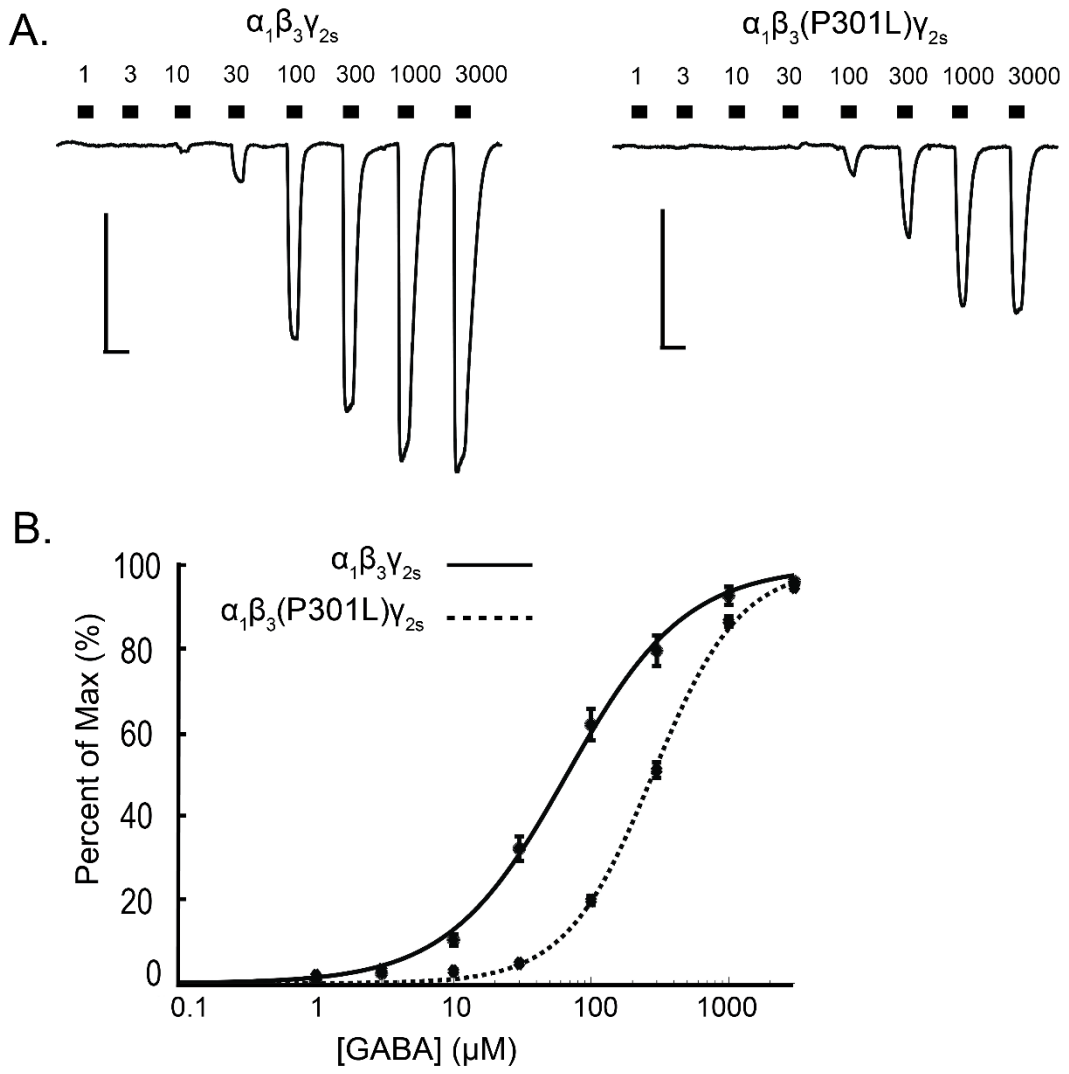

Figure 4.5. $\alpha_1\beta_3$(P301L)γ_{2s} receptors are less sensitive to GABA activation. (A) Example trace of GABA concentration-response assays (1-3000 μM) for $\alpha_1\beta_3\gamma_{2s}$ and $\alpha_1\beta_3$(P301L)γ_{2s} receptors expressed in HEK293T cells. Scale bars: horizontal = 5sec, vertical = 500pA. (B) GABA concentration-response curves for $\alpha_1\beta_3\gamma_{2s}$ (solid line, n = 21 cells) and $\alpha_1\beta_3$(P301L)γ_{2s} (dotted line, n = 20 cells) receptors. Points are mean ± SEM and error bars are not shown where bars are smaller than points. The drawn line is a representative fit based on the average GABA concentration responses.

4.5 Discussion

$GABA_A$ receptors are important regulators of neuronal inhibition, and mutations in *GABRs* have been associated with various types of epilepsy. While pathogenic variants in *GABRB3* were recently reported in individuals with mild and severe forms of epilepsy (Janve et al., 2016; Moller et al., 2017), there has only been a single report of a pathogenic variant in *GABRA2* (Orenstein et al., 2018). Furthermore, there have been no reports of disease-causing variants in *GABRA5,* although Hernandez and colleagues recently identified three *GABRA5* missense variants (p.V204I, p.W280R, and p.P453L) from individuals with epilepsy that resulted in gating defects and/or reduced current production. However, without familial segregation information, the authors were unable to determine pathogenicity and concluded that these variants might act as modifiers of epilepsy susceptibility (Hernandez et al., 2016). In this study, we describe three individuals with epilepsy and developmental delay who were found to carry heterozygous missense variants in *GABRA5*, *GABRA2*, and *GABRB3*, and present the effects of these variants on channel function.

GABRA5 is highly expressed in the hippocampus and contributes to extrasynaptic tonic inhibition in the brain (Caraiscos et al., 2004). The *GABRA5* gene is located on chromosome 15, along with *GABRB3* and *GABRG3*. This genomic region has been implicated in the neurological features of Prader-Willi syndrome, Angelman syndrome, and autism spectrum disorder; three disorders that often include seizures (Fatemi et al., 2010; Hogart et al., 2007). Brain slices from mice lacking *Gabra5* exhibit increased excitability of hippocampal pyramidal neurons (Bonin et al., 2007), and reduced *Gabra5* mRNA and protein expression has been observed in experimental rat models of

pilocarpine-induced temporal lobe epilepsy (Fritschy et al., 1999; Houser and Esclapez, 2003).

We identified the novel *de novo GABRA5* variant (p.V294L) in Patient 1, who presented with severe epilepsy and delayed motor and cognitive development. Interestingly, a *de novo* valine-to-leucine change was observed at the homologous position of *GABRA1* (p.V287L) in a patient with early-onset epileptic encephalopathy (Kodera et al., 2016). Although the group who identified the *GABRA1* p.V287L variant did not examine the effects of the variant on channel function (Kodera et al., 2016), this observation suggests that substitutions at this position may be associated with severe epilepsy phenotypes.

Whole-cell patch-clamp recordings of α_5(V294L)$\beta_2\gamma_{2s}$ receptors revealed a 10-fold increase in the GABA apparent-affinity, but with a concurrent reduction in maximal current. The observed properties of α_5(V294L)$\beta_2\gamma_{2s}$ channels are consistent with previous mutagenesis of the homologous position in the α_2 subunit (denoted as V260W), where mutant receptors became 10 times more sensitive to GABA but had decreased maximal responses (Ueno et al., 2000). Although the α_5(V294L)$\beta_2\gamma_{2s}$ receptors are more sensitive to GABA, this likely causes more channels to accumulate in the desensitized state, removing them from the pool of activatable receptors and thus leading to less neuronal inhibition. During desensitization, the receptors enter a state in which the channel is closed and chloride current is blocked, despite the receptor being activated. In the presence of higher GABA concentrations, the population of activated $GABA_A$ receptors increases, and the probability of those receptors entering a desensitized state also increases. Indeed, when we measured the degree of receptor desensitization from the

GABA concentration-response assays, we found that α_5(V294L)$\beta_2\gamma_{2s}$ receptors desensitized significantly more than wild-type receptors as GABA concentration increased (**Fig. 4.3D**). The impact of this deficit is predicted to be more pronounced for extrasynaptic receptors, such as α_5, where the constant presence of GABA would not allow receptors to recover from desensitization.

