Potassium Channel Gene Mutations in Epileptic Encephalopathy

A thesis submitted to fulfill the requirements for the degree of

Doctor of Philosophy

By

Umesh Nair B.Biomed (Hons)

The Florey Institute of Neuroscience and Mental Health
Department of Medicine, Dentistry and Health Science
University of Melbourne

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PREFACE

This thesis is submitted for the degree of Doctor of Philosophy at the University of Melbourne. The work described in this thesis was carried out on a full-time basis by the candidate under the supervision of Prof. Steven Petrou, Dr. Melody Li and Dr. Snezana Maljevic at the Florey Institute of Neuroscience and Mental Health, Victoria, Australia.

The studies described in this thesis have not been submitted for a degree at this or any other University. The experiments undertaken are my own original work except where due acknowledgment has been made.

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*Co-author*


*Co-author*

ABSTRACT

The worldwide prevalence of epilepsy is between 2-3 % with many unmet clinical and health policy needs, especially in developing countries. Current estimates suggest that as many as 70 % of all epilepsy syndromes have a robust genetic etiology although the mechanisms of disease genesis are largely unknown. The improvement in genetic sequencing and testing has uncovered many genes associated with various forms of epilepsy, especially syndromes with an early onset. Many of these genes encode various ion channels, including the family of voltage-gated potassium channels, which are crucial in setting the afterhyperpolarization in neurons, preparing them to fire more action potentials. Mutations in these channels therefore bring about imbalance in the excitatory-inhibitory homeostasis in neural networks, leading to seizure development. Therefore the aim of this thesis was to characterize novel mutations of two different potassium channels encoded respectively by the KCNT1 and KCNC1 gene. The goal was to identify the biophysical changes brought about by KCNT1 and KCNC1 mutations, which were previously not reported. This thesis also looks at the effect of quinidine, which was previously reported as a possible therapeutic for KCNT1-related epilepsy, to determine the drug’s efficacy on these novel KCNT1 mutations. This thesis reports that the novel KCNT1 mutations identified from patients with autosomal dominant nocturnal frontal lobe epilepsy and epilepsy of infancy with migrating focal seizures produced potassium currents with large amplitudes and altered kinetics, similar to previously reported KCNT1 mutations in the literature; however, there was no clear genotype-phenotype pattern identified. Novel KCNT1 mutations in myoclonic atonic epilepsy and Lennox-Gastaut Syndrome, where changes in the KCNT1 gene were previously unreported, produced similar biophysics to WT, therefore indicating that KCNT1 may not be the underlying
cause of epilepsy in these patients. The inclusion of single nucleotide polymorphisms in these experiments acted as controls as well as emphasized that not all genetic alterations are detrimental. The application of quinidine on some of the variants showed varied effects with some mutations having reduced and some increased currents upon its application, indicating that the drug may not be a magic-bullet treatment for all KCNT1-related epilepsies. This thesis also looked at characterizing novel KCNC1 mutations identified in patients with epileptic encephalopathy or intellectual disability without seizures. These de novo variants were compared against the recurrent p.Arg320His mutant, which was previously identified in patients suffering from progressive myoclonus epilepsy. The results obtained show varied levels of loss of function for the analysed mutants with some of them also showing changes in voltage dependence of activation and a dominant-negative effect.

Therefore the results obtained in this thesis, provide a basic insight into the changes caused by newly identified mutations in two voltage-gated potassium channel genes. The data act as a framework, which can assist in the development in more complex experimental models to further understand the biophysical effects of the mutation as well as drug-protein interactions. Nonetheless, this work also emphasizes the need for in vitro experiments as a way of breaking down complex disorders such as epilepsy.
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<td>ADNFLE</td>
<td>Autosomal Dominant Nocturnal Frontal Lobe Epilepsy</td>
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<tr>
<td>AED</td>
<td>Anti-Epileptic Drug</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid</td>
</tr>
<tr>
<td>Array-CGH</td>
<td>Array-Comparative Genomic Hybridization</td>
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<tr>
<td>ASO</td>
<td>Antisense oligonucleotides</td>
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<tr>
<td>Ca$^{2+}$</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CAE</td>
<td>Childhood Absence Epilepsy</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Cl$^{-}$</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy Number Variants</td>
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<tr>
<td>DRG</td>
<td>Dorsal Root Ganglion</td>
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<tr>
<td>EE</td>
<td>Epileptic Encephalopathy</td>
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<td>EEG</td>
<td>Electroencephalograms</td>
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<td>FMRP</td>
<td>Fragile X Mental Retardation Protein</td>
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<td>FXS</td>
<td>Fragile X syndrome</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration, US</td>
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<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric Acid</td>
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<td>GGE</td>
<td>Generalised Genetic Epilepsy</td>
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<tr>
<td>hKCNT1</td>
<td>Human KCNT1</td>
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<tr>
<td>IGE</td>
<td>Idiopathic Generalized Epilepsy</td>
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<tr>
<td>ILAE</td>
<td>International League Against Epilepsy</td>
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<tr>
<td>I$_{\text{max}}$</td>
<td>Maximum current</td>
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<tr>
<td>JME</td>
<td>Juvenile Myoclonic Epilepsy</td>
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<tr>
<td>K$_{(\text{Na})}$</td>
<td>Sodium activated Potassium</td>
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<tr>
<td>K$^{+}$</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>Kir</td>
<td>Inwardly rectifying Potassium channel</td>
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<td>Kv</td>
<td>Voltage-gated Potassium channel</td>
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<tr>
<td>LGS</td>
<td>Lennox-Gastaut Syndrome</td>
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<tr>
<td>M$_{1}$R</td>
<td>Muscarinic Receptor</td>
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MAE  Myoclonic Atonic Epilepsy
MEAK  Myoclonus Epilepsy and Ataxia due to a Potassium channel mutation
mGluR1  Metabotropic Glutamate Receptor
MMPSI  Malignant Migrating Partial Seizures of Infancy
Na+  Sodium ion
nAChR  Neuronal Nicotinic Acetylcholine Receptor
NAD+  Nicotinamide Adenine Dinucleotide
NFE  Non-Lesional Focal Epilepsy
NFLE  Nocturnal Frontal Lobe Epilepsy
NGS  Next Generation Sequencing
NMPS  N-Methyl-D-Aspartate
OS  Ohtahara Syndrome
PIP2  Phosphatidylinositol 4,5-Biphosphate
PKA  Protein Kinase A
PKC  Protein Kinase C
PMA  Phorbol 12-myristate 13-Aacetate
PME  Progressive Myoclonus Epilepsy
Po  Probability of ion channel opening
RCK  Potassium conductance domain
REM  Rapid Eye Movement
rkcnt1  Rat kcnt1
SAD  Seizure-Alert Dog
SLACK  Sequence Like A Calcium-Activated Potassium
SLICK  Sodium-Activated Potassium
SNP  Single Nucleotide Polymorphism
TM  Transmembrane
TPA  12-O-tetradeanooylphorbol-13-acetate
ULD  Uverricht-Lundbord disease
WES  Whole Exome Sequencing
WGS  Whole Genome Sequencing
WHO  World Health Organization
CHAPTER 1

General Introduction

1.1 Epilepsy: prevalence and characteristics

Epilepsy is characterized by spontaneous recurrent seizures that can cause motor, sensory, cognitive, psychic or autonomic disturbances. It is estimated to affect 65 million people at any one time in the world (1-3). Traditionally, the diagnosis of epilepsy is defined as 2 unprovoked seizures that occur 24 hours apart, which could last from a few seconds to minutes, either as an isolated event or in a series (3, 4). However, in 2005 the International League Against Epilepsy (ILAE), a global organization promoting awareness of this neurological disorder, altered this definition (4). In the new definition, a person was diagnosed of having epilepsy if they had at least one epileptic seizure and had predispositions to generate epileptic seizures (4). In 2013, ILAE updated this definition to include one unprovoked seizure and a probability of further seizures similar to the general recurrence risk, which is approximately 60%, after two unprovoked seizures, occurring over the next 10 years. The organization also included in its criteria of having epilepsy if the patient is diagnosed with an epilepsy syndrome (5).

Defined by the World Health Organization (WHO) as the most common serious brain disorder, the incidence of epilepsy has doubled in developing countries compared to their developed counterparts. An estimated 2.4 million new cases of epilepsy are reported each year globally. Epilepsy Action Australia report that of these 2.4 million, approximately 250,000 patients live in Australia. It is of particular importance that the scientific and medical fields work towards a cure, and improved treatment options for
The overriding hypothesis for seizure formation focuses on the imbalance between excitation and inhibition in the highly interconnected neuronal networks of the brain, giving rise to this circuit-like phenomenon called epilepsy (3, 6). Two mechanisms have been proposed for this imbalance; the first being that a localized hyperexcitability moves into surrounding neuronal networks and overcomes the counterbalance of the inhibitory neurons, it then recruits more excitatory neurons to bring about a clinically visible seizure phenotype (3). The second mechanism, which is seen in a form of epilepsy known as absence seizures, indicates that an abnormal synchronization of the neuronal networks brings about the seizure phenotype (3).

1.2 Classifying Human Epilepsies: the complexities

Classification is crucial in diagnosis of epilepsy type in patients to ensure correct treatment is administered at the right time. The ever evolving and complex clinical phenotypes of epilepsy have led to the incorporation of more basic biological inputs into the classification of human epilepsies (7, 8). The ILAE set up a task force in 2003 to analyze the possibilities of shifting from the traditional paradigm of diagnosis that simply uses clinical symptoms and electroencephalograms (EEGs) to a more dynamic criterion that also incorporates pathophysiological information of the disease itself (8).

A common method for distinguishing certain epilepsies, involves determining the location of onset of the seizure and these can be broadly divided into two distinct divisions, namely generalized and focal (partial) syndromes (7). Generalized
epilepsies, as seen on EEGs, are seizures generated from both hemispheres of the brain (6). About 30% of all epilepsy cases reported are generalized epilepsies, which can occur at any time throughout life and have a characteristic discharge pattern on an EEG. Examples of such generalized epilepsies include childhood absence epilepsy (CAE) and juvenile myoclonic epilepsy (JME) (7). In CAE, patients experience absence episodes and are unaware of the event having taken place. In JME, patients experience muscle myoclonic contractions during the seizure, which result in rhythmic jerking motions called epileptic spasms or tonic-clonic seizures (6, 7).

Focal epilepsy, as its name suggests, tends to have a clear onset within a localized area of the brain (6, 7). Also known as partial epilepsies, they comprise about 60% of all epileptic cases and seizures are most commonly identified in the temporal or frontal lobes (7). Furthermore, a subset of focal epilepsies portray an autosomal dominant inheritance pattern, therefore adding an additional layer of complexity in the classification of epilepsy (7).

Apart from the localization of the seizure, classification of epilepsy can also be determined by its etiology. The coining of two terms, “idiopathic” and “symptomatic” are now a commodity in epilepsy literature, with idiopathic indicating no underlying cause but with a strong genetic backing (3), while symptomatic conversely indicates having a underlying structural cause with possible neurological deficits (7). Though still used in the literature, the boundaries between the two are becoming less clear as knowledge is accumulated about the disorder (7).

Given the complexity and inter-conjunctions of the various epileptic syndromes, classifying the syndromes either by their localization or etiology may not be sufficient. As a result of this, the ILAE has released a list of criteria identifying
factors such as seizure type, age of onset, prognosis and the nature of the disease as well as the genetic basis of the epileptic syndrome, providing a more comprehensive way to classify the 40 or more epileptic syndromes that have been reported (8).

In 2017, the ILAE further updated the classification of epilepsy to enable clinicians to make better diagnosis. This new classification fleshed out various contributing factors to the epileptic syndromes, therefore allowing better judgment on clinical diagnosis (Figure 1). Under the new classification, a patient can be diagnosed based on the individual or combination of the following criteria: type of onset seizures, epilepsy type (based on the 2014 classification), etiology, comorbidities and epilepsy syndromes (9)

1.3 Genetics in Epilepsy: when genes get altered

Research in the recent years has unraveled the involvement of mutations in an array of genes as the underlying etiology of many epileptic syndromes. Approximately 30% of all epilepsy syndromes have genetic involvement (10).

The impact of genes in epilepsy has created awareness of genetic testing, especially among parents who are planning on starting families. The other benefactors from genetic testing are epilepsy patients. Once candidate genes are identified in these patients, proper prognosis and tailored therapies can be administered, under the realms of personalized medication (11).

Indeed, the area of personalized medication has stemmed from the involvement of genes in epilepsy; the idea that certain drugs work best with a particular genotype. And given that 30% of all epilepsy syndromes are pharmacoresistant to available
anti-epileptic drugs (AEDs), the discovery of genes and personalized medication will improve treatment of severe epilepsy (12-14).

There has been a vast development of genetic tools to scan and detect abnormalities in gene sequences. Previously used techniques such as karyotyping, which determines changes in chromosomal arrangements, has been revamped to detect micro-deletions and duplications as well. Known as array-comparative genomic hybridization (array-CGH), this technique also has the capacity to determine the exact genomic location where changes have occurred (15-17).

Next-generation sequencing (NGS), a high-throughput technique for detecting changes in either exome or genome, has replaced the standard Sanger sequencing method of determining DNA sequences. Given the rapid and cost effective sequencing of multiple genes concurrently, NGS has become a regular choice in large exome or gene panel studies, across the world. It is safe to say that NGS has enriched the literature on the origins and pathophysiology of various epilepsy syndromes (18-21).

Given the increasing number of gene diagnostic tests being made available (Table 1), the generation of such valuable, yet large data sets, requires useful interpretations. This is crucial for clinicians to make the right call in determining correct tailored therapies for patients with specific genotypes, emphasizing the need for collaboration between clinicians and basic scientists (22).

1.4 Epileptic Encephalopathies: more than just seizures

Epileptic encephalopathies (EEs) are defined as severe, unmanageable seizures, which can contribute to developmental dysfunction and have poor prognosis (23-25). Mainly
encompassing early-onset epilepsy syndromes, epileptic encephalopathies are devastating and can lead to death, due to the combination of epilepsy and the neuro-comorbidities.

Many different factors have been identified as plausible causes of neurocognitive deficiency seen in EE patients, with predisposition to epilepsy as the largest reason (26). Statistics prove that patients inclined to have epilepsy are at higher risk of neurocognitive comorbidities (27).

Genetics has a major role in EE; high throughput sequencing tools such as NGS have been able to identify novel genes and mutations in affected probands (24). These genetic variations observed tend to be de novo mutations, in genes encoding ion channels, which are involved in regulation of neuronal excitability or in genes involved in cortical development (24, 25, 28). This complex involvement of genes as an etiology in both EE and/with neurodevelopment, encouraged the ILAE to introduce a new term, ‘developmental and epileptic encephalopathy’, to better categorize EE patients (9).

Increased seizure frequency in EE patients also contributes to neurocognitive dysfunction (25). The constant imbalance in excitation and inhibition leads to neurodevelopment deficiencies in these early-onset patients. Post-mortem brains obtained from rodent models with EE, show loss of spines in pyramidal cells of the CA3 region in hippocampus, altered neurogenesis and synaptic reorganization (29, 30).

The strong urge to reduce the seizure burden in EE patients encourages many clinicians to treat these patients with an array of AEDs at high doses. However, the high toxicity of these medications, together with the ongoing neuronal imbalance
contributes further to the decline in the overall neurocognitive function in these patients (25). Common AEDs such as phenobarbital, phenytoin and lamotrigine have been observed to cause neuronal death in rats (31).

Given the catastrophic effects and complexity of EEs, it is vital to study these syndromes in depth; understand their etiologies and therefore determine suitable therapies, to assist these patients, as young as neonates.

1.5 Early onset Epilepsies: the building blocks to neurological deficits

Many different forms of epilepsy have been reported clinically, from as young as the neonatal period up to old age (8). Epilepsy is not necessarily lifelong; patients may outgrow or have remission from seizures, however there are cases of infancy or childhood onset epilepsies progressing into adulthood and having a severe phenotype such as Lennox-Gastaut Syndrome (LGS) (1).

In recent times, there has been widespread use of genetic testing to determine underlying genes that bring about particular epileptic encephalopathies. Parents of young neonates and infants with epilepsy want to know what the genetic basis of their child’s epilepsy is; possibly to determine suitable treatments specific to the genotype of epilepsy.

Large studies have been performed to determine mutations in common genes associated with epilepsy. This has led to the development of consortia and large group studies across the world, to better understand the interplay of genetics and environment.
There are well over 30 gene described now with an epilepsy phenotype, including many with co-morbidity such as intellectual disability. Many are de novo and overall are “individually rare, but collectively common” (32). However, among these novel genes identified, certain genes seem to appear more frequently, indicating its prevalence in epilepsy (Table 2) (32).

Along with understanding the genetic background of the epilepsy, these new genetic techniques have the ability to determine the mode of inheritance of these mutations. Whole-exome sequencing (WES) of both parents and affected child are able to identify, if the mutation is de novo, happening at gameto- or embryogenesis or in fact is of X-linked inheritance or due to somatic mosaicism, specifically gonadal mosaicism (33).

