Genetic and molecular studies of skeletal muscle channelopathies

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Declaration

I, Siobhan Durran, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Acknowledgements

I would like to thank my supervisors for their kind help and support throughout my studies. Special thanks go to Professor Steve Cannon for inviting me to spend 6 weeks in his laboratory to learn electrophysiology techniques which were important for the experiments of this thesis and for his ongoing advice and collaboration in relation to the D1420G experiments. I would also like to thank Roope Mannikko and Michael Thor for their help in obtaining and analysing patch clamp experimental data for D1420G and R222Q/W. Finally I would like to thank everyone in the 7th floor office/lab for their friendship and support over the last four years, it has been greatly appreciated and has helped keep me going.

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Abstract

This thesis investigates the genetic and molecular aspects of the skeletal muscle channelopathies, in particular periodic paralysis. A genetic study was conducted to identify causative mutations in a cohort of patients who did not have a genetic diagnosis following routine diagnostic screening. Through screening of the coding regions of SCN4A 9 mutations were identified, 6 of which were novel. Additionally, exome sequencing in a PMC family identified 10 variants in 7 genes, although only three genes of interest: RYR-1, AGRN and COL6A3. However, further work is needed to confirm the variants found. Two SCN4A mutations identified in this thesis were studied in vitro using two-electrode voltage clamp and patch clamp in *Xenopus laevis* oocytes and HEK-293 cells, respectively. D1420G is associated with a Hypo PP phenotype and is located within the S3 segment of DIV. No Hypo PP mutation has been associated with this region of the channel. D1420G was found to produce a gating pore current which is activated by negative voltages. This is the first Hypo PP mutation outside of the S4 voltage sensor to produce a gating pore current and support the notion that D1420G is a pathogenic mutation. R222Q was identified in a patient showing a myotonic phenotype. R222Q affects the S4 R2 gating charge of DI. A known Hypokalemic Periodic Paralysis mutation, R222W, affects the same residue. Both R222 mutations were compared in order to determine how two mutations affecting the same residue can cause different phenotypes. R222Q was found to cause a 16 mV hyperpolarizing shift in the voltage dependence of channel activation, which is consistent with a myotonic phenotype, whilst R222W had no effect. Both mutations were found to produce a gating pore current. This is the first time a myotonia related mutation has been found to cause a gating pore current.
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Chapter 1: Introduction

1.1 Skeletal Muscle

Skeletal muscle forms a part of the locomotor system which is responsible for all voluntary movement, maintains posture and stabilises joints (Franzini-Armstrong & Jorgensen, 1994; Huijing, 1998; Lieber & Friden, 2000). It is a precise and effective machine composed of a number of interlinking and intricate networks which are vital for contractile activity.

An individual muscle is composed of muscle fibre bundles. Muscle fibres are long, cylindrical and multinucleated cells. Each muscle fibre contains numerous tubular structures called myofibrils (McComas, 1996). Myofibrils contain filaments which run the length of the muscle fibre and are vital for the contractile ability of the muscle (H.E. Huxley, 1963; HUXLEY & HANSON, 1954).

The cell membrane that surrounds each muscle fibre is known as the sarcolemma. It contains an orchestra of ion channels and pumps which maintain the negative resting membrane potential and generate action potentials. Invaginations of the sarcolemma, which run between the myofibrils, form a membrane network known as the T-tubule. The function of the T-tubule is to allow the quick distribution of action potentials from the surface to the centre of the cell (Frankenhaeuser & Huxley, 1964; Franzini-Armstrong, 1999; A. Huxley & Stampfli, 1951; Huxley 1969). It is found in close contact with the sarcoplasmic reticulum which is the organelle responsible for storage and release of Ca$^{2+}$. Arrival of an action potential induces the release of Ca$^{2+}$ from the sarcoplasmic reticulum, triggering muscle contraction (Franzini-Armstrong, 1999; H.E. Huxley & Brown, 1967).
Pathogenic mutations of the genes encoding the individual components that make up the skeletal muscle network results in disruption to normal contractile activity and consequently a wide range of disorders such as the muscular dystrophies, congenital myopathies and the skeletal muscle channelopathies (Cannon 2006; Jurkat-Rott et al., 2002).

1.2 Electrical properties of skeletal muscle

Most cells maintain a negative resting membrane potential (A. Huxley & Stämpfli, 1951). In excitable cells the membrane potential changes in response to different stimuli. Changes to the membrane potential occur when ion channels open, allowing the permeation of the ions across the otherwise impermeable plasma membrane (Kimizuka & Koketsu, 1963).

Excitable cells use electricity to relay signals. The main unit of electrical signals is known as the action potential. The action potential is rapidly propagated along the membrane of axons or muscles and is regenerative and self propagating (Frankenhaeuser & Huxley, 1964; Guy & Seetharamulu, 1986). An action potential is controlled by the opening and closing of ion channels, specifically voltage gated Na\(^+\) and K\(^+\) channels, although in muscle Cl\(^-\) channels also play an important role in shaping the action potential (Barnett & Larkman, 2007; Eisenberg & Gage, 1969; Hodgkin & Katz, 1949; Institutet, 1964; Karin Jurkat-Rott, Fauler, & Lehmann-Horn, 2006). The electric signal is transduced into a cellular or mechanical response e.g. muscle contraction.

In muscle, action potentials are initiated at the neuromuscular junction when a stimulus from the motor neuron depolarises the sarcolemma above the threshold for activation of voltage gated sodium channels. Activation of voltage gated sodium channels results in the rapid inflow of Na\(^+\) into the cell, which results in the depolarisation of the membrane potential, to a peak potential of \(~+30\) mV. Depolarisation of the membrane potential results in activation of voltage gated potassium channels, increasing membrane permeability to K\(^+\),
allowing for efflux of K\(^+\) from the cell down the electrochemical gradient, which helps to repolarise the cell (Guy & Seetharamulu, 1986).

Skeletal muscle has an unusually high Cl\(^-\) conductance compared to other cells – accounting for up to 85\% of all membrane conductance at rest (Bryant & Morales-Aguilera, 1971; Palade & Barchi, 1977). A large Cl\(^-\) conductance plays an important role in stabilising the membrane potential and in the repolarisation of the membrane potential after an action potential, enabling muscle activity to be maintained during repeated stimulation. Following an action potential, K\(^+\) builds up within the T-tubules. Repeated action potentials can cause a increase in the T-tubule K\(^+\) concentration, which is large enough to shift the equilibrium potential of K\(^+\) (\(K_{eq}\)), leading to depolarisation of the skeletal muscle membrane potential. This promotes more action potentials and further depolarisation. The presence of a high Cl\(^-\) conductance within the skeletal muscle negates the depolarising effect of K\(^+\) build up within the T-tubules (Aromataris & Rychkov, 2006).

The action potential propagates along the sarcolemma to the T-tubules, where the excitation-contraction coupling machinery is found. Arrival of the action potential activates the voltage gated calcium channel. Conformational changes within the channel upon activation lead to activation of the ryanodine receptor within the sarcoplasmic reticulum (Bannister, 2007; Block, Imagawa, Campbell, & Franzini-Armstrong, 1988; Franzini-armstrong & Jorgensen, 1994; Franzini-Armstrong, 1999). Activation of the ryanodine receptor results in release of Ca\(^{2+}\) from the sarcoplasmic reticulum into the cytoplasm (Frankenhaeuser & Huxley, 1964; A. Huxley & Stämpfli, 1951; H E Huxley, 1969). The sudden increase in Ca\(^{2+}\) within the cytoplasm triggers activation of the contractile apparatus leading to muscle contraction. Relaxation of the muscle occurs through removal of the cytoplasmic Ca\(^{2+}\) via Ca\(^{2+}\) pumps (Berchtold, Brinkmeier, & Müntener, 2000; Karin Jurkat-Rott et al., 2006).
Figure 1-1 Diagrammatic representation of muscle action potential initiation (diagrammatic concept adapted from Cannon 2006 review)
1.3 Ion Channels

1.3.1 What are Ion channels?

The hydrophobic plasma membrane acts as an electrostatic barrier for the movement of small inorganic ions such as Na⁺, K⁺, Ca²⁺ and Cl⁻. A variety of different ion-transporting proteins (ion channels and ion pumps) have evolved to allow the movement of specific ions across the plasma membrane. This highly controlled movement of ions into and out of a cell is important for numerous biological processes such as regulation of cell volume or pH, muscle contraction, conduction of nerve impulses, control of heartbeat, sensory reception and ciliary clearing of pathogens (Hille, 2001).

Ion pumps move ions across the plasma membrane against the concentration gradient and require energy to do so. Within the extracellular space there is a high concentration of Na⁺ and Cl⁻ ions while inside the cell a high K⁺ concentration is found. Cytoplasmic Ca²⁺ is kept at very low concentrations (nanomolar range) by sequestration into intracellular storage organelles (Barnett & Larkman, 2007).

Ion channels form an aqueous pore within the plasma membrane that provides a pathway for the passive movement of ions along the electrochemical gradient, at a rate that is often close to that of free diffusion. The flow of ions through channels transports ions from one compartment to another, e.g. across the epithelial cells in the kidneys and generates an electrical current that is used to relay electrical signals. The activity of ion channels is controlled by specific biological signals, allowing for the tightly controlled movement of ions in response to an appropriate stimulus.

1.3.2 Classification & function of ion channels

Ion channels can be classified by a number of functional or structural characteristics, for example: the mode of gating (e.g. voltage-, mechano- or ligand gated channels), the type
of ion they are permeable to (e.g. sodium or potassium ions), the number of pores (e.g. two-pore channels), where the channel is expressed (e.g. P (purkinje)-type calcium channels), rectification (inward- or delayed rectifier K⁺ channels) and conductance (large and small conductance K⁺ channels).

Voltage gated ion channels are a particular class of ion channels found in excitable cells such as neurons and muscle cells. Their activity is controlled by changes in the membrane potential. Channels are activated by a change in membrane potential, above a threshold, which causes a conformational change within the channel and subsequent opening of the channel, allowing ions to flow into or out of the cell (Kew & Davies, 2009). For the purpose of this work further focus will be placed on the voltage gated cation channels and more specifically voltage gated sodium channels.

1.3.2.1 Potassium channels

Potassium channels make up a large family of ion channels that are widely expressed throughout almost all organisms and have one basic function: to allow K⁺ ion movement across the cell membrane. The voltage gated potassium channels mediate the movement of K⁺ out of the cell following depolarisation of the membrane potential. They play a crucial role in the repolarisation of the membrane during an action potential. The inwardly rectifying K⁺ (Kir) channels allow for the movement of K⁺ ions into the cell and are involved in setting and resetting the resting membrane potential (Hibino et al., 2010).

Members of the potassium channel family show a diverse range of roles in excitable and non excitable cells, from cell growth and survival in prokaryotes to cellular signalling in eukaryotes. The cellular processes in which these channels play a role varies with the localisation of the channel, for example, in the heart they help to regulate the heartbeat; in the pancreas they enable insulin release from beta-cells; and in renal epithelium they
enable cellular recycling of $K^+$ to regulate the electrolyte balance (Miller, 2000; Shieh et al., 2000).

Potassium channels are categorised based upon the primary structure of the channel. There are two main types of potassium channel: Voltage gated potassium Channels ($K_v$) or Inwardly rectifying potassium channels ($Kir$). The $Kir$ family of potassium channel is subdivided into 4 groups: Classical $Kir$ channels ($Kir2.x$, constitutively active), $G$ protein gated $Kir$ Channels ($Kir3.x$), ATP-sensitive potassium channels ($Kir6.x$) and potassium transport channels ($Kir1.x$, $Kir4.x$, $Kir5.x$ and $Kir7.x$).

**1.3.2.2 Sodium channels**

Voltage gated sodium channels are highly selective to sodium ions, and are found in numerous organisms in which they are highly conserved. They play a key role in the generation and propagation of action potentials in excitable cells. The role of sodium channels within the initiation of an action potential means they are critical for the transmission of information between excitable cells allowing for co-ordination of processes and networks.

To date nine sodium channels have been characterized which are expressed in a variety of tissues. Four sodium channels ($Na_v1.1, 1.2, 1.3$ and $1.6$) are primarily expressed within the central nervous system, three channels within the peripheral nervous system ($Na_v1.7, 1.8$ and $1.9$), one in skeletal muscle ($Na_v1.4$) and one in cardiac muscle ($Na_v1.5$). These sodium channels show different biophysical and pharmacological properties, cellular functions and tissue localisation. For example, $Na_v1.4$ is expressed in skeletal muscles, is tetrodotoxin sensitive, and is important for muscle contraction, whilst $Na_v1.8$ is expressed within peripheral sensory nerves, is tetrodotoxin insensitive, and is important for nociception (Catterall et al., 2012; Marban et al., 1998).
1.3.2.3 Calcium channels
Voltage gated calcium channels are the hallmarks of excitable cells. They mediate the influx of calcium in response to membrane depolarisation allowing Ca\(^{2+}\) to act as a secondary messenger to electrical signalling. The activity of these channels is vital for the transduction of membrane potential changes into a variety of physiological events such as muscle contraction, endocrine and synaptic secretion, and gene expression. Signal transduction in various cell types involves different voltage gate calcium channels which show differential tissue expression as well as variations in physiological and pharmacological properties. For example, Ca\(_{\alpha 1.1}\) is highly expressed in skeletal muscle T-tubules, is dihydropyridine sensitive, and acts as the voltage sensor for the coupling of muscle depolarisation to the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR).

Ten calcium channel genes have been identified which encode channels with different localisation, pharmacological and physiological properties. These channels can be grouped into three families on their molecular properties (see Dolphin 2006 for review): (1) the high-voltage activated (HVA) dihydropyridine-sensitive (L-type, Ca\(_{\alpha 1.x}\)) channels; (2) the HVA dihydroyridine-insensitive (P/Q, N & R type, Ca\(_{\alpha 2.x}\)) channels and (3) the low–voltage activated (T-type, Ca\(_{\alpha 3.x}\)) channels (Birnbaumer et al., 1994; Ertel et al., 2000; Perez-Reyes, 1998)(Dolphin, 2006).

1.3.2.4 Chloride channels
As with other ion channels, chloride channels are ubiquitously expressed and are found in all living organisms. Chloride channels allow the passive diffusion of Cl\(^{-}\) ions across the plasma membrane along the electrochemical gradient. Whilst they are permeable to many anions, Cl\(^{-}\) is the permeating ion within these channels as Cl\(^{-}\) is the most abundant ion within cells (Maduke et al., 2000). They play a vital role in a variety of different biological processes from maintaining skeletal muscle excitability, cell volume regulation or ion homeostasis.
Three different families of chloride channel have been identified thus far: CLC, CFTR and ligand gated Cl⁻ channels. Of these the CLC channels are the most characterised and well known. There are 9 CLC family members, which show different localisation and functions (Jentsch et al., 2002; Mindell et al., 2001). Members of this subfamily show 50-80% sequence identity (Jentsch et al., 2002; Maduke et al., 1999). Interestingly some members have been since been shown to act as Cl⁻/H⁺ exchangers but still share similar molecular architecture with other members of the family (Accardi & Miller, 2004).
1.3.3 Structural Features of cation channels

Due to a common evolutionary relationship, the voltage gate cation channels share number of structural features, including the structure of the central pore and voltage sensing domain (VSD).

The inwardly rectifying potassium channels form the simplest structural motif of the voltage gated cation channels (Figure 1-1). Kir subunits are composed of only two transmembrane segments, M1 and M2, which together with a re-entrant P-loop form the pore domain (Hibino et al., 2010). The voltage gated potassium channels are large proteins where the structure of these channels can be subdivided into two functional sub-domains: the pore domain (S5-S6) and the voltage sensing domain (VSD; S1-S4). The S5 and S6 segments are analogous the M1 and M2 helices of the Kir channels. Voltage gated potassium channels (Kv) are composed of tetramers of α subunits (Ho et al., 1993; Yang et al., 1995a).

The α subunit of the voltage gated sodium (Na, ) and calcium (Ca, ) channels do not form tetramers but are large multi domain proteins. Each Na, or Ca, α subunit is composed of four homologous domains (I-IV). Each domain is equivalent to a single subunit of a voltage gated potassium channel (Doyle et al., 1998; George at al. 1992; Hille & Schwarz, 1978; Noda et al. 1984; Takahashi et al., 1987; Trimmer et al., 1989; Yang et al., 1995b).
Figure 1-2 Schematic representation of potassium channels.

Topology of the a single α subunit for A) voltage gated potassium channels or B) inward rectifiers and C) four subunits forming a functional channel.
1.3.3.1 Pore domain of the voltage gated cation channels

The ionic pore is the central component of voltage gated cation channels. It not only forms the permeation pathway but provides the gating mechanisms for opening and closing the channels as well as controlling the selectivity of the channel. Within cation channels the permeation pathway can be found at the interface of four individual subunits or domains along the axis of symmetry. The pore is formed by four re-entrant P loops (the loop region linking the S5 and S6 segments) together with the S5 and S6 segments (Chahine et al., 1998; Heinemann et al., 1992; Noda et al., 1989; Pérez-García et al., 1996).

All the channels share a common pore architecture which is formed by a narrow selectivity filter, found towards the extracellular end of the channel; a large central cavity; and separate activation and inactivation gates, which can be found at the intracellular end of the channel (Doyle et al., 1998; Jan & Jan, 1997; Kuo et al., 2003; Long et al., 2005; Payandeh et al., 2011). The crystal structure of the tetrameric bacterial sodium channel from A. butzleri, NavAb, showed an extracellular funnel formed by the P2 helix which is not present in potassium channels (Payandeh et al., 2011). It is thought that the funnel may be specific to sodium and calcium channels. In sodium channels, there are four lateral openings from the membrane into the lumen of the pore (Payandeh et al., 2011). These fenestrations allow a connection between the pore cavity and the membrane lipids but are not present in potassium channels.
The α-subunit of voltage gated sodium and calcium channels is composed of four homologous domains each containing 6 transmembrane helices. Each of the four domains contributes towards the formation of the central ionic pore.
1.3.3.1.1 Selectivity filter

Ion selectivity is central to the proper functioning of the voltage gated cation channels. Ion selectivity is in the most part determined by the selectivity filter. The selectivity filter is found at the narrowest part of the pore and formed by the re-entrant P loops which line the pore. The amino acid residues within the P loops interact with the permeating ions to control the selectivity of the channel.

Whilst the overall pore structure of sodium, potassium and calcium channels is similar, the structures of the selectivity filters differ and provide means for selecting for permeation of a distinct ion.

In potassium channels the selectivity filter is formed by the conserved sequence: T-X-G-(Y/F)-G (Choe, 2002; Heginbotham et al., 1994; Mackinnon, 1995). Oxygen atoms from the carbonyl backbone of the selectivity filter, stabilise the movement of K$^+$ ions through the pore in a nearly fully dehydrated state (Doyle et al., 1998; Roux & MacKinnon, 1999; Zhou et al., 2001).

In sodium channels, there are four residues which are critical for ion selectivity: aspartate-glutamate-lysine-alanine (DEKA), one provided by each domain (Heinemann et al., 1992; Payandeh et al., 2011; Yang et al., 1993). Interaction with these residues allow Na$^+$ ions to travel through the selectivity filter in a partially dehydrated state, in contrast to the mechanism for K$^+$ channels (Payandeh et al., 2011).

Despite the fact that Na$^+$ and Ca$^{2+}$ ions have an almost identical diameter (~2 Å) and that the extracellular Na$^+$ concentration is 70 fold higher than Ca$^{2+}$, calcium channels can prevent the permeation of Na$^+$ ions. Calcium channel selectivity is determined by the presence of four glutamate residues (EEEE motif) within the selectivity filter. Substitution of just two of these residues has been shown to confer Na$^+$ selectivity to calcium channels (Heinemann et al., 1992; Yang et al., 1993). The structural basis of selectivity of Ca$^{2+}$, and
block of Na⁺, was recently determined by modifying the selectivity filter of the NavAb channel to that of a calcium channel (Tang et al., 2014). The crystal structure of this modified NavAb channel showed that calcium selectivity is determined by the amino acid side chains of the selectivity filter. Selectivity of calcium over sodium is due to the presence of two high affinity binding sites within the selectivity filter to which the more electropositive Ca²⁺ ion binds tightly preventing Na⁺ permeation.

1.3.3.1.2 Channel Gating
Gating of the voltage gated cation channels involves two types of gate: activation and inactivation gates. They enable the controlled flow of ions into or out of the cells for the length of a given stimulus and prevent an excess of ionic movement.

Gating of the cation channels involves structural rearrangements of the activation and inactivation gates (Baukrowitz & Yellen, 1995; Panyi & Deutsch, 2006). At rest, the activation gate is closed whilst the inactivation gate is open. Upon depolarisation the voltage sensor induces a conformational change which opens the activation gate within the pore, allowing the permeation of the specific cation (Catterall, 2010). Following activation, many channels inactivate rapidly (through closure of the inactivation gate) to prevent further permeation of ions. Inactivation can be both time and voltage dependent and results in the transition of an open channel into an inactivated, non-conducting state. The conformation of this state is distinct from the closed, non-conducting state. After a given time period the channel recovers from inactivation (the activation gate closes and the inactivation gate opens) restoring the channel to the closed state from which it can be activated.

*Activation gate*
The activation gate is formed by the cytoplasmic convergence of the four S6 transmembrane segments which line the pore (del Camino et al., 2000; del Camino & Yellen, 2001; Liu et al., 1997). In the closed state, the S6 helices form a bundle that blocks the pore
and prevents the entrance of ions into the central cavity of the pore. The activation gate of voltage gated cation channels is coupled to the voltage sensor via the S4-S5 linker (Lu et al., 2002). Opening of the activation gate occurs following depolarisation of the membrane potential and involves rearrangement of the intracellular end of the S6 segment (del Camino et al., 2000; del Camino & Yellen, 2001; Hackos et al., 2002; Holmgren et al., 1998; Holmgren et al., 1997; Liu et al., 1997). For many channels, a glycine residue within the S6 segment forms a hinge. This hinge enables the movement of the N-terminal part of the S6 segment, to open or close the activation gate (Ader et al., 2009; Doyle et al., 1998; Holmgren et al., 1998; Jiang et al., 2002; Kelly & Gross, 2003; Liu et al., 1997; Perozo et al., 1998).

**Inactivation Gates**

Kv and Nav channels show two main types of inactivation: fast or slow, whilst for Cav channels inactivation is more complex. Fast inactivation of Kv channels involves the occlusion of the internal vestibule of the pore by cytoplasmic domains, in a ball and chain mechanism. Site directed mutagenesis of amino acids within the N-terminal domain of Shaker Kv channels were found to disrupt channel inactivation, indicating that the N-terminus plays a role in channel inactivation (Hoshi et al., 1990). Application of a peptide containing the first 20 amino acids of the N terminus was able to restore inactivation to non-inactivating Kv channels (Zagotta et al., 1990). The gate region for the N-terminal “ball” lies within the hydrophobic central cavity and inner pore of the potassium channel (Zhou et al., 2001).

Kv channel slow inactivation is sensitive to cation concentrations and involves the rearrangement of the outer vestibule and the selectivity filter (Yellen, 2002). The selectivity filter of potassium channels has been found to have two conformations: activated or inactivated. In low potassium concentrations the selectivity filter of KcsA channels was
found to undergo a conformational transition into the inactivated state (Cordero-Morales et al., 2006; Imai et al., 2010).

Fast inactivation of Nav channels occurs via a “hinged lid” mechanism in which an inactivation gate blocks the intracellular side of the pore (analogous to ball-and-chain mechanism of Kv channels) whilst slow inactivation of sodium channels occurs via a global conformational change in channel structure, which is less characterised. Fast inactivation is mediated by the highly conserved loop between DIII and IV, which acts as the inactivation gate. A short hydrophobic cluster of three amino acids, Ile-Phe-Met (IFM motif), in the DIII-DIV loop is a crucial part of the inactivation gate (Vassilev et al., 1988; Vassilev et al., 1989). Substitution of these three residues to glutamine removes inactivation completely (West et al., 1992). Small peptides containing the IFM motif are sufficient to restore inactivation in mutant non-inactivating channels, suggesting the gate has a docking site on the intracellular site of the pore (Eaholtz et al. 1994). The docking site of the gate is thought to include DIV S4-S5 intracellular loops (Lerche et al., 1997; McPhee et al. 1998), DIII S4-S5 loops (Smith & Goldin, 1997) and the intracellular side of DIV S6 (McPhee et al., 1995), mutations of residues in these areas result in impaired inactivation.

Inactivation of Cav channels is important for the tight regulation of intracellular calcium levels which enables Ca$^{2+}$ to act as a secondary messenger. Inactivation of calcium channels appears to be more complex than seen in potassium or sodium channels. Calcium channels undergo three different types of inactivation: calcium dependent inactivation and fast or slow voltage dependent inactivation.

Calcium dependent inactivation provides a negative feedback loop to Ca$^{2+}$ influx, as it is triggered by a high intracellular Ca$^{2+}$ concentration. It has been shown to only occur in HVA channels and is mediated by calmodulin (CaM) which acts as a Ca$^{2+}$ sensor. Binding of Ca$^{2+}$ to CaM enables Ca-CaM to bind to an IQ-like motif on the C terminus of the channel and
induces rapid channel inactivation (Budde et al., 2002; Lee et al., 1999; Peterson et al., 1999; Zühlke et al., 1999). Channel chimera and site direct mutagenesis experiments were used to establish the regions of the Cav channel which are involved in the fast inactivation. The S6 transmembrane segment, D1–DII, DII-III and DIII-IV linker regions and the C terminus have all been shown to play a role in channel fast inactivation (Berjukow et al., 2001; Soldatov et al., 2012; Soldatov et al., 1998; Stotz et al., 2001; Zhang et al., 1994). Inactivation is thought to occur via a “hinged lid” mechanism similar to that seen in sodium channels (An & Zamponi, 2005; Kubalová, 2003; Stotz et al., 2000).

Slow inactivation occurs following a prolonged duration of membrane depolarisation (~1 minute). It is mediated by a group of hydrophobic amino acids located near the cytoplasmic ends of the S6 transmembrane segments (Shi & Soldatov, 2002).

1.3.3.2 Voltage sensing domain

The voltage sensing domain of voltage gated cation channels is formed from the S1-S4 segments and enables these channels to activate in response to a change in voltage (MacKinnon, 1991). The S4 segment acts as the main “voltage sensor” of the channel. It contains a conserved sequence of positively charged residues at every third amino acid position (the first charged residue on at the N-terminal end of the S4 is denoted R1, the second R2 and so forth). These charged residues act as gating charges that are sensitive to changes in transmembrane voltage. Their movement in response to a change in voltage controls channel gating. Neutralisation of these positive charges disrupts the voltage dependence of channel gating, highlighting their important role in conferring voltage sensitivity (Aggarwal et al., 1996; Seoh et al., 1996; Starace & Bezanilla, 2001).

The localisation of the gating charges is unusual since it incurs a high energetic cost to place charged residues within the hydrophobic environment of the phospholipid membrane. The voltage sensor is located within the aqueous crevice of a channel structure called the gating
pore which is formed by the remaining segments of the voltage sensing domain, S1-S3 (Sato et al., 2001). Counter charges within the gating pore (from the S2 and S3 segments) help to stabilise the positive charges, reducing the energetic cost of placing charged residues in a hydrophobic environment (DeCaen et al., 2009; DeCaen et al., 2008; Papazian et al., 1995; Seoh et al., 1996; Tiwari-Woodruff et al., 2000). In support of this is the crystal structure of NavAb, which shows that there are two clusters of negatively charged residues with which the S4 segment interacts (Payandeh et al., 2011). These clusters are called the extracellular negative-charge cluster (ENC) and the intracellular negative-charge cluster (INC). Each cluster contains highly conserved residues that form electrostatic interactions or hydrogen bonds with the S4 gating charges forming a network of interactions that help to stabilise S4 segment movement (Payandeh et al., 2011). Furthermore, the crystal structure has shown there is a large aqueous cleft that extends ~10 Å into the membrane region to a point called the hydrophobic constriction site (HCS). The HCS contains three highly conserved residues (Ile, Phe & Val) which act to seal the voltage sensing domain against aberrant ionic leakage during the movement of the S4 segment (Payandeh et al., 2011).

Upon membrane depolarisation the S4 voltage sensor translocates outward through the gating pore, triggering a conformational change within the protein, which is transmitted through the S4-S5 linker resulting in opening of the main pore. Movement of the charged residues through the electric field of the membrane generates a transient gating current. The movement pattern of the voltage sensor has been supported by substituted cystine or histidine accessibility method (SCAM or SHAM) and toxin exposure experiments (DeCaen et al., 2008; Mantegazza & Cestèle, 2005; Pérez-García et al., 1996; Starace & Bezanilla, 2001). These experiments found that R2 and R3 are accessible to modification by cysteine specific reagents from the inside of the cell when the channel is in the resting state and become accessible from the outside after depolarisation (Catterall, 2000; Gandhi et al., 2003; Larsson et al., 1996; Männikkö et al., 2002; Yang et al., 1996; Yang & Horn, 1995). This supports the
idea that the voltage sensor moves in an outward motion during depolarisation. Similar experiments with the S3 segment have shown that the S3 doesn’t move during activation of the Na_{1.4} (Nguyen, 2002) or the Drosophila Shaker potassium channel (Gandhi et al., 2003).

### 1.3.3.3 Chloride channels

Voltage gated CLC chloride channels are the outliers of the voltage gated ion channels. They do not share the molecular architecture of the voltage gated cation channels. CLC channels are formed by two subunits. Each subunit is composed of 18 intramembrane α helical segments (A-R) and contributes a single pore to the dimer. The presence of two independently gate pores gives CLC channels a characteristic double barrelled structure.

Chloride channels have two independent pores (one from each subunit). The formation of each pore from a single subunit is in vast contrast to other voltage gated ion channels in which the pore is formed along the axis of symmetry between the different subunits (or domains in the case of Ca_{v} and Na_{v} channels). The pore lining residues come from different regions within the primary sequence of the channel (Ludewig, Pusch, & Jentsch, 1996, 1997; Middleton, Pheasant, & Miller, 1996).

