Title Page

The skeletal muscle channelopathies: phenotype, genotype and pathogenesis

Emma Louise Matthews

UCL

Submission for PhD examination

Declaration

I, Emma Louise Matthews 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.

Abstract

The skeletal muscle channelopathies are a group of inherited disorders due to the dysfunction of voltage gated channels in the sarcolemma resulting in abnormal membrane excitability. Simplistically they are broadly divided into those that result from an “over excited” membrane (the non-dystrophic myotonias) and those due to an inexcitable one (the periodic paralyses). Skeletal muscle channelopathies were described clinically long before they were genotyped or hypotheses regarding pathogenesis fully evolved. This thesis explores all three, the phenotype, the genotype and recent insights into the pathogenesis.

Detailed clinical and neurophysiologic examination of a large group of patients identified new aspects of the phenotype including neonatal presentations with important implications for early life care. Morphological findings are also expanded with the presence of inflammatory infiltrates, not previously described in the channelopathies.

Extensive DNA sequencing of causative genes was undertaken in a carefully genotyped cohort. In hypokalaemic periodic paralysis an exclusive relationship between mutations and the channel voltage sensor emerged which relates closely to recent electrophysiological evidence of a “gating pore” disease mechanism. A small but significant minority of cases remain however where no mutation is found. The implication of other potential genetic mechanisms or even undescribed genes in these cases is discussed.

Current drug therapies are also examined in three separate cohorts and evidence suggests acetazolamide, a commonly prescribed treatment, may only be effective in 50-60% of those with hypokalaemic periodic paralysis. A tentative relationship between efficacy and genotype also emerges.

Patch clamp studies show significant loss of function of the main alpha pore of the sodium channel in periodic paralysis but the implications of this in light of the “gating pore” hypothesis are discussed. Tentative explorations are made as to the viability of performing future studies in myoctes as opposed to the traditional HEK cell model with early experiments illustrating limitations.

Acknowledgements

Firstly I must thank the patients and families I met during this thesis. The “skeletal muscle channelopathies” are a small community but one filled with individuals who meet their problems with unending humour and who gave me their time and experience so generously.

The entire Neurogenetics Laboratory staff also receives a big thank you. There is not a single member of the department who didn’t help me in some way during my thesis. All the staff at the MRC Centre for Neuromuscular diseases are equally deserving of thanks. It must be said that both departments not only helped me in practical terms but extended a very warm welcome and injected frequent humour into the often long and lonely hours.

Some individuals however cannot go without special mention. Anne Grayson – I will never forget the day you walked with me to Gower Street in the rain. Mary Davis – those hours spent behind the door in your office were very special and I miss you. Mary Sweeney, I have said it before and will say it again, there would have been no thesis without you but it is your friendship that I treasure most.

My supervisors, Stephanie Schorge, who taught me that the best way to survive electrophysiology is to always, have wine in the fridge! Prof Hanna –who taught and inspired me enormously but especially for that first opportunity and continued belief I will always be grateful.

Finally, to Mr Matthews whose selflessness was infinite, and this thesis can only be dedicated to my very precious family.

Abbreviations

ACZ**:** acetazolamide

ATP: adenosine triphosphate

ATS: Andersen Tawil syndrome

CA: carbonic anhydrase

CMAP: compound muscle action potential

CK: creatine kinase

DM1: myotonic dystrophy type 1

DM2: myotonic dystrophy type 2 (or PROMM proximal myotonic myopathy)

DMEM: Dulbecco's Modified Eagle Medium

ECG: electrocardiogram

EMG: electromyography

FBS: Foetal bovine serum

GOSH: Great Ormond Street Hospital

HEK: human embryonic kidney

HyperPP: hyperkalaemic periodic paralysis

HypoPP: hypokalaemic periodic paralysis

IRK: inward rectifying potassium channel

MC: myotonia congenita

NCG: National Commissioning Group

NDM: non-dystrophic myotonia

NHNN: National Hospital for Neurology and Neurosurgery

PAM: potassium aggravated myotonia

PCR: polymerase chain reaction

PMC: paramyotonia congenita

RyR: ryanodine receptor

RyR1: isoform of ryandine receptor primarily expressed in skeletal muscle

SCM: sodium channel myotonia

TBE: Tris Borate Ethylenediaminetetraacetic acid

TTX: tetrodotoxin

WT: wild type

Contents

Introduction

The skeletal muscle channelopathies are a rare group of episodic neuromuscular disorders including the periodic paralyses and the non-dystrophic myotonias. Mutations in different genes are responsible for each sub-group but they are all genes that code for voltage gated skeletal muscle ion channels. These ion channels regulate muscle membrane excitability, and consequently these diseases share a common pathology of altered sarcolemmal excitability. The resultant symptoms include episodes of muscle stiffness as well as episodes of weakness. The stiffness, or myotonia is associated with hyper-excitability of the membrane. In contrast, weakness, or paralysis, arises when muscle membranes become inexcitable.

Given these two major types of symptoms, these channelopathies can be classified in two broad groups: The first are the non-dystrophic myotonias, in which myotonia is the predominant symptom, this group encompasses myotonia congenita, paramyotonia congenita and the sodium channel myotonias. The second group is characterised predominantly by paralysis rather than myotonia, and includes the primary periodic paralyses hyperkalemic periodic paralysis, hypokalaemic periodic paralysis and Andersen-Tawil Syndrome.

Skeletal Muscle Channelopathy	Disease Causing Gene	Mode of Inheritance
Myotonia Congenita	CLCN-1	Autosomal Dominant or Recessive
Paramyotonia Congenita	**SCN4A**	**Autosomal Dominant**
Sodium Channel Myotonia	**SCN4A**	**Autosomal Dominant**
Hyperkalaemic Periodic Paralysis	**SCN4A**	**Autosomal Dominant**
Hypokalaemic Periodic Paralysis	**SCN4A (10% of cases) CACNA1S (70% of cases)**	**Autosomal Dominant**
Andersen-Tawil Syndrome	KCNJ2	Autosomal Dominant

Table 1: Skeletal muscle channelopathies associated with voltage-gated ion channels and their causative genes

This thesis focuses primarily on the muscle channelopathies due to mutations of the SCN4A and CACNA1S genes. For practical reasons I will consider these in two categories:

1. SCN4A only: Paramyotonia congenita, sodium channel myotonia and hyperkalaemic periodic paralysis

2. SCN4A or CACNA1S: Hypokalaemic periodic paralysis

1.0 The role of voltage-gated channels in muscle cells

Skeletal muscle is an electrically excitable tissue. Changes in the muscle membrane potential due to the movement of charged ions in and out of the cell stimulate a cascade of reactions that result in co-ordinated muscle contraction and subsequent relaxation. Nav1.4, the voltage gated sarcolemmal sodium channel encoded by SCN4A supports the rising phase of action potentials in skeletal muscle (Fig 1). In cardiac muscles, Nav1.5 (SCN5A) predominates, while in neurons several different isoforms of sodium channel are used, but not Nav1.4. Thus mutations or drugs targeting Nav1.4 can be expected to have relatively specific effects on skeletal muscle excitability.
CACNA1S encodes the skeletal muscle voltage-dependent calcium channel, Cav1.1. Like Nav1.4, expression of Cav1.1 is predominantly restricted to skeletal muscle, with cardiac muscle expressing Cav1.2 (CACNA1C), and neurons utilising a diverse group of voltage gated calcium channels but not Cav1.1. Upon depolarisation by Nav1.4, Cav1.1 channels are activated. These channels, although designated voltage-gated calcium channels, are physically coupled to ryanodine receptors (RyR1) in the sarcoplasmic reticulum, and the activation of Cav1.1 leads to opening of RyR1 and release of calcium from intracellular stores (Fig 2). It is thought that the activation of RyR1 via physical coupling, not via calcium influx, is the main function of Cav1.1 in healthy muscle cells(Catterall., 1995; Brini., 2004).

The other two genes associated with similar muscle disorders are CLCN1, which encodes the voltage-gated chloride channel ClC-1, and KCNJ2, which encodes an inward rectifying potassium channel, Kir2.1. ClC-1 is restricted to skeletal muscle cells where it is thought to help set the muscle resting potential and facilitates repolarisation when potassium accumulates in T-tubules(Cannon., 2006). As is consistent with its restricted expression in skeletal muscles, mutations in CLCN1 lead to a purely muscular disorder, myotonia congenita.

KCNJ2, in contrast, is expressed in a variety of cell types, including cardiac myocytes, and mutations in this gene lead to Andersen-Tawil syndrome which has symptoms associated with this relatively wide distribution, including long QT, facial dysmorphia, and a muscle phenotype of periodic paralysis. In skeletal muscle cells, it has recently been proposed that mutations in KCNJ2 that disrupt the expression of Kir2.1 lead to an elevation of the resting membrane potential of myoblasts, indicating that in healthy cells this channel may be required to maintain resting membrane potential(Sacconi *et al*., 2009).

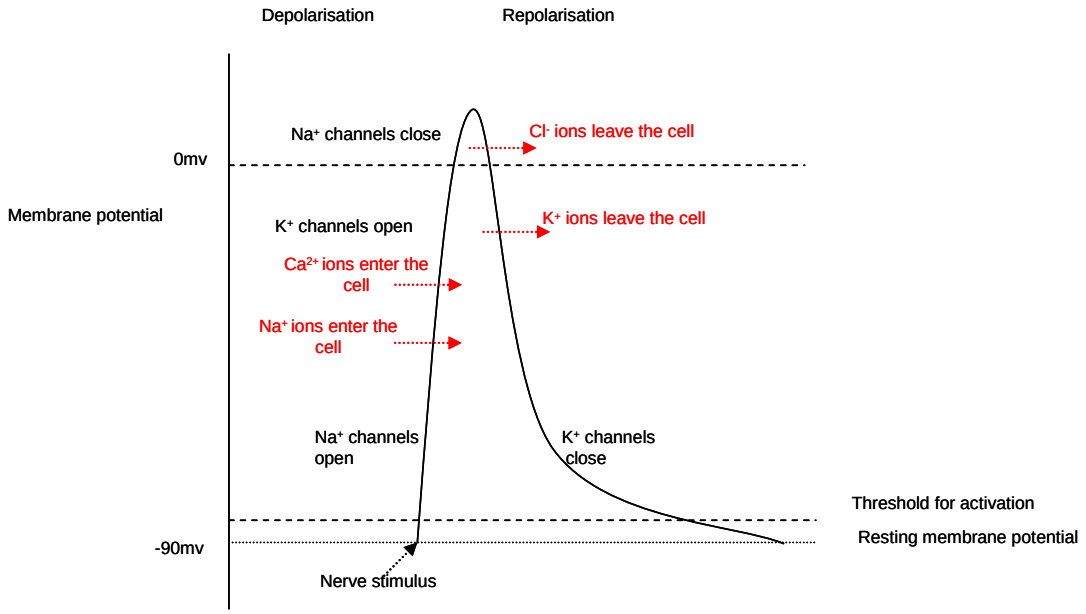


Fig 1: Schematic representation of a muscle action potential

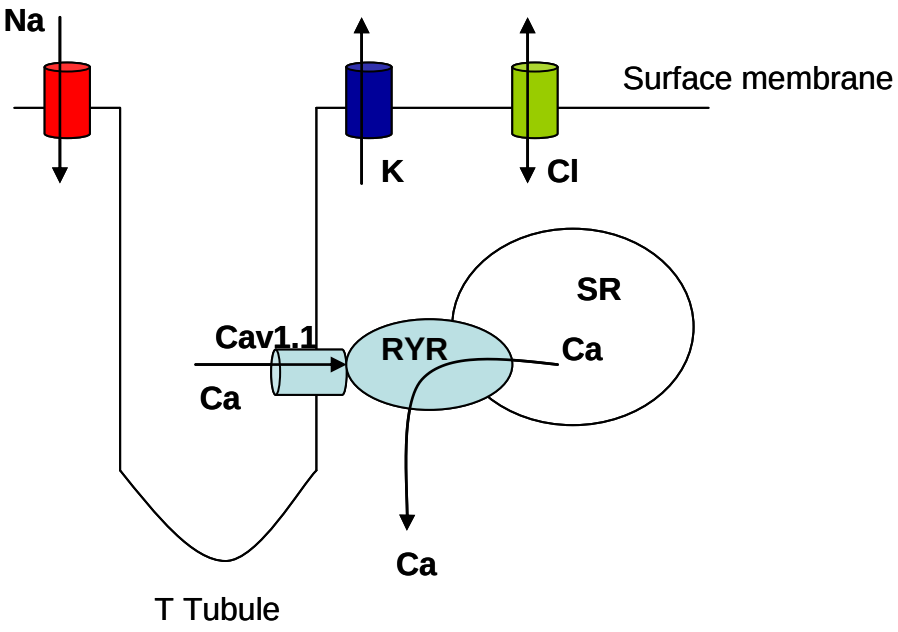


Fig 2: Schematic representation of the skeletal muscle voltage gated ion channels

1.1 Structure of Nav1.4 and Cav1.1

Nav1.4 and Cav1.1 channels both consist of a core α subunit and one or more accessory subunits. In both cases, the α-subunits form the ion conducting pore of the channel and thus far only mutations in the α-subunits have been associated with the skeletal muscle channelopathies. As such only the α-sub-units are considered in detail here. The α sub-units of Nav1.4 and Cav1.1 have similar structures consistent with a shared evolutionary history linked to a single ancestral channel(Cannon., 2007). Both subunits are comprised of four domains, each of which contains six transmembrane segments. The four domains fold together to form a single ion-conducting pore (Fig 3).

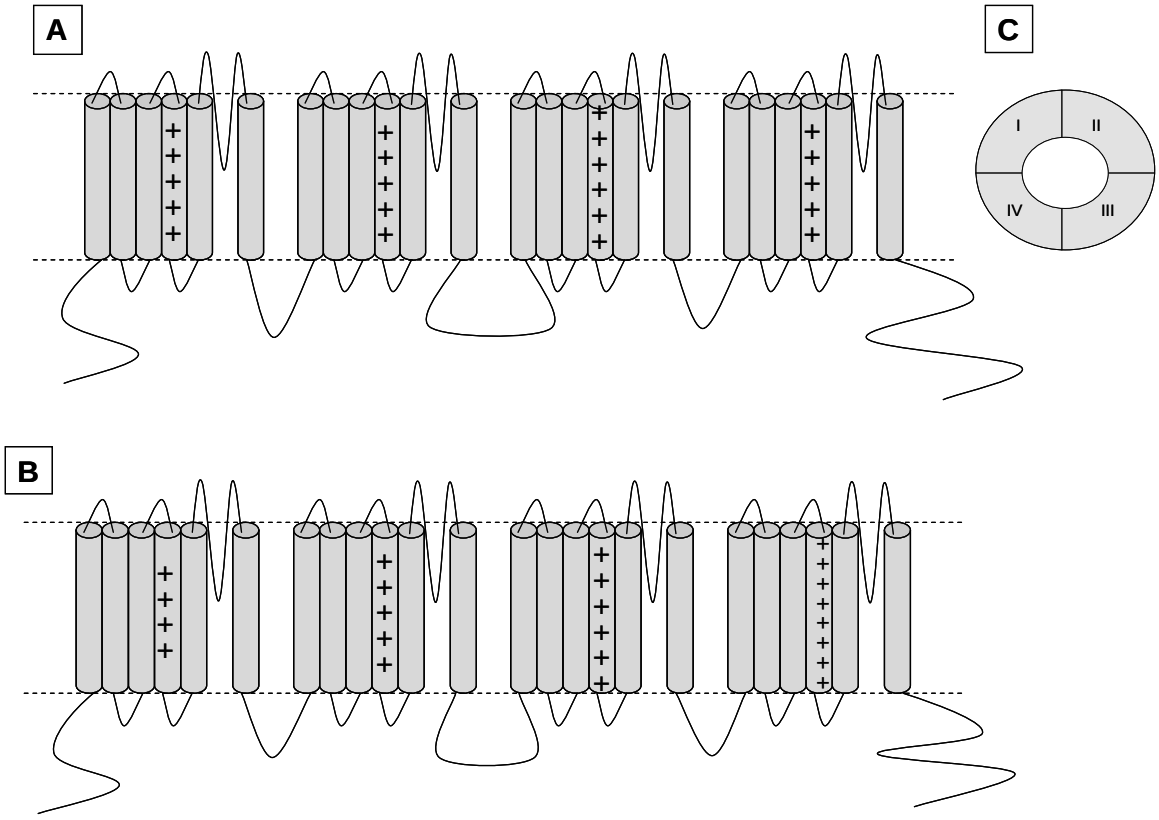


Fig 3: Schematic representation of the A:Cav 1.1 and B: Nav 1.4 channels. C: representation of the alignment of the four domains to form a single ion selective pore.