Mosaicism involves the presence of two populations of cells in the body, where one contains the mutation and the other is normal, hence the person appears to not suffer drastically from the condition (34). Gonadal mosaicism involves the specific presence of the mutation in the gametes, therefore when combined with another mutated gamete, brings about a major devastating effect (35).

Other than increasing the severity of the epileptic syndrome, mosaicism adds an additional layer of complexity to the genetics, where the affected offspring has polygenic causes of the epilepsy (36). This may affect the ability of tailored therapeutics, which then may require the combination of two or more therapies or lifestyle changes.

The complexity of treating early-onset epilepsy is no different from adulthood epilepsy; resistance to AEDs. As previously mentioned, 30% of epilepsy patients remain unresponsive to available AEDs, especially patients with early-onset epileptic
encephalopathies (37). The vast resistance to AEDs has pushed researchers to reinvent new therapeutics, as well as repurpose drugs used for other conditions to determine efficacy with epilepsy.

Combinational therapy with different AEDs has also been introduced in children with epilepsy, with the aim of providing additional relief from seizure activity and increase the quality of life of these children (38). Changes in diet have also brought about some benefits to children with epilepsy. Clinical trials of the ketogenic diet have improved the severe conditions of children with EEs, with significant reduction in seizure frequency (39, 40).

Nonetheless, encephalopathic effects of epilepsy during the developmental phases in infancy have been seen to be more severe and potentially irreversible (1). Childhood onset epilepsy patients reportedly do less well academically or in their careers often with problematic personal lives, which is evident in areas such as marriage and parenting skills (41). Furthermore, these patients complain about depression, sleep disturbances and pain (1).

Therefore, there is a dire need to identify and start appropriate treatments at early stages of life, to enable these children to have an improved life quality.

This literature review will focus on two forms of early-onset epileptic encephalopathy; Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE) and Epilepsy of Infancy with Migrating Focal Seizures (EIMFS) both associated with mutations in several genes including *KCNT1*. 
1.5.1 Autosomal Dominant Nocturnal Front Lobe Epilepsy

ADNFLE is a rare familial frontal lobe focal epilepsy that occurs in childhood and is characterized by clusters of brief episodes of motor seizures that tend to arise during non-rapid eye movement (REM) sleep (3, 42, 43). These motor seizures are defined to be thrashing hyperkinetic activity or tonic stiffening with superimposed clonic jerking (3). The disorder has been seen to show considerable variation in age of onset and severity within families (3). ADNFLE is generally seen to develop within the first two decades of life and continues through to adulthood (43, 44).

ADNFLE was first described in 1994 by Scheffer and colleagues as the first focal epilepsy to follow single gene inheritance. Data obtained from six affected families showed characteristic frontal lobe seizures with brief motor attacks in sleep, and segregation analysis strongly inclined to an autosomal dominant inheritance defined in the pedigree chart of one of the affected families (Figure 2) (45).

Scheffer and colleagues published an extensive report on ADNFLE in 1995 to further support the publication made the previous year. This report, which involved 47 affected individuals from 5 different families, provided a clear description of the main clinical (Table 3) as well as electroencephalographic and genetic features of ADNFLE.

The pathogenesis of the disorder in approximately 10% of the families is caused by mutations in genes encoding the different subunits of the neuronal nicotinic acetylcholine receptor, nAChR (43, 44, 46). An p.Ser248Phe mutation in the α4-subunit gene, CHRNA4, was the first reported mutation from a linkage analysis study.
in 1995 performed in an Australian pedigree that had 27 affected individuals in six generations (3, 47).

Following this, further mutations were identified including some in other genes such as CHRNA2 that encodes the \( \alpha \)-2 subunit and CHRNA2 that encodes the \( \alpha \)-2 subunit (3, 42, 47). These mutations in the nAChR subunits were postulated to either reduce calcium dependence of the nAChR or increase sensitivity to acetylcholine (3, 42).

Recent rodent studies have identified that change in control of nAChR over the release of Gamma-Aminobutyric Acid (GABA) may lead to the pathogenicity seen in ADNFLE (48).

Heron and colleagues recently identified mutations in the KCNT1 gene, which encodes a sodium-gated potassium channel, to cause severe ADNFLE (42, 43). A genome-wide linkage analysis study of six affected members from a multi-generational Australian family identified a linkage peak that corresponded to a region on chromosome 9q34.3, which contains the KCNT1 gene (2, 42).

Whole exome capturing and sequencing of two of the six affected members revealed a missense mutation in the KCNT1 gene; c.2782C>T that encoded a p.Arg928Cys alteration (2, 42). KCNT1 missense mutations in 2 other ADNFLE affected multi-generational families as well as a de novo mutation causing severe nocturnal frontal lobe epilepsy (NFLE) was also identified (2, 42).

These mutations (Table 4) were predicted to be damaging by an in silico tool (PolyPhen-2), which predicts changes in protein structure and function caused by amino acid substitutions. Due to highly evolutionary conserved amino-acid residues
being altered, it was predicted that these mutations in \textit{KCNT1} brought about pathogenicity.

Interestingly, the 3 ADFNLE affected families lacked any of the known ADNFLE-associated n\textit{AChR} gene mutations, therefore highlighting \textit{KCNT1} mutations as the basis of the severe ADNFLE in these particular families (2, 42). It would therefore appear that mutations in two very different gene families could lead to ADNFLE. Mutations in the \textit{KCNT1} gene were observed to be 100% penetrant and cause a more severe form of ADNFLE with an earlier age of onset compared to n\textit{AChR} mutations. ADNFLE caused by \textit{KCNT1} mutations were also associated with major comorbidities of varying intellectual disability (mild to severe) as well as psychiatric features such as psychosis, catatonia and aggression, hence the focus has now shifted to understanding the mechanism of ADNFLE caused by \textit{KCNT1} mutations (42, 43, 49).

In 2014, Milligan and colleagues published data of human \textit{KCNT1} (h\textit{KCNT1}) mutants implicated in ADNFLE expressed in \textit{Xenopus} oocytes. Data from the studies indicated a characteristic gain-of-function property in channel activity for all mutants as compared to wild type (WT) channels (Figure 3) (50). The group also tested the effects of quinidine; an antiarrhythmic drug that has been reported to block rodent \textit{KCNT1} (r\textit{kcnt1}) channels (29), on these ADNFLE associated mutations. Results obtained showed a clear block in current activity for all ADNFLE mutants with the application of 300 \textmu M quinidine (Figure 4) (50).

Data from the ADNFLE functional studies provided a good understanding of the mutant channel physiology as well as the effect of quinidine on these mutants.
1.5.2 Epilepsy of Infancy with Migrating Focal Seizures

In 1995, Coppola and colleagues reported, for the first time, an epileptic condition characterized by multifocal seizures in 14 infants (51). No proper etiology or familial recurrence was determined and the condition was seen to be intractable with conventional AEDs (51). Death was reported in 3 of the infants and neuropathological investigation uncovered severe neuronal loss in the hippocampus that was accompanied by gliosis (Figure 5) (51).

The syndrome was first named Malignant Migrating Partial Seizures of Infancy (MMPSI), but recently has been changed to Epilepsy of Infancy with Migrating Focal Seizures (EIMFS) (52). EIMFS is a rare, sporadic and early onset encephalopathy of multiple seizure types that are pharmacoresistant and have an overall poor prognosis (52-55). It has been included in the ILAE Classification of epilepsy and epilepsy syndromes as a childhood epilepsy syndrome (53).

Infants before the age of 6 months with EIMFS are seen to have nearly continuous polymorphous focal seizures (55). These seizures occur randomly from independent areas of both hemispheres of the brain and ‘migrate’ from one half to the other (52, 55, 56). Multifocal ictal discharges are observed in EEG recordings (Figure 6) from these cases and a progressive deterioration of psychomotor development has also been reported (53, 54).

Seizure control is necessary for the infants to survive into childhood. Yet as most of the cases of EIMFS are resistant to the available AED’s, prognosis of the disease is extremely poor (53). Some of the observed outcomes of EIMFS are mental retardation, inability to speak or walk or reach for objects and death before the end of
the first year of life (53). Though majority of the cases have been seen to be drug resistant, Merdariu and colleagues reported prolonged seizure control in only one case of EIMFS using a combination of clonazepam, levetiracetam and stiripentol, suggesting that a more dynamic treatment option is required to treat this devastating disorder (53, 54).

Given its rarity, with only up to 100 cases being reported to date, the underlying cause of the EMIFS is not well understood (52). However, with the growing evidence of genetic etiology in such early onset encephalopathies, screening for mutations of particular genes has been the adopted way to understand such diseases better (55).

In an attempt to find the genetic basis for the condition, Barcia and colleagues in 2012 performed exome sequencing in 12 probands that fulfilled the standard criteria for diagnosis with EIMFS. Given that the disease was sporadic and not familial, de novo dominant mutations were identified and compared to unaffected parents of the probands as well as other control data sets (55). Interestingly, in 6 of the 12 probands tested, de novo missense mutations in the C-terminal domain of KCNT1 were identified that were absent in the control group (Table 5) (57).

In an attempt to understand the pathophysiological mechanism of EIMFS causing KCNT1 mutations, Barcia and colleagues performed functional experiments using tkcnt1 mutations in a Xenopus laevis oocyte assay (57, 58), given that rat and human KCNT1 proteins share 92.0% sequence identity. The group embarked on evaluating two specific rat mutations, which align with the human mutations p.Arg428Gln (p.Arg409Gly in rat) and p.Ala934Thr (p.Ala913Thr in rat) and compared them to WT tkcnt1 channels (55). Electrophysiological data obtained from the oocyte
expression system showed higher current amplitude for both \textit{rkcnt1} mutants as compared to WT (55).

Regulation of current conductance of the \textit{KCNT1} channel occurs via Protein Kinase C (PKC) phosphorylation and 13 putative PKC sites have been identified in the C-terminal part of the channel (55). When activated by phosphorylation, \textit{KCNT1} channels are seen to produce a two- to three-fold increase in current amplitude as compared to WT channels (55). They hypothesized that the EIMFS mutated channels were mimicking the active state caused by PKC phosphorylation, accounting for the higher current amplitudes observed (55, 57).

To further test this hypothesis, they performed additional mutagenesis by replacing each serine and threonine residue at the 13 putative PKC phosphorylation sites with an alanine, hence producing 13 different mutants. The mutant channels were expressed similarly in the \textit{Xenopus} oocytes and electrophysiological analysis was performed in response to 100 nM of 12-O-tetradecanoylphorbol-13-acetate (TPA), a known PKC activator (55).

Data obtained showed that all, except for one mutant, p.Ser407Ala showed increased current amplitude in response to incubation with TPA (55). They proposed that the p.Ser407Ala mutation, adjacent to the p.Arg409Gly mutation, would by itself produce higher current amplitudes by locking the channel in a phosphorylated-like state and therefore the application of TPA would not contribute to any further increase (55).

The group compared WT and p.Ser407Ala current traces and showed that the p.Ser407Ala mutant did not produce an increase in current amplitude with the application of 100 nM TPA (55). They also showed that both the p.Arg409Gly and p.Ala913Thr \textit{rkcnt1} channels did not have a further increase in current amplitude in
the presence of 100 nM TPA, hence therefore concluding that these \( rkcnt1 \) mutants mimicked the phosphorylated state of the channel (55).

However, Milligan and colleagues refuted this theory by presenting data showing inhibition of current amplitudes of mutant \( hKCNT1 \) channels with the application of Phorbol 12-myristate 13-acetate (PMA), a potent PKC activator, at a concentration of 100 nM (Figure 7) (50). This lead to the discovery of using PKC activators as possible therapeutics in \( KCNT1 \) derived epilepsies.

### 1.6 Epilepsies as Channelopathies

Over 400 human genes have been discovered to encode ion channels, which are sophisticated transmembrane proteins that allow passage of specific ions, at speeds close to the limit of diffusion, yet under very strict regulation (59, 60).

Ion channels are fundamental components of cell membranes and are involved in most physiological processes. They are the gateway through which most charged ions pass through the hydrophobic membranes of cells. The excitatory and inhibitory properties of neurons depend on the various ion channels present in their membranes. A proper balance between excitation and inhibition is required to maintain normal brain activity (3, 61).

Mutations in genes that code for ion channels or their accessory subunits contribute disproportionately to the development of idiopathic epilepsies, where the imbalance between excitatory and inhibitory signaling leads to seizure onset (3, 61).

Two main mechanisms have been identified as the effect of mutations on genes encoding ion channels. Mutations that result in reduced ion flow across the channel
are classified as loss-of-function, while those with increased ion passage are defined as gain-of-function mutations (60).

However, over the years, researchers have come to realize that these two terminologies may not completely reflect all channelopathies, especially in epilepsy, where clinical phenotypes have been observed to be opposite to the underlying effect of the genotype (60).

Given that idiopathic epilepsies have specific genes underlying its etiology, which in turn, encodes a specific ion channel, it is important to have clear understanding of the physiology and functionality of the ion channel implicated.

Given the implication of potassium channels in ADNFLE and EIMFS discussed here, the focus of this literature review shifts towards understanding potassium channels and their impact on these early-onset EEs with or without seizures.

### 1.6.1 Potassium channels: a brief overview

Potassium (K⁺) channels encompass a large group of ion channels, found across cell membranes in the human body, including the brain. More than 80 genes that code for K⁺ channels have been identified and molecularly cloned so far. Given their broad expression and many important physiological roles, it is no wonder that mutations in these K⁺ channels have been implicated in idiopathic epilepsies (62).

K⁺ channels in neurons are heavily involved in maintaining excitation as well as setting the resting membrane potential (63). These channels can be classified into 4 main categories that perform various functions in cells: voltage-gated (Kv), inwardly rectifying (Kir), sodium (Na)-activated channels and calcium (Ca²⁺)-activated channels (64) (Table 6).
K⁺ channels can also be classified by the number of transmembrane domains each subunit has. Each of Kv, Na-activated and Ca²⁺-activated K⁺ channel subunits consists of 6 transmembrane domains (S1-S6), a re-entering loop between S5 and S6 that when combined in the tetrameric channel, forms the central-pore of the channel, and an N and C termini, respectively. Kir channel subunits, on the other hand, consist of only 2 transmembrane domains that flank the pore-forming domain (Figure 8) (64). Kv and Na-activated channels have an additional feature of a voltage sensor (S4), which detects the voltage changes in the cell membrane, which leads to conformational changes within the protein that result in opening of the pore, i.e. channel activation. To increase diversity and improve functionality, Kv channels can further co-assemble with a range of auxiliary subunits. Na-activated channel subunits contain different domains in their C-termini, which assists in the channel’s kinetics (65-67).

The existence of diverse types of K⁺ channels, together with their various subunit types and additional features, emphasizes the role of this group of ion channels in regulation of excitability of cell membranes. Hence, mutations in genes encoding these proteins can cause detrimental effects and result in different disorders related to altered cell membrane excitability, from cardiac arrhythmias to epilepsy.

1.6.1.2  **KCNT1 a Sodium Activated Potassium Channel**

Mutations in the **KCNT1** channel have been reported to be a common factor in both ADNFLE and EIMFS; hence an understanding of the implicated channel is of great importance. **KCNT1** encodes for a sodium-activated potassium channel (KNa), which is sensitive to internal sodium (Na⁺) concentrations (42, 55). These channel currents are also seen to increase with increasing depolarization, indicating that they also have
a degree of voltage sensitivity (68-71). KCNT1 channels have been reported to be synergistically activated by increased intracellular chloride (Cl⁻) and Na⁺ levels (68-70, 72).

KCNT1 is also known as Slack (Sequence like a calcium-activated K⁺), although this is a bit puzzling as only 7% of its domain structures are similar to the Slo1 family of calcium-activated potassium channels (68, 70, 73, 74) well below random similarity. KCNT1 channels are known to produce a slow afterhyperpolarization effect following a train of bursting action potentials; therefore it has been suggested that they set the resting membrane potential and control the basal excitability of neurons (55, 68, 72, 74-76). Furthermore, under physiological conditions KCNT1 also contributes to the total delayed outward current in neurons (68, 69, 71, 77). Recent studies have identified the role of KCNT1 channels in increasing the firing rate of neurons. Cells of electric organs from electric fishes, which express KCNT1 channels, have the capacity to produce firing frequencies of up to 500 Hz. With the ability of KCNT1 to repolarize very large action potentials, these electric organ cells can therefore fire action potentials at higher rates (78).

Many potassium channels with various firing patterns have been observed in the plasma membranes of cells, and to date, there are 77 known genes, which encode the α-subunits of these channels (68, 79). The general subunit configuration of such channels tends to be homo- or heterotetrameric and alternative splicing has been identified in most RNA encoding for potassium channels, indicating the vast variety of channels present in this family each with their own unique kinetic and voltage properties (68, 76).
$K_{Na}$ channels were first reported 25 years ago in cardiomyocytes and subsequently they have been identified throughout the nervous system as well as in diaphragm muscle and gastric myocytes (68, 69, 76). Not surprisingly, $K_{Na}$ channels are located in close proximity to sources of internal Na$^+$ such as where voltage-gated sodium channels and ionotropic ligand-gated receptors such as α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid (AMPA) and N-Methyl-D-aspartic acid (NMDA) glutamate receptors are expressed. Indeed, co-immunoprecipitation studies in rats have illustrated this co-localization, especially with $KCNT1$ and GluR2/3 AMPA receptors (68).