There is no conserved charged sequence within CLC channels that corresponds to the voltage sensor as in the cation channels. In fact, the voltage dependence of CLC channel gating does not arise from the movement of charged residues within the protein as seen in channels with voltage sensing domains. Instead voltage sensitivity is derived from the movement of the permeating Cl\(^-\) ion (Jentsch et al., 2002; Christopher Miller, 2003).
Figure 1-4 Schematic diagram of a single CLC monomeric subunit

The individual subunits are composed of 19 helices, which are indicated by blocks labelled A-R.
1.3.4 Ion Channels & Disease

Considering the important role ion channels play in biological processes it is no surprise that mutations in these proteins are known to cause human disease and disability. Diseases that develop due to a defect in ion channel function, either through genetic or acquired factors, are known as channelopathies. Disruption of ion channel function leads to a change in the electrical excitability of excitable cells or the cellular ion homeostasis. Channelopathies affect a range of different areas within the body, causing a range of conditions including epilepsy, migraine, blindness, deafness, cardiac arrhythmia, diabetes, cystic fibrosis and more.
1.4 Skeletal Muscle Channelopathies: clinical features

Mutations in the voltage gated ion channels expressed in skeletal muscle cause disruption to sarcolemma electrical excitability resulting in two main phenotypes; myotonia and episodic attacks of weakness, which are two ends of the spectrum of electrical disruption (See Vicart et al. 2005 for review). The two phenotypes can be broadly separated into the non-dystrophic myotonias and the periodic paralyses, although significant overlap of symptoms can occur between these groups. The non-dystrophic myotonias include myotonia congenita (MC), paramyotonia congenita (PMC) and sodium channel myotonia (SCM) where the principal symptom is muscle stiffness. The periodic paralyses are divided into hyperkalemic periodic paralysis (Hyper PP), hypokalemic periodic paralysis (Hypo PP) and Anderson-Tawil syndrome (ATS) all of which show episodes of flaccid muscle paralysis as the main symptom.

Skeletal muscle channelopathies have provided naturally occurring models to study the role, function and structure of skeletal muscle ion channels.

1.4.1 Non Dystrophic Myotonia

The non-dystrophic myotonias are a group of rare disorders grouped by the common symptom of myotonia. This group of disorders includes myotonia congenita, paramyotonia congenita and sodium channel myotonia.

Disruption to normal ion channel function results in enhanced muscle excitability that manifests as myotonia. In normal muscle fibres a single stimulus from the neuromuscular junction will result in a single action potential that initiates a single muscle contraction. However, in muscle affected by myotonia a voluntary contraction produces a sustained burst of action potentials that persist even after the initial stimulus has finished. This results
in delayed muscle relaxation following contraction which patients often describe as muscle stiffness. It has been shown to have a worldwide prevalence of approximately ~1 in 100,000 although there are variations between countries (Emery, 1991; Horga et al., 2013).

1.4.1.1 Myotonia Congenita
Myotonia congenita presents with transient attacks of muscle stiffness from infancy up until as late as the fourth decade. Myotonia occurs on the first voluntary movement often following rest after exercise but shows improvement with repetition of movement. This is known as the warm up phenomenon. There are two forms of myotonia congenita which are distinguished by the form of inheritance: Thomsen’s disease (OMIM 160800), which is dominantly inherited; and the recessively inherited Becker’s disease (OMIM 255700). Thomsen’s disease usually presents in early childhood often before the age of three years with generalised myotonia that affects the legs, arms and hands, making falls a frequent occurrence and grasping objects difficult. Onset of Becker’s disease occurs slightly later between the ages of 8 and 12 years old with much the same symptoms as Thomsen’s disease although patients show much more severe myotonia that has a greater impact on their daily lives.

Upon clinical examination patients can show muscle hypertrophy with normal muscle strength which can allow them to participate in sports that favour muscle strength over speed (Matthews et al., 2010; Wakeman et al., 2008). Percussion myotonia – where the muscle is pressed leaving an indent for several seconds - can also be noted in many patients. Electromyography (EMG) needle testing of resting muscle in patients with either form of myotonia congenita shows myotonic bursts in all muscles tested. Myotonia can be triggered by rest after exercise, hunger, stress or fatigue. A large amount of phenotypic variability is seen within myotonia congenita even amongst family members.
1.4.1.2 Paramyotonia Congenita

Paramyotonia congenita (PMC, OMIM 168300) is an autosomal dominant disorder which shows onset during early childhood and is established by the first decade of life. For PMC the prominent symptom is muscle stiffness that unlike other forms of myotonia is made worse by repeated muscle activity (paramyotonia) and exposure to cold (Fournier et al., 2006). Muscle stiffness is brought on during exercise rather than after exercise as with other forms of myotonia. Cold sensitivity in PMC can be dramatic and can make it difficult for patients to cope during cold weather. Myotonia mainly affects the upper limbs, face, tongue and hands with eyelid lag a notable common feature in patients (Miller et al., 2004). In addition, myotonia may be accompanied by weakness and in some cases full attacks of paralysis. As with the attacks of myotonia, weakness can also be exacerbated by cold and exercise (McClatchey et al., 1992; Wagner et al., 1997). Myotonia is often short lived, lasting seconds to minutes whereas weakness lasts much longer, on the scale of hours or days. Unlike myotonia congenita, PMC is less frequently associated with muscle hypertrophy although it is thought to be present in about 30% of patients (Matthews et al., 2008a). During EMG testing patients exhibit a gradual and persistent reduction in compound muscle action potential (CMAP) during a short exercise test which can be enhanced by both cooling and repetition.

1.4.1.3 Sodium Channel Myotonia

Sodium channel myotonia is clinically distinct from both myotonia congenita and PMC (Table 1-1). Initially it was described as three separate diseases: myotonia fluctuans, myotonia permanens and potassium aggravated myotonia. These disorders have been combined to form sodium channel myotonia, as they all have a similar phenotype and shared genotype (Matthews et al., 2010; Raja Rayan & Hanna, 2010). Patients show autosomal dominant inheritance with a purely myotonic phenotype that sometimes shows severe potassium sensitivity. Cold sensitivity is not always present but can be with varying
severity (Colding-Jørgensen et al., 2006; Heine et al., 1993; Orrell et al., 1998; Rossignol et al., 2007). Weakness is never shown in these patients and if present will rule out SCM as a diagnosis. Myotonia is generalised throughout the body with transient attacks of muscle stiffness ranging in severity and length of time. In addition, some cases may show the warm-up phenomenon while others show paradoxical myotonia (Lee et al., 2009). Due to the pure myotonia phenotype, SCM can be difficult to distinguish clinically from dominant myotonia congenita.
<table>
<thead>
<tr>
<th></th>
<th>Myotonia Congenita</th>
<th>Paramyotonia Congenita</th>
<th>Sodium Channel Myotonia</th>
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</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
<td><em>CLCN1</em></td>
<td><em>SCN4A</em></td>
<td><em>SCN4A</em></td>
</tr>
<tr>
<td><strong>Mode of Inheritance</strong></td>
<td>Dominant &amp; Recessive</td>
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<td>Dominant</td>
</tr>
<tr>
<td><strong>Age of onset</strong></td>
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<td>1st decade</td>
<td>1st decade</td>
</tr>
<tr>
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<td>Face &amp; upper limbs</td>
<td>Face &amp; upper limbs</td>
</tr>
<tr>
<td></td>
<td>Dominant = upper limbs</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sensitivity to Cold</strong></td>
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<td>Yes</td>
<td>Variable</td>
</tr>
<tr>
<td><strong>Warm up</strong></td>
<td>Yes</td>
<td>No</td>
<td>Sometimes</td>
</tr>
<tr>
<td><strong>Episodic weakness</strong></td>
<td>In recessive form</td>
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<td>Paradoxical myotonia</td>
<td>Painful myotonia &amp; K⁺ sensitivity</td>
</tr>
</tbody>
</table>

Table 1-1 Clinical features of the non dystrophic myotonias
1.4.2 Periodic Paralysis

The periodic paralyses encompass disorders characterised by transient attacks of weakness. This abnormal electrical excitability is limited only to skeletal muscle, except in the case of Andersen-Tawil Syndrome. There are four known forms of periodic paralysis: Hyperkalemic periodic paralysis (OMIM 170500), Hypokalemic periodic paralysis (OMIM 170400), Andersen-Tawil Syndrome (OMIM 170390) and Thyrotoxic periodic paralysis (TPP, OMIM 188580). Hyper PP and Hypo PP can be distinguished by differences in the potassium levels during attacks of paralysis: (Hyper PP = high, Hypo PP = low, see Table 1-2).

In between transient attacks patients retain normal muscle strength and reflexes, and in mild cases attacks can be circumvented through life style changes that avoid known triggers such as carbohydrate rich meals and stress for Hypo PP or high potassium and rest after exercise for Hyper PP. Regardless of the severity and frequency of attacks many patients can develop progressive proximal myopathy that can make daily life difficult. Mobility aids are needed in later life for ~50% of periodic paralysis patients (Meyer et al., 2008).

1.4.2.1 Hyperkalemic Periodic Paralysis

Hyper PP is an autosomal dominant disorder that presents with recurrent paralytic attacks usually in the first decade. Following the second decade attacks decrease in frequency over time. Attacks of weakness can occur at any time of the day and are relatively short in duration (10 minutes to 4 hours) and will normally be followed by periods of normal muscle activity (Jurkat-Rott & Lehman-Horn, 2007). A diagnosis can be made by noting attacks of weakness associated with elevated serum potassium (>6 mEq/L) although some patients may have normal levels. Paralysis can be precipitated by ingestion of potassium rich foods and rest after exercise but can be improved by intake of glucose. Hyper PP shows significant
overlap of symptoms with PMC, with 50-75% of patients are affected by myotonia (Miller et al., 2004; Plassart et al., 1994). On clinical examination myotonia is most notable in the tongue, as well as facial and hand muscles, and can be measured through EMG needle testing.

1.4.2.2 Hypokalemic Periodic Paralysis
Hypo PP is the most common form of periodic paralysis and is inherited in an autosomal dominant fashion but shows reduced penetrance in women (Miller et al., 2004). Onset of Hypo PP is later than seen in Hyper PP, beginning in the second decade. The main feature of Hypo PP is recurring attacks of weakness that are associated with low serum potassium levels (<3 mEq/L), that are of longer duration than Hyper PP, and can last for several hours or days. Myotonia is never present in Hypo PP. Thus, the presence of myotonia in a patient with periodic paralysis directs the diagnosis towards Hyper PP. In most cases of Hypo PP, the paralytic attacks take place in the early morning with the patient unable to move their limbs although facial muscles are spared. The frequency and severity of these transient attacks varies significantly between patients and variability in severity is even seen between family members (Bulman, 1997; Hong et al., 2010). Attacks are triggered by exercise, stress, some medications such as insulin, and carbohydrate rich meals. Hypo PP attacks can be ameliorated by ingestion of oral potassium.

1.4.2.3 Andersen-Tawil Syndrome (ATS)
Andersen-Tawil syndrome (ATS) is a distinct and rare variant of periodic paralysis that affects less than 10% of all periodic paralysis patients. It differs from the other periodic paralyses as it is a multisystem disorder that shows a triad of symptoms: periodic paralysis, cardiac abnormalities, and distinctive facial and skeletal characteristics (Haruna et al., 2007; Meola et al. 2009). Whilst the triad of symptoms is a defining feature of disease, the presence of all three features is variable, with lack of one often confusing diagnosis.
(Modoni et al., 2011). Onset occurs during the first or second decade often with episodes of weakness before the development of cardiac features. Attacks of paralysis are triggered by the same precipitating factors as other forms of periodic paralysis and recovery normally takes 24 to 48 hours. As with other forms of periodic paralysis attacks of weakness can occur upon awakening in the morning.

1.4.2.4 Thyrotoxic Periodic Paralysis (TPP)

Thyrotoxic periodic paralysis (TPP) is the only sporadic form of periodic paralysis to be identified. This form of periodic paralysis is found in thyrotoxic individuals. Attacks of weakness can sometimes be associated with hypokalemia and can be indistinguishable from those seen in Hypo PP patients. A higher prevalence of disease is seen in Asian and Hispanic populations - 2% compared to 0.1% in non Hispanic Caucasians (Kelley et al., 1989; Okinaka et al., 1957; Ryan et al., 2010)
1.5 Genetic Causes of Skeletal Muscle Channelopathies

1.5.1 Non-Dystrophic Myotonia

1.5.1.1 Myotonia Congenita

Myotonia congenita is caused by mutations in the gene *CLCN1* which encodes the skeletal muscle chloride channel CLC-1 (George et al. 1993; Koch et al., 1992). Over 150 mutations have been found in *CLCN1* to date associated with the dominant or recessive forms of myotonia congenita (Brugnoni et al., 1999; Mailänder et al., 1996; Meyer-Kleine et al., 1995; Plassart-Schiess et al., 1998; Sangiuolo et al., 1998; Zhang et al., 1996). *CLCN1* is composed of 23 exons with mutations causing myotonia congenita spread widely across the gene. One potential mutation hotspot has been highlighted for dominant MC in exon 8 whilst no hotspot has been identified thus far for recessive mutations (Fialho et al., 2007; Matthews et al., 2010).

Most causative mutations in dominant myotonia congenita have been found to be heterozygous missense mutations, although, two nonsense mutations: Arg894X and Glu193X, have been identified (George et al., 1994; Wu et al., 2002). Dominant mutations are localised to the subunit interface and result in the disruption of dimer interaction (Jurkat-Rott et al., 2005). In contrast, recessive mutations show more diversity in the type of mutation as indels, splice site alterations, truncations, missense or nonsense errors have been identified to date.

Recessive myotonia congenita can be caused by homozygous or compound heterozygous mutations which can result in dimers where both subunits contain a mutation. The impact of a single recessive mutation is often not sufficient to cause disease which is highlighted by heterozygous carriers of *CLCN1* recessive mutations who are often asymptomatic. The
inheritance of two recessive mutations (both subunits contain a mutation) results in a larger disruption of channel functioning which results in myotonia.

Many cases of recessive MC have been reported to have only a single mutation despite full sequencing the coding region of the gene. In addition, ~25% of patients lack any mutations in CLCN1 following full screening of the gene (Meyer-Kleine et al., 1995; Zhang et al., 1996). Raja Rayan et al. 2012 showed that 6.7% of recessive MC patients have a partial or whole exon deletion in CLCN1. Tandem analysis of CLCN1 together with SCN4A identified mutations in 93% of cases in a cohort of NDM patients (Trip et al., 2008). This highlights the importance of investigating other genes in suspected myotonia congenita cases.

Unusually, some mutations have been reported to cause both dominant and recessive forms of MC (such as R894X, G230E, T268M and R317Q), despite both forms showing different mechanisms of disease (Sun et al., 2001; Zhang et al., 1996). No clear explanation has been found for the presence of dual inheritance mutations although a number of hypotheses, such as different allelic expression, have been suggested.

### 1.5.1.2 Paramyotonia congenita and Sodium Channel Myotonia

Paramyotonia congenita and sodium channel myotonia are allelic disorders which are caused by mutations in the SCN4A gene, which encodes the voltage gated sodium channel, Na\(_{v}\),1.4 (McClatchey et al. 1992; Ptacek et al., 1991, 1993).

To date 35 pathogenic mutations within SCN4A have been identified in relation to PMC or SCM, with most causing a missense change. Mutations are widely distributed across the gene. The majority of PMC mutations are found within exons 22 and 24. The same is true for SCM although to a lesser extent (Matthews et al., 2008a; Trip et al., 2008). The most common PMC mutations have been identified as T1313M and R1448C (Jurkat-Rott et al., 2010; McClatchey et al., 1992; Ptacek et al., 1993; Ptácek et al., 1992; Yang et al., 1994).
whilst for SCM they are V1589M and G1306E (Lerche et al., 1993; Matthews et al., 2008b).

Only one indel of SCN4A has been identified thus far. Kubota et al 2011 found a small indel c.3912+6_3912+10del inG within the intronic region between exon 21 and exon 22 causing aberrant splicing of the gene. The aberrantly spliced isoform encodes a functional channel with a 35 amino acid insertion that elongates the DII-DIV loop of Na\textsubscript{v}1.4, leading to a gain of function within the channel.

About 20% of PMC and SCM cases have no identifiable mutation in SCN4A following full sequencing of the whole coding region. There is a possibility that this could be the result of unidentified intronic mutations such as the one identified by Kubota et al 2011; mutations in known genes such as CLCN1; mutations of promoter sites; or mutations within unidentified genes.

1.5.2 Periodic Paralysis

1.5.2.1 Hyperkalemic Periodic Paralysis

Hyperkalemic periodic paralysis was the first skeletal muscle channelopathy for which a causative gene was identified. Through the linkage analysis of two Hyper PP families the loci which maps to chromosome 17q23-25 was highlighted (Ptácek et al., 1991; Rojas et al., 1991). This loci contains the gene SCN4A which encodes the α subunit of the voltage gated sodium channel Na\textsubscript{v}1.4 (Fontaine et al., 1990). 10 mutations have been identified to date in relation to Hyper PP. Causative mutations are, thus far, all missense mutations which are spread across the gene; although a large number of the mutations are located within exons 13 and 24. Hyper PP mutations are located in areas that play an important role in channel gating. Clusters of mutations are seen within S4-S5 loop and S6 DIV, which have been identified as potential docking sites for the inactivation gate. The most common
Hyper PP mutations are T704M and M1592V (exons 13 and 24, respectively), which account for 60% and 30% of Hyper PP cases respectively, (Vicart et al., 2005).

A large amount of overlap is seen between Hyper PP and PMC in terms of phenotype and genetic causes. A number of SCN4A mutations, for example T704M, A1156T, M1360V and R1448C/H, which have been reported with overlapping phenotypes between PMC and Hyper PP (Brancati et al., 2003; Hayward et al., 1999; McClatchey et al., 1992; Vicart et al., 2005; Wagner et al., 1997).

Following sequencing of the whole coding region of SCN4A, ~20% of Hyper PP cases have no identified causative mutation (Matthews et al., 2008a). This might be explained by the presence of intronic SCN4A mutations, such as the intronic indel identified in PMC (Kubota et al., 2011); or mutations in currently unidentified genes.

1.5.2.2 Hypokalemic Periodic Paralysis
Hypokalemic periodic paralysis has been associated with mutations in two different genes. Initially, genetic analysis linked hypokalemic periodic paralysis to missense mutations in the gene CACNA1S. This gene encodes the α1 subunit of skeletal muscle voltage gated calcium channel CaV1.1 (Elbaz et al. 1994; Jurkat-Rott et al., 1994). However, mutations in CACNA1S alone could not explain all cases of Hypo PP. Linkage in Hypo PP families with no CACNA1S mutation revealed mutations within SCN4A (referred to as Hypo PP2) (Bulman et al., 1999).

The presence of two causative genes for Hypo PP can be in some part explained by the fact that these proteins have homologous structures. Mutations in both CACNA1S and SCN4A are not located to a particular exon, but map to the conserved S4 segment of the voltage sensing domain. Out of 21 Hypo PP mutations, 18 have been shown to cause neutralisation of the positively charged residues of the voltage sensor (Matthews et al., 2009). Three mutations have been found outside of the voltage sensor of either channel. V876E and
H916Q in the calcium channel and P1158S of the sodium channel were found in patients with a conventional Hypo PP phenotype. For V876E mutation regions of the gene corresponding to the voltage sensors were not ruled out.

*CACNA1S* accounts for 70% of Hypo PP cases whilst just 10-20% of affected individuals have *SCN4A* mutations (Miller et al., 2004; Venance et al., 2006). In 10-20% of cases the genetic cause is unaccounted for which indicates the possibility of an unidentified causative gene.

### 1.5.2.3 Andersen-Tawil Syndrome

Andersen-Tawil Syndrome is caused by mutations within the potassium channel gene, *KCNJ2*, which encodes the inwardly rectifying potassium channel Kir2.1 (Plaster et al., 2001). ~70% of ATS cases can be accounted for by mutations in *KCNJ2* (Smith et al., 2006; Weir et al., 2011). Over 60 mutations have been identified in *KCNJ2*, to date, in relation to ATS, most of which are loss of function mutations. However following sequencing of *KCNJ2*, 30% of patients remain without a genetic diagnosis. Recently, a new gene has been associated with ATS – *KCNJ5* which encodes the inwardly rectifying potassium channel Kir3.4. Mutations in *KCNJ5* have been previously associated with LQT syndrome. Kokunai et al 2014 identified a *KCNJ5* mutation in an ATS family which was found to mimic a *KCNJ2* loss of function mutation. Currently, no genotype-phenotype correlation has been identified within ATS patients due to the high variability in symptoms (Kimura et al., 2012).

### 1.5.2.4 Thyrotoxic Periodic Paralysis

Thyrotoxic periodic paralysis has been associated with mutations in a newly indentified ion channel gene, *KCNJ18* which encodes the inwardly rectifying potassium channel Kir2.6 (Cheng et al., 2011; Ryan et al., 2010). However, the genetic cause of TPP is often unresolved as less than 50% of TPP cases are accounted for by mutations in *KCNJ18*. Thus far, seven mutations have been identified in *KCNJ18* in association with TPP (Ryan et al., 2010). Two further mutations within the gene have been identified in sporadic periodic
paralysis (SPP), a non familial form of periodic paralysis without hyperthyroidism (Cheng et al., 2011). The majority of mutations within \textit{KCNJ18} affect the C terminal region of the protein. A single nucleotide deletion has been identified leading to a frameshift and stop codon (c.428del C, p.Ile144fs). The \textit{KCNJ18} gene is transcriptionally regulated by the thyroid hormone (Ryan et al., 2010).
<table>
<thead>
<tr>
<th></th>
<th>Hypo PP</th>
<th>Hyper PP</th>
<th>ATS</th>
<th>TPP</th>
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<td>\textit{SCN4A}</td>
<td>\textit{KCNJ2}</td>
<td>\textit{KCNJ18}</td>
</tr>
<tr>
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<td>Dominant</td>
<td>Dominant</td>
<td>Dominant</td>
</tr>
<tr>
<td><strong>Age of onset</strong></td>
<td>1\textsuperscript{st} or 2\textsuperscript{nd} decade</td>
<td>1\textsuperscript{st} decade</td>
<td>1\textsuperscript{st} or 2\textsuperscript{nd} decade</td>
<td>1\textsuperscript{st} or 2\textsuperscript{nd} decade</td>
</tr>
<tr>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Length of Attacks</strong></td>
<td>Hours to days</td>
<td>Minutes to hours</td>
<td>Minutes to days</td>
<td>Minutes to days</td>
</tr>
<tr>
<td><strong>Ictal K+ Levels</strong></td>
<td>Low</td>
<td>High or normal</td>
<td>Low, normal or high</td>
<td>Low, normal or high</td>
</tr>
<tr>
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<td>Potassium</td>
<td>Rest after exercise</td>
<td>Rest after exercise</td>
</tr>
<tr>
<td></td>
<td>Rest after exercise</td>
<td>Rest after exercise</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Yes</td>
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<td>Sometimes</td>
</tr>
<tr>
<td><strong>Myotonia</strong></td>
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<td>Sometimes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
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<td>No</td>
<td>Dysmorphic Features &amp; cardiac conduction defects</td>
<td>Hyperthyroidism</td>
</tr>
</tbody>
</table>

Table 1-2 Clinical features of periodic paralysis
1.6 Treatment for skeletal muscle channelopathies

Patients with skeletal muscle channelopathies have symptoms which continue throughout adulthood, can worsen with age leading to permanent muscle weakness and can impact greatly on the quality of life. In addition, there is a range of severity experienced by patients resulting in a need for a variable approach to treatment depending on the patient.

The current treatments for the skeletal muscle channelopathies are imperfect. For the main treatments of the skeletal muscle channelopathies, adverse side effects pose a problem especially due to involvement of cardiac muscle seen in ATS and following administration of anti-arrhythmic Mexiltine for treatment of myotonia. In addition, little is known about the mechanism of action or efficacy of the therapies used and too few randomised control trials have been conducted. Further research is needed in order to fully understand the mechanism by which the therapies work. The ideal treatment would involve targeting the specific protein dysfunction that results in the attacks of myotonia or paralysis in patients. This would hopefully lead to better efficacy in treatments with fewer side effects due to tissue specific molecular targets.

1.6.1 Non-dystrophic myotonia

In some mild cases treatment of myotonia can simply include avoidance of triggering factors; such as exposure to cold or demanding exercise, however this is not sufficient for those who are more severely affected.

The current treatments available for NDM involve the use of off label drugs with sodium channel blocking activity, such as anti-epileptic and anti-arrhythmic drugs. A number of drugs used have been given anecdotal support for the use for treating myotonia such as quinine and phenytoin (Matthews et al., 2010; Raja Rayan & Hanna, 2010; Trivedi et
The most common drug used to treat myotonia is mexiletine which is an anti-arrhythmic drug that blocks sodium channels of both skeletal and cardiac muscle in a use dependent manner.

Few controlled trials have been conducted looking at the efficacy of treatments for the Non-Dystrophic Myotonias. A 2006 Cochrane review found that there was insufficient data available in order to consider any treatment safe and effective for NDM (Trip et al., 2006). Difficulties arise in performing randomised trials for such a rare disorder which shows a vast amount of genetic and phenotypic variability. Until 2012 Mexiletine had only shown to be effective through an uncontrolled clinical trial (Kwieciński et al., 1992). The first randomized controlled trial of Mexiletine for the treatment of myotonia was conducted by CINCH (the consortium for clinical investigation of neurologic channelopathies). This trial showed that Mexiletine significantly improved patient stiffness although some adverse cardiac events were reported (Statland et al., 2012; Trivedi et al., 2012).

### 1.6.2 Periodic paralysis

An important part of treatment of periodic paralysis is avoiding factors that can trigger an attack of weakness. In Hyper PP this involves avoiding potassium rich foods and fasting whilst for Hypo PP, patients are recommended to eat small regular meals to avoid carbohydrate loading.

As with treatment of the non-dystrophic myotonias, most drugs used have been shown to work through case reports or anecdotal evidence. Onset of attacks of paralysis can be treated through the intake of oral potassium, in Hypo PP, or inhalation of salbutamol, in Hyper PP. For both forms of periodic paralysis, prophylactic treatment mainly occurs through the administration of the carbonic anhydrase inhibitors: acetazolamide or
dichlorphenamide. These drugs have been shown to have beneficial effects on attack frequency and muscle strength.

Acetazolamide and dichlorphenamide have been shown to activate of calcium activated potassium channels – BK channels. Both drugs have been shown to prevent paralysis and weakness in potassium depleted rats by activating the BK channels and enabling the repolarisation of skeletal muscle fibres (Tricarico et al., 2006). Increased hyperpolarizing K+ current would counteract the abnormal depolarization of the muscle fibres and may account for some of the beneficial effects of these drugs. However, it is still not fully understood how carbonic anhydrase inhibitors work to improve Hypo PP symptoms. It has been suggested that they may act to help deal with the acidic environment of the skeletal muscle which could be caused by the gating pore current (Matthews & Hanna, 2010).

It has been reported that acetazolamide is unable to prevent or in fact worsens attacks of paralysis in some Hypo PP patients (Matthews et al., 2011). Furthermore acetazolamide has been shown to prevent myopathy in potassium depleted rats, although the ability to avert progressive myopathy of Hypo PP patients is unclear (Tricarico et al., 2008).

Recent studies of the mouse models of hypo periodic paralysis (Hypo PP Na\textsubscript{v}1.4 R669H & Ca\textsubscript{v}1.1 R528H) have shown bumetanide could be a potential new treatment for Hypo PP. Bumetanide acts upon the Na-K-2Cl transporter within skeletal muscle (van Mill et al., 1997). It was shown that bumetanide prevents development of weakness and restores force during an attack (Wu et al., 2013a; Wu et al., 2013b). Trials are now underway in order to establish the efficacy of bumetanide in the treatment of the human disease. Bumetanide may act to prevent muscle depolarization by altering the intracellular chloride concentration which is thought to predispose the skeletal muscle to paradoxical depolarization (Geukes Foppen et al., 2002; van Mil et al., 1997; Wu et al., 2013).
There have been very few randomised control trials that study the effectiveness of drugs for the treatment of periodic paralysis. A 2008 Cochrane report found one randomised control study of dichlorphenamide which was shown to be effective in the prevention of attacks of weakness in both hypo and hyperkalemic periodic paralysis. Two small studies provided evidence that acetazolamide could improvement of muscle strength. The report concluded that there was still insufficient evidence in order to provide guidelines for the treatment of periodic paralysis (Sansone et al., 2008).

Treatment of ATS is complicated by the necessity of treating the triad of symptoms present. As with other forms of periodic paralysis, treatments are based on current anecdotal evidence rather than detailed controlled studies. It has been suggested that the carbonic anhydrase inhibitors may have a beneficial effect on the frequency of attacks for ATS patients, but the effect on the cardiac muscle is still unclear. Combining treatment with oral potassium has been shown to have a beneficial effect on cardiac muscle (Sansone & Tawil, 2007). The standard treatment for the cardiac symptoms of ATS is through the use of beta-adrenergic blockers such as propranolol or calcium channel blockers such as amlodipine and nifedipine (Sansone & Tawil, 2007). Treatment of the cardiac symptoms of ATS through the use of anti-arrhythmic drugs can result in the exacerbation attacks of paralysis in some cases.

For TPP the main form of treatment is treatment of the thyrotoxocosis as there are no specific treatments for attacks of paralysis. Acetazolamide cannot be used in TPP patients because it is thought to worsen symptoms experienced in these patients (Tricarico & Camerino, 2011).
1.7 Pathomechanism of Skeletal Muscle Channelopathies

1.7.1 Hyperkalemic Periodic Paralysis, Paramyotonia Congenita and Sodium Channel Myotonia

The allelic diseases of Hyper PP, PMC and SCM show a spectrum of phenotypes from enhanced excitability (myotonia) to reduced excitability (periodic paralysis) with fluctuation and overlap between the two.