Given the enhanced sensitivity of α_5(V294L)-containing receptors to GABA, the patient harboring this variant is unlikely to receive therapeutic benefit from traditional positive allosteric modulators targeting $GABA_A$ receptors, such as benzodiazepines and barbiturates, as these types of drugs would be expected to further increase the number of desensitized receptors. Patient 1, who had the *GABRA5* p.V294L variant, experienced increased seizure frequency while on the barbiturate phenobarbital; however, it was hard to differentiate whether this was the natural course of the patient's epilepsy or whether this was due to the medication. Treatment with the benzodiazepine clonazepam had no effect on seizure frequency but caused sedation. Interestingly, Patient 1 achieved seizure freedom on a combination of zonisamide, levetiracetam, and oxcarbazepine. These anti-epileptic drugs act predominantly through non-GABAergic mechanisms, such as inhibiting voltage-gated sodium and calcium channels.

GABRA2 is located on chromosome 4 in a gene cluster with *GABRB1, GABRA4,* and *GABRG1*. *GABRA2* is also highly expressed in the hippocampus but is localized to the cell soma to mediate synaptic transmission (Prenosil et al., 2006; Tian et al., 2005). In a meta-analysis of 12 genome-wide association studies, an intergenic single nucleotide polymorphism (rs535066) near the 3' end of *GABRA2* was associated with increased risk for epilepsy (International League Against Epilepsy Consortium on Complex Epilepsies.

Electronic address, 2014). Hawkins and colleagues demonstrated that decreased expression of *Gabra2* was correlated with greater mortality of heterozygous *Scn1a*$^{+/-}$ mice that serve as a model of Dravet syndrome (Hawkins et al., 2016). Finally, *Gabra2* mRNA expression was observed to be reduced in several brain regions following kainic acid-induced seizures in rats (Drexel et al., 2013).

Patient 2, harboring the *de novo GABRA2* p.T292K variant, presented with intractable epilepsy, profound intellectual disability, and severe cerebral palsy. Recently, Orenstein and colleagues reported a *de novo* missense variant in *GABRA2* (c.1003A>C, p.N335H) in an individual with early-onset epileptic encephalopathy, choreiform movement disorder, and visual impairment (Orenstein et al., 2018). The clinical presentation of their patient is strikingly similar to that of Patient 2, suggesting that pathogenic variants in *GABRA2* may be associated with severe disease.

Beyond *GABRA2*, a threonine-to-lysine change was reported at the homologous position of *GABRB2* (p.T284K) from a patient with early myoclonic encephalopathy who passed away at 17 days of age (Hamdan et al., 2017). Additionally, a threonine-to-isoleucine substitution at the homologous position in *GABRA1* (p.T292I) was identified in two unrelated patients with Dravet syndrome and infantile spasms, respectively (*de novo* in both cases) (Allen et al., 2013; Johannesen et al., 2016). The individual with infantile spasms also shared many features with Patient 2, including poor vision, microcephaly, hypotonia, and cognitive and motor delay (Allen et al., 2013). Therefore, substitution of this highly conserved threonine residue lining the pore of the receptor appears to be associated with severe epilepsy phenotypes.

The α_2(T292K) variant occurs at the 10' position in the M2 domain, which is conserved across the human α, β, and γ subunits. A previous study examined this position of the α_2 receptor via tryptophan scanning mutagenesis. The α_2(T292W) mutant (numbered as T265W in that study) led to spontaneous channel openings, with no detectable GABA-evoked responses (Ueno et al., 2000). Furthermore, an induced threonine-to-lysine substitution at the homologous position in the ρ_1 subunit (T302K) resulted in channels that exhibited small GABA-evoked currents and high background leak (Wotring and Weiss, 2008). These experiments show that substitution of this highly conserved threonine, especially with bulky, positively charged residues, can alter the gating of the channel.

Functional studies of α_2(T292K)$\beta_2\gamma_{2s}$ receptors revealed channels that did not produce GABA-evoked currents, consistent with previous studies. Although we saw that expression of the mutant receptor was reduced, protein was detectable in the biotinylated surface fraction, indicating that receptors are present at the cell surface. Therefore, reduction in α_2(T292K)$\beta_2\gamma_{2s}$ receptor expression alone cannot completely explain the lack of GABA-evoked currents observed in the GABA concentration-response assays. Cells expressing α_2(T292K)$\beta_2\gamma_{2s}$ receptors also exhibited larger basal leak currents, which could be suppressed with increasing concentrations of the channel blocker picrotoxin. These results are consistent with a receptor that is expressed at the cell surface but unable to transition between open and closed states. A channel that is caught in the open state would be expected to increase the amount of tonic inhibition, which is usually set by a low frequency of spontaneous channel openings in the absence of GABA, or by activation at basal GABA concentrations (<1 μM). Furthermore, this receptor would have

reduced ability to respond to temporally-specific GABA stimulation. Consequently, neurons expressing these receptors would pass indiscriminate $GABA_A$ receptor-mediated currents, whose polarity would depend on the reversal potential of chloride in the neuron. During early development, the intracellular concentration of chloride is higher, causing chloride ions to move out of the cell when receptors become activated, thereby depolarizing neuronal membranes. Later in development, expression of the KCC2 potassium-chloride cotransporter lowers the intracellular concentration of chloride, allowing $GABA_A$ receptors to hyperpolarize cells in response to activation by GABA (Hubner et al., 2001; Rivera et al., 1999). Therefore, α_2(T292K) could result in aberrantly high levels of depolarization signals elicited during early brain development when GABAergic synaptic development precedes glutamatergic development (Ben-Ari, 2006). This would likely promote the development of seizures since proper tonic GABAergic currents are important during development (Lee and Maguire, 2014). Also, because *GABRA2* expression is highest during early development and declines with age, it is reasonable to expect that the α_2(T292K) variant might confer the highest vulnerability to the brain during development (Laurie et al., 1992).

Pathogenic epilepsy variants in *GABRB3* were first reported by the Epi4K Consortium and the Epilepsy Phenome/Genome Project in 2013 (Allen et al., 2013). Since then, several *GABRB3* variants have been identified in individuals with a spectrum of epilepsies, ranging from mild febrile seizures to severe epileptic encephalopathy (Moller et al., 2017). We identified the *GABRB3* p.P301L variant in a patient with intractable epilepsy and developmental delay. Although we were unable to determine variant inheritance, the same variant was recently reported as *de novo* in a patient with

focal epilepsy (Moller et al., 2017). Additionally, *GABRB3* p.Y302C was previously identified in three unrelated patients with phenotypes that include focal epilepsy and intractable epileptic encephalopathy (Allen et al., 2013; Moller et al., 2017). The M2-M3 linker, where P301L and Y302C are located, is a highly conserved region that is known to be involved in coupling binding of the agonist to the gating of the channel, a function important for conferring ligand efficacy (O'Shea et al., 2009).

