DEVELOPMENT OF A NOVEL CONCATEMER TECHNOLOGY USING POTASSIUM CHANNEL (KV1.1) HOMOTETRAMER AS A FRAMEWORK

by

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ABSTRACT

Voltage gated potassium channels (Kv) belong to a larger family of proteins called ion channels. Kv channels occur ubiquitously in the body and play a range of essential roles in cellular processes such as signal propagation and cellular excitability. Kv are made up of four subunits, each of which is translated individually, followed by assembly and expression on the cell surface. Kv1.1 is a Kv family member capable of assembling as either a homotetramer or a heterotetramer. In particular, when Kv1.1 subunits assemble with other Kv1.x subunits, the former shape the activation threshold and kinetics of macroscopic current of a channel, and play an important role in the trafficking and surface expression of other Kv1.4 channels. Dysfunction of Kv1.1 has been linked to an autosomal dominant neurological disorder, episodic ataxia (EA-1), that affects both the central nervous system and peripheral nervous system. Patients with EA-1 are 10 times more likely to develop epilepsy than normal individuals. Kv1.1 channels have also been implicated in sudden unexpected death in epilepsy (SUDEP) and Alzheimer’s disease. Dysfunction of Kv1.1 channels has been characterized by studying specific mutations in KCNA1 genes. Current techniques for generating Kv1.1 channels in heterologous expression systems for subsequent biophysical characterizations include coexpression and dimer construction methods, and the creation of tandem dimer-linked concatamers. The latter provides the greatest control over stoichiometry and arrangement of subunits; however, generation of each concatemer is extremely labour- and time-intensive.

This thesis focuses on the development of a new concatemer system built on an inhouse plasmid (pICDNA), with an intentionally designed linker sequence that physically concatenates four Kcna1 genes. The Kv1.1 homotetramer concatemer system has been developed to permit flexibility, such that each gene (or multiple genes) in the concatemer can be targets for future cloning. The development of the Kv1.1 homotetramer system will facilitate the examination of the role Kv1.1
channels play, independently of its main partners, Kv1.2 and Kv1.4. The Kv1.1 concatemer platform can be used in the future as a backbone upon which future Kv1.x heterotetramers can be developed more easily.
LAY SUMMARY

Epilepsy is a neurological syndrome that affects 60 million people of all ages worldwide, and is characterized by recurrent and unprovoked seizures, accompanied by characteristic brain electrical changes, mediated by the abnormal firing of large numbers of nerve cells in the brain. Symptoms of a seizure can vary from illusory sensations to sudden interruption in consciousness and behavior, or a convulsion where some or all the limbs contract and/or shake violently. Genetic mutations that alter the structure of "potassium channel" proteins have been strongly linked to some forms of epilepsy. In laboratory settings, current models for characterizing the dysfunction of potassium channels have technical challenges that have slowed efforts to understand epilepsy. My research, which developed a novel method to build potassium channel proteins from their constituent parts, helped overcome some of these challenges, allowing researchers to explore questions that they were unable to before due to limitations in technology.
PREFACE

This thesis is based on work I performed in the laboratories of Dr. Tara Klassen and Dr. Abby Collier. The human samples were collected from tissues with consent for future experimentation from non-surviving organ donors, provided by the University of Hawaii Biorepository. This use of these tissues was approved by the University of British Columbia Clinical Research Ethics Board (H14-00092). The biophysical characterizations and data were kindly performed and provided by Victoria Baronas, a trainee in Associate Professor Harley Kurata’s Laboratory at the University of Alberta.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>Cav</td>
<td>Voltage gated calcium channel</td>
</tr>
<tr>
<td>EA-1</td>
<td>Episode ataxia 1</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>GABA G</td>
<td>Gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>SUDEP</td>
<td>Sudden unexpected death in epilepsy patient</td>
</tr>
<tr>
<td>Kv</td>
<td>Voltage gated potassium channel</td>
</tr>
<tr>
<td>LBA</td>
<td>Luria Broth Agar</td>
</tr>
<tr>
<td>LGIC</td>
<td>Ligand gated ion channel</td>
</tr>
<tr>
<td>LM</td>
<td>Mouse fibroblast</td>
</tr>
<tr>
<td>Nav</td>
<td>Voltage gated sodium channel</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>P2X</td>
<td>Receptor ATP-gated P2X receptor cation channel</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>rSap</td>
<td>Shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOC</td>
<td>Super Optimal Broth with Catabolite repression</td>
</tr>
<tr>
<td>VSOP</td>
<td>Voltage-sensor domain only-protein</td>
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This thesis and the work has been the result of a lot of love poured into by many people in my life. I would like to thank first and foremost two wonderful supervisors. Dr. Tara Klassen who started me on this journey, helped me realise that my dreams do not necessarily have to be dreams. Next, I would like to thank Dr. Abby Collier who supported me throughout the rest of my degree. She was the bedrock that I could always rely on, always believing in me and successfully championing me to what seems like a fairy-tale chapter ending. Thank you for everything the two of you!

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Ion channels

Ion channels are proteins that span cell membranes, and contain a central pore that controls the passage of charged particles (ions). DNA sequences encoding ion channels account for ~1% of the human genome, with over 400 distinct genes expressing these proteins in all types of cells\(^1\). Ion channels play a range of roles in the human body, including (but not limited to) electrical signalling in the central and peripheral nervous systems, and regulating homeostasis of a variety of biological processes, such as epithelial transport of nutrients, immune system responses, and insulin release\(^2\). These critical functions are vital for life itself and every tissue in the body expresses its own specific and unique set of ion channels that enables it to carry out its physiological functions. Thus the disruption of the expression or functioning of ion channels is a frequent cause of a number of human disease classes, including cardiac arrhythmias, epilepsy, cystic fibrosis, deafness, and skeletal muscle disorders\(^3\). Many pharmacological agents have primary or secondary effects on ion channels that can sometimes prevent or ameliorate symptoms resulting from ion channel dysfunction (or conversely, in some cases, may induce them as undesired off-target effects). Ion channels are oligomeric complexes comprised of pore forming subunits and regulatory or accessory subunits, encoded by single and/or multiple genes\(^4\). Subunit composition, which can be homo- or heteromeric, plays a part in tuning the biophysical properties of ionic conduction through the membrane, thereby controlling excitability profiles and channel functions. It should come as no surprise therefore that within the macromolecule complex, the composition of the individual components and/or subunits, is often specific to particular cells, tissues, or organs, having been shaped and optimized by evolutionary forces\(^5\).
The classification of ion channels can be broadly categorized based on their selective permeability to cations; e.g. potassium (K+), sodium (Na+), calcium (Ca2+), and magnesium (Mg2+), or anions, e.g. chloride (Cl-), and/or by their gating mechanisms (e.g. ligand, leak, and voltage-gated). In ligand-gated channels such as the ionotropic receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), gamma-Aminobutyric acid (GABA), the binding of a molecule induces pore opening. Leak channels are constitutively open at resting membrane potential (e.g. Potassium channel subfamily K member KCNK). Voltage-gated channels both regulate and respond to changes in transmembrane voltage (e.g. sodium voltage gated Nav, calcium voltage gated Cav, potassium voltage Kv), and regulated by an integral voltage sensor domain.

1.1.1 Ligand gated channels

Ligand gated ion channels (LGIC, commonly referred as ionotropic receptors) mediate intercellular communication when a neurotransmitter binds, by converting this chemical signal into a ion flux (Na+, K+, Ca2+, Cl-) in the post synaptic membrane. LGIC have been studied largely at biophysical, pharmacological and electrophysiological levels because their gating has immediate electrical consequence. It has recently been discovered that LGIC do not function independently but interact with intercellular proteins that link them to the cytoskeleton and signal transduction pathway. This may be important for the immobilization and clustering of the receptors and or modulation of receptors by kinases, phosphatases and other regulatory proteins. The LGIC can be broadly categorized into three super families: Cys-Loop receptors, ionotropic glutamate receptors and ATP gated channels.
1.1.1.1 Cys loop receptors

The Cys loop receptors are named after the formation of disulphide bond between two cysteine residues in the N terminal extracellular domain\(^\text{10}\). The extracellular N-terminal ligand binding domain can give receptor specificity for a range of ligands including acetylcholine, serotonin, glycine, glutamate and \(\gamma\)-aminobutyric acid (GABA). The receptors are usually pentameric where each subunit contains conserved extracellular N-terminal domain, three highly conserved transmembrane domains, variable size cytoplasmic loop and a fourth transmembrane domain with a short and variable extracellular C terminal domain\(^\text{11}\).

1.1.1.2 Ionotropic glutamate receptors

The ionotropic glutamate receptors are activated by the neurotransmitter glutamate. These receptors can be further divided into four subtypes on the basis their ligand binding properties as well as sequence similarity: AMPA receptors, kainate receptors, NMDA receptors and delta receptors\(^\text{12}\). Ionotropic glutamate receptors are made up of four subunits, each of which share the same architecture: four domains made up of two extracellular domains which have a clamshell like architecture called the N-terminal domain (NTD) and ligand binding domain, the transmembrane domain that forms the ion channel and intracellular C-terminal domain\(^\text{12}\).
1.1.1.3 ATP-gated channels

The ATP-gated P2X receptor cation channel (P2X Receptor) belong to a larger family of receptors known as the EnaC/P2X superfamily. These cation-permeable ligand gated ion channels open in response to the binding of extracellular adenosine 5’-triphosphate (ATP). The subunits for P2X receptors are capable of assembling as homo- or hetero-tetramers. The subunits share a common topology, however the subunits for ATP gated channels is different from the cys loop receptors and ionotropic glutamate receptors because they possess two transmembrane domains, an extracellular loop and intracellular carboxyl and amino termini. Some of the physiological processes that P2X receptors are involved in include modulation of cardiac rhythm and contractility, macrophage activation and apoptosis.