The overall putative structure of the $KCNT1$ channel is similar to that of a voltage-dependent potassium channel ($K_v$), which has six hydrophobic membrane spanning domains (S1-S6) with a pore (P-domain) between S5 and S6. However, $KCNT1$ is larger than the typical $K_v$ channel, comprising approximately 1237 amino acids and lacks the S4 voltage-sensor motif (Figure 9) (68, 71, 77, 80).

$KCNT1$ has a large cytoplasmic C-terminal domain where, two domains regulating potassium (K$^+$) conductance (RCK1 & 2) are predicted to be present (68, 81). The RCK2 domains is also sensitivity to internal Na$^+$ concentrations, therefore linking the concentration of internal Na$^+$ to activation of $KCNT1$ (68).

With its ability to regulate K$^+$ conductance, the $KCNT1$ channel has been found to have multiple subconductance states (68, 74). Previous studies have observed large unitary conductance of $KCNT1$, when excised into solutions of equal concentration of potassium internally and externally. However a 50% drop in conductance is seen in the same channels when they are placed in physiological solutions (68).
Alternative splicing in KCNT1 RNA does occur and so far five different transcripts of the rkcnl gene have been identified. These are predicted to produce channels that differ mainly in their cytoplasmic amino termini (68, 74). Two of the well-studied isoforms, Slack-A and Slack-B, are good examples of how alternative splicing brings about variation. Comparatively, Slack-A subunits have a smaller N-terminal region in contrast to Slack-B subunits, which are currently the largest potassium channel subunits due to their longer N-terminal domain (2, 68, 74). As well as structural differences, the two isoforms have different activation kinetics, with Slack-A channels activating rapidly upon depolarization while Slack-B channels take over hundreds of milliseconds to activate (68, 74). Interestingly, immunocytochemical experiments showed Slack-B subunits were able to co-assemble with Slick (Sequence like an intermediate potassium channel) subunits (Figure 10) (76). These heteromeric channels were observed to produce 18-25 fold increased current amplitudes, as compared to homomeric channels (42, 55, 68, 71, 76, 77, 82).

The localization of rkcnl channels has been well studied in the rat model (30). Robust tools such as in-situ hybridization and immunocytochemistry have made identifying proteins such as KCNT1 possible. Northern blot analysis identifying rat RNA transcripts, identify a prominent distribution of rkcnl channels in the brain, as well as in the kidney and heart although in lower concentrations (42, 70, 77).

In situ hybridization using a specific probe to rkcnl RNA depicted a regional distribution of rkcnl in the rat brain (Figure 11) (71). Areas such as the brainstem, especially within the cell bodies and axonal fibers of neurons found in the trigeminal system and reticular formation showed strong signals of rkcnl presence (71). A strong presence of the channel was also reported in neurons at the vestibular,
oculomotor nuclei as well as medial nucleus of the trapezoid, which is involved in
detecting location of sound stimuli (68, 71, 82). Other areas in the rat brain that
reported similar signals included the olfactory bulb, deep cerebellar nuclei, thalamus,
substansia niagra, amygdala and at the frontal cortex (71).

The presence of *rkcnt1* subunits has also been identified in the periphery especially at
the plasma membranes of the cell bodies and axons of nociceptive Dorsal Root
Ganglion (DRG) neurons (81)

Although *KCNT1* in the nervous system has only been an area of interest by
researchers in recent times, the channel has been studied in other regions, such as in
cardiac cells, where a variety of pharmacological assays have been performed (68, 73, 75).

Anti-arrhythmic compounds such as bepridil, clofilium and quinidine have been
reported to reversibly inhibit *KCNT1* channels of cardiac origin in a concentration-
dependent manner (68, 70, 73, 75). Quinidine an optical isomer of quinine, obtained
from the extract of the cinchona tree bark, has been reported to produce a 90% block
of *rkcnt1* (70, 72).

Activators of *KCNT1* have also been reported including bithionol, a non-selective
reversible potassium channel activator, which increases the current amplitude of
*KCNT1* channels. Furthermore other compounds, such as riluzole, loxapine and
niclosamide, increase *KCNT1* current amplitudes (68, 73, 75, 83). Loxapine, an
antipsychotic drug has also been reported to be a more selective activator of $K_{Na}$
channels than bithionol (68, 75, 83). The interest in *KCNT1* channel modulators is a
particular area of research in analgesic pharmacology, where increased activity of this
channel in the peripheral nervous system decreases DRG excitability and therefore decreases nociceptive pain signaling (83).

Given the importance of KCNT1 function in various systems, regulation of the channel is a key determinant in maintaining homeostasis. A variety of regulatory signaling pathways have been reported to assist channel function. Na⁺ and Cl⁻ sensitivity play key roles in KCNT1 regulation, particularly roles involving hypoxia where the cytoplasmic concentrations of these two ions are elevated (68). Activation of KCNT1 hyperpolarizes neurons preventing any further damage, thus conferring a protective mechanism against hypoxia (68, 72, 74, 75, 80).

PKC plays an important role in increasing current amplitude, where phosphorylation at particular sites in KCNT1 C-terminal region increases current conductance by 2- to 3-fold (42, 55, 68, 80). Interestingly, PKC produces a profound suppression in current conductance in the Slick/Slack-B heteromeric channels, indicating how heteromeric formations of different channel proteins differ in channel kinetics as compared to the kinetics of their single subunits (76). Similarly, activation of muscarinic (M1) and metabotropic glutamate receptors (mGluR1) as well as phosphatidylinositol 4,5-biphosphate (PIP2), a phospholipid located in the inner lipid leaflet of the plasma membrane, increase current conductance in KCNT1 (68, 80).

Furthermore, decrease in current amplitude, together with internalization of KCNT1 channels from the plasma membranes of DRG neurons have been reported to be modulated by Protein Kinase A (PKA), via elevated cyclic AMP (cAMP) levels as seen in hyperalgesic situations due to DRG hyperexcitability (68, 81).

The open probability of KCNT1 channels is another area that has been studied a great deal. Research has identified estradiol found in the lipid bilayer, NAD⁺ an
intracellular component and the RCK2 domain, for which putative binding sites have been identified, as modulators of open probability. This increase in the probability of channel opening is required especially in ischemic situations (55, 68, 75).

Activity at the C-terminal of \textit{KCNT1} is not limited to just phosphorylation or sensitivity to ions; interactions with other proteins have also been discovered at the site. One such protein that has been identified from co-immunoprecipitation experiments in yeast two-hybrid systems is the Fragile X Mental Retardation Protein (FMRP) (55, 68). FMRP, an RNA-binding protein, if absent, is known to cause Fragile X syndrome (FXS), which is the most common form of hereditary intellectual disability in humans (82).

Further data from patch-clamping studies of \textit{KCNT1} have determined that the presence of the N-terminal region of FMRP, reversibly increases the channel opening probability by two- to three-fold, indicating that FMRP is a potent activator of \textit{KCNT1} channels (68, 82).

\textbf{1.6.1.3 }\textit{KCNC1} a Voltage Activated Potassium Channel

The \textit{KCNC1} gene, located on chromosome 11p15 in humans, encodes a voltage-gated potassium channel subunit, known as Kv3.1. The gene, which was initially studied in the Drosophila, was first identified via in situ hybridization of the rodent NGK2 gene to human cDNA (84).

The Kv3.1 channel subunit with six transmembrane domains (S1-S6) follows the typical topology of voltage-gated potassium channels; (\textbf{Figure 12}). Four such subunits make a functional Kv3.1 channel, with S5 and S6 domains of each subunit
forming the ion pore and the S4 domains of each subunit sensing the changes in the membrane potential (85).

Studies have identified two alternative splice forms of the Kv3.1 subunits, Kv3.1a and Kv3.1b, which differ at their C-termini. This difference has been identified to regulate the subcellular trafficking of the two forms of Kv3.1 channel in neurons, without affecting their biophysical properties (86).

The distinguishing factor of Kv3.1 channels is the voltage dependence of channel opening and fast deactivation kinetics (87). The main function of the Kv3.1 channel is to allow for after-spike hyperpolarization in neurons that fire short duration action potentials at high frequencies (87, 88).

With the combination of voltage dependence, which sets a low threshold for action potential initiation, and fast deactivation, Kv3.1 channels reduce after-spike hyperpolarization and therefore allow for sustained high-frequency action potential firing (89). Given this combination, Kv3.1 channels have been identified in various neuronal pathways to assist with high throughput action potential firing.

Kv3.1 is highly expressed in the auditory neuronal pathways of mice and birds, including the medial nucleus of the trapezoid body, to provide high frequency temporal information used to identify the position of sounds in space (90-92). The channel has also been identified in the mouse retina on starburst amacrine cells, which assist in determining direction of stimuli (93). In the CNS, Kv3.1 has been described in parvalbumin positive interneurons in the cortex, in deep cerebellar nuclei and in purkinje cells (94-96)
1.6.1.4 *KCNC1* and its implication in epilepsy

A recurrent mutation in the *KCNC1* gene (p. Arg320His; c.959 G>A) has been implicated in a subtype of progressive myoclonus epilepsy (PME) named myoclonus epilepsy and ataxia due to a potassium channel mutation (MEAK) (97-99).

MEAK is a rare and grievous form of epilepsy that is characterized by action myoclonus, tonic-clonic seizures, gradual neurological deterioration and ataxia, with some cases having additional features such as dementia (97). Clinical diagnosis of MEAK is complicated due to its similarities to other forms of PME including Unverricht-Lundbord disease (ULD) ([Table 7](#)) (97, 99).

Unlike other cases of PME that are inherited autosomal recessively or in some cases with autosomal dominant or mitochondrial inheritance, MEAK is caused by a recurrent *de novo* mutation p.Arg320His found in several dozens of unrelated patients in the world (97).

Interestingly, MEAK patients were seen to have better symptom management at elevated body temperatures such as fever. This was further characterized in cells, where a shift was observed in the voltage dependence of WT Kv3.1 cells with increased temperature, while cells co-expressing Kv3.1 WT and p.Arg320His mutant has no significant change(99).

The shift in voltage dependence that brings about increased current amplitudes could balance the reduction in current brought about by the mutation, which may alleviate the symptoms seen in MEAK patients by increasing interneuron firing to balance the overall network excitability (99).
1.7 Future of Epilepsy Research: what holds beyond?

There has been great progress in the past two decades in terms of epilepsy research; specifically in understanding the genetic basis, which poses as a major challenge in epilepsy research (100). Mutations in genes coding for ion channels as well as receptors for neurotransmitters have been identified as causes of epileptic syndromes, yet the mechanisms behind how these mutations cause the various epileptic syndromes are still unclear (100).

The establishment of international epilepsy consortia has enabled genetic studies in large cohorts of epilepsy patients. As an example, Epi4K consortium aimed to sequence and analyze a minimum of 4000 subjects with highly selected and well characterized epilepsies (100). The consortium put in place 4 different projects to be completed within a span of 5 years. Data for Project One focused on the genetics of two common EEs, infantile spasms and Lennox-Gastaut syndrome (LGS), (100, 101).

Project Two of the consortium aimed to use whole genome sequencing (WGS) to identify genomic variations that contribute to the risk for common forms of idiopathic generalized epilepsy (IGE) as well as non-lesional focal epilepsy (NFE), found in families with two or more cases, in order to study the genetic influences on epilepsy (100). Project Three, has been set up to test the hypothesis that genetic variation influences the possible responses to specific AEDs. This project aimed to use established and well-characterized epilepsy cohorts as well as seizure control data to try and understand the relationship between genetic variation and prognosis (100).

Lastly, the primary aim of Project Four was to apply Epi4K patients in order to detect epilepsy-associated copy number variants (CNVs) that play an important role in the genetic risk and etiology of epilepsy (100).
With such large scale efforts there is hope that a better understanding of the causes of many forms of idiopathic and cryptogenic epilepsies is achieved (100). The data generated from large-scale projects, such as the Epi4K project, should drive the research and development of novel anti-seizure drugs and hopefully result in better interventions to modify or even prevent epilepsy (100).

1.8 Aims of thesis; understanding novel potassium channel mutations

This main focus of this thesis is to characterize novel human mutations in two different voltage-gated potassium channels, *KCNT1* and *KCNC1*. Using the *Xenopus laevis* oocyte system, alterations in electrophysiological patterns such as current amplitudes and rate of activation will be compared between WT and mutant cRNA-injected oocytes, using an automated two-electrode voltage clamp system, the Roboocyte (Multi Channel Systems, Reutlingen, Germany). This data will provide the preliminary understanding of the effect of these novel mutations in these two potassium channels as well determine which novel mutations should be studied further by developing specific mutant mouse model, where compared to oocytes, provide a more realistic picture of the epilepsy type, given the complexity of rodent systems.

As a secondary aim, a drug screening assay, using quinidine, an established *KCNT1* channel blocker, will be performed on selected novel KCNT1 mutations. Data from these experiments will provide the basis to translational clinical trials on patients with the same mutation genotype.
### 1.9 Tables

<table>
<thead>
<tr>
<th>Diagnostic test</th>
<th>Description</th>
<th>When to use it</th>
</tr>
</thead>
<tbody>
<tr>
<td>Array-CGH</td>
<td>Identify single nucleotide polymorphisms (SNP arrays) or to determine</td>
<td>Epilepsy with developmental delay, dysmorphism, ASD</td>
</tr>
<tr>
<td></td>
<td>chromosomal rearrangements submicroscopic (array-CGH) as CNVs.</td>
<td></td>
</tr>
<tr>
<td>Single gene sequencing</td>
<td>Detects changes in the gene and if it causes amino acid alterations.</td>
<td>Suspected single-gene defect (e.g. SLC2A1 in Glut-1 deficiency)</td>
</tr>
<tr>
<td>Duplication deletion of a single gene</td>
<td>CNV of a single gene</td>
<td>Suspicous of a single gene defect when sequencing is inconclusive</td>
</tr>
<tr>
<td>Research of a specific mutation</td>
<td>Sequencing of a specific mutation</td>
<td>On parents to understand if an unknown mutation is pathological disease with</td>
</tr>
<tr>
<td>Targeted-resequencing</td>
<td>Sequencing and duplication/deletion research of a gene panel for a specific</td>
<td>more genes involved</td>
</tr>
<tr>
<td>Karyotype</td>
<td>disease</td>
<td></td>
</tr>
<tr>
<td>Fluorescent in situ hybridization (FISH)</td>
<td>Probes that analyse specific chromosome’s portions</td>
<td>Confirmation of a duplication/deletion</td>
</tr>
<tr>
<td>Whole-exome and genome sequencing</td>
<td>Analysis of all chromosome for big duplication/deletion</td>
<td>Patients with dysmorphism and/or multiorgan dysfunction</td>
</tr>
<tr>
<td></td>
<td>Sequencing of all DNA only for codifying regions (exons) or all regions</td>
<td>Suspected genetic aetiology with otherwise normal investigations</td>
</tr>
<tr>
<td></td>
<td>(genome)</td>
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**Table 1** Available diagnostic tests for epilepsy patients (22).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Epilepsy Phenotype</th>
<th>Discovery</th>
</tr>
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<tbody>
<tr>
<td><em>SCN1A</em></td>
<td>Dravet Syndrome</td>
<td>2000</td>
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<tr>
<td><em>SCN2A</em></td>
<td>Infantile spasms neonatal epileptic encephalopathies</td>
<td>2002</td>
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<td><em>CDKL5</em></td>
<td>Atypical Rett syndrome early-onset epileptic encephalopathy</td>
<td>2003</td>
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<tr>
<td><em>STXBP1</em></td>
<td>Infantile spasms Ohtahara syndrome</td>
<td>2008</td>
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Table 2 Examples of genes for epileptic encephalopathies [modified from (32)].
<table>
<thead>
<tr>
<th>Childhood onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistence through adult life</td>
</tr>
<tr>
<td>Clusters of seizures occurring in sleep</td>
</tr>
<tr>
<td>Partial seizures</td>
</tr>
<tr>
<td>Aura nonspecific</td>
</tr>
<tr>
<td>Vocalization at onset</td>
</tr>
<tr>
<td>Prominent motor features: hyperkinetic, tonic</td>
</tr>
<tr>
<td>Awareness retained</td>
</tr>
<tr>
<td>Occasionally secondarily generalized</td>
</tr>
<tr>
<td>Normal intelligence</td>
</tr>
<tr>
<td>Normal neurological examination</td>
</tr>
<tr>
<td>Interictal EEG usually normal</td>
</tr>
<tr>
<td>Ictal EEG bifrontal discharges in some</td>
</tr>
<tr>
<td>CT and MRI of the brain normal</td>
</tr>
<tr>
<td>Often responsive to carbamazepine monotherapy</td>
</tr>
</tbody>
</table>

Table 3 Common features of ADNFLE seen in the 47 individuals reported by Scheffer and colleagues (102)
Table 4 Clinical and molecular features of families with KCNT1-associated ADNFLE listed by Heron and colleagues. Listed also are the specific missense mutations causing the amino acid swap, the number and origin of patients as well as predicted severity of each specific mutation (42)
Table 5 6 de novo missense mutations identified at the C terminal of KCNT1 in the probands by Barcia and colleagues. The location and amino acid swap is listed for each mutation as well as all mutations were identified to be possibly damaging (55)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Ancestry</th>
<th>Genomic position (bp)</th>
<th>Mutation</th>
<th>Protein alteration</th>
<th>Polyphen-2 prediction</th>
<th>SIFT prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>European (France)</td>
<td>138671275</td>
<td>c.2800G&gt;A</td>
<td>p.Ala934Thr</td>
<td>Possibly damaging</td>
<td>Deleterious</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>European (France)</td>
<td>138657552</td>
<td>c.1283G&gt;A</td>
<td>p.Arg428Gln</td>
<td>Probably damaging</td>
<td>Deleterious</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>European (France)</td>
<td>138657552</td>
<td>c.1283G&gt;A</td>
<td>p.Arg428Gln</td>
<td>Probably damaging</td>
<td>Deleterious</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>European (France)</td>
<td>138657552</td>
<td>c.1283G&gt;A</td>
<td>p.Arg428Gln</td>
<td>Probably damaging</td>
<td>Deleterious</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>European (France)</td>
<td>138660694</td>
<td>c.1421G&gt;A</td>
<td>p.Arg474His</td>
<td>Probably damaging</td>
<td>Deleterious</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>European (Ukraine)</td>
<td>138667192</td>
<td>c.2280C&gt;G</td>
<td>p.Ile760Met</td>
<td>Probably damaging</td>
<td>Deleterious</td>
</tr>
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</table>

M, male; F, female.
Table 6 Summary of human $\text{K}^+$ channels subfamilies [modified from (62)].