1.2 Elements determining the ion selectivity of Nav1.4 and Cav1.1

Although Nav1.4 and Cav1.1 are structurally related, they are permeable to different ions. In both channels the S5 and S6 segments from each domain assemble together to create a central channel pore through which ions enter the cell(Varadi *et al*., 1999; Catterall., 1995). However, Nav1.4 is highly selective for sodium, while Cav1.1 is most permeable to calcium ions. The loops between segments S5 and S6 are known as the P loops and site-directed mutagenesis experiments have revealed that a small number of amino acid residues in these P loops determine the ion selectivity for each respective channel. For calcium channels four glutamic acid residues (EEEE) have been identified as the key amino acids in determining selectivity for calcium(Tang *et al*., 1993; Mikala *et al*., 1993).

The amino acids occupying the comparable positions in the voltage gated sodium channels (aspartate, glutamate, lysine, alanine, DEKA) have similarly been implicated in setting the selectivity of sodium channels(Heinemann *et al*., 1992; Favre *et al*., 1996)(See Figs 4 and 5).

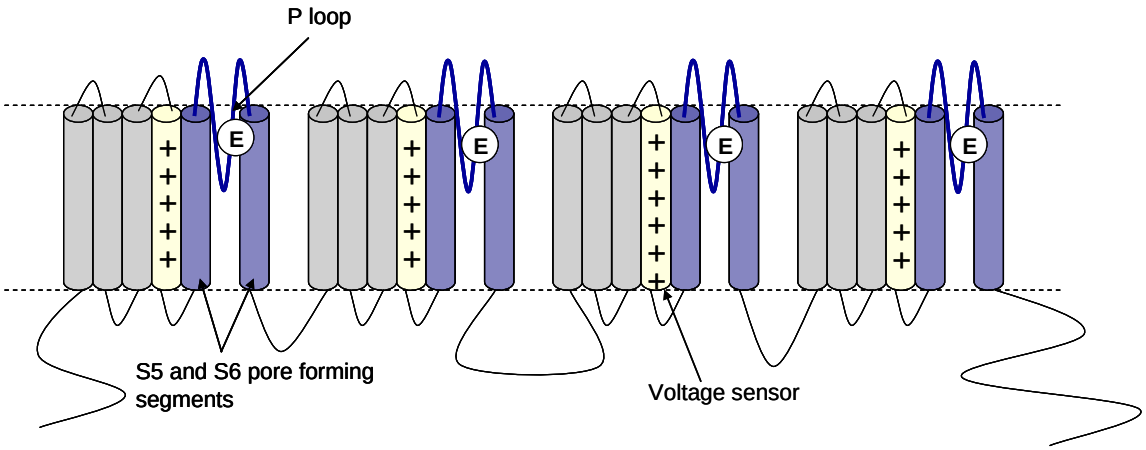


Fig 4: Schematic representation of Cav1.1 highlighting amino acid residues responsible for channel ion selectivity.

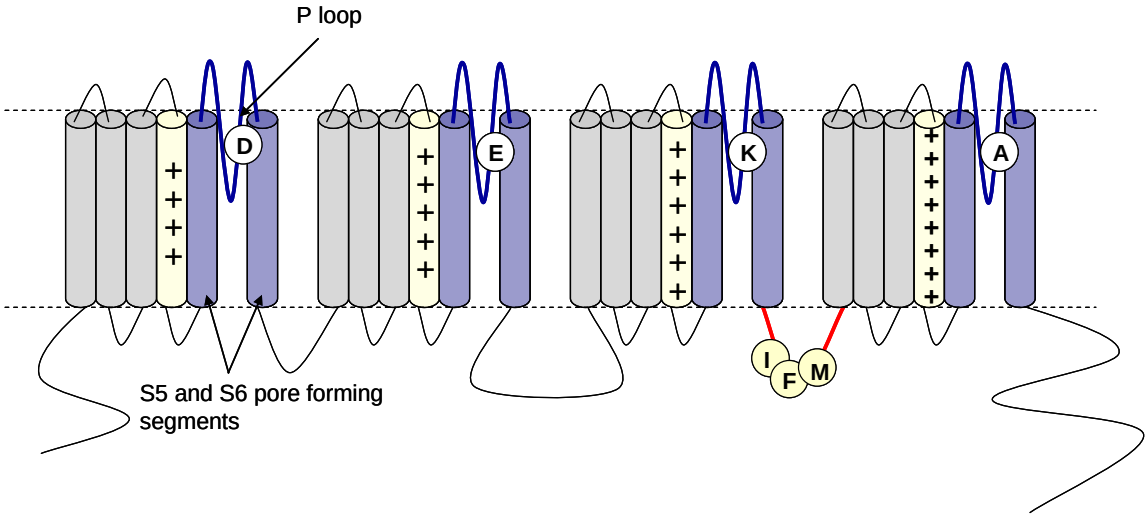


Fig 5: Schematic representation of Nav1.4 highlighting amino acid residues responsible for channel ion selectivity and the IFM motif/inactivation gate.

1.3 Channel activation and inactivation

Both Nav1.4 and Cav1.1 are activated by depolarisation of the muscle membrane. In the case of Nav1.4 the activation is rapid, and the main role of the channel is to allow sodium ions to enter the cell, further depolarizing it, and allowing more voltage-gated channels to open. In the case of Cav1.1, it appears the structural change upon activation is sufficient to mediate the main effect, a physical activation of the ryanodine receptors, however Cav1.1 proteins still contain a channel pore that is opened in response to depolarization, and which allows calcium to enter the muscle cell.

In common with many voltage gated channels, both Nav1.4 and Cav1.1 use positively charged segments within the pore-forming α subunits to detect changes in membrane voltage(Catterall., 2010). The S4 segments of both channels contain positively charged residues (arginines or lysines) at every third position surrounded by hydrophobic residues. These segments with their abundance of electrical charge behave as the voltage sensors(Yang *et al*., 1996a) by moving towards the cytoplasmic side of the membrane in response to membrane depolarisation. It is this outward movement that produces a conformational change at the intracellular surface of the channels(Catterall., 1995), and opens the central pore of the channel to allow influx of ions (Fig 6).

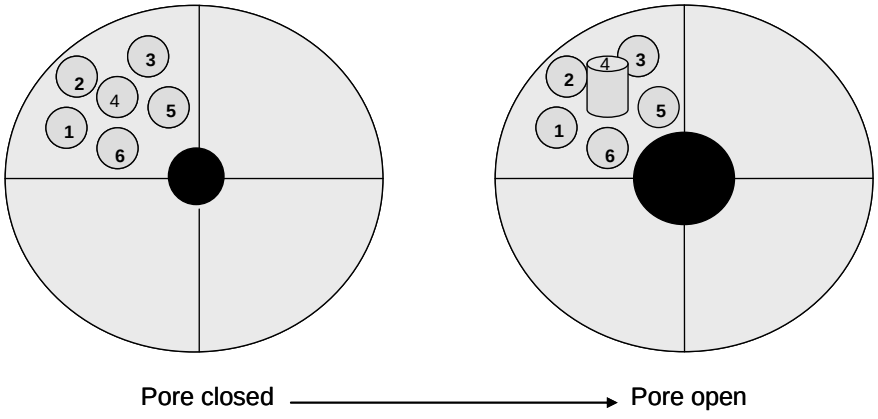


Fig 6: Outward movement of the S4 segment in response to depolarization produces a conformational change in the channel which opens the central pore.

Although significant advances in understanding the function of the S4 segments as the voltage sensors have been made the exact role of each segment is not fully understood(Catterall., 2010). It is likely that individual segments may have slightly different roles e.g. in the sodium channel evidence suggests the S4 segments of domains III and IV play a more significant role in fast inactivation(Cha *et al*., 1999).
In basic terms the channels can be in one of three states: closed, open or inactive. When the sarcolemma is at its resting potential the channels are closed and allow no movement of sodium or calcium across the membrane. In response to depolarisation the S4 segments (voltage sensors) move outwards and the pore undergoes a responding conformational change such that the channel moves into the open state and allows the influx of ions through the central pore. With sustained depolarisation, the channels inactivate stopping the flow of ions. As the sarcolemma repolarises the channels return to the closed state and recover from inactivation (see Fig 7).

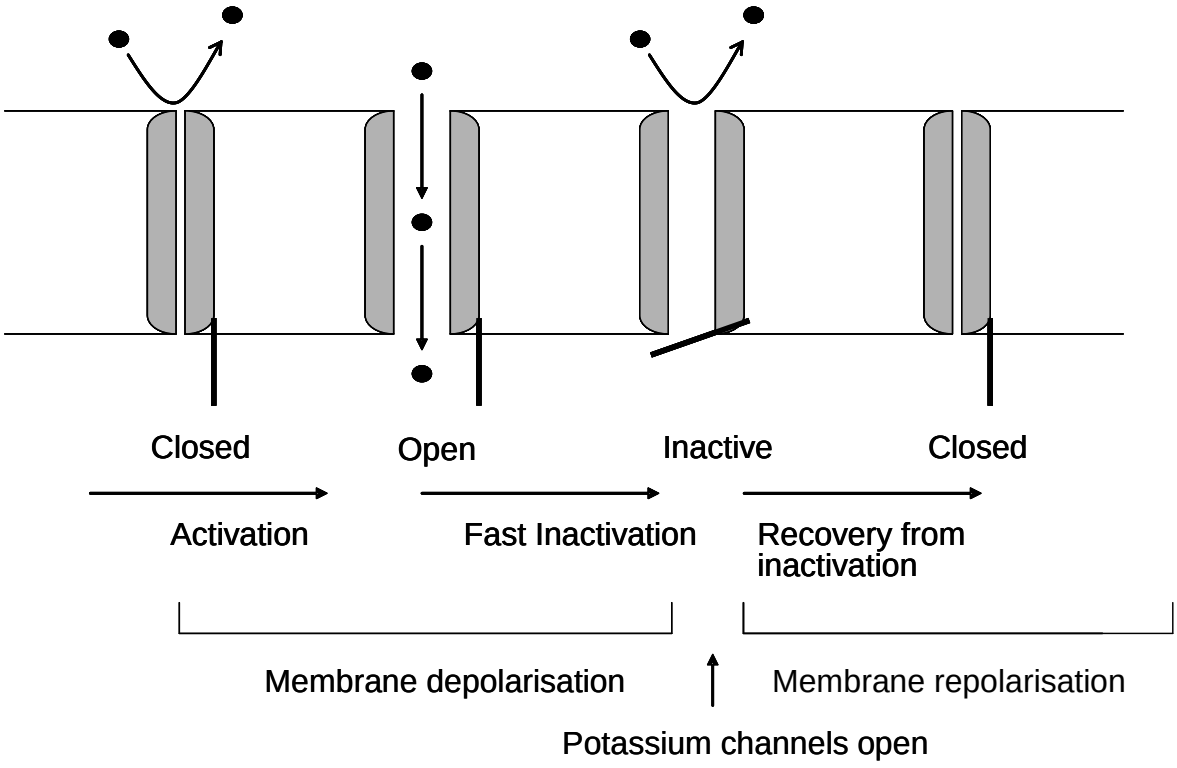


Fig 7: States of activation and inactivation of Nav1.4 in response to changes in membrane potential.

The sodium channels can undergo two forms of inactivation. After a brief depolarisation of the muscle membrane the channel undergoes fast inactivation which occurs in milliseconds and is mediated by the cytoplasmic linker between domains III and IV that acts as a hinged "inactivation gate" swinging across and blocking the cytoplasmic side of the pore to inactivate the channel. The specific inactivation particle consists of three residues in the DIII-IV linker (see Fig 5) known as the "IFM motif" (isoleucine, phenylalanine, methionine) that are thought to bind to an acceptor site on the cytoplasmic mouth of the pore blocking the passage of sodium ions(Vassilev *et al*., 1988; Patton *et al*., 1992; McPhee *et al*., 1994). After longer depolarisations the channel inactivates over a period of seconds to minutes by a process of slow inactivation. Fast and slow inactivation are thought to be structurally independent processes, but the mechanism for slow inactivation is less well understood and no specific structural part of the channel has been identified as a slow inactivation gate(Vedantham *et al*., 1998).

While ion selectivity, activation and inactivation are thought to be processes which are intrinsic to α subunits the full physiological function of these channels, as is common to most ion channels, is dependent on the assembly of several proteins, including multiple accessory subunits(Catterall., 1995). However since this project is focused on the clinical manifestation of diseases which are so far only associated with mutations in the α subunits, the accessories will not be considered here.

1.4 Clinical features of diseases associated with voltage gated ion channel dysfunction in skeletal muscle

The non-dystrophic myotonias

Paramyotonia Congenita

Eulenberg first described and named Paramyotonia Congenita (PMC) in 1886 after studying six generations of a German family affected with the disease(Eulenberg A., 1886). PMC is due to mutations of the SCN4A gene and is inherited in an autosomal dominant manner.

Symptoms usually present in the first decade. Affected individuals experience episodes of muscle stiffness (myotonia) and weakness or paralysis that are markedly worsened or precipitated by cold environments and with periods of exercise. The muscles of the face and hands tend to be most severely affected. The myotonia commonly lasts seconds to minutes but the paralysis can last hours.