The common mechanism behind these disorders is the presence of gain of function mutations which lead to an increase in the inflow of Na⁺ ions and subsequent increase in the depolarisation of the muscle membrane (Jurkat-Rott et al., 2010). This depolarisation activates more sodium channels leading to repetitive firing of action potentials; the hallmark of myotonia. Myotonic activity of the muscle leads to increased sodium inflow and depolarisation. If the depolarisation is large enough the sodium channels may enter an inactivated state. Inactivation of sodium channels prevents cells from firing action potentials, resulting in flaccid, inactive muscle; the hallmark of periodic paralysis (Jurkat-Rott et al., 2010).

Sodium channels mutations often disrupt fast inactivation or increase channel activation (Cannon, 2000). Whilst different mutations target the same gating mechanisms, the impairment varies depending on the disorder which results in the difference in phenotypes despite similar mutation defects. The extent of depolarisation is predicted to be smaller for mutations causing myotonia compared to Hyper PP mutations which is why the main symptom is myotonia with some transient weakness (Bendahhou et al., 1999; Cannon, 2000; Rojas et al., 1999).
Most PMC and SCM mutations cause a reduced rate of channel fast inactivation but do not affect the extent of the inactivation. In addition, mutations also result in an enhanced rate of recovery from inactivation. These defects lead to an increase in the number of sodium channels available for activation following an action potential, increasing muscle excitability (Bouhours et al., 2004; Mitrovic et al., 1996; Plassart-schiess et al., 1998).

Hyper PP mutations have been reported to disrupt both channel inactivation and/or activation. Some mutations, such as M1592V, cause a slower rate of channel inactivation and prevent complete fast inactivation (Bendahhou et al., 2002; Hayward et al., 1999; Rojas et al., 1999). Following a prolonged depolarisation, 1.5-5% of mutant channels remain open, whilst the majority of WT channels undergo full fast inactivation with only 0.1% of channels remaining open (Cannon et al., 1991). This extended opening of the sodium channel persists until the channel closes upon repolarisation. The persistent current depolarizes the membrane, causing inactivation of the sodium channels rendering the muscle inexcitable. For some Hyper PP mutations the voltage dependence of fast inactivation is shifted to depolarising direction, which results in increased Na\(^+\) currents, muscle depolarisation, inactivation of the sodium channels and muscle inexcitability (Cannon, 2002).

Other Hyper PP mutations, such as T704M and I693T, cause a hyperpolarising shift of the voltage dependence of channel activation (Bendahhou et al., 2002, Bendahhou et al., 1999; Brancati et al., 2003; Yoshinaga et al., 2012). These mutant sodium channels open at potentials where the channel would normally be closed. A small hyperpolarising shift (~5mV) in the voltage dependence of activation can cause a large depolarisation of the membrane potential (Cannon, 2002; Trivedi, Cannon, & Griggs, 2014b).
Skeletal muscle channelopathies show a spectrum of electrical excitability due to significant amount of overlap in terms of causative gene and disease mechanism. This leads to further overlap in terms of patient symptoms. Adapted from Cannon 2006.
1.7.2 Hypokalemic Periodic Paralysis

Early functional studies of Hypo PP mutations using heterologous expression systems demonstrated reduced current density and small shifts in channel kinetics (i.e. a small shift in voltage dependence of inactivation), suggesting a loss of channel function (Bulman et al., 1999; Kuzmenkin et al., 2002; Struyk et al., 2000). However, small loss of function defects could not explain the paradoxical depolarisation of Hypo PP muscle fibres in low potassium or the pathophysiological basis of paralytic attacks of weakness in low potassium.

The key to elucidating the mechanism for Hypo PP came through structure-function voltage sensor experiments of the Shaker K+ channel which revealed that mutations of the S4 arginine residues provided a pathway for cations to move through the voltage sensing domain (Starace & Bezanilla, 2001; Tombola et al., 2005). These aberrant currents were described as “gating pore currents”- or “omega currents”. Coincidently, Hypo PP mutations affect the conserved arginine residues within the S4 voltage sensor of both NaV1.4 and CaV1.1 causing loss of these charged residues. Thus, it was hypothesised that Hypo PP mutations might cause an aberrant ionic leak current through the gating pore of the CaV1.1 and NaV1.4 VSDs resulting in the depolarization seen in Hypo PP muscle fibers (See Figure 1-6).

An aberrant gating pore current in Hypo PP mutant channel was first reported by Sokolov et al. 2007 who demonstrated a hyperpolarization activated cationic leak through the voltage sensing domain in the Hypo PP mutations R672G, R672H and R669H of NaV1.4. The findings on R669H mutant channels were confirmed by Struyk et al. 2007. This current was shown to be carried by protons rather than other cations, such as Na+ ions, which act as the charge carriers for the R672G mutation. In addition, the domain III mutations R1132Q and R1135C/H have been reported to cause gating pore currents. R1132Q is activated at
potentials more negative than -60 mV and has a preference Na⁺ ions as the charge carrier (Francis et al., 2011). In contrast R1135C/H mutations result in outward gating pore currents at positive potentials but inward gating pore currents following S4 immobilization during channel inactivation (Groome et al., 2014). A further type of periodic paralysis, referred to as normokalemic periodic paralysis, is caused by R675G mutations in NaV1.4. As seen with R1135C/H, R675G mutant channels carry gating pore currents at depolarising potentials but also cause inward currents following channel inactivation (Sokolov et al., 2008).

In addition to a depolarising gating pore current, it has been suggested that sarcolemmal depolarisation is a result of impairment of the potassium conductance which governs the resting membrane potential (V_{rest}). In vitro studies of Hypo PP muscle fibres have shown the muscle fibres depolarise in the presence of low external potassium, whereas normal muscle hyperpolarises when exposed to the same conditions (Rudel et al., 1984; Ruff, 1999). Additionally, inhibition of the outward currents from inward rectifying potassium (Kir) channels have been shown to cause paradoxical depolarisation of muscle fibres exposed to low potassium (Jurkat-Rott et al., 2009; Struyk & Cannon, 2008). It is suggested that a modest impairment of Kir conductance increases the susceptibility of the muscle to depolarisation in response to low extracellular potassium concentrations (Struyk & Cannon, 2008). The presence of a depolarising gating pore current is thought to increase the probability that low extracellular potassium will cause depolarisation of V_{rest} (Jurkat-Rott et al., 2009; Jurkat-Rott et al., 2012; Struyk et al., 2008).

However it is not fully understood how disruption to CaV1.1 or NaV1.4 reduces the Kir conductance. One suggestion is that the gating pore current disrupts the sarcolemma intracellular pH homeostasis, leading to intracellular acidification which is known to inhibit Kir channels (Matthews & Hanna, 2010).
Figure 1-6 Schematic representation of gating pore domain and ionic leak currents for normal and R2 mutant sodium channels.
1.8 Aims of Project

The overall aim of this thesis was to improve our fundamental understanding of the genetic heterogeneity and mechanism of disease of the skeletal muscle channelopathies, in particular, periodic paralysis. My work had two main components: a genetic study of skeletal muscle channelopathy cohorts and a functional analysis of mutant skeletal muscle ion channels with the specific aims of:

1. Investigating the genetic causes of periodic paralysis in cohorts without the common mutations (Chapter 3).

2. Investigating the genetic causes of myotonia in cohorts without the common mutations within SCN4A and mechanisms of dual mode of inheritance in myotonia congenita (Chapter 4).

3. Assessing the functional impact of novel sodium channel mutations which cause Hypo PP (Chapter 5).

4. Structure- function studies of the gating pore domain (Chapter 5).

5. Assessing the functional impact of novel sodium channel mutations which cause myotonia (Chapter 6).
Chapter 2: Methods

2.1 Patient Cohort

The MRC Centre for Neuromuscular Diseases is the NHS England centre for channelopathies and provides the national diagnostic clinical screening service. Patients were chosen from the database of cases that had been referred to the MRC Centre for Neuromuscular Diseases with a suspected skeletal muscle channelopathy but were negative for the common mutations. Informed consent was given for all samples used.

2.1.1 Periodic paralysis cohort

A large cohort of periodic paralysis patients who lacked mutations in the known genes (CACNA1S, or SCN4A) was identified. The cohort was then divided into two sub groups: well characterised cohort and the less characterised cohort. Those patients in the well characterised cohort had a well characterised phenotype, which followed the documented phenotype for periodic paralysis. All patients in this cohort had supportive electrical studies and were thought to have a high likelihood of finding a genetic cause. Patients in the second cohort had a wide range of phenotypes with some uncharacterised symptoms for periodic paralysis. Not all patients had supportive electrical testing.

2.1.2 Myotonia cohorts

Two cohorts of myotonia patients were chosen for candidate gene screening. The first contained patients with a well characterised PMC or SCM phenotype and supportive electrical studies. Mutations in SCN4A and CLCN-1 had been previously ruled out. The second cohort consisted of 10 dominant myotonia congenita families where a single
mutation in \textit{CLCN-1} had been identified through genetic screening. For all families the mutation identified had been previously reported as showing dominant and recessive patterns of inheritance.

\section*{2.2 Genetic Screening of Patient Cohorts}

\subsection*{2.2.1 Exonic Sequencing}

Sequencing of four candidate genes: \textit{SCN4A}, \textit{CACNA1S}, \textit{KCNJ18}, MT-ATP6 and MT-ATP8 was undertaken to identify new causative mutations in the cohort of patients. Primers for the target exons were designed against \textit{SCN4A}, \textit{CACNA1S} and \textit{KCNJ18} (GenBank accession numbers NM_000334, NM_000069 & FJ434338 respectively) using Primer3 (See Appendix for primer sequences).

\subsection*{2.2.1.1 \textit{SCN4A} \& \textit{CACNA1S} PCR reactions}

To amplify the desired exons for \textit{SCN4A} and \textit{CACNA1S} a PCR mastermix was made containing 12.5 µl of AmpliTaq Gold 360 mastermix (Applied Biosystem ABI, USA), 10pmol/µl primers and DNA in a 25µl reaction. PCR conditions consisted of an initial denaturing step of 95 °C for 10mins followed by 30 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for 30 seconds and a final step of 72 °C for 7 minutes. Samples were then run on a 2 % agarose gel for 30minutes at 60 V to determine the success of the reaction. Following PCR, samples were diluted to 100 µl with nanopure water, transferred to a PCR cleanup filter plate (Millipore, USA) and placed on a vacuum for 5minutes. 50 µl of water was then added to resuspend and the plate was placed on a shaking platform for 30minutes.
2.2.1.2 *KCNJ18* PCR Reaction

The whole coding exon of *KCNJ18* was amplified by PCR using 12.5 µl of AmpliTaq Gold 360 mastermix (Applied Biosystem ABI, USA), 10pmol/µl primers, 1.5 µl of GC enhancer and 2 µl of DNA in a 25µl reaction. PCR conditions consisted of an initial denaturing step of 95 °C for 10mins followed by 30 cycles of 95 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 2 minutes and a final step of 72 °C for 7 minutes. Samples were then run on a 0.8% agarose gel. Following PCR, samples were diluted to 100 µl with nanopure water, transferred to a PCR cleanup filter plate (Millipore, USA) and placed on a vacuum for 5minutes. 50 µl of water was then added to resuspend and the plate was placed on a shaking platform for 30minutes.

2.2.1.3 Mitochondrial genes: MT-ATP6 & MTP-8 PCR Reaction

The mitochondrial genes MT-ATP6 and MT-ATP8 were amplified through PCR using three primer pairs. PCR mastermix was made containing 6.5 µl of AmpliTaq Gold 360 mastermix (Applied Biosystem ABI, USA), 5pmol/µl primers and DNA in a 12µl reaction. PCR conditions consisted of an initial denaturing step of 95 °C for 10mins followed by 30 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for 30 seconds and a final step of 72 °C for 7 minutes. Samples were then run on a 2 % agarose gel for 30minutes at 60 V to determine the success of the reaction. Following PCR, samples were diluted to 100 µl with nanopure water, transferred to a PCR cleanup filter plate (Millipore, USA) and placed on a vacuum for 5minutes. 75 µl of water was then added to resuspend and the plate was placed on a shaking platform for 30minutes.

2.2.1.4 Sequencing of PCR products

Sequencing of PCR products was done using ABI Big Dye terminator kit version 1.1 (ABI), primers (3.2pmol/µl) and 2 µl of clean PCR product. M13 primers were used for *CACNA1S*
and SCN4A, whilst primers designed against the gene sequence were used for KCNJ18, MT-ATP6 and MT-ATP8 (see primer sequences).

PCR conditions consisted 25 cycles of 95 °C for 10 seconds, 50 °C for 5 seconds, 60 °C for 4 minutes. Samples were then diluted to 20µl with nanopure water and a dye terminator kit (Thermo Scientific, USA) was used to remove excess dye from the sequencing reaction. Samples were then run on a 3730x sequencing machine and the sequencing data obtained was analysed using SeqScap V2.5 (Applied Biosystems, USA). In silico analysis using alamut software and PolyPhen was used to determine whether mutations were likely to be pathogenic (Adzhubei et al. 2010). Mutations found were confirmed by repeating from initial PCR.

2.2.2 Whole-Exome Sequencing

Samples were selected on the basis of good family history and exclusion of mutations in known genes. Genomic DNA was characterized by whole-exome sequencing by Neuromics, who dealt with data acquisition and primary data analysis. Data was then analysed using the online DeCode Health software (sequence miner and sequence analyser). Synonymous and non-coding variants that didn’t affect splice sites were filtered out. An autosomal dominant inheritance pattern was predicted for the family and thus variants which had a frequency of >0.01 using 1000genomes were filtered out. Additionally, a skeletal muscle gene list was used to filter out any variants which are not expressed in the skeletal muscle. SNPs in public databases, read dept and predicted impact on protein were also used to filter variants. The Decode Health software focuses on filtering for candidate genes or known variants. Candidate genes were selected based on the association with the known symptoms of the patients. All known pathogenic variants were also highlighted by the software. All other variants were then filtered based on read dept, inheritance pattern and predicted impact on the protein.
2.3 Functional Analysis of mutations

2.3.1 Plasmids

The wild type human SCN4A and rat SCN4A clones were gifts from Prof. Steve Cannon (University of Texas Southwestern Medical Center, Dallas, USA). The human SCN4A vector is a mammalian expression vector, pRc/CMV. The rat SCN4A vector is a pGEMHE vector and the untranslated regions of a Xenopus β-globin gene for expression within *Xenopus* oocytes. It contains a NHeI restriction site for linearization during RNA preparation and a T7 promotor site for RNA transcription.

2.3.2 Transformation of chemically competent cells & DNA Prep

30µl of Top10 competent cells (Invitrogen, USA) were mixed with at least 50ng of plasmid DNA in round bottomed tubes (BD Biosciences, USA) and incubated for 30 minutes on ice. Tubes were then heat shocked at 42 °C for 40 seconds in water bath before returning to ice for a further 2 minutes. 200 µl of fresh LB was added to each tube, which were then incubated at 37 °C shaking at 200rpm. Following incubation 100 µl was then plated onto LB agar plates with 100 µg/µl of ampicillin and incubated overnight at 37 °C. Tubes containing 2-6 ml of LB with 100µg/µl of ampicillin were then inoculated with single colonies and incubated shaking at 37 °C overnight. Transformed plasmid DNA was then extracted using a GenElute Plamid mini prep kit (Sigma-Aldrich, USA). The concentration of purified DNA was measured using a nanodrop spectrophotometer.

2.3.3 Mutagenesis

Either human or adult rat wild type skeletal muscle Na⁺ channel α subunit (hNav1.4 or rNav1.4) subcloned into the mammalian expression vector pRC-CMV or the Xenopus expression vector pGEMHE respectively (a kind gift from Prof Cannon, UT Southwestern,
USA), were used as a template for site directed mutagenesis using the QuickChange Mutagenesis kit (Stratagene, USA). Primers were designed to introduce the substitution mutation by altering the appropriate codons. Direct sequencing was used to confirm correct point mutation had been introduced.

### 2.3.4 Functional analysis within oocytes

#### 2.3.4.1 RNA preparation & Oocyte Injection

Circular plasmid DNA containing the rat SCN4A insert was linearized with the restriction enzyme Nhel (NEB, USA) (10 µg of DNA, 10µl of 10x buffer 2 (NEB), 100x BSA, 5 µl Nhel in a total volume of 100 µl at 37 °C for 2 hours). Following linearization the DNA was cleaned using Geneclean II kit (MP bio) or QIAquick PCR purification kit (Qiagen, Netherlands). 2µg of linearised DNA was used for in vitro transcription of cRNA using Ambion T7 mMessage mMachine kit (Life Technologies, USA). The reaction mixture contained: 10µl 2x NTP, 2 µl 10x Reaction buffer, 2 µl Enzyme mix and 2µg of linear template DNA made up to 20 µl with water. Following incubation at 37 °C for 2 hours, 1 µl of DNAse was added to the reaction and incubated at 37 °C for a further 15minutes. Lithium chloride precipitation was used to extract the RNA. 30 µl of H2O and 30 µl of LiCl precipitation solution was added to the reaction. The mixture was put at -20 C overnight. The mixture was then centrifuged for 15mins at 13,000rpm and 4 °C. The supernatant was removed, 500µl of 70% ethanol added and spun again at 4 °C. The supernatant was removed and the pellet was resuspended in 20 µl of RNAse free H2O. RNA concentration was measured using nanodrop spectrometer.

Stage V-VI Xenopus laevis oocytes were harvested from female Xenopus laevis frogs and stored in MBS media (87 mM NaCl, 1 mM KCl, 1.68 mM MgSO4, 10 mM Heps, 0.94 mM NaNO3, 2.4 mM NaHCO3, 0.88 mM CaCl2). The follicular layer was removed by incubation with 2mg/ml Collagenase A (Roche diagnostics, Switzerland) in MBS for 90minutes. Healthy
Stage V–VI oocytes were selected and stored in OR-2 media (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM Hepes) with 100 U/ml penicillin-streptomycin and stored at 14-16 °C. 1 to 3 days after harvest oocytes were injected with ~50 ng of cRNA using a Drummond Nanoject microinjector pipette (Drummond Scientific Company, USA). 3.5” glass capillaries (Nanolitre replacement tubes, Drummond Scientific) were formed using a multistage-stage puller (Zeitz DMZ Universal Puller, AutoMate Scientific, USA). The tip was broken manually and the electrode backfilled with mineral oil (Sigma-Aldrich, USA). For Naᵥ1.4 experiments, WT or mutant channels were mixed in equal volumes with β1 subunit and injected into the oocyte cytoplasm. Injected oocytes were incubated at 18 °C in L-15 medium supplemented with Horse Serum, Pen/strep and Gentamycin.

2.3.4.2 Gating Pore Current Analysis

To analysis gating pore currents in Hypo PP mutations, two different techniques were used: Cut open voltage clamp (COVC) and Two Electrode Voltage Clamp (TEVC). COVC was used to validate the data obtained through TEVC experiments.

2.3.4.3 Two Electrode Voltage Clamp

A two electrode voltage clamp set up was used to investigate the mutations expressed in Xenopus oocytes 2-6 days after injection. The voltage clamp was controlled by an GeneClamp 500B amplifier (Axon Instruments, USA). The amplifier was controlled and signals recorded using pClamp 7 software together with a DigiData1200A A/D converter (Axon Instruments, USA). Microelectrodes were fabricated from borosilicate glass (Harvard Apparatus, USA) using a multi stage puller (AutoMate Scientific, USA) to an O.D of 3-5µm for the voltage electrode and 7-9 µm for the current electrode. The tip resistance of the electrodes were <2MΩ and <1MΩ respectively with both electrodes filled with 3M KCl.
Ag+/AgCl coated electrodes were connected the amplifier headstage and glass agar bridges used to connect the bath electrodes to the main camber.

Ionic currents were measured in response to ms voltage steps from the holding potential of -100 mV to test potentials of -100 to +45mV in 5 mV increments. Steady state currents were measured in response to 300 ms voltage steps from the holding potential of -100 mV to test potentials of -140 mV to + 50 mV. The holding potential was varied for some experiments was changed to 0 mV.

In experiments studying the voltage dependence of gating pore currents a Cl- free Na+ bath solution (referred to as NaMes: 120 mM NaMes, 1.8 mM CaSO4 and 10mM Hepes, pH7.4 with NaOH) was used. 1 μM TTX was present to block sodium currents. For ion selectivity experiments, Names was substituted for different solutions: Kmes (120 mM potassium methanesulfonate, 1.8M calcium sulfonate & 10 mM Hepes, pH7.4 with KOH), Guanidinium (120 mM Guanidinium methanesulfonate, 1.8M calcium sulfonate & 10 mM Hepes, pH7.4 with NaOH), NMDG-Mes (120 mM NMDG, 1.8M calcium sulfonate & 10 mM Hepes, pH7.4 with methanesulfonic acid) and NMDG-Cl (120 mM NMDG, 1.8M calcium sulfonate & 10 mM Hepes, pH7.4 with HCl).

2.3.4.4 Cut Open Voltage Clamp

Oocytes were voltage clamped in the cut open configuration and controlled by a CA-1B amplifier (Dagan Corportation, USA). The amplifier was controlled and signals recorded using pClamp 7 software together with a DigiData1200 A/D converter (MDS Analytical Technologies, USA). The lower oocyte membrane was permeabilized with 0.1% saponin to allow ionic control over the intracellular solution. Electrodes were fabricated from borosilicate capillary glass (1.5mm OD thin wall with filament; WPI) using a multi-stage puller (Sutter Instrument Co., USA) and were filled with 3M KCl.
The external bath solution contained to approximate the mammalian physiologic monovalent cation gradient contained: 112 mM NaOH, 3 mM KOH, 1.5 mM CaOH$_2$, 4.5 mM BaOH$_2$, 10 mM HEPES, pH 7.4 with methane sulfonic acid. The external solution contained 1 µM TTX. The internal solution contained: 103 mM KOH, 7 mM NaOH, 10 mM EGTA, 10 mM HEPES, pH 7.4.

Currents were filtered at 5kHz and sampled at 100kHz for gating charge displacement measurements or filtered at 1kHz and sampled at 10kHz for steady state currents.

Gating pore currents were measured following a 30 ms voltage step from a holding potential of -60 mV to test potentials of -130 to +40 mV.

2.3.4.5 Data Analysis

Gating pore currents were analysed by removing non-specific leak currents through leak subtraction at linear using the linear component of currents occurred after recording to remove background non-specific leak from across the oocyte membrane allowing isolation of gating pore currents. The peak Na$^+$ currents were recorded for all cells before the addition of TTX to the bath solution. Gating pore currents were normalised to the peak Na$^+$ currents in order to compensate for differences in expression levels. Data was analysed using a combination of ClampFit 9 (Molecular Devices), Excel (Microsoft) and Origin Pro 9 (OriginLab) software packages.

2.3.5 Functional analysis in HEK cells

2.3.5.1 HEK cell culture & Transfection

HEK293 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich, USA) with 10% fetal bovine serum within T25 flasks. using GFP as a marker, hSCN4A DNA was transfected into HEK293 with Lipofectamine 2000(Invitrogen, USA) as per the
manufactures instructions. After 24 hours, cells were passaged onto 10 mm round coverslips.

2.3.5.2 Whole cell patch clamp

Whole cell patch recordings were made at room temperature, 1-3 days following transfection, using an Axopatch 200B amplifier (Axon Instruments, USA). All data were sampled at 50kHZ and filtered at 10kHZ. Any junction potential errors were not corrected for. Electrodes were formed from borosilicate glass tubes ((G150F, Harvard Apparatus) using a multistage puller (AutoMate Scientific, USA). The external bath solution contained: 145 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM Hepes, pH 7.4 with NaOH. The internal pipette solution contained: 145 mM CsCl, 5 mM NaCl, 10 mM EGTA, 10 mM HEPES, pH 7.35 with CsOH. A custom in house written program was used in LabView software for data acquisition (DM Kullmann, Institute of Neurology, UCL).

2.3.5.3 Protocols

The voltage dependence of activation was measured from voltage steps from holding potential of -80 mV to test potentials of -130 mV to +60 mV in 10 mV increments. The voltage dependence of fast inactivation was measured using a 300 ms conditioning voltage step from -130 mV to 60 mV in 10 mV increments, followed by a constant test pulse step to -10 mV. The voltage dependence of slow inactivation was measured using a 10s conditioning prepulse to test voltages (-130 mV to -60 mV), then a 20 ms step to -100 mV to allow recovery from fast inactivation, followed by a 20 ms test pulse to 0 mV.

2.3.5.4 Data Analysis

Any recordings resulting in series resistance errors greater than 5 mV were discarded straight away. All data were fitted to a Boltzmann function in order to obtain voltage values.
at which half of the channels were activated (or inactivated) \( (V_{0.5}) \) and the offset current \( (C) \). The resulting current responses were analyzed using LabView, Clampfit (Axon Instruments) and Origin9.0 (Originlab).
Chapter 3: Sequencing of Patients with Periodic Paralysis

3.1 Summary

In periodic paralysis about 20-30% patients remain without a genetic diagnosis following sequencing of the known common mutations. Normal diagnostic testing is targeted to specific areas containing the common mutations in either the sodium or calcium channel, which leaves a large part of the gene unsequenced. In addition, mutations have been identified which are located outside of the common sequencing areas suggesting that further areas require screening. The aim of this study was to identify novel mutations that cause periodic paralysis in order to further the understanding of the genetic heterogeneity present in the disease.

The S4 segments of CACNA1S and the whole coding region of SCN4A were screened in a small cohort of patients. Five novel mutations were found in SCN4A, two causing Hypo PP (c.502T>G, p.Try168Asp and c.4259A>G, p. Asp1420Gly) and three associated with Hyper PP (c.665G>A, p.Arg222Gln; c.1207A>C, p.Met403Leu and c1957A>G, p.Ser653Gly). Both Hypo PP mutations were found in areas of the channel not normally associated with disease; indicating that Hypo PP mutations are not restricted solely to the voltage sensor. The Hyper PP mutations identified were all located in areas close to known mutations and together with the Hypo PP mutations support the notion that diagnostic screening should be extended to the whole gene.

Three candidate genes were screened in a larger cohort of patients: MT-ATP6, MT-ATP8 and KCNJ18, in order to investigate whether pathogenic mutations in these genes could cause a pure periodic paralysis phenotype. 60 samples were screened for mutations in
these genes. No mutations were found in MT-ATP6 or MT-ATP8. One mutation was identified in KCNJ18, although this is unlikely to be the causative mutation as the patient was also found to have a normokalemic periodic paralysis SCN4A mutation, p.Arg675Gln. These findings indicate that mutations in these genes could not be a common cause of periodic paralysis phenotype for MT-ATP6 and MT-ATP8 or a non Asian population for KCNJ18.
3.2 Introduction

Mutations in two genes have been identified to be the main cause of periodic paralysis – SCN4A and CACNA1S. Mutations identified to date within these genes account for ~75% of all periodic paralysis cases. Targeted testing for diagnostics of periodic paralysis involves the sequencing of only four exons: two SCN4A exons (exons 13 & 24) and two exons in CACNA1S (exons 11 & 30). Hypo PP is caused by mutations in both SCN4A and CACNA1S, whilst Hyper PP is caused by mutations only in SCN4A (Jurkat-Rott et al. 1994; Jurkat-Rott et al. 2000; Sternberg et al. 2001; Davies et al. 2001; Miller et al. 2004; Vicart et al. 2004).

Whilst for most cases the causative mutation is found in these areas, the full genetic and mutation spectrum of the disease is not known. This is highlighted by studies which have identified mutations areas outside the hotspots, indicating the importance of expanding the screening area in the causative genes(Sugiura et al. 2003; Matthews et al. 2009; Lee et al. 2009; Ke et al. 2009; Fan et al. 2013).

Recent studies have discovered three new genes which play a role in the causation of periodic paralysis: KCNJ18 and MT-ATP6 and MT-ATP8. KCNJ18 encodes the potassium channel, Kir2.6. Mutations in this gene have been shown to result in thyrotoxic periodic paralysis and sporadic periodic paralysis in Asian populations(Ryan et al. 2010; Cheng et al. 2011). While these studies are focused solely on patients within the Asian population, it is possible that mutations in KCNJ18 could cause periodic paralysis in other populations. MT-ATP6 and MT-ATP8 are mitochondrial genes that have been recently associated with an atypical periodic paralysis like phenotype(Auré et al. 2013). This is the first non channel gene to be associated with transient attacks of paralysis and offers the exciting potential to further our understanding of the causative pathways of the disease. Both KCNJ18 and the mitochondrial genes highlight the likelihood that some patients will not have a mutation in
the known genes but could have mutations in currently unknown genes which can be investigated through next generation sequencing techniques such as exome sequencing.
3.2.1 Aims

The aims of this study were to identify novel mutations in the known genes associated with periodic paralysis, in two well defined cohorts, through screening which encompasses the areas of the gene normally not covered during diagnostic sequencing. In addition, new genes associated with rare or atypical forms of periodic paralysis were screened in order to expand the current understanding of genetic causes. This offers the potential to further the understanding of the genetic heterogeneity of periodic paralysis.
3.3 Periodic Paralysis Patient Cohort

In order to expand our knowledge of periodic paralysis, genetic analysis was undertaken in a cohort of 68 patients who were negative for the common periodic paralysis mutations. This large cohort was divided into two groups in order to enable two different approaches to be used (See Figure 3-1 for overview of cohort investigation strategy). The first group consisted of 21 patients who have a well characterised phenotype consistent with either Hypo PP or Hyper PP, with positive electrical tests that support the clinical examinations. Patients in this group were then divided into Hypo PP and Hyper PP and the associated genes were screened. This enabled the screening of areas of the gene which are not normally screened during routine diagnostic testing. The second group was composed of 47 patients with more variable phenotypes. This group contained a broad spectrum of phenotypes which were less well defined, and were rated based on the likelihood of finding an ion channel mutation. A different approach for genetic screening of this group was undertaken since there was a lower chance of success for screening the known genes through Sanger sequencing of individual exons. Samples were run on a customised TSCA ion channel gene panel to rule out mutations in the known genes before undertaking a candidate gene screening of three recently identified periodic paralysis genes, KCNJ18, MT-ATP6 and MT-ATP8. In the future, those patients with no mutations following these steps will be selected for exome sequencing depending on the availability of other family members.
Figure 3-1 Strategy for investigating causative mutations in periodic paralysis cohort
3.4 Well Defined Cohort: Hypokalemic periodic paralysis

3.4.1 CACNA1S Sequencing in Hypokalemic Periodic Paralysis

Mutations in Ca\(_{\text{v}1.1}\) S4 segments have been shown to cause 70% of Hypo PP cases (Davies et al. 2001; Matthews et al. 2009) with all known mutations causing loss of charge of an arginine residue. However, only mutations in residues R1-R3 in domains II, III and IV have been identified. To identify novel mutations in Hypo PP cases where the known causative mutations had been ruled out, all the exons which encode for S4 segments of the calcium channel were sequenced using the Sanger sequencing method. Following sequencing of exons 4, 11, 21 and 30 of CACNA1S no mutations were identified in the 13 samples screened. It is possible that the causative mutation for some of these patients is located in other areas of the channel or another gene. CACNA1S consists of 44 exons therefore to use Sanger sequencing to investigate the whole gene is costly. In cases where no mutation was found the DNA was then sequenced on a custom designed TSCA gene panel that covers all the known skeletal muscle channelopathy genes (SCN4A, CACNA1S, KCNJ2 & CLCN-1), this enabled the whole coding region of CACNA1S to be covered. No mutations were identified following full CACNA1S screening.