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Main functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage-gated $\text{K}^+$ channels ($\text{K}_\text{v}$)</td>
<td>Regulation of outward $\text{K}^+$ currents and action potentials, modulation of neurotransmitter release, control of both excitability and electrical properties of neurons</td>
</tr>
<tr>
<td>Inwardly rectifying $\text{K}^+$ channels ($\text{K}_\text{ir}$)</td>
<td>Maintenance of the resting membrane potentials and regulation of the cell excitability</td>
</tr>
<tr>
<td>Sodium-activated $\text{K}^+$ channels ($\text{K}_\text{Na}$)</td>
<td>Regulation of delayed outward currents $I_{\text{K}_\text{Na}}$ and contribution to adaptation of firing rate</td>
</tr>
<tr>
<td>Calcium-activated $\text{K}^+$ channels ($\text{K}_\text{Ca}$)</td>
<td>Regulation of neuronal firing properties and circuit excitability</td>
</tr>
<tr>
<td>Characteristic</td>
<td>MEAK</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>First symptom</td>
<td>Myoclonus (or “tremor”)</td>
</tr>
<tr>
<td>Disease onset, yr, mean</td>
<td>10</td>
</tr>
<tr>
<td>Disease onset, yr, range</td>
<td>3–15</td>
</tr>
<tr>
<td>Progressive features</td>
<td>Myoclonus, ataxia</td>
</tr>
<tr>
<td>Cognitive decline</td>
<td>Mild or absent</td>
</tr>
<tr>
<td>Mode of inheritance</td>
<td>Autosomal dominant, usually de novo</td>
</tr>
<tr>
<td>Gene</td>
<td>\textit{KCNC1}</td>
</tr>
<tr>
<td>Protein function</td>
<td>Ion channel</td>
</tr>
</tbody>
</table>

\(\text{MEAK} = \text{myoclonus epilepsy and ataxia due to K}^+\ \text{channel mutation};\ \text{ULD} = \text{Unverricht–Lundborg disease.}\)

\textbf{Table 7} Comparison of typical MEAK characteristics to classical ULD (99)
1.10 Figures

Figure 1 Framework for classification of the epilepsies. *Denotes onset of seizure (9).
Figure 2 Pedigree of Australian family with 11 affected members. This was one of the six affected families reported by Scheffer and colleagues to suffer from ADNFLE. Autosomal dominance was the observed pattern of inheritance of this disorder (45).
Figure 3 Electrophysiological characterisation of KCNT1 WT and mutant currents recorded in Xenopus oocytes. (A) Average peak currents at a voltage step to +10 mV for WT (n= 94), M896I (n= 28), R398Q (n= 34), Y796H (n= 63) and R928C (n= 42) channels. (B) Averaged and normalised current-voltage relationships for WT (n= 105), M896I (n= 36), R398Q (n= 58), Y796H (n= 92), R928C (n= 78) channels. Currents were normalized to the value at a test potential of +80 mV ($I_{max}$). (C) Pooled WT (black, n= 158) and epilepsy syndrome ADNFLE (blue, n= 160) at +10 mV (50).
Figure 4 Quinidine inhibition of KCNT1 currents expressed in *Xenopus* oocytes. (A) Current records from WT (black) R928C (blue) mutant in vehicle and in the presence of 300 μM quinidine. (B) Comparison of average current amplitude for WT and mutant channels illustrating the degree of block by 300 μM quinidine (grey) (WT n= 13; M896I n= 5; R398Q n= 8; Y796H n= 19; R928C n= 14 derived from measurements made at +10 mV. (C) Average time to reach peak current amplitude for WT (n = 28), M896I (n= 5), R398Q (n= 8), Y796H (n= 9), R928C (n= 13) mutant channels at +10 mV before and after application of quinidine (300 μM). (*p < 0.05, **p < 0.01) (50)
Figure 5 Hippocampal section of deceased infant with EIMFS. Neuronal loss (arrows) with mild gliosis (arrowhead) identified in CA1 region (51)
Figure 6 EEG recording of a 5-month-old boy with EIMFS. Ictal discharges observed at left temporooipital region initially, which end when discharges begun to appear at right frontocentral and temporal areas. Frequency of the ictal discharges seen to be different between the two areas reported (53).
Figure 7 PMA inhibition of hKCNT1 and rkcntl currents expressed in Xenopus oocytes. (A) Average percent inhibition at 110mV of wild-type (WT; n= 58), M896I (n= 18), R398Q (n= 25), Y796H (n= 47), R928C (n= 34), R428Q (n= 9), A934T (n= 25), and P924L (n= 17) hKCNT1 channels by PMA (100nM). WT= black; ADNFLE= light gray; EIMFS= dark gray. (B) Representative current traces obtained from oocytes expressing WT and mutant R907C rkcntl channels. Scale bars apply to all traces. (C) Average peak currents at 110mV for WT (n= 51) and R907C (n= 37) rkcntl channels in the presence of vehicle and then following application of PMA (100nM). The peak currents for WT and R907C rkcntl in the presence of vehicle were compared to each other and to the peak currents in the presence of PMA (100nM). *p < 0.05, ****p < 0.001 (50).
Figure 8 K⁺ channel families classified by different transmembrane (TM) domains [modified from (64)].
Figure 9 Putative structure of the KCNT1 protein and corresponding table of amino acid locations from UniProt. It has a small N-terminal domain, six transmembrane domains and a large C-terminal domain. The C-terminal domain contains the NAD$^+$-binding domain and two tandem RCK domains [modified from (42)].
Figure 10 Immunocytochemical labeling of Slick and Slack (KCNT1) subunits at (A) medial nucleus of the trapezoid body, (B) oculomotor nuclei and (C) CA1 region of the hippocampus of adult rats showing coassembly of both channels in these regions (76).
Figure 11 In situ hybridization of rkcnt1 RNA transcripts using anti-sense probe derived from the 3’ non-coding region of rkcnt1 cDNA at cortex (b), hippocampus (c), brainstem (d) and medial nucleus of the trapezoid body (e) in rats (71)
Figure 12 Cartoon structure of the KCNC1 channel subunit and corresponding table of amino acid locations in different regions of the protein obtained from UniProt [modified from (97)]
Chapter 2

Functional characterization of novel KCNT1 variants related to severe early-onset epilepsy

2.1 Introduction

Mutations in the KCNT1 gene, which encodes a sodium-activated potassium channel, have been determined as possible causes for a range of epileptic syndromes with different outcomes. The two most common syndromes are autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) and epilepsy of infancy with migrating focal seizures (EIMFS).

Recent studies have also uncovered mutations in the KCNT1 gene in patients suffering from Ohtahara and West syndromes, both of which are early-onset epileptic encephalopathies. Sequencing experiments using PolyPhen-2, uncovered these mutations to be pathogenic, and thus most plausible contributors to the epilepsy observed in these probands (103, 104).

Whole genome sequencing exercises have also identified mutations in the KCNT1 gene in syndromes such as nocturnal frontal lobe epilepsy (NFLE), myoclonic atonic epilepsy (MAE), Lennox-Gastaut syndrome (LGS) and generalized genetic epilepsy (GGE), which were previously unidentified. However, it has yet to be determined if these KCNT1 mutations are in-fact causative of these syndromes as a majority of them have also been identified in the general population as single-nucleotide polymorphisms (SNPs).

Therefore, the goal of this chapter is to understand the contribution of these different variations in the KCNT1 gene to its functional outcome. The KCNT1 variants
analyzed and presented in this chapter have been obtained from clinical collaborators. These variants were screened using the gnomAD browser, to determine for their presence in the general population (Table 8).

The aim is to determine any characteristic changes in the biophysical activity of \( KCNT1 \) brought about by the variations, hence providing some insight into its pathogenicity.

The other aim is to determine the pharmacogenetic effect of quinidine with some of these novel \( KCNT1 \) mutations. A single dose of 300 µM was chosen as this was the efficacious dose for \( KCNT1 \) previously published by Milligan et al, where blocking effects were observed in both WT and mutant \( KCNT1 \) channels characterized in that study (50). Therefore, the goal was to determine if the same blocking effect would be observed at 300 µM quinidine with some of these novel \( KCNT1 \) mutant channels.

### 2.2 Methods

#### 2.2.1 \( KCNT1 \) Mutagenesis and \textit{In Vitro} Transcription

Variants were introduced into human \( KCNT1 \) (h\( KCNT1 \)) expression construct (50) using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s instructions. Construct fidelity was confirmed by DNA sequencing. cDNAs were transcribed \textit{in vitro} (mMessage mMACHINE; Ambion, Austin, TX).

#### 2.2.2 Oocyte Preparation

\textit{Xenopus} oocytes (Dumont stage V or VI) were surgically removed from \textit{Xenopus laevis} and prepared as described previously (105). Oocytes were kept in ND96 solution and stored at 17°C. Fifty nanoliters of capped cRNA was injected into each
oocyte using the Roboocyte system (Multi Channel Systems, Reutlingen, Germany).
A total of 10 ng of cRNA was injected into each oocyte.

2.2.3 Electrophysiology Recording

2-electrode voltage clamp recording was performed using the Roboocyte System (Multi Channel Systems) after 14 – 48 hours of expression. Electrodes containing 1.5M K-acetate and 0.5M KCl were used to impale oocytes that were held at −90 mV and perfused with bath solution containing (in millimolars) 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES, pH 7.5. Recording frequency was 1 kHz, and temperature was maintained between 20 and 22°C. To record expressed membrane currents, oocytes injected with M896K, F502V, V271F, F346L, L274I and R950Q variant, were held at −90 mV, and test depolarizations lasting for 600-milliseconds were applied at 10 mV increments, from −110 mV to +80 mV, at 5 second intervals. The same protocol, but with a longer test depolarizations, lasting for 1000-milliseconds was applied on R474H, R474C, Q206R, P1053H, T581I, I627V, Q543R, R910Q, P1227S and T465M variants. Contemporaneous measurement of WT currents was performed with the same batch of oocytes injected with variant channels to maintain an internal control of possible variability in expression from different batches of oocytes. Quinidine (Sigma, St Louis, MO) was dissolved in ethanol to a concentration of 300 µM and was perfused continuously to the oocytes for 1 minute, following with 5 minute incubation. Currents were recorded before and after the application of the compound.

The data obtained was compiled to depict functional differences and effect of quinidine on the KCNT1 variants in relation to WT. Relative current traces, current voltage relationship curves and time to 50% peak were undertaken to determine
functional differences between WT and variants. To determine effect of quinidine, in addition to relative current traces, the difference in normalized current-voltage relationship, before- and after- quinidine application and the percent of inhibition with 300 μM quinidine for variants and WT was evaluated.

AxoGraph (AxoGraph Scientific, Sydney, Australia) was used to analyze the electrophysiological data while statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Data is presented as a mean ± standard error of the mean. Statistical significance for time to 50% peak and percent inhibition of 300 μM quinidine at +80 mV was determined using one-way ANOVA with Bonferroni post hoc analysis.

2.3 Results

2.3.1 Functional assessment of novel KCNT1 variants

Evaluation of the effects of these novel KCNT1 variations and SNPs were undertaken using the Xenopus laevis oocyte expression system. cRNA was injected into oocytes at a set volume and concentration to allow direct comparison between WT and the variants. Automated two-electrode voltage clamping was performed to determine alterations in electrophysiological measurements in oocytes that were expressing these channels individually.

All EIMFS-causing KCNT1 variants were observed to produce larger current amplitudes, with some mutants producing up to 25 μA current (Figure 13). Similarly, the R950Q variant that was present in both ADNFLE and EIMFS patients, produced close to 15 μA current, which was larger than the 6 μA current produced by WT channels (Figure 13). Interestingly, P1053H and T581I, obtained from MAE and
LGS patients respectively, had similar current amplitudes, compared to WT and the SNPs (Figure 13).

To further evaluate the differences in current amplitudes, the raw current-voltage relationship curves were compiled and compared. As observed in the current traces, all EIMFS and ADNFLE- causing KCNT1 variants produced larger current outputs, as compared to WT (Figure 14A). The mutant EIMFS and ADNFLE curves were seen to have a left shift, where many of these mutants were seen to be activated at lower potentials (Figure 14A). A major difference in current output can be observed at approximately 10 mV where the minimum current in the mutants are approximately 3 µA, while WT is approximately 1 µA (Figure 14A).

P1053H had quite similar current-voltage relationship curves to WT, although a slight left shift was observed in the T581I mutant (Figure 14B) and the SNPs had the same current outputs as WT, seen by the overlay of their current-voltage curves with WT (Figure 14C).

Normalized current-voltage relationship curves of all EIMFS and ADNFLE-causing KCNT1 variants were observed to have altered kinetics as compared to WT (Figure 15A). Most EIMFS-causing variants were observed to produce more sigmoidal curve which produced larger currents at lower voltage inputs. ADNFLE-causing variant, R950Q and EIMFS-causing variant Q206R were observed to produce more linear current-voltage curves compared to WT (Figure 15A). Comparing the remaining variants in this study, the kinetics of T581I was slightly altered while P1053H and the SNPs were similar to WT (Figure 15B and 15C).

To determine if these channel kinetic differences were significant, the time taken to achieve 50% peak was analyzed (Figure 16). Reiterating the normalized data, all
**EIMFS**-causing *KCNT1* variants took a significantly shorter time to reach 50% peak vs. WT. The **ADNFLE**-causing variant, **R950Q**, was also observed to reach 50% peak significantly quicker compared to WT. **T581I** and **P1053H** variants reached 50% peak in significantly shorter time as well, outlining changes in kinetics, that were not observed in the normalized data. All **SNP** variants reached 50% peak within similar timeframes as WT.

### 2.3.2 Effect of quinidine on **EIMFS** and **ADNFLE**-causing **KCNT1** variants

Quinidine at a concentration of 300 µM had variable current blocking effects in the different *KCNT1* variant channels. Current traces obtained before and after the application of quinidine showed various levels of blocking across the set of variants tested (**Figure 17**); **M896K** showed a large reduction in current with application of 300 µM of quinidine, while **F346L** showed increase in current with quinidine application.

The difference in normalized current-voltage relationships before and after application of 300 µM of quinidine was compared between WT and the variants (**Figure 18**). Interestingly, all *KCNT1* variants (**EIMFS** and **ADNFLE**-causing) had smaller differences in current with the application of quinidine, as compared to WT.

The percent of inhibition at +80 mV of WT and variant *KCNT1* channels, reiterated the observations of both the current traces and difference in normalized current-voltage relationships (**Figure 19**). Inhibition with 300 µM of quinidine was significantly lesser across all variants compared to WT. Strikingly, a wide range in the percentages of inhibition was observed among the variants as well, with no
correlation to the epilepsy type. The range of inhibition varied between -20 to 25%, where the F346L variant was observed to have no effect with 300 µM quinidine.

2.4 Discussion

The presence of the KCNT1 channel is extensive in the human brain and plays a crucial role in maintaining the firing of action potentials in neuronal circuits. The channel, which conducts K+ ions out of neurons; allows for slow afterhyperpolarization recovery in neurons, following firing of action potentials, ultimately preparing the neuron to fire again (66, 74). With its large C-terminal, KCNT1 channels have been identified to mediate various protein-protein interactions, such as with FMRP and other critical proteins involved in cognitive development (55, 66, 106, 107)

Mutations in the KCNT1 gene have been identified previously in early-onset epilepsy such as EIMFS and ADNFLE (42, 50, 55). These mutations bring about increased seizure activity and more importantly, debilitating co-morbidities, such as low IQ and even death (42, 55). With the availability of modern high-throughput, NGS and molecular techniques, more information about novel KCNT1 mutations have been uncovered, leading the pathway towards developing precision therapeutics for KCNT1-related epilepsy.