Sodium Channel Myotonia

Before the recognition of SCN4A as the disease causing gene for paramyotonia congenita there were many reports of other myotonic phenotypes that did not fit the typical presentation for PMC. These phenotypes were united by features such as a delayed onset of myotonia following rest after exertion, little or no cold exacerbation but significant exacerbation following potassium ingestion. They were purely myotonic phenotypes with no associated episodes of muscle paralysis. Individually they were described as acetazolamide responsive myotonia congenita(Trudell *et al*., 1987; Ptacek *et al*., 1994b), myotonia fluctuans(Ricker *et al*., 1990; Lennox *et al*., 1992; Ricker *et al*., 1994) and myotonia permanens(Lerche *et al*., 1993; McClatchey *et al*., 1992b). Together due to their shared features they are termed the potassium aggravated myotonias (PAM).
Other phenotypes were reported that had overlapping features of PMC and PAM in that they were purely myotonic and often exacerbated by potassium ingestion but also by exposure to cold(Heine *et al*., 1993; Koch *et al*., 1995; Wu *et al*., 2001).
The distinguishing feature between all these collective phenotypes and PMC has been that, unlike PMC, they are purely myotonic disorders with a lack of any associated episodes of muscle paralysis. As all of these phenotypes have been shown to be allelic to PMC and due to mutations in SCN4A some authors collectively use the term sodium channel myotonia (SCM)(Fournier *et al*., 2004) to encompass all of these purely myotonic phenotypes.
Predominantly it is the face and limb muscles affected by these two groups of myotonic disorders (PMC and SCM) but respiratory compromise which can be severe has been reported as has dysphagia(Lerche *et al*., 1993);(Colding-Jorgensen *et al*., 2006).
The sodium channel myotonias can be generally relatively easily separated from paramyotonia congenita by their absence of episodic weakness but they can have

considerable clinical overlap with the dominant form of myotonia congenita (MC) due to mutations in the CLCN-1 gene. Table 2 outlines the pertinent clinical features for each sub group of non-dystrophic myotonia. The major features of overlap between SCM and dominant MC are the presence of the "warm-up" phenomenon used to describe an improvement in myotonia with repetitive activity. This is often described as the distinguishing feature of myotonia congenita but it can be present in the sodium channel myotonias. If so however, it often fluctuates with paramyotonia, (myotonia that worsens with repetition as is seen in PMC), whereas in myotonia congenita only the warm up phenomenon will be seen. The distribution of muscle involvement can also be very similar for SCM and dominant MC and often episodic weakness is also absent in dominant myotonia congenita. The recessive form of myotonia congenita is not easily confused with sodium channel myotonia but the clinical features are outlined in Table 2 for comparative purposes.

Development of a progressive proximal myopathy is described in PMC and SCM although whether the onset or severity of this correlates to age or episodes of myotonia and/or paralysis is unknown(Schoser *et al*., 2007).

	Recessive MC	Dominant MC	Paramyotonia Congenita	Sodium Channel Myotonia
Inheritance	Recessive	Dominant	Dominant	Dominant
Causative gene	CLCN-1	CLCN-1	SCN4A	SCN4A
Myotonia distribution	Lower limbs more than upper limbs	Upper limbs more than lower limbs Facial muscles may be involved	Upper limbs and face more than lower limbs	Upper limbs, face , extraocular, more than lower limbs
Myotonia cold sensitivity	None or minimal	None or minimal	Yes – often dramatic	Variable – ranging from none to severe
Warm up phenomenon	Present	Present	Absent	May be present
Paradoxical myotonia	Absent	Absent	Present	May be present
Delayed onset myotonia after exercise*	Absent	Absent	Absent	May be present Characteristic of myotonia fluctuans
Episodic muscle weakness	Common, develops on initiation of movement but transient and improves rapidly	Uncommon	Common often exacerbated by cold and/or exercise and frequently prolonged for several hours	Not reported
Eyelid myotonia	Infrequent	Infrequent	Common	Common

*Myotonia typically develops after a short period of exercise e.g. 10 minutes or on resting after a period of exercise.

Table 2: Pertinent clinical features of the non-dystrophic myotonias

The Periodic Paralyses

Hyperkalaemic Periodic Paralysis

The periodic paralyses are thought to be the most common of the skeletal muscle channelopathies although the exact incidence is unknown(Venance *et al*., 2006). Hyperkalaemic periodic paralysis is due to mutations in SCN4A and is inherited in an autosomal dominant manner. Onset of symptoms is usually within the first decade. The prominent symptom is episodic muscle paralysis frequently occurring after exercise or following the ingestion of potassium rich foods e.g. bananas and tomatoes. Attacks usually last from a few minutes to hours and myotonia can occur. ECG abnormalities secondary to raised serum potassium levels may be seen during an attack of paralysis. Paramyotonia congenita, sodium channel myotonia and hyperkalaemic periodic paralysis are all allelic disorders due to mutations of the SCN4A gene. There are often overlapping clinical features and it has been proposed that these disorders represent different ends of a spectrum rather than being individual diseases(Cannon., 2000).

Hypokalaemic Periodic Paralysis

Hypokalaemic periodic paralysis (hypoPP) is also inherited in an autosomal dominant manner. It is characterised by attacks of flaccid skeletal muscle paralysis in association with reduced serum potassium levels. Onset is commonly in the first or second decade although presentation in the third decade has been described(Miller *et al*., 2004). Attacks of muscle paralysis are precipitated by factors that reduce serum potassium levels such as a large carbohydrate meal. Attacks occur most commonly following strenuous exercise or during the night or early morning. They typically last in the region of hours to days although some patients will report it is weeks to months before full muscle strength returns.

It is predominantly the limb muscles which are affected in hypoPP but occasionally severe respiratory muscle involvement is described(Kil *et al*., 2009b; rzel-Hezode *et al*., 2009b). Cardiac muscle is not inherently affected but potassium levels outside the normal range can cause ECG changes(Kim *et al*., 2005; Kil *et al*., 2009a; Hecht *et al*., 1997) (flattened ST segments, U waves, prolonged QT interval) that may be pro-arrhythmic and

require cardiac monitoring while potassium levels are restored. One case of severe sinus bradycardia requiring temporary pacemaker has been reported during an episode of hypokalaemia(Maffe *et al*., 2009) and another case report has described sudden death amongst relatives of a genetically confirmed individual with hypoPP(Hecht *et al*., 1997). Whether such severe outcomes are directly or exclusively attributable to hypokalaemia is not fully established.

Many patients with hyperPP and hypoPP will function independently between attacks of paralysis but in a significant number a fixed myopathy develops that can be debilitating(Fouad *et al*., 1997; Miller *et al*., 2004; Biemond *et al*., 1934). It is of note that three independent series all reported abnormal morphological findings in 100% of hypoPP patients who had been biopsied(Fouad *et al*., 1997; Sternberg *et al*., 2001; Miller *et al*., 2004). In a minority these were non-specific myopathic changes with vacuolar myopathy and tubular aggregates accounting for the majority. The reports of permanent muscle weakness were more varied from 25% to 72% of genotyped individuals. The mechanism of this myopathy is not understood. It is postulated that it is independent of paralytic attack frequency or severity(Buruma *et al*., 1978; Links *et al*., 1990) although there is evidence that increasing age is associated with the myopathy(Links *et al*., 1990).

Only 10% of cases of hypokalaemic periodic paralysis are due to mutations of the SCN4A gene with the majority being caused by mutations in the CACNA1S gene(Fouad *et al*., 1997; Miller *et al*., 2004; Sternberg *et al*., 2001). Approximately 20% remain genetically undefined. There is no clear clinical distinction between hypoPP arising from the different genetic sources.

Andersen-Tawil Syndrome

Andersen-Tawil syndrome is the only muscle channelopathy in which the causative gene, KCNJ2(Plaster *et al*., 2001), is expressed in tissue other than skeletal muscle. As such the triad of periodic paralysis, dysmorphic features and cardiac conduction defects comprise the characteristic phenotype(Andersen *et al*., 1971; Tawil *et al*., 1994). As with nearly all the channelopathies it is an autosomal dominant disorder. The type of periodic paralysis can be either hyper or hypokalaemic although most commonly it is hypoPP(Davies *et al*., 2005). The dysmorphic features include mandibular micrognathia, short stature,

clinodactyly, syndactyly, hypertelorism and low set ears(Andersen *et al*., 1971; Tawil *et al*., 1994; Davies *et al*., 2005; Haruna *et al*., 2007) but can be very subtle and easily missed. Likewise there are often no cardiac symptoms and unless an ECG is performed for unrelated circumstances the cardiac conduction defects are often undetected. For these reasons hypokalaemic periodic paralysis may be the only obvious presentation and some individuals may be clinically misdiagnosed. This can have significant consequences as the cardiac conduction defects described include abnormal U waves, prolonged QUc interval, prolonged QTc interval, bigeminy and bidirectional VT. Rarely sudden death can occur(Tawil *et al*., 1994; Davies *et al*., 2005; Zhang *et al*., 2005; Haruna *et al*., 2007).

There is very little data as to the natural history of any of the skeletal muscle channelopathies, particularly with regards to the proximal myopathy. Additionally, there are few phenotype-genotype correlations, something which is exacerbated by significant variability in the severity of the phenotype amongst individuals with the same mutation. There are even examples of this occurring amongst members of a single kindred. The skeletal muscle channelopathies are generally considered to be more severe in males than females. A significant worsening of symptoms is also commonly reported during pregnancy. Both of these factors imply there may be a hormonal influence on the phenotype. There is some suggestive in vitro evidence to support this(Fialho *et al*., 2008) but it is yet to be fully established or understood.

An allied disease where hormones are clearly shown to be of significance is thyrotoxic periodic paralysis. This is most common in Asian males and represents a form of hypokalaemic periodic paralysis in which attacks only occur in relation to deranged thyroid function. It is not to be missed as treatment of the thyrotoxicosis and return to a euthyroid state will abolish the attacks of muscle paralysis. Equally although the thyroid function may be abnormal, the patient may not be obviously thyrotoxic and diagnosis requires a degree of suspicion.

The first aim of this thesis is to study the phenotypes among a large cohort of genotyped channelopathy patients to explore any additional recurring features and assess phenotype-genotype correlations.

	Hyperkalaemic PP	Hypokalaemic PP	Andersen-Tawil Syndrome
Causative gene	SCN4A	CACNA1S SCN4A	KCNJ2
Inheritance	Autosomal dominant	Autosomal dominant	Autosomal dominant
Episodic skeletal muscle paralysis	Yes	Yes	Yes
Duration of paralysis	Commonly minutes to hours	Commonly hours to days	Variable, minutes to days
Ictal potassium levels	High	Low	Low, high or normal
Precipitators of paralysis	Potassium rich foods Rest after exercise	Large carbohydrate load Rest after exercise	Dependant on ictal potassium
Typical time of attacks	Any time of day	During night or early morning	Any – dependant on ictal potassium
Cardiac conduction defects	Only those attributable to severe hyperkalaemia; resolve with restoration of normal potassium values	Only those attributable to severe hypokalaemia; resolve with restoration of normal potassium values	Common especially abnormal u waves, prolonged QUc interval and ventricular arrhythmias, irrespective of potassium levels
Dysmorphic features	No	No	Common especially short stature, mandibular hypoplasia, clinodactyly and low set ears

Table 3: Summary of clinical features of the primary periodic paralyses

1.5 Clinical Neurophysiology

Recently specialized clinical neurophysiology protocols have aided precise diagnosis in muscle channelopathies by directing genetic testing based on genotype specific electrophysiological patterns. Sarcolemmal excitability can be measured indirectly as the variability of the compound muscle action potential (CMAP) following different stimuli. The CMAP size varies in skeletal muscle channelopathies in response to short (10-20seconds) or long (3-5 minutes) exercise tests (Streib EW., 1982; McManis *et al*., 1986). Using these exercise protocols in combination with muscle cooling distinct electrophysiological patterns, termed patterns I, II, III, IV and V are now recognized for the major skeletal muscle channelopathy sub-groups (Fournier *et al*., 2004; Fournier *et al*., 2006). The details of these patterns will be discussed for each channelopathy below. For clinical diagnosis the repeat short exercise test with muscle cooling is of particular value in the non-dystrophic myotonias.

Neurophysiologic patterns in the non-dystrophic myotonias

Patients with paramyotonia congenita (PMC) typically have a gradual and prolonged decrement in CMAP after exercise, termed pattern I(Fournier *et al*., 2004). This decrement is exacerbated with repeat testing and muscle cooling (Fig 8A) reflecting the clinically observed cold- and exercise-induced weakness. Some genotypes only display this typical pattern when the short exercise test is performed with the muscle cooled(Fournier *et al*., 2006).

The sodium channel myotonias are separated clinically from PMC by their lack of weakness. This is illustrated by pattern III, normal responses to all provocative tests (Fig 8B) and EMG myotonia is usually the only positive electrophysiological finding. This is the characteristic finding in SCM but is not absolute and there are some variations for certain genotypes(Fournier *et al*., 2006).

Patients with chloride channel myotonia (MC) can show one of two patterns. The most common is pattern II(Fournier *et al*., 2004) in which at room temperature there is an

immediate CMAP decrement after the short exercise test which recovers quickly and diminishes with repetition, reflecting the transient weakness observed clinically (Fig 8C). This pattern is most frequently seen in recessive MC but can be observed in any muscle ion channel disorder in which there is a loss of sarcolemmal chloride conductance. It is therefore also seen in dominant MC and in both DM1 and DM2. There is now clear evidence that the myotonia in DM1 and DM2 is secondary to reduced chloride conductance(Charlet *et al*., 2002). In recessive MC cooling has little further effect (Fig 8C). However, in dominant MC the CMAP decrement may be worsened or only seen with cooling(Fournier *et al*., 2006) making it essential to perform the short exercise test at both room temperature and with the muscle cooled (Fig 8D). However, some patients with dominant MC show a normal response (pattern III)(Fournier *et al*., 2006) to all provocative tests (Fig 8B), even with muscle cooling which is indistinguishable electrophysiologically from sodium channel myotonia. Clinical history and examination usually helps to distinguish between the two and guide genetic analysis but both of these can have considerable overlap between the two groups as discussed earlier (see Table 2). Table 4 outlines the most common electrophysiological pattern observed in the repeat short exercise test with muscle cooling for each of the NDMs. Variability exists and where muscle cooling has already proven useful in improving diagnosis, repetitive nerve stimulation may have a future role to play in distinguishing the sub-types of NDM. There is some evidence that a reduction in CMAP may be provoked by repetitive nerve stimulation in certain cases of recessive MC where exercise testing even with the muscle cooled has failed to produce any such decrement(Michel *et al*., 2007). In this way repetitive nerve stimulation may become an additional future tool to guide the genetic analysis towards recessive MC in cases that may otherwise be thought to be dominant MC or SCM.

Neurophysiologic patterns in the primary periodic paralyses

For hyperkalaemic periodic paralysis and hypokalaemic periodic paralysis due to calcium channel mutations electrophysiological patterns IV and V were determined (Table 4). In summary, pattern IV describes an increase in CMAP that is exaggerated by repetition in

the short exercise test, and an immediate increase in CMAP followed by a later reduction in CMAP in the long exercise test. Pattern V illustrates the lack of change observed in CMAP during the short exercise test and the prolonged decrement in CMAP during the long exercise test of patients with HypoPP due to calcium channel mutation. It is important to note that while these patterns are undoubtedly useful in each group they were performed in a relatively small number of individuals all carrying the same causative gene mutation (6 T704M and 13 R528H). Only two hypoPP patients with sodium channel mutations were studied each with a different mutation and each showing a different electrophysiological pattern. No large scale studies have been performed in those with Andersen-Tawil syndrome. As a result the electrophysiological patterns are perhaps less clearly defined for the periodic paralyses than the myotonic disorders.