1.1.1.4 Leak channels

The commonly referred to leak channels are the tandem pore domain potassium channels which have 15 members and it’s α subunit is composed of four transmembrane segments with two pore loops. These channels are regulated by several mechanisms including molecular oxygen, cyclic nucleotides, serotonin and GABA. Potentially the most well-known of the leak channels is the Na⁺/K⁺ pump. This channel helps regulate resting membrane potential and osmotic balance within a cell by ensuring that the intracellular concentration of Na⁺ ions remains low. Thus K⁺ ions play a key role in balancing the gradient created by the negative fixed anions present in a cell by not only being actively pumped into the cell but also by moving freely in and out through K⁺ leak channels in the cell membrane.
1.1.1.5 Voltage gated channels

Voltage gated channels are the class of ion channels that are activated by changes in electrical membrane potential near the channel. At resting potential, the probability of an open state voltage gated channel is very low and as the membrane depolarizes it cause a change in the conformation of the channel leading to channel opening. These channels play crucial roles in excitable cells all over the body including: the nervous, cardiovascular, respiratory, endocrine, urinary, and immune systems. Voltage gated ion channels are generally ion specific and they can be classified based on ion permeation through the channel (Na\(^+\), K\(^+\), Ca\(^{2+}\) and Cl\(^-\)) \(^4\)

1.2 Voltage gated channels and disease

Disorders caused by inherited mutations of ion channels are collectively called channelopathies. Genetic mutations in over sixty ion channel genes are known to cause a range of clinical syndromes involving the brain (e.g, Dravet syndrome), muscle (hyperkalemic periodic paralysis), kidneys (Bartter syndrome), endocrine (neonatal diabetes mellitus) and immune systems (myasthenia gravis)\(^19\). Mendelian mutations have been linked to single channel in familial episodic and degenerative disorder in nervous, cardiovascular and immune systems but de novo channel variants are also emerging as prime candidates for risk in complex psychiatric, metabolic and metastatic disease\(^20\)–\(^25\). Channelopathies display both phenotypic heterogeneity and genetic heterogeneity. In phenotypic heterogeneity (or pleiotropy), different mutations in the same gene can cause different disease (i.e, mutations in SCN4A can result in hyperkalemic periodic paralysis, hypokalemic periodic paralysis, paramyotonia congenital and potassium-aggravated myotonia)\(^19\). In genetic (locus) heterogeneity, mutations in different genes can result in the same disease phenotype (an example is Dravet syndrome, a severe form of epilepsy most commonly resulting from mutations in SCN1A, but can also be caused by mutations in GABRG2)\(^19\).
Additionally, channelopathies may result from either abnormal gain, or loss of function mutations. A number of well-characterized channelopathies result in clinical skeletal muscle disorders. Loss of function mutations in \textit{CLCN1} results in myotonia (inappropriate persistence of muscle contraction after initiation of a voluntary movement, or triggering by mechanical stimuli, associated with muscle hyper-excitability), whereas gain of function mutations in \textit{SCN4A} can cause myotonia or flaccid (hypotonic) paralysis (muscle hypo-excitability) \(^{19}\). Thus, channelopathies are a heterogeneous group of disorders united by genetically determined defects in ion channels.

1.2.1 Architecture of voltage gated channels

Evolutionary analysis of disparate and related species has provided insight into the diversity and origins of the human voltage channels. Resulting from gene duplications and divergence of an ancestral Kv gene, all organisms studied to date express one or more Kv forms within their genome, including \textit{C. elegans}, which lacks a voltage gated sodium channel. The most basic functional and highly conserved component of voltage gated channels is the ion selective pore\(^{26}\). The pore forming domains consist of intracellular N- and C- termini, two transmembrane segments (2TM) and an extracellular loop that dips partially back into the pore, forming a \(K^+\) selectivity filter with a characteristic TTVGXG sequence. All Kv channels have a variation or elaboration of this basic template \(^{27}\).

Although bacterial ion channels (KcSA) and inward rectifying (Kir) channels are functional 2TM tetramers, the largest group of Kv channels are the six transmembrane structures where gating of the 2TM (designated S5- pore loop – S6) is controlled by a voltage sensor domain (designated as S1-
The voltage sensor is upstream of the pore encoding region and the resulting polypeptide includes four transmembrane segments of the voltage sensor on the N-terminus (6TM)\textsuperscript{26}. This includes a highly conserved S4 voltage sensor domain in which every third amino acid is a basic positively charged residue (arginine or lysine) that translocates in response to the changing transmembrane voltage and is stabilized by salt bridge formation with corresponding acidic residues (glutamate or aspartate) in the S2 and S3 helices\textsuperscript{28}.

Further gene duplications of the 6TM template has resulted in the typical 24TM structure that is observed in voltage gated sodium (Nav) and calcium (Cav) channels\textsuperscript{27}. The pore forming α subunit of the 24TM channels is comprised of four homologous repeat domains (DI-DIV), each consisting of six transmembrane segments (S1-S6) all encoded as a single transcript forming a pseudotetramer once translated\textsuperscript{29}. In contrast, the single potassium channel transcript encodes for a single subunit\textsuperscript{26}, therefore, four subunits have to be translated independently before it can be assemble as a tetramer that forms the functional Kv channel that is expressed on the plasma membrane.

### 1.2.2 Voltage gated sodium channels

The voltage gated sodium channels have nine known α subunit members (Nav1.1 – 1.9) that are prevalent in central nervous system and/or peripheral nervous system\textsuperscript{30}. The β subunits are transmembrane glycoproteins involved in the regulation of the α subunits, while the pore of sodium channels contains a selectivity filter made of primarily negatively charged amino acid residues. This pore forms the most constricted part of the pore (geometrically) allowing only a single Na\textsuperscript{+} ion with its associated water molecule to pass through\textsuperscript{31}.
1.2.3 Voltage gated calcium channels

Voltage gated calcium channels (Cav) are key signal transducers that mediate Ca\textsuperscript{2+} influx in response to an action potential, thereby serving as secondary messengers that initiate cellular events\textsuperscript{32}. The Cav proteins are complexes that can consist of an \(\alpha_1\) subunit that is sufficient to produce functioning Ca\textsuperscript{2+} channel but with low expression and abnormal kinetics\textsuperscript{33}. It was later discovered experimentally that the auxiliary subunits \(\alpha_2\delta\) and \(\beta\) helped increase levels of channel expression and renormalized the properties to Ca\textsuperscript{2+} and that the \(\gamma\) subunit had smaller effects\textsuperscript{34,35}. Some of the signalling processes that is modulated by Cav include gene transcription, neurotransmitter release, neurite outgrowth and the activation of calcium dependent enzymes such as Calmodulin-dependent protein Kinase II and Protein Kinase C\textsuperscript{36}.

1.2.4 Voltage gated chloride channels

Voltage gated chloride channels are involved in the permeation several anions in addition to Cl\textsuperscript{-} such as HCO\textsubscript{3}\textsuperscript{-}, I\textsuperscript{-}, SCN\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-}\textsuperscript{37}. The CLC gene family is a group of chloride channel that have voltage dependence and is thought to have 10-12 transmembrane domains; however, this model has been revised based on a 3D crystal structure of bacterial CLC protein\textsuperscript{38,39}. Chloride channels have a multitude of function such as stabilization of membrane potential, cell volume regulation, neutralization of H\textsuperscript{+} ions either in the cell membrane and/or intracellular compartments\textsuperscript{37}.

1.2.5 Voltage gated proton channels

The \textit{HVCN1} gene codes for a protein called Hv1 or VSOP (Voltage-sensor domain only-protein) that was recently discovered as a proton channel. The Hv1 protein consists of a N-terminal cytoplasmic domain, and a C-terminal cytoplasmic domain that has a coiled-coil motif and four transmembrane
domains analogous to the S1-S4 of voltage sensor in potassium channels. The bacterial proton channel is a dimer that is connected by coiled-coil interactions in the C terminal domain, thus containing a pore within each protomer\textsuperscript{40,41}. Some of the functions of proton channels include volume regulation, setting membrane potential, pH regulation in airway epithelium and histamine release from basophils\textsuperscript{42–45}

1.3 Voltage gated potassium channel super family

\textit{The voltage gated potassium channel super family in general, and the channel Kv1.1 specifically, are the focus of this thesis.}

The human genome contains around 80 potassium channels, with 40 genes encoding voltage gated potassium channels\textsuperscript{46}. The voltage gated potassium channel family is grouped into 12 classes based on sequence homology (Kv 1-12)\textsuperscript{46} (Figure 1) . The diversity of Kv channels occurs due to the ability of Kv mixing and matching with each other. The Kv1, Kv2, Kv3, Kv4, and Kv7 tertiary structures are capable of forming homo- and hetero-tetramers which display a range of functional properties\textsuperscript{46,47}. It has been shown that Kv2 units are more likely to assemble with Kv5, 6, 8 or 9 subunits and are expressed almost exclusively in nervous system and smooth muscle cells\textsuperscript{48}. The unique properties of voltage dependence and kinetics of Kv channels leads to their differential contribution to action potential modulation. The Kv1, Kv4 and Kv7 proteins require low levels of membrane depolarization, which affects the threshold for action potential generation as well as the number of action potentials generated. In contrast, Kv2 and Kv3 require much higher voltage for depolarization\textsuperscript{47}. Thus, the conformation of Kv channels influence the ways in which their kinetics differ and how they contribute to action potential generation. The voltage gated potassium channel family occur
ubiquitously in the body and play a range of essential roles in cellular processes such as: signal propagation and cellular excitability, apoptosis, cell differentiation and growth, maintenance of cardiac, skeletal and endocrine systems as well immune and inflammatory pathways⁴⁹.
Figure 1: Phylogenetic tree for Kv1-12 families based on amino acid sequence alignment of human Kv proteins. Source: Carlos Gonzalez et al. 2012\textsuperscript{46}. 

\textsuperscript{46} Carlos Gonzalez, et al. (2012).
1.3.1 Classification of voltage gated potassium channels

The voltage gated potassium channels can be classified based on sequence homology or physiological function. In terms of physiological function, voltage gated potassium channel can be grouped as: delayed rectifier, A-type potassium channel, outward rectifying, inwardly rectifying, slowly activating and modifiers/silencers.

1.3.1.1 Delayed rectifier

The term “delayed rectifier” was adopted from the classic experiments performed by Hodgkin and Huxley that described the outward K⁺ ion that activated later than Na⁺ currents. Classically, these channels help terminate the action potential, and restore the resting membrane potential. The typical feature that underlies delayed rectifiers is the property of slow inactivation or non-inactivation of current in voltage patch clamp experiments.

Several members of Kv1, Kv2, Kv3, Kv7 and Kv10 can be categorized as delayed rectifiers. In particular for Kv3, the Kv3.1 and Kv3.2 members are delayed rectifiers that are found in specialized neurons that fire short action potential at very high rates such as in the auditory system. They can also be found in fast spiking neurons in cerebellum, skeletal muscle, arterial smooth muscle and germ cells. The Kv7 channel (also known as KCNQ in humans) is another example of a delayed rectifier channel. The KCNQ1 (Kv7.1) channel is found abundantly in the heart, pancreas, kidney, lung, and placenta, and mutations in this gene give rise to the most common form of long QT syndrome. Long QT syndrome is a rare congenital heart condition which predisposes to cardiac arrhythmias. The symptoms can range from fainting to seizures and sudden death due to ventricular fibrillation, which begins with a characteristic electrocardiographic pattern, torsade de pointes.
1.3.1.2 The A-type Potassium Channel

Typical members of this class of inactivating channels include Kv1.4, Kv3.3, Kv3.4, Kv 4.1, Kv4.2 and Kv4.3. The key characteristics of A-type Potassium Channel are rapid inactivation, resulting in transient responses. Physiologically, the role of fast inactivation channels is in setting the action potential interval, because the repolarization phase of an action potential gets shorter, and thus neurons are ready to fire faster. Studies have shown that Kv 4.1-4.3 subunits are capable of forming heterotetramers and, together with auxiliary β subunits, modulate other Kv subunits by enhancing plasma membrane expression and inactivation.