Therefore the emphasis of this chapter is to determine the functional changes brought about by these KCNT1 variations and their response to quinidine, an FDA (US) approved antiarrhythmic, which was previously reported to significantly reduce overactive KCNT1 variants towards a WT phenotype (50).
The variants analyzed in this chapter were divided into three broad groups; (i) EIMFS & ADNFLE; (ii) others, which included other epilepsy types that had no KCNT1 mutations previously identified and; (iii) SNPs. These variant channels were analyzed using the *Xenopus laevis* expression system which is a known technique to study ion channel physiology.

Reiterating results that were previously reported in other publications, all of the EIMFS causing variants as well as the ADNFLE variant in this study, produced larger current amplitudes as compared to WT. Mapping these variants onto the *KCNT1* channel subunit, identifies two main clusters in which these variants occur, in the C-terminal and the S5-pore region of the subunit (Figure 20). As previously discussed, the C-terminal is a hotspot for various protein-protein interactions occurring; therefore variants such as F346L, R474H, R474C and R950Q may affect interactions within the protein network, therefore possibly contribute to the other comorbidities observed such as lower IQ and speech difficulties as well as increasing the seizure burden in these severe epileptic patients. Interestingly, both the F502V and M896K variants are located in the RCK domain, which regulates the K⁺ conductance across the channel. Hence, mutations in this region may affect the homeostatic regulation of ions, therefore leading to irregular firing of action potentials and thus contributing to epileptogenesis. Q206R, V271F and L274I were mapped to the S5-pore region of the *KCNT1* subunit, which contributes to the formation of the pore in the channel. The increase in current amplitudes in these 3 variants is indicative that these changes may have caused the channel’s pore to be restrained in an open state, therefore accounting for the increased current amplitudes.
These EIMFS and ADNFLE variants took a significantly shorter time to achieve 50% peak current as compared to WT. Given that the time to 50% peak current is a standard measure to determine changes in channel kinetics, the data obtained explains the increased current outputs observed in these channels. The quicker these variant channels reach peak current, the faster they allow the neuron to fire another action potential, therefore increasing the firing capacity of the neurons. This increased firing capacity contributes to a large imbalance in the neuronal networks, i.e. imbalance in excitability and inhibition; and thus, the onset of seizures.

Previous literature had also reported a phenotype-genotype relationship between KCNT1 mutations identified in ADNFLE and EIMFS patients. A distinct 3-fold increase in current amplitudes was observed in ADNFLE mutations, while a 5-fold increase was observed in EIMFS mutations (50).

However, no genotype-phenotype relationship could be established with the EIMFS and ADNFLE variants in this study. The range of the current amplitudes within the EIMFS variants differed, with some variants producing up to 5-fold increase in current amplitude while others only slightly more, as compared to WT. The normalized current-voltage curves also showed striking differences between the EIMFS variants, where majority of them produced close to a sigmoidal shaped curve, except for the Q206R variant, which produced a similar curve to that of WT and R950Q, the ADNFLE variant. Interestingly, the ADNFLE variant (R950Q) was also identified in patients with EIMFS, therefore refuting the genotype-phenotype theory.

Two novel variants from epilepsy types that were previously not reported to have KCNT1 mutations were analyzed in this chapter. P1053H from an MAE patient and T581I from an LGS patient were identified from WGS studies and were functionally
characterized. The overall results from the functional analysis of these two variants showed no differences to WT; current amplitudes were close to WT and no change of shape was observed in normalized current-voltage relationship curves. However, the time taken to 50% peak was significantly shorter for both as compared to WT. Of interest, the presumptive position of T581I was identified to be in RCK domain, which regulates K$^+$ conductance across the channel. P1053H was mapped to be located towards the end of the C-terminal in the KCNT1 channel subunit (Figure 20). Hence given the functional outcome of both mutations and their locations in the channel, these two mutations may be positively assisting in maintaining the K$^+$ homeostasis, similar to WT. Therefore, the data obtained from this study conclude that P1053H and T581I variants in the KCNT1 gene are most likely not the cause of epilepsy seen in these two patients.

The inclusion of SNPs in this chapter was to provide a direct comparison of novel disease-causing variants to variants found in the general public that generally have no negative outcome. The positions of the SNPs in this study were mainly located in the C-terminal, with some positioned in critical regions such as the RCK domains (Q543R and I627V), which regulates K$^+$ conductance and NAD$^+$ binding domain (R910Q), which modulates channel opening probability. However, functional analysis identified no differences between the SNPs and WT, with SNPs having identical current amplitudes, current-voltage curve patterns as well as channel kinetics to WT. Therefore, the inclusion of SNPs in this study assisted in emphasizing that not all genetic changes have to be detrimental.

One of the initial breakthroughs for precision medication in epilepsy was the discovery of quinidine’s effect on KCNT1 variants obtained from ADNFLE and
EIMFS patients (50). These variants were observed to have significantly reduced current amplitudes and channel kinetics, close to WT with the application of quinidine that works as a channel blocker. The clinical use of quinidine proved success in an EIMFS patient (R428Q) where reduction in seizures was observed and quality of life improved (108).

However, more clinical trials with quinidine uncovered its ineffectiveness in many other KCNT1-related epilepsies, therefore indicating the variability of effectiveness of quinidine (109-111). This chapter aimed to look at the functional effect of quinidine on selected EIMFS variants as well as the ADNFLE variant obtained.

A variety of outcomes was observed with the application of 300 µm quinidine amongst the selected variants. The before- and after- quinidine application current traces, of variants including M896K and F502V depict substantial reduction in current, while variants such R474C is observed to have no change in the current amplitudes. Interestingly the F346L variant was seen to be further activated with the application of 300 µm quinidine.

Each of the variants analyzed had a different response to quinidine in comparison to WT, as observed in the difference in normalized current. Furthermore, the percentage of inhibition by 300 µm quinidine was significantly different between the variants and WT. This therefore indicates that although quinidine was able to reduce current amplitudes in these variants, the scalability of this reduction was nowhere close to that of WT, suggesting that quinidine might not be a magic bullet for all KCNT1-related epilepsies.

A possible explanation to the failure of quinidine treatment in some these variants could be due to their position in the channel. Variants L274I and V271F have been
mapped to the S5-pore region of the KCNT1 subunit, which forms the pore of the channel. Given that quinidine blocks voltage-gated potassium channels by lodging into the pore of the channel, structural changes brought upon by variations in the amino acids on the pore region in KCNT1, may result in the instability of quinidine lodging in the variant channel’s pore (112).

In conclusion, this chapter reiterates the importance of functional testing of novel mutations to characterize and understand them better and to provide foresight into therapeutic outcomes, based on the results obtained. Functional characterization of these novel mutations disproved the theory of a genotype-phenotype relationship between EIMFS & ADNFLE variants. Furthermore, this chapter provides striking findings on the effect of 300 µm quinidine on novel KCNT1 variants.
### 2.5 Table

<table>
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<th>Coding DNA sequence</th>
<th>Protein change</th>
<th>Expression construct</th>
<th>Epilepsy type</th>
<th>Present in population (SNPs)</th>
<th>Allele count in population</th>
<th>Allele frequency in population</th>
<th>Polyphen-2 prediction</th>
<th>SIFT prediction</th>
<th>CADD score</th>
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<tbody>
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<td>EIMFS</td>
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<td>-</td>
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<td>Affect protein function (0.00)</td>
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<td>-</td>
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<td>Affect protein function (0.01)</td>
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<td>V271F</td>
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<td>-</td>
<td>-</td>
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<td>31</td>
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<tr>
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<td>p.Phe346Leu</td>
<td>F346L</td>
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<td>-</td>
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<td>Benign (0.02)</td>
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<td>L274I</td>
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**Table 8** List of KCNT1 variants undertaken in chapter 2. Variants were obtained from patients via clinical collaborators and were checked for their presence in the general population using the gnomAD browser. Data of SNP variants were defined by the allele count and frequency in the general population, using data obtained in gnomAD browser. Polyphen-2 predictions were calculated using an online tool ([http://genetics.bwh.harvard.edu/pph2/](http://genetics.bwh.harvard.edu/pph2/)) to determine the probability of variants being benign, possibly damaging or probably damaging. SIFT predictions were calculated using an online tool ([https://sift.bii.a-star.edu.sg/](https://sift.bii.a-star.edu.sg/)), which determined if the changes in amino acids affected protein function and lastly the CADD score was calculated using [https://cadd.gs.washington.edu/snv](https://cadd.gs.washington.edu/snv), where scored below 10 were likely benign and above 20 were pathogenic.
2.6 Figures

**Figure 13** Representative current traces obtained from *Xenopus* *laevis* oocytes expressing *KCNT1* WT or variant channels. Oocytes were held at -90 mV and stepped from -110 mV to +80 mV for 600 milliseconds (M896K, F502V, V271F, F346L, L274I and R950Q) or for 1000 milliseconds (R474H, R474C, Q206R, P1053H, T581I, I627V, Q543R, R910Q, P1227S and T465M) every 5 seconds. H2O injected oocytes were included as positive controls to the experiments. Respective scale bars apply to all traces within the set.
Figure 14 Comparison of raw current–voltage relationships between KCNT1 WT (n = 37) and variants; (A) EIMFS-causing [M896K (n = 23), F502V (n = 20), V271F (n = 20), F346L (n = 20), L274I (n = 20), R474H (n = 20), R474C (n = 20) and Q206R (n = 20)] and ADNFLE-causing [R950Q (n = 20)] KCNT1 mutations; (B) P1053H (n = 20) and T581I (n = 20) variant and (C) SNP variants [I627V (n = 20), Q543R (n = 20), R910Q (n = 20), P1227S (n = 20) and T465M (n = 20)].
Figure 15 Comparison of normalized current–voltage relationships between KCNT1 WT (n = 37) and variants. All currents were averaged, then normalized to the value at a test potential of +80mV (Imax); (A) EIMFS-causing [M896K (n = 23), F502V (n = 20), V271F (n = 20), F346L (n = 20), L274I (n = 20), R474H (n = 20), R474C (n = 20) and Q206R (n = 20)] and ADNFLE-causing [R950Q (n = 20)] KCNT1 mutations; (B) P1053H (n = 20) and T581I (n = 20) variant and (C) SNP variants [I627V (n = 20), Q543R (n = 20), R910Q (n = 20), P1227S (n = 20) and T465M (n = 20)].
Figure 16 Time to 50% peak of currents for KCNT1 WT (n = 20) and variants [EIMFS: M896K (n = 20), F502V (n = 20), V271F (n = 20), F346L (n = 20), L274I (n = 20), R474H (n = 20), R474C (n = 20) and Q206R (n = 20), ADNFLE: R950Q (n = 20), P1053H (n = 20), T581I (n = 20) and SNPs: I627V (n = 20), Q543R (n = 20), R910Q (n = 20), P1227S (n = 20) and T465M (n = 20)]. One-way ANOVA followed by Bonferroni post hoc analysis. *p < 0.1, ***p < 0.001, ****p < 0.0001.
Figure 17 Representative current traces obtained from Xenopus oocytes expressing KCNT1 WT or variant channels, each with application of vehicle (ND 96) before and 300 μM quinidine after. Oocytes were held at -90 mV and stepped from -110 mV to +80 mV for 600 milliseconds (M896K, F502V, V271F, F346L, L274I and R950Q) or for 1000 milliseconds (R474H and R474C) every 5 seconds. Respective scale bars apply to all traces within the set.
Figure 18 Difference in $I_{\text{max}}$ obtained from subtracting $I_{\text{max}}$ (current) before and after application of 300µM quinidine for KCNT1 WT (n = 37) and variants [EIMFS: M896K (n = 23), F502V (n = 20), V271F (n = 20), F346L (n = 20), L274I (n = 20), R474H (n = 20), R474C (n = 20) and ADNFLE: R950Q (n = 20)]. All currents were averaged, then normalized to the value at a test potential of +80mV ($I_{\text{max}}$) before and after quinidine application.
Figure 19 Average percent inhibition at +80 mV of KCNT1 WT (n = 37), EIMFS- causing [M896K (n = 23), F502V (n = 20), V271F (n = 20), F346L (n = 20), L274I (n = 20), R474H (n = 20), R474C (n = 20)] and ADNFLE-causing [R950Q (n = 20)] KCNT1 channels after application of 300 µM quinidine. One-way ANOVA followed by Bonferroni post hoc analysis. ****p < 0.0001.
Figure 20 Schematic of KCNT1 subunit with approximate positions of variants undertaken in this study [modified from (42)]
Chapter 3

In vitro case study of KCNT1 variants obtained from two patients

3.1 Introduction

Epilepsy of Infancy with Migrating Focal Seizures (EIMFS) is a severe early-onset epileptic encephalopathy (EE) with high morbidity. Seizure onset in patients with EIMFS most commonly occurs in the neonatal or early infantile period with a clinical course characterized by intractable epilepsy and severe intellectual disability (51, 52). Seizures in EIMFS are prolonged, multifocal and arise independently and sequentially from both hemispheres, which can occur many times each day.

The most common known cause of EIMFS is mutations in the KCNT1 gene, which encodes for a sodium-activated potassium (K\textsubscript{NA}) channel (55, 77). Activation of the KCNT1 channel results in K\textsuperscript{+} efflux, contributing to the delayed outward current of the action potential (113). Gain-of-function mutations in KCNT1 have been identified previously in patients with EIMFS, where changes were identified in output current amplitudes and channel kinetics.

Seizures in EIMFS have been identified to be refractory to nearly all conventional antiepileptic drugs, except for some which have been reported to respond to potassium bromide or levetiracetam (114-116). The discovery of the antiarrhythmic drug quinidine’s ability to reverse KCNT1 channel over-activity in vitro led to its rapid clinical application in a patient with EIMFS and a R428Q variant with reported in vivo efficacy (50, 108). Subsequently, a significant reduction of seizure frequency were observed in additional patients with mutations in KCNT1, however, treatment failures in other patients have also been reported, therefore highlighting the need for genotypic functional analysis prior to clinical administration (109, 117).
The previous chapter characterized novel KCNT1 mutations derived from varied epileptic (ADNFLE, EIMFS and Others) and non-epileptic (SNPs) patients, obtained from clinical collaborators. The chapter also characterized the effect of 300 μM quinidine only on some of these novel KCNT1 mutants.

This chapter focuses on two novel KCNT1 variants obtained via a different clinical collaborator, from two individual patients (Patient 1 and 2) with EIMFS. Hence, although both chapters characterize novel mutant KCNT1 mutations, given the differences in required data as well as source of mutations, the data is presented as two individual chapters in this thesis.

Patient 1 was identified as a combined heterozygote for two KCNT1 variations; a novel in-frame deletion in the 550\textsuperscript{th} amino acid; c.1649-1651del (Del550), Glutamine, inherited maternally and also the paternally inherited R1114W (c.3340C>T) \{Polyphen-2 prediction: Probably damaging [1.000], SIFT prediction: Affect protein function [0.03] and CADD score: 24\}, a single-nucleotide polymorphism (SNP) determined using the gnomAD browser (Figure 21).

Patient 2 presented a novel de novo substitution mutation, A259D (c.776C>A) \{Polyphen-2 prediction: Possibly damaging [0.860], SIFT prediction: Tolerated [0.71] and CADD score: 28.7\}, in the KCNT1 protein (Figure 21). Contrary to that, a change from Alanine (A) to Valine (V) at amino acid position 259 (c.776C>T) \{Polyphen-2 prediction: Possibly damaging [0.843], SIFT prediction: Tolerated [0.12] and CADD score: 28.7\} was identified as a SNP in gnomAD, indicating the importance of the right amino acid present at that position to determine pathogenicity.
Therefore, the aim of this chapter was to functionally characterize these variants to understand the effects of these changes in the \textit{KCNT1} gene and to also determine the potency of quinidine on these variants, as a potential therapeutic for these patients.

3.2 Methods

3.2.1 \textit{KCNT1} Mutagenesis and \textit{In Vitro} Transcription

\textbf{A259D} and \textbf{A259V} constructs were obtained from Genewiz in a Miniprep form. \textbf{R1114W} and \textbf{Del550} mutations were introduced into human \textit{KCNT1} expression construct (50) using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s instructions. Construct fidelity for all four mutants were confirmed by DNA sequencing. cDNAs were transcribed \textit{in vitro} (mMessage mMMachine; Ambion, Austin, TX).

3.2.2 Oocyte Preparation

\textit{Xenopus} oocytes (Dumont stage V or VI) were surgically removed from \textit{Xenopus laevis} and prepared as described previously (105). Oocytes were kept in ND96 solution and stored at 17°C. Fifty nanoliters of capped cRNA was injected into each oocyte using the Roboocyte system (Multi Channel Systems, Reutlingen, Germany). A total of 10 ng of cRNA was injected into each oocyte.

3.2.3 Electrophysiology Recording

2-electrode voltage clamp recording was performed using the Roboocyte System (Multi Channel Systems) after 14 – 48 hours of expression. Electrodes containing 1.5M K-acetate and 0.5M KCl were used to impale oocytes that were held at –90 mV and perfused with bath solution containing (in millimolars) 96 NaCl, 2 KCl, 1.8 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 5 HEPES, pH 7.5. Recording frequency was 1 kHz, and
temperature was maintained between 20 and 22°C. To record expressed membrane
currents, oocytes were held at −90 mV, and test depolarizations lasting for 1000-
millisecond were applied at 10 mV increments, from −110 mV to +80 mV, at 5
second intervals. Contemporaneous measurement of WT currents was performed with
the same batch of oocytes injected with mutant channels to maintain an internal
control of possible variability in expression from different batches of oocytes.
Quinidine (Sigma, St Louis, MO) was dissolved in ethanol to a concentration of 300
µM and was perfused continuously to the oocytes for 1 minute, following with 5
minute incubation. Currents were recorded before and after the application of the
compound.