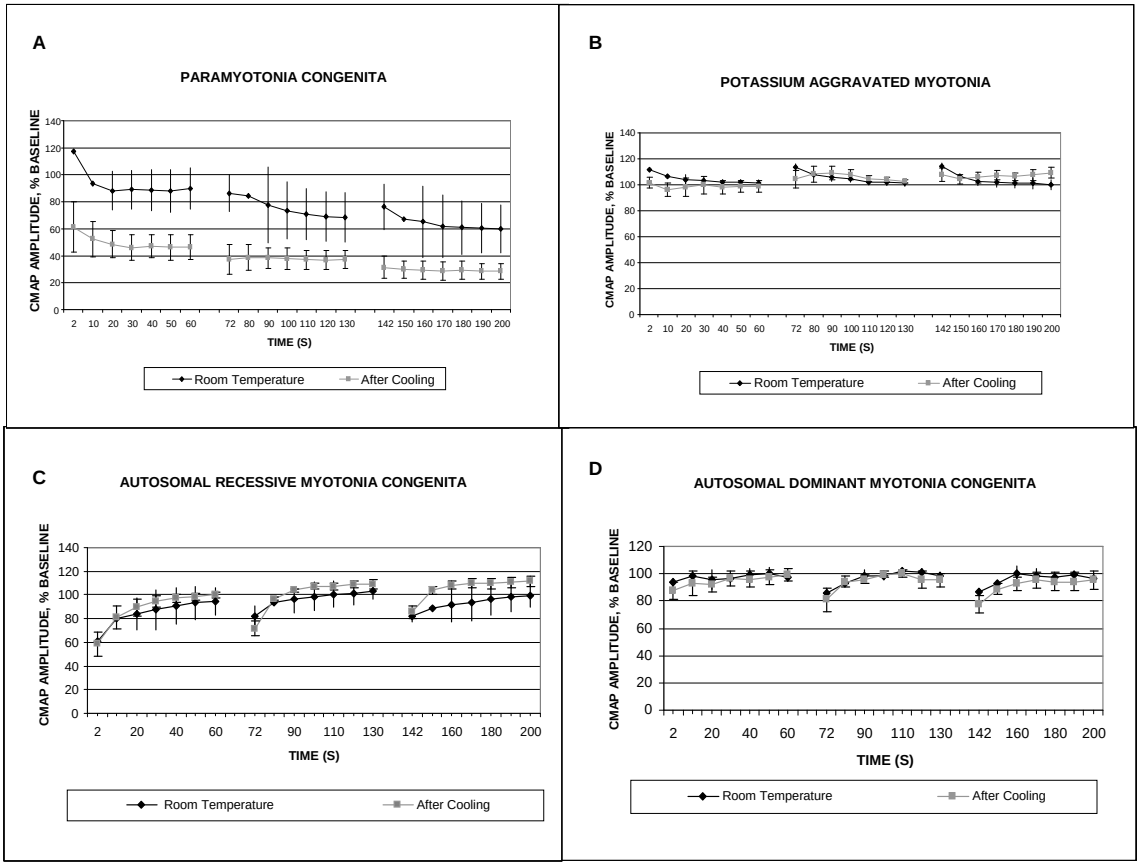


Fig 8: Common electrophysiological patterns seen in the repetitive short exercise test at room temp and with the muscle cooled in the NDMs (produced by Dr Tan)

	Paramyotonia congenita	Sodium channel myotonia	Dominant MC	Recessive MC	HyperPP	HypoPP
Myotonic potentials	Yes	Yes	Yes	Yes	Yes – less frequent/florid than NDMs	No
Repeat SET at room temp	Gradual and persistent reduction in CMAP Enhanced by repetition	No significant change of the CMAP from baseline*	Little or no decrement in CMAP	Early decrement in CMAP with rapid recovery Improves with repetition	Increase in CMAP exaggerated by repetition	No change in CMAP
Repeat SET with muscle cooling	Gradual and persistent reduction in CMAP enhanced further by cooling (see Fig)	No significant change of the CMAP from baseline * (see Fig)	Early decrement with rapid recovery and reduction with repetition may be seen (see Fig)	Cooling has little further effect (see Fig)	ND	ND
Long exercise test	Prolonged decline in CMAP	No change in CMAP	No change in CMAP or slight early decrement only	No change in CMAP or slight early decrement only	Early increase in CMAP followed by prolonged decline in CMAP	Prolonged decrease in CMAP
Fournier pattern	I	III	II/III	II	IV	V

*Note: this same pattern may be observed in dominant MC SET: short exercise test, CMAP: compound muscle action potential

Table 4: Electrophysiological patterns seen in the non-dystrophic myotonias and the primary periodic paralyses

1.6 Genetics of the skeletal muscle channelopathies

The Skeletal Muscle Sodium Channelopathies

The SCN4A gene has 24 exons that code for the α-subunit of the voltage gated skeletal muscle sodium channel, Nav1.4. All of the disorders due to mutations in this gene are inherited in an autosomal dominant manner. Every mutation, with the exception of one small deletion, associated with the sodium skeletal muscle channelopathies has been a point mutation. Mutations have been reported throughout the gene although exons 13, 22 and 24 are recognised as "hotspots". Table 5 outlines all reported SCN4A mutations including the described phenotypes and functional consequences. Briefly, the most common mutations associated with PMC are T1313M and substitutions at the R1448 position in exons 22 and 24. The most frequently occurring SCM mutations are G1306A and V1589M in exons 22 and 24.The mutations most commonly seen in hyperkalaemic periodic paralysis are the T704M and M1592V mutations in exons 13 and 24. Although these exons represent hotspots (Fig 9), mutations have been reported throughout the gene and there is no clustering or discernible pattern to their position in the protein (Fig 10).

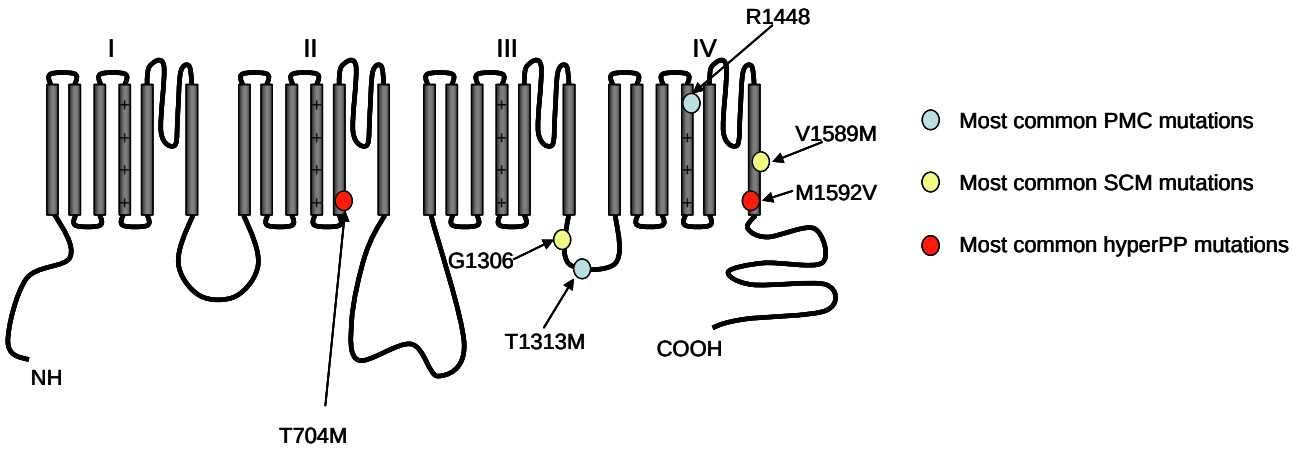
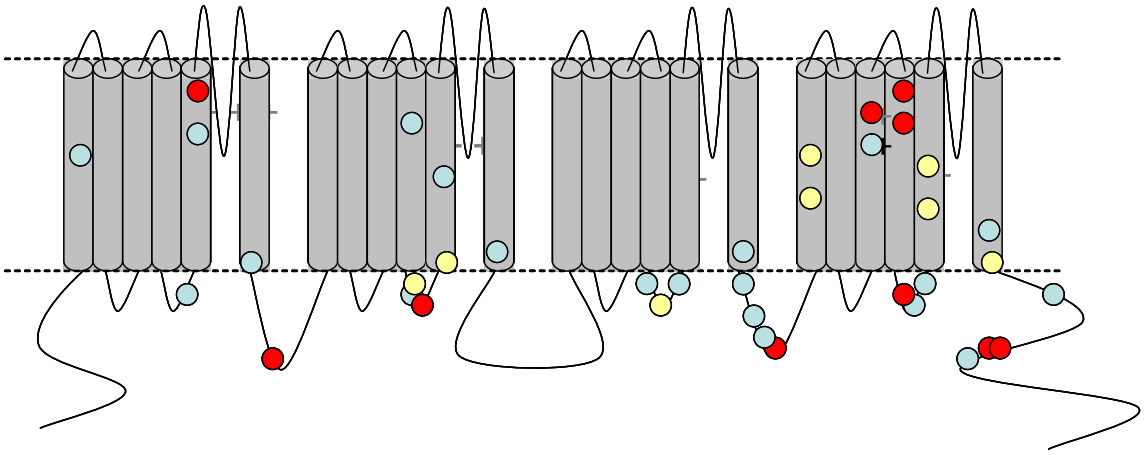


Fig 9: Common Nav1.4 mutations associated with PMC, SCM and HyperPP.



SCM		PMC	HyperPP
I141V	V1293I	Q270K	L689I
L250P/V	N1297K	I693T	T704M
L266V	G1306A/E/V	T1313A/M	A1156T
V445M	I1310N	L1433R	M1360V
F671S	L1436P	R1448C/H/L/P/S	M1370V
L689F	M1476I	G1456E	F1490L+M1493I
A715T	A1481D	F1473S	I1495F
S804F/N	V1589M	Del 1700-1703	M1592V
A1152D	Q1633E	E1702K	
I1160V	F1705I		

Fig 10: Distribution of all point mutations in Nav1.4 associated with PMC, SCM and HyperPP

Mutation	Exon	Protein Position	Phenotype	Clinical Features	Major Pathomechanism	Reference
I141V	4	DI/S1	SCM	Myotonia following rest after exertion Cold exacerbated myotonia No weakness	Enhanced activation Enhanced slow inactivation	(Petitprez *et al*., 2008)
L250P/V	6	DI/S5	SCM	Myotonia with warm up phenomenon No response to cooling	Unknown	(Trip *et al*., 2008)
L266V	6	DI/S5	SCM	Cold exacerbated myotonia No weakness	Impaired fast inactivation Accelerated recovery from fast inactivation	(Wu *et al*., 2001)
Q270K	6	DI/S5	PMC	Cold exacerbated myotonia Cold exacerbated paralysis	Unknown	(Fournier *et al*., 2006)
V445M	9	DI/S6	SCM – painful congenital myotonia	Painful myotonia No weakness	Enhanced activation Impaired fast inactivation Enhanced slow inactivation	(Rosenfeld *et al*., 1997; Takahashi *et al*., 1999)
E452K	9	DI-II	PMC	Myotonia Episodic weakness No cold exacerbation clinically	Unknown	(Dupre *et al*., 2009)
F671S	12	DII/S4	SCM	Severe myotonia Generalised hypertophy and joint retraction No weakness reported	Unknown	(Dupre *et al*., 2009)
L689I	13	DII/S4-5	HyperPP	Episodic muscle weakness especially following rest after exercise Positive K challenge Pseudomyotonic discharges on EMG, no myotonic discharges	Enhanced activation Impaired slow inactivation	(Bendahhou *et al*., 2002)
L689F	13	DII/S4-5	SCM	Myotonia with warm up No response to cooling	Unknown	(Trip *et al*., 2008)

I693T	13	DII/S4-5	PMC	Cold and exercise induced weakness Myotonia on EMG	Enhanced activation Impaired slow inactivation	(Plassart *et al*., 1996; Plassart-Schiess *et al*., 1998; Hayward *et al*., 1999)
I693T Different kindreds				Neonatal hypotonia Cold and exercise induced myotonia and weakness		(Matthews *et al*., 2008a)
T704M	13	DII/S5	HyperPP	Episodic muscle weakness exacerbated by rest after exercise Positive K challenge	Enhanced activation Impaired slow inactivation	(Ptacek *et al*., 1991; Bendahhou *et al*., 1999a)
A715T	13	DII/S5	SCM	Cold exacerbated myotonia Myotonia worse after rest Warm up phenomenon No weakness	Unknown	(Fournier *et al*., 2006)
S804F	14	DII/S6	SCM	Cold exacerbated myotonia Severe myotonia after anaesthesia	Impaired fast inactivation	(McClatchey *et al*., 1992a; Green *et al*., 1998)
S804F different kindred			SCM – myotonia fluctuans	Fluctuating myotonia Delayed onset myotonia following rest after exercise No cold exacerbated myotonia No weakness		(Ricker *et al*., 1994)
S804N	14	DII/S6	SCM	Cold exacerbated myotonia Myotonia worse after rest Warm-up phenomenon No weakness	Unknown	(Fournier *et al*., 2006)
A1152D	19	DIII/S4-5	SCM*	Cold exacerbated myotonia No weakness	Impaired fast inactivation	(Bouhours *et al*., 2005)
A1156T	19	DIII/S4-5	HyperPP	Episodic muscle weakness following rest after exertion associated with raised	Impaired fast inactivation Slow inactivation indistinct from WT	(McClatchey *et al*., 1992a; Hayward *et al*.,

				serum K levels Muscle stiffness (hands and face) EMG myotonia		1999)
I1160V	19	DIII/S4-5	SCM – acetazolamide responsive myotonia	Fluctuating severe painful myotonia Potassium aggravated myotonia Paradoxical myotonia and warm up phenomenon No weakness Marked response to acetazolamide	Enhanced recovery from fast inactivation Slower deactivation	(Trudell *et al*., 1987; Ptacek *et al*., 1994b; Richmond *et al*., 1997)
V1293I	21	DIII/S6	SCM*	Cold exacerbated myotonia Occasional myotonia after heavy exercise No weakness	Enhanced activation Accelerated recovery from fast inactivation	(Koch *et al*., 1995; Green *et al*., 1998)
N1297K	21	DIII-DIV	SCM-severe neonatal non-dystrophic myotonia	Neonatal onset Cold induced severe myotonia and weakness Hypoxia from resp muscle myotonia Psychomotor retardation Fatal outcome	Unknown	(Gay *et al*., 2008)
G1306A	22	DIII-DIV	SCM – myotonia fluctuans	Myotonia of fluctuating severity and frequency Myotonia exacerbated by potassium, rest after exertion and anaesthesia No cold exacerbation No weakness	Impaired fast inactivation	(Lerche *et al*., 1993; Ricker *et al*., 1994; Mitrovic *et al*., 1995)
G1306E	22	DIII-DIV	SCM – myotonia permanens	Severe permanent myotonia Respiratory muscle myotonia causing episodic hypoxia and acidosis without treatment No weakness	Impaired fast inactivation Enhanced activation	(Lerche *et al*., 1993; Mitrovic *et al*., 1995)