Because the *GABRB3* p.P301L substitution was not functionally characterized previously, we performed a GABA concentration-response assay on $\alpha_1\beta_3$(P301L)γ_{2s} receptors. Mutant receptors demonstrated a reduction in GABA apparent-affinity and GABA-evoked current amplitude. This would lead to a receptor that does not respond as strongly to GABA signals as receptors expressing wild-type β_3, likely causing reduced neuronal inhibition. The observed reduction in function of β_3(P301L) receptors is similar to the reduction seen for receptors containing other *GABRB3* epilepsy variants, particularly the adjacent β_3(Y302C) variant (Janve et al., 2016; Moller et al., 2017), providing support for the pathogenicity of this variant.

In addition to the three variants that we investigated functionally, we identified 17 other heterozygous *GABR* variants from individuals with epilepsy (**Table 4.1**). The frameshifting *GABRR2* c.57_67delCCTCACAGATG variant, which was absent from the gnomAD database, was not considered to be causative of disease since multiple heterozygous LOF *GABRR2* variants are listed in gnomAD. Similarly, *GABRA6* appears to be tolerant of heterozygous LOF variation based on the presence of multiple frameshifting, splice-site, and premature stop variants in gnomAD. Therefore, we excluded the *GABRA6* p.W305X variant from further study. The *GABRG1* p.I279V

variant is also unlikely to be casual since the affected individual carried a pathogenic frameshifting variant in *SCN1A*. On the other hand, *GABRG2* p.D231N was classified as a variant of uncertain significance. This variant affects the N-terminal ligand-binding domain of the receptor and is predicted to be deleterious by several prediction algorithms; however, it was observed four times in the gnomAD database.

The remaining 13 *GABR* variants, observed five or more times in gnomAD (**Table 4.1**), are unlikely to act as monogenic causes of epilepsy since a recent study showed that dominant pathogenic epilepsy variants were typically absent from or observed only once in the ExAC database, even for milder forms of epilepsy such as GEFS+ and benign familial neonatal-infantile epilepsy (Bennett et al., 2017). However, we cannot exclude the possibility that some of these variants could contribute to the risk for developing epilepsy. Hernandez and colleagues evaluated *GABR* variants identified from individuals with genetic epilepsies and healthy controls and showed that several variants altered channel gating kinetics (Hernandez et al., 2016). Therefore, it is possible that some of the rare variants presented in Table 4.1 could also contribute to epilepsy susceptibility through complex and polygenic inheritance.

In summary, we present *de novo* variants in *GABRA5* and *GABRA2* as contributors to early-onset epilepsy. We provide multiple lines of evidence to support the role of the *GABRA5* p.V294L and *GABRA2* p.T292K substitutions in disease, including that the variants are *de novo*, absent from population controls, affect highly conserved and important regions of the $GABA_A$ receptor, and alter the function of α_5- and α_2-containing receptors. Additionally, we provide functional evidence of pathogenicity for the identified *GABRB3* p.P301L variant. This study highlights how different epilepsy-

associated variants in *GABRs* can reduce neuronal inhibition via a range of alterations in $GABA_A$ receptor function. The β_3(P301L) variant produced functional changes that were consistent with several published mutations (Janve et al., 2016; Moller et al., 2017), while the α_2(T292K) and α_5(V294L) variants produced unexpected changes in receptor function that have not been previously reported for human $GABA_A$ receptor variants. Further studies are needed to determine whether pathogenic variants in *GABRA2* and *GABRA5* are associated with distinct epilepsy phenotypes or whether they will result in a spectrum of phenotypes, as observed for *GABRA1, GABRG2*, and *GABRB3*.

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CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Summary

When I began my studies in 2014, over one hundred genes had been associated with epilepsy and seizures; however, variants in these genes were typically identified in less than a quarter of epilepsy patients tested, leaving more than 75% of patients without a genetic diagnosis. While 20-30% of epilepsy cases result from acquired neurological insults (e.g., stroke, head trauma), there was an expectation that additional genetic factors were contributing to the development of epilepsy in a large proportion of these individuals. Furthermore, even among the known epilepsy genes, there were several for which only a small number of variants had been reported, and identification of additional disease variants would strengthen the association with epilepsy.

The overarching goal of my graduate research was to further our understanding of the genetics of epilepsy with a focus on novel genes and variants. The data presented in this dissertation furthers our knowledge of the known epilepsy genes through the evaluation of genetic variants detected from the ESD panel in more than 300 epilepsy patients. Additionally, I used the Mendeliome data to identify novel variants in epilepsy genes not included in the ESD panel (e.g., *CHD2, SLC6A1*), genes associated with other neurological disorders (e.g., *POGZ, ANKRD11*), and genes not currently associated with disease (e.g., *GABRA2*).

5.2 Improving gene panel analysis for epilepsy

Based on our experience using the available sequence data from the ESD panel, we identified several factors that could improve the diagnostic yield of gene panel testing for epilepsy. First, although parental samples are not routinely included when performing

gene panel analysis, follow up testing on parental samples is critical for the reclassification of VUS identified in the proband. It would be beneficial to collect parent samples at the same time as the proband sample when sending for testing in order to avoid delays in follow up of variants that require further analysis. Greater availability of parental samples might have increased the yield in our cohort by allowing for the identification of *de novo* variants and phasing of recessive variants.

Second, the diagnostic yield would also likely have been higher if more samples were accompanied by clinical information since such knowledge can facilitate variant interpretation by allowing for comparisons with previously reported cases. This information is also helpful for identifying individuals with atypical clinical presentations that may expand the phenotypic spectrum of a given gene. The expansion of the clinical spectrum has already been demonstrated for many epilepsy genes, including *SCN2A* and *KCNQ2* (Nakamura et al., 2013; Weckhuysen et al., 2012).

Finally, although recent publications have demonstrated the ability of next-generation sequencing (NGS) to detect intragenic copy number variation, these algorithms are still in the development stage and need confirmatory testing (Epilepsy Phenome/Genome Project Epi, 2015). Therefore, exon level copy number analysis for single and multiple exon deletions and duplications is still beneficial for identifying variants that may be missed by NGS. For instance, there were three individuals in our cohort of 339 patients that had concurrent deletion/duplication testing that detected copy number variants that could explain their epilepsy.

One major theme that emerged from my work was the importance of frequently updating gene panels to incorporate newly identified epilepsy genes. Through my

analysis of the Mendeliome data, I identified candidate variants in many genes that have been reported in the literature as being associated with epilepsy. These genes, including *CACNA2D2*, *CHD2*, *DEPDC5*, *GABRB3*, *GRIN2B*, and *SLC6A1* (see Appendices), should be added to the ESD panel. Many diagnostic laboratories use Mendeliome-type libraries but only analyze a subset of those genes according to the test ordered, similar to EGL. Therefore, newly-associated genes that are already present in the larger sequencing libraries could easily be added to epilepsy panels to keep them up-to-date. Furthermore, in negative cases, with appropriate consent, the remainder of the Mendeliome gene set could be evaluated to identify candidate variants in genes outside of the epilepsy panel. Even as whole-exome and -genome sequencing approaches become more commonly used for genetic testing, it will remain important to have up-to-date epilepsy gene lists to aid in variant prioritization and phenotype definition.

In addition to the novel epilepsy genes that I identified, my analysis of variants from the ESD panel also revealed several genes that should be removed from current epilepsy panels, including multiple genes whose original "disease" variants turned out to be common polymorphisms present in the general population. Removing these questionable genes from analysis has the potential to reduce the number of variants that must be classified by diagnostic labs and reduce the number of VUS reported to clinicians and patients. Although it was not initially my intention to modify the ESD panel, it became apparent that the knowledge I gained from my research had the potential to greatly benefit the ESD panel. As a result, I worked with directors at EGL to develop an improved panel that both incorporates new genes and removes genes whose association with epilepsy failed to be replicated.