The data obtained was compiled to depict functional differences of variants (A259D,
A259V, R1114W and Del550) and effect of quinidine on variants (A259D, R1114W
and Del550) in relation to WT. Relative current traces, current voltage relationship
curves and time to 50% peak curves were employed to determine functional
differences between WT and variants. To determine effect of quinidine, in addition to
relative current traces and current voltage relationship curves, the percent of
remaining current for variants (A259D, R1114W and Del550) and WT was evaluated,
after exposure to the compound.

AxoGraph (AxoGraph Scientific, Sydney, Australia) was used to analyse the
electrophysiological data while statistical analysis was performed using GraphPad
Prism 6 (GraphPad Software, La Jolla, CA). Data is presented as a mean ± standard
error of the mean. Statistical significance for current at +80 mV for all variants, time
to 50% peak for all variants and average percent inhibition with 300 µM quinidine for
WT, A259D, R1114W and Del550 were determined using One-way ANOVA with
Bonferroni post hoc analysis.
3.3 Results

3.3.1 Functional analysis of KCNT1 variants

To study the consequences of these novel KCNT1 mutations as well as SNP variants, the *Xenopus laevis* oocyte expression system was adopted. Set concentrations and volumes of cRNA were injected into oocytes to facilitate direct comparison between WT and variants. To determine changes in electrophysiological outcomes in the oocytes, automated two-electrode voltage clamping was performed on injected oocytes.

Current traces of the novel EIMFS-causing A259D and Del550 KCNT1 mutants showed larger current amplitudes as compared to WT (Figure 2). Comparatively, the A259V and R1114W SNP current traces were similar to WT. This was indicative of a gain of function attribute for both the novel KCNT1 mutants.

To reiterate the current trace findings, the raw current-voltage relationship curves were compared, and it was evident that both, A259D and Del550 had a much larger current output as voltage increased (Figure 23). Strikingly, the Del500 mutant produced a sigmoidal current-voltage curve as compared to A259D, indicating possible changes in channel kinetics in the former. The A259V and R1114W SNPs were observed to have similar current-voltage curves as to WT.

The current amplitudes of all variants at maximum voltage (+80 mV) were compared to determine if any of the changes observed was significant (Figure 24). Both EIMFS-causing KCNT1 mutants, A259D and Del550 produced significantly increased current at +80 mV as compared to WT, confirming therefore that these were gain-of-function mutations. The two SNPs, A259V and R1114W had no significant differences from WT.
To further investigate if these variations in the KCNT1 gene also altered the channel’s kinetics, the time to 50% peak for WT, A259D, R1114W and Del550 variants was analyzed and compared (Figure 25). The time to 50% peak for Del550 was inaccurate, given the instant activation of the mutant KCNT1 channel, as observed in the linear current traces. Strikingly, the other EIMFS-causing mutant, A259D, took a significantly shorter time to reach 50% peak current as compared to WT. There was no difference observed between the R1114W SNP and WT.

3.3.2 Effect of quinidine on KCNT1 variants

To determine the efficacy of quinidine on KCNT1 WT and A259D, R1114W and Del550 KCNT1 variants, 300 μM quinidine was perfused over the injected oocytes and two electrode voltage clamp recording was performed after 5 minute incubation. The current traces obtained before and after the application of 300 μM quinidine show varied outcomes (Figure 26). The largest reduction in current was observed in R1114W, with A259D, while Del550 was observed to have very minimal changes in current amplitudes.

The difference in normalized current voltage curves, before and after the application of quinidine reiterated further the variable difference in outcomes across the 3 variants (Figure 27). Similar to what was observed in the current traces, the highest difference in normalized current was observed in R1114W while the lowest was seen in Del550.

To significantly differentiate the variability in outcomes to the exposure of 300 μM quinidine, the average percentage inhibition at maximum voltage (+80 mV) of A259D, R1114W and Del550 were compared (Figure 28). All three variants had significantly lower percentages of blocking with application of quinidine as compared
to WT. As seen in the current traces and difference in normalized current voltage curves, \textbf{R1114W} had the highest percentage of inhibition while \textbf{Del550} had the lowest.

3.4 Discussion

The vast availability and decreasing cost of genetic testing has led to an increased reporting of specific mutations in epileptic patients (118). This chapter reports two such novel \textit{KCNT1} mutations which were identified from two different patients. Patient 1 was a combined heterozygote with two \textit{KCNT1} mutants; a novel in-frame deletion, \textbf{Del550} and SNP, \textbf{R1114W}, while patient 2 had a novel \textit{de novo} substitution mutation, \textbf{A259D}. A search for \textit{KCNT1} SNPs on gnomAD, uncovered a population variant also at the same amino acid position as patient 2, \textbf{A259V}.

Therefore the aim of the chapter was to functionally understand and concurrently, determine the efficacy of quinidine on these variants.

In order to understand the genotype-phenotype complexity of patient 1, both mutations were analyzed individually to determine individual effects of each variant. The paternally inherited \textbf{R1114W} variation produced current traces and current-voltage curves identical to WT. No significant difference in current amplitudes was observed between \textbf{R1114W} and WT at maximum voltage (+80 mV). To determine if the \textbf{R1114W} variation altered channel kinetics, the time taken to achieve 50% peak current was analyzed and no significant difference observed between \textbf{R1114W} and WT.

Given the understanding that \textbf{R1114W} is a polymorphism, with an allele count of 61, the results obtained from this study support its presence as a non-pathogenic variant of the \textit{KCNT1} gene. The similarity in biophysical properties shared by the variant and
WT KCNT1 channels strongly indicate that the R1114W did not bring about the detrimental effects of EIMFS as determined in patient 1.

The novel in-frame deletion at 550 in the KCNT1 channel was presumed to have a loss of function effect, given that a deletion may render a channel defective. Surprisingly, the traces obtained from oocytes injected with Del550 cRNA, produced currents double to WT; close to 12 μA. The current amplitude at +80 mV was significantly higher for the Del550 mutant, compared to WT.

Another intriguing feature of the Del550 currents was the immediate activation of the channel, indicated by both the linear traces and current-voltage relationship curves. Therefore, the deletion at 550 suggests a possible loss of voltage-sensitivity to the channel, which could render the channel permanently open.

Position 550 in the KCNT1 channel is located in the C-terminal of the protein, which interacts with secondary proteins, such as FMR1. Furthermore, the C-terminal contains features such as the RCK domains, which regulate potassium conductance and also a NAD binding domain (Figure 21). The NAD binding domain, located approximately at the 800 residue of the protein, determines channel opening probability. A KCNT1 protein with a deletion at position 550 could therefore alter the control on channel opening, which reiterates the electrophysiology data obtained.

Functional analysis of patient 2’s mutation, A259D depicted the classic gain of function property of KCNT1 mutations leading to EIMFS. Current traces of A259D show larger currents as compared to WT. When compared to WT, current-voltage relationship curves of the A259D mutant was observed to produce bigger gains in current output. As well, the current output at +80mV of A259D mutant was significantly larger compared to WT. This significant increase in current in A259D
mutants is identical to previously reported literature on EIMFS mutants causing a gain-of-function in the KCNT1 channel (50, 119).

Differences in the time taken to 50% peak current between WT and A259D indicate changes in channel kinetics. The significantly lower time required by A259D is intuitive of altered channel kinetics, as the channel is activated quicker at lower voltages. Given that A259 is found in the T3-T4 linker region, mutations at that position may alter the channel’s voltage sensitivity; however further protein homology studies are required to determine this (Figure 21).

To determine the effect of amino acid substitutions occurring at position A259 on the KCNT1 subunit, a SNP, A259V, with allele count of 1, was cloned and injected into oocytes. Similar electrophysiological measurements were performed on the A259V injected oocytes.

Current traces of the A259V SNP were observed to be similar to WT. Similarly, current-voltage relationship curves were tightly matching to that of WT curves; superimposing the WT curves. Reiterating this close resemblance to WT, there was no significant difference in current amplitude at +80 mV. Therefore, the switch between a charged aspartic acid to a hydrophobic valine, at position 259 in the KCNT1 subunit, renders the current output back to WT levels.

Application of 300 μM quinidine on oocytes injected with WT, R1114W, Del550 and A259D cRNA, produced a variety of observable outcomes. Current amplitude reduction was observed in R1114W with quinidine application. However, the percentage of inhibition of R1114W mutant channels with 300 μM quinidine was significantly lesser than WT. Hence, although R1114W was identified to be similar to WT, the variation clearly altered the SNP’s channel sensitivity to quinidine.
300 μM of quinidine was ineffective on the Del550 channels. A minute reduction in current was observed in the difference in current (I_{max}) curve, which was the difference between normalized currents before and after application of quinidine. The percentage of inhibition indicated a block of only 20% with 300μM quinidine for Del550. This is significantly different to the percent inhibition observed in WT KCNT1 channels.

Hence, these experiments provide strong evidence of Del550’s insensitivity to quinidine, indicative that patients with this mutation should not be treated with quinidine. A plausible explanation to this failure could be the effect of the deletion which increases the KCNT1 channel’s activity. The blocking effect of quinidine may therefore be insufficient to cope with the significantly large increase in current amplitude in these Del550 channels.

The application of quinidine at 300 μM showed reduced current amplitudes of A259D, as observed in the current traces. The difference between normalized, before and after I_{max} curve uncovered a reduction in current close to half the maximum current. Although the percentage of inhibition was significantly lesser to WT, the application of 300 μM quinidine on A259D injected oocytes produced a positive response as compared to the other novel KCNT1 mutant, from patient 1, in this study.

Given its position in the S5-pore region of the channel, it could be assumed that the A259D mutant did not alter the pore region completely, therefore allowing the lodging of the pore blocker quinidine, but not to the extent of the WT KCNT1 channel pore (Figure 21). To decipher this further, a detailed homology study would be required to understand the intricacies of the KCNT1 protein.

In conclusion, this chapter reports the unique case of EIMFS-caused by a combined heterozygous KCNT1 patient, with a SNP and novel deletion, as well as a de novo
substitution in the 259\textsuperscript{th} amino acid of the \textit{KCNT1} protein. The functional data appreciates the biophysical and kinetic changes in the \textit{KCNT1} channel, brought about by these variations. The in-vitro application of quinidine to these variants provides a strong basis on the negligible outcome of treatment of these patients with quinidine, therefore emphasizing the importance of undertaking such studies.
3.5 Figures

Figure 21 Schematic of KCNT1 subunit with approximate positions of variants undertaken in this study [modified from (42)]
**Figure 22** Representative current traces obtained from *Xenopus laevis* oocytes expressing *KCNT1* WT, A259V, A259D, R1114W and Del550 *KCNT1* channels. H2O injected oocytes were included as positive controls to the experiments. Oocytes were held at -90 mV and stepped from -110 mV to +80 mV for 1000 milliseconds every 5 seconds. Scale bars apply to all traces.
Figure 23 Comparison of raw current–voltage relationships between KCNT1 WT (n = 37) and A259V (n = 13), A259D (n = 25), R1114W (n = 25) and Del550 (n = 27).
Current (µA) at +80 mV

- WT
- A259V
- A259D
- R1114W
- Del550
- H₂O
Figure 24 Current amplitudes at +80mV compared across *KCNT1* WT (n = 37) and **A259V** (n = 13), **A259D** (n = 25), **R1114W** (n = 25) and **Del550** (n = 27). One-way ANOVA followed by Bonferroni *post hoc* analysis. **p < 0.01.
**Figure 25** Time to 50% peak of currents for *KCNT1* WT (n = 20), A259D (n = 20) and R1114W (n = 20). One-way ANOVA followed by Bonferroni post hoc analysis. **p < 0.01.** Time to 50% peak for Del550 mutant was not accurate due to quick activation of channel.
**Figure 26** Representative current traces obtained from *Xenopus laevis* oocytes expressing *KCNT1* WT or variant channels, each with application of vehicle (ND 96) before and 300 µM quinidine after. Oocytes were held at -90 mV and stepped from -110 mV to +80 mV for 1000 milliseconds every 5 seconds. Respective scale bars apply to all traces within the set.
Figure 27 Difference in current ($I_{\text{max}}$) obtained from subtracting $I_{\text{max}}$ before and after application of 300 µM quinidine for KCNT1 WT ($n = 37$), A259D ($n = 15$), R1114W ($n = 16$) and Del550 ($n = 14$). All currents were averaged, then normalized to the value at a test potential of +80 mV ($I_{\text{max}}$) before and after quinidine application.
Figure 28 Average percent inhibition at +80 mV of KCNT1 WT (n = 37), A259D (n = 15), R1114W (n = 16) and Del550 (n = 14) KCNT1 channels after application of 300 µM quinidine. One-way ANOVA followed by Bonferroni post hoc analysis. *p < 0.1, **p < 0.01, ****p < 0.0001.
Chapter 4

Functional analysis of novel KCNC1 mutations involved in epilepsy and neurodevelopmental disability

4.1 Introduction

The implication of potassium channels has been previously described in various epilepsy syndromes. Given their high expression rates in rapidly firing neurons, mutations in genes encoding potassium channels, such as Kv3.1, may contribute to epileptogenesis (120-123). The implication of members from the Kv3 family of potassium channels generated large interest in brain excitability research, due to their relatively large conductance, swift channel kinetics and high activation threshold (87).

A missense mutation in the KCNC1 gene, which encodes the voltage-gated K+ channel, Kv3.1 was first identified by Muona et al., in 2015 (97). The mutation, c.959G>A (p.Arg320His) was obtained from whole exome sequencing (WES) of eighty-four patients who suffered from progressive myoclonus epilepsy (PME). Functional analysis in Xenopus laevis oocytes confirmed that the mutant had a dominant-negative, loss-of-function effect.

In 2017, Oliver et al., coined a new terminology, myoclonus epilepsy and ataxia due to potassium channel mutation (MEAK) and extensively explained both clinical and electrophysiological outcomes of the p.Arg320His mutation in the KCNC1 gene (99). Interestingly, the group identified that an increase in core body temperature, improved the symptoms of p.Arg320His patients. Correspondingly, a leftward shift in channel activation was observed in patch-clamp recordings of WT Kv3.1 expressed in cells.
Following this discovery, the inclusion of the *KCNC1* gene in many gene panels in sequencing studies has identified novel mutations, previously unknown. A novel recurrent *KCNC1* missense mutations, p.Ala421Val was identified in patients with epileptic encephalopathies (EEs).

Four cases of c.1262C>T, (p. Ala421Val) {Polyphen-2 prediction: Probably damaging [1.000], SIFT prediction: Affected protein function [0.00] and CADD score: 26.8} were identified from sequencing studies done in the UK, Germany, France and US. Clinical features of these identified patients include early-onset epilepsy, as early as at birth in some cases, atypical absences, focal onset generalized tonic-clonic and myoclonus seizures with delayed neurological development. Additionally, two patients were observed to have hypotonia, gastro-intestinal disabilities and problems with sleep. Mild ataxia was also observed in two of the four patients and interestingly, fever in two of the patients was seen to exacerbate the symptoms as opposed to p.Arg320His patients, which were observed to get better with fever.

An interesting case of a single missense mutation, c.949G>A (p. Arg317Ser) {Polyphen-2 prediction: Probably damaging [1.000], SIFT prediction: Affected protein function [0.01] and CADD score: 32}, in the *KCNC1* gene was obtained from a fifteen-year-old boy in China. The patient presented with episodic generalized tonic-clonic seizures and episodic lower limb myoclonus seizures. He was seen to be intellectually impaired and have a family history of ataxia, with his mother also suffering the same. The patient’s epilepsy was controlled with a combination of valproate, oxcarbazepine, levetiracetam and clonazepam.
Although the focus so far has been on *KCNC1*’s implication in epilepsy, there have been novel mutations reported in patients without epileptic seizures as well. Poirier *et al.*, in 2017, identified three probands from the same family, carrying a c. 1015C> T (p. Arg339*) {CADD score: 39} nonsense mutation, which resulted in truncated *KCNC1* gene products. All three patients had delayed motor and speech abilities as well as hypotonia, intellectual disability and dysmorphic features such as epicanthal folds, ptosis and protruding ears. Significant reduction of mRNA transcripts was found in the three, from quantification studies using fibroblasts of the patients (124).

Another novel mutation in the *KCNC1* gene was identified in a patient with Wilms tumour. A nonsense mutation in c.1474 C>T (p.Gln492*) {CADD score: 39} was identified in the patient who suffered from intellectual disability, which was inherited from the patient’s intellectually disabled mother.

One patient with a novel missense mutation, c.950G>A, p.Arg317His {Polyphen-2 prediction: Probably damaging [1.000], SIFT prediction: Affected protein function [0.01] and CADD score: 32}, in *KCNC1* was identified, who presented with intellectual disability and autism.