G1306E Different kindred			SCM	Exercise, cold and potassium exacerbated myotonia No significant respiratory myotonia No weakness		(Colding-Jorgensen *et al*., 2006)
G1306V	22	DIII-DIV	SCM	Cold exacerbated myotonia or chronic myotonia (different kindreds) No weakness	Impaired fast inactivation	(Van den Bergh P *et al*., 991; McClatchey *et al*., 1992b; Mitrovic *et al*., 1995)
G1306V Different kindred			SCM	Exercise exacerbated myotonia	Impaired fast inactivation	(Lerche *et al*., 1993)
I1310N	22	DIII-DIV	SCM	Cold exacerbated myotonia Myotonia worse after rest Warm-up phenomenon No weakness	Unknown	(Fournier *et al*., 2006)
T1313A	22	DIII-IV	PMC	Cold and exercise induced myotonia Occasional cold and exercise induced paralysis	Impaired fast inactivation	(Bouhours *et al*., 2004)
T1313M	22	DIII-IV	PMC	Cold and exercise exacerbated myotonia and weakness	Impaired fast inactivation Enhanced recovery from inactivation	(McClatchey *et al*., 1992b; Ptacek *et al*., 1993; Hayward *et al*., 1996; Yang *et al*., 1994)
M1360V	23	DIV/S1	HyperPP	Episodic weakness especially early morning and following rest after exercise Clinical weakness observed following K challenge and cooling forearm muscles EMG myotonia	Impaired inactivation	(Wagner *et al*., 1997)

M1370V	23	DIV/S1	HyperPP	Episodic limb weakness Muscle stiffness face and hands when exercising in cold Positive K challenge EMG myotonia	Unknown	(Okuda *et al*., 2001)
L1433R	24	DIV/S3	PMC	Cold and exercise exacerbated myotonia Paralysis induced by exercise when muscles cooled	Impaired fast inactivation Enhanced recovery from inactivation	(Ptacek *et al*., 1993; Yang *et al*., 1996b; Yang *et al*., 1994)
L1436P	24	DI/S3	SCM	Cold and exercise exacerbated myotonia	Unknown	(Matthews *et al*., 2008b)
R1448C	24	DIV/S4	PMC	Cold exacerbated myotonia and paralysis	Impaired fast inactivation Impaired deactivation Enhanced recovery from inactivation	(Ptacek *et al*., 1992; Yang *et al*., 1994; Featherstone *et al*., 1998)
R1448H	24	DIV/S4	PMC	Cold exacerbated myotonia and paralysis Potassium aggravated myotonia following potassium challenge No potassium aggravated weakness	Impaired fast inactivation Enhanced recovery from inactivation	(Ptacek *et al*., 1992; Yang *et al*., 1994)
R1448L	24	DIV/S4	PMC	Cold and exercise exacerbated myotonia	Unknown	(Matthews *et al*., 2008b)
R1448P	24	DIV/S4	PMC	Cold exacerbated myotonia and weakness	Impaired fast inactivation Impaired deactivation	(Featherstone *et al*., 1998; Wang *et al*., 1995)
R1448S	24	DIV/S4	PMC	Myotonia Exercise exacerbated weakness	Impaired fast inactivation Impaired deactivation	(Bendahhou *et al*., 1999b)
G1456E	24	DIV/S4	PMC	Cold and exercise exacerbated myotonia Cold induced weakness	Unknown	(Sasaki *et al*., 1999)

F1473S	24	DIV/S4-S5	PMC	Cold and exercise exacerbated myotonia Cold induced weakness	Impaired fast inactivation	(Mitrovic *et al*., 1996; Fleischhauer *et al*., 1998)
M1476I	24	DIV/S4-S5	SCM**	Variable myotonia, majority of cases asymptomatic (myotonia on EMG) or mild symptoms Cold exacerbated and in some painful myotonia Rare paralytic episodes in 1/44 patients	Unknown	(Rossignol *et al*., 2007)
M1476I			SCM	Mild to asymptomatic myotonia (detected on EMG) Warm up present No weakness clinically and no CMAP decrement with SET	Unknown	(Dupre *et al*., 2009)
A1481D	24	DIV/S4-S5	SCM	Cold aggravated myotonia Myotonia profoundly worse after anaesthetic Occasional exercise exacerbated myotonia No weakness	Unknown	(Schoser *et al*., 2007)
F1490L_M1493I	24	DIV/S5	HyperPP	Episodic muscle weakness especially on waking Myotonia muscles of hands and face EMG myotonia	Enhanced slow inactivation	(Bendahhou *et al*., 2000)
I1495F	24	DIV/S5	HyperPP	Episodic muscle weakness following rest after exertion Positive K challenge	Enhanced slow inactivation	(Bendahhou *et al*., 1999a)
V1589M	24	DIV/S6	SCM	Cold and potassium aggravated myotonia Myotonia also exacerbated by anaesthetic	Enhanced recovery from inactivation	(Heine *et al*., 1993; Mitrovic *et al*., 1994)

				No weakness		
V1589M Different kindred			PMC	Cold and exercise exacerbated myotonia Subjective cold and exercise exacerbated weakness EMG reduced CMAP after repeat exercise but no CMAP decrement with muscle cooling		(Ferriby *et al*., 2006)
M1592V	24	DIV/S6	HyperPP		Impaired slow inactivation	
Q1633E	24	C-terminus	SCM	Cyanotic attacks in infancy Myotonia exacerbated by cold and potassium No paralytic attacks	Impaired fast inactivation	(Kubota *et al*., 2009)
T1700_E1703 del	24	C-terminus	PMC	Paradoxical myotonia of hand muscles Significant reduction in CMAP with muscle cooling	Unknown	(Michel *et al*., 2007)
E1702 K	24	C-terminus	PMC	Very brief symptoms,10-15secs several times a day or night	Unknown	(Miller *et al*., 2004)
F1705I	24	C-terminus	SCM	Cold induced myotonia Warm up phenomenon described No weakness	Impaired fast inactivation	(Wu *et al*., 2005)

The distinction used to classify phenotype as PMC or SCM was myotonia with the presence (PMC) or absence (SCM) of episodic muscle weakness or paralysis. Classification was based only on available published data. If episodic paralysis was the predominant/only feature the phenotype was classified as HyperPP.

* Denotes phenotypes that were originally reported as PMC but that have been classified as SCM using the above criteria.

** This phenotype was considered overall to be classified as SCM based on the lack of weakness reported in all but 1/44 individuals.

Table 5: Summary of clinical features and pathophysiological consequences of reported SCN4A mutations associated with PMC, SCM and HyperPP.

Hypokalaemic Periodic Paralysis

Only approximately 10% of cases of hypokalaemic periodic paralysis are due to mutations in the SCN4A gene(Sternberg *et al*., 2001). This less frequent genotype is sometimes referred to as HypoPP 2. The majority of cases, estimated at between 60-70%, are due to mutations in the CACNA1S gene that codes for the voltage gated skeletal muscle calcium channel Cav1.1 (the dihydropyridine receptor). Approximately 20% of cases are genetically undefined(Fouad *et al*., 1997; Miller *et al*., 2004; Sternberg *et al*., 2001). All mutations that have been reported in either gene associated with hypokalaemic periodic paralysis have been substitutions of positively charged arginine residues in the voltage sensors (S4 segments) of the channels(Ptacek *et al*., 1994a; Jurkat-Rott *et al*., 1994; Bulman *et al*., 1999; Jurkat-Rott *et al*., 2000; Bendahhou *et al*., 2001; Sternberg *et al*., 2001; Davies *et al*., 2001; Kim *et al*., 2004; Wang *et al*., 2005). In total substitutions of only 5 arginine residues have been reported in association with hypokalaemic periodic paralysis. Substitution of a sixth arginine residue has been reported as causing a potassium sensitive normokalaemic periodic paralysis (Fig 11).

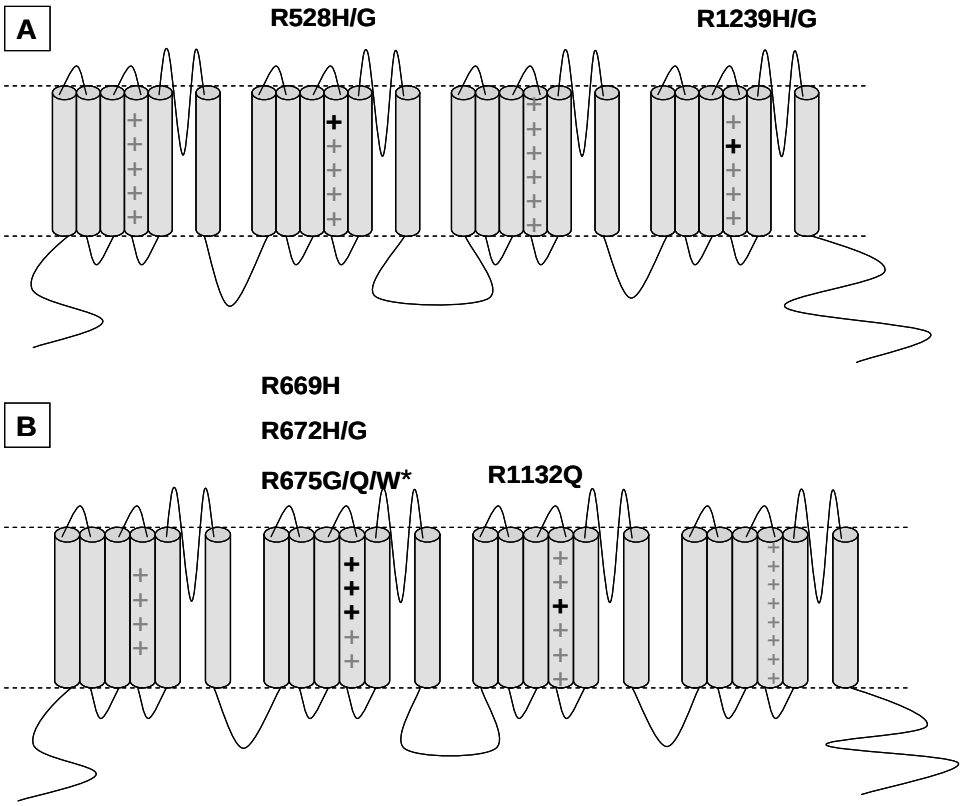


Fig 11: Voltage sensor mutations of A: Cav1.1 and B: Nav1.4 associated with HypoPP. *These mutations are associated with a different phenotype described as potassium sensitive normokalaemic periodic paralysis.

Mutation	Gene	Exon	Protein position	Reported Phenotype	Major Pathomechanism	Reference
R528G/H	CACNA1S	11	DII/S4	Hypokalaemic periodic paralysis	Slower kinetics of activation Reduced current density	(Lapie *et al*., 1996; Morrill *et al*., 1999)
R1239G/H	CACNA1S	30	DIV/S4	Hypokalaemic periodic paralysis	Slower kinetics of activation Reduced current density	(Morrill *et al*., 1999)
R669H	SCN4A	12	DII/S4	Hypokalaemic periodic paralysis	Enhanced slow inactivation Enhanced fast inactivation Reduced current density	(Struyk *et al*., 2000; Kuzmenkin *et al*., 2002)
R672G/H	SCN4A	12	DII/S4	Hypokalaemic periodic paralysis	Enhanced slow inactivation (R672G only) Enhanced fast inactivation Reduced current density	(Kuzmenkin *et al*., 2002)
R675G/Q/W	SCN4A	13	DII/S4	Potassium sensitive normokalaemic periodic paralysis	Unknown	(Vicart *et al*., 2005)
R1132Q	SCN4A	18	DIII/S4	Hypokalaemic periodic paralysis	Enhanced fast and slow inactivation	(Carle *et al*., 2006)

Table 6: Summary of pathophysiological consequences of voltage sensor mutations associated with HypoPP

The second aim of this thesis is to address whether a carefully phenotyped cohort of channelopathy patients can be 100% genotyped by exomic sequencing of known disease causing genes.

1.7 Pathogenesis of the skeletal muscle channelopathies

The skeletal muscle sodium channelopathies

The mutations in the SCN4A gene associated with PMC, SCM and HyperPP are scattered throughout the gene affecting all domains of the Nav1.4 channel (see Fig 10). The functional consequence of all these mutations is to produce a "gain of function" effect of channel activity. This is achieved either by enhanced activation or impaired inactivation of the sodium channel (see table 5).

Normally when the sodium channel is activated from its resting state by depolarization, the channel opens rapidly, allowing an influx of sodium ions through the sodium selective central pore into the cell. This rapid influx of positively charged sodium ions depolarises the cell, and produces an action potential that triggers muscle contraction. Sodium channels usually inactivate rapidly, stopping the influx of sodium ions, and, in conjunction with potassium and chloride channels, promoting a return to resting potentials (see Fig 1).

Normally a single action potential in a neuron leads to a single contraction in its target muscle fibre. Myotonia results when a sequential train of action potentials is evoked in the muscle in response to a single action potential nerve stimulus. Mutations that cause myotonia do so by delaying inactivation of the sodium channel (see Table 5 and Fig 12) which results in a hyper-excitable muscle cell. When inactivation is delayed the sodium channel is open for longer and the resulting influx of sodium ions consequently persists for longer. The persistent inward depolarising sodium current produces a comparable increase in the outward repolarising potassium current which leads to a greater accumulation of potassium ions in the t-tubules. This accumulation of positive charge

makes the sarcolemma susceptible to further spontaneous depolarisations with accompanying muscle contraction.

In contrast to the hyper-excitable sarcolemma associated with myotonia the episodes of flaccid muscle paralysis experienced in the sodium skeletal muscle channelopathies reflect an unexcitable sarcolemma. This occurs due to incomplete inactivation of the sodium channel (see Table 5 and Fig12). Once the sodium channel has opened there is large persistent inward sodium current due to the inability of the channel to close. This persistent current leads to sustained depolarisation of the sarcolemma and an inability to support any further action potentials.

The key difference between these two situations is that in the unexcitable state, the sodium channels fail to shut, preventing the cell from repolarising, while in the hyper excitable state the channels do inactivate completely, only more slowly than normal.

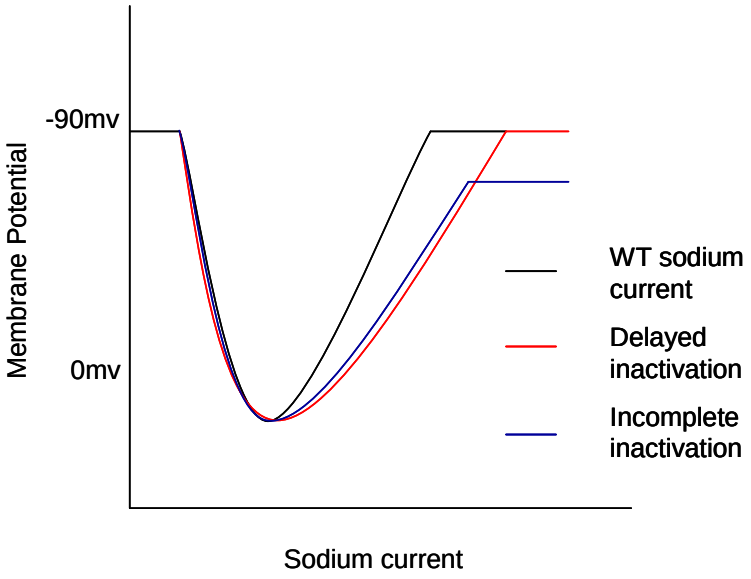


Fig 12: Diagrammatic representation of impaired inactivation of Nav1.4 in PMC and HyperPP.