1.3.1.3 The outward rectifying channel

The nomenclature of outward rectifying comes from voltage gated K+ channels of plants, where the channels facilitate K+ flux outwards across the plasma membrane. The channels only open at high concentrations of intracellular K+, thus driving the efflux of K+ ions outwards. This nomenclature was adopted when characterizing Kv10.2 channel encoded by the gene KCNH5 (also referred to as Eag) in mammalian cells. The characteristics of Kv10.2 are thus described as outward rectification with no inactivation, but fast deactivation and slow activation. The Kv10.2 channel has only been observed in the CNS and in humans, is the only member of the group with these physiological characteristics.
1.3.1.4 The inward rectifying channel

The potassium inward rectifying channel includes Kv11.1, Kv11.2 and Kv11.3 encoded by Erg genes. Experimentally, these channels pass current more easily into the cell from outside. These channels should not be confused with the two transmembrane Kir channels (also called inward rectifying K+ channels), which gain rectification properties through voltage-dependent block and not by the movement of the voltage sensor. These channels also differ from potassium channels that are responsible for repolarizing a cell following an action potential, such as the delayed rectifier and A-type potassium channel, which preferentially carry outward potassium currents at depolarized membrane potential. The three members of the Kv11 family are capable of forming heteromultimers, and their mRNA expression has been reported in many organs including heart, brain, nerves, kidney, liver, uterus, and prostate.

1.3.1.5 Slowly activating channel

These channels received their name from physiological characterization of a group channels that displayed a slow activating current on the application of a membrane potential. There are three members of the Kv channel: Kv12.1, Kv12.2, and Kv12.3. These channels are only capable of forming heterotetramers, and have been found in the central nervous system, colon and lung.

1.3.2 Modifiers/ Silencers of Kv family channels

Members of the Kv family that are in this group include Kv5, Kv6, Kv8 and Kv9. Despite sharing the same general structure with other Kv family members, they do not form functional homotetramers and have been nominally labelled as silent subunits. They have been shown to assemble with Kv2 and Kv 3 subunits and help modulate their biophysical properties.
1.4 The specific shaker-related Kv1 super family

Kv1.x channels form a major subfamily, and play critical roles in adjusting bio-electrical activity of neurons when defective channel function is a known cause of disease. The functional versatility of Kv1.x channels is due to their molecular diversity and precise regulation. Since the cloning of first Kv1 gene, shak, (also known as Kv1.1) in Drosophila in 1987, eight members of the family (Kv1.1-Kv1.8) encoded by the KCNA1-KCNA8 genes have been identified.

All members in this family are delayed rectifier channels, with the exception of Kv1.4. In eukaryotes, it was discovered that when Kv1.1, Kv1.2, Kv1.3 and Kv1.5 were expressed with auxiliary Kvβ1.1 they become rapidly inactivating. The Shaker family of Kv1.x is expressed in a tissue specific manner and have diverse roles. For example; in rats, Kv1.1 is expressed in primarily in the brain but also found in the heart, retina, skeletal muscle; while Kv1.2 is expressed primarily in brain, spinal cord, schwann cells, atrium, ventricle, retina, and smooth muscle. Additionally, the Kv1.3 subtype is expressed in brain and lung, but also has a major role in the immune system with expression seen in thymus, spleen, lymph nodes, B and T lymphocytes, and macrophages. In particular, researchers have examined Kv1.3 as a therapeutic target for immunosuppressants: a Kv1.3 inhibitor was demonstrated to inhibit calcium signaling, cytokine production, and proliferation of T cells in vitro and T cell motility in vivo. The Kv1.5 channel is expressed in hippocampus, aorta, colon, kidney, stomach, smooth muscle and pulmonary artery, and has properties similar to ultra rapidly activating IK\textsubscript{UR} current (involved in the repolarization of the human atrium). Recently, the theory has been advanced that Kv1.5 modulation may have potential therapeutic utility in the management of atrial fibrillation by blocking IK\textsubscript{UR} current.
1.4.1 Kv1.1 role in regulating other Kv1.x subunits

The specific channel Kv1.1 is a member of the Kv1.x family encoded by the gene KCNA1. Figure 2 shows the homology model of Kv1.1 channel. The protein can form both homo and hetero-tetramers in its quaternary structure in heterologous expression systems and previous reports have shown that all heterologous combinations of Kv1 subunit produce K+ currents. Analysis of crude brain extracts demonstrated that Kv1.2 is the most abundant subunit followed by Kv1.1, Kv1.6, Kv1.4, and Kv1.3, respectively. Furthermore studies with α-dendrotoxin (Kv1.2 specific mamba snake toxin) show that vast majority of Kv1.1, Kv1.4, and Kv1.6 oligomerize with Kv1.2 to form functional channels. This indicates that that assembly of Kv1 channels within intact neurons is unlikely to be promiscuous, but rather; tightly regulated to tune biophysical properties of discrete neurons.

The Kv1.1 channel, in particular, appears to play an important role in the trafficking and surface expression of other Kv1.x channels. Critically, Kv1.1 supersedes other regulatory motifs like the cytoplasmic C-terminal (VXXSL forward trafficking signal) of other Kv1.x subunits, and also negates the regulatory effects of the Kvβ2 auxiliary subunit. Thus, Kv1.1 is dominant over these additional regulatory signals and is, therefore; capable of overriding their effects when combined within a heteromeric channel.

The presence of a Kv1.1 subunit shapes the activation threshold and kinetics of macroscopic current in expression systems. This has been shown in vitro through production of a synthetic concatenated Kv1.1/Kv1.2 hetero-dimer or hetero-tetramer; where an increase in the number of Kv1.1 subunit in a tetramer enhanced activation kinetics of macroscopic currents and shifted V_{1/2} towards more negative potential. Considering that Kv1.2 is the most abundant subunit in the brain,
these studies suggest that the overriding effect of Kv1.1 may not necessarily be due to its abundance, but to its localization and companion proteins.

Figure 2. Homology model of the 6TM mKv1.1 developed on pdb 2A79 Rat Kv1.2 crystal structure. (A) Single subunit representation of transmembrane Kv1.1 at the interface of the membrane. (B) Arrangement of single subunit of Kv1.1 in 3D space. (C) Extracellular view, perpendicular to the membrane. (D) View of Kv1.1 parallel the cell membrane.
1.4.2 (Dys)function of KCNA1 gene (Kv1.1)

Potassium channels are important determinants of seizure susceptibility. In particular, mutations of KCNA1 result in neurological syndromes like episodic ataxia. Episodic ataxia type 1 (EA1) is a monogenic autosomal dominant disorder that affects both the central nervous system and peripheral nervous system mapped to Kv1.1 mutations\textsuperscript{52}. The hallmark symptoms of episodic ataxia include continuous rippling movements of muscles (myokymia), and episodic attacks of ataxia (incoordination of limb movements, unsteady gait and balance associated with cerebellar dysfunction). Spontaneous and kinesogenic (movement-induced) jerking movements of the head, arms, and legs are prominent during episodes. Vertigo, blurred and double vision, nausea, headache, sweating, clumsiness, stiffening of the body, dysarthria (slurred speech), and difficulty in breathing can also be seen. Calf muscle hypertrophy can be seen, and shortened Achilles tendons may result in tiptoe walking\textsuperscript{82}. Patients with EA-1 are 10 times more likely develop epilepsy than normal individuals, thus implicating KCNA1 mutations as potential pathogenic susceptibility factors for epilepsy\textsuperscript{83}.

KCNA1 mutations have also been implicated in sudden unexpected death in epilepsy (SUDEP). In SUDEP, patients with epilepsy who are otherwise healthy die unexpectedly for unknown pathological reasons, at a rate up to 24-times greater than the population\textsuperscript{84}. A proposed potential mechanism involves pathogenic neural signalling between the brain and the heart leading to lethal cardiac arrhythmias\textsuperscript{85}. Therefore, ion channels that co-express both in brain and heart are logical candidates for SUDEP, as defects in these channels could underlie both epilepsy and cardiac arrhythmias\textsuperscript{86}. Consistent with this hypothesis, mice lacking the KCNA1 gene exhibited severe seizures and die prematurely due to cardiac abnormalities\textsuperscript{87}. 
Autosomal dominant hypomagnesemia with myokymia (also associated with episodic muscle cramping and tetany, tremor, and distal limb weakness) was mapped in a Brazilian family to a heterozygous missense mutation in *KCNA1* that resulted in a non-functional channel associated with a dominant negative effect on wild-type Kv1.1 channel function in a human kidney cell line, demonstrating a potential role for *KCNA1* in magnesium homeostasis.

Understanding *KCNA1* function and its derangements may provide insights into the pathophysiology of common disorders such as Alzheimer’s disease (AD). For example, Aβ(1-42) peptide suppressed Kv1.1 currents in dose-dependent and calcium-independent fashion, probably through direct protein-protein interactions. An interaction with the neurofilament protein tau, phosphorylated aggregates of which accumulate in AD brains, has also been reported. In Kcna1 null mice, additional knockout of *Mapt* (which codes for tau) not only decreased seizures and mortality, but also the megacephaly and hippocampal hypertrophy that accompanies *Kcna1* knockout.

These examples, along with the evidence of the critical role of Kv1.1 subunits in regulating other Kv1.x subunits, suggest that studying the molecular aberrations and perturbation of protein function associated with mutations in *KCNA1* genes may lead to new insights into the pathophysiology of diverse disorders, including those other than the classical channelopathies.
1.4.3 Current methods to develop and test pathogenic variants of Kv1.x

Characterization of pathogenic variants of Kv1.1 has previously been performed via the co-expression method\textsuperscript{91}, which involves transfecting both wildtype (wt) and mutant versions of the Kv1.1 single subunit encoding gene contained within the expression plasmid in equimolar amounts. The assumption has been that the different subunits will be equally expressed within the test cell and will co-assemble as they would within a neuron. However, these may be erroneous assumptions as the experimental method relies on subunits randomly associating with each other in identical fashion for all channels interrogated\textsuperscript{92,93}. Hence, there is little control over the stoichiometry or arrangement of mutation subunits in relation to the wild type subunit (FIGURE 3A). This confounds the investigation of pathogenic variants in hetero-tetrameric channels, as the subunits might assemble randomly and in different configurations, and even express on the cell surface (FIGURE 3B).
Figure 3A (Top): Schematic representation of possible combinations of assembly after co-transfection a wild type plasmid and mutant plasmid of Kv1.1
Figure 3B (Bottom): Schematic representation of possible combination of assembly after co-transfection of a Kv1.1 plasmid and mutated Kv1.2 plasmid
In recognition of this limitation, other researchers have attempted to circumnavigate the issue using wild type mutant genes (wt-mut), mutant genes linked to mutant genes (mut-mut) or wild type genes linked to wildtype genes (wt-wt) into a plasmid expression system\textsuperscript{94}. This combination of three “dimer types” could be co-expressed in *Xenopus laevis* in oocytes (a heterologous expression system) where the Kv1.1 channel would be more uniform in stoichiometry and orientation within the tetramer (FIGURE 4). A persistent criticism of this method has been that there is no way for the bench scientist to ensure equal expression of different dimer constructs similar to the co-expression method. Additionally as seen by Figure 3, the mutated subunit can express in two arrangements (cis and trans to each other).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Diagram 1" /></td>
<td><img src="image2" alt="Diagram 2" /></td>
</tr>
<tr>
<td><img src="image3" alt="Diagram 3" /></td>
<td><img src="image4" alt="Diagram 4" /></td>
</tr>
</tbody>
</table>

Figure 4: Schematic representation of dimer constructs and translated assembled *Kcna1* channel protein
To overcome these limitations, concatemer expression vectors for ion channel studies have been developed, offering greater control of stoichiometry and arrangement of subunits in heterotetramers. Traditionally, these expression vectors have been generated via the tandem construct method where a dimer (wt-mut) is generated and then ligated to another dimer (wt-wt or mut-wt)\textsuperscript{95}. This system ensures equal expression between the wild type and mutant containing plasmid constructs and the technology has also been used to generate heterotetramers of Kv1.1 with other Kv1.x subunits (Kv1.2 and Kv1.4) to study the activation/inactivation kinetics of these channels\textsuperscript{96}. Notably, concatemers have offered new insights into how the stoichiometry of mutants in a homotetramer can have additive functional effects\textsuperscript{97}. Similarly, concatemer-generated heterotetramereric Kv1.x channels show intermediate biophysical phenotypes compared their respective homotetramer counterparts\textsuperscript{98}. However, this recombination of dimers does not permit direct investigation of the arrangement, stoichiometry, and combinatorial effects of common and rare de novo genetic variants. Thus, there is a compelling reason to build a concatemer model that provides precise control over the arrangement and stoichiometry of subunits of Kv1.x subunits. In this thesis, we propose to develop and validate a potassium channel pseudotetramer model consisting of four Kcna1 genes linked together, that can be used as a potential tool for studying combinatorial effects of common and rare de novo genetic variants.