5.3 What defines an "epilepsy" gene?

As more individuals with epilepsy are screened using whole-exome sequencing, it is becoming very clear that many neurodevelopmental disorders (epilepsy, autism, intellectual disability, ADHD, schizophrenia) share common underlying genetic factors.

In our cohort of clinically-referred epilepsy patients, I identified candidate variants in genes that were first associated with other disorders, including autism and intellectual disability (ex. *SHANK3*, *POGZ* – see Appendix B). Similarly, large sequencing studies for other neurodevelopmental disorders have identified variants in genes first reported in epilepsy (ex. *SCN2A*, *GABRB3*) (Chen et al., 2014; Sanders et al., 2012; Wang et al., 2016). In fact, pathogenic *SCN2A* variants have now been identified in individuals ascertained for epilepsy, autism, intellectual disability, and schizophrenia (Li et al., 2015).

Therefore, what qualifies a gene to be an "epilepsy" gene? Can a gene also be considered an "autism" gene or an "intellectual disability" gene? These questions become important when considering how to design gene panels and interpret the results of genetic testing. Targeted gene panels are useful for quickly diagnosing individuals with variants in known disease genes. However, how do diagnostic laboratories decide what genes should be included when designing or updating a gene panel? Similarly, how do you interpret a variant that is identified in an individual with epilepsy when the affected gene has only been associated with autism up to that point? Epilepsy and autism are often co-morbid, especially in individuals that also have intellectual disability (Amiet et al., 2008). This means that individuals referred for genetic testing for epilepsy often have autism and/or intellectual disability in addition to seizures. We suspect this is likely the case for

many of the individuals in our epilepsy cohort (**Table 1.4**). On one hand, it is the responsibility of the referring physician to order the correct type of genetic testing. But on the other hand, diagnostic test providers should recognize and account for the genetic overlap between neurodevelopmental conditions.

In the future, it will likely be beneficial for diagnostic laboratories to offer broader "Neurodevelopmental Disorder" gene panels that can be ordered for individuals with a combination of unexplained neurological symptoms, including epilepsy, autism, and intellectual disability. This type of panel would contain all genes known to be associated with neurological and neurodevelopmental disorders. When the test is ordered, the physician could indicate which clinical features (seizures, autism, etc.) are most prominent, allowing the testing laboratory to prioritize which genes should be examined first. If the initial list of genes is negative for pathogenic variants, then the laboratory could easily expand their analysis to examine the rest of the neurodevelopmental genes for variants. In theory, this type of testing set-up could still be quicker than whole-exome analysis as it would involve fewer genes.

5.4 The move towards WES and WGS

WES has the potential to result in a higher diagnostic yield compared to gene panels; however, clinical laboratories are only able to assign pathogenicity to variants in known epilepsy genes. This means diagnostic yields will remain similar between gene panels and WES as long as gene panels are kept up-to-date with all of the known epilepsy genes. Eventually, it will become more cost-effective to perform whole-exome or even whole-genome sequencing rather than periodically updating targeted sequencing

libraries. In fact, some diagnostic laboratories are already sequencing the whole exome of patients and just analyzing a subset of the genes for their targeted panels. However, the best way to utilize the rest of the WES data remains to be determined. For example, if a specific gene panel is ordered but variant data is available for the whole exome, when should the referring physician or diagnostic laboratory decide to expand their analysis beyond the ordered gene panel? WES is most effective when performed on a trio (proband plus both biological parents), as this allows for the identification of *de novo* variants. But most gene panels are only performed on the proband, with secondary Sanger sequencing of the parents to determine the inheritance of candidate variants. As mentioned above, it would be advantageous for diagnostic laboratories to request parent samples be collected at the same time as the proband's sample. In this way, gene panel testing of the proband could easily reflex to trio-based WES following a negative panel result.

5.5 Solving the missing genetics of epilepsy

Currently, genetic factors are predicted to play a role in approximately 70-80% of epilepsy cases; however, the diagnostic yield of CMA and NGS technologies remains around half this amount. This begs the question: What are we missing?

5.5.1 Missed coding variants

As sequencing technologies and bioinformatic tools continue to improve, we should see increases in read depth and read length, which will increase our ability to detect variants and increase our confidence in variant calls. This will undoubtedly allow

for the identification of additional epilepsy genes, although each new gene is only likely to account for a small percentage of cases. With increased read depth, we will also have improved capability to detect mosaic mutations. These are variants that are not present in every cell of the body and are therefore harder to detect and distinguish from sequencing errors. There is an increasing awareness of the contribution of mosaic variants to disease, including epilepsy (Stosser et al., 2017). A recent study showed that 7.5% of pathogenic *de novo SCN1A* variants were mosaic in the proband (de Lange et al., 2018). Similarly, mosaic variants in genes of the mTOR pathway have been detected in the brains of individuals with a range of epileptogenic malformations, including focal cortical dysplasia, hemimegalencephaly, and megalencephaly (D'Gama et al., 2017; Mirzaa et al., 2016).

Improved bioinformatic tools may also allow for the detection of exons for alternative transcripts of known epilepsy genes. For example, we hypothesized that an alternative exon of *SCN8A*, which was not being analyzed by diagnostic laboratories, could harbor pathogenic epilepsy variants. This theory was later confirmed through reanalysis of WES data from more than 3,700 individuals by the Epilepsy Genetics Initiative, which led to the identification of three *de novo* variants in the 5A exon of *SCN8A* (Epilepsy Genetics, 2017). Thus, it is possible that variants in other important alternative exons are currently being overlooked and may be recognized in the future.

5.5.2 Noncoding variation

Variation in the noncoding regions of the genome may also contribute to epilepsy. This includes variants in promoter and enhancer sequences, as well as those affecting

noncoding RNAs (e.g., microRNAs) and their binding sites. There have been a small number of reports of variants identified in noncoding regions of DNA from individuals with epilepsy, including a point mutation in the 5' region of *SCN1A* (Gao et al., 2017) and a duplication upstream of *GRIA3* that reduces mRNA expression (Bonnet et al., 2012). Similarly, microRNAs have been observed to be dysregulated in patients and animal models of epilepsy (Shao and Chen, 2017); however, a direct causal relationship has not been established. Few studies to date have examined the noncoding regions of the genome in epilepsy patients; however, increased use of WGS should allow for the identification of candidate noncoding variants. Currently, noncoding variants are more difficult to interpret and require experimental testing to determine if they have a functional effect.

5.5.3 Polygenic inheritance, Modifiers, and Dual diagnoses

Most of the discussion to this point has considered monogenetic causes for disease, but many cases of epilepsy are likely to be the result of variants at multiple loci across the genome. Polygenic factors have long been predicted to play a role in common forms of epilepsy, such as genetic generalized epilepsies and focal epilepsies (Berkovic et al., 1998; Ottman et al., 1996). However, there has been very little success identifying such factors. The largest meta-analysis of epilepsy GWA studies to date, which included 8,696 cases and 26,157 controls, only identified three loci that met genome-wide significance (International League Against Epilepsy Consortium on Complex Epilepsies. Electronic address, 2014). Therefore, while a small number of common variants (minor allele frequency (MAF) > 5%) may contribute to epilepsy, these types of variants remain difficult to detect. On the other hand, there is increasing evidence for rare variants (MAF

< 1%) with small-to-medium effect sizes as susceptibility alleles for epilepsy (Hernandez et al., 2016; Klassen et al., 2011). For example, a study performed by the Epi4K Consortium and Epilepsy Phenome/Genome Project identified an excess of ultra-rare (MAF < 0.05%) variants in known epilepsy genes in cases with common epilepsies compared to controls (Epi and Epilepsy Phenome/Genome, 2017). Thus, genes that cause rare monogenetic forms of epilepsy may also play a role in the common epilepsies. Despite this discovery, it is still unclear how many genetic "hits" a person requires before he or she develops epilepsy or how environmental factors play into the risk for disease.