Sequencing studies uncovered the presence of the novel mutation c.1294G> A (p.Val432Met) {Polyphen-2 prediction: Probably damaging [1.000], SIFT prediction: Affected protein function [0.00] and CADD score: 27.8} in the *KCNC1* gene from epilepsy patients. Information obtained from the NHLBI Exome Sequencing Project, indicated that the variant was rare and not found in the general population. No clinical information was made available for publication in this chapter.

Therefore, the aim of this chapter was to functionally analyze these novel mutations (p.Ala421Val, p.Arg317Ser, p.Arg317His, p.Arg339*, p.Gln492* and p.Val432Met)
(Figure 29) to determine their individual contribution to the change in biophysical properties of the Kv3.1 channel and thus the disease phenotype.

4.2 Methods

4.2.1 KCNC1 Mutagenesis and In Vitro Transcription

Mutations were introduced into human KCNC1 expression construct (Muona et. al., 2015) using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s instructions. Construct fidelity was confirmed by DNA sequencing. cDNAs were transcribed in vitro (mMessage mMachine; Ambion, Austin, TX).

4.2.2 Oocyte Preparation

Xenopus oocytes (Dumont stage V or VI) were surgically removed from Xenopus laevis and prepared as described previously (Petrou, Ugur et al. 1997). Oocytes were kept in ND96 solution and stored at 17°C. Fifty nanoliters of capped cRNA was injected into each oocyte using the Roboinject system (Multi Channel Systems, Reutlingen, Germany). Concentration of cRNA was adjusted to the lowest concentration obtained across WT and mutants.

4.2.3 Electrophysiology Recording

2-electrode voltage clamp recording was performed using the Roboocyte2® (Multi Channel Systems) after 48 hours of expression. Electrodes containing 1.5 M K-acetate and 0.5 M KCl were used to impale oocytes that were held at −90 mV and perfused with bath solution containing (in millimolars) 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2 and 5 HEPES, pH 7.5. Recording frequency was 1 kHz, and temperature was
maintained between 20 and 22°C. To record expressed membrane currents, oocytes were held at −90 mV, and test depolarizations lasting for 500 ms were applied at 10 mV increments, from −60 mV to +60 mV, followed by a step to -90 mV for 500 ms to analyze tail currents.

Contemporaneous measurement of WT currents was performed with the same batch of oocytes injected with mutant channels to maintain an internal control of possible variability in expression from different batches of oocytes.

4.2.4 Data Analysis

Maximum current amplitudes at +60 mV were compared and presented as mean values ± s.e.m. Student’s t-test was used to determine statistical significance between WT and each mutant. Tail current amplitudes at -90 mV were used to determine voltage-dependence activation of the channel. A Boltzmann function was fit to the current-voltage relationships, $I/V - I_{\text{max}}/(1 - \exp[(V - V_{0.5})/k]) + C$, where $I_{\text{max}}$ is the maximum tail current amplitude at test potential $V$, $V_{0.5}$ is the half-maximal activation potential, $k$ is a slope factor reflecting characteristics of voltage-dependent channel gating and $C$ a constant.

Electrophysiological recordings from the Roboocyte2® were extracted using Roboocyte2+ (Multi Channel Systems) and analyzed using AxoGraph (AxoGraph Scientific, Sydney, Australia). Plotting of graphs and statistical analysis was performed using Prism 6 (GraphPad Software, La Jolla, CA).
4.3 Results

To determine the changes brought about by the mutations in the Kv3.1 channel, the *Xenopus laevis* oocyte expression system and automated two-electrode voltage clamping was used. cRNA of WT and mutants were diluted to the lowest concentration among them and a set volume of cRNA was injected per oocyte.

Initial inferences of difference in WT and mutant Kv3.1 subunits were understood by observing current traces that were obtained from a set voltage protocol of depolarizing pulses up to +60 mV ([Figure 30A; [a-f]]). The maximum current amplitudes were analyzed at the end of the current trace obtained at maximum voltage (+60 mv) and normalized for all mutants to WT recorded on the same day, therefore providing the relative current amplitude ([Figure 30B; [a-f]]).

*KCNC1* WT injected oocytes produced the largest current amplitudes compared to all the mutants in this study. Interestingly, both A421V and R339* mutants produced very minimal current in the range of water-injected oocytes, which acted as negative-controls for these experiments ([Figure 30A, B; [a-b]]).

The R317S mutant, which generated larger current traces as compared to A421V, produced however significantly lower relative current amplitudes, when compared to WT Kv3.1 channels ([Figure 30A, B; [c]]).

In contrast to R317S, the R317H mutant produced much smaller current traces compared to both WT and R317S ([Figure 30A; [d]]). This was statistically confirmed as the R317H relative current amplitudes were significantly lower compared to WT ([Figure 30B; [d]]).
Similar to R317S mutant, both Q492* and V432M mutants produced large current traces, comparable to the other mutants in this study (Figure 30A; [e-f]). However, when comparing their relative current amplitudes with WT, both mutants had significantly lesser current amplitudes at maximum voltage (Figure 30B; [e-f]).

The tail current amplitudes at -90 mV were analyzed to determine voltage dependence of activation for all mutants and WT and determine V_{0.5}, the voltage at which half of the available KCNC1 channels would be open. The V_{0.5} for R317S and Q492* was significantly more negative, while V432M was similar to WT (Figure 31; [a-c]). The current-voltage relationships for A421V, R339* and R317H were not obtained as they produced very low currents.

To determine heterozygous effects of mutant and WT subunits, co-expression experiments were performed, where both WT and mutant cRNA of the same concentration were injected at a ratio of 1:1. Similarly, to allow direct comparison, WT was co-injected with water at a ratio of 1:1. Following which, electrophysiological measurements were done, similar to the previous set of experiments, with the same voltage protocol.

Among all the mutants involved in this study, five were observed to produce significantly lower current amplitudes due to co-expression of mutant and WT together in the oocytes. Co-expression of WT+A421V produced a small drop in current amplitudes as compared to WT (Figure 32A, B; [a]). Similarly, current amplitudes of WT+Q492*, produced a significantly small drop (Figure 32A, B; [e]). However, co-expression of WT+R339*, WT+R317S as well as WT+R317H produced significantly lower current amplitudes; up to 50% as compared to WT + water co-
injected oocytes (Figure 32A, B; [b-d]). The co-expression of WT+V432M did not show reduced current amplitudes, but rather an increase (Figure 32A, B; [f]).

Tail current amplitudes at -90 mV for all co-expressed mutants were compared to WT to determine voltage dependence of activation and \( V_{0.5} \). Most of the mutants produced similar current-voltage relationship curves as WT, with the exception of WT+R317H, which had a slight leftward shift in voltage, therefore indicating a plausible dominant negative effect (Figure 33). Interestingly, WT+V432M produced a rightward shift in its current voltage relationship curve (Figure 33; [f]).

4.4 Discussion

The advancement in genetic testing for diseases such as epilepsy has uncovered mutations in various genes, especially those that encode ion channels, present throughout in neurons.

The discovery of the R320H mutation in patients with PME identified \( KCNC1 \) as a novel epilepsy gene (97). Further functional and clinical analysis on the R320H mutation identified more detailed properties of the epilepsy form, thus rebranding it to be known as MEAK (99).

This chapter aimed to study novel \( KCNC1 \) mutations obtained from patients with and without epileptic outcomes and comorbidities and to establish any similarities or differences among these mutations in relation to the disease phenotype. The expression model used in this study was the \( Xenopus laevis \) oocyte system, similar to what was used in the published \( KCNC1 \) functional studies.

Interestingly, two mutations in this study, R317S and R317H were identified to be mapped very closely to R320H on the \( KCNC1 \) channel subunit (Figure 29). These
mutations are localized in the S4 region, which presides as the voltage sensor of the channel. Individual expression of the mutants in oocytes determined low current amplitudes for both mutations (Figure 30A, B; [c-d]). Similarly, when co-expressed with WT at a ratio of 1:1, R317S and R317H produced significantly lower current amplitudes, similar to R320H (Figure 32A, B; [c-d]) (97). However, the identified phenotypes only partially overlapped (R317S) or were very different (R317H) from MEAK.

Interestingly, tail current analysis revealed differences in voltage dependence of activation between R317S and R317H. Individual tail currents of R317S showed significant difference in $V_{0.5}$ as compared to WT (Figure 31; [a]), however when co-expressed with WT, no significant differences were observed (Figure 33; [c]). Co-expression of WT+R317H showed a leftward shift in the current-voltage curve, signifying a dominant negative effect brought about by the change of arginine to histidine at amino acid 317 (Figure 33; [d]). This was the similar effect observed in R320H, with a leftward shift in the current-voltage curve (97).

Although both histidine and serine are polar amino acids, there are slight differences in their structure, with histidine having an imidazole side chain and carboxylic acid group while serine has a hydroxymethyl side chain and carboxyl group. These differences contribute to the individual structure of the two amino acids, therefore varied structures in the overall protein, however further studies in structural biology are required to understand these differences.

The R339* was mapped to be found in the intracellular linker protein between the S4 and S5 region (Figure 29). The addition of a stop codon at amino acid position 339 produces a truncated protein that is not functionally expressed and yields completely
diminished currents in oocytes (Figure 30A, B; [b]) and markedly reduced currents when co-expressed with the WT (Figure 32A, B; [b]).

No difference was observed in voltage dependent activation between the tail currents of WT and WT+R339*, indicating that the truncation was probably reducing the expression of the WT in surface membrane (Figure 33; [b]).

As previously published, the R339* mutation was identified in 3 patients with intellectual disability and dysmorphic features (124).

The A421V, Q492* and V432M mutants were observed to be mapped to the S6 region of the channel (Figure 29). Although found on the same region, unique differences was uncovered with the mutations. Individual expression of A421V mutant channels produced a significantly large reduction in current amplitudes (Figure 30A, B; [a]). Q492* and V432M expression showed significant reduction (Figure 30A, B; [e-f]). Tail current data of Q492* showed significant difference in $V_{0.5}$ compared to WT (Figure 31; [b]).

Co-expression of WT+A421V and WT+Q492* produced significantly lesser current amplitudes, while WT+V432M showed no difference as compared to WT (Figure 32A, B; [a, e-f]). The tail current data of all three mutants showed varied outcomes; A421V and Q492* produced similar conductance-voltage relationships to WT, but a rightward shift was observed in the WT+V432M mutant (Figure 33; [a, e-f]).

Based on the results obtained for these 3 mutations, the substitution of alanine to valine at amino acid position 421 and the truncation at amino acid 492 expressed alone caused a major reduction in current amplitude; however, co-expression with the WT yielded only slightly reduced currents compared to the control condition. In
contrast, the V432M mutation, although by itself, produces reduced current amplitudes, when co-expressed with WT did not show any current reduction.

Although both valine and methionine amino acids share similar properties, including being non-polar and hydrophobic, the presence of additional non-hydrogen substituent attached to the alpha-carbon of valine, increases its presence in the backbone of a protein (125).

In silico predictions have identified the V432M mutation to be likely pathogenic due to the substitution occurring at a position conserved across various species; hence a higher probability of damage to the protein structure/function. More functional and structural analysis, combined with clinical observations is required to further investigate the outcome of this particular variant.

In conclusion, this functional study has been able to provide insight into the biophysical changes brought about by the various KCNC1 mutations. However it is insufficient to conclude the overall contributions of these mutations to the various epileptic and non-epileptic outcomes observed clinically. The complexities of gene-gene and protein-protein interactions take prominence over the simple functional analysis performed in this chapter.

Yet, this functional characterization in oocytes has uncovered interesting features and hotspots, which show diverse effects of disease variants on the function of the KCNC1 channel. The comparison at amino acid position 317 in the KCNC1 channel has identified the varied effect of substituting amino acids at the same position.

Furthermore, the clinical data obtained from patient’s whose mutations were analyzed in this study are indicative of co-morbidities such as intellectual disability,
dysmorphic features and delayed motor function. These concurrent outcomes are suggestive of the role played by Kv3.1 alternative to preparing mere enabling fast spiking neurons to fire rapidly. Previous literature has identified Kv3.1’s role in early neural development stages, involving cell proliferation, neuronal growth and regulation of neuronal and glial function, the processes that can contribute to the disease phenotype (126-128).

To further understand the complexity of each mutation, more in-depth functional and structural studies are required to provide insights into disease mechanism and possibly provide basis for targeted and more efficient treatment of KCNC1-related disorders.
Figure 29 Schematic of KCNC1 subunit with approximate positions of mutations involved in this study [modified from (97)].
Figure 30 Functional characterization of KCNC1 mutants. A (a-f) Representative traces of whole-cell currents recorded in *Xenopus laevis* oocytes, injected with the same amount of cRNA encoding Kv3.1 wild-type (WT) or mutant, during 0.5 s voltage steps ranging from −60 mV to +60 mV. B (a-f) Current amplitudes analyzed at the end of the voltage step to +60 mV and normalized to the mean current amplitude of WT (n = 136) and mutants (a, A421V [n = 62]; b, R339* [n = 48]; c, R317S [n = 45]; d, R317H [n = 32]; e, Q492* [n = 30]; f, V432M [n = 32]) (Mann-Whitney non-parametric test; ****P < 0.0001, **P < 0.01).
Figure 31 Current-voltage relationships of WT and KCNC1 mutant channels. Lines represent fits of a Boltzmann function. Comparison of WT [$V_{0.5}: 22.6 \pm 1.9 \text{ mV (n = 37)}$, slope factor $k: 12.7 \pm 0.6$] with (a) R317S [$V_{0.5}: 16.0 \pm 2.6 \text{ mV (n = 18)}$ {Mann-Whitney non-parametric test; *P < 0.1}, slope factor $k: 12.1 \pm 0.7$], (b) Q492* [$V_{0.5}: 15.5 \pm 3.0 \text{ mV (n = 19)}$ {Mann-Whitney non-parametric test; *P < 0.1}, slope factor $k: 12.7 \pm 0.8$] and (c) V432M [$V_{0.5}: 17.2 \pm 2.1 \text{ mV (n = 30)}$ and the slope
factor $k : 13.1 \pm 0.4]$. Current-voltage relationships for A421V, R339* and R317H were inaccurate due to their low current amplitudes, and therefore not analysed.

a)

b)

c)
Figure 32 Co-expression analysis of KCNC1 WT and mutants. A (a-f) Representative traces of whole-cell currents recorded in *Xenopus laevis* oocytes, injected with cRNA encoding Kv3.1 wild-type (WT) and water or WT and mutants at a ratio of 1:1, during 0.5 s voltage steps ranging from −60 mV to +60 mV. B (a-f) Current amplitudes analyzed at the end of the voltage step to +60 mV and normalized to the mean current amplitude of WT+H2O (n = 111) and WT+mutant (a, A421V [n = 62]; b, R339* [n =
Figure 33 Current-voltage relationships of WT and co-expression of WT with KCNC1 mutant channels. Lines represent fits of a Boltzmann function. Comparison of WT [$V_{0.5}$: 18.3 ± 1.4 mV (n = 27), slope factor $k$: 12.4 ± 0.4] with (a) WT+A421V [$V_{0.5}$: 16.6 ± 1.8 mV (n = 36), slope factor $k$: 15.2 ± 0.4 {Mann-Whitney non-parametric test; ****$P < 0.0001$}], (b) WT+R339* [$V_{0.5}$: 15.7 ± 3.1 mV (n = 15), slope factor $k$: 12.2 ± 1.1], (c) WT+R317S [$V_{0.5}$: 21.7 ± 2.9 mV (n = 17), slope factor $k$: 12.8 ± 0.9], (d) WT+R317H [$V_{0.5}$: 11.5 ± 5.3 mV (n = 4), slope factor $k$: 15.3 ± 3.6], (e) WT+Q492* [$V_{0.5}$: 24.7 ± 3.2 mV (n = 12), slope factor $k$: 13.9 ± 0.5].
(Mann-Whitney non-parametric test; *P < 0.1) and (f) WT+V432M [$V_{0.5}$: 31.2 ± 1.7 mV (n = 12) (Mann-Whitney non-parametric test; ****P < 0.0001), slope factor $k$: 12.9 ± 0.7]
5.1 General Discussion

5.1.1 Genetic aetiology underlying epilepsy

Early-onset epileptic encephalopathies (EEs) are debilitating conditions with detrimental co-morbidities. These patients, as young as neonates, suffer long term mental and cognitive issues, including low IQ, inability to speak or even attend major milestones across their life. To determine plausible therapies for these patients, a vast knowledge on the underlying causes of such detrimental conditions is required.

The quick development of high throughput genetic sequencing and testing has shifted the paradigm in epilepsy diagnosis. The presence of a genetic underlying in epilepsy goes back to more than 25 years ago, when the first gene linked to seizures was identified (129). Since then, there has been an exponential growth in the number of causative mutations in genes detected in large sequencing consortia.

The development of sequencing technology has provided researchers a large amount of data. In-depth analysis of this sequencing data has provided researchers with more information on specific genes involved in disease outcomes, including epilepsy. Strikingly, majority of the genes identified in epilepsy encode ion channels, which are crucial proteins that maintain cellular homeostasis (7, 130).