Hypokalaemic Periodic Paralysis

Hypokalaemic periodic paralysis is due to dysfunction of two voltage gated skeletal muscle ion channels, the sodium channel Nav1.4 and the calcium channel Cav1.1. These channels have similar structures and it has been noted that the only mutations shown to cause the hypoPP phenotype are substitutions of arginine residues in the voltage sensors of both channels(Cannon., 2006). In contrast to the myotonic phenotypes all of these mutations were initially thought to cause a "loss of function" effect on channel gating (see Table 6).

Attacks of paralysis occur in conjunction with reduced serum potassium levels in hypoPP. In vitro studies of muscle fibres from individuals affected by hypoPP have been shown to paradoxically depolarise when placed in low potassium solution (in contrast to muscle fibres from normal controls which hyperpolarise)(Ruff., 1999; Rudel *et al*., 1984). Early functional studies of the voltage sensor mutations demonstrated reduced current density and relatively small shifts in the voltage dependence of inactivation(Struyk *et al*., 2000; Kuzmenkin *et al*., 2002) suggesting that a loss of channel function may be important. However, this mild loss of function did not explain the paradoxical depolarization seen in native muscle and did not explain how the episodes of paralysis in lowered extracellular potassium were triggered.

The gating pore in hypokalaemic periodic paralysis

Recently studies examining the role of the S4 voltage sensors of the sodium channel in more detail revealed a potential additional pathomechanism for hypoPP. The S5 and S6 segments of each of the four domains of the sodium channel fold together to line the sodium selective central, or 'alpha' pore of the channel. In response to a depolarising stimulus the S4 segments undergo a conformational change moving outwards from the sarcolemma. This movement is coupled to the opening of the alpha pore that is lined by the S5/6 segments (see Fig 6).

The S4 segment itself however contributes to a second pore known as the omega pore, which is formed by its interaction with the surrounding S1, S2 and S3 segments (Fig 13). The S4 segments have an abundance of positively charged amino acids, either an arginine or a lysine occurring at every third position in the segment. The total number of positive

charges varies for each S4 segment. The omega pore is not uniform in diameter and the narrowest portion is occupied by different positively charged residues depending on whether the membrane is resting (S4 down) or depolarised (S4 up).

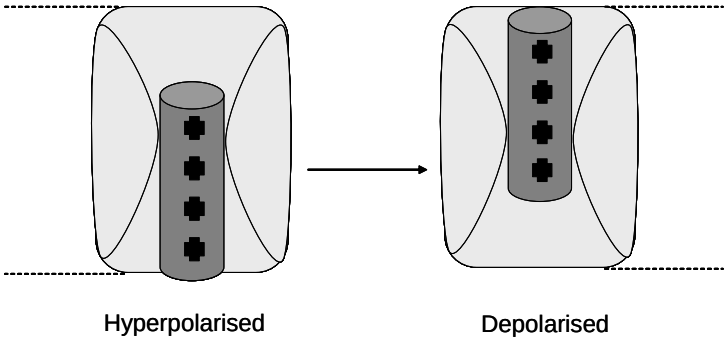


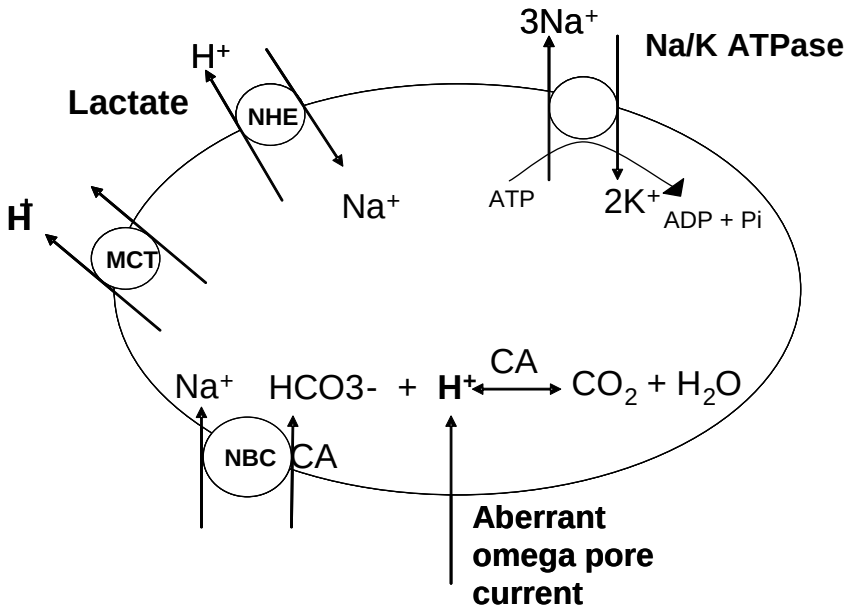
Fig 13: The movement of the S4 segment through the omega pore

Under normal conditions the charged residues in the S4 segments form salt bridges with other segments which effectively block any free ions from flowing through the omega pore. Recent work has shown that neutralisation of either of the two outer arginine residues in the DII/S4 segment of SCN4A has led to a loss of integrity of the omega pore and allows monovalent cations to "leak" through the omega pore and into the cell when the cell is hyperpolarised.

It is known that the amino acid that is substituted for the arginine residue affects the size of this leak. If the arginine is replaced by a histidine, a proton leak through the omega pore is favoured. Any other amino acid substitution at this site allows less selectivity and a greater variety of monovalent cations can pass including sodium(Sokolov *et al*., 2007; Struyk *et al*., 2007).

While the omega pore leak has been hypothesised as an additional pathomechansim in hypoPP the precise consequences of this "gain of channel function" on muscle cell homeostasis and sarcolemmal excitability are not yet fully understood. It is suggested that movement of protons and other ions including sodium via the gating pore may disrupt pH homeostasis and lead to intracellular sodium accumulation via activation of several ion transporters including the sodium-hydrogen anti-port exchanger (see Fig 14)(Struyk *et al*., 2008b; Jurkat-Rott *et al*., 2009). However, the cause of the paradoxical membrane depolarisation in low potassium solution and the stimulus for the low serum potassium

itself that occurs in HypoPP remains less clear although possible mechanisms are discussed.



MCT: monocarboxylate transporter **NHE:** Sodium hydrogen anti-port exchanger
NBC: sodium dependant bicarbonate transporter **CA**:carbonic anhydrase

Fig 14: An aberrant current permeable to protons could stimulate the NHE and NBC transporters with a resultant increase in intra-cellular sodium ions. Stimulation of the monocarboxylate transporter would result in increased lactate efflux from the cell which has been proposed as contributory to vacuolar formation in HypoPP.

A further aim of my thesis was to examine the functional consequences of Nav1.4 hypoPP mutations in light of the recently described gating pore current.

Role of inward rectifying potassium channels in hypokalaemic periodic paralysis

Muscle fibre resting membrane potential ($V_{REST)}$ is controlled by the membrane permeability to K^+ ions. It has been suggested that inhibition of the outward component of the inward rectifying potassium channels could account for the abnormal membrane response to low serum potassium and also for the lowered serum potassium itself due to intracellular accumulation of potassium(Hofmann *et al*., 1970; Ruff., 1999). Furthermore, barium which blocks inward rectifying potassium channel current(Standen *et al*., 1978) produces reduced twitch force in the skeletal muscles of mammals in vitro in low potassium solution.(Gallant., 1983) Reduced ATP dependant potassium channel (a subgroup of IRK channel) current has also been identified in vitro from muscle biopsies of hypoPP patients.(Tricarico *et al*., 1999) The aberrant inward depolarizing gating pore current has recently been shown to contribute to the probability of the membrane paradoxically depolarising in the presence of low potassium solution and also to the reduced outward current component of the IRK channels.(Jurkat-Rott *et al*., 2009; Struyk *et al*., 2008a) However, it is not clear why the potassium conductance should be reduced by dysfunction of the voltage sensors of Cav1.1 or Nav1.4. One possible explanation is that a proton selective gating pore causes an acidic intracellular environment and IRK channels are known to be inhibited at this pH(Struyk *et al*., 2008a).

1.8 Treatment of the skeletal muscle channelopathies

Some patients with the skeletal muscle channelopathies feel their symptoms are not restrictive enough to take medication and will manage their illness by avoiding precipitating factors as far as possible. When drug therapy is considered a number of therapeutic agents exists.

Myotonic disorders: early treatments

Where treatment is considered numerous membrane stabilising agents have been employed in the myotonic disorders. These are currently mainly use dependant blockers of sodium channels e.g. anti-convulsants, local anaesthetics or anti-arrhythmics. As these

target persistent sodium currents they are ideal to inhibit the abnormal sodium channels with impaired inactivation seen in PMC, SCM and hyperPP but have little or no effect on the functioning of wild type sodium channels.

In early studies, procainamide, quinine and glucocorticosteroids were employed. A small randomized double blind trial compared the efficacy of each of these treatments in relation to placebo in 20 individuals with myotonic disorder (16 myotonic dystrophy, 4 myotonia congenita). The diagnosis was made on a clinical basis without genetic confirmation. The trial lasted 12 weeks and all participants received each of the four treatments for a 3 week period with no washout period. An end-point of at least a 50% reduction in the duration of hand grip myotonia, measured by EMG and timed clinically, was employed. Using this endpoint, 6/20 participants taking quinine, 15/20 taking procainamide, 15/19 taking prednisone (one patient did not receive prednisone) and 0/20 taking placebo showed improvement(Leyburn *et al*., 1959). This study, although imperfect, illustrated a low efficacy of quinine. Despite the suggested benefits of procainamide and prednisone the side effect profile of both these drugs restricts their use and they are no longer recommended as therapeutic agents in the non-dystrophic myotonias.

The carbonic anhydrase inhibitor acetazolamide is commonly used in the periodic paralyses and has been reported to be beneficial in the non-dystrophic myotonias.(Trudell *et al*., 1987; Ferriby *et al*., 2006) In a small series of nine patients with myotonia, seven diagnosed clinically with myotonia congenita and two with paramyotonia congenita, all cases reported a subjective and objective (timed measurements of myotonia) improvement in myotonia with acetazolamide. However, one individual with paramyotonia congenita developed quadriparesis 12 h after the ingestion of acetazolamide.(Griggs *et al*., 1978) Larger studies of acetazolamide use in the non-dystrophic myotonias have not been performed, and while there is evidence of some benefit, it is not generally considered as a first line agent for the treatment of myotonia.

The anti-convulsant phenytoin has been shown to improve the righting time of myotonic mice turned onto their backs.(Aichele *et al*., 1985) Ricker et al.(Ricker *et al*., 1978) reported subjective improvement in muscle stiffness and an improved timed walk in one patient with myotonia congenita and a dose dependant improvement in isometric force in

another. The lidocaine derivative tocainide gave encouraging results initially(Rudel *et al*., 1980; Streib., 1987) but was eventually withdrawn from the market due to the risk of potentially fatal agranulocytosis.(Volosin *et al*., 1985) Synthesis of tocainide analogues has been attempted in vitro and may be of value for future study as anti-myotonic agents if the efficacy and side effect profiles are favourable.(Catalano *et al*., 2008)

Myotonic disorders: Class I anti-arrhythmics

More recently class I anti-arryhthmics have offered potential for treatment. Flecainide, a class Ic anti-arrhythmic, has been shown to be effective in vitro(Aoike *et al*., 2006) although its use in clinical practice as an anti-myotonic agent is rarely reported.(Rosenfeld *et al*., 1997) An improvement in clinical symptoms and cold induced EMG findings with propafenone, another class Ic anti-arrhythmic has been reported in a single case of paramyotonia congenita.(Alfonsi *et al*., 2007)

The class Ib anti-arrhythmic mexiletine is generally considered to be the first-line treatment of choice by myologists but a randomized controlled trial is required. It is usually well tolerated with only minor side effects reported. Importantly it has proarrhythmic potential and therefore pre- and post-treatment ECGs are essential to ensure satisfactory QT interval. More extensive cardiac evaluation prior to commencement is important if there is an abnormal baseline ECG or a history of cardiac disease. Single case reports have shown that mexiletine is effective in treating myotonia in both sodium and chloride channel disorders.(Ceccarelli *et al*., 1992; Jackson *et al*., 1994) However, a recent Cochrane review highlighted the lack of adequate randomized double blind placebo controlled trials to prove efficacy.(Trip *et al*., 2006)

The ability to conduct such trials is partly hampered by the difficulty in quantitating myotonia(Torres *et al*., 1983; Hammaren *et al*., 2005; Logigian *et al*., 2005; Moxley, III *et al*., 2007; Hogrel., 2009) and in recruiting adequate numbers of patients to achieve statistical power. A recent study employed trunk sway analysis to measure the warm up phenomenon in recessive myotonia congenita and proposed that with further evaluation this may offer an alternative potential end-point for therapeutic trials.(Horlings *et al*., 2009) Sodium MRI has also recently been proposed as a possible outcome measure in

patients with sodium channel diseases. An increase in intramuscular sodium content was demonstrated to accompany muscle weakness following exercise of cooled muscles in paramyotonia congenita. In a small group of patients this increase was significantly reduced following treatment with mexiletine.(Weber *et al*., 2006) It is possible this technique could be used to monitor response to treatment in both a clinical and research setting.

In vitro studies continue to identify pharmacological agents that preferentially block sodium channels in the open state, thereby targeting persistent sodium currents.(Wang *et al*., 2008b; Wang *et al*., 2008a) These studies may identify future therapies. No safe drugs are currently available which specifically act on the ClC-1 channel. A number of experimental approaches may have future implications for the treatment of myotonia congenita including oligonucleotides to promote exon skipping, trans-splicing and restoration of normal protein trafficking.(Cleland *et al*., 2008)

Hypokalaemic periodic paralysis: potassium supplements and potassium sparing diuretics

In the 1930s it was recognised that lowered potassium levels were associated with attacks of paralysis(Biemond *et al*., 1934) prompting treatment of these episodes with potassium supplements and dietary modification to limit carbohydrate intake. It was realised however that although often effective in aborting or reducing the duration and severity of paralytic attacks, potassium supplementation had no effect on their frequency or on the development of a permanent muscle weakness.

In the late 1950s Conn performed extensive experiments on 2 patients with hypokalaemic periodic paralysis and reported that the attacks of paralysis were not only associated with hypokalaemia but also hypernatraemia. He proposed that it was even possible the hypernatraemia was the primary stimulus for the paralytic attacks with hypokalaemia being secondary(Conn *et al*., 1957). While he did not suggest hyperaldosteronism was the cause *per se* he did identify raised aldosterone levels during attacks in his patients. However others did not corroborate these findings(Poskanzer *et al*., 1961). Conn suggested a low sodium diet was beneficial in reducing both paralytic attacks and

myopathy. He also proposed carbonic anhydrase inhibitors may be effective due to their ability to increase sodium excretion in the urine. In practical terms the restrictive sodium diet proposed by Conn was difficult for patients to tolerate and others did not find sodium restriction to be as beneficial(Poskanzer *et al*., 1961).