1.5 Hypothesis

We hypothesize that a Kcna1 concatemeric construct where four α subunits of kv1.1 are joined by a randomized β globulin linker sequence, will have the same biophysical properties as self-assembled homotetramer generated from wild type Kcna1 plasmid expression system.
1.6 Aims and objectives

1.6.1 Aim 1: *Design and develop a concatenated pseudotetramer kv1.1 plasmid expression vector*

1.6.1.1 Rationale and Objectives

Kv1.1 has been shown to assemble as homotetramers and heterotetramers when expressed in heterologous expression systems yielding K⁺ currents\(^{79,80}\). However studies have shown that homotetramers of Kv1.1 are not found in the brain and human nervous system\(^{99}\), but rather co-assemble primarily with Kv1.2 and Kv1.4 to form functional channels. Thus the development of a concatenated Kv1.1 is not reflective of actual biology. However the reason for the development of a Kv1.1 pseudotetramer is that it provides a unique opportunity to study a Kv1.1 model in isolation of its main partners of Kv1.2 and Kv1.4. Thus the Kv1.1 concatemer could be used for future mutagenesis studies looking to elucidate the specific role of Kv1.1 in different cellular processes.

Furthermore, studies utilizing heterologous expression systems for studying Kv1.1 have shown that alterations in biophysical parameters such as activation threshold, alterations of activation and deactivation kinetics associated with pathogenic EA1 mutations correlate with neurological deficits\(^{100-105}\).

Finally, the Kv1.1 homomeric concatemer would be a proof of concept for the development of future iterations of Kv1.1/Kv1.2 and Kv1.1/Kv1.4 heteromeric concatemers created by switching Kv1.1 subunits in the homomeric concatemer with wild type or mutant Kv1.2 or Kv1.4 subunits.
1.6.2 Aim 2: Biochemical and Biophysical validation of pseudotetramer Kv1.1 against a wild type Kv1.1 channel

1.6.2.1 Rationale and objectives

As mentioned, pseudotetramer potassium channels are not reflective of natural biology. This makes it critical that the Kv1.1 concatemer is validated against a natural self-assembled wild type Kv1.1 when expressed in a heterologous expression system such as the mouse fibroblast. This would be a foundational piece of research in ensuring the construct is valid for scientific study and that it can be used as a base structure upon which future iterations of hetrotetramer concatemers can be reliably built. Essentially, we are providing a functional tool for further study.

The two ways of validating this concatemeric construct involved: (1) biochemical and (2) biophysical methods. For the functional biochemistry, immunoblotting was performed to confirm the expression of Kv1.1 concatemer. For functional biophysical analysis, patch-clamp techniques allowed for the measurement of parameters that included channel opening, channel inactivation and recovery from inactivation. These measures had been chosen as they are the critical processes that define characteristics of a voltage gated channel.
1.7 Significance and impact of this Work

Current expression plasmids are ill-equipped to generate concatemers due to the limited number of unique restriction sites that are present in commercial plasmid expression systems. Specifically, there are several unique restriction sites within the vector plasmid that are rendered unusable because those cut sites are frequently present within the KCNA genes themselves, limiting the number of usable restriction sites for molecular biology, such that dimeric rather than tetrameric expression vectors have been generated. Thus, to make the system work, one must sacrifice some level of control of the stoichiometry and arrangement of subunits being expressed.

A recent advancement, specifically our development of the pICDNA vector designed for easy cloning and mutagenesis of mammalian ion channels, has overcome this limitation, with the construction of a custom special multiple cloning site (MCS) that incorporates both common restriction sites and those that cut only a few select channels internally. This vector was developed by Dr. Alexander Smith and incorporates the sequence analysis of human, mouse, and rat sodium, potassium, calcium, and chloride ion channels (n=428) (personal communication). To generate the vector, the multiple cloning site of pcDNA3.1, a mammalian expression vector, was replaced with a novel MCS containing restriction sites for 35 different enzymes that cut a nominal (<20%) percentage of ion channel genes. This enabled the development of a concatemer system where three linker sequences physically interlink four Kv1.x subunits to each other. These linkers are conveniently flanked with unique restriction sites which, when translated, act as bridging peptide linkers between the subunits, similar to the structure of the 24TM Nav and Cav channels.
Importantly, the concatemer plasmid would be truly modular in nature, as the restriction sites between subunits are unique, such that each domain of this concatenated pseudotetramer can be interchanged and modified with subsequent sequence validation. This would provide unprecedented control over the precise arrangement and stoichiometry of compound pathogenic variants in homotetramers or heterotetramers and explorations of allelic dosage as a phenotype modifier.

Finally, development of this concatemer model will provide insight into rare de novo mutations found in EA1. Electrophysiological studies from a heterologous expression system have already shown that for some EA1 mutations, the extent of changes in certain macroscopic Kv1 currents correlate with the severity of the neurological phenotypes\textsuperscript{106–108}. This implies a link between alterations of the specific parameters of Kv1 currents and neurological symptoms and therefore there is utility in developing and studying Kv1.1 homotetramers in the context of EA1. Ultimately, this tool would allow for the characterization and understanding of complex Kv1.x channelopathies that have not yet been studied due to technological limitations.
CHAPTER 2: METHODS AND MATERIALS

2.1 Materials

All chemicals were purchased from VWR (Edmonton, AB, Canada) unless stated otherwise.

The pICDNA-Kcna1 (pICDNA-A1) upon which the concatemer was built was generated by Dr Alex Smith (Figure 5 A&B).

Figure 5A. Shows the multiple cloning site of pICDNA plasmid vector that was developed in house. Figure 5B. Visualization of pICDNA-A1 construct, developed from cloning a Kcna1 amplicon into pICDNA plasmid vector.
2.2 Linker design and production

The three linker sequences developed are a modified version of the 5’-untranslated region of *Xenopus* β-globulin gene used by Akhtar et al.\textsuperscript{109}. The changes were made to introduce vector-specific flanking restriction sites, as well as randomizing the amino acid sequences to create unique sequences to help differentiate between the different linkers for subsequent downstream Sanger sequencing (Figure 6). The sequences for the three linkers (linker 1, linker 2 and linker 3) were as follows:

5’-ACTGCTAGCGATACGAAGGAGTTCCGAAACCTCAGCGATCAACCGAAGACTTAAGTGCTC-3’

5’-TGGGTACCGATACGAAGGAGACGCTCACTTCCGACGATCAACCAGCGATACGCCAGT-3’

5’-TCAGGATCCGATACGAAGGAGCGAGGAAACCTCTTCCGTCACGCAACCAGCGGCGCAGT-3’

The sequence for each of the linkers and their reverse complement was custom-synthesized (Integrated DNA Technologies, Coralville, IA, United States).

![Figure 6](image.png)

Figure 6. Visualization of linker amino acid sequence to be inserted into concatemer model. The unique sequence is indicated in red.
The single stranded sequences for the linker and their respective reverse complements were made up to concentration of 50 µm in double distilled water (ddH2O), then 18 µl of the linker and 18 ul of the reverse complement was mixed together with 4 µl of Cutsmart buffer (50 mM Potassium Acetate, 20 mM Tris-Acetate, 10 mM Magnesium-Acetate, 100 µg/ml BSA, pH 7.9 at 25°C, New England Biolabs). The samples were vortexed for 15 seconds and centrifuged at x800 g for 30 seconds. The mixed samples were then added to heated 95 °C heatblock for 15 minutes, following which the samples were allowed to cool slowly to room temperature.
2.2 Digestion and purification of linkers and pICDNA vector

Linker 1, 2 and 3 (2 µg of each) were double digested at 17 °C overnight using 1 µl of restriction enzymes: AflIII and NheI-HF, KpnI-HF and AsiSI & BamHI-HF and Fsel respectively (New England Biolabs, Ipswich, MA, United States) in 30 µl total volume.

After overnight digestion, the linkers were purified using a spin column (E.Z.N.A Cycle pure PCR clean up kit, Omega biotek, Norcross, GA, United States). The CP buffer (150 µl) supplied with the kit was mixed with 12 µl of 100% isopropanol and then added to each of the digested products. The mixture was vortexed for 15 seconds and centrifuged to help retain the linkers with the column. The columns were spun down with wash buffer provided in the kit twice at 21000 xg for 1 minute. The wash buffer was discarded and the column was dry spun for 2 minutes at 21000 xg to allow for the removal of any residual ethanol on the columns. The linkers were eluted in the 10 mM Tris HCl pH 8.5 in 20 µl volume. The linkers were stored at 4 °C until they were ready to be used.

The pICDNA vector is an inhouse plasmid with a pcDNA3.1 backbone and custom built multiple cloning site (MCS). Initially 2 µg of pICDNA vector was double digested with the corresponding restriction enzymes at 17 °C overnight along with 1 µl of shrimp alkaline phosphatase (rSAP, New England Biolabs) in 30 µl total volume. The digested pICDNA vector was purified using a spin column, similar to the linkers described above. Since the vector is >200 bp, no isopropanol was added.

Quantification of purified digested pICDNA vector and digested linkers was performed using Nanodrop 2000 (Thermofisher Scientific, Waltham, MA, United States) to estimate concentrations after a purification step. The digested pICDNA vector and linkers were stored at 4 °C until they were ready to be used.
2.3 Ligation of linker into pICDNA vector and subsequent transformation

Digested pICDNA vector (20 ng) was mixed with linker in molar insert:vector ratios of 1:10 and 1:100 with 1 µl of T4 DNA Ligase Reaction Buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DDT, pH 7.5 at 25°C, New England Biolabs), 1 µl of 10 mM ATP (Cell Signaling Technologies) and 1 µl of T4 DNA Ligase. The mixture was vortexed for 15 seconds and centrifuged at x800g for 30 seconds and incubated overnight at 17 °C.