Therefore, the aim of this thesis was to understand the contributions of different mutations in two different voltage-gated potassium channels $\textit{KCNT1}$ and $\textit{KCNC1}$; found abundantly expressed in neurons in the central nervous system as well as in other cell types in the peripheral nervous system. Given their major role in regulating
the excitation and inhibition in neural networks, the effects of potassium channel mutations may have severe consequences. Hence to understand these effects, functional studies were performed using Xenopus oocytes, to determine the biophysical changes caused by these novel mutations.

5.1.2 Outcome from novel KCNT1 in vitro studies

The KCNT1 gene, found on human chromosome 9, position 34.3, encodes a sodium-activated potassium channel also known as SLACK. The main responsibility of the KCNT1 channel is to allow for the slow afterhyperpolarization, following bursts of action potentials. The channel has been identified in both the central as well as in the peripheral nervous system and also in other tissues, such as the heart and kidney (66, 74). KCNT1 channels interact with a vast variety of proteins, such as the Fragile X related protein (FMRP) and Protein Kinase C (PKC), emphasising the channel’s plausible role in development and cognition (55, 66, 80, 82, 106, 107).

Mutations in the KCNT1 gene has been reported in a wide range of epilepsies, including early onset encephalopathies such as EIMFS, ADNFLE and Ohtahara Syndrome (OS) (20, 42, 52, 55, 101, 103, 104, 107-109, 117, 131-138). Chapter 2 and 3 of this thesis aimed to functionally characterise novel KCNT1 variants obtained from EE patients.

The results obtained in chapter 2 and 3 are in agreement with previously published literature, where pathogenic KCNT1 variants display a gain-of-function effect, by producing increased current amplitudes (50, 55, 104, 137, 139). However, chapter 3 demonstrates the intricacy of genes, where a deletion in the protein, does not necessarily render the protein inactive. The Del550 mutant analysed in chapter 3,
produced large current amplitudes, therefore emphasizing the importance of functional studies to understand novel mutations.

Although no one mechanism has been identified to explain the increase in current amplitudes with \( KCNT1 \) mutations, a few plausible explanations have been discussed in previous literature. The gain in current amplitudes in pathogenic \( KCNT1 \) variants is the result of increased opening probability of the channel (Po). A plausible cause of this increased Po has been linked to mutant channel cooperativity, where it is perceived that individual mutant channels interact and cooperatively enhance the \( K^+ \) current (107).

Altered voltage sensitivity may also result in increased Po, given that the channel can open at more depolarised potentials, hence allowing for a persistent hyperpolarising current, with a resultant inter-neuronal disinhibition, as reported in \( SCN1A \)-related epilepsy (140). On the contrary, the increased repolarisation of excitatory neurons allows for more frequent and rapid firing of action potentials, therefore causing an imbalance in neuronal networks (139, 141).

Other putative disease mechanisms have been described from protein homology studies, where some pathogenic \( KCNT1 \) variants have been identified to have abnormal channel gating or protein instability within the pore-forming region (119). An in-silico study of \( KCNT1 \) variants, predicted that the G288S variant would produce detrimental effects (134), while the Y775H variant would alter the channel’s sensitivity to sodium, therefore contributing to altered channel function (139). Nonetheless, further in-depth studies are required to decipher the mechanism of action of these pathogenic \( KCNT1 \) variants.
\textit{KCNT1}-variants obtained from patients with epilepsies that were not previously related to the gene, were also analysed in chapter 2. Functional analysis determined that these changes were unlikely to be the underlying cause of epilepsy seen in these patients. The inclusion of SNPs in both chapter 2 and 3, accentuates that not all genetic changes are detrimental. Strikingly, the comparison between A259D and A259V, displays the efficacy of a single amino acid substitution to render a detrimental variant to a non-pathogenic state.

Previous literature has attempted to demonstrate relationships between the severity of the epilepsy type (phenotype) with the degree of gain of-function caused by the \textit{KCNT1} mutation (genotype) (50, 104). However, recent studies have disproved such relationships (107), and the results obtained from both chapters 2 and 3 shows no evidence of this phenotype-genotype relationship. The \textit{KCNT1}-EIMFS mutations identified in both chapters display varied current amplitudes, where some produced up to 5-fold increase in current amplitude, as compared to WT, while some just a 1-fold increase. Given these varied distinctions within one particular phenotype (EIMFS) renders no logic comparing it to the other \textit{KCNT1}- phenotypes (ADNFLE, OS).

\textbf{5.1.3 Quinidine in \textit{KCNT1}-related epilepsy}

Both chapters 2 and 3 also reviewed the effect of quinidine, an antiarrhythmic, on these novel pathogenic \textit{KCNT1} variants. Quinidine was previously identified in the literature as a potential treatment for \textit{KCNT1}-related epilepsy. \textit{In vitro} data showed significant reduction in both mutant and WT \textit{KCNT1} current amplitudes (50).
The variants analysed in both chapters 2 and 3, showed varied effects to a set concentration of quinidine. Some variants such as M896K and F502V showed significant reduction in current amplitudes, while variants such as Del550 showed no change with application of 300 µM quinidine. Interestingly, the F346L variant in chapter 2 was seen to produce higher current amplitudes with the application of quinidine. In silico modelling of the F346L variant identified the abnormal channel opening dynamics due to the mutation, which deters quinidine’s ability to block the channel (119). Therefore, the data obtained from both chapters 2 and 3 rebukes the notion of quinidine as a magic bullet for KCNT1-related epilepsy.

Clinical translation of this potential therapy was successful for one patient with the R428Q variant, where quinidine therapy controlled seizures and there was a significant improvement in neurodevelopment (55, 108). However, there have been more trials involving quinidine administration to epilepsy patients with KCNT1-mutations, where there was little or no response noted to the therapy; albeit in vitro studies of the same mutations showing reduced current amplitudes with the application of quinidine (117). Interestingly, another patient with the same R428Q variant did not have a favorable response to the quinidine treatment (109).

The mismatch in the in vitro and in vivo responses to quinidine could be due to inadequate tissue concentrations of the drug. The oral administration of quinidine results in cerebrospinal fluid concentrations between 9-15 nM. This is much lower than 300 µM; which is the minimum concentration of quinidine required to produce significant reduction in current amplitudes in KCNT1 injected oocytes (50). However, this should not advocate increasing the quinidine dosage, given the drug’s side effects.
Given quinidine’s ability to elongate QT intervals, which could lead to cardiac arrest and systemic vasculitis, the administration of the drug to patients with KCNT1-epilepsy must be done with caution (142). Accurate dosage calculations as well as constant cardiac monitoring must be performed while administrating quinidine to these patients. Recent clinical trials on a patient with West syndrome had a good response with quinidine, however only with a higher dose of 60 mg/kg/day, which significantly increased the possibility of cardiac failure (103). In another trial, one patient developed a severe pulmonary vasculopathy, following which quinidine administration was discontinued (119).

Given quinidine’s mixed response from various clinical trials, the literature however, suggests the vigilant use of the drug in patients with KCNT1-related epilepsy, where there is no effective therapy currently. Rather than denying these patients a possible therapy, clinicians should inform suitable patients of the therapy, as well as educate them on the possible adverse effects. Monitoring these patients for any adverse effects is crucial and guidelines in administration and dosage must be strictly adhered to (119).

The data obtained from both chapters 2 and 3 have functionally characterised as well as determined the pharmacogenetic variability of quinidine response for KCNT1 variants which have not been published previously. These experiments add to the emphasis of in vitro studies for all new variants providing more insights on the outcome, mechanism and effect of possible therapeutic treatments.
5.1.4 Outcome from in vitro studies of KCNC1 mutations

The relationship between mutations in the KCNC1 gene and epilepsy were identified very recently. Muona et. al. had identified a missense mutation (R320H) in the KCNC1 gene, which encodes the Kv3.1 channel, in a large cohort of progressive myoclonus epilepsy (PME) patients, (97). This generated great interest in understanding this gene and its protein product, and in 2017, Oliver et. al., introduced a new terminology for epilepsy patients with this specific KCNC1 mutation: myoclonus epilepsy and ataxia due to potassium channel mutation (MEAK) (99).

Until recently, R320H was the only mutation identified from various gene panel experiments conducted across various cohorts of epileptic patients. A truncated variant, R339* was reported in 2017, but not in epilepsy patients. However, recent sequencing exercises have identified novel KCNC1 variants from different epilepsy types.

Therefore chapter 4 of this thesis focused on functionally characterising these novel KCNC1 variants, including R339* and determining if they were biophysically similar to R320H, which had been extensively studied, both in vitro and clinically. Functional analysis of these novel variants identified similarities and differences between them. The R317H variant was identified as the most similar to R320H, with the low current amplitudes and presence of the dominant negative effect, when co-expressed with WT, even though the mutation carrier presented with only intellectual disability and autism without seizures.

Interestingly, similar outcomes were observed from two other variants analysed in this chapter. The R339* and R317S variants produced low current amplitudes and
exerted a dominant-negative effect when co-expressed with WT. In contrast the effect of A421V and Q492* on WT currents was only minor or not at all present as observed in V432M.

The data obtained from chapter 4 hence identified the novel biophysical properties of these newly identified KCNC1 variants, which have not been published previously. Although these are preliminary results from oocyte experiments, they provide the platform for drug screening assays as well as a framework to develop more complex models of epilepsy.

5.1.5 *Xenopus laevis* oocytes; the limitations

Experimental models play a crucial role in research to understand the physiology of a disease. The adoption of oocytes from the South African clawed frog *Xenopus laevis*, has been widely documented in electrophysiology-literature. Going back more than 30 years, *Xenopus* oocytes have been the chosen expression system for many functional studies including receptor and ion channel proteins (143-149). The robustness and size of these oocytes make them suitable for impalement with electrodes, therefore enabling the recording of currents from these cells (150).

With the development of *in vitro* molecular cloning techniques, the study of ion channel proteins has gained new experimental techniques, such as electrophysiological characterization via *Xenopus* oocyte expression. The ability to translate RNA into protein and also perform post-translational modifications increases the edge of using *Xenopus* oocytes as an expression system (151).

Furthermore, this expression system works hand-in-hand with the two-electrode voltage-clamp (TEVC), where two electrodes are impaled into the injected oocytes,
one measuring the membrane potential and the other injecting currents at various potentials, which are recorded.

With the automation of TEVC, such as the Roboocyte system by Multi Channels Systems (Reutlingen, Germany) both the accuracy and reproducibility of experiments have improved as well as the productivity, given the rapid turnover of experiments that can be performed (152).

However, the *Xenopus* oocytes expression system has its limitations as well. Parameters such as water temperature, age of the female frogs as well as their nutrition are crucial in maintaining healthy colonies of frogs, which in turn produce healthy oocytes. Yet, variability has been observed between different batches of oocytes, mainly during changes in seasons, therefore emphasizing the need for contemporaneous controls per experiment (153-156).

A major drawback to the use of *Xenopus* oocytes is the presence of endogenous channels and modulatory proteins, which must be accounted and explained for. These channels and proteins assist to maintain homeostasis of the oocytes and therefore may interfere in the experimental outcome (151, 157).

However, it has been previously identified that the currents recorded from oocytes expressing target proteins tend to be larger than endogenous currents (157). Nonetheless, the presence of WT and water injected oocytes provide an additional level of control to the experiments.

Yet the most crucial disadvantage of such a basic expression system is the correlativity to higher organisms. The *Xenopus* oocyte system lacks the complex
interactions between proteins and therefore may not be able to mirror the actual outcome observed in humans.

The protein of interest may not express as well in the oocytes as compared to a kidney cell or neuron, which is its native cell of expression. Also, the effect of pharmacological agents on oocytes may differ as compared to a more complex model such as mammalian cells or rodents. It is therefore common to use the oocyte expression system as preliminary tool, to which further studies will be based upon in more complex organisms, such as rodents (151, 157).

The future of deciphering functional changes in ion channels implicated in epilepsy, as well as developing therapeutics for the disease, may require a more complex and multileveled model organism as compared to the *Xenopus* oocytes. As identified by Oyrer *et. al.*, (Figure 34) the pathway toward obtaining understanding and therefore therapies, requires a multi layered approach; from *in vitro* to *in vivo* and finally to clinical success (158). Therefore, the results obtained from this thesis provide the first layer of the framework, where an *in vitro* profile of these novel *KCNT1* and *KCNC1* variants have been identified. An attempt to understand possible effects of molecular therapy has also been pursued, with the quinidine experiments performed in chapter 2 and 3.

**5.1.6 Going forward**

Moving forward, the study of the genetic epilepsies needs to shift into the *in vivo* domain, such as rodent model systems. The use of genetically modified mice has proven successful in many forms of diseases, including those of immunology, development and recently of the central nervous system. Given their proximate
genetic makeup to humans as well as the development of various techniques, which allow easy manipulation of their genetic make-up, mice prove to be the chosen animal model system to represent complex diseases, such as cancer and also epilepsy (159).

Furthermore, various forms of data types can be obtained from mice; be it understanding behaviors from tests such as the Y-maze test, to determine the explorative nature of the altered mice or to mice brain slice histology, to determine concentrations of a desired protein using fluorescence in-situ hybridization (FISH).

This requires the development of rodents with specific KCNT1 or KCNC1 mutations, using tools such as CRISPR/Cas9, which performs genome editing to ensure the introduction (knock-in) or removal (knock-out) of gene mutations can occur. Many of the available rodent epileptic models have been developed using the CRISPR/Cas9 technology, including the SCN1A-KO mice, that mimics the devastating Dravet syndrome in humans (160).

In 2015, Lu et al, had published data using Slack knock-out (KO) mice, where the KCNT1 gene was ablated globally across the rodent’s genome. The group discovered the role of the channel in neuropathic pain in mice and was coincidently was the first ever data published using KCNT1 mice (161). A.E Bausch et al, in 2018, determined the importance of Slack in social behavior in mice, using the same Slack-KO mice. The group analyzed the relationship of Fragile-X-Mental-Retardation-Protein (FMRP); the loss of which causes Fragile-X-Syndrome and Slack, using both Slack-KO mice and FMRP-deficient mice (162).

There has yet to be any studies in the area of epilepsy using these available Slack-KO mice models. Neither has there been reports of KCNT1 missense mutation mice, where the mice produce the mutated form of the protein, just as in humans or in the
Xenopus oocytes. The availability of such epilepsy-rodent models allow physical parameters such as electroencephalography as well as behaviour to be observed; a major difference from simple model systems such as the Xenopus oocytes.

Given their close genetic similarity to humans, rodent studies would hopefully provide a good illustration of these epilepsies; as close to the human phenotypes, and therefore boost the effort to develop more personalised therapies for various genetic epilepsies.

The quinidine data obtained from this thesis contributes to the push of repurposing “orphaned” drugs for other diseases. The concept of “orphaned” drugs arise from their nasty adverse effects, therefore being removed from federated safe-drug lists, such as the FDA (US). With the availability of both in vitro and in vivo drug screening assays, these drugs can be screened across different diseases to determine any positive effects. Furthermore, the use of available drugs to treat new diseases substantially reduces the total cost and time to develop newer therapeutics. Thus, moving forward, the development of large drug libraries, including “orphaned” drugs, together with such high throughput experimental models, will provide more opportunities for treatment to severe diseases, including epilepsy (163).

The search for new therapeutics for drug-resistant epilepsy is ongoing and has uncovered many novel discoveries available naturally. Previous literature has identified the positive effects of cannabis in many neurological disorders, including epilepsy (164). A recent clinical trial involving the administration of cannabidiol to Dravet patients resulted in overall reduction in seizure frequency, with overall improvement in patient condition (165). These findings open a new realm of
therapeutics for severe drug-resistant epilepsy, and therefore need to be investigated further.

A futuristic goal in epilepsy treatment would be gene-therapy, where corrections of the mutant gene is rectified at the genetic level. Antisense oligonucleotides (ASOs) which are short, synthetic, single-stranded oligodeoxynucleotides can modify RNA and therefore alter the amount of RNA produced, and thus alter protein expression. Currently, two such therapies have been approved by the FDA (US) for the treatment of Duchenne muscular dystrophy and spinal muscular atrophy (166).

Thus the development of both in vitro and in vivo models will allow greater understanding of the genetic epilepsies, which in turn will provide more possibilities of understanding and developing personalised therapies which can then translate into the clinic.

5.1.7 Conclusion

In conclusion, this thesis has been able to identify the differential effects of novel human mutations in two different K+ channels, KCNT1 and KCNC1. This thesis has shown that not all mutations in the KCNT1 gene are gain of function in nature. The analysis of KCNT1 SNPs in this thesis, provide understanding for the basis of non-pathogenic variants present in the general population. This thesis goes on to establish the varied effect of the repurposed drug, quinidine on novel KCNT1 mutations. These quinidine experiments assist in the prediction of the clinical effect of quinidine in patients with these novel mutations. Novel mutations in the KCNC1 gene were characterized in this thesis. These characterized mutants had varied biophysical properties as compared to WT KCNC1 and the previously published p.Arg320His
mutant. Lastly, this thesis highlights the importance of *in vitro* characterization of mutations and drug assays, which provide the structure to translate into more complexed organisms and personalized therapies.
5.2 Figure

Figure 34 Framework to understand and develop targeted therapeutics. This involves multiple layers of research, both in vitro and in vivo before moving into the clinic (158).
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