Genetic modifiers are alleles that can alter the clinical presentation produced by another allele of large effect size (i.e., a pathogenic disease variant), either improving or worsening the clinical phenotype. Genetic modifiers are often cited to explain phenotypic variability, especially between individuals harboring the same genetic variant. However, modifier alleles have been challenging to identify because of the large amount of genetic variation that exists in the human genome. It is for this reason that most modifiers of epilepsy have been identified in mice and other model systems, where genetic background can be easily controlled (Bergren et al., 2005; Miller et al., 2014). Current literature suggests that rare variants with modest-to-moderate effect sizes could act as modifiers to influence epilepsy presentation (Hammer et al., 2017). These are likely the same alleles that contribute to polygenic inheritance of epilepsy, but act as modifiers when combined with pathogenic epilepsy mutations, such as truncating *SCN1A* variants.

One interesting observation that has come out of several large sequencing studies is the identification of individuals with two or more pathogenic variants that appear to independently cause disease. These variants can contribute to "dual diagnoses" or

"blended phenotypes" in the patient, which can occasionally be mistaken for a new disease entity or the expansion of the phenotypic spectrum of a single gene (Posey et al., 2017). In some cases, the two variants are associated with distinct disorders affecting different organ systems. In other cases, both genes are associated with overlapping clinical presentations. On average, 4.3% of cases solved using WES were found to have two or more genetic diagnoses, although these studies were not specific to epilepsy (Balci et al., 2017; Farwell et al., 2015; Posey et al., 2017). Unlike modifier alleles and polygenic causes of epilepsy, variants involved in dual diagnoses may be easier to identify in patient genomes since these variants will be absent from healthy controls. In the examination of data from the Mendeliome, there were several cases in which I identified candidate variants in multiple genes. However, without detailed clinical information or parental samples to determine inheritance, it was challenging to assign pathogenicity to one variant, let alone both. As a future direction, it would be interesting to examine the Mendeliome data from individuals that had a pathogenic variant identified from the ESD panel to determine if any of these individuals had a second genetic diagnosis.

5.6 Future Directions

There are several obvious future directions that emerge from my work using the available variant data from epilepsy patients.

From our investigation of *SCN8A* variants detected from gene panel testing, I discovered that a known alternative exon (5A) was not being analyzed by diagnostic laboratories. I speculated that variants in this exon could contribute to the development of

epilepsy and had planned to Sanger sequence exon 5A in our cohort of epilepsy patients with negative gene panel results. However, prior to beginning our screen, the Epilepsy Genetics Initiative reported three variants in exon 5A detected from WES (Epilepsy Genetics, 2017), confirming my hypothesis.

Now that pathogenic epilepsy variants have been identified in the 5A exon of *SCN8A*, it would be interesting to compare the functional properties of the two $Na_v1.6$ channel isoforms encoded by the 5N and 5A exons, which only differ at two amino acid positions. This type of analysis has already been performed for $Na_v1.1$ and $Na_v1.2$, revealing differences in channel properties (Fletcher et al., 2011; Gazina et al., 2015; Xu et al., 2007). It would also be intriguing to compare how variants in the two alternate exons affect $Na_v1.6$ channel function to cause disease. Two of the variants identified thus far in the 5A exon (p.V211A and p.R223G) have also been reported in the 5N exon of epilepsy patients (de Kovel et al., 2014; Epilepsy Genetics, 2017), allowing for direct comparison.

A future direction that the Escayg Lab is actively pursuing is the functional evaluation of other candidate variants identified from the Mendeliome library. We have already established collaborations to assess the functional impact of variants identified in newer epilepsy genes, including *CACNA2D2* and *SLC6A1*. Moreover, I identified interesting single variants in *ATP6V0C* and *HUWE1*, two genes with unclear epilepsy associations, that warrant follow-up. As more patients are tested using the ESD panel, we will be able to screen their sequence data for additional variants in our candidate genes, which could strengthen our argument for disease association.

We functionally investigated two *de novo* variants detected in *GABRA2* and *GABRA5*, genes not previously associated with epilepsy, using whole-cell patch-clamp electrophysiology. Recordings from HEK293T cells expressing the mutant $GABA_A$ receptor subunits revealed changes in functional properties that hinted at both loss and gain-of-function mechanisms. For example, receptors containing α_5(V294L) subunits were significantly more sensitive to GABA but also desensitized more quickly compared to wild-type receptors. Similarly, α_2(T292K)-containing receptors were expressed at a lower level relative to wild-type, but mutant receptors at the cell surface were also constitutively open, allowing ions to pass indiscriminately. Consequently, it would be fascinating to study both of these variants in an animal model to examine the *in vivo* effects of these missense changes. Although mice would be the best model system because of the presence of orthologous *Gabra5* and *Gabra2* genes, *Drosophila melanogaster* could also be employed as flies have been used to study human epilepsy genes previously (Parker et al., 2011; Song and Tanouye, 2008).

Despite the existence of mouse strains with null *Gabra2* and *Gabra5* alleles, spontaneous seizures have not been reported in these mice. Furthermore, it does not appear that anyone has examined their seizure susceptibility. The lack of overt seizure phenotypes in these mice could suggest that the patient missense variants I identified may produce more complex effects than just reduced receptor function. Aside from seizures, both affected patients experienced other clinical features, including vision, motor, and behavioral impairments, which could be investigated further in a mouse model.

Lastly, the approaches I used to examine the genetic architecture of epilepsy could be extended to other disorders for which gene panel testing is routinely ordered at

EGL (ex. autism spectrum disorder, neuromuscular disorders, ciliopathies, etc.) since all of the gene panels at EGL utilize the Mendeliome library.

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Appendix A

Identification of Compound Heterozygous Variants in *CACNA2D2*

Introduction

The voltage-dependent calcium channel auxiliary subunit *CACNA2D2* encodes a single protein that is post-translationally cleaved into two smaller peptides, alpha-2 and delta-2, which are covalently linked by disulfide bonds. The alpha2-delta2 (α2δ2) complex functions to modulate the activation and inactivation kinetics of the alpha-1 (α1) pore-forming subunit of the calcium channel. It is also involved in the assembly, trafficking, and localization of the α1 subunit.

There have been two reports of patients with epileptic encephalopathy due to homozygous *CACNA2D2* variants. Edvardson and colleagues reported a consanguineous family in which three affected siblings were reported to have intractable seizures, global developmental delay, axial hypotonia, intermittent choreiform movements, vision issues, and cerebellar atrophy (Edvardson et al., 2013). Using homozygosity mapping and whole exome sequencing (WES), the authors identified two missense variants that were homozygous in all three affected individuals but heterozygous in the unaffected parents and an unaffected sibling. The variants occurred in *CACNA2D2* and *CELSR3*, which are both proximally located on chromosome 3. The authors focused on the *CACNA2D2* c.3119A>G (p.L1040P) variant since the patient phenotypes were similar to those reported for mouse models of *Cacna2d2* dysfunction, including “ducky” and “entla” mice, which exhibit absence seizures, spontaneous convulsive seizures, cerebellar atrophy, ataxia, paroxysmal dyskinesia, and premature mortality (Brill et al., 2004; Meier, 1968). Analysis in *Xenopus* oocytes showed that expression of α2δ2 subunits

containing the p.L1040P variant were comparable to wild-type but the mutant subunit was unable to enhance the currents produced by α1 channels, suggesting a loss-of-function mechanism (Edvardson et al., 2013).