3.4.2 SCN4A Sequencing

Following CACNA1S S4 segment sequencing, the whole coding region of the SCN4A gene, which encodes the sodium channel Na\(_{\text{v}1.4}\), was sequenced. Mutations in Na\(_{\text{v}1.4}\) account for ~10% of HypoPP cases, with 18 out of 21 reported mutations causing a loss of charge of an arginine residue. All 24 coding exons of SCN4A were sequenced for mutations using the Sanger sequencing method. 3 out of 13 samples (23%) were found to have a missense mutation in areas of SCN4A not normally covered by diagnostic screening of the common...
mutations (Table 3-1& Table 3-2). Of the mutations identified two were found to be novel and predicted to be pathogenic through in silico analysis using PolyPhen (Adzhubei et al. 2010), while the other was a known well characterized Hypo PP mutation, p.Arg669His. The first novel mutation identified was p.Tyr168Asp which is located in the S2 segment of DI while the second was p.Asp1420Gly found within the S3 segment of DIV. Both mutations are found in areas of the sodium channel which have not been associated with Hypo PP before and require functional investigations in order to determine the effect they might have on the functioning of the normal channel.

In the remaining samples of the cohort, in which no mutation was identified (77%), a number of different approaches were taken to identify a causative mutation, including custom gene panel sequencing.

3.4.2.1 Patient and Mutation overview

Patient 1

Patient 1 is a 30 year old man who showed onset of symptoms at the age of 2 years old when he had difficulty walking. Throughout childhood he showed problems with running and jumping with his first paralytic attack appearing at the age of 11. This occurred during the night affecting his arms and legs with recovery by the morning. Two nights later he suffered another attack which resulted in hospitalization where his potassium level was measured and found to be low during attacks. The attacks can be triggered by cold weather, rest after exercise and eating of a carbohydrate rich meal. He is able to abort the onset of attacks by taking Sando-K. On physical examination the patient was revealed to have mild proximal weakness of arms and legs. The patient has an affected mother who shows no distinct attacks, with onset during pregnancy where her arms and legs were affected for 8 hours (Figure 3-2A). She now has severe proximal weakness and requires a
wheelchair for long distances. Muscle biopsies have been taken of both patients and show extensive vacuolation of muscle fibres, typical of periodic paralysis. The condition of both patient and mother is made worse when treated with acetazolamide and daranide.

Sequence analysis of DNA from this patient revealed a T to G substitution at nucleotide 502 of SCN4A which results in a Tyrosine to Aspartic Acid change at position 168 (p.Tyr168Asp) of the sodium channel Na,1.4 (Figure 3-2B). The same mutation was also found in the patient’s affected mother. The mutation was found to be absent in over 300 control chromosomes as well as dbSNP, 1000 genomes and NHLBI exome sequencing project. The amino acid is located in the transmembrane S2 segment of domain I and is highly conserved amongst several species. Through in silico analysis using PolyPhen p.Tyr168Asp was predicted to be possibly damaging with a score of 0.557.

In 2011 Payandeh et al. used NavAb to determine the first crystal structure of a sodium channel. The bacterial channel, NavAb, is formed from four identical subunits which correlate to a single Nav1.4 domain. Sequence alignment using ClustalW of the human Na,1.4 amino acid sequence to NavAb revealed Tyr168 aligns with the NavAb Phe56 residue (Figure 3-2C). Phe56 together with three other residues forms part of the hydrophobic constriction site, indicating that Tyr168 might also be involved in this role.
Figure 3-2 SCN4A mutations in Hypo PP

A & D, Pedigrees of families, arrow indicates proband mutation was originally identified in. B & E, Chromatograms of partial sequences of SCN4A showing on heterozygous point mutation with Thymidine 502 changes to Guanine resulting amino acid substitution Tyrosine to Aspartic Acid and one heterozygous point mutation of Adenine 4259 to Guanine leading to an amino acid substitution Aspartic Acid to Glycine.  C & F, Amino acid sequence alignment of Na\textsubscript{v}1.4 domains with bacterial channel NavAb.
Patient 2

This patient is a 19 year old man who before the onset of symptoms had reached all major childhood milestones without problems and was able to keep up with peers during sporting activities at school. His first attack of paralytic weakness affecting his arms and legs appeared at the age of 14 years old following a period of extensive exercise. During this attack he was admitted to hospital and recovered after 3-4 days. A second attack followed a week later where his potassium levels were measured and found to be 1.2. Since this, he experiences a number of severe episodes a year for which he has to be admitted to hospital, all of which are associated with low potassium. Due to the frequency of attacks he had a Portacath inserted to allow for emergency potassium supplementation. In between the severe attacks, the patient experiences milder attacks, with weakness observed in some limbs upon waking in the morning, which lasts a few hours and is followed by full recovery. On examination the patient showed positive for the McManus long exercise test with a maximum decrement of 62% with no myotonia. All family members of the patient are healthy and show no symptoms.

Upon sequence analysis of the SCN4A gene, it was found that the patient had a missense mutation c.2006G>A which results in the amino acid change p.Arg669His. This is a known mutation of the S4 segment in domain II of the skeletal muscle sodium channel. Previous studies have shown that this mutation results in a gating pore current which is suggested to be the main pathomechanism behind Hypokalemic periodic paralysis (Sokolov et al. 2007; Struyk et al. 2008).
<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age of onset (years)</th>
<th>Affected Family Members</th>
<th>Clinical Features</th>
<th>Phenotype</th>
<th>SCN4A mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>11</td>
<td>Mother</td>
<td>Periodic paralysis with muscle weakness affecting arms and legs associated with hypokalemia; attacks of weakness triggered after strenuous exercise and carbohydrate.</td>
<td>Hypo PP</td>
<td>p.Tyr168Asp</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>14</td>
<td>Not known</td>
<td>Periodic paralysis with generalised muscle weakness associated with hypokalemia; attacks triggered by exercise and carbohydrate.</td>
<td>Hypo PP</td>
<td>p.Arg669His</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>13</td>
<td>Brother</td>
<td>Periodic paralysis with weakness affecting arms and legs associated with hypokalemia. Attacks triggered by exercise, stress and glucose</td>
<td>Hypo PP</td>
<td>p.Asp1420Gly</td>
</tr>
</tbody>
</table>

Table 3-1 Clinical Features of Hypo PP patients
Patient 3

Patient 3 is a 28 year old male who shows episodes of weakness affecting arms and legs that began at the age of 13. The patient experiences a mixture of severe and minor attacks, with minor attacks occurring once a week with normal strength returning within 3 to 4 hours. The minor attacks mainly result in some proximal weakness and rarely full paralysis. Severe attacks that affect the neck, arms and legs occur every few months lasting on average 7-8 hours. Administering of Sando-K is often enough to ameliorate attacks but in some cases this is not sufficient and hospitalisation occurs. The longest attack of weakness experienced lasted 27 hours. Potassium levels have been found to be low during attacks of weakness. A McManus exercise test has been conducted during an attack, showing a drop in CMAP and loss of reflexes. Additional McManus tests during normal muscle functioning have also been positive. Interestingly, on examination the patient shows no myotonia but appears to have calf muscle hypertrophy. The proband has a possibly affected younger brother while both parents are thought to be unaffected (Figure 3-2D).

In order to find a causative mutation, sequence analysis of the whole coding region of the SCN4A gene was undertaken. Upon sequencing of exon 23, a substitution c.4259A>G was found which leads to an amino acid change Aspartic Acid to Glycine at position 1420 (p.Asp1420Gly). The amino acid change was absent from over 300 control chromosomes as well as the dbSNP, 1000 genomes and NHLBI exome sequencing project databases. The aspartic acid residue is a highly conserved amino acid down to Tetradon and is located in the transmembrane segment S3 in domain IV of the sodium channel, Na1.4. Polyphen was used for in silico analysis to determine if the mutation was likely to be pathogenic. Asp1420Gly is predicted to be probably damaging with a score of 1.000.

To gain a further understanding of the importance of the Aspartic Acid residue involved in this mutation, the human Nav1.4 sequence was aligned against the NavAb bacterial
channel sequence using Clustal W. Through alignment to this channel it was found that Asp1420 aligns to the NavAb residue Asp80 (Figure 3-2F). Asp80 is found within the intracellular negative charge cluster (INC) and interacts with the R4 residue of the S4 when the voltage sensor is in the active position. This indicates that Asp1420 would have a similar role although functional data would be needed to confirm this.
<table>
<thead>
<tr>
<th>DNA Change</th>
<th>Location within Gene</th>
<th>Amino Acid Change</th>
<th>Location within Protein</th>
<th>Pathogenic (In silico Analysis)</th>
<th>Known mutation?</th>
<th>Functional Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.502T&gt;G</td>
<td>Exon 4</td>
<td>p.Tyr168Asp</td>
<td>Domain II S2</td>
<td>Yes</td>
<td>No</td>
<td>To be done, see Chapter 6</td>
</tr>
<tr>
<td>c.665G&gt;A</td>
<td>Exon 5</td>
<td>p.Arg222Gln</td>
<td>Domain I S4</td>
<td>Yes</td>
<td>No</td>
<td>To be done, see Chapter 6</td>
</tr>
<tr>
<td>c.1207A&gt;C</td>
<td>Exon 8</td>
<td>p.Met403Lue</td>
<td>Domain I S5-S6 Loop</td>
<td>Yes</td>
<td>No</td>
<td>To be done</td>
</tr>
<tr>
<td>c.1957A&gt;G</td>
<td>Exon 12</td>
<td>p.Ser653Gly</td>
<td>Domain II S3</td>
<td>Yes</td>
<td>No</td>
<td>To be done</td>
</tr>
<tr>
<td>c.2006 G&gt;A</td>
<td>Exon 13</td>
<td>p.Arg669His</td>
<td>Domain II S4</td>
<td>n/a</td>
<td>Yes</td>
<td>Known to cause an aberrant ionic leak called a gating pore current (Bulman et al. 1999; Struyk &amp; Cannon 2007)</td>
</tr>
<tr>
<td>c.4259A&gt;G</td>
<td>Exon 23</td>
<td>p.Asp1420Gly</td>
<td>Domain IV S3</td>
<td>Yes</td>
<td>No</td>
<td>To be done, see Chapter 5</td>
</tr>
</tbody>
</table>

Table 3-2 SCN4A mutations found in periodic paralysis
3.5 Well Defined Cohort: Hyperkalemic Periodic Paralysis

3.5.1 SCN4A Sequencing

Mutations in SCN4A account for ~ 60% of Hyper PP cases, with the remaining cases without a genetic diagnosis. With 40% of cases without a genetic diagnosis, it is likely another gene is involved but no other gene has been identified to date. During diagnostic testing only two exons, containing the common mutations, are routinely sequenced (exons 13 and 24), resulting in the possibility of missing causative mutations in SCN4A. Therefore, to expand the current knowledge of Hyper PP causative mutations, the whole SCN4A coding region was sequenced in a well defined cohort of Hyper PP patients in order to identify new areas of the sodium channel involved in Hyper PP. Furthermore, any new mutations in SCN4A found in novel locations would provide us with further insight into the workings of the voltage gated sodium channel.

All 24 exons of SCN4A were analysed through Sanger sequencing for 8 well defined Hyper PP patients who all had electrical examinations consistent with periodic paralysis. 3 out of 8 patients (37.5% of samples) were found to have a novel missense mutation in SCN4A, in an area of the gene not normally covered by routine genetic testing (Table 3-2 & Table 3-3). These mutations were predicted to be pathogenic by in silico analysis by Polyphen (Adzhubei et al. 2010). The first mutation is p.Met403Leu located in the loop region of S5 and S6 segments of Domain II, the second p. Ser653Gly located in S3 segment of Domain II, and the final mutation is p.Arg222Gln located in S4 segment of Domain II. Arg222Gln is particularly interesting as this mutation is located in a region of the channel not normally associated with Hyper PP but with Hypo PP.
In the remaining samples of the cohort, in which no mutation was identified (62.5% of cohort), a number of different approaches were taken to identify a causative mutation or novel genes involved, including custom gene panel sequencing.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age of onset (years)</th>
<th>Affected Family Members</th>
<th>Clinical Features</th>
<th>Phenotype</th>
<th>SCN4A mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>14</td>
<td>Son</td>
<td>Periodic paralysis with muscle weakness lasting for a few weeks; brief myotonia like runs of single fibre discharges; attacks induced by extremes of temperature.</td>
<td>Hyper PP</td>
<td>Met403Leu</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>13</td>
<td>Mother</td>
<td>Periodic paralysis with attacks lasting several hours along with myotonia</td>
<td>Hyper PP</td>
<td>Ser653Gly</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>14</td>
<td>Maybe mother’s brother &amp; Father’s sister</td>
<td>Myotonia with possible episodes of weakness. Also has a silent <em>CLCN-1</em> mutation.</td>
<td>PMC/Hyper PP</td>
<td>Arg222Gln</td>
</tr>
</tbody>
</table>

Table 3-3 Clinical Features of Hyper PP Patients
3.5.1.1 Patient & mutation overview

Patient 1

Patient 1 is a 56 year old male affected by Hyper PP. Onset of symptoms began at 14 years old with episodes of weakness and stiffness. Before treatment with acetazolamide was started the patient experienced night time attacks occurring every couple of days taking a couple of days to weeks to reach full recovery. Upon treatment the patient now experiences fewer night time attacks, with a frequency of once or twice a year, affecting his arms and legs and occasionally his face. He also experiences daytime attacks several times a year. Triggers of attacks include extremes of temperature and a heavy breakfast. EMG showed brief myotonia like runs of single fibre discharges. A 43% decrement was found on testing of the long exercise test. Serum potassium levels were measured and found to be within the normal range. The patient has a son who presents with a similar phenotype.

Through sequencing of the whole SCN4A coding region it was found that this patient had a missense mutation in exon 8. The A>C change at position 1207 results in an amino acid change of a methionine to a leucine at position 403 in the sodium channel (p.Met403Leu, Figure 3-3B). This mutation is located in the loop region between the S5 and S6 segments of domain I (Figure 3-4). A methionine residue at this position is highly conserved between many species. In addition, the mutation was found to segregate with disease (Figure 3-3 A). In silico analysis showed that p.Met403Leu is predicted to be possibly damaging, suggesting that this mutation is probably the causative mutation for this patient.
Figure 3-3 Novel mutations found in Hyper PP cohort.
A, C & D, Pedigrees of families, arrow indicates proband with affected members are shaded in. B, D & E, Chromatograms of partial sequences of SCN4A showing one heterozygous point mutation of Adenosine for Cytosine resulting in an amino acid change of Methonine to Leucine, one heterozygous point mutation of Adenine to Guanine which causes the amino acid change Serine to Glycine and finally a Guanine to Adenine change causing the amino acid change Arginine to Glutamine.
Patient 2

Patient 2 is a 51 year old male who shows symptoms of Hyper PP with PMC overlap. The patient showed normal development during childhood with onset of symptoms noted at the age of 13 years old with cramps in legs. Symptoms present as short episodes of weakness that last between 1–4 hours as well as intermittent weakness affecting the eyes and hands. These attacks can be triggered by tiredness and exertion, stress and cold temperatures. The patient also has type II diabetes. Laboratory tests show the patient has a CK level of 116 and TSH of 6.89. Upon electrophysiological testing the patient showed myotonic bursts and had a normal short exercise test at room temperature. The CMAP was shown to fall upon cooling of the muscle and then slowly recovered. Furthermore, testing revealed possible mild myopathy of some muscles. His mother is also affected with attacks of weakness affecting her arms and legs as well as her grip (Figure 3-3C). These attacks are short in duration lasting at most 10 minutes. In addition to this she also experiences stiffness and cramps. First symptoms appeared at the age of 12 years old but she did not experience her first attack of weakness until she was 17 years old during her first pregnancy.

Through sequencing of the patient DNA they were found to have a mutation in exon 12 at position 1957 of SCN4A substituting an A to G, resulting in the amino acid change p.Ser653Gly (Figure 3-3D). This highly conserved Serine residue is located in the S3 segment of domain II, an area that doesn’t contain any known Na,1.4 mutations. p.Ser653Gly is predicted to be pathogenic through in silico analysis which predicts the amino acid change would not be tolerated. In addition, the mutation was found to segregate with disease.
Patient 3

Patient 3 is a 26 year old Kurdish male from a consanguineous family who is affected by myotonia with some episode muscle weakness (Figure 3-3E). At the age of 14 years this patient began to experience stiffness of muscles. He experiences grip myotonia without eyelid myotonia. In addition, he has had gradual onset of weakness in his right thigh. Symptoms are thought to be worse in cold. The potassium serum levels of this patient were measured and found to be normal. EMG shows the patient has short bursts of myotonia which are consistent with his symptoms of muscle stiffness, he also showed a positive long exercise test with an abnormal amplitude decrement from maximum CMAP of >40% and an abnormal amplitude decrement from baseline of >30%.

This patient was initially thought to have myotonia congenita and underwent whole \textit{CLCN-1} sequencing where he was found to have a heterozygous synonymous variant c.1650G>A which is predicted to affect splicing and has been associated with myotonia congenita, in a homozygous state (Horga et al. 2013). It is not known what effect this mutation has and whether it has an impact on channel function in the heterozygous state and no muscle sample from the patient was available for confirmation of correct splicing of the \textit{CLCN-1} gene. Following this analysis of common \textit{SCN4A} mutations that cause myotonia were found to be negative. Therefore, the patient’s DNA was analysed for mutations in the entire coding region of \textit{SCN4A} through Sanger sequencing and was found to have a missense mutation in exon 5 c.665G>A which results in a change in the arginine residue at position 222 to a glutamine residue (Figure 3-3F). Arg222Gln is predicted to be possibly damaging by PolyPhen with a score of 0.640. The Arg222 residue is a highly conserved amino acid located within the S4 segment of domain I of Na,1.4.
Figure 3-4 Location of Novel Periodic Paralysis Mutations on the Sodium Channel α Subunit

A schematic representation of the Na\textsubscript{v}1.4 α subunit transmembrane domains I-V and transmembrane segments S1-S6 with location of novel mutations.

- Hypo PP: Tyr168Asp, Asp1420Gly
- Hyper PP: Arg222Gln, Met403Leu, Ser653Gly
3.6 New Periodic Paralysis Genes

To further the understanding of the genetic causes of periodic paralysis, both groups of the periodic paralysis cohort had the coding region of three potential candidate genes: \textit{MT-ATP6}, \textit{MT-ATP8} and \textit{KCNJ18}. These genes have been associated with periodic paralysis in recently published papers with phenotypes that differ slightly from the standard clinical symptoms of Hyperkalemic periodic paralysis or Hypokalemic periodic paralysis. This cohort of patients consisted of 60 patients who were diagnosed with Hypo PP, Hyper PP or a general periodic paralysis phenotype but were not found to have mutations in the known genes. Known genes were previously ruled out through the use of a custom skeletal muscle channel gene panel (designed, run and analysed by A. Gardiner). Patients were also rated on the strength of their phenotype with a likelihood of finding a mutation.

3.6.1 MT-ATPase6/8 Sequencing

For sequencing of the mitochondrial genes \textit{MT-ATP6} and \textit{MT-ATP8}, 60 samples were analysed. Following this, no mutations were found in the cohort of patients in either gene, suggesting that the full periodic paralysis phenotype does not fully expand to this specific subset.

3.6.2 \textit{KCNJ18} Sequencing

For \textit{KCNJ18} sequencing, 60 samples were analysed for mutations using Sanger sequencing. Due to the high sequence identify of \textit{KCNJ18} with \textit{KCNJ12} (99%), it can be difficult to determine which gene has been amplified during PCR. All samples were analysed to check that the target gene had been amplified and sequenced correctly through the presence of 7 variations that distinguish \textit{KCNJ18} from \textit{KCNJ12}(Table 3-4). In all 60 samples \textit{KCNJ18} was correctly amplified. Out of 60 samples, one sample was found to have a possible mutation.
in KCNJ18. The variation, c.35 T>C is located at the beginning of the third exon of KCNJ18, which is the only coding exon of the gene, and results in an amino acid change of Isoleucine to Threonine at position 12 (p.Ile12Thr). This mutation is predicted to be pathogenic through in silico analysis, however due to the homology of this gene with KCNJ12 and its relatively new discovery there is no information available on polymorphisms in the online databases such as NLHBI, db SNP and 1000 genomes. Ptacek et al highlighted 20 SNPs in KCNJ18 that they came across during identification and sequencing of the gene. However, c.35T>C is not present on the list.

The patient is a 45 year old male who has experienced transient attacks of paralysis since the age of 15 following normal childhood milestones. Since his first attack this patient has experienced only a few episodes of mild muscle weakness affecting the lower limbs and occasionally arms. The attacks last for up to an hour before the restoration of full muscle strength. No apparent triggers have been noted. The patient has a long family history of attacks of paralysis with 6 affected individuals, whom all suffer from intermittent weakness usually affecting the lower limbs and sometimes arms for an hour or more. These symptoms and family history are suggestive of Hyperkalemic periodic paralysis, although the frequency of attacks and lack of triggers are atypical.

Before investigating this variant further, a MiSeq custom panel screen was used to rule out other mutations. Following this the patient was found to have a known Na,1.4 mutation, p. Arg675Gln. This mutation is located within DII S4 segment and is a well established normokalemic periodic paralysis mutation.
Table 3-4 Variations that distinguish between *KCNJ12* and *KCNJ18*

<table>
<thead>
<tr>
<th>Nucleotide Residues</th>
<th>Nucleotide Position</th>
<th>Amino Acid Residues</th>
<th>Amino Acid Position</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KCNJ12</strong></td>
<td><strong>KCNJ18</strong></td>
<td><strong>Kir2.2</strong></td>
<td><strong>Kir2.6</strong></td>
</tr>
<tr>
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<td>T</td>
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<td>Ser</td>
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<tr>
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<tr>
<td>A</td>
<td>G</td>
<td>1290</td>
<td>Glu</td>
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3.7 Discussion

3.7.1 Further Screening of Known Genes
In order to further understand the genetic spectrum of periodic paralysis, a cohort of 68 periodic paralysis patients negative for the common mutations were investigated. This cohort was subdivided into two groups: the well defined cohort comprised of 21 samples and the less defined cohort comprised of 47 samples. The first group contained patients with clear, well defined phenotypes for both Hypo PP and Hyper PP. For this group of samples Sanger sequencing of the known genes was undertaken in order to find a causative mutation.

In the Hypo PP group, 13 patients were analysed for mutations in both causative genes, SCN4A and CACNA1S. In the case of CACNA1S sequencing, it was only possible to sequence those exons relating to the S4 voltage sensors using Sanger sequencing, due to the large size of this gene. No mutations were identified in these exons of CACNA1S for all the samples tested. In order to be sure that there were no mutations in this gene; patients were put on a customised gene panel which covered the whole coding region of the CACNA1S gene, as well as other known channelopathy genes. Of the 13 samples tested, none were found to have mutations in this gene. One explanation for these negative results could be that mutations in this gene are only localised to the regions of the gene tested for in routine genetic screenings. Thus, this cohort would have been negative selection for mutations in this gene due to selection of samples that have already been screened for the common mutations. However, this is unlikely to be the case as there have been two mutations reported to date which are located outside the S4 segment of CACNA1S that do not cause neutralisation of a gating charge, suggesting that future sequencing of the whole CACNA1S gene might identify causative mutations in regions outside the S4 segment. Sequencing of the whole coding region of CACNA1S on a customised gene for this cohort
showed that no mutations were located outside the S4 region. Therefore, mutations in other genes or within intronic or promoter regions are likely to explain the phenotypes of this cohort.

Following sequencing of the whole coding region of *SCN4A*, 3 out of 13 patients, were found to have a likely causative mutation (Table 3-2). Of these three mutations, two were identified as novel mutations located in areas of the channel which have not previously been associated with disease. The first mutation is a known pathogenic variant, p.Arg669His, and was found in a patient who presents with a phenotype of transient attacks of weakness affecting his arms and legs associated with low potassium levels. This mutation has been reported as pathogenic through functional analysis and is clearly the causative mutation (Bulman et al. 1999; Struyk et al. 2000; Struyk & Cannon 2007). In the remaining two cases, novel mutations, p.Tyr168Asp and p.Asp1420Gly, were identified in patients with Hypo PP phenotypes. The former, p.Tyr168, is located in domain I within the S2 segment of the channel (Figure 3-4). It was found in a patient who presents with attacks of paralysis that have led to hospitalisation and are associated with low potassium. In this case the tyrosine residue at position 168 is highly conserved throughout species and is predicted to be pathogenic. The latter, p.Asp1420Gly, is located within the S3 segment of domain IV and affects a highly conserved negatively charged residue (Figure 3-4). The mutation was found in a male patient who experiences short attacks of paralysis lasting 7-8 hours which have been shown to be associated with low potassium levels. Both mutations were not found in over 300 control chromosomes and were absent from the known databases, including 1000 genomes, NLHBI and dbSNP. In addition, these mutations both affect highly conserved residues and in silico analysis predicts them to be pathogenic, indicating that these amino acid changes are likely to be the causative mutation of Hypo PP
in these cases. However, how these mutations might fit in with the current knowledge of the Hypo PP mechanism of disease requires further discussion.

It is now well established that Hypo PP mutations localise to the voltage sensor of Ca,1.1 or Na,1.4 resulting in neutralisation of positively charged arginine residues, known as gating charges (Bulman et al. 1999; Matthews et al. 2009). The positive charges of the voltage sensor are enclosed within the voltage sensing domain, which is formed by S1, S2 and S3 segments. Negatively charged residues within the surrounding segments form electrostatic interactions with the voltage sensor gating charges (Seoh et al. 1996; Tiwari-Woodruff et al. 2000; DeCaen et al. 2008; DeCaen et al. 2009). Substitution of the positive charge is known to disrupt the integrity of the voltage sensing domain through loss of ion pair interactions, providing an alternative pathway for ionic movement across the channel (Tombola et al. 2005; Sokolov et al. 2007; Struyk & Cannon 2007). This aberrant ionic leak is thought to be the mechanism by which muscle depolarisation occurs resulting in the Hypo PP phenotype.

Both the novel mutations in question, p.Try168Asp and p.Asp1420Gly, are located outside the voltage sensor. However they are found within the S2 and S3 segments respectively, which play an important role in maintaining the integrity of the voltage sensing domain and preventing leak through the domain.

Located in the S2 segment of Domain I, p.Tyr168Asp aligns to Phe56 of NavAb, the bacterial sodium channel used to elucidate the first crystal structure of the sodium channel (Figure 3-2). Tyrosine and Phenylalanine are both polar residues which are highly similar as both contain a side chain of a 6 carbon aromatic ring (benzene ring), suggesting that both could perform the same function when substituted for one another within a protein. Phe56 together with three other residues (Ile33, Ile96 and Val97) is suggested to form part of the hydrophobic restriction site (HSC) of the voltage sensing domain. These residues line the narrowest part of the gating pore domain, forming a hydrophobic seal (Payande et al.
The hydrophobic constriction site (HCS), is thought to prevent aberrant ionic leakage through the gating pore (Payandeh et al. 2011). No other Hypo PP mutations have been identified within the HCS of Na\textsubscript{v}1.4. It is possible that substitution of the tyrosine residue to the smaller negatively charged residue of aspartic acid could result in disruption to the HCS through unwanted electrostatic interactions of the negative charge, providing an alternative pathway for ions and thus result in a gating pore current. As the HCS is not involved in the movement of the voltage sensor it is possible that any gating pore current produced by this mutation might not show voltage dependence making it difficult to detect through the electrophysiological methods normally used. Furthermore, it is difficult to know whether loss of one residue would be sufficient to disrupt the HCS as the importance of each residue in the formation of the HCS is not known. Functional analysis is needed to look at the effect of Tyr168Asp on Na\textsubscript{v}1.4 to determine whether it causes a gating pore current or affects the main pore.