Based on his research however spironolactone, a known inhibitor of aldosterone, was trialled as therapy for hypokalaemic periodic paralysis. In the initial patient studied, subjective and objective observations of not only reduced paralytic attacks but also improved inter-attack muscle strength were noted. This treatment with spironolactone did produce a slight increase in sodium excretion with normal potassium serum levels being maintained but the authors argued the effects on sodium and potassium balance were too minimal to adequately explain the full mechanism of action of spironolactone in producing such significant symptom reduction in hypokalaemic periodic paralysis(Poskanzer *et al*., 1961). Spironolactone and other potassium sparing diuretics are still used today as therapy for hypoPP although often not as first line agents(Kim *et al*., 2001; Bendahhou *et al*., 2001; Kim *et al*., 2005; Kim *et al*., 2007). Gynaecomastia can limit the tolerability of spironolactone for male patients.

Carbonic anhydrase inhibitors

Acetazolamide was first used in 1962 to lower the elevated potassium levels associated with paralytic attacks in hyperkalaemic periodic paralysis(McArdle., 1962). A few years later, despite seeming paradoxical in terms of potassium balance, it was also noted to be an effective prophylactic agent in hypokalaemic periodic paralysis(Resnick *et al*., 1968). An observational study further suggested acetazolamide may improve inter-attack muscle strength in some patients(Griggs *et al*., 1970). Although no randomised controlled trials have been performed using acetazolamide a plethora of case reports and series support its beneficial effects (see Table 13 in Results). Another carbonic anhydrase inhibitor, dichlorphenamide is the only therapy for hypoPP to have undergone a randomised double blind placebo controlled cross over trial. The results of this trial showed a significant efficacy of dichlorphenamide in reducing attack frequency although the inclusion criteria were based on clinical diagnosis of hypoPP and not genetic confirmation(Tawil *et al*.,

2000; Sansone *et al*., 2008). Others have reported a benefit of dichlorphenamide on inter-attack weakness(Dalakas *et al*., 1983). There are no trials comparing the use of dichlorphenamide to acetazolamide in periodic paralysis.

Acetazolamide rapidly became the treatment of choice for hypokalaemic periodic paralysis, and almost half a century later remains the frontline treatment. However with genetic advances and the discovery of two causative genes it was noted that some patients did not respond to acetazolamide treatment or indeed found it to be detrimental(Bendahhou *et al*., 2001; Sternberg *et al*., 2001). It was initially suggested that it was only the minority of patients with hypoPP 2 due to mutations in SCN4A who reacted adversely to acetazolamide therapy but other reports of hypoPP 2 patients who benefited from acetazolamide refuted this(Bulman *et al*., 1999; Kim *et al*., 2004; Venance *et al*., 2004). Despite its popularity as a therapeutic agent for hypokalaemic periodic paralysis the disease specific mechanism of action of acetazolamide is not understood. Importantly despite some suggestive case reports it is not established in either the myotonic disorders or hypokalaemic periodic paralysis if currently available treatments have any influence on the development of permanent myopathy.

In my thesis I attempt to quantify the efficacy of acetazolamide in hypokalaemic periodic paralysis and whether genotype has any influence on treatment response.

1.9 Mechanisms of action of acetazolamide

Carbonic anhydrase inhibition

Carbonic anhydrase is an enzyme that catalyses the reversible reaction converting carbon dioxide and water into protons and bicarbonate.

$$CO_2 + H_20 \longleftrightarrow H2CO3 \longleftrightarrow H^+ + HCO_3$$

Acetazolamide is a sulphonamide that inhibits carbonic anhydrase (CA) and it is generally considered that its main therapeutic mechanism of action in hypoPP is

somehow linked to this capacity. Inhibition of CA in the renal tubules by acetazolamide leads to increased urinary loss of bicarbonate, sodium and some potassium with a resultant metabolic acidosis (Fig 15). It remains unclear how this alteration of pH could reduce paralytic attacks or prevent the development of myopathy. One study did examine the effect of pH on the R669H SCN4A mutation and pH on the R672H/G SCN4A mutations expressed in a HEK cell system. The deleterious effects of the histidine substitutions could be ameliorated by a more acidic pH whereas the glycine substitution was insensitive to alterations in pH. The authors proposed this may predict that those hypoPP patients with a glycine substitution would not benefit from acetazolamide (Kuzmenkin *et al*., 2002).

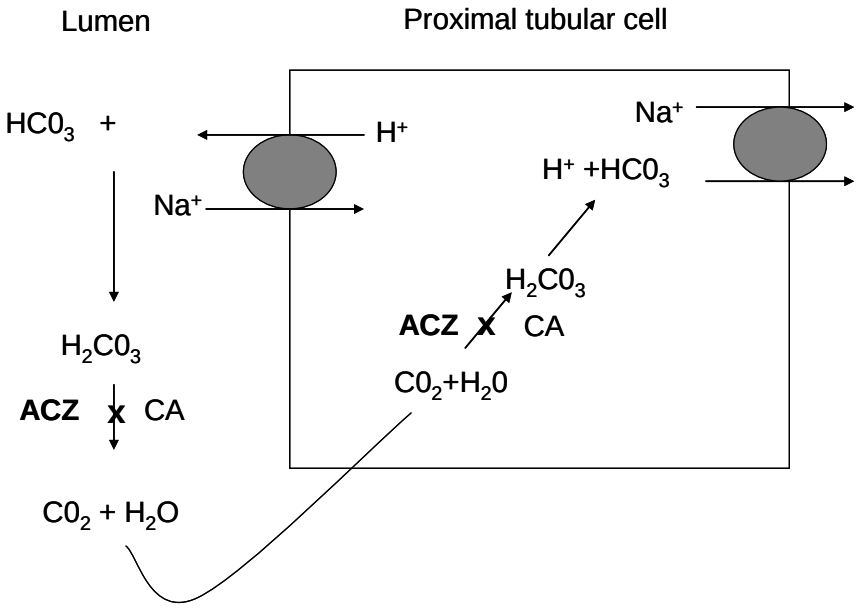


Fig 15: Acetazolamide(ACZ) inhibits the carbonic anhydrase(CA) present in the tubular lumen preventing the conversion of H_2CO_3 to CO_2 and H_2O. H_2CO_3 dissociates to H^+ and HCO_3. Bicarbonate is lost in the tubular lumen producing a metabolic acidosis. Within the proximal tubular cell itself ACZ blocks the CA preventing the conversion of CO_2 and H_20 to H^+ and HCO_3. The reduction in protons reduces the re-absorption of sodium ions which are excreted in the urine.

Carbonic anhydrase isoenzymes

At least 14 isoenzymes of CA exist in humans. Different isoenzymes are preferentially expressed in the cytosol, cell membranes or mitochondria of different tissues. Each isoform demonstrates a different degree of catalytic activity and affinity for sulfonamides.(Clare *et al*., 2006; Supuran *et al*., 2000)

The subcellular localisation of several isoforms has been studied in animal models and shown to be present at variable levels in the sarcolemma and sarcoplasmic reticulum of skeletal muscle(Wetzel *et al*., 1998; Wetzel *et al*., 2007; Scheibe *et al*., 2008). The presence of different isoforms of CA in skeletal muscle raises questions about how the contribution of each isoform may influence CA treatment response and how this might be influenced by the proposed proton permeable gating pore. Intracellular isoforms of CA can only be reached by the lipophilic membrane permeable sulphonamides. Hydrophilic sulphonamides such as acetazolamide or the alternative carbonic anhydrase inhibitor dichlorphenamide, which is also used in hypoPP, cannot easily cross the sarcolemma and would not be expected to have any significant direct effect on the intracellular isoforms. This suggests any benefit derived from acetazolamide relies on inhibition of extracellular carbonic anhydrase or carbonic anhydrase on the extracellular surface of the membrane.

Acetazolamide and activation of sarcolemmal calcium activated potassium channels [K_{Ca2+}]

Tricarico et al used the potassium depleted rat as an animal model of hypoPP to explore the hypothesis that the mechanism of action of acetazolamide and other carbonic anhydrase inhibitors was not exclusively related to their inhibition of CA(Tricarico *et al*., 2004). A dose dependant increase of calcium activated potassium channel [KCa^{2+}] activity and restoration of the serum potassium levels to within the normal range was observed in the muscle fibres of potassium depleted rats in whom treatment with acetazolamide prevented insulin induced attacks of paralysis(Tricarico *et al*., 2000; Tricarico *et al*., 2004). The ability of acetazolamide to enhance the sarcolemmal conductance of potassium seems particularly relevant when considered in light of the studies discussed that implicate inhibition of IRK channel conductance in the pathomechanism of hypoPP.

Effects of acetazolamide on inter-attack weakness

The reported clinical benefit of acetazolamide in preventing or improving inter-attack weakness has also been explored using the K depleted rat model. Vacuoles are a common morphological finding in primary and secondary hypokalaemic periodic paralysis. They are considered to represent localised swelling and vacuolation of the t-tubules secondary to increased osmolarity caused by the local accumulation of ions or metabolites (including lactate). The rat model demonstrated a vacuolar myopathy and an increased efflux of lactate from muscle fibres in vitro. Muscle biopsies from rats treated with acetazolamide demonstrated significantly reduced vacuoles and lactate efflux(Tricarico *et al*., 2008).

These observations are particularly interesting when considered in light of the proposed proton leak described in hypoPP. Accumulation of intracellular protons could produce an increased efflux of lactate by stimulating the proton linked monocarboxylate transporter (Fig 14). Potentially this pathway may partly explain the reports of acetazolamide ameliorating inter-attack muscle weakness.

1.10 Mechanisms of muscle degeneration in the skeletal muscle channelopathies

Patients with periodic paralysis have been frequently reported to exhibit vacuoles and/or tubular aggregates on muscle biopsy(Venance *et al*., 2006) and as discussed there is some evidence suggesting lactate efflux may contribute to this. However, the myopathological findings in NDMs are not defined well and often reported to be non-specific(Miller *et al*., 2004). Furthermore, with the characteristic clinical history and examination findings coupled with the recent advances in electrophysiological techniques a diagnosis of NDM is usually apparent and it is now rare that a muscle biopsy will be performed in such patients other than as a research procedure.

It is clear muscle damage can occur in the NDMs but its pathomechansim and frequency are unknown. It has been postulated that the abnormally prolonged intramuscular influx of sodium that is known to occur via the mutant sodium channels may be responsible for muscle degeneration(Bradley *et al*., 1990) but the specifics of such a mechanism have not

been shown. However, it is of note that there is evidence for increased intracellular sodium contributing to cell necrosis in the mouse model of Duchenne muscular dystrophy(Hirn *et al*., 2008). One recent study has employed ultrasound to assess permanent muscle changes in the NDMs. Using ultrasound measurements of eight muscles, (four upper limb and four lower limb) in a group of 63 genetically confirmed NDM patients an increase in the mean echo intensity compared with controls from all muscles examined except the rectus femoris was observed. The ultrasound changes were considered to indicate structural muscle damage such as fatty infiltration or fibrosis. This change was most marked in the forearm flexors where the increased echogenicity correlated negatively with muscle power. There was no positive correlation between echo intensity and age for individual muscles except the rectus femoris although the sum of the scores did show a significant positive correlation(Trip *et al*., 2009b).

Recently, a mouse model of hyperkalaemic periodic paralysis has been engineered by introducing the murine equivalent of the SCN4A mis-sense mutation M1592V(Hayward *et al*., 2008). This mutation causes both myotonia and paralysis in humans(Rojas *et al*., 1991; Kelly *et al*., 1997) and was demonstrated to produce these same symptoms in the mouse verifying its use as a model of the human disease. At a few months of age the mice heterozygous for this mutation already displayed subtle myopathic changes. In those that were homozygous significant muscle abnormalities were seen including an increase in fibre size variability, frequent internal nuclei and large scattered vacuoles. These early changes were present at a few months of age before any spontaneous episodes of paralysis had been observed. Furthermore, they were shown to increase with age in the heterozygotes while muscle force generation declined(Hayward *et al*., 2008). This supports the clinical observations that myopathy increases with age in humans(Links *et al*., 1990; Plassart *et al*., 1994) and may be independent of paralytic attacks in the periodic paralyses(Buruma *et al*., 1978; Links *et al*., 1990).

Although this animal model is of a hyperkalaemic periodic paralysis genotype the same symptoms of myotonia and muscle weakness occur in the allelic disorders PMC and SCM. The pathomechanism is also a gain of function of the sodium channel in both. It is likely that future insights into muscle degeneration gained from the study of this model will also have implications for our understanding of PMC and SCM. The possibility that

myopathy develops independently of symptom frequency or severity may influence future approaches to therapy which is currently aimed at relieving symptoms. As such, many patients with minimal or manageable symptoms decline pharmacological treatment.

1.11 Morbidity in the skeletal muscle channelopathies

Very little is known about the impact of the muscle channelopathies on quality of life and these disorders have often been regarded as benign. A single study has recently examined this in a group of 62 genetically confirmed NDM patients and found painful myotonia and fatigue to be the best predictors of poor general health perception and physical functioning(Trip *et al*., 2009a). In this study painful myotonia was reported in 28% of those with myotonia congenita and 57% with sodium channelopathy. In addition, there are numerous case reports where pain which is often severe, is described in the NDMs(Vicart *et al*., 2004; Rosenfeld *et al*., 1997; Ptacek *et al*., 1994b; Colding-Jorgensen *et al*., 2006; Fialho *et al*., 2007; Walsh *et al*., 2007; Wang *et al*., 2008c). This suggests that pain is a frequent symptom that may have been previously under-recognised and possibly undertreated in the NDMs.

1.12 Summary of Aims

The specific aims of this thesis were:

1. To examine the phenotype of the skeletal muscle channelopathies in patients referred to the NCG service for channelopathies at the NHNN to determine
    a. if there were any clinically useful phenotype-genotype correlations that could be made and
    b. if there were more extensive aspects to the phenotypes than is currently recognised.

2. To genetically characterize patients to assess if new mutations in known genes existed and if these genes could account for 100% of a carefully phenotyped cohort or if new genes may be implicated.

3. To ascertain the efficacy of acetazolamide therapy in hypokalaemic periodic paralysis and if genotype has any influence on treatment response.

4. To functionally characterize mutations in hypokalaemic periodic paralysis in light of the recently described gating pore seen in some of the Nav1.4 mutations.

Methods

2.0 Examining the phenotype of the skeletal muscle channelopathies

Information on the phenotype of patients with skeletal muscle channelopathies was examined from several sources. Where possible, patients were seen and examined personally through the specialist neuromuscular clinic at the MRC Centre for Neuromuscular Diseases (under the government funded NCG scheme for rare diseases) when they attended for clinical care or when they participated in a natural history trial of non-dystrophic myotonias. The natural history trial was a collaborative effort between the MRC Centre for Neuromuscular Diseases and five other American and Canadian centres. All participants consented to the study which had ethical approval from local ethics committee and included DNA analysis to allow correlation of genotype with any emergent phenotypic features. Data collected included history of illness and symptoms, relevant past medical history, family history and drug therapy. Patients were examined for the presence of myotonia, and muscle strength was recorded by Elizabeth Dewar (senior physiotherapist) using standardised tests. All patients seen at the centre also underwent electrophysiological testing with Dr Veronica Tan including standard NCS, EMG and specialised short and long exercise tests. All patients enrolled in the natural history trial also completed quality of life questionnaires.