Using similar methods, Pippucci and colleagues identified a homozygous frameshifting *CACNA2D2* variant (c.1295delA, p.N432fsX) in an affected proband from a consanguineous relationship. The affected individual exhibited intractable convulsive epilepsy, absence seizures, axial hypotonia, dyskinetic movements, vision issues, cerebellar atrophy, microcephaly, and facial dysmorphisms (Pippucci et al., 2013). The proband also had a homozygous missense variant in *CELSR3*; however, the authors felt that this variant was likely incidental due to the close proximity of the two genes. A muscle biopsy from the proband revealed reduced *CACNA2D2* mRNA and protein levels.

Here, I describe an individual with compound heterozygous missense variants in *CACNA2D2* and epileptic encephalopathy. Unlike previous reports, this individual did not have any variants in the *CELSR3* gene.

Case Report

The proband was referred to the Emory Genetics laboratory for genetic testing on the ESD panel, and later for trio-WES. Testing was indicated by the presence of epileptic encephalopathy, intractable absence epilepsy, cerebellar atrophy, developmental delay, fatigue, difficulty seeing, and gastrointestinal issues in the proband. No pathogenic variants were identified by gene panel analysis or by WES. This prompted us to perform research-based reanalysis of the data from this patient. During reanalysis, it was noted that the proband had two rare missense variants in the *CACNA2D2* gene, which were each inherited from one parent (**Fig. A1**).

Methods

Gene panel analysis was performed as described in the Chapter 2 Methods. Whole exome sequencing was performed on DNA from the proband and his parents using the Agilent V5 Plus exome enrichment kit (Agilent Technologies, Santa Clara, CA). DNA from the proband and both parents were obtained to confirm the variant inheritance, by Sanger sequencing, after written consent was given. The following mRNA and protein Refseq accessions were used to annotate the *CACNA2D2* variants: NM_006030.2 and NP_006021.2. The following primer pairs were used for PCR amplification and sequence analysis of the two variants: P261L_F-GTGGCTGAGGGAGGAGAGAA, P261L_R-CCTGGATAGGCCGAGAACAG, L1046P_F-GTCGCGTTGTAGTCGAAGCA, L1046 _R-CTCGGTAAACGCCTCCTACA. This study was approved by the Institutional Review Board of Emory University.

Results and Discussion

We focused on the two *CACNA2D2* variants during our reanalysis of the variant report for several reasons. First, the patient had two variants in a gene that is associated with autosomal recessive disease. Second, the p.L1046P variant is absent from the gnomAD database, while p.P261L is only observed once out of 245,184 alleles in gnomAD, indicating that both variants are extremely rare. Third, both variants were predicted to be deleterious by *in silico* algorithms, including CADD, PolyPhen-2, and SIFT. Fourth, both variants affected annotated functional domains of the α2δ2 protein

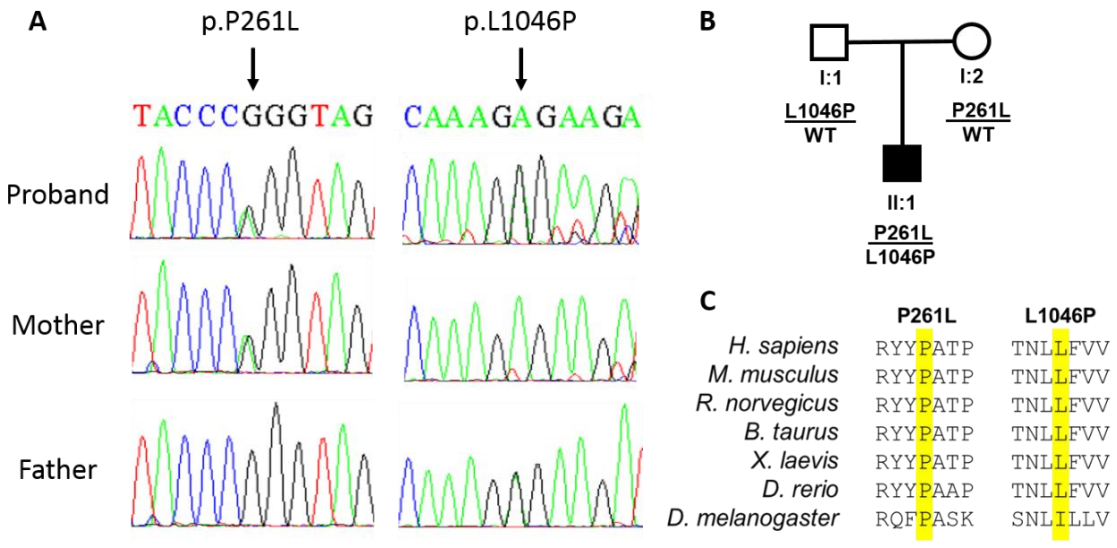

Figure A1 Compound heterozygous *CACNA2D2* variants.

Sanger sequencing traces (A) show that the *CACNA2D2* c.782C>T (p.P261L) variant was inherited from the unaffected mother while the c.3137T>C (p.L1046P) variant was inherited from the unaffected father. Note: The *CACNA2D2* gene is encoded by the minus strand of DNA. (B) Pedigree showing the inheritance pattern of the two variants. (C) Species protein alignment showing the conservation of the two amino acid positions.

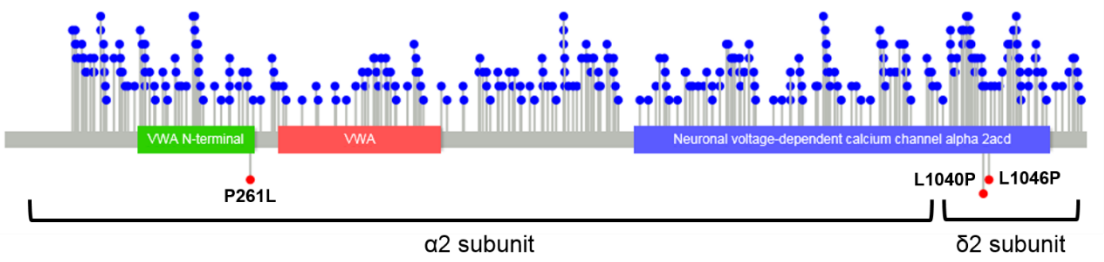

Figure A2 *CACNA2D2* variants affect different domains of the α2δ2 protein. The lollipop plot shows the distribution of population (blue) and epilepsy (red) missense variants across the α2δ2 protein structure. The population variants for *CACNA2D2* were extracted from the ExAC database. The P261L variant affects the α2 domain, while the L1046P variant affects the δ2 domain. The L1040P variant detect by Edvardson *et al.* is shown for reference. The protein structure is based on UniProt accession Q9NY47.

Table A1 Clinical Features of Individuals Carrying Recessive *CACNA2D2* variants

	This Study	Edvardson *et al*. 2013	Pippucci *et al.* 2013
CACNA2D2 Variant[a]	p.P261L/p.L1046P	p.L1040P	c.1295delA
CELSR3 variant	none	p.M2630I	p.G2136D
Sex	Male	2 Males, 1 Female	Male
Seizure Onset	NA	20-60 days	5 months
Epileptic Encephalopathy	+	+	+
Developmental delay	+	+	+
Absence seizures	+	-	+
Intellectual disability	+	+	+
MRI	Cerebellar atrophy	Cerebellar (vermian) atrophy	Cerebellar atrophy
Other features	Hypotonia, difficulty seeing, gastrointestinal issues, fatigue	Axial hypotonia, choreiform movements, no eye contact	Axial hypotonia, dyskinetic movements, no eye contact, tremor, facial dysmorphisms, small head
Seizure types	NA	Atonic, clonic, tonic, focal	Focal, tonic-clonic
Refractory seizures	+	+	+
EEG	NA	Slow background rhythm with multifocal spikes and slow waves	Multifocal spikes over the right centro-temporal and left parieto-occipital regions, slowed background activity

[a]*CACNA2D2* variants annotated according to Refseq NP_006021.2. NA, not available; EEG, electroencephalogram; MRI, magnetic resonance imaging.