The second Hypo PP mutation identified was p.Arg1420Gly, located in S3 segment of DIV. It is the first Hypo PP mutation identified which results in loss of a negative charge rather than a positive gating charge. Loss of the negative charge at position 1420 to the smaller uncharged glycine residue is likely to cause a disturbance in the protein structure, which could destabilise the movement of the voltage sensor and disrupt the integrity of the voltage sensor domain as is seen with conventional Hypo PP mutations.

The Asp1420 residue aligns with Asp80 from the sodium channel NavAB. Asp80 is one of many negatively charged residues within the intracellular negative charge cluster (INC) which interacts with R4 when the voltage sensor is activated (Payandeh et al. 2011). The INC forms ion-pair interactions with the gating charges which catalyse voltage sensor movement by providing a low energy pathway for movement (Catterall 1986; DeCaen et al. 2008; DeCaen et al. 2009; Catterall 2010). In conventional Hypo PP mutations loss of a
positively charged arginine residue causes loss of the electrostatic interactions with the negative charges in ENC and INC of the surrounding voltage sensing domain. Disruption of these interactions by loss of one positive charge is sufficient to allow for an aberrant pathway for ionic leak across the channel (Sokolov et al. 2007; Struyk & Cannon 2007; Sokolov et al. 2008; Struyk et al. 2008; Francis et al. 2011). Thus, it is possible that loss of a negatively charged aspartic acid residue to the small uncharged glycine residue will cause similar disruption to the voltage sensing domain through interruption of the electrostatic interactions that hold it together. If Asp1420G were to result in a gating pore current as seen in known Hypo PP mutations, it would be the first non S4 segment Hypo PP mutation to cause a gating pore current. Such a discovery would help to confirm and further establish the gating pore current as the causative mechanism of disease in Hypo PP.

To date, all but one substitution mutation of an S4 arginine residue has been shown to disrupt the voltage sensing domain by opening up the tight pore to allow a gating pore current (Francis et al. 2011). Francis et al. 2011 showed that the PMC mutation Arg1448Cys located in Domain IV does not result in a gating pore current. Interestingly, this mutation is in a homologous location to the R4 in the NavAb channel which Asp80 and so Asp1420 interacts with (Payandeh et al. 2011; Vargas et al. 2012). However, this interaction occurs when the voltage sensor is in the activated position suggesting that Asp1420 is likely to interact with other residues enabling a gating pore current to be produced upon loss of the negative charge.

A similar analysis approach was taken for genetic screening of the Hyper PP group. This group contained eight patients all of whom had a strong Hyper PP phenotype with some possible myotonia overlap. In this approach we screened the whole SCN4A gene in order to indentify the causative mutation in this cohort. Following sequencing of all 24 exons of SCN4A, 3 out of 8 samples (37.5%) were found to have a novel mutation in SCN4A (Table
All three mutations were predicted to be pathogenic through in silico analysis, were absent from over 300 control chromosomes and are not present in the known genome databases including 1000 genomes. This indicates that these mutations are likely to be the causative mutations in these cases, although functional analysis is needed to confirm this.

The first mutation, p.Ser653Gly, was found in a middle aged man with a clear Hyper PP phenotype with some PMC overlap and was observed to segregate with disease. The serine residue at position 653 is a highly conserved residue which is found within the S3 segment of Domain II. The S3 segment forms part of a larger structure of the sodium channel called the voltage sensing domain (VSD). The VSD is composed of the S4 voltage sensor which is surrounded by the gating pore formed by S1-S3 segments (Catterall 1988; Sato et al. 2001; Payandeh et al. 2011). Movement of the S4 segment within the gating pore occurs through ion pair exchange with negatively charged residues in the S1-S3 segments (Yang et al. 1996; Pérez-García et al. 1996; Starace & Bezanilla 2001; Gandhi 2002; Mantegazza & Cestèle 2005). These interactions are supported by hydrogen bonds between the S4 and the carbonyl backbone of certain residues of the VSD (Payandeh et al. 2011). Ser653 aligns to Ser 87 of the bacterial NavAb. From the crystal structure of NavAb, Ser87 was highlighted as one of the gating pore residues that forms the network of hydrogen bonds and specially interacts with R3 (Payandeh et al. 2011).

Hyper PP mutations are known to result in disruption to channel activation or inactivation and are often located in areas of the channel which are known to be associated with channel gating (Cannon 2006). To date, the mutations found with Na1.4 DII are located in either the S4 segment (Hypo PP) or the S4-S5 loop (Hyper PP). Only two mutations within Na1.4 have been reported within a S3 segment, Leu1433Arg and Leu1436Pro, which are found within the S3 of DIV and are associated with PMC (Ptacek et al. 1993; Matthews et al. 2008). Functional studies of Leu1433Arg have found that it results in slower inactivation of
the channel, a common effect of Hyper PP or PMC mutations, indicating that Ser653Gly might have the same effect on Na$_{1.4}$ gating (Yang et al. 1994; Ji et al. 1996). However, it is known that the individual domains of Na$_{1.4}$ have distinct functions within the channel, for example, it has been shown that DIII and DIV voltage sensors are associated with fast inactivation, whilst DI, DII and DIII are associated with activation (Cha et al. 1999; Chanda 2002; Chanda et al. 2004). This functional asymmetry seen between the voltage sensors of different domains is likely to be present in other parts of the channel, thus functional work is needed to determine the effect of Ser653Gly on channel functioning.

The second novel mutation, p.Met403Leu, was identified in a patient who presents with a typical Hyper PP phenotype and was found to segregate with disease. Met403Leu is located in the loop region between S5 and S6 segments of Domain I and affects a highly conserved methionine residue. The S5-S6 interlinking loops are often referred to as the P loops and help to form the central pore of the channel. P loops have been shown to be important for conductance and selectivity of channels (Zagotta & Aldrich 1990; Qu et al. 1999; Catterall 2012). These loops have been found to partially re-enter the membrane, with MTS cysteine experiments showing that some residues within the S5-S6 loop are buried within the protein (Pérez-García et al. 1996). This enables the S5-S6 loops from each domain to come together to form the outer vestibule of the channel and part of the narrow ion selectivity filter (Fozzard & Hanck 1996; Marban et al. 1998; Ertel et al. 2000). A number of Hyper PP and PMC mutations are located within the pore forming areas of Na$_{1.4}$. In particular, the two most common Hyper PP mutations, p.Thr704Met and p.Met1592Val, are located in the DII S5 and DIV S6 segments, respectively. Both mutations have been shown to cause disruptions to channel inactivation resulting in a persistent Na$^+$ current (Rojas et al. 1999; Bendahhou et al. 1999). Moreover, two mutations are located nearby Met403Leu within DI S6 segment: p.Asn440Lys and p.Val445Met (Trip et al. 2007; Lossin et al. 2012; Lehmann-
Horn et al. 2011). Functional analysis has shown p.Asn440Lys results in a depolarised shift in voltage dependence of fast inactivation and increase recovery from inactivation resulting in a persistent Na$^+$ current (Lossin et al. 2012). Ergo, it is highly likely that p.Met403Leu is the causative mutation within this patient and will result in disruption to Na$_v$1.4 gating, further functional studies are necessary to confirm this hypothesis.

Finally, p.Arg222Gln was found in a 26 year old Kurdish man from a consanguineous family who presents with myotonia as a main symptom. This patient has a history of myotonia from childhood but in recent years has experienced episodic weakness in legs, suggesting Hyper PP with a PMC overlap. Initial genetic investigations revealed a heterozygous silent sequence variant in $CLCN$-1 c.1650G>A, which is predicted to effect gene splicing and is associated with myotonia congenita within a homozygous recessive family. This variant has not been studied functionally to determine the effect on channel functioning and it is not known whether the variant would have an effect in the heterozygous state. It is possible that the variant might act as an exacerbating factor to the phenotype without being the causative mutation. Further analysis of other known genes revealed the mutation p.Arg222Gln which is located within the S4 segment of Domain 1 and affects a highly conserved residue. Most reported Hyper PP mutations are widely spread across Na$_v$1.4, in areas important for channel gating such as loops regions, and have been shown to result in disruption of fast inactivation or activation, leading to a persistent inward Na$^+$ current. Interestingly, Arg222Gln affects the R2 gating charge of the S4 voltage sensor – normally the target for Hypo PP mutations. Hypo PP voltage sensor mutations are known to give rise to a gating pore current due to disruption of the ion-pair interactions which hold the gating pore domain together and protect the channel from unwanted ionic leak (Sokolov et al. 2007; Struyk & Cannon 2007; Struyk et al. 2008; Sokolov et al. 2008; Sokolov et al. 2010; Francis et al. 2011). It is possible that substitution of the Arginine residue at position 222 for
the uncharged polar glutamine could cause a gating pore current, especially as a Hypo PP mutation has been found at the same residue, p.Arg222Trp. Matthews et al. reported Arg222Trp in two unrelated patients with a clear Hypo PP phenotype with no atypical features. Although no further studies have been conducted to establish what effect this particular mutation could have on the channel, it has recently has been reported that neutralisation of the Arg222 results in a gating pore current. Holzherr et al. showed that the mutation Arg222Gly produces a gating pore at voltages negative to -50 mV. Furthermore, similar mutations to Arg222Gln/Trp have been found in the gene SCN5A; which encodes the cardiac sodium channel Na\textsubscript{v}1.5. Na\textsubscript{v}1.5 mutations are spread throughout the channel and cause a complex array of disorders including cardiomyopathy and Brugada Syndrome, with a variety of defects in channel functioning. Of particular interest are Na\textsubscript{v}1.5/Arg219His and Na\textsubscript{v}1.5/Arg222Gln, which affect Domain 1 R1 and R2 residues respectively (Gosselin-Badaroudine et al. 2012; Mann et al. 2012; Nair et al. 2012). Gosselin-Badaroudine et al. showed that p.Arg219His results in a conduction pathway through the VSD which allows a proton carrying gating pore current. This leads to questions as to how Arg222Gln causes a predominantly myotonic phenotype when the affected residue is implicated in a case of Hypo PP. The studies highlighted suggest that neutralisation of Arg222 is likely to cause a gating pore current; however this would not fit in with the established mechanisms of disease for Hyper PP mutations.

There have been a few Hyper PP and PMC mutations which result in the neutralisation of positive S4 gating charges, without the formation of a gating pore current. Francis et al. 2011, studied the PMC mutation p.Arg1448Cys and found that neutralisation of this Domain IV R1 arginine had no effect on the structure of the gating pore domain, but rather causes a gain-of-function defect manifested as a reduced rate of inactivation for the Na current through the main pore. Similarly, Lee et al. reported a mutation affecting the R3
residue of the Domain I S4, p.Arg222Trp, in a case of mild non-painful myotonia. However, no further studies have occurred to show what effect this mutation has on the channel. Arg222Gln and Arg222Trp offer an interesting opportunity to study the connection between the disease mechanisms for Hyper PP and Hypo PP as well as giving insight into the effect different amino acids can have on the integrity of the voltage sensor domain. Prior studies of gating charge mutation suggest that both Arg222Gln and Arg222Trp should result in an aberrant ionic leak through the VSD despite resulting in different in phenotypes. Can the difference in amino acid between Arg222Gln and Arg222Trp explain the difference in phenotype? Further investigations are needed to determine how this mutation causes disease and whether it might break the established consensus that only Hypo PP voltage sensor mutations can result in a gating pore current.

3.7.2 Implications for future diagnostics strategy

Through sequencing of the known genes patients who were negative for the common mutations but have a well defined phenotype, mutations were identified in ~30 % of patients. In the case of the Hypo PP samples, this has identified mutations in areas of the gene which have not previously been associated with the disease. Both p.Tyr168Asp and p.Asp1420Gly defy the current consensus that Hypo PP mutations are solely localised to the S4 voltage sensor. Through functional work it will be possible to determine whether these mutations will also challenge the current proposed mechanism of disease – the gating pore current. However, for the Hyper PP samples screened, all 3 mutations identified were located within areas of the channel that have been previously associated with the disease. Of the 3 identified, p.Arg222Gln offers a slight deviation from the previously described mutations as it is affects a residue that has been previously associated with Hypo PP.
All mutations found within the periodic paralysis well defined cohort are located in areas not routinely investigated during diagnostic genetic screening. Accordingly, this study highlights the importance of full screening of the whole coding region of at least SCN4A and possibly other genes which are involved in periodic paralysis.

At the time of the study routine diagnostic genetic screening for periodic paralysis focused on targeted exon sequencing of only four exons of SCN4A. Since the findings of this study, a screen has been developed to enable the sequencing of the whole gene through a customised gene panel. This targets all the known channelpathy genes and sequences all exons.

3.7.3 Screening of new genes
Three genes have been recently associated with episodic weakness or periodic paralysis with secondary symptoms. These are MT-ATP6 and MT-ATP8 and KCNJ18 (Ryan et al. 2010; Cheng et al. 2011; Auré et al. 2013). These genes were sequenced in a larger cohort of patients in order to investigate if the phenotypes associated with these genes could be expanded to include periodic paralysis without secondary symptoms. In addition, KCNJ18 is associated with periodic paralysis in Asian populations, thus this study provided an opportunity to determine if KCNJ18 mutations can be identified in a non Asian population.

The first genes investigated were MT-ATP6 and MT-ATP8 which are mitochondrial genes which have recently been associated with a phenotype of episodic weakness mimicking periodic paralysis with later onset of distal motor neuropathy (Auré et al. 2013). MT-ATP6 is normally associated with Leigh Syndrome and NARP (Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa) as well as CMT type 2 (Charot Marie Tooth), Spastic paraplegia and Cerebellar ataxia (Holt et al. 1990; Santorelli et al. 1996; Y et al. 1997; Moslemi et al. 2005; Verny et al. 2011; Pitceathly et al. 2012; Synofzik et al. 2012; Craig et al. 2007; Pfeffer et al. 2012). The MT-ATP6 and MT-ATP8 genes encode components of the large protein
complex, FOFO1 ATP synthase (complex V), the final enzyme in the oxidative phosphorylation pathway. ATP synthase drives the synthesis of ATP from ADP + phosphate (Pi) using energy provided by the proton gradient across the mitochondrial membrane.

Auré et al. reported six families with episodic weakness and distal motor neuropathy which showed homogeneity in terms of onset of symptoms and trigger factors. Following genetic investigations in which they ruled out the known periodic paralysis genes, mutations were found in \textit{MT-ATP6} and \textit{MT-ATP8} genes which segregated with disease in all families. Molecular studies of patient fibroblasts revealed oxidative defects with reduced ATP production and decrease complex V and I activity. Furthermore, whole cell patch clamp recordings showed the fibroblasts had a partially depolarised membrane with a reduced outward $K^+$ current density. These findings offer support for the mitochondrial genes causing the episodic weakness phenotype. Thus, the periodic paralysis cohort was screened for mutations within these genes in order to determine whether the \textit{MT-ATP6} and \textit{MT-ATP8} associated phenotypes could be further expanded to include periodic paralysis without secondary symptoms. 60 patients were screened (Figure 3-1); however no mutations were identified in either gene. This suggests that with the current cohort it is not possible to expand the phenotypes associated with \textit{MT-ATP6} and \textit{MT-ATP8} mutations further to include a purely periodic paralysis phenotype.

\textit{KCNJ18}, the final gene screened in the periodic paralysis cohort, encodes the inwardly rectifying potassium channel Kir2.6. Kir2.6 is expressed within the skeletal muscle and is vital for muscle functioning due to its role in stabilizing the resting membrane potential. \textit{KCNJ18} has been associated both Thyrotoxic Hypo PP (TPP) and Sporadic Periodic Paralysis (SPP) (Ryan et al. 2010; Cheng et al. 2011). TPP is associated with attacks of weakness, hypokalemia and thyrotoxicosis and is highly prevalent in men from Asian and Latin American population. 10% of men in these populations develop TPP compared with 0.1% of
Caucasians (Okinaka et al. 1957; Kelley et al. 1989; Kung 2006). SPP is a non familial form of periodic paralysis which is normally found within the Asian population who are not affected by altered thyroid hormone levels (Cheng et al. 2011). Ryan et al. identified KCNJ18 in TPP following genetic screening of 151 patients within the Asian population. Subsequently, Cheng et al found KCNJ18 mutations within a cohort of Asian SPP patients. Both reported mutations that caused non functional channels, reduced surface expression, decrease current density and disrupted interaction with PIP₂.

KCNJ18 was screened for 60 patients with periodic paralysis in order to determine if KCNJ18 can cause periodic paralysis in non Asian populations. The majority of the patients in the cohort screened are from a Caucasian background, with a few from other non Asian backgrounds. Before analysis of sample sequences occurred it was confirmed that KCNJ18 was amplified correctly rather than the homologous KCNJ12, with which it shares 99% sequence identity. Following this it was found that one sample out of 60 had a variant in exon 3 of KCNJ18, p.Ile12Thr. The mutation is located in the N-terminal end of the protein which has previously been associated with the SPP mutation p.Arg43Cys. However, due to the relatively recent discovery of KCNJ18, there is little information available on the channel and polymorphisms of the gene, making it difficult to determine the effect of the mutation without functional characterisation. Thus, the MiSeq custom gene panel data for this patient was checked for mutations in the known genes. A known mutation in SCN4A, p.Arg675Gln, was identified. Arg675Gln is a known normokalemic periodic paralysis mutation that has been shown to cause a gating pore current which is activated by depolarised potentials (Sokolov et al. 2008). The symptoms experienced by this patient fit in with the phenotype associated with p.Arg675Gln, indicating that this is the causative mutation. Subsequently, as the patient does not present with an atypical phenotype it was concluded that no further investigations should occur for the KCNJ18 p.Ile12Thr mutation.
due to the presence of the known SCN4A mutation and difficulty in determining the likelihood of pathogenicity for the variant. This indicates that for this cohort of periodic paralysis patients, it is not possible to expand the KCNJ18 phenotype to include periodic paralysis in familial and non-Asian populations.

3.7.4 Limitations of sequencing approach

Although a number of mutations were identified during this study, there is still a significant portion of the cohort sequenced who do not have a genetic diagnosis. Whilst this may be explained by mutations in as yet unidentified genes; it is possible that the strategy used has resulted in some mutations within the known genes being missed. Since the channelopathies in question are known to have a dominant inheritance pattern, the present study focused on exonic sequencing and the identification of non-synonymous mutations within the genes sequenced. Only a small part of the intronic region of each gene was sequenced to ensure full coverage of the exon. Furthermore, any synonymous mutations not located near the end of an exon were not investigated further. As a result, it is possible that synonymous variants or intronic splice site variants were missed which could disrupt channel functioning. Although such mutations are not normally associated with a dominant inheritance within ion channels, it is possible that they may be able to effect channel functioning in a way consistent with mechanism of disease. For example, Kubota et al identified an intronic mutation in SCN4A which affect channel splicing and caused disruption to channel gating. Accordingly, it is possible that investigating intronic mutations may result in identification of further mutations within the ion channel genes.

3.8 Conclusion

From sequencing of SCN4A in a periodic paralysis cohort, 6 mutations were identified; five of which were novel. Two mutations found within Hypo PP cases were located in areas of
the channel which have not normally been associated with disease. This genetic study highlights the importance of full sequencing of the known genes in order to provide the patient with a genetic diagnosis. Next generation sequencing methods such as targeted gene panels and exome sequencing provide cost effective methods to sequence all the known genes. In addition, this study has widen the current knowledge of mutations associated with Hypo PP.

3.9 Further Work

- A number of cases within the cohorts where no mutation was identified. In order to find the causal mutation within the remainder of this cohort, patients with a strong phenotype and family history should be selected and exome sequencing used. This would enable identification of the genetic cause of disease.
- The mutation Y168D needs to be functionally characterised in order to determine if it is the pathogenic mutation.

3.10 Acknowledgements

Dipa Raja-Ryan and Emma Matthews clinically evaluated the patients screened. Alice Gardner performed and analysed MiSeq data.
Chapter 4: Sequencing of Patients with Myotonia

4.1 Summary

The non-dystrophic myotonias are caused by mutations in two ion channels: SCN4A and CLCN-1. Over 150 CLCN-1 pathogenic mutations have been identified in relation to myotonia congenita. Interestingly, 10 of these mutations have been reported as having both dominant and recessive inheritance, leading to questions as to the mechanism behind this. Furthermore, ~20% of paramyotonia congenita and sodium channel myotonia patients remain without a genetic diagnosis following routine testing of the causative gene, SCN4A. The aim of this study is to address these questions, investigate the causative mutations of myotonia and the mechanism of dual mode of inheritance of mutations in myotonia congenita.

The whole coding region of SCN4A was screened in a small cohort of patients with well characterised PMC or SCM phenotypes who had undergone routine testing and were found to be negative for the known common mutations. 3 out of 8 patients were found to have a mutation in SCN4A. Of these, two mutations were known, well characterised mutations (p.Val1293Ile and p.Val1589Met) with one novel mutation (p.Ser1159Pro). The novel mutation is located in the S4-S5 loop near other PMC mutations, and is predicted through in silico analysis to be pathogenic, which is indicative of p.Ser1159Pro being the causative mutation of PMC in this case. The second study investigated the mechanism behind dual inheritance mutations in myotonia congenita. 10 dominant MC pedigrees with CLCN-1 mutations which have been reported as having a dual mode of inheritance were screened for mutations in three known myotonia genes: SCN4A, DMPK5 and ZNF9. No mutations were found all three genes. These findings indicate that in this cohort, mutations in other genes cannot explain dual inheritance of the CLCN-1 mutations. Finally, exome sequencing
was undertaken to identify the causative mutation for a large PMC family without any mutations following fully SCN4A screening.
4.2 Introduction

Both paramyotonia congenita and sodium channel myotonia are caused by mutations in SCN4A and show autosomal dominant inheritance. Studies have shown that the most common PMC mutations are p.Thr1313Met and p.Arg1448Cys (McClatchey et al., 1992; Ptacek et al., 1993; Ptácek et al., 1992; Yang et al., 1994) whilst for SCM they are p.Val1589Met and p.Gly1306Glu (Heine et al. 1993; Lerche et al., 1993; Mitrović et al., 1995). These gain of function mutations, along with all other SCM/PMC mutations, have been shown to cause a defect of channel inactivation or enhanced activation. This results in an increased level of sodium entering the muscle which causes an increase of the sarcolema excitability (Cannon, 2006; Jurkat-Rott et al., 2002). Routine diagnostic testing often only involves the targeted screening of exons 22 and 24 which cover the common mutations. However, over 45 mutations, spread widely across the gene, have been identified in relation to these disorders, leading to a negative diagnostic test for some patient.

In contrast to the other forms of NDM, myotonia congenita is caused by CLCN-1 mutations with over 150 mutations reported in this gene. Interestingly, MC inheritance has been reported as both autosomal dominant and recessive. The dominant and recessive forms of MC can be distinguished based on onset of disease and severity of symptoms but the main distinguishing point is the causative mutation. Mutations causing dominant MC are largely missense mutations often found in one hotspot in exon 8 of CLCN-1 (Fialho et al., 2007), while recessive MC mutations show greater variety in the type of mutation and are widely distributed across the gene. Myotonia congenita genetics is complicated by the knowledge that 10 CLCN-1 MC mutations to date, have been reported to have both dominant and recessive inheritance (Table 4-1). The presence of dual mode of inheritance mutations is an unusual and almost unique feature of myotonia congenita. This phenomenon is interesting
due to the vastly different effects on channel function dominant and recessive mutations have been shown to have. Possible explanations include incomplete penetrance of dominant mutations, differential allelic expression, unidentified modifying factors or mutations in other genes. However, Dunø et al 1994 studied the relationship between inheritance pattern and mRNA levels in pedigrees carrying p.Arg894X and found no difference with dominant and recessive pedigrees. Interestingly, within the ALS associated gene SOD-1, one mutation has been reported with dual inheritance, in which a common founder was identified providing a protective factor (Al-Chalabi et al., 1998). As mentioned one possible explanation for dual inheritance mutations is the presence of mutations in other genes. The most likely genes to play a role would be the other myotonia related genes: SCN4A, DMPK5 and ZNF9 which cause paramyotonia congenita, muscular dystrophy type 1 and muscular dystrophy type 2, respectively. Misdiagnosis or phenotype overlap is well documented between mutations in these genes and CLCN-1 for example, Suominen 2008 showed that 5% of patients with the DM2 ZNF9 repeat expansion also had a CLCN-1 mutation (Suominen et al., 2008). Although this finding might be explained by experimental bias, it highlights the possibility that MC might be caused by mutations in other genes, both known or unknown.
<table>
<thead>
<tr>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Effect on Channel</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.598G&gt;A</td>
<td>Gly200Arg</td>
<td>Dominant shift of po</td>
<td>(Mailänder et al. 1996; Wollnik et al. 1997; Zhang et al., 1996)</td>
</tr>
<tr>
<td>c.689G&gt;A</td>
<td>Gly230Glu</td>
<td>Slightly dominant negative; altered gating</td>
<td>(Brugnoni et al., 1999; Fahlke et al., 1997; Meyer-Kleine et al., 1995; Plassart-Schiess et al., 1998; Steinmeyer et al., 1994; Zhang et al., 1996)</td>
</tr>
<tr>
<td>c.803C&gt;T</td>
<td>Thr268Met</td>
<td>n/a</td>
<td>(Brugnoni et al., 1999)</td>
</tr>
<tr>
<td>c.937G&gt;A</td>
<td>Ala313Thr</td>
<td>Dominant negative shift of po</td>
<td>(Kubisch et al., 1998; Plassart-Schiess et al., 1998; Sun et al., 2001)</td>
</tr>
<tr>
<td>c.950G&gt;A</td>
<td>Arg317Gln</td>
<td>Dominant negative shift of po</td>
<td>(Esteban et al., 1998; Meyer-Kleine et al., 1995; Pusch et al., 1995)</td>
</tr>
<tr>
<td>c.1013G&gt;A</td>
<td>Arg338Gln</td>
<td>Slight shift in po</td>
<td>(Meyer-Kleine et al., 1995; Zhang et al., 1996; Zhang, Bendahhou, Sguinetti, &amp; Ptácek, 2000)</td>
</tr>
<tr>
<td>c.1592C&gt;T</td>
<td>Ala531Val</td>
<td>n/a</td>
<td>(Papponen et al., 1999; Sun et al., 2001)</td>
</tr>
<tr>
<td>c.1655A&gt;G</td>
<td>Gln552Arg</td>
<td>Dominant shift of po</td>
<td>(Meyer-Kleine et al., 1995; Pusch et al., 1995)</td>
</tr>
<tr>
<td>c.1667T&gt;A</td>
<td>Ile556Asn</td>
<td>Recessive shift of po</td>
<td>(Kubisch et al., 1998; Plassart-Schiess et al., 1998; Saviane, Conti, &amp; Pusch, 1999)</td>
</tr>
<tr>
<td>c.2680C&gt;T</td>
<td>Arg894X</td>
<td>Slightly dominant negative</td>
<td>(Dunø et al., 2004; George et al., 1994; Meyer-Kleine et al., 1995)</td>
</tr>
</tbody>
</table>

Table 4-1 Dual Inheritance myotonia congenita mutations and their effect on the channel

Po = open probability
4.2.1 Aims

This study investigated two cohorts of patients which had a main symptom of myotonia. The patients were clinically diagnosed with Myotonia Congenita; Paramyotonia Congenita or Sodium Channel Myotonia, but following routine diagnostic testing had been none of the common mutations. For the PMC/SCM cohort we aimed to identify new causative mutations, through the screening of non-routinely tested areas. Whilst with the MC cohort, dominant families were sequenced for the known genes in order to further our understanding of the presence of mutations described as both dominant and recessive. Furthermore, next generation sequencing techniques was used in the case of a well defined PMC family, who were found to have no SCN4A mutations, in order to indentify novel genes involved in the disease.
4.3 Myotonia Patient Cohorts

Two small cohorts of patients with myotonia as the main overriding symptom were selected for two different gene screening studies. The first contained eight patients who have been clinically diagnosed with either PMC or SCM who did not have the common mutations within SCN4A, following routine diagnostic testing. The second cohort was composed of 10 dominant families who have been clinically diagnosed with myotonia congenita and have been found to have one CLCN-1 mutation. However, these mutations have been reported to show dual inheritance even though dominant and recessive forms of myotonia congenita showing different disease mechanisms. Thus, it is possible that another causative mutation in the known genes might be found in these dominant families. In this cohort, SCN4A was initially screened, followed by the myotonic dystrophy genes: DMPK5 and ZNF9. Finally, exome sequencing was used to identify possible causative mutations in a PMC family, who had previously been sequenced for both CLCN-1 and SCN4A, and found to be negative.

4.4 PMC and SCM Cohort

4.4.1 Screening SCN4A in PMC and SCM patients

In order to identify new mutations in the PMC/SCM cohort of patients, the whole coding region of SCN4A was screened. DNA from the cohort was amplified using primers targeted to all 24 SCN4A coding exons and then sequenced. 3 out of 8 patients were found to have mutations in SCN4A. Of these mutations, one was found to be novel and predicted to be pathogenic through in silico analysis, c.3475T>C, p.Ser1159Pro. The other two mutations, c.4765G>A, p.Val1589Met and c.3877G>A, p.Val1293Ile, are known well characterised mutations.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age at onset (years)</th>
<th>Affected Family Members</th>
<th>Clinical Features</th>
<th>Phenotype</th>
<th>SCN4A mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>Infancy</td>
<td>Father &amp; Brother</td>
<td>Nonpainful myotonia mainly affecting eyes and grip; slight muscle hypertrophy; myotonia provoked by cold exposure, tiredness and alcohol</td>
<td>SCM</td>
<td>Ser1159Pro</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>Childhood</td>
<td>Maybe Mother</td>
<td>Myotonia affected by cold and exercise.</td>
<td>SCM</td>
<td>Val1589Met</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>3 years old</td>
<td>Father, sister &amp; daughter</td>
<td>Myotonia on EMG. Also has a $CLCN-1$ mutation.</td>
<td>MC</td>
<td>Val1293Ile</td>
</tr>
</tbody>
</table>

Table 4-2 Clinical features of myotonia patients
Patient 1

Patient 1 is a 36 year old female who has shown symptoms of myotonia from infancy with eyelid myotonia as a baby. While growing up she always noted impaired relaxation of her muscles associated with intermittent stiffness. Cold, alcohol and tiredness exacerbate her symptoms particularly eyelid myotonia. She has a 63 year old father and 26 year old brother who are similarly affected (Figure 4-1A). On examination she shows clear eyelid myotonia which improves on repetition as well as grip and percussion myotonia. Detailed electrophysiological testing has been conducted showing profuse myotonic discharges in all muscles. On the short exercise test there was a small increase in CMAP on cooling while the long exercise test is negative. These symptoms along with electrophysiological tests suggest the patient has sodium channel myotonia.