For transformation, 200 µl of 10Gf’ E.coli competent cells (Lucigen, Middleton, WI, United States) were incubated with 1 µl of ligation product for 20 minutes on ice. The competent cells were heat shocked at 42°C for 45 seconds and then allowed to rest for 2 minutes. The competent cells were then incubated with 800 µl of Super Optimal Broth with Catabolite repression (2% w/v tryptone, 0.5% w/v Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 10 mM MgSO₄, 20 mM Glucose; SOC) in a shaking incubator for 60 minutes at 37 °C to allow for recovery. After recovery, the media with the cells were spun down at 3200 xg for 5 minutes. The media was discarded and the spun down cells were plated in Luria Broth Agar (10 g tryptone, 5 g yeast, 10 g NaCl, 15 g Agar in 1000 ml ddH₂O; LBA). The plates were incubated overnight at 37 °C and the transformed colonies were counted the next day. The plates with transformed colonies were stored at 4 °C until ready for colony PCR.
2.4 Polymerase chain reaction (PCR) primer design

The primers were designed with OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html).

The linker-specific forward and reverse primers were designed from the randomized sequence within each linker. The Kcna1 gene sequence was imported into Geneious 7.0 software (New Zealand Pharmaceuticals, New Zealand) from National Centre for Biotechnology Information (NCBI, Bethesda, MD, United States). The primers were designed using the 5’ and 3’ flanking regions of the gene along with the relevant restriction sites (shown in underline in table). The stop codon was appropriately removed from the Kcna1 gene amplicons.
Table 1. All primers and expected product sizes for all polymerase chain reactions performed. Pre Sanger checks were used to very insertion of linkers/mKCNA amplicons when developing the concatemer.

<table>
<thead>
<tr>
<th>Gene Description</th>
<th>Primer Sequence , Sense and Anti-Sense (5’ – 3’)</th>
<th>Predicted Product Size (bp)</th>
<th>Predicted Annealing Temp. (°C)</th>
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</thead>
<tbody>
<tr>
<td>Linker 3 Pre-Sanger Check Colony PCR</td>
<td>AGCGAGGAAACCTCTTCACG</td>
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<td>55</td>
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<tr>
<td></td>
<td>CCTGCTCAGCTATCTCCGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linker 2 Pre-Sanger check Colony PCR</td>
<td>AGACGCTCAACTTGGGACG</td>
<td>920</td>
<td>55</td>
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<tr>
<td></td>
<td>CCTGCTCAGCTATCTCCGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linker 1 Pre-Sanger check Colony PCR</td>
<td>GAGTTCCGAAACCTCACGG</td>
<td>976</td>
<td>55</td>
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<tr>
<td></td>
<td>CCTGCTCAGCTATCTCCGTG</td>
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<td></td>
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<tr>
<td><em>Kcna1</em> (3rd Domain) amplicon generation</td>
<td>AACCAAGCGATCAGATGACGGTGATGTCGGGCATCGGGATTTACACAGGCAGGTCAGGAGCTTG</td>
<td>1503</td>
<td>72</td>
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<tr>
<td><em>Kcna1</em> (2nd Domain) amplicon generation</td>
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<td>TATCGGTACCAACATCGCGTCAGGAGGAGCTTG</td>
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<tr>
<td><em>Kcna1</em> (1st Domain) amplicon generation</td>
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<td>CGTAAAGAGGTTTTCCTCCT</td>
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<tr>
<td><em>Kcna1</em> (2nd Domain) Pre-Sanger Check Colony PCR</td>
<td>GAGTTCCGAAACCTCACGG</td>
<td>1556</td>
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<td>CGTCCAAGGTTGAGCGTCT</td>
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<td></td>
<td>GAGTTCCGAAACCTCACGG</td>
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</table>
2.5 Colony polymerase chain reaction

Colony PCR was performed to screen and check for positive hits before sending for Sanger Sequencing. For Colony PCR, random transformed cells were picked and swirled in 20 µl of ddH$_2$O. 1 µl of this water was plated onto LBA and given a number tag for identification. All the colonies streaked on the plate were then incubated overnight at 37 °C for subsequent DNA extraction steps. The rest of the 19 µl was boiled at 100 °C for 10 minutes to help break open the cells and release the plasmid. 5 µl of the plasmid was mixed with 2µl of commercial 10x reaction buffer (100 mM Tris-HCl pH9.0, 500 mM KCl, 1% Triton X-100; Lucigen), 1.5 mM MgCl$_2$, 0.2 mM deoxyribose nucleoside triphosphate (dNTPs), 0.2 µM relevant forward/reverse primers, 1 Unit of EconoTaq (Lucigen) making up a final volume of 20 µl. The PCR conditions for colony PCR is shown

Table 2: PCR conditions for colony PCR to screen insertion of linkers into pICDNA vector

<table>
<thead>
<tr>
<th>Cycle conditions</th>
<th>Temperature – Time</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>95C – 3'</td>
<td></td>
<td>1x</td>
</tr>
<tr>
<td>95C – 20''</td>
<td></td>
<td>33x</td>
</tr>
<tr>
<td>57C – 20''</td>
<td></td>
<td>33x</td>
</tr>
<tr>
<td>72C – 1'</td>
<td></td>
<td>33x</td>
</tr>
<tr>
<td>72C – 10'</td>
<td></td>
<td>1x</td>
</tr>
<tr>
<td>10C – Hold</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: PCR conditions for colony PCR to screen insertion of Kcna1 amplicons into pICDNA vector

<table>
<thead>
<tr>
<th>Cycle conditions</th>
<th>Temperature – Time</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95C – 3’</td>
<td>1x</td>
</tr>
<tr>
<td></td>
<td>95C – 20”</td>
<td>33x</td>
</tr>
<tr>
<td></td>
<td>57C – 1:30”</td>
<td>33x</td>
</tr>
<tr>
<td></td>
<td>72C – 1’</td>
<td>33x</td>
</tr>
<tr>
<td></td>
<td>72C – 10’</td>
<td>1x</td>
</tr>
<tr>
<td></td>
<td>10C – Hold</td>
<td></td>
</tr>
</tbody>
</table>

2.6 Visualization of PCR products

The buffer for gel electrophoresis is Lithium Acetate Buffer (5mM LiOAc, 1mM EDTA-LiOH pH8.0).

The 80 ml of buffer was mixed 0.8% w/v Agarose RA (Amresco, Dallas, TX, United States) and brought to a boiling temperature. Ethidium bromide (50 µg) was then added to the mixture (Amresco), mixed briefly and then poured into 14 cm x 8 cm tray to cool with 1.5 mm 27 well combs or 1 mm 10 well comb depending on the number of samples. Upon cooling, the LiOAc agarose gel is placed in gel electrophoresis containing LiOAc Buffer

For colony pcr products, 6 µl of pcr product was mixed 4 µl of ddH₂O with 2 µl of 6x loading buffer (10 mM Tris-HCl pH 7.4, 60 mM EDTA pH 8.5, 60% glycerol, 0.03% w/v bromophenol blue, 0.03% w/v xylene cyanol) to make a total volume of 12 µl. The 12 µl was then loaded onto the wells and 4 µl of 2 Log DNA Ladder (New England Biolabs) were added to either side help identify the size of the PCR product. The running gel conditions were 150 V for 30 minutes, following which the bands were visualized using UV dual transilluminator (VWR) to identify positive hits.
2.7 DNA extraction of positive clones from Colony PCR and Sanger sequencing

The positive clones were picked and transferred to 5 ml of Luria Broth and incubated at 37°C overnight with ampicillin (200 µg/ml). The cells were then centrifuged at 3200 xg for minutes and media was removed. DNA plasmid extraction was performed using a spin column (E.Z.N.A. Plasmid Mini Kit I V-Spin, Omega Biotek), following the recommended kit protocol. The final elution was done in 10mM Tris Hcl pH 8.5 to a final volume of 50 µl.

Positive hits were subsequently sent for Sanger sequencing to the DNA sequencing facility, NAPS unit, University of British Columbia. Sequencing was performed using BigDye Terminator v3.1 Sequencing Chemistry and an Applied Biosystems 3703S 48-capillary DNA Analyzer with 50 cm array. Sequencing results were imported into Biogeneious 7.0 to view chromatograms, and confirm verification of insertion of the linker or Kcna1 amplicon.

2.7 Polymerase chain reaction of Kcna1 amplicons

Another set of PCR reactions was performed to generate a Kcna1 amplicon that would have corresponding flanking restriction for cloning. 50 ng of pICDNA-A1 was added to 50 µl PCR reaction mix that contained 10 ul of 5x Q5 reaction buffer, 0.2 mM dNTP, 0.5 µM relevant oligonucleotide primer (sense and antisense), 0.04 unit of Q5 High Fidelity DNA Polymerase and ddH2O was used to make up to final volume.
The PCR was carried out using Mastercycler Nexus (Eppendorf, Hamburg, Germany). The condition for PCR are as follows:

Table 4: PCR conditions for generating flanking restriction sites to Kcna1 amplicon

<table>
<thead>
<tr>
<th>Cycle conditions:</th>
<th>Temperature - Time</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>98°C – 30”</td>
<td></td>
<td>1x</td>
</tr>
<tr>
<td>98°C – 10”</td>
<td></td>
<td>31x</td>
</tr>
<tr>
<td>72°C – 15”</td>
<td>31x</td>
<td></td>
</tr>
<tr>
<td>72°C – 40”</td>
<td>31x</td>
<td></td>
</tr>
<tr>
<td>72°C – 10’</td>
<td></td>
<td>1x</td>
</tr>
<tr>
<td>10°C – Hold</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.8 Digestion and purification of Kcna1 amplicons to build mKCNA1 concatemer

2 µg Kcna1 amplicons with flanking restriction sites BamHI-HF/AsiSI, KpnI-HF/AfIII and HindIII-HF/Nhel-HF were double digested at 17 °C overnight. At this point, all the linkers had been iteratively cloned into pICDNA-A1 to form pICDNA-linker1-linker2-linker3-Kcna1 (pICDNA-L1-L2-L3-A1). The pICDNA-L1-L2-L3-A1 (2 µg) was also double digested with the relevant restriction sites at 17 °C with rSAP overnight. Digested products were purified similar to digestion purification methods described above. The digested Kcna1 amplicons were stored 4°C until they were ready to be used.
2.9 Ligation and transformation of Kcna1 amplicons to generate Kcna1 concatemer

Digested (20 ng) pICDNA-L1-L2-L3-A1 was mixed with digested Kcna1 amplicons in molar insert:vector ratios of 3:1 and 10:1 ratio with 1 µl of T4 DNA Ligase Reaction Buffer (50 mM Tris-Hcl, 10 mM MgCl₂, 1 mM ATP, 10 mM DDT, pH 7.5 @ 25°C, New England Biolabs), 1 µl of 10 mM ATP (Cell Signaling Technologies, Danvers, MA, United States) and 1 µl of T4 DNA Ligase. The mixture was vortexed for 15 seconds and centrifuged at 800 xg for 30 seconds and incubated overnight at 17 °C.