(**Fig. A2**) and the affected amino acid residues were evolutionarily conserved across species (**Fig. A1**). Finally, one of the proband's variants, p.L1046P, was proximal to the previously reported disease variant, p.L1040P, which was demonstrated to reduce the function of the α2δ2 protein (Edvardson et al., 2013). Additionally, we did not identify any other potentially causative variants from the analysis of the WES data.

When we compared the clinical presentation of the proband to the previous two reports by Edvardson and Pippucci (**Table A1**), we found striking similarities between the affected individuals (Edvardson et al., 2013; Pippucci et al., 2013). The observed cerebellar atrophy is also reported in several *Cacna2d2* mouse models. While all of the available evidence supports the possible pathogenicity of these variants, functional testing is still needed.

Unlike the two previous reports, the proband in the current study did not have any variants in the *CELSR3* gene. The observed phenotypic similarities amongst all of the affected individuals indicates that altered *CACNA2D2* function is the major cause of disease and that the previously identified *CELSR3* variants are most likely incidental.

Because *CACNA2D2*-related disease is rare, the optimal treatment regime for these individuals is currently unclear. The disease mechanism appears to be reduced α2δ2 function, leading to reduced α1 cell surface expression and function (Edvardson et al., 2013). This would explain the partial phenotypic overlap observed between *CACNA2D2*- and *CACNA1A*-related disorders. However, there does not appear to be a consensus for the best treatment for *CACNA1A*-related disorders as different mutations produce different functional and phenotypic effects. As whole-exome and genome sequencing technologies are applied to individuals with different diseases, it will be interesting to see

if *CACNA2D2* variants are identified in individuals with other disease presentations, such as ataxia or migraine, as has been seen for *CACNA1A*, or if *CACNA2D2* will be associated with a very specific clinical presentation as has been the case so far.

In conclusion, I present the third report of a patient with epileptic encephalopathy and recessive variants in *CACNA2D2*. Importantly, I present the first case with compound heterozygous *CACNA2D2* variants and no additional candidate disease variants (e.g. *CELSR3*).

Appendix B

Positive Findings in Genes from the Mendeliome

Summary

This is a subset of the variants I identified from my analysis of the Mendeliome data from individuals with negative or inconclusive ESD panel results. I have divided the variants into two tables. The first table includes variants from genes that have been demonstrated to cause epilepsy when mutated but are not yet included in the ESD panel. These are genes that should be added to future iterations of the epilepsy panel and I am confident that the variants presented in **Table B1** are pathogenic.

The second table (**Table B2**) contains variants from genes that are not specifically associated epilepsy; however, many of these genes are associated with disorders that include seizures. These variants are potentially causative but are only likely to be identified by WES analysis since the affected genes are not often included in epilepsy panels.

Methods

Mendeliome Analysis

Variants from the Mendeliome were filtered using Python scripts to remove synonymous variants, variants with low coverage (<20X), and those observed at a frequency above 1% in EVS and/or ExAC databases. Variants were further filtered to remove those with skewed nucleotide base calls ($5\% < x < 35\%$ or $65\% < x < 95\%$) which did not fit with zygosity expectations (e.g., heterozygous or homozygous). Additionally, I removed any variant that was observed to be homozygous in ExAC.

SeqAnt 2.0 (Shetty et al., 2010) was used to generate CADD, PhyloP, and PhastCons scores for the variants that passed filtering. These prediction scores were used to tentatively rank variants by likelihood to be damaging.

Variant Analysis

Individual genes and variants were analyzed using available information to determine their likelihood to contribute to disease. Below is a list of online resources with the information each resource provides for analysis and the current URL address.

OMIM Disease-Gene associations, clinical phenotypes, gene expression patterns, biological functions; http://omim.org/

HGMD Previously published disease variants, variant classifications, disease phenotypes; https://portal.biobase-international.com/hgmd/pro/start.php

ClinVar Variants reported from clinical genetic testing, variant classifications; https://www.ncbi.nlm.nih.gov/clinvar/

EmVClass EGL's variant classification (if previously observed); http://www.egl-eurofins.com/emvclass/emvclass.php

ExAC Population frequency of variants (precursor to gnomAD); http://exac.broadinstitute.org/

gnomAD Population frequency of variants; http://gnomad.broadinstitute.org/

NextProt Protein structural domains; https://www.nextprot.org/

COBALT Protein alignments to assess amino acid conservation; https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?CMD=Web

Polyphen-2 Prediction of functional effect of variants; http://genetics.bwh.harvard.edu/pph2/

SIFT Prediction of functional effect of variants; http://sift.jcvi.org/

PubMed Primary literature; https://www.ncbi.nlm.nih.gov/pubmed

Table B1 Pathogenic Variants in Epilepsy Genes From the Mendeliome

Case	**1**	**2**	**3**	**4**	**5**
Gene	***CHD2***	***CHD2***	***CHD2***	***DEPDC5***	***GRIN2B***
OMIM Disease Association (MIM)	Childhood-onset epileptic encephalopathy (615369)			Familial focal epilepsy with variable foci (604364)	Early-infantile epileptic encephalopathy 27 (616139)
RefSeq	NM_001271.3			NM_001242896.1	NM_000834.3
mRNA Change	c.5035C>T	c.1503-2A>G	c.4771_4772 delCT	c.3662_3663delTC	c.2011-1G>T
Protein Change	p.R1679*		p.L1591Dfs *32	p.I1221Tfs*8	
Variant Notes	Variant reported twice in HGMD	Variant reported previously in individual with Lennox-Gastaut Syndrome	Variant not previously reported but loss of function associated with disease	Variant not previously reported but loss of function associated with disease	Variant reported once in HGMD
Clinical Information Provided	Not provided	Epilepsy and developmental delay	Partial complex seizures and elevated ammonia	Not provided	Convulsions and developmental delay

Table B2 Candidate Variants Identified from Mendeliome Analysis

Case	**6**	**7**	**8**	**9**
Candidate Gene	***ACO2***	***ANKRD11***	***ANKRD11***	***CACNA1G***
OMIM	100850	611192		604065
OMIM Disease Association (MIM)	Infantile cerebellar-retinal degeneration (614559)	KBG Syndrome (148050)		Spinocerebellar ataxia (616795)
Inheritance	AR	AD		AD
Clinical Synopsis from OMIM	Failure to thrive, eye abnormalities, developmental delay, intellectual disability, ataxia, seizures, hypotonia, cortical/cerebellar atrophy, hyporeflexia, peripheral demyelinating neuropathy	Short stature, characteristic facies, macrodontia, skeletal anomalies, developmental delay, intellectual disability, seizures, EEG anomalies		Eye anomalies, spinocerebellar ataxia, gait instability, dysarthria, cerebellar atrophy, tremor, hyperreflexia, spasticity, slow-progressing
mRNA Change (Refseq)	c.12661G>A, c.2278G>A (NM_001098.2)	c.6472G>T (NM_013275.5)	c.3224_3227delAAAG (NM_013275.5)	c.832G>A (NM_018896.4)
Protein Change	p.E421K, p.E760K	p.E2158*	p.E1075Gfs*242	p.G278S
Zygosity	Presumed Compound Heterozygous	Heterozygous	Heterozygous	Heterozygous
Variant Notes	E421K absent from ExAC, E760K seen 42x but never homozygous.	Not previously reported in ExAC or in disease databases but loss of function associated with disease	Not previously reported but loss of function associated with disease	Not in ExAC, highly conserved
Clinical Information Provided	Unspecified epilepsy, seizures, nystagmus, myopia, hypotonia, developmental delay, lack of coordination, wide-based gait	Not provided	Epilepsy NOS with intractable epilepsy	Cerebellar ataxia, history of episodic ataxia, lack of coordination
Sex	Female	Male	Female	Male