Sequencing of the coding exons of SCN4A for the proband found a substitution mutation at position 3475 of a T to a C resulting in an amino acid change Ser1159Pro (Figure 4-1B). Ser1159Pro is a novel mutation located in the loop region between the S4 and S5 segments of domain III and affects a highly conserved amino acid. Furthermore, in silico analysis predicts this mutation to be probably damaging with a score of 0.999. The mutation was found to segregate with disease in the proband’s family suggesting that it is the causative mutation.
Figure 4-1 SCN4A mutations identified in the sodium channel related myotonia cohort

A, C & E, Pedigrees of families, arrow indicates proband and affected members are shaded in. B, D & F, Chromatograms of partial sequences of SCN4A showing one heterozygous point mutations.
Patient 2

Patient 2 is a 46 year old woman who has been affected with stiffness from childhood. She experiences muscle stiffness with no warm up and is not affected by episodes of muscle weakness. The stiffness is brought on by cold temperatures and exercise. From electrophysiological testing the patient was found to have profuse myotonia in all muscles tested but showed normal long and short exercise tests. This is consistent with sodium channel myotonia. It is thought that the patient’s mother is also affected although she is unavailable for testing (Figure 4-1C).

All coding exons in SCN4A were sequenced revealing a substitution in exon 24 at position 4765 of a G to A resulting in an amino acid change p.Val1589Met (Figure 4-1D). Val1589Met is a known Na,1.4 mutation which has previously been reported to cause Sodium Channel Myotonia (Heine et al. 1993; Orrwll et al. 1998). It is located in S6 of domain IV and is found nearby the Hyper PP causing mutation p.Met1592Val (Ptacek et al., 1993; Rojas et al., 1991).
<table>
<thead>
<tr>
<th>DNA Change</th>
<th>Location within Gene</th>
<th>Amino Acid Change</th>
<th>Location within Protein</th>
<th>Pathogenic (In silico Analysis)</th>
<th>Known mutation?</th>
<th>Functional Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.3475T&gt;C</td>
<td>Exon 19</td>
<td>p.Ser1159Pro</td>
<td>DIII S4-S5 loop</td>
<td>Yes</td>
<td>No</td>
<td>To be done</td>
</tr>
<tr>
<td>c.4765G&gt;A</td>
<td>Exon 24</td>
<td>p.Val1589Met</td>
<td>DIV S6 Segment</td>
<td>n/a</td>
<td>Yes</td>
<td>Defect of inactivation(Heine et al., 1993; Orrell et al. 1998)</td>
</tr>
<tr>
<td>C.3877G&gt;A</td>
<td>Exon 21</td>
<td>p.Val1293Ile</td>
<td>DIII S6 Segment</td>
<td>n/a</td>
<td>Yes</td>
<td>Defect of inactivation and shift in voltage dependence of activation(Koch et al. 1995)</td>
</tr>
</tbody>
</table>

Table 4-3 SCN4A mutations identified in myotonia patients
Patient 3

Patient 3 is a 51 year old male affected with myotonia congenita with dominant inheritance. During childhood he reached all normal milestones with symptoms occurring at the age of 3 years old with stiffness in legs. The patient’s symptoms are not made worse by cold temperatures but are exacerbated by lack of sleep and emotional stress. Examination of the patient shows mild myotonia of the face especially around the eyes. In addition, the patient is being treated for hypothyroidism with thyroxine. The family history suggests a dominant pattern of inheritance with both father and grandfather of the patient potentially affected. The patient has two younger sisters, one of which shows mild symptoms although not as severely as her brother, and two young daughters; one affected and one unaffected (Figure 4-1E).

An initial investigation into the causative mutation of the patient revealed a deletion in CLCN-1 c.1872delG in exon 16 which results in a frame shift mutation p.Glu624fs. A single frame shift mutation of this kind in CLCN-1 usually causes recessive myotonia congenita. Interestingly, this mutation was also found in the patient’s apparently unaffected daughter but none of the other family members tested. Therefore, the patient was put forward for further sequencing of other genes. Through sequencing of the all the coding exons of SCN4A it was found that this patient had a mutation in exon 21, c.3877G>A which results in an amino acid change p.Val1293Ile (Figure 4-1F). Val1293Ile is located near the cytoplasmic face of S6 in Domain III and is a known variant which has been previously reported to cause a milder form of PMC where paralysis does not occur even upon cooling (Koch et al. 1995). The mutation was also found in the patient’s sister who shows mild symptoms but not in the unaffected sister or daughter. Unfortunately, the patient’s affected daughter is unavailable for testing.
Figure 4-2 Schematic diagram of the sodium channel α subunit showing the localisation within the channel of the SCN4A mutations identified in the myotonia cohort.
4.5 Dominant Myotonia Congenita Cohort

4.5.1 Screening for SCN4A mutations

In order to identify a secondary mutation in dominant myotonia congenita families with a dual inheritance CLCN-1 mutation, the whole coding region of SCN4A was screened through amplification of individual exons from patient DNA which were then sequenced. 10 samples were screened for SCN4A mutations, however, no mutations were found in this gene in all samples following analysis.

4.5.2 Screening Myotonic Dystrophy Genes

Following screening of SCN4A, the dominant MC cohort were screened for expansions in both myotonic dystrophy genes, DMPK and ZNF9, which cause myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2). Following fragment analysis of both genes no samples were found to have expansions in either gene which are sufficient to cause disease.
4.6 Exome Sequencing

Exome sequencing provides a helpful tool for the identification of a causative mutation with the potential of finding a new gene for those patients who have a clear phenotype and a strong family history, yet are found to be negative for mutations in the known genes. For this study exome sequencing was used to identify the causative mutation within a family with a history of paramyotonia congenita, without any mutations in the known genes. The family is a large Sicilian family with a number of members who show symptoms of paramyotonia congenita. Samples were obtained from three affected family members: Mother (proband) and two children.

The proband is a middle-aged Sicilian woman who has experienced muscle stiffness and paralysis from infancy. Onset occurred at the age of three with muscle stiffness upon exposure to cold temperatures. Myotonia is present in her peripheral muscles, eyelids and extraocular muscles. Without treatment the proband experiences muscle cramp and pain upon waking in the morning. Triggers for attacks include old temperatures, exercise and some foods. EMG reveals extensive myotonia especially upon cooling. The proband has responded well to a low dose of mexiletine. Other family members are known to be similarly affected although details and samples are only available for the proband’s son and daughter who are exhibit similar symptoms.

DNA from each family member was sequenced with Neuromics and the data obtained was analysed and filtered using Neuromics Decode software. Variants were filtered based on low predicted protein impact, presence in databases, frequency within the general population and read dept. Variants within all members were then filtered based on the inheritance pattern, relation to known symptoms and diseases and against a skeletal muscle gene list.
Following multistep filtering 10 variants were identified. Of these three genes were identified as clinically relevant with potentially causative variants: RYR-1, AGRN and COL6A3 (see Table 4-4 for variant list). RYR-1 is a vital component of the excitation-contraction coupling machinery and is associated with malignant hyperthermia and central core disease. Mutations of RYR-1 have been shown to be inherited in a dominant and recessive manner. In this family three RYR-1 mutations were identified which all have been previously reported and associated with disease, c.4711A>G, p.1571I>C (rs146429605), c.10097G>A, p.3366R>H (rs137932199) and c.11798A>G, p.3933Y>C (rs147136339). These mutations have been previously shown to co-segregate with disease; Klein et al 2011 and Tammaro et al have shown that c.4711A>G is inherited in cis with c.11798A>G whilst Duarte et al 2011 showed that c.10097G>A and c. 11798A>G are inherited together(Duarte et al., 2011; Klein et al., 2012; Tammaro et al., 2011). AGRN is a component of the synaptic basal lamina and is crucial for the formation of the neuromuscular junction. Mutations of AGRN have been associated within limb girdle congenital myasthenic syndrome. In this family we identified a novel variant, c.497C>G, p.166P>R and is predicted to be pathogenic. Finally, COL6A3 forms one of three α chains of type VI collagen and forms a part of the extracellular matrix which surrounds muscle cells. COL6A3 is necessary for muscle cell stability, growth and movement. Mutations associated with COL6A3 cause Bethlem myopathy or Ullrich congenital muscular dystrophy and are inherited dominantly or recessively, respectively. A novel variant, c.4510G>A, p.1504R>W, was identified and is predicted to be pathogenic.

In order to establish which variants are responsible for disease in this family, it is necessary to confirm the presence of the mutation through Sanger sequencing and to check for segregation within the rest of the family members. Unfortunately, due to time constraints it has not been possible to confirm these mutations via Sanger sequencing.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Function</th>
<th>Expressed in skeletal muscle</th>
<th>Associated with disease</th>
<th>Variants identified</th>
<th>Known variant?</th>
<th>Clinically Relevant</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGRN</td>
<td>Agrin</td>
<td>Component of the synaptic basal lamina</td>
<td>Yes</td>
<td>Limb-girdle congenital myasthenic syndrome</td>
<td>c.497C&gt;G, p.P166R</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>CLCNKB</td>
<td>CIC-kb</td>
<td>Important for the reabsorption of salt from urine into the bloodstream</td>
<td>No – Kidney</td>
<td>Bartter Syndrome</td>
<td>c.403C&gt;G, p.P135A</td>
<td>Yes – SNP rs1057857 (Allele frequency = 0.6207)</td>
<td>No</td>
</tr>
<tr>
<td>TTN</td>
<td>Titin</td>
<td>Important for contraction of striated muscle</td>
<td>Yes</td>
<td>Hereditary myopathy with respiratory failure Limb-girdle muscular dystrophy type 2J Tibial muscular dystrophy</td>
<td>c.13316T&gt;C, p.Y4439C</td>
<td>Yes – rare SNP rs72648942 (Allele frequency = 0.005848)</td>
<td>Yes</td>
</tr>
<tr>
<td>COL6A3</td>
<td>Collagen, type VI, alpha 3</td>
<td>Forms part of the extracellular matrix surrounding muscle cells.</td>
<td>Yes</td>
<td>Bethlem myopathy Ullrich congenital muscular dystrophy</td>
<td>c. 4510G&gt;A, p.R1504W</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>COL9A3</td>
<td>Collagen, type IX, alpha 3</td>
<td>An important component of cartilage Cartilage</td>
<td>No – Hyaline Cartilage</td>
<td>Multiple Epiphyseal dysplasia</td>
<td>c.3008G&gt;A, p.R103Q c.520-6C-T</td>
<td>Yes – both rare SNPs rs142639450 rs45476191 (Allele frequency = 0.01459 rs0.02267)</td>
<td>No</td>
</tr>
<tr>
<td>GARS</td>
<td>Glycine tRNA ligase</td>
<td>Important for protein synthesis</td>
<td>Low expression</td>
<td>Distal hereditary motor neuropathy type V CMT type 2D</td>
<td>c.11C&gt;T, p.P4L</td>
<td>Yes – rare SNP rs62636572 (Allele frequency = 0.02066)</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 4-4 Table showing variants identified through whole exome sequencing following filtering of data

Allele frequency data taken from Exome Aggregation Consortium (ExAC) database.
4.7 Discussion

The non-dystrophic myotonias are a group of disorders characterised by increased muscle excitability due to mutations within the ion channel genes: CLCN-1 and SCN4A. In order to further our understanding of the genetic causes of non-dystrophic myotonia two small cohorts of myotonia patients were sequenced. The first consisted of patients with clear, well characterised phenotypes of either: myotonia that worsens with cold and occasional weakness (PMC) or myotonia with no weakness and some potassium sensitivity (SCM). These patients had undergone routine diagnostic testing without a causative mutation being identified. The second cohort contained 10 families with dominant myotonia who had been found to carry a single mutation in CLCN-1 which has been reported as either dominant or recessive. For both myotonia cohorts, Sanger sequencing of SCN4A was undertaken in order to expand the current knowledge of the genetic causes of myotonia. In the dominant MC cohort the myotonic dystrophy genes ZNF9 and DMPK were also sequenced. Finally, exome sequencing was undertaken for a family with a myotonia phenotype, without mutations in any of the known channelopathy genes in order to further the genetic spectrum of channelopathy genes.

4.7.1 Sequencing of known genes in myotonia

The skeletal muscle sodium channel gene, SCN4A was analysed in a cohort of eight patients with either PMC or SCM. Following sequencing of all 24 exons within SCN4A, 3 out of 8 samples (37.5%) were found to have a likely causative mutation. Of the three mutations identified two were previously reported and associated with a myotonia phenotype: p.Val1589Met and p.Val1293Ile (Table 4-2 and Table 4-3). The final mutation is a novel variant, p.Ser1159Pro which was absent from controls and is not present in the online databases including 1000 genomes, NLHBI and HGMD.
The first mutation, p.Val1589Met, was identified in a patient who presents with muscle stiffness without weakness which can be exacerbated cold temperatures and exercise. The mutation is located near the cytoplasmic region of the DIV S6 segment, which has been implicated as a potential docking site for the inactivation gate of the channel. p.Val1589Met is a known pathogenic mutation which has previously been associated with a myotonia phenotype which is aggravated by cold and potassium (Heine et al., 1993; Orrell et al., 1998). This phenotype is consistent with that of the proband. Whole cell and single channel recordings of HEK293 cells expressing both WT and Val1589Met mutant have shown that the mutant causes a defect in normal channel inactivation (Mitrović et al., 1994). This results in a clear instability of the inactivated state as the mutant channels show faster recovery from inactivation and a larger steady-state currents compared with the WT channel (Mitrović et al., 1994). Thus, it is possible to conclude that p.Val1589Met is the causative mutation in this patient.

The second known mutation, p.Val1293Ile, was identified in a middle aged male who has presented with myotonia since the age of three years old and does not experience any weakness or aggravation with cold or potassium. The patient’s family history is suggestive of dominant inheritance with a mildly affected sister and one affected daughter. Val1293Ile is located within the DIII S6 segment which has been shown to play a critical role in fast inactivation (McPhee et al. 1995). This is a known pathogenic mutation which has previously been associated with a mild myotonia phenotype which is aggravated by cold but shows no paralysis (Koch et al., 1995). Studies in expression systems have shown that p.Val1293Ile causes a reduced rate of fast inactivation, accelerated recovery, altered voltage dependence of inactivation as well as a small hyperpolarised shift in activation (Green et al. 1997). These disruptions to channel gating although mild are sufficient to cause the myotonic bursts responsible for the phenotype. This sodium channel
mutation appears to segregate with disease for this family suggesting it is at least partially accountable for the symptoms these patients experience. Interestingly, upon initial investigations a deletion in \textit{CLCN-1} exon 16, c.1872delG, which causes a frameshift, p.Glu624fs, was identified in the patient. However, this mutation did not segregate with disease as the proband's affected sister did not carry the variant whilst the unaffected daughter did. It is feasible that the proband experiences more severe symptoms in comparison to his affected sister due to the combination of the \textit{CLCN-1} mutation with the \textit{SCN4A} mutation.

The final mutation found within the PMC/SCM cohort was a novel variant, p.Ser1159Pro. The proband carrying this mutation presents with eyelid, grip and percussion myotonia that is exacerbated by cold without paralysis. Ser1159Pro is located near the COOH terminal end of the DIII S4-S5 loop, an area which has been shown to be important for the fast inactivation of the channel. The mechanism for inactivation is suggested to occur through a ball and chain mechanism whereby an inactivation gate occludes the central pore through binding of an inactivation gate receptor site (Armstrong & Bezanilla, 1977). It has been shown that the loop between DIII and DIV forms the inactivation gate with three critical residues which form the IFM (Ile-Phe-Met) motif. The exact structure of the inactivation gate receptor is not known, however a number of amino acids within the sodium channel DIII and DIV S4-S5 loops have been highlighted for their interaction with the IFM motif (McPhe et al. 1998; Popa et al. 2004; Smith & Goldin, 1997). Additionally, Ser1159Pro is located near a number of other myotonia mutations: Ala1152Asp, Ala1156Thr and Ile1160Val (Figure 4-2) (Bouhours et al., 2005; McClatchey et al., 1992; Ptacek et al., 1993; Richmond et al. 1997; Yang et al., 1994b). These mutations have been previously studied to identify the effect they have on the sodium channel. All three have been shown to cause an altered rate of inactivation, accelerated recovery and altered voltage dependence of
inactivation. Ile1160Val and Ala1152Asp also showed an accelerated rate of deactivation (Richmond et al. 1997; Bouhours et al. 2005). These are common defects amongst myotonia mutations and have been well documented to result in a prolonged influx of Na⁺ ions leading to the increased excitability which results in myotonia. Thus, it is possible that loss of the polar serine residue for a non polar cyclic proline could cause sufficient disruption to the S4-S5loop to effect channel inactivation, as seen in the nearby myotonia mutations, which cause a similar phenotype to that seen in the proband. Functional work is needed to confirm that this mutation results in the expected defect of inactivation.

Following screening of the whole SCN4A gene in this myotonia cohort, 37.5% of patients were found to have a mutation that would not have been identified during routine diagnostic testing where just two exons are sequenced (exon 22 and 24). This result suggests that mutation screening for myotonia patients should extend to the whole gene as now happens with CLCN-1. However, during routine investigations it can be time consuming and costly to sequence large genes when a large proportion of mutations are found within two exons. Thus, the development of next generation sequencing techniques such as customised gene panels which is run on Illumina’s MiSeq and enables the quick sequencing of genes, will greatly improve diagnostic output in the future.

For PMC/SCM it is well documented that 20% of patients are still without a causative mutation following sequencing of SCN4A (Horga et al., 2013; Matthews et al., 2008). Conversely, in this study we found that the proportion of patients without a mutation following SCN4A sequencing was actually 62.5%. This proportion is unlikely to be representative of the wider patient population due to positive bias for patients without an SCN4A mutation during the selection of patients who had had the common mutations already ruled out. However, we cannot completely rule out mutations in SCN4A from this
study as only the exonic regions were sequenced. Kubota et al. have shown that PMC can be caused by mutations within the intronic regions of SCN4A; therefore it is possible that further mutations would be indentified in SCN4A by screening intronic areas. This was not conducted during this study due to time and cost required to screen large intronic regions within this gene. The negative findings within this study give further support to the notion that other genes, which remain as of yet unidentifed, are likely to play a role in the mechanism of disease of the NDMs. Thus next generation sequencing such as exome sequencing will be important for the identification of mutations within this proportion of patients and enable more understanding of the mechanism of disease.

4.7.2 Dominant families

In order to further understand the presence of dual mode of inheritance mutations in myotonia congenita, a small cohort of dominant families that were found to have dual inheritance mutations in CLCN-1 were investigated to see if the phenomenon of dual inheritance mutations can be explain by the presence of mutations in other genes. 10 individuals were sequenced for mutations within known genes which have been associated with myotonia: SCN4A, DMPK and ZNF9.

Initially the whole SCN4A coding region was sequenced in 10 samples. No mutations were identified in SCN4A gene for all cases screened. This indicates that in this cohort, sodium channel mutations cannot explain the disease as seen in other dominant MC patients who have been shown to carry mutations in both CLCN-1 and SCN4A (Hehir et al. 2013; Trip et al., 2008). Following this, the muscular dystrophy genes were investigated. Muscular dystrophy type 1 is caused by repeat expansions of a CTG trinucleotide repeat in DMPK. DM1 is caused by between 50 to 5,000 copies of this unstable motif with disease severity correlating with the copy number of the repeat. All patients in this cohort were found to have CTG repeat numbers within the normal limit. A similar disease mechanism has been
identified in DM2, which is caused by expansion of the repeat CCTG in intron 1 of ZNF9 within a complex repeat motif. In order to cause disease the number of CCTG repeats in expanded alleles has an average of 5,000 repeats. As with DMPK all samples analysed were found to have repeats within the healthy range. Thus in this cohort expansions within the muscular dystrophy genes are not accountable for disease causation. This was surprising as some cases have been reported with patients with a single CLCN-1 gene were also found to carry a repeat expansion associated with one of the muscular dystrophies. In these cases the presence of a CLCN-1 mutation is thought to intensify the myotonic symptoms of muscular dystrophy leading to initial misdiagnosis of myotonia congenita (Suominen et al., 2008). However, following screening of this cohort it is clear that repeat expansions in these genes cannot explain the description of these CLCN-1 mutations as dominant and recessive.

Although no mutations were identified in this dominantly inherited MC family cohort it does not completely rule out the hypothesis that the phenomenon is due to dominant families carrying a CLCN-1 that is in fact a recessive mutation. It is possible that there are mutations within intronic or promoter regions as well as in currently unidentified genes which may play a role within this phenomenon. Furthermore, other mechanisms such as modifying factors, incomplete penetrance or mutations in unknown genes which have not been investigated in this study might be better placed to explain the role of mutations in the causation of both dominant and recessive forms of the same disease. For example, it has been suggested that the mutation p.Ala531Val could be a dominant mutation that shows reduced penetrance, however, it is not clear due to the small number of pedigrees it has been reported in (Colding-Jørgensen, 2005; Papponen et al., 1999; Sun et al. 2001). On the other hand, p.Arg894X has been identified in four highly penetrant pedigrees and in 10 recessive pedigrees and has been shown through expression studies to have a dominant
style effect on channel functioning which is suggestive of a tightly associated modifying factor (Colding-Jørgensen et al. 2003; Colding-Jørgensen, 2005; George et al., 1994; Meyer-Kleine et al. 1995; Papponen et al., 1999; Sun et al. 2001; Zhang et al., 1996). Interestingly, in the case of the dual mode of inheritance mutation identified in ALS within the SOD-1 gene, p.Asp90Ala, the recessive families were found to share a common founder suggesting a tightly linked protective factor (Al-Chalabi et al., 1998). These mutations are suggestive of multiple explanations for dual inheritance mutations that could be specific to the individual mutations and the pedigrees in which they are identified.

4.7.3 Exome Sequencing

For this study whole exome sequencing was chosen to further investigate the causal variants for a family with a history of paramyotonia congenita who were found to be negative for the known genes. Affected members experience muscle stiffness which can be triggered by cold which is suggestive of paramyotonia congenita. Three affected individuals were available for analysis (Mother and two children).

Exome sequencing was conducted by Neuromics and analysed using the DeCode Health software. Variants were filtered based upon low predicted protein impact, presence in databases, frequency within the general population and read dept. Following filtering, 10 variants were identified. After looking at expression patterns of all genes, only three genes were found to be clinically relevant due to expression within skeletal muscle: RYR-1, AGRN and COL6A3.

RYR-1 is a large gene composed of 105-107 exons and encodes the ryanodine receptor which plays a crucial role in excitation-contraction coupling within skeletal muscle. The ryanodine receptor interacts with the calcium channel Ca,1.1 and regulates the release of calcium from the sarcoplasmic reticulum in response to an action potential. Mutations
within RYR-1 are associated with the congenital myopathies: central core disease, multiminicore disease and malignant hyperthermia susceptibility. Central core disease (CCD) often presents in infancy (although adult onset can occur) with hypotonia, muscle weakness and susceptibility to malignant hyperthermia. Muscle biopsies from CCD patients show a characteristic histology of central cores – areas of reduced oxidative enzyme activity. CCD is an autosomal dominant disease. Multiminicore disease shows more variability than CCD in the clinical presentation. Muscle biopsies show multiple cores within the muscle fibre which do not extend along the entire fibre length (Jungbluth, 2007). RYR-1 mutations associated with Multiminicore Disease (MmD) are inherited in an autosomal recessive manner. Malignant hyperthermia is triggered in susceptible patients during exposure to aesthetic agents. Upon exposure to triggers, patients experience muscle rigidity, tachycardia, increased temperature and hypermetabolism due to defective calcium homeostasis resulting in an unregulated increase in the intracellular calcium concentration. Continued muscle contraction depletes ATP levels and leads to rhabdomyolysis and hyperkalemia. Unless aborted, malignant hyperthermia is fatal.

Over 300 RYR-1 variants have been identified to date, with three hotspot regions within the protein: N-terminal, central and C-terminal. However, only a small number have been fully characterised (Brislin & Theroux, 2013; Kraeva et al., 2013; Roesl et al., 2014; Schiemann et al., 2014; Shepherd, Ellis, Halsall, Hopkins, & Robinson, 2004). Functionally characterised mutations have been shown to result in a disruption to the calcium flux through the channel through three main mechanisms: loss of calcium conductance due to reduced protein levels, increased permeation of calcium and uncoupling of calcium release to sarcolemma depolarisation (Bannister, 2007; Brislin & Theroux, 2013). The three variants identified in the PMC within the RYR-1 gene have been previously reported and are associated with central core disease and malignant hyperthermia susceptibility (Duarte et
al., 2011; Klein et al., 2012; Tammaro et al., 2011). The functional consequences of these mutations have not been studied to date. Due to the important role of RYR-1 within excitation-contraction coupling within the muscle it is possible that the variants identified within RYR-1 are the causative of disease in the family studied, however it is not possible to determine without further investigation.

AGRN is composed of 36 exons and encodes a heparin sulphate proteoglycan, Agrin, which is a basal lamina protein important for the formation and stabilisation of the neuromuscular junction(Bezakova & Ruegg, 2003; Gautam et al., 1996; Singhal & Martin, 2011). Agrin is a multidomain protein synthesized by motor neurons and secreted at the site of contact with muscle fibers to stimulate AChR aggregation (Cohen & Godfrey, 1992; Nitkin, Smith, & Magill, 1987; Reist et al., 1992; Rupp et al., 1992).

Mutations within AGRN are associated with congenital myasthenic syndrome (CMS). Congenital myasthenic syndromes are a heterogenous group of disorders resulting from defects in signalling within the neuromuscular proteins. CMS is caused by mutations in a number of neuromuscular junction proteins, with each genetic cause forming a distinct subtype. Patients exhibit a wide variety of symptoms with a common symptom of muscle weakness upon exertion. AGRN mutations form a rare subtype of recessive CMS with only a few cases reported worldwide(Huzé et al., 2009; Maselli et al., 2012; Nicole et al., 2014). Patients with AGRN mutations exhibit a limb-girdle pattern of involvement and age of onset usually occurs between infancy and 5 years (Barišić et al., 2011; Huzé et al., 2009; Maselli et al., 2012; Nicole et al., 2014). Symptoms include difficulty walking during childhood, bilateral ptosis, and muscle weakness, which worsen during pregnancy. Muscle biopsies show disorganised neuromuscular junctions.
Within the PMC family studied, a novel AGRN variant was identified which is predicted to be pathogenic. The mutation is located within the NtA (N-terminal Agrin) domain of the protein and is known to be required for binding laminin and connecting to the basal lamina (Denzer et al., 1997; Ruegg et al., 1992). Binding of agrin to laminin is important for the clustering of AChR receptors at the neuromuscular junction. Due to the important role of agrin within skeletal muscle it is possible that the novel variant identified could be the causal variant within the family. Although, AGRN has only been associated with recessive inheritance, it is not impossible for a gene to be associated with autosomal dominant and recessive disorders. Further investigation is needed to determine whether the AGRN variant identified is a causal mutation within the family and if so determine the effect of this variant on the protein functioning.

COL6A3 is one of three chains which form collagen type VI, a microfibrillar component of the extracellular matrix that interacts with a number of matrix molecules. Collagen type VI is thought to have a role in binding cells with the extracellular matrix and plays an important role within muscle (Bönnemann, 2011; Haliloglu & Topaloglu, 2011). The three chains of collagen type VI fold into a complicated polymeric structure. Mutations within COL6A3 are associated with the collagen VI-related myopathies: Bethlem myopathy and Ullrich congenital muscular dystrophy (Bönnemann, 2011; Jöbsis et al., 1999; G. Jöbsis, Keizers, & Vreijling, 1996; T. C. Pan et al., 1998; Yonekawa & Nishino, 2014). Both disorders represent a spectrum of disease ranging from Ullrich congenital muscular dystrophy at one end and the milder form Bethlem myopathy at the other. Patients experience muscular weakness, hypotonia, joint contractures affecting ankles and elbows. Mutations result in the formation of abnormal collagen VI and disruption to the extracellular matrix (G. J. Jöbsis et al., 1999; G. Jöbsis et al., 1996; T.-C. Pan et al., 2013).
A novel COL6A3 mutation was identified through exome sequencing of the three PMC family members. The mutation is located with the N-terminal globular domain, N2, which resembles a vWA (vWF type A) domain. These domains are thought to mediate cell-cell, or cell-matrix interactions. Disruption to one of the three collagen type VI chains has been shown to prevent correct assembly of collagen type VI causing disruption to the extracellular matrix (T. C. Pan et al., 1998). Thus, it is possible that the COL6A3 mutation identified could be the casual mutation within the PMC family described and further investigation is needed.

Due to the overlap in symptoms between PMC and the diseases associated with the identified genes it is possible that the family have been originally misdiagnosed. Interestingly, a number of cases have been reported where exome sequencing has lead to the correct clinical diagnosis following identification of the casual variant. For example, Choi et al used exome sequencing to identify mutations in the gene SLC26A3 in a case with an initial diagnosis of Bartter syndrome. Identification of the SLC26A3 mutations resulted in a change of the clinical diagnosis to congenital chloride diarrhea - a disease already known to be caused by mutations of the gene. It is important to determine if this is the case as it can help with the clinical management of disease of the patients. The presence of the variants identified within this study has not been confirmed via Sanger sequencing due to time constraints. Further work is needed to confirm the presence of these variants, to determine whether they segregate with disease and the functional consequences of the casual variant.