The majority of patients were personally examined by me, e.g. all of the participants in the natural history trial, the English adults detailed in the neonatal phenotypes and all others who attended the clinic who have been described in this thesis. When it was not possible to personally examine individuals (e.g. the family in Australia, the individuals with inflammatory biopsies) information was obtained from a clinical and genetic database of all those referred to the diagnostic NCG service. In selected cases the referring clinician was contacted to request supplemental information and in some cases, a muscle biopsy and DNA samples. Muscle biopsies were reviewed with Dr Janice Holton (consultant pathologist). DNA samples were analysed as detailed in the following sections. Data gathered on patients seen during the thesis was added to the clinical and

genetic database held at the MRC Centre for Neuromuscular Diseases. A record was kept of all unusual phenotypes and endeavours made to obtain complete details as above.

2.1 Gene Sequencing

Direct automated sequencing of all 24 exons of SCN4A, 44 exons of CACNA1S and one exon of KCNJ2 was carried out for all patients with available DNA and no pre-existing genetic diagnosis. Selected samples were additionally analysed for the presence of the quadruplet nucleotide repeat expansion in intron 1 of the ZNF9 gene associated with myotonic dystrophy type 2 (DM2). A small number of samples had direct automated sequencing performed of all exons of CLCN 1 associated with myotonia congenita and the RYR 1 gene. These two genes were analysed by the diagnostic labs at the NHNN and GOSH.

Primer Design

KCNJ2 primers had been previously designed and optimised by the diagnostic genetic laboratory at the NHNN. Aliquots of these primers were kindly given to me by the lab. Primers for SCN4A and CACNA1S were designed and PCR conditions optimised as follows.

Gene sequences were identified using the Ensemble database: http://www.ensembl.org/index.html. Using these sequences forward and reverse primers were designed for each exon using the Primer 3 web-based programme: http://frodo.wi.mit.edu/primer3/. Optimal primers were designed to begin within approximately 100 base pairs from the beginning or end of each exon and to produce a fragment size of approximately 450 base pairs. Primers were then blasted against the human genome to ensure specificity using another website: http://genome.ucsc.edu/cgi-bin/hgPcr?org=Human&db=hg18&hgsid=90948004. Any non-specific primers were redesigned. Finally all primers were analysed for the presence of single nucleotide polymorphisms (SNPs) using:http://ngrl.man.ac.uk/SNPCheck/SNPCheck.html. Any primers containing SNPs were also re-designed. Once optimised, primers were tagged with M13 universal primer sequences before ordering from SIGMA.

Exon	Forward Primer	Reverse Primer
M13	TGTAAAACGACGGCCAGT	CAGGAAACAGCTATGACC
Ex1	GCACATCTCCCAGTCCTGAT	TGGATGGCAGACAGACAGAG
Ex2_3	CTCTGTCTGTCTGCCATCCA	CCACACAGAGGTGCAAACAC
Ex4	GCCACCTGAGTGGCATATTT	GGGCTGCCTCATGTGATTAT
Ex5	AGGAGCTTTGGGGGTGTCT	ATCTGCCCTCTGGTCACG
Ex6	CAAGCCTTGAAGATGGAGTAGG	GAGGCTACCCTAGGGACTGG
EX7_8	GTGTCCATGTGGGTGACTTG	CCAGCTGGAAGAGGTTCTCC
Ex9	ATGTATGGAAAGGGGCACTG	ACCCTGGGTCCTCTATCTCC
Ex10	CTTTGGTCCCTACCCTGTCA	TCCTCCTGAATCCAGTCCAG
Ex11	GCTTGGAAGGTGGAACAAAA	CATACAGCCAGACAGCTCCA
Ex12	CTCTGTGACAGGGCCTCATG	TCCTCACCCCACCCCCATCC
Ex13	CTGCCTTGGGTGGTGGTC	TTGGGGAGATAGAAAACAGTCA
Ex14a	TGCCCAACATGTGGTAGTTT	GCGGCTCCTTCTTCTCATC
Ex14b	CCCAAGGACATCATGCTCA	GCTCCAGGTCACAGGAGAAT
Ex15	AGGGCTGGATGAATGAATGA	GCTCCAAGCTAGGTCTGCAC
Ex16	TGTTGGATCCAGCTGTGAAG	CCCTTCCTGTGTGTGGAGAC
Ex17	ATGGCAATTTGCTGTTGTTG	TGACAGCCTCTGGATGTAGC
Ex18	GTGCCAGGCTCCTACAGGT	CTCTGGTGGTGGTTGGAGTA
Ex19	AGGAAGCCCTCCCTAATTGA	AGTGACACTGGGGTTGGGTA
Ex20	GTTGTACCCAACCCCAGTGT	CTGAGGGCAGGACCCATC
Ex21	CGTTCTGTCCCCGAGACTT	GGGTTTGTGCAATGGAGAGT
Ex22	GCTGCCTTAAAGGTGAGGT	GGGTGGAAGGCAGGAAAC
Ex23	CTGCCTGTCCATGTCCTGT	GTCTTCCCGAGTGCCTCA
Ex24a	CTCTGAGACTTGAGCAGAGCAC	TCTCGAACAGGCAGATGATG
Ex24b	TTGCCTACGTCAAGAAGGAGTC	AAGTCTGAGAGGCGGCTGTA
Ex24c	TCTCCTTCCTCATCGTGGTC	ACCTCCTCGTGCTTCCTCTT
Ex24d	AAGCAGACCATGGAGGAGAA	AGGCACAGTCCCAGATTCAA

Table 7: SCN4A Primer sequences

Exon	Forward Primer	Reverse Primer
M13	TGTAAAACGACGGCCAGT	CAGGAAACAGCTATGACC
1	GGCTGGGTGGAATGACAG	TGTGATCACCCCGAATCC
2	GGCATCAGAGTCACAGTCCA	GACCAATGAGTGCGTGCTTA
3	TTCCATCAGCAAGTGCAAAC	TGATCCACTCTCCACCAACA
4	TATTTGCCGTCTCTCCCCTA	TAGGAAGGGGACCCAGAACT
5	AGTTCTGGGTCCCCTTCCTA	CCAGGAGAAACCCATTCTCA
6	TTCACTGTCACCGTCTGCTC	GTGGTCACGCAAGTCAGAGA
7	CAGGGTTGGGTGGAGTCTTA	AATTGAAGGGCTCCTGTGTG
8	GGTGAGCAGGAAGTGAGAGG	ACCATTTTGAGCCATTTTGC
9	TGGGCTCTTTAGCTCTTGGA	GTCCCTCAGAGCAGGAACTG
10	GCCTGGAGTTTGTCTGAAGG	GGGTATGTTGCTTTGCCACT
11	GGGAGTCAGGAGAAGGGAAG	AACCTGCACAGATCCCAGAC
12	CCCTCCCTCTGTGTCACATC	CCCACCTTGATCTTGAAGGA
13	AGTAACAGGCGTGAGCCACT	GGGCCTGGCTACCTAGAAAC
14	TGAGGGCCTTCCACTATGTC	CCAGAAGGCCAACTTGTCTC
15	GCCCTCTCACCACAGAGAAG	AGGAGTCCCTGGAGATGGAT
16	CCAAGCAGTGGGTATGTGAA	ATGGAGGGGTACAGGTAGGG
17	CAACATGCAGCCACAAAGTT	AGATGAGAGCCGCATCAATC
18	GGAGCAGGAGGTGTATTCCA	CAGCATAAAGCAGGCAGTCA
19	CTTCACCCTCTCTGCCACTC	TGCCAGTCTCCACCTCTTTT
20	TGTTCTCCAAGCTCCTCCAT	CTGTGGGTGGCTAGAAGCTC
21	GGAAGGTGGGAGTGTTTTCA	AGACTTGCTGCCTCCTGATG
22	AGGAATGGGAGGGGTTACAG	GGCTCCTTGTGCTTGAGAGT
23	GAGCTGTGGGAATGAACGAT	AGCCTAAAGGCTGAGCTTCC
24	AGGAGGGACACTCACAGAGG	AGGAGGGACACTCACAGAGG
25	CTTCCCAGCCTGACTCTCTG	CACCCTTAGGCCTCTCTTCC
26	TTCACAGTCCTCAGCCACAG	TCCATGTAGCACCTCAGCAC
27	CCCCTCCCCTTCTGTGTTAT	GAGCAAGTTGGGAGCAGAAG

28	AAGAGCAAAGGGGAGAGGAG	AGTTACCTCTCTGCCCAGCA
29	CCCACAGAGCTTCAGTCCTC	AAGCCGCTATATCCATGCAC
30	AGTTCCTCCATGCCACAAAC	GAGCGCCAGTCAGTGTCTTA
31	GACCCTGGAAACTGCACGTA	ATCTGACACTCCAGCCATCC
32	CCCCCACACATTGACTTACC	CAGGTCACACACCCATGAAG
33	AGGCAGGAGCAGATAGGTGA	TGGCAGCCTCTCTGGAGTAT
34	GTGTGATGTGGGTGGTTCAG	AAATGAAGGGGGAAGGAGAA
35	TCCCATATCTGAGGGAATGC	CCATCAGGTCCTCACCAGTT
36	TCAGCAATGTCACCCAACTC	TGGGGTCCTCCCTCTACTTC
37	GCCTGTGGGGAGAAAGTACA	CTGGTCCGTTCTCAGATTCC
38	CGGACCAGATCCTCCATAAA	CTTCTGGGCTTCCTTTTTCC
39	TCTCCTGGGCTGGAGAGATA	TGGTAGGGAAATGTGGGAAA
40	CCATCCTCCTTTGGCCTTAT	GCTGACATTGTCCTCCCAGT
41	TGAGGGAAAAGGATGTTTGG	TTAGCATGAATGGGCTTTCC
42	AAGCGAGCCTCTGTTGACTC	CCACCAATGAGCAAAATTCC
43	CCTGGCCTGATACTTGGAGT	CTGTTGGCCCTACCCTCTCT
44	AGAGGGTAGGGCCAACAGAG	TTTTGAGGTGGTTCCTGACC

Table 8: CACNA1S Primer Sequences

PCR Reagents and Reactions

PCR reactions to amplify each exon of SCN4A and CACNA1S were performed using a number of different reagents and conditions but the optimal methods most commonly used were:

1. A 25µL reaction contained 200ng genomic DNA, 12.5µL of Amplitaq 360 MasterMix (Applied Biosysytems), 10pmol of each primer (forward and reverse) and 9.5µL of ddnH20.
2. A 25µL reaction contained 200ng genomic DNA, 5µL of 10 x PCR buffer without MgCl2 (Applied Biosystems), 4µL of 25mM MgCl2, 5µL of 2mM dNTPs, 15pmol of

each primer (forward and reverse), and 2.5 units of AmpliTaq Gold polymerase (Applied Biosystems).

For each method all constituents except the DNA were pipetted into a 1.5ml eppendorf and vortexed for 5 secs. 24μL were then pipetted into each well of a 96 well PCR plate. 1μL of DNA was added to each well at this stage. A control well substituting sterilized H20 for DNA was included for each exon (i.e. each set of primers). The 96 well plate was sealed with a transparent self-adhesive seal and centrifuged for 1min at low speed to collect samples at the bottom of the wells.

9700 PCR machines were used. Variable PCR cycling conditions were also trialed but the optimum conditions consisted of an initial denaturing step of 95°C for 10 minutes followed by 30 cycles of 95°C for 30 seconds, 55°C(for CACNA1S primers)/60°C(for SCN4A primers) for 30 seconds, 72°C for 30 seconds, and a final extension step of 72°C for 7 minutes.

Agarose Gels

PCR products were run on 2% agarose gels. Gels were made using 2g of agarose powder per 100ml of 1 x TBE (Tris Borate EDTA) which was microwaved on full power for 2-3 mins to dissolve. Ethidium bromide (5μL) was added to the gel. The gel was stood to cool for a further 3-5 mins before being poured into a tray with gel combs and end plates and allowed to set for 1 hour. 5μL of PCR product from each well of the 9700 PCR plate was mixed with 3μL of orange G (as a loading dye) and transferred to the gel chambers. A DNA size marker was placed in the first well of each row of samples. Additional 1 x TBE was poured over the gel as a buffer and the lid placed on the tray. Electrodes were connected to the power supply and 65Volts run through the tray. Gels were left to run for 45mins or until the loading dye had run ¾ of the length of the tray. The gel was then carefully removed from the tray and placed under UV light in a light box to identify the presence of DNA fragments of expected size (using the DNA size marker for comparison) bound to ethidium bromide and hence success or failure of the PCR reaction.

PCR Clean Up

If the PCR was successful the remainder of the PCR product was cleaned to remove all constituents except the DNA fragments. The PCR plate was first centrifuged for 1min to collect samples at the bottoms of wells. 30µL of sterilized water was added to each well and the plate was resealed and re-spun on the centrifuge for a further 1min. The diluted product was then transferred to a 96 well PCR clean up plate and placed on a vacuum for 5-10mins or until dry. The dry DNA was re-constituted with 40µL of sterilized water and placed on a rotator for 30mins before being transferred to a clean 96 well PCR plate.

Sequencing Reagents and Reactions

Samples were sequenced (bidirectionally) using the ABI Big Dye Terminator Sequencing Kit version 1.1, M13 universal primers, and an ABI Model 3730xl Automated DNA Sequencer.

Each 10µL sequencing reaction contained the following reagents: 1µL of Big Dye, 2µL of 5 x sequencing buffer, 10pmol of M13 universal primer (forward or reverse), 3.5µL of the clean PCR product and 2.5µL of sterilized ddnH20. All constituents with the exception of PCR product were pipetted into a 1.5ml eppendorf in volumes adequate for 100 reactions and vortexed for 5secs. 6.5µL was then pipetted into each well of a 96 well 9800 PCR plate and 3.5µL of PCR product added to the sequencing reagents. The plate was sealed and centrifuged for 1 min to collect samples at the bottom of the wells.

A 9800 PCR machine was used and the following cycling conditions: an initial step of 96°C for one minute, followed by 25 cycles of 96°C for 10secs, 50°C for 5secs, 60°C for 1min 15secs, and then held at 4°C.

After sequencing the samples were cleaned in order to remove all reagents. The clean up plates were centrifuged for 3mins at 2150rpm and the flow though discarded. The samples were centrifuged for 1 min at low speed to ensure they collected in the bottom of the wells. 10µL of sterilized ddnH20 was added to each well, the plate resealed and centrifuged for a further 1 min. All samples were then transferred to the clean up plate which was placed on top of a clean PCR plate. Plates were spun together for 3 mins at 2150rpm so that the cleaned sequencing product was filtered into the clean PCR plate.

The samples were sequenced bidirectionally using an ABI Model 3730xl automated DNA sequencer. All primers were initially tested and all conditions optimized on control DNA samples that were known to be of good quality before proceeding to research samples.

2.2 Analysis of genetic data

Data was analyzed using the version 2.5 SeqScape Analysis software