For transformations, in addition to 10Gf’ E.Coli, Stbl2 E.Coli competent cells were also used. Stbl2 competent cells are temperature sensitive; therefore, incubation temperature for recovery and plates overnight was set to 30 °C for these cells. All other steps were the same as for the transformation of 10 Gf’ competent cells described above. Similarly, subsequent steps of colony PCR, gel electrophoresis, DNA visualization, DNA quantification and sequencing were as described above.

2.10 Transformation of mouse fibroblast cell lines

These transformations were performed by Victoria Baronas in the laboratory of Dr. Harley Kurata at the University of Alberta. Transformations proceeded as follows:

The day before transfection, mouse fibroblast (LM) cells were plated in 35mm well in 2ml of DMEM without antibiotics such that it reaches 50-70% confluency with a cell count of 1-2 x 10⁵ cells on the day of transfection.
Mouse fibroblast cells were maintained in culture in a 5% CO$_2$ incubator at 37°C in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were transfected with channel cDNAs using Polyplus Jetprime (Polyplus transfection, NY, United States) and, 12 h later, split onto sterile glass coverslips. Cells were then cotransfected with fluorescent proteins to allow identification of cells for recording by epifluorescence.

2.11 Electrophysiology for active voltage gated channels

The electrophysiology was performed by Victoria Baronas in the laboratory of Dr. Harley Kurata at the University of Alberta. The method is as follows:

Recordings were done 1–2 d after transfection. Patch pipettes were manufactured from borosilicate capillary glass (World Precision Instruments) using a Sutter Instruments P-97 puller. When filled with standard recording solutions, pipettes had a tip resistance of 1-3 MΩ. Recordings were filtered at 5 kHz, sampled at 10 kHz, with manual capacitance compensation and series resistance compensation between 70 and 90%, and stored directly on a computer hard drive using Clampex software (Molecular Devices, Sunnyvale, CA, USA).

Bath solutions had the following composition (in mM): 135 NaCl, 5 KCl, 1 CaCl$_2$, 1 MgCl$_2$, and 10 HEPES, adjusted to pH 7.4 with NaOH. Pipette solution had the following composition (in mM): 135 KCl, 5 K-EGTA, and 10 HEPES, adjusted to pH 7.2 using KOH. Chemicals were purchased from Sigma-Aldrich (Burlington, ON, Canada) or ThermoFisher Scientific (Waltham, MA, United States). All data are shown as mean ± SD.
2.12 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Cell lysates from transfected LM cells were harvested in NP40 buffer 3 days after transfection. BCA protein assay was performed to estimate total protein concentration of cell lysates.

SDS-PAGE and western blot were used to confirm transformation of the mouse fibroblast cell line, including negative controls of untransformed cells, and cells transformed with empty vector and a positive control human heart atrium sample. These tissues were collected with consent for future experimentation from non-surviving organ donors by the University of Hawaii Biorepository, and supplied in de-identified fashion. This use of these tissues was approved by the University of British Columbia Clinical Research Ethics Board (H14-00092). No clinical information is available for this human tissue sample, except that the donor was a 42 year old Caucasian male.

Ten percent acrylamide resolving gel was made up to total volume of 10.05 ml (0.375 M Tris pH 8.8, 27% Acrylamide, 29:1 Bis Acrylamide, 10% SDS, 65 µl ammonium persulphate, 6.5µl Tetramethylethylenediamine). The gel was poured and allowed to set in gel apparatus (BioRad) for 40 minutes. 2-Proponol was pipetted on top of the resolving gel to reduce exposure of air to resolving gel. Once the gel has been set, 2-proponol was discarded and the top of the gel was rinsed with ddH₂O.
A 4% acrylamide stacking gel was made up to total volume of 10.05 ml (0.375 M Tris pH 6.8, 27% Acrylamide, 29:1 w/v Bis Acrylamide, 10% SDS, 50 µl ammonium persulphate, 10 µl TEMED). The stacking gel was poured on top of resolving gel, following which a 0.75 mm 10 well comb was placed and the gel was allowed to set for 40 minutes. For both gels, ammonium persulphate and TEMED were added last.

The Kcna1 wildtype mouse fibroblast lysates, pICDNA mouse fibroblast lysates, Kcna1 concatemer mouse fibroblast lysates, non transfection mouse fibroblast lysates were resolved at a concentration of 20 µg each along with 2 µg of Kcna1 antigen (positive control). Samples were mixed with 5x loading buffer to make up a total volume of 15 µl (0.5 M Tris pH 6.8, 20% w/v glycerol, 10% SDS 10 mM beta mercapto-ethanol, 0.05% w/v bromophenol blue) and heated at 95 °C for 5 minutes. The samples were then transferred to the wells on the stacking gel and 12 ul of molecular weight marker (Rainbow Marker) was added directly to one well to help identify molecular weights of the proteins. The gel was run at 200V for 1 hour.

After gel electrophoresis, the stacking gel was removed and the gel was incubated and agitated in transfer buffer (25 mM Tris, 190 mM glycine, 20% v/v methanol, 0.0375% SDS) at room temperature for 30 minutes. An appropriate sized polyvinylidene fluoride (PVDF) membrane was preincubated in methanol for 5 seconds and then gently agitated in ddH₂O for 5 minutes, following which the membrane was agitated in transfer buffer for 30 minutes.

A sandwich was assembled using blotting paper, filter paper, gel, PVDF membrane, filter paper and blotting paper. The blotting paper and filter paper were prewet in Tris buffered saline and Tween 20 (20mM Tris, 150mM NaCl, 0.001 v/v tween-20, TBS-T). The layers were put carefully on top of each
other and air bubbles were carefully removed. Protein transfer from the gel to PVDF membrane was performed in a semi dry transfer Transblot Apparatus. The transfer was carried out at (12 V) for 1 hour.

After the transfer was complete, the layers were removed carefully. The gel was stained with Coomassie blue (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) for 20 min. The gel was destained using Coomassie destain (40% methanol, 10% glacial acetic acid). The solution was replenished several times until background of gel was destained. The gel was used to confirm that protein loading was even. The membrane was washed 3x 5 minutes with TBS-T, following which the membrane was incubated and gently agitated with rabbit anti-kv1.1 antibody (Alomone Laboratories, Jerusalem, Israel) diluted 1:1000 in 5 ml of TBS-T and 10% skim milk overnight. The membrane is then washed 3x 6 minutes with TBS-T and then incubated with donkey anti-rabbit horseradish peroxidase (HRP) secondary antibody which was diluted 1:5000 in 5 ml of TBS-T, 5% skim milk and 2% normal donkey serum. After gentle agitation for an hour, the membrane was washed 3x 6 minutes with TBS-T.

Next 2 ml of Amersham enhanced chemiluminescence (ECL) prime western blotting reagent (GE healthcare, Chicago, IL United States) was added to the PVDF membrane and incubated for a minute. The excess ECL was then removed with the membrane being placed in Cassette and exposed to an X-ray film for 75 – 90 minutes. Following exposure, the X-ray film was developed using Medical Film processor SRX-101A for 5 minutes. Bands were identified by molecular weight comparison as compared to positive control tissues (human heart atrium, as described above).
<table>
<thead>
<tr>
<th>Step 0</th>
<th>Vector before Kcna1 gene insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>The vector initially already had one Kcna1 gene incorporated. Therefore, even before the Kcna1 gene was inserted individually, the three 60 bp linkers had to digested, ligated and transformed into the vector. Sanger sequencing was performed to confirm successful insertion of linker into pICDNA vector.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Generation of 3rd domain of Kcna1 pseudotetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward primer (contains AsiSI): 5'-AACCAAGGATCGCATGAGGGTGATGTCG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer (contains BamHI): 5'-ATCGGATCC'AACATCGGTACGGACGTTGC-3'</td>
</tr>
<tr>
<td></td>
<td>The primers were designed to ensure that unique flanking restriction sites were added to the Kcna1 gene. The start codon for the gene is shown in red and the * represents that the stop codon was removed the end of the gene.</td>
</tr>
<tr>
<td></td>
<td>A touchdown PCR protocol was used to generate a single PCR fragment that contained the restriction sites and gene with stop codon removed</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 2</th>
<th>Digestion, Ligation &amp; Transformation of 3rd domain of Kcna1 pseudotetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>The PRC fragment and the vector were first subjected to overnight digestion to ensure complete digestion.</td>
</tr>
<tr>
<td></td>
<td>Next, the PCR fragment insert was ligated into the vector at 3 different molar ratios if 1:1, 3:1, 7:1. Since this not been done before, the ligation protocol had to be optimized.</td>
</tr>
<tr>
<td></td>
<td>Finally, the ligated products were transformed into 10Gf' chemically competent cells. These cells now generated a vector with linkers and 3rd domain incorporated.</td>
</tr>
</tbody>
</table>

| Step 3 | |
|--------| |
To screen which transformants contained successful inserts, a colony PCR was performed where primers from a unique site on the linkers were used to confirm the insertion of PCR fragment insert (shown as one-sided arrow).

The same primers were employed for sequencing to verify that the gene was inserted successfully, and that there were no PCR induced mutations.

**Step 4**

**Generation of 2nd domain of Kcna1 pseudotetramer**

Forward primer (contains AfIII):
5' ACCGAACCTTAAGATGACGGTGATGTCGGGG-3'

Reverse primer (contains KpnI):
5'-TATCGGTACC’AACATCGGTCAGGAGCTTTGC-3'

Done the same way as step 1.

**Step 5**

**Digestion, Ligation & Transformation of 2nd domain of Kcna1 pseudotetramer**

Done the same way as step 2

**Step 6**

**Sanger sequencing of 2nd domain of Kcna1 pseudotetramer**

Done the same way as step 3
### Table 5: Step by step development of Kv1.1 homotetramer

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 7</strong></td>
<td>Forward primer (contains HindIII): 5’-CCC[AAGCTT]ATGACGGTGATGTCGGGG-3’&lt;br&gt;Reverse primer (contains NheI): 5’-ATCGCTAGC’AACATCGGTCAGGAGCTTGC-3’&lt;br&gt;Done the same way as step 1</td>
</tr>
<tr>
<td><strong>Step 8</strong></td>
<td>Done the same way as step 2&lt;br&gt;Digestion, Ligation &amp; Transformation of 1st domain of Kcna1 pseudotetramer</td>
</tr>
<tr>
<td><strong>Step 9</strong></td>
<td>Done the same way as step 3&lt;br&gt;Sanger sequencing of 1st domain of Kcna1 pseudotetramer</td>
</tr>
</tbody>
</table>
CHAPTER 3: RESULTS

3.1.1 Cloning of Linker 3 into pICDNA Kcna1 vector

In Figure 7, a colony PCR using a primer pair that included a linker 3 specific primer showed three positive hits. The expected band size is ~900, and the intensity of the three bands are different, indicating differing level of plasmid replication in 10Gf’ competent cells. The bright band of ~500 bp in Lane 8 was used initially as a positive control to confirm PCR conditions were optimized for detection of DNA from colony PCR. The positive control reaction was using a wild type Kcna1 plasmid with primer pairs that had similar predicted annealing temperatures to the primer pairs used in the colony PCR of the samples. The two brightest bands were validated with Sanger sequencing to generate pICDNA Kcna1: Linker 3- Kcna1 (pICDNA Kcna1: L3-A1)

![Image of gel photo of colony PCR showing insertion of linker 3 into vector. Lanes are marked from left to right. Molecular weight has been denoted in base pairs (bp).](image-url)
3.1.2 Cloning of Linker 2 into pICDNA Kcna1: L3-A1

A colony PCR using a primer pair that included linker 2 specific primer showed sixteen positive hits that incorporated linker 2 (Figure 8). The expected band is 900 bp and there are different band intensities indicating different levels plasmid expression in 10Gf’ competent cells. Only two of the brightest bands were sent for Sanger validation to ensure successful insertion of linker 2 into pICDNA Kcna1: L3-A1 to generate pICDNA Kcna1 : Linker 2 – Linker 3 – Kcna1 (pICDNA Kcna1 : L2-L3-A1).