4.8 Conclusion

From sequencing the whole coding region of SCN4A in a myotonia cohort, three mutations were identified, one of which was novel. The novel mutation was identified in a part of the channel which contains a number of other myotonia related mutations and is likely to be
pathogenic, although functional data is needed to support this. In addition, dominant myotonia congenita families were screened for mutations in known genes in order to investigate the presence of CLCN-1 mutations which are reported as having dual inheritance. No mutations were identified in any of the genes studied indicating that in these families mutations in other genes cannot explain the dual inheritance of the mutations. Finally, exome sequencing was used to identify the causative mutation in a PMC family which did not have mutations in the known genes. Variants were identified in three possible genes, however Sanger sequencing is needed to confirm the presence of these mutations.

This genetic study highlights the importance of full sequencing of the known genes in order to provide the patient with a genetic diagnosis. Next generation sequencing methods such as targeted gene panels and exome sequencing provide cost effective methods to sequence all the known genes.

4.9 Further Work

- A number of cases within the cohorts where no mutation was identified. In order to find the causal mutation within the remainder of this cohort, patients with a strong phenotype and family history should be selected and exome sequencing used. This would enable identification of the genetic cause of disease.

- The variants identified in the PMC family through exome sequencing need to be confirmed by Sanger sequencing. In order to confirm which gene holds the causal variants, confirmation of the mutations via Sanger sequencing needs to occur. Following this DNA needs to be collected from other family members who are both affected and unaffected in order to look at segregation of the variants with disease for the family.
• Ser1159Pro needs to be functionally characterised in order determine whether they are pathogenic mutations.

4.10 Acknowledgements

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Chapter 5: Functional Analysis of a possible Hypo PP mutation and other related Na\textsubscript{v}1.4 S3 segment negative charge mutations

5.1 Summary

Hypokalemic periodic paralysis is caused by mutations in two genes: CACNA1S and SCN4A which encode the voltage gated calcium (Ca\textsubscript{v}1.1) and sodium channels (Na\textsubscript{v}1.4). Mutations within these channels have been found to localise to the S4 voltage sensor of both channels. 18 out of 21 mutations are found with the voltage sensor and cause loss of positively charged arginine residues called gating charges. Studies of Hypo PP arginine mutations revealed an aberrant ionic leak called a gating pore current which is activated by negative voltages and is carried by cations (Sokolov et al., 2007a, 2010; A. F. Struyk & Cannon, 2007a; Struyk et al., 2008).

Through Sanger sequencing a novel mutation (D1420G) was identified in a Hypo PP patient. D1420G is located in the S3 segment of DIV and is predicted to interact with the R4 residue when the voltage sensor is activated. Analysis of the biophysical effect of D1420G revealed a small hyperpolarised shift of 4 mV in the voltage dependence of fast inactivation; however this is not the disease causing mechanism. D1420G was found to produce a gating pore current activated by negative voltages which is permeable to Na\textsuperscript{+}. The currents were still present with only NMDG\textsuperscript{+} as the extracellular cation, unlike for gating pore currents in S4 arginine mutant channels. An additional Cl\textsuperscript{−} component was identified within the D1420G gating pore current. These findings suggest that D1420G is indeed a pathogenic mutation.

Additionally, the role of homologous negatively charged residues within other Na\textsubscript{v}1.4 domains was investigated. S3 segment negative charges from DI, DII and DIII, were substituted to a glycine and steady state currents were measured. All three mutations were
found to cause a gating pore current, although differences were identified between domains. D197G and D1094G revealed a gating pore current activated by positive voltages which is carried solely by Cl ions. In contrast, D640G showed a gating pore current that had similar voltage dependence and ion selectivity to D1420G.

From this study it can be seen that negatively charged residues of the voltage sensing domain, which form part of the electrostatic network of interactions to stabilise the movement of the S4 voltage sensor, occlude the gating pore from ionic leak. Mutations affecting these residues may lead to gating pore currents and could play a role in causation of disease.
5.2 Introduction

Hypokalemic periodic paralysis is caused by mutations within Na,1.4 and Ca,1.1. 18 out 21 mutations are located within the S4 voltage sensor, and result in the neutralisation of the gating charges. Expression studies of seven Hypo PP Na,1.4 arginine mutations have shown that they cause an ionic leak current through the gating pore which is activated by hyperpolarised potentials. Furthermore, studies of muscle fibres from a R669H mouse model and human fibres have provided further evidence for the gating pore current as the mechanism of disease (Jurkat-Rott et al., 2009; Rudel et al.,1984; Wu et al.,2011). Until recently no experimental evidence has been available to link Ca,1.1 mutations with the gating pore current hypothesis. In 2012, Wu et al, showed a small anomalous inward current present at the resting potential in the mouse model of the Ca,1.1 R528H mutation. In addition, Fan et al., have shown that the Ca,1.1 mutation R1242G results in a gating pore current with two phases: an outward current at positive potentials and an inward current present at negative potentials(Fan et al., 2013).

Whilst the mechanism has been identified for Hypo PP S4 arginine mutations, there is still no viable explanation for the 3 mutations (V876E, H916Q (Ca,1.1) and P1158S (Na,1.4)) which have been identified outside of the S4 voltage sensor. No functional studies have been conducted to date on a Hypo PP mutation located outside the S4 segment. It is not known whether mutations outside of S4 voltage sensor could cause a sufficient disruption to the integrity of the voltage sensing domain to cause the consequential aberrant ionic pathway. Until this has been done the gating pore current Hypo PP mechanism cannot be fully validated.
5.3 Aims

The aim of this study was to determine if the novel Hypo PP mutation D1420G a pathogenic mutation. Hypo PP mutations are normally localised to the S4 voltage sensor, neutralise positively charged residues and cause an aberrant ionic leak. Therefore, D1420G was investigated in order to determine if it causes a gating pore current.

In addition, mutations of homologous negatively charged residues to D1420 in other domains were studied in order to determine if other S3 negative charges are important for the stability of voltage sensing domain.
5.4 Functional Analysis of Hypo PP mutation D1420G

As discussed in Chapter 3, a novel mutation in SCN4A was identified within a patient with a Hypo PP phenotype. This mutation causes loss of a negative charge of an S3 segment aspartic acid (D1420G). D1420G is predicted to be pathogenic through in silico analysis (using PolyPhen) and was found to segregate with disease (see Chapter 3). However, this is not sufficient to determine whether D1420G is the causative mutation in this case. Functional analysis is needed to determine the mechanism by which this mutation could act. It is possible that loss of a negative charge such as D1420G could result in disruption of the voltage sensing domain and leading to an aberrant pathway for ionic leak as is seen in Hypo PP S4 arginine mutations. Here the functional consequences of D1420G are determined through analysis of the effects on alpha currents and gating pore currents.

5.4.1 Characterization of alpha pore currents

To determine the mechanism by which D1420G may cause Hypo PP WT and mutant sodium channels were transiently expressed in HEK-293 cells to allow whole cell recordings of $\alpha$ currents. HEK cells are a widely used heterologous expression system for ion channels. They were used for this study since they provide a similar cellular environment to the natural ion channel environment and allow for the detection and measurement of sodium currents as endogenous currents can easily be blocked and are not selective to sodium ions. However, the expression levels can be difficult to control and therefore HEK cells were only used to measure the sodium channel kinetics.

As can be seen from the G-V curves in Figure 5-1, D1420G has no effect on the voltage dependence of activation ($WT V_{0.5} = -20.6\pm0.8mV, n=19; D1420G V_{0.5} = -19.5\pm0.7mV, n=15, p<0.05$).
It has been shown that some Na\textsubscript{v}1.4 mutations associated with Hypo PP cause a small shift in the voltage dependence of fast inactivation. Therefore, the voltage dependence of D1420G channel fast inactivation was measured. Figure 5-1 shows the inactivation curve for WT and mutant channels. D1420G induced a small but significant hyperpolarised shift of 4 mV in the voltage dependence of fast inactivation (WT $V_{0.5} = -66.5 \pm 0.9$ mV, $n=20$; D1420G $V_{0.5} = -70.2 \pm 0.8$ mV, $n=15$, $p<0.005$). This results in enhancement of fast inactivation, follows what has been seen in other Na\textsubscript{v}1.4 Hypo PP mutations.

Finally, as some periodic paralysis Na\textsubscript{v}1.4 mutations have also previously been shown to disrupt steady state slow inactivation, the effect of D1420G on this was measured. As it can be seen from Figure 5-1C, D1420G has no effect on the slow inactivation showing WT –like curve (WT $V_{0.5} = -46.2$ mV $\pm 1.7$ mV, $n=7$; D1420G $V_{0.5} = -48.9$ mV$\pm 0.7$ mV, $n=6$, $p<0.05$).
**Figure 5-1 Central pore Na⁺ currents for Na₁.4 channels**

Whole cell currents from WT and D1420G transfected cells. 

**A** = Conductance/voltage relationship of activation. Whole cell Na⁺ currents were evoked by a series of voltage steps from -130 to +60 mV from a holding potential of -80 mV. 

**B** = Voltage dependence of fast inactivation measured using a 300 ms conditioning voltage step from -130 mV to 60 mV in 10 mV increments, followed by a constant test pulse step to -10 mV. Normalised averages were fitted with a Boltzmann equation. 

**C** = The voltage dependence of steady state slow inactivation induced by a 10s conditioning prepulse to test voltages, with a 20 ms step to -100 mV to allow recovery from fast inactivation, followed by a 20 ms test pulse at 0 mV. Data was normalised and fitted using a Boltzmann equation.
5.4.2 Gating pore current

The small loss-of-function effect found in the functional analysis of the alpha-currents cannot explain depolarization of the muscle fiber often associated with Hypo PP. For most Na$_{1.4}$ Hypo PP mutations it has been shown that these effects on alpha current are secondary to the gating pore current. These mutations cause loss of integrity to the voltage sensing domain, providing a pathway for ionic leak through the channel independent to the main pore.

Since D1420G was identified in a Hypo PP patient and is located within the voltage sensing domain, the effect of the mutation on the voltage sensing domain was investigated. The D1420G mutation was introduced into the rat ortholog skeletal muscle sodium channel (rNa$_{1.4}$-D1413G, rat numbering used from here onwards). Xenopus oocytes were injected with RNA encoding either wild type or mutant rNa$_{1.4}$ together with the rat β1 subunit. The biophysical properties of WT and mutant were measured using two electrode voltage clamp technique (TEVC). The main pore was blocked using 1 μM tetrodotoxin (TTX) to allow detection of gating pore currents.

Xenopus oocytes were used for this part of the study since they enable the control of ratio of channel subunits, have a short expression time and enable high expression levels. However, these cells have calcium dependent chloride currents which can make ion selectivity studies difficult in some circumstances.

Figure 5-2A shows currents measured in response to 300 ms voltage steps from a holding potential of -100 mV to test potentials of -140 mV to +50 mV, with a Cl$^-$ free Na$^+$ bath solution. The current amplitudes at hyperpolarised voltages were larger for D1413G expressing channels compared to wild type channels. Studying the current-voltage relationship reveals that the mean current of wild type and D1413G channels are similar at
depolarised voltages (between +50 mV to -40 mV) but at voltages negative to -40 mV, D1413G expressing channels show larger currents compared to wild type channels.

Leak subtraction was performed on data for both WT and mutant using the linear component at positive voltages (0 to +20 mV), and the leak-subtracted gating pore current was then normalised to the peak sodium currents for each cell and averaged (WT n=9, D1413G n=23). D1413G shows an inwardly rectifying current that is not present in WT expressing cells. Figure 5-2C shows no significant change in the steady state currents of WT expressing cells, even at extreme voltages. In contrast, D1413G shows a clear inward current which is activated at voltages negative to -40 mV, which suggests that the gating pore current will be present at physiological conditions. At potentials negative to -100 mV the current saturates, which is similar to other Hypo PP Na\(_v\)1.4 mutants studied by Struyk et al 2008.

It has been possible in favourable conditions to measure the peak Na\(^+\) currents without TTX followed by measuring the D1413G gating pore current, with TTX, from the same cell. This enables normalisation of gating pore currents to peak Na\(^+\) currents. In our setup the D1413G gating pore currents were found to be between 1\% and 5\% of the peak Na\(^+\) currents, which is much larger than previously described gating pore currents (Figure 5-3C), and what was seen in Chapter 6 for S4 arginine mutant channels.

Previous studies of gating pore currents in Na\(_v\)1.4, have been investigated using the cut open voltage clamp (COVC) technique rather than TEVC. The COVC technique shows faster voltage clamp compared to TEVC and allows intracellular solution control, enabling higher resolution recordings of the small gating pore currents. Thus, to validate the gating pore current results obtained through TEVC, COVC was also used to measure the gating pore currents of D1413G. Both COVC and TEVC recordings showed similar voltage dependence for D1413G gating pore currents (See Figure 5-2 & Figure 5-3 for comparison). TEVC was
used for the rest of the characterisation for D1413G since the COVC set-up was not available at the time for use in further characterisation of the mutation.
Figure 5-2 Voltage dependence of gating pore currents in D1413G measured with TEVC

Currents were recorded in the presence of 1µM TTX in response to 300 ms voltage steps ranging from -140 mV to +50 mV from a holding potential of -100 mV. External bath solution contained 120 mM NaMes, 1.8 mM CaSO₄ & 10 mM HEPES, pH7.4 with NaOH. A) The representative steady-state currents for oocytes expressing WT or D1413G channels. B) Representative voltage dependence of currents before leak subtraction. C) Voltage dependence of mean leak subtracted gating pore currents for WT (Red circles) and D1413G (Black Squares). Error bars show SEM.
Figure 5-3: Voltage dependence of gating pore currents in D1413G measured with COVC

Voltage dependence of gating pore currents measured using COVC in WT or D1413G expressing cells for A) non leak subtracted data or B) leak subtracted (0 to +30 mV) data normalised to maximal gating charge displacement. Currents measured following a 30 ms voltage step from holding potentials of -60 mV to test potentials of -130 to +40 mV.
5.4.2.1 Ion Selectivity of Gating pore current

In order to further characterise the gating pore currents produced by D1413G, the ion selectivity of the currents was studied by substituting Na⁺ within the bath solution for other cations in the presence of 1µM TTX. A holding potential of 0 mV was used in order to improve the size and stability of the gating pore currents recorded. The currents measured from holding voltage of 0 mV show larger amplitude but similar voltage dependence to the currents measured using holding voltage of -100 mV.

Figure 5-4 shows averaged data from D1413G cells in different bath solutions, normalised to the peak Na⁺ currents measure in the same cell. With K⁺ as the extracellular cation, currents were observed showing the same voltage dependence as seen in Na⁺, although currents are smaller (p<0.05 at -100 mV).

Guanidinium can be used in Na,1.4 S4 arginine mutations to increase the size of the gating pore up to 10 fold (Sokolov et al.,2007b; Sokolov et al., 2010). Gating pore currents for D1413G expressing cells in the presence of guanidinium did not increase. In comparison to Na⁺, guanidinium was found to decrease the currents (Figure 5-4B, p<0.05 at -100 mV).

The large organic molecule NMDG⁺ has previously been shown to be impermeable through the gating pore caused by S4 arginine mutation, since the size of the molecule means it is unable to pass through the gating pore. In the presence of NMDG, large magnitude gating pore currents were still observed in cell expressing D1413G channels, although the currents were smaller than those seen in Na⁺ and guanidinium (p=0.013 at -100 mV).

There are two possible reasons why currents remain when NMDG is the only extracellular cation. First, NMDG can pass through the channel. Second, there is another component to the currents is present which is not carried by cations. Since the cation NMDG is very large, the second possibility was investigated.
Figure 5-4 Ion selectivity of gating pore currents in D1413G

Leak subtracted gating pore currents of D1413G in response to 300 ms voltage steps from -140 mV to +50 mV from a holding potential of 0 mV, following substitution of the external bath solution to contain different cations in the presence of the anion methansulfonate:  

A) Na⁺ (black squares) or K⁺ (red circles),  
B) Na⁺ (black squares) or 60 mV Guandinium (red circles),  
C) Na⁺ or NMDG (red circles). Data are normalised to peak Na⁺ currents with error bars denoting s.e.m.
5.4.3 Chloride component of D1413G gating pore current

The gating pore currents of the D1413G channels remain of considerable amplitude even when the only extracellular cation is NMDG. NMDG is a very large cation and is unable to pass through the gating pore pathway formed by other mutations in the voltage sensing domain. It is thus possible that the gating pore currents may be carried by other ions.

As D1413G results in the loss of a negatively charged residue, it is possible that negatively charged ions such as chloride ions may pass through the aberrant pathway with the voltage sensing domain. To investigate the possibility that Cl⁻ might pass through the voltage sensor domain and carry a part of the gating pore current, the anion within the extracellular solution was changed whilst the cation was kept constant i.e. the sodium ion remained constant whilst the chloride ion was replaced by methanesulfonate. The raw I-V curves revealed a difference in the currents seen in Cl vs Mes solutions. At positive voltages the slope of I-V curve becomes steeper in the presence of NMDG-Cl compared to both NMDG-Mes and NaMes solutions (Figure 5-5A). WT expressing cells did not show such a large difference difference between Cl⁻ and Mes solutions. Furthermore, it was found that currents were the same even in the cations were Na⁺ or K⁺ (Figure 5-5B).
Cl\(^-\) component of D1413G gating pore currents was investigated by exchanging the anion present in the bath solution to contain Cl\(^-\). Raw currents in the presence of: A) NaCl or NaMes, B) KCl or KMes, C) NMDG with Methanesulfonate or NMDG with Cl Currents. D) Raw WT currents in the presence of NaCl or NaMes.
5.5 Do other intracellular negative charge cluster mutations cause gating pore currents?

Since D1413G was found to result in a gating pore current, it is possible that homologous negative charges within the other domains of Na\(_{\text{1.4}}\) also have a role in maintaining the electrostatic interactions which protect the voltage sensing domain from ionic leak. Therefore, the role of homologous negatively charged residues in preventing ionic leak through the voltage sensor domain was investigated.

5.5.1 S3 negative charge mutations

Three S3 segment aspartic acids in DI, DII and DIII were identified as being in a homologous position within the S3 segment to D1413 (D197, D640 & D1100). Each mutation was introduced into the rat ortholog of Na\(_{\text{1.4}}\) (rD197, rD640 & D1094), all mutations will be referred to by the rat homolog numberings from now on. Voltage dependence and ion selectivity of gating pore currents were analysed for all three mutations.

5.5.1.1 Domain I – D197G

To detect possible gating pore currents, the central pore was blocked with 1 µM TTX and currents were recorded in response to a 300 ms voltage step from a holding potential of -100 mV to test potentials of -140 to +50 mV. In Figure 5-6A, a difference can be seen between WT and D197G steady state currents, with NaCl as the main salt of the bath solution. WT non-leak subtracted currents show an almost linear voltage-current relationship over the full range studied, while in contrast, the I-V curve for D197G shows a non-linear component at depolarised voltages, revealing a gating pore current through the voltage sensor domain which is different from that of D1413G (
Figure 5-6B). To determine the voltage dependence of the gating pore current, leak subtraction was conducted using the linear component at hyperpolarised potentials.

Figure 5-6C shows the I-V curves for D197G and WT following leak subtraction. D197G shows an outward current activated at potentials positive to -50 mV. This activation occurs at a similar voltage to the activation of the central pore.

Next, the gating pore currents in D197G expressing cells was further characterised by determining the ion selectivity. Figure 5-6 shows the results of substitution experiments of the main ions in the external bath solution. Currents were recorded in the presence of NaCl or NaMes extracellular recording solutions. Gating pore currents in both solutions followed the same voltage dependence, with activation around -50 mV. In the presence of NaCl currents were 6 times larger compared to NaMes currents at +50 mV. This shows a clear Cl⁻ selective component of D197G gating pore currents (Figure 5-6). Oocytes have endogenous current Cl⁻ currents which can be activated by a high concentration of Cl⁻ within bath solutions. However, no such difference was seen between the solutions was observed in WT expressing cells, suggesting that endogenous currents could not explain the difference.
Figure 5-6 Voltage dependence of D197G gating pore currents.

Currents were recorded in the presence of 1µM TTX in response to 300 ms voltage steps ranging from -140 mV to +50 mV from a holding potential of -100 mV. External bath solution contained 120 mM NaCl, 1.8 mM CaSO₄ & 10 mM HEPES, pH7.4 with NaOH. For some recordings NaCl was substituted with NaMes. A) The representative steady-state currents for oocytes expressing WT or D197G channels. B) Representative recordings of voltage dependence of currents before leak subtraction. C) Voltage dependence of leak subtracted gating pore currents for WT (Red circles) and D197G (Black Squares). Data are normalised to peak Na⁺ currents. D) Averaged raw data for D197G expressing cells in the presence of NaCl (black squares) or NaMes (Red circles). E) Voltage dependence of leak subtracted gating pore currents in the presence of NaCl (black squares) or NaMes (Red circles). Error bars denoting s.e.m.
5.5.1.2 Domain II – D640G

Figure 5-7 shows currents measured from WT and D640G expressing cells in response to the voltage protocol used to study the gating pore currents. Steady state currents observed in D640G deviated from WT currents. I-V curves for WT expressing cells show a linear relationship for all ranges tested in the presence of a Cl⁻ free Na⁺ extracellular solution. In contrast, D640G currents were found to have a non-linear component present at hyperpolarised potentials, similar to D1413G. Leak subtraction was performed on data for both WT and mutant using the linear component at positive voltages. The gating pore current amplitude was normalised to the peak sodium currents for each cell and averaged (Figure 5-7, WT n=9, D640G n=9). D640G expressing cells show an inward current which is activated at voltages negative to -30 mV. The voltage dependence of D640G gating pore currents is similar to that seen for D1413G. However, the D640G currents were often very small and difficult to record consistently.

The gating pore currents of D640G were further characterised by determining the ion selectivity. The steady state currents were measured with holding potential of 0 mV. When Na⁺ in the bath solution was substituted for K⁺, gating pore currents were observed with the same voltage dependence but with reduced amplitude (n=1)(Figure 5-7D). This observation was similar to D1413G currents but differed from those of D197G.

Finally, the possibility that D640G gating currents also have a Cl⁻ component was explored. Currents were measured in Na⁺ bath solutions with and without Cl⁻ (NaCl and NaMes). A Cl⁻ component of the gating pore currents is observed for D640G. However this is only shown with an n of two and more recordings are necessary to confirm this. (Figure 5-7D).
Figure 5-7 Voltage dependence of D640G gating pore currents.

Currents were recorded in the presence of 1µM TTX in response to 300 ms voltage steps ranging from -140 mV to +50 mV from a holding potential of 0 mV. External bath solution contained 120 mM NaMes, 1.8 mM CaSO₄ & 10 mM HEPES, pH7.4 with NaOH. In some experiments NaMes was substituted for NaCl. A) The representative steady-state currents for oocytes expressing WT or D640G channels. B) Representative recordings of voltage dependence of currents before leak subtraction. C) Voltage dependence of leak subtracted gating pore currents for WT (Red circles) and D640G (Black Squares), data are normalised to peak Na⁺ currents. In order to determine the whether D640G shows a Cl⁻ component, D640G gating pore currents were measured in the presence of different extracellular bath solutions D) NaMes or NaCl. Error bars denoting s.e.m.
5.5.1.3 Domain III – D1094G

Figure 5-8 shows steady state currents measured in WT and D1094G expressing cells following a gating pore current protocol with NaCl as the main extracellular salt. Raw I-V curves for D1094G show a non linear component at positive voltage which is not present in WT expressing cells. Following leak subtraction using the linear component at negative voltages and normalisation to peak Na\textsuperscript{+} currents, D1094G shows an outward gating pore current which is activated at voltages positive to -50 mV(Figure 5-8C). The voltage dependence of this outward gating pore current is similar to currents observed in D197G expressing cells. Steady state currents of WT expressing cells do not show this outward current.

As D1094G currents show similar characteristics to D197G, the possibility that D1094G gating pore currents are carried by Cl\textsuperscript{-} ions was explored. Currents were measured using Na\textsuperscript{+} bath solutions with methanesulfonate or Cl\textsuperscript{-} as the anionic species. Larger gating pore currents were observed in the presence of NaCl compared to NaMes (Figure 5-8). This data is similar to D197G.
Figure 5-8 Voltage dependence of D1094G gating pore currents.

Currents were recorded in the presence of 1µM TTX in response to 300 ms voltage steps ranging from -140 mV to +50 mV from a holding potential of -100 mV. External bath solution contained 120 mM NaCl, 1.8 mM CaSO₄ & 10 mM HEPES, pH7.4 with NaOH. A) The representative steady-state currents for oocytes expressing WT or D1094G channels. B) Voltage dependence of currents before leak subtraction. C) Voltage dependence of leak subtracted gating pore currents for WT (Red circles) and D1094G (Black Squares). In order to confirm that currents were conducted by Cl⁻ ions, currents were recorded in the presence of NaCl or NaMes, voltage dependence of currents is shown D) raw currents and E) Leak subtracted normalised to peak Na⁺ currents. Error bars denoting s.e.m.
Figure 5-9 Location of D1420 within channel

A) Schematic diagram showing location of D1420 residue B) Shows the VSD of domain IV: The six outermost arginine residues are shown in red spheres. D1420 is shown in blue sphere, based on homology model of rat NaV1.4 constructed by D Kuzmin, UCL (unpublished).
5.6 Discussion

5.6.1 Is D1420G a pathogenic mutation?
A novel Na\(_{1.4}\) mutation, D1420G was identified in a Hypo PP patient during a genetic screen of Hypo PP patients who lack the common mutations. The patient is 28 year old male who presented with attacks of paralysis associated with low potassium levels (see Chapter 3 for the detailed phenotype). Attacks of paralysis mainly affect arms and legs and last on average 7 to 8 hours. The probands younger brother is similarly affected and was also found to carry the D1420G mutation.

Unusually for a Hypo PP mutation D1420G is located outside of the S4 segment. D1420G is found within the S3 segment of DIV resulting in the loss of a negative charge. The negative charge at position 1420 is highly conserved and is part of the intracellular negative charge cluster (INC) which stabilises S4 arginine residues in the hydrophobic transmembrane region (DeCaen et al., 2008; Payandeh et al., 2011; Yarov-Yarovoy et al., 2012). This study aimed to investigate whether D1420G was the causative mutation for the Hypo PP family studied within Chapter 3. It was hypothesized that loss of a negative charge within the voltage sensor domain could cause a similar gating pore current as conventional S4 arginine Hypo PP mutations.

D1420G mutant channels expressed in HEK-293 cells showed a small but significant hyperpolarising shift in the voltage dependence of fast inactivation. This was the only effect the mutation was found to have on the central pore alpha currents. Similar loss of function changes have been observed for Hypo PP arginine mutations, for example the Na\(_{1.4}\) mutation R669H causes hyperpolarized shift in the voltage dependence of slow inactivation, whilst R672H/G causes a hyperpolarized shift in the voltage dependence of fast inactivation and reduced current density (Sternberg et al., 2001; Struyk et al., 2000).
Such loss of function changes have been shown to be insufficient to cause the attacks of paralysis associated with hypokalemia (Bendahhou et al., 2001; Bulman et al., 1999; Carle et al., 2006; Fan et al., 2013; Jurkat-Rott, Groome, & Lehmann-Horn, 2012; Kuzmenkin et al., 2002; Lapie et al., 1996; Sokolov et al., 2007; Struyk et al. 2000; Struyk & Cannon, 2007a). Thus, the shift in the voltage dependence of fast inactivation caused by D1420G is unlikely to be sufficient to cause the attacks of paralysis observed in the Hypo PP family.

Next the presence of gating pore currents was studied in D1420G expressing cells. It was found that D1420G causes an aberrant gating pore current activated at voltages negative to -40 mV. Basic selectivity experiments showed that this hyperpolarizing gating pore current is carried primarily by Na\(^+\) ions. These findings are consistent with previous studies of Hypo PP arginine mutations which show that loss of the positive arginine residues results in a gating pore current carried by cations and activated at hyperpolarised potentials. Such aberrant inward currents have been shown to contribute towards depolarisation of the skeletal muscle. The gating pore currents produced by D1420G are likely to be active at physiological conditions in the resting skeletal muscle. Thus, these D1420G gating pore currents are likely to contribute to the attacks of paralysis associated with the Hypo PP phenotype observed.

Unexpectedly, gating pore currents at hyperpolarized voltages were clearly evident in absence of small monovalent cations in extracellular solution. It is possible that at least a part of this current is carried by outflow of chloride ions. However, due to the limitations of TEVC, with regards to controlling the intracellular ionic concentrations, further work is needed to characterise this part of the D1420G gating pore current. By controlling the intracellular concentration, it would be possible to fully determine the presence of an inward Cl\(^-\) flow.
Furthermore, these findings are consistent with the newly identified role of chloride conductance in the development of attacks of paralysis. Both bumetanide (a blocker of sodium potassium chloride transporter) and 9-anthracene carboxylic acid (9-AC, a CLC-1 blocker) have been shown to prevent depolarization in muscle fibres (van Mil et al., 1997; Wu et al., 2013; Wu et al., 2013). It has also been shown that a high intracellular [Cl⁻] predisposes the skeletal muscle to paradoxical depolarisation (Geukes Foppen et al., 2002; van Mil et al., 1997; Wu et al., 2013). It is thought that the inward flow of chloride ions at depolarized voltages might hyperpolarize the cell and counteract the sodium driven action potentials. However, it is unclear if this would contribute to the development of the episodes of periodic paralysis that are commonly associated with depolarization of the muscle fibre, although increased chloride conductance might, in D1420G muscles, depolarize the reversal voltage of chloride ions and consequently lead to depolarization of the muscle membrane.