Table B2 Continued

Case	**10**	**11**	**12**	**13**
Candidate Gene	***CLCN4***	***DLG3***	***KIAA1109***	***KIF4A***
OMIM	302910	300189	611565	300521
OMIM Disease Association (MIM)	X-Linked Mental Retardation (300114)	X-Linked Mental Retardation (300850)	Alkuraya-Kucinskas Syndrome (617822)	X-Linked Mental Retardation (300923)
Inheritance	XLR	XLR	AR	XLR
Clinical Synopsis from OMIM	Hypotonia, delayed psychomotor development, intellectual disability, poor or absent speech, seizures, cortical atrophy	Eye anomalies, hypotonia, delayed psychomotor development, mild to severe intellectual disability, seizures, behavioral problems, ADHD	Brain atrophy, clubfoot, arthrogryposis, severe global developmental delay, syndactyly, hypotonia	Nonspecific facial dysmorphism, mild to moderate intellectual disability, poor speech, seizures
mRNA Change (Refseq)	c.823G>A (NM_001830.3)	c.1057C>T (NM_020730.2)	c.911C>T, c.3764C>T (NM_015312.3)	c.2137C>T (NM_206883.2)
Protein Change	p.V275M	p.H353Y	p.P304L, p.S1255F	p.R713C
Zygosity	Hemizygous	Hemizygous	Presumed Compound Heterozygous	Hemizygous
Variant Notes	Variant previously reported in disease in female patient with seizures	Located in the guanylate kinase domain, position is highly conserved. Observed once in ExAC as heterozygous	Both variants present in ExAC but neither homozygous. Drosophila model "Tweek" has seizures and motor dysfunction	This variant is seen 1x in ExAC. It is highly conserved across species down to plants and insects, variant is in the stalk/coiled coil domain.
Clinical Information Provided	Not provided	Convulsions, developmental delay, and delayed milestones	Not provided	Epilepsy and hypotonia
Sex	Male	Male	Male	Male

Table B2 Continued

Case	14	15	16	17
Candidate Gene	***PACS1***	***PACS1***	***POGZ***	***POGZ***
OMIM	607492		614787	
OMIM Disease Association (MIM)	Schuurs-Hoeijmakers Syndrome (615009)		White-Sutton Syndrome (616364)	
Inheritance	AD		AD	
Clinical Synopsis from OMIM	Facial dysmorphisms, feeding difficulties, hypotonia, delayed psychomotor development, intellectual disability, language delay, poor or absent speech, seizures, cerebellar hypoplasia, behavioral abnormalities, aggressive behavior, autistic features		Short stature, facial dysmorphisms, visual abnormalities, feeding difficulties, hypotonia, delated psychomotor development, intellectual disability, sleep difficulties, self-injurious behavior, autistic features	
mRNA Change (Refseq)	c.607C>T (NM_018026.3)	c.607C>T (NM_018026.3)	c.1689G>C (NM_015100.3)	c.1741G>T (NM_015100.3)
Protein Change	p.R203W	p.R203W	p.K563N	p.D581Y
Zygosity	Heterozygous	Heterozygous	Heterozygous	Heterozygous
Variant Notes	Recurrent disease mutation	Recurrent disease mutation	Absent from ExAC, proximal to p.I564F reported in HGMD, located in the zinc finger domain of the protein.	Absent from ExAC, proximal to p.I564F and p.Y597C reported in HGMD, located in the zinc finger domain of the protein.
Clinical Information Provided	Seizures and developmental delay (Patient 5 from Chapter 3)	Seizures and hypotonia	Epileptic encephalopathy, tonic-clonic and absence seizures, ataxia, and a brother with autism	Seizures, anemia, small testicles, sparse hair growth, high palate, and broad thumbs
Sex	Female	Male	Male	Male

Table B2 Continued

Case	**18**	**19**	**20**	**21**
Candidate Gene	***SHANK3***	***SHANK3***	***SMARCA2***	***SMARCA2***
OMIM	606230		600014	
OMIM Disease Association (MIM)	Phelan-McDermid Syndrome (606232)		Nicolaides-Baraitser Syndrome (601358)	
Inheritance	AD		AD	
Clinical Synopsis from OMIM	Autism, intellectual disability, seizures, hypotonia, developmental delay, speech/language delay and/or impairment, variable facial dysmorphism		Short stature, poor growth, facial dysmorphisms, skeletal anomalies, skin anomalies, sparse hair, intellectual disability, early-onset seizures, poor speech, tantrums and aggression	
mRNA Change (Refseq)	c.3108_3109dupGG (NM_001080420.1)	c.3727dupG (NM_001080420.1)	c.2744C>G (NM_003070.3)	c.2564G>A (NM_003070.3)
Protein Change	p.E1037Gfs*58	p.A1243Gfs*69	p.A915G	p.R855Q
Zygosity	Heterozygous	Heterozygous	Heterozygous	Heterozygous
Variant Notes	Not previously reported but loss of function associated with disease	Not previously reported but loss of function associated with disease	Variant not in ExAC, position is highly conserved, disease mutations located between AAs 730-1100.	Variant previously reported in Nicolaides-Baraitser syndrome
Clinical Information Provided	Motor delay, regression, grand mal seizures, and complex partial seizures	Developmental delay with regression and seizures	Personal and family history of myoclonic dystrophy	Psychomotor epilepsy without intractable epilepsy, autistic disorder
Sex	Female	Female	Female	Female

Table B2 Continued

Case	**22**	**23**	**24**	**25**
Candidate Gene	***SATB2***	***SMC1A***	***SMC1A***	***SMC1A***
OMIM	608148	300040		
OMIM Disease Association (MIM)	Glass Syndrome (612313)	Cornelia de Lange Syndrome 2 (300590)		
Inheritance	AD	XLD		
Clinical Synopsis from OMIM	Short stature, growth retardation, facial dysmorphisms, delayed psychomotor development, intellectual disability, seizures, poor speech, broad-based gait, hyperactivity, aggression, happy demeanor	Growth retardation, characteristic facies, gastroesophageal reflux, skeletal anomalies, delayed psychomotor development, cognitive impairment, poor speech, seizures, enlarged ventricles		
mRNA Change (Refseq)	c.1244T>A (NM_015265.3)	c.1900C>T (NM_006306.3)	c.3592G>A (NM_006306.3)	c.3619-2A>G (NM_006306.3)
Protein Change	p.L415Q	p.Q634X	p.E1198K	
Zygosity	Heterozygous	Heterozygous (skewed)	Hemizygous	Heterozygous
Variant Notes	Absent from ExAC, located in the CUT1 domain of the protein where missense mutations are reported to cluster in patients.	Loss of function in females reported to cause epilepsy and developmental delay	Nearby variants reported in disease, absent from ExAC, top CADD score, highly conserved	Absent from ExAC, gene intolerant to variation, located before the last exon of gene.
Clinical Information Provided	Abnormal EEG and MRI and recurrent ataxia. Also has a reported pathogenic deletion of the X-linked STS gene	Not provided	Developmental delay, epilepsy NOS with intractable epilepsy	Infantile spasms, epilepsy, developmental delay, autism
Sex	Male	Female	Male	Female