Figure 8. Gel photo of colony PCR showing insertion of linker 2 into vector. Lanes are marked from left to right. Molecular weight has been denoted in base pairs (bp).
3.1.2 Cloning of Linker 1 into pICDNA Kcna1: L2-L3-A1

A colony PCR using primer pair that included linker 1 specific primer showed three positive hits that incorporated linker 1 into pICDNA Kcna1: L2-L3-A1 (Figure 9). The expected band size is ~900 and the intensities of bands indicate different levels of expression of plasmid in 10Gf’ competent cells. The bright band at ~500 bp is the positive control (using the wild type Kcna1 plasmid and primer pairs that have similar annealing temperature). The two brightest bands were validated using Sanger sequencing to generate pICDNA Kcna1: Linker 1 – Linker 2 – Linker 3 – Kcna1 (pICDNA Kcna1: L1-L2-L3-A1).

Figure 9. Gel photo of colony PCR showing insertion of linker 1 into vector. Lanes are marked from left to right. Molecular weight has been denoted in base pairs (bp).
3.1.3 PCR of *Kcna1* amplicons with unique restriction sites

In Figure 10, three *Kcna1* amplicons with different unique flanking restriction sites were generated using PCR. The results show successful generation of all three *Kcna1* amplicons with flanking ends of HindIII/Nhel, KpnI/AflIII and BamHI/AsiSI respective at ~1500 bp. There were some faint non-specific bands for the *Kcna1* amplicon with HindIII/Nhel; however they did not seem to affect downstream cloning.

![Figure 10. Gel Photo of PCR amplification of *Kcna1* amplicons with restriction sites. Lane 1 *Kcna1* (HindIII/Nhel), Lane 2 (KpnI/AflIII) and Lane 3 (BamHI/AsiSI) were loaded left to right. Molecular weight has been denoted in base pairs (bp).](image-url)
3.1.4 Cloning of *Kcna1* amplicon with BamHI/AsiSI restriction into pICDNA *Kcna1*: L1-L2-L3-A1

A colony PCR showed five positive hits, demonstrating successful incorporation of the *Kcna1* amplicon with BamHI/AsiSI into pICDNA *Kcna1*: L1-L2-L3-A1 to generate pICDNA *Kcna1*: Linker 1 – Linker 2 – *Kcna1* – Linker 3 – *Kcna1* (pICDNA *Kcna1*: L1-L2-A1-L3-A1) as seen by Figure 11. The expected band size is around ~1500 bp, and the positive hits had relatively similar band intensity. Two of the five positive hit bands were sent for Sanger sequencing to confirm the presence of mKCNA amplicon. These results showed there were no PCR-induced mutations from the generation of *Kcna1* amplicon with BamHI/AsiSI.

![Figure 11. Gel photo of colony PCR showing insertion of *Kcna1* amplicon (BamHI/AsiSI) into vector. Lanes are marked from left to right. Molecular weight has been denoted in base pairs (bp).](image)
3.1.5 Cloning \textit{Kcna1} amplicon with KpnI/AflII restriction sites into pICDNA \textit{Kcna1}: L1-L2-A1-L3-A1

A colony PCR showed four positive hits that successfully incorporated \textit{Kcna1} amplicon with KpnI/AflII into pICDNA \textit{Kcna1}: L1-L2-A1-L3-A1 to generate pICDNA \textit{Kcna1}: Linker 1 – \textit{Kcna1} – Linker 2 – \textit{Kcna1} – Linker 3 – \textit{Kcna1} (pICDNA \textit{Kcna1} : L1-A1-L2-A1-L3-A1) as seen by Figure 12. The expected band size is around \textasciitilde1500 bp and the positive hits had relatively similar band intensity. Two of the four positive hit bands were sent for Sanger sequencing to confirm the presence of \textit{Kcna1} amplicon. The Sanger sequencing results showed there were no PCR-induced mutations from the generation of \textit{Kcna1} amplicon with KpnI/AflII.

![Figure 12. Gel photo of colony PCR showing insertion of \textit{Kcna1} amplicon (KpnI/AflII) into vector. Lanes are marked from left to right. Molecular weight has been denoted in base pairs (bp).]

The colony PCR showed eleven positive hits that successfully incorporated Kcna1 amplicon with HindIII/NheI restriction sites into pICDNA Kcna1: L1-A1-L2-A1-L3-A1 to generate the full concatemer pICDNA Kcna1: Kcna1 - Linker 1 – Kcna1 – Linker 2 – Kcna1 – Linker 3 – Kcna1 (pICDNA Kcna1 : A1-L1-A1-L2-A1-L3-A1) as seen by Figure 13. The expected band size is ~1500 bp, however, high expression of plasmid from 10 Gf’ resulted in the bright broad bands seen in the gel. This makes estimation of band size more difficult. However, Sanger sequencing helped confirm and validate that Kcna1 amplicon with HindIII/NheI was successfully incorporated into the concatenated vector with no PCR induced mutations from the generation process.

Figure 13. Gel photo of colony PCR showing insertion of Kcna1 amplicon (HindIII/AflIII) into vector. Lanes are marked from left to right. Molecular weight has been denoted in base pairs (bp).
3.1.7 Confirmation of concatenated genes using restriction digests

A restriction digest (shown in Figure 14) was performed on the \textit{Kcna1} concatemer at different cut sites to get generate linear DNA of four, three, two and one concatenated genes of \textit{Kcna1}, respectively. The four concatenated genes of \textit{Kcna1} are approximately 6000 bp in length, three concatenated genes of \textit{Kcna1} are approximately 4500 bp, two concatenated genes of \textit{Kcna1} are 3000 bp and a single gene of \textit{Kcna1} measures approximately 1500 bp. The Figure 14 shows in lane 2, the presence of band size around 6000 bp; in lane 3, the presence of band size around 4500 bp; in lane 4 and 5, the presence of band around 3000 bp and lanes 6-9 showing the presence of band around 1500 bp, thereby confirming the presence of a fully intact concatemer.

![Figure 14. Gel photo of restriction digest of a full Kcna1 concatemer](image_url)

Figure 14. Gel photo of restriction digest of a full \textit{Kcna1} concatemer. lane 1 \textit{Kcna1} concatemer was cut using the restriction enzyme pair HindIII/Ascl; lane 2 \textit{Kcna1} concatemer was cut using the restriction enzyme pair HindIII/KpnI; lane 3 \textit{Kcna1} concatemer was cut using the restriction enzyme pair HindIII/KpnI; lane 4 \textit{Kcna1} concatemer was cut using the restriction enzyme pair AsISI/AsClI; lane 5 \textit{Kcna1} concatemer was cut using the restriction enzyme pair HindIII/Nhel; lane 6 \textit{Kcna1} concatemer was cut using the restriction enzyme pair KpnI/AfIII; lane 7 \textit{Kcna1} concatemer was cut using the restriction enzyme pair AsISI/BamHI and lane 8 \textit{Kcna1} concatemer was cut using the restriction enzyme pair Fsel/Ascl. Molecular weight has been denoted in base pairs (bp).
3.2 Validation of Kv1.1 antibody using human atrium samples

The results show expression of Kv1.1 subunit at around 52kDa using immunoblotting. The coomassie blue stain on the gel showed even protein loading. Too much ECL had been left on the PVDF membrane when it was being exposed to the X ray film. This accounts for the smearing that is seen at Figure A at the top of lane 1. Both lanes are replicates of the same human atrium sample, and this set of experiments was performed to optimize conditions for validation of expression of KV1.1 in mouse fibroblasts. There was an unidentified band around ~75 KDa that appears as well, which is consistent with the manufacturer’s product sheet which showed two bands at 50 kDa and 75 kDa respectively. The results confirm similar results that had been found by Glassock et al. where they detected Kv1.1 in human atrium^{110}.

Figure 15A. X Ray film showing expression of Kv1.1 using immunoblotting in human atrium samples. + is human atrium sample used a positive control.

Figure 15B. Coomassie Blue gel stain of corresponding blot. Molecular weight is shown in Kilo Daltons (KDa).
3.3 Expression of Kv1.1 in mouse fibroblasts

The results show expression of a protein around 50kDa which indicates Kv1.1 subunit generated from wild type \textit{Kcna1} and concatemer \textit{Kcna1}. There is an unidentified band at \(\sim 75\text{kDa}\), which is consistent with the band seen for the human atrium samples. There is also other nonspecific binding at molecular weight of less than 36KDa across all the samples. The comassie blue stain on the gel showed roughly even protein loading.

Figure 1A. X Ray film showing expression of Kv1.1 using immunoblotting. \(-\) is negative control, \(+\) is human atrium sample used a positive control, \textit{wt} is wild type Kv1.1, \textit{con} is Kv1.1 concatemer. Molecular weight is shown in Kilo Daltons (KDa).

Figure 1B. X Ray film showing expression of Kv1.1 using immunoblotting. \(+\) is human atrium sample used a positive control, \textit{wt} is wild type Kv1.1, \textit{con} is Kv1.1 concatemer. Molecular weight is shown in Kilo Daltons (KDa).
Figure 17A&B. Coomassie Blue gel stain of corresponding immunoblots seen in Figure 14A and 14B. – is negative control, + is human atrium sample used a positive control, wt is wild type Kv1.1, con is Kv1.1 concatemer.
3.4 Steady state activation curves of wild type Kv1.1 and Kv1.1 concatemer.

The voltage dependence of Kcna1 activation was examined by plotting fractional conductance as function of test depolarization. The fractional conductance is a measure of percentage of Kcna1 channels that can be found in the open state. The figure shows very similar steady state activation curves of both Kcna1 concatemer and wild type Kcna1. At -60mV, the channel is closed for both, and by 0mV, the channel is fully open for both. The V_{1/2} of wild type Kv1.1 and Kv1.1 concatemer was -32.9 mV (+ 2.7), and -32.6 (+ 4) mV, respectively.

![Graphs indicating the open probability of wild type Kv1.1 and Kv1.1 concatemer at specific membrane potential during activation.](image)

Figure 18. (Top) Graph indicating the open probability of wild type Kv1.1 at specific membrane potential during activation. (Bottom) Graph indicating the open probability of Kv1.1 concatemer at specific potential during activation. Errors are shown in SD. These images are kindly performed and provided by Victoria Baronas, a trainee in Associate Professor Harley Kurata’s Laboratory at the University of Alberta. All work was performed in collaboration.
3.5 Steady state inactivation curve of wild type Kv1.1 and Kv1.1 concatemer

The steady state inactivation for both wild type Kv1.1 and Kv1.1 concatemer are very similar. At -80mV both wild type and concatemer channels are open but with increasing voltage, the channels inactivate at a similar rate. The $V_{1/2}$ for wild type Kv1.1 and Kv1.1 concatemer are -41.2 (± 2.6) mV and -40.0 (± 0.4) mV respectively.

Figure 19. (Top) Graph indicating the open probability of wild type Kv1.1 at specific membrane potential during inactivation. (Bottom) Graph indicating the open probability of Kv1.1 concatemer at specific potential during inactivation. Errors are shown in SD. These images are kindly performed and provided by Victoria Baronas, a trainee in Associate Professor Harley Kurata’s Laboratory at the University of Alberta. All work was performed in collaboration.
3.6 Rate of recovery from inactivation of wild type Kv1.1 and Kv1.1 concatemer

The rate of recovery from inactivation for wild type Kv1.1 and Kv1.1 concatemer appeared to be significantly different. The average tau for wild type Kv1.1 was 46.6 (+11.8) seconds compared to the average tau for Kv1.1 concatemer which was much faster at 17.1 (+10) seconds. To understand this difference, the current density of cells expressing their respective channels was measured. Figure shows the average current density for wild type Kv1.1 is 28 (+4.8) pA/µF compared to the current density for Kv1.1 concatemer of 284 (+255) pA/µF.

![Figure 20](image)

Figure 20. (Top) Graph indicating the time it takes for wild type Kv1.1 to recover from inactivation. (Bottom) Graph indicating the time it takes for Kv1.1 concatemer to recover from inactivation. Errors are shown in SD. These images are kindly performed and provided by Victoria Baronas, a trainee in Associate Professor Harley Kurata’s Laboratory at the University of Alberta. All work was performed in collaboration.
Figure 21. Graph and table showing the current density of four recordings wild type Kv1.1 compared to Kv1.1 concatemer. Capacitance was measured in μF and error shown in SD. These images are kindly performed and provided by Victoria Baronas, a trainee in Associate Professor Harley Kurata’s Laboratory at the University of Alberta. All work was performed in collaboration.
CHAPTER 4: DISCUSSIONS

4.1 General Discussion

Concatemers have been used previously in studying the functional role of stoichiometry and position of ion channels\textsuperscript{111-114}. The development of the concatemers in Kv1.x was performed by linking two genes to generate a dimer, and then linking with another dimer set. A possible reason for this method of development was the lack of convenient and unique restriction sites. While this method allowed the study of stoichiometry and position of subunits in ion channels, the concatemer itself did not facilitate the generation of other concatemeric constructs; i.e, if a Kv1.1/K1.2 concatemer was generated using tandem dimers linked together, there would be no way to reuse the concatemer to test for a Kv1.1/Kv1.2 mutant heterotetramer without having to rebuild a new concatemer from scratch, a significant drawback, given that the molecular biology of developing each concatemer is a labour-intensive and time consuming process.

Similarly, a Kv1.1/Kv1.2 concatemer could not be easily reused to develop a Kv1.1/Kv1.4 concatemer due to the nature of tandem linking of dimers. The ease of development of a concatemer of heterotetramers of Kv1.x is key consideration because it mimics the heteromeric expression of Kv1.x found in the human brain. For example, Kv1.1 is expressed with Kv1.2 in cerebellum, cerebral cortex and brainstem as heterotetramers, but with Kv1.4 in the hippocampus. Therefore, to explore the role of stoichiometry or arrangement of pathogenic variants of any Kv1.1, Kv1.2 or Kv1.4 found in patients would require development of a new concatemer every time.

One of the key distinguishing factors of the Kv1.1 concatemer employed in our work is that this novel technology was built to be flexible. In contrast to other concatemers, which rely on tandem construction of dimers linked together, this concatemer is constructed by individually cloning all the
subunits into the plasmid vector. This flexibility is enabled by our in-house plasmid vector developed by Dr. Alexander Smith as a tool to improve cloning of ion channels in general. The vector has unique restriction sites, which nominally cut other ion channels, and allow for the insertion not only of Kv1.1 subunits, but other Kv1.x subunits in a modular fashion. Table 5 shows that for the development of the Kv1.1 homotetramer, four Kcna1 genes were physically linked. In Figure 14, the restriction digest following gel electrophoresis utilized different restriction enzymes to demonstrate that the genes are physically linked together, but each subunit can be cloned, or multiple concatenated subunits can be targets for future cloning.

The genes themselves are linked by three different short linking sequences (the amino acids for the sequences are: DTKEFRNLGTST, DTKETLNFRGST, DTKERGNSFTST) that are especially important for this concatemer system, because they allow each of the “domains” in the pseudotetramer to be distinguished separately (Figure 2, Table 5). By contrast, linkers used in concatemers produced in other laboratories are simple repeats of glutamine residues, or of other amino acid combinations, such as alanine-glycine-serine triplets\textsuperscript{112,115}. The rationale for avoiding long repeats of identical amino acids is that the linkers may result in depletion of transfer RNA molecules, with consequent possible termination of synthesis.

The other consideration is the size of the linker itself. A linker that is too small in size can cause structural deformities in the subunits that prevent correct assembly\textsuperscript{114–116}. Conversely, longer linkers can cause variability in subunit arrangement\textsuperscript{115,116}. Thus, the ideal linker length is 20-40 amino acids for properly functioning constructs. In our work, the ~20 amino acid linker was designed carefully to contain a short unique sequence that can be used by primers for Sanger sequencing. The
unique sequence in each linker was verified to ensure it didn’t match with any sequence within members of the Kv1.x family.

The utility of the unique sequence is seen from the results of the colony PCR, which used primers against the unique sequence of the linkers to screen for insertion of Kcnal genes into the vector for subsequent Sanger sequencing. The development of these intentionally designed unique linkers has not been performed in any other concatemer models described in literature thus far and is novel. The use of the plasmid vector expression system and the linker in combination is what allows for the construction of this concatemer in a modular fashion. Our approach facilitates the use of Kv1.1 concatemer as a technology that can be used as a backbone structure upon which other subunits can be reliably inserted to create Kv1.x heterotetramers.

The development of any new concatemer requires validation; this was performed both biochemically and biophysically for the Kv1.1 concatemer. Results from Figure 16 A and B show immunoblotting using Kv1.1 specific antibodies to reveal the expression of Kv1.1 subunit in mouse fibroblasts. However, this only provides insight into presence and absence of Kv1.1 subunits being translated. Numerous studies have shown that concatemers are expressed as intended \(^{117-119}\), but there has also been a report that they may not remain intact at the plasma membrane \(^{120}\). It is quite possible that these degraded channels found on the surface assembled as intact concatemers, but were later degraded by proteases to lose their covalent connections, and thus a convincing validation based on simple biochemical characterization alone is difficult. Therefore, to validate that the Kv1.1 concatemer is functional at the cell surface, electrophysiological characterization was performed.
The results from Figure 18, 19 and 20 show parameters for steady state activation (channel opening), steady state inactivation (after prolonged stimulation) and recovery from inactivation (returning to baseline and ready for activation). The results demonstrate that steady state activation and steady state inactivation is very similar to the wild type self-assembled Kv 1.1. However, there was a significant difference between the recovery from inactivation between the concatemer and the wild type (Figure 20). The difference in the recovery from inactivation is potentially revealed by examining the difference in peak current density seen between Kv1.1 concatemer and wild type Kv1.1 (Figure 21). The 10-fold difference in peak current density indicates more aggregate channel activity (which could result from increased channel numbers, per-channel activity or a combination of both) in Kv1.1 concatemers compared to the wild type Kv1.1.

4.2 Limitations of the study

Weak cell surface expression of Kv1.1 is consistent with evidence that Kv1.1 contains an endoplasmic reticulum retention (ERR) signal. Our Kv1.1 concatemer still retains the ERR signal, however, it appears that this signal does not affect the surface expression of the concatemer Kv1.1 as much and it is not clear why this happens in our non-natural construct. The ERR signal has been localized to the deep pore region. Therefore, in conjunction with the aforementioned marked difference in peak current density between concatemer and wild type channels, we cannot exclude the possibility that one or more of our custom linkers has resulted in an unwanted conformational change in the channel pore, potentially due to constraints imposed by the linker linking S6 region with the subsequent subunit’s S1 region. If this was indeed the case, then it seems to have that only the recovery of inactivation biophysical parameter was affected as channel activation and inactivation...
were unchanged. Further finer biophysical characterizations might need to be performed to better understand this phenomenon.

Another possibility could be that the linkers themselves have directly altered the trafficking and/or membrane targeting of the concatemer. A report showed that that a single amino acid substitution of threonine 330 in the S1-S2 linker region of Kv1.4 channels dramatically altered their surface expression. The authors suggested that threonine-330 helped interlock the voltage sensing domain and gating domains of adjacent monomers. The linkers in our concatemer could be playing a similar role in yielding a structure competent for the surface expression of functional tetramers. However, experiments exploring the trafficking of the concatemer would have to be performed before such conclusions can be made.

Similarly, it might be important to explore whether the ion channels being expressed are forming clusters on the cell surface or distributed randomly. Given the high variance seen in the current density for Kv1.1 concatemer as seen in Figure 21, a potential follow up would be to perform single channel recordings of the Kv1.1 using inside out patch clamp techniques, for example, to investigate if there are differences in cell density. Thus, for the reasons stated above, it will be important before extending this concatemer model to study heterotetramers and the effects of mutations on channel function, to ensure that the linker sequences do not substantially alter channel structure, function, and localization.

4.3 Future directions and experiments

Dr. Harley Kurata and his trainee Dr. Victoria Baronas have demonstrated that heterodimers (Kv1.1/Kv1.2) and homodimers (Kv1.2/Kv1.2) both have unique properties (like binding) with respect to novel cellular regulatory proteins involved in neuronal signaling and homeostasis (unpublished data, personal communication Dr Harley Kurata)
Future experiments will involve testing the stoichiometric binding of the Kv1.1 concatemer to chaperone proteins by selectively mutating known binding sites (that was already being explored in their existing channel models). This may provide insights into the role of Kv1.1 (partnered with other Kv1.x subunits) in binding with regulatory proteins of interest, thereby flagging novel targets for future therapeutics.

Another set of experiments could explore the biophysical alterations and potential functional significance entailed by compound mutations involving more than one Kv1.x subunit, after developing heterotetramers that use the current Kv1.1 homotetramer as a base.

4.4 Conclusions and significance

The Kv1.1 concatemer will help answer specific questions about the role of stoichiometry of a Kv1.1 model, independent of its main partners, using mutagenesis studies. Furthermore, the development of Kv1.1 homotetramer concatemer was a proof of concept for an exciting novel technology that will ease the development of future heterotetramer of Kv1.x concatemers by reducing time and labour involved in developing them. In the process of doing so, it will allow for the exploration of nuanced questions like allelic dosage that were previously unexplored due to limitations in technology.
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