5.6.2 D1420G and Hypo PP

Over the last 10 years, the mechanism of Hypo PP has been unravelled. Through a series of studies we now know that 18 out of 21 Hypo PP mutations are localised to the S4 segment of Ca_{1.1} or Na_{1.4}. Of those Hypo PP S4 mutations studied, all have shown to bring about a pathway permeable to cations. Hypo PP arginine mutations cause a sustained inward current with steep voltage dependence at hyperpolarised potentials (Francis, Rybalchenko, Struyk, & Cannon, 2011; Groome et al., 2014; Jurkat-Rott et al., 2009; Sokolov et al., 2010; A. F. Struyk & Cannon, 2007b). (Francis et al., 2011; Jurkat-Rott et al., 2012; Matthews et al., 2009; Sokolov et al., 2007a, Sokolov et al., 2010; Starace & Bezanilla, 2004; Struyk & Cannon, 2007a). However, the three non S4 mutations associated with Hypo PP have not been characterised.
In this study, the first non S4 arginine Hypo PP mutation has been studied and shown to cause gating pore currents. The observations made for the novel D1420G mutation are consistent with previously studied Hypo PP mutations, with the exception of the possible Cl⁻ component. Furthermore, the functional properties of D1420G suggest that it is likely to be the causative pathogenic mutation for the family described. The findings described in this thesis lend further support to the hypothesis that the Hypo PP is caused by a common dysfunction of the voltage sensing domain. Additionally, the presence of a gating pore current in a non S4 segment mutation will hopefully lead to characterisation of the three non S4 segment mutations: V876E, H916Q (Ca₁,1) and P1158S (Na₁,4). Identification of gating pore currents within these mutations will complete the picture of the Hypo PP mechanism of disease.

The data presented in this chapter has shown that Hypo PP mutations cannot be solely isolated to the S4 voltage sensor. In fact, it is reasonable to suggest that Hypo PP could be caused by any mutation which sufficiently disrupts the stability of the voltage sensor domain to induce the creation of a novel conduction pathway. Thus, following the exclusion of S4 segment mutations, genetic screening in Hypo PP should be extended to all parts of Na₁,4 and Ca₁,1.

5.6.3 S3 negative charges within the Voltage Sensing Domain and implications for other channelopathies

Following the characterisation of D1420G, mutations were created in this conserved aspartate residue in the S3 segment of each domain (D197G, D640G & D1094G). Through expression of the mutant channels in *Xenopus* oocytes, gating pore currents were characterised in each mutant channel. Two different types of gating pore current were identified: D640G & D1413G produce an inward gating pore current which is active at
hyperpolarized voltages whilst D197G and D1094G produce outward gating pore currents active at depolarized voltages.

It was found that gating pore currents in D197G and D1094G channels are carried by Cl⁻ ions flowing into the cell since their substitution by methanesulfonate largely eliminates the currents. D640G and D1413G channels also display a depolarization activated gating pore current component carried by chloride. However, it is not understood why loss of the negatively charged residue might enable Cl⁻ to permeate through the channel. The findings in this study highlight the significance of the highly conserved S3 negative charge residues within the voltage sensing domain. Mutations leading to loss of the INC aspartate residue all cause disruption to the voltage sensing domain and allow for an aberrant ionic leak to be conducted through the channel.

Due to the presence of endogenous Cl⁻ currents within oocytes it is difficult to confirm whether the Cl⁻ component is due to the mutation or the endogenous currents. Attempts have been made by recording WT currents with and without Cl⁻ in the bath solution. It appears that the difference in currents observed in the mutant channels is not replicated in WT expressing cells. Further experiments are needed to fully characterise these currents. In addition, it has been difficult to fully characterise the mutant channels since the expression levels in oocytes were often too low for gating pore currents to be detectable.

Although gating pore currents have been observed in a number of ion channels including the Shaker K⁺, K,1.2, K,3.2, K,7.4, H,1, Na,1.2, Na,1.4, Na,1.5 and Ca,1.1 channels(Delemotte et al.,2012; Delemotte et al.,2010; Fan et al., 2013; Francis et al., 2011; Khalili-Araghi et al.,2012; Klassen et al.,2008; Miceli et al.,2012; Ramsey et al.,2006; Sasaki et al.,2006; Sokolov et al.,2005; Sokolov et al., 2007a; Starace & Bezanilla,2001; Starace et al.,1997; Starace & Bezanilla,2004; A. F. Struyk & Cannon, 2007b; A. F. Struyk et al., 2008; Tombola et al.,2005), gating pore currents have only been associated with the pathogenesis
of three disorders: Hypo PP (Ca\textsubscript{v}1.1 & Na\textsubscript{v}1.4) Brugada Syndrome (Nav1.5 R219H) and Benign Familial Neonatal Seizurers (BFNS, Kv7.4) (Gosselin-Badaroudine et al., 2012a; Miceli et al., 2012; Sokolov et al., 2007a; A. F. Struyk & Cannon, 2007b).

A number of disease related mutations have been identified within the voltage sensing domain (Table 5-1). These mutations have been shown to cause a variety of effects on channel functioning (e.g. reduced current density, shifts in the voltage dependence for activation or inactivation and altered recovery from inactivation), but few studies have looked for a gating pore current. Of particular interest are the non S4 arginine mutations, located in the S3 segments of the ion channels. Na\textsubscript{v}1.1 and Na\textsubscript{v}1.2, Na\textsubscript{v}1.5, Na\textsubscript{v}1.7, Ca\textsubscript{v}1.4, K\textsubscript{v}11.1 and K\textsubscript{v}7.1 all contain disease associated mutations which are located in homologous positions to the mutations studied in this chapter. It would be interesting to confirm the hypothesis that these mutations also cause a gating pore current. Identification of gating pore currents in ion channels associated with other diseases would provide an exciting mechanistic link between a diverse group of diseases.

5.6.4 Use of TEVC for measuring gating pore currents
The identification of gating pore currents in five negative charge mutations in this study was conducted using the TEVC set up. Most previously reported gating pore currents have been measured using the more sophisticated set up – cut open voltage clamp. The advantages of using COVC are that recordings provide a higher temporal resolution enabling better detection of small currents such as gating currents or gating pore currents and more accurate description of the Na\textsuperscript{+} central pore currents. The COVC also provides opportunity for intracellular solution exchange for detailed description of ion selectivity. However, the disadvantage for COVC is the complexity of the system which is highly time consuming to set up for each recording. This means that the set up is impractical for screening a large number of mutations. Whilst TEVC does not provide the high resolution
recordings of COVC, this study has shown that it can be used for the basic characterisation of gating pore currents. Our TEVC data is qualitatively similar to the preliminary COVC data. However, further COVC recordings are required to confirm and amplify our gating pore current findings in TEVC.

The disadvantage of TEVC is the inability to control the intracellular ion concentration which resulted in conclusive characterisation of the D1413G selectivity. Additionally, WT recordings were not always found to be linear due to endogenous currents which could not be fully eliminated by removing the ions responsible for these currents.

TEVC can be used for the basic characterisation of Na\textsubscript{v}1.4 gating pore currents and so could be useful for quick screening of pharmacological compounds and novel mutations. This will be helpful in the future for determining the functional consequences of novel Na\textsubscript{v}1.4 HypoPP mutations located outside of the S4 segment and for more large scale structure-function studies.

5.7 Conclusion

D1420G was identified in a HypoPP family that present with attacks of paralysis lasting on average 8 hours. Functional studies have confirmed that D1420G is the causative mutation for this family. Interestingly, D1420G is the first non-S4 arginine mutation associated with HypoPP which has been shown to cause a gating pore current. In addition, further experiments showed that loss of the conserved aspartate residue in other domains also results in a gating pore current. This supports the notion disruption to the stability of the voltage sensing domain provides a pathway for the conduction of an aberrant ionic leak current which can contribute towards disease.
5.8 Further Work

- During this study it has not been possible to fully confirm the presence of the Cl− current component. However, due to the presence of endogenous Cl− currents within oocytes it is difficult to confirm whether the Cl− component is due to the mutation or the endogenous currents. In order to confirm the presence of the Cl− component in D1420G, steady state currents were compared between mutant and WT expressing cells in the presence of a Cl− solution. A difference was observed between WT and the mutant. However, further work is needed to confirm the presence of a Cl− component. Control experiments are needed to confirm this:
  - Niflumic acid, 9-AC and DIDS can be used to block the endogenous Cl− currents present in oocytes. Blockage of the Cl− currents would show that the currents are in fact endogenous currents rather than as a result of the mutations.
  - A picospritzer pipette can be used to inject EGTA or BAPTA into an oocyte to chelate Ca2+ ions and prevent activation of oocyte Ca-Cl currents. Any alteration to the Cl− currents would suggest it a role of the endogenous Cl− currents.

- For two negative charge mutations, D640G and D1094G for some conditions the number of cells were clear recordings were obtained was too low for statistical analysis. Further experiments are needed to reach a minimum of 5 cells per condition for each mutation.

- TEVC was used to studying the gating pore currents for the negative charge mutations. Further experiments using COVC are needed to fully confirm the TEVC findings and confirm that TEVC can be used in place of COVC for screening novel mutations.
5.9 Acknowledgements

Roope Mannikko collected and analysed patch clamp data for D1420G. In addition, R.M helped with the experimental design for TEVC experiments. David Francis and Volodymyr Rybalchenko of the Cannon Laboratory UT Southwestern, Dallas, USA, collected and analysed data for D1420G COVC experiments.
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<td></td>
</tr>
<tr>
<td>p.K1641N</td>
<td>S4 DIV</td>
<td>Seizures, benign infantile</td>
<td>(Zara et al., 2013)</td>
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**SCN5A**  
**Na\(_{1.5}\)**

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<tr>
<th>p.E161Q/K</th>
<th>S2 DI</th>
<th>Brugada syndrome</th>
<th>(Gui et al., 2010; Kapplinger et al., 2010; Smits et al., 2002, 2005)</th>
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</thead>
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<tr>
<td>p.E1240Q</td>
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<td>(Priori et al., 2002; Risgaard et al., 2013)</td>
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<td>p.E1253G</td>
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<td>Brugada syndrome</td>
<td>(Kapplinger et al., 2010)</td>
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<tr>
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<td>(Groenewegen et al., 2002; Gui et al., 2010)</td>
</tr>
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<td>(Nguyen et al., 2008; Olson et al., 2005; Wang et al., 2002)</td>
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**SCN9A  Na\_1.7**

| p.E1160Q | S3 DIII | Dravet syndrome | (Singh et al., 2009) |

**CACNA1A  Ca\_2.1**

| p.E147K   | S2 DI  | Episodic ataxia 2 | (Imbrici et al., 2004) |
| p.E533K   | S2 DII | Episodic ataxia 2 | (Scoggan et al., 2006), |

**CACNA1F  Ca\_1.4**

| p.D944Y   | S3 DIII | Night blindness, congenital stationary | (Zeitz et al., 2009) |
### Table 5-1 VSD mutations affecting charged residues (S2-S3 segments) in other voltage gated cation channels

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<tr>
<th>Channel</th>
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<th>Mutation</th>
<th>Disease</th>
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Chapter 6: Comparison of two S4 arginine mutations which affect the same residue but cause different phenotypes.

6.1 Summary

A novel Na$_{1.4}$ mutation, R222Q, was identified in a patient with a myotonic phenotype through Sanger sequencing. R222Q is located within the S4 segment (voltage sensor) of DI and causes the neutralization of a positively charged residue. A prior mutation at this location, R222W, has also been identified, which causes a Hypo PP phenotype. In fact, mutations targeted to voltage sensor gating charges are normally associated with a Hypo PP phenotype and cause a gating pore current. Myotonia is not found with a Hypo PP phenotype nor has a gating pore current been shown to cause it. Myotonic phenotypes are normally associated with gain of function mutations that cause disruption to inactivation or activation of the sodium channel. R222Q was studied to determine if it is a pathogenic mutation. Additionally, the effects of both R222Q and R222W were compared in order to determine how they result in different phenotypes.

The effects of both R222 mutations were compared in two different heterologous expression systems: HEK-293 and *Xenopus* oocytes. The effect of the mutations on the biophysical properties of the central pore α currents was studied using whole cell patch clamp of HEK-293 cells. R222Q was found to cause a 16 mV hyperpolarizing shift in the voltage dependence of channel activation. This would be consistent with a myotonic phenotype. In comparison, R222W showed no difference in the voltage dependence of channel activation compared to WT, but a reduction in the current amplitude. Next, both mutations were studied to determine if they cause a gating pore current, using Two Electrode Voltage Clamp with expressed in *Xenopus* oocytes expressing the mutant
channels. Both R222Q and R222W produced small non linear steady state currents which are consistent with a gating pore current. Addition of guanidinium (mimics the guanidinium group of the arginine side chain) to the bath solution increased the amplitude of the gating pore currents – a phenomenon seen in other S4 arginine mutations. R222W was found to conduct gating pore currents consistent with a Hypo PP phenotype. However, the myotonia mutant channel R222Q was found to also conduct gating pore currents but these were smaller than for R222W channel. R222Q is the first myotonia related mutation which has been shown to conduct gating pore currents similar to those found for Hypo PP mutations. In addition, these findings confirm that R222Q is the causative mutation for an individual with a myotonic phenotype.
6.2 Introduction

Myotonia and Hyper PP mutations are most often found in areas important for channel gating where they cause defects in inactivation or activation of the channel, leading to either an increased number of sodium channels available for activation following an action potential, or a persistent depolarizing Na⁺ current. Hypo PP mutations affect the voltage sensing domain and target the positive gating charges. Neutralization of these positive arginine residues disrupts the tight interactions which occlude the gating pore, creating a conduction pathway which allows leak currents.

Mutations causing Hyper PP and PMC, show both phenotypic and pathomechanism overlap, with the same mutations known to cause different either disease, for example, T704M, A1156T and R1448C are both associated with either Hyper PP or PMC (Brancati et al., 2003; Hayward et al., 1999; McClatchey et al., 1992; Vicart et al., 2005; Wagner et al., 1997).

However, no phenotypic or mechanistic overlap with Hypo PP has been identified thus far. Only a handful of myotonia mutations have been identified within the S4 segment: R1448C/S/P/H/L and R225W (Bendahhou et al., 1999; Jarecki et al., 2010; Lee et al., 2009; Matthews et al., 2008; Ptácek et al., 1992). Of these only R1448C/S/H have been functionally characterized. R1448 mutations have been shown to cause only small effects on channel activation but considerably increase the time constant of fast inactivation (Bendahhou et al., 1999; Dice et al., 2004; Ji et al., 1996; Yang et al., 1994). What's more, Francis et al. have shown that R1448C, which affects the outermost arginine residue of DIV, does not cause a gating pore current. This highlights that currently there is no known mechanistic overlap between Hypo PP and PMC or Hyper PP.
6.3 Aims

The aim of this study was to determine whether R222Q is the causative mutation for an individual with a myotonic phenotype. Additionally, the functional effect of R222Q was compared to that of the Hypo PP mutation R222W in order to determine how two Na\(_{\text{V}}\),1.4 mutations affecting the same arginine residue cause different phenotypes. This was done by studying the effect of the mutations on channel function to determine if both mutations cause a gating pore current as expected for S4 arginine mutations.
6.4 Functional analysis of R222Q and R222W Nav1.4 mutations

Two mutations affecting the same arginine residue have been identified. These cause different clinical phenotypes which are associated with different pathomechanisms. R222Q and R222W affect the R2 of DI S4 segment and are associated with PMC and Hypo PP, respectively. Mutations affecting the S4 arginines are usually associated with Hypo PP and the gating pore current. Two approaches were taken to determine the mechanism through which these mutations act. First, whole cell patch clamp was used to study the biophysical characteristics of the central pore α currents of mutant channels expressed in HEK-293 cells. Second, two electrode voltage clamp was used to investigate the presence of gating pore current in mutant channels expressed in Xenopus oocytes.

6.4.1 Characterization of alpha pore currents

To characterize the alpha pore currents of the mutations, mutant Na,1.4 channels were transiently expressed within HEK-293 cells. Properties of channel α currents were analyzed using whole cell patch clamp. Figure 6-1 shows sodium current traces produced by WT, R222Q, and R222W expressing channels. Few cells expressing R222W channels were found to have measurable sodium currents. When sodium currents were measured in R222W expressing cells they had a reduced current amplitude compared to WT and R222Q expressing cells. On the other hand, R222Q channels expressing cells showed currents with WT-like current amplitude, although these currents were activated at more hyperpolarized voltages than WT channels.

The voltage dependence of activation was examined using a voltage step protocol from holding voltages of -80 mV to test potentials of -130 to +90 mV. R222Q channels showed a 16mV hyperpolarising shift of in the voltage dependence of activation (WT $V_{0.5} = -$
18.6±3.4 mV, n=13; R222Q $V_{0.5} = -34.2±3.4$ mV, n=12, $p=2\times10^{-9}$). No change in the voltage dependence of activation was observed for R222W channels.

A number of studied Hyper PP mutations have been shown to cause disruptions to fast inactivation; therefore the voltage dependence of fast inactivation was examined in both mutations. Both mutations were found to have a small hyperpolarizing shift in fast inactivation (WT $V_{0.5} = -63.7±2.6$ mV, n=10; R222Q $V_{0.5} = -68.5±4.8$ mV, n=14; R222W $V_{0.5} = -67.0±2.7$, n=6). However, the 3.4 mV shift seen in R222W is not statistically significant ($p=0.16$).
Figure 6-1 Biophysical properties of Nav1.4 α currents of DI arginine mutations

Whole cell currents from WT, R222Q and R222W transfected cells. Whole cell Na⁺ currents were evoked by a series of voltage steps from -130 to +60 mV from a holding potential of -80 mV. Sodium current traces for A) WT, B) R222Q and C) R222W. D) Conductance/voltage relationship of activation. E) Voltage dependence of fast inactivation measured using a 300 ms conditioning voltage step from -130 mV to 60 mV in 10 mV increments, followed by a constant test pulse step to -10 mV. Normalised averages were fitted with a Boltzmann equation. Data was normalised and fitted using a Boltzmann equation. Error bars represent SEM.
6.4.2 Characterization of gating pore currents

The presence of gating pore currents was investigated in both R222W and R222Q. The cRNA of mutant channels was injected into Xenopus oocytes and the currents measured 3-7 days after injection.

Figure 6-2 shows the mean current amplitude measured during the last 200 ms of a 300 ms voltage step from a holding potential of -100 mV to test potentials of -140 to +50 mV. Both mutations show a non-linear component at hyperpolarised potentials which is consistent with gating pore currents observed in the previously studied Hypo PP mutations. Following leak subtraction at positive voltages, between +5 and -20 mV, small gating pore currents could be observed for both mutations. Due to the small size of the gating pore currents and limited sensitivity of TEVC set up, it was difficult to distinguish the gating pore currents in every oocyte.

Guanidinium has been used as a pharmacological tool to study gating pore currents as it can increase the size of arginine mutation gating pore currents up to 10 fold. Therefore, increasing fractions of the Cl\(^-\) free Na\(^{+}\) bath solution were substituted with guanidinium methanesulfonate in order to increase the amplitude of the gating pore current and provide further support for the presence of the gating pore currents. In the presence of increasing fractions of guanidinium, larger gating pore currents could be seen for both mutations (Figure 6-3). R222W gating pore currents are activated by currents negative to -30 mV (Figure 6-3A). R222Q mutant channels conduct smaller gating pore currents when compared to R222W (P< 0.05 at – 60mV, Figure 6-3C). These currents are activated by currents negative to -50 mV (Figure 6-3B). This suggests that the Hypo PP mutation R222W and the PMC mutation R222Q both cause an aberrant ionic current through the voltage sensing domain.
Currents were recorded in the presence of 1µM TTX in response to 300 ms voltage steps ranging from -140 mV to +50 mV from a holding potential of -100 mV. External bath solution contained 120 mM NaMes, 1.8 mM CaSO₄ & 10 mM HEPES, pH7.4 with NaOH. A) Representative voltage dependence of R222Q, R222W and WT currents before leak subtraction. B) Representative voltage dependence of R222Q (red circles), R222W (Blue triangles) and WT (black squares) currents after leak subtraction between +5mV and -20 mV.

Figure 6-2 Gating pore currents of R222Q & R222W channels without Guanidinium
Figure 6-3 Gating pore currents produced in the presence of Guanidinium

Currents were recorded in the presence of 1µM TTX in response to 300 ms voltage steps ranging from -140 mV to +50 mV from a holding potential of -100 mV in the presence of two different concentrations of guanidinium (30 mM Gn, red circles & 90 mM Gn, blue triangles) within the external bath solution. A) Voltage dependence of R222Q currents following leak subtracted currents, B) Voltage dependence of R222W currents following leak subtracted currents, C) Comparison of R222Q (red circles) and R222W (blue triangles) currents at the highest concentration of guanidinium (90 mM). Error bars represent SEM.
Figure 6-4 Location of R222 residue within channel

Figures are based on homology model of rat NaV1.4 constructed by D Kuzmin, UCL (unpublished). A) Shows VSD of Domain 1 from side: R222 is highlighted in red with the Intercellular Negative charge Cluster (INC) S3 aspartate in blue sphere. Red and blue sticks denote the other S4 arginines and INC negative charges. B) Show the VSD in relation to the pore domain.
6.5 Discussion

6.5.1 Is R222Q a pathogenic mutation?

The R222Q mutation was identified during a genetic screen of myotonia patients (See Chapter 4 for further details). The patient is a young male from a consanguineous family who present with severe muscle stiffness and pain with onset at age 14. His symptoms worsen with cold. The patient has reported one possible episode of weakness lasting for 35 minutes, but has not had episodes of paralysis. Myotonia was clearly present on EMG. No family members were available for testing. In addition, the patient was found to have a heterozygous synonymous mutation in CLCN-1 (c.1650G>A). The mutation is predicted to create a novel splice site in exon 15 and has been previously associated with myotonia congenita. However, no muscle sample was available from the patient to confirm correct splicing of the CLCN-1 gene in the muscle.

R222Q mutant channel expressed in HEK-293 cells showed a large hyperpolarising shift in the voltage dependence of activation. A smaller hyperpolarizing shift was found in the voltage dependence of fast inactivation. In addition, R222Q mutant channels were found to conduct a gating pore current when expressed in Xenopus oocytes which showed similar voltage dependence to gating pore currents conducted by Hypo PP mutant channels. The gating pore currents measured were smaller than the gating pore currents which have been detected in other mutations studied in the work of this thesis.

A negative shift in the voltage dependence of activation, as observed for R222Q, is consistent with a PMC phenotype. The Na\textsubscript{v}1.4 mutations I693L and T704M, associated with episodic myotonia with paralysis, have been shown to cause a similar defect in the voltage dependence of activation (Brancati et al., 2003; Cannon, 2002; Yoshinaga et al., 2012). Such
mutation cause increased electrical excitability of the muscle and repetitive firing of action potentials present as myotonia in the patient.

A PMC phenotype has not been previously associated with gating pore currents. Gating pore currents have been thought to solely cause Hypo PP. In fact, Hypo PP is defined as attacks of paralysis without myotonia and no previously mechanistic link between Hypo PP and myotonia has been established. Studies of the PMC DIV S4 arginine mutation, R1448C, showed that R1448C causes a defect in fast inactivation without causing a gating pore current, leading to the notion that only Hypo PP mutations conduct a gating pore current. In addition, gating pore currents are not necessary to cause a PMC phenotype since other PMC mutation affect the central pore domain and disrupt channel gating. Therefore, it is unlikely that the gating pore currents observed in the R222Q mutant channel contribute towards the myotonic symptoms present in the patient.

Interestingly, the patient has normal potassium levels and does not experience attacks of paralysis despite R222Q conducting a depolarising gating pore current. In addition to the sodium channel mutation, the patient is also heterozygous for a synonymous CLCN-1 mutation, which is predicted to disrupt normal gene splicing. This mutation has been previously described in association with recessive myotonia congenita case, where the proband was a homozygous carrier of the mutation. This suggests the mutation maybe pathogenic loss-of-function mutation. However, no functional studies have been conducted to confirm this mutation reduces chloride currents. It is possible that the loss of function mutation of the chloride channel may provide protection against episodic attacks of paralysis which are normally associated with hyperpolarisation activated gating pore currents. This hypothesis is further supported by studies of the Hypo PP mouse model which showed that reduction of chloride conductance using bumetanide prevented attacks
of muscle weakness and paradoxical depolarisation of muscle fibres in the presence of low potassium.

6.5.2 Is R222W a pathogenic mutation?

The R222W was previously reported by Matthews et al. 2009 in two unrelated Hypo PP kindreds but has not been functionally studied. R222W mutant channels displayed robust gating pore currents at negative membrane potentials. The gating pore currents observed from R222W mutant channels are consistent with those seen in other Hypo PP mutations such as R669H, R672G and R1132H (Sokolov, Scheuer, & Catterall, 2007; Struyk & Cannon, 2007a). These findings are consistent with the notion that the depolarising currents contribute towards the Hypo PP phenotype. In addition, R222W mutant channels were found to have a reduced Na⁺ current amplitude with little effect on the gating of central pore Na⁺ currents when expressed in HEK-293 cells. It is not known if the R222W mutation leads to reduced functional expression of the channel in the muscle of the patient. Previous studies of Hypo PP mutations have shown that such loss of function mutations are not sufficient to cause the Hypo PP phenotype (Matthews et al., 2009, 2010; Struyk & Cannon, 2007). Thus, the gating pore currents conducted by R222W channels are the likely pathomechanism resulting in attacks of paralysis in the patients.

6.5.3 R222Q vs R222W

The effects of the R222 mutations on channel functioning were found to differ with the nature of the substituting residue. Both mutant channels conducted gating pore currents with similar voltage dependence. However, gating pore currents conducted by R222Q channels were much smaller gating pore currents than seen for R222W.
One explanation for the difference is that the different substituting residues cause different size conduction pathways through the gating pore. Substitution of the conserved R222 with the bulky tryptophan residue resulted in large gating pore currents indicating that the tryptophan residue is unable to prevent aberrant ionic leak through the voltage sensing domain. In contrast, substitution with glutamate resulted in smaller gating pore currents. It is possible that R222Q forms a smaller conductance pathway within the gating pore due to the smaller amino acid side chain.

An alternative explanation is that the hyperpolarized shift in the voltage dependence of R222Q channel activation may result in the destabilisation of resting state which conducts the gating pore current. This would cause the voltage sensor in R222Q channels to favour the activated 'up' conformation. Thus, the R222Q voltage sensor is less likely to be found in the resting conformation which conducts the gating pore current, leading to a reduction in the size of the currents. This is further supported by studies of homologous Kv10.2 mutation, R327H, associated with epileptic encephalopathy, which showed the R2 residue is important for stabilising the closed state of the channel (Yang et al., 2013). It is possible that R222Q may destabilise the closed resting state, resulting earlier activation of the voltage sensor compared to wild type channels causing a hyperpolarizing shift in the activation curve.

The differences observed between the mutations are sufficient to explain the difference in phenotypes seen. Previous studies looking at the structure-function relationship of the voltage sensor of the Shaker and Kv1.1 potassium channels have shown that different mutations of the S4 R2 residue result in differential effects on channel gating. For example, R2Q of Shaker and Kv1.1 causes a hyperpolarising shift in the voltage dependence of activation whilst R2C of Shaker causes a depolarising shift in the voltage dependence of activation (Larsson et al., 1996; Liman et al., 1991; Logothetis et al., 1992; Papazian et
al., 1991; Stühmer et al., 1989). This data shows the importance of interactions of the arginine residues with the surrounding environment of the voltage sensor domain.

### 6.6 Conclusion

R222Q is the first myotonia mutation to be associated with a gating pore current. However, the patient studied did not present with episodic attacks of weakness and the gating pore currents are unlikely to cause the myotonic phenotype presented by the patient. R222W was found to cause a gating pore currents similar to those already observed in Hypo PP mutations. These findings support the notion that gating pore currents are solely associated with attacks of paralysis. Additionally, it is thought that the different phenotypes associated with the two mutations studied due to the different substituting residues which result in different effects on channel functioning.

### 6.7 Further Work

- To confirm the protective role of the synonymous CLCN-1 mutation a muscle biopsy sample is needed from the patient in order to determine whether the chloride channel is erroneously spliced. In addition, functional studies are needed to confirm that the mutation results in reduced chloride currents as is currently expected.
- To further characterise the gating pore currents conducted by R222Q and R222W, ion selectivity studies are needed. Using TEVC the gating pore currents were only fully measured in the presence of guanidinium. Therefore, it would be preferable that these experiences were conducted using the cut open voltage clamp set up which is more sensitive.
6.8 Acknowledgements

Patch clamp recordings for R222Q and R222W were performed and analyzed by R. Mannikko and M. Thor. In addition, R.M and M.T helped with the collection of some gating pore current recordings.
## Appendix

Table of primers used for Sanger sequencing.

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KCNJ18  Outer  ATGCTGCTCTCTCTGTC  GTTCTTTTTGCCCTA
### Nest 1
- TCTCCATCGAGACGCAGAC
- CACTCTCCGTACACACGC

### Nest 2
- CATCTTGCATGAATTGACGA
- TTCCAGGATGACCACGATCT

### Nest 3
- CCAGGCATGACTTTGACAGA
- ATCTCTGACCCCCGTCTGTA

**Table of primers used for SCN4A mutagenesis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>Rat</td>
<td>SCN4A Y168D</td>
<td>CACCTTCACGGGATCGATACCTTTGAGTCT</td>
<td>AGGGACTCAAGGTATCGATCCCCGTGAAGGTGCT</td>
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<td></td>
<td>D197G</td>
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<td>R222Q</td>
<td>CTGAGGACCTTCAGGTGCTGCGGCCC</td>
<td>GGGCGGCAGCACCCTGGAAGGTCTCAG</td>
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<tr>
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<td>R222W</td>
<td>CCTGAGGACCTTCTGGTGCTGCGGCCC</td>
<td>GGGCGGCAGCACCAGAAGGTCTCAGG</td>
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<td>D640G</td>
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<tr>
<td></td>
<td>E1072G</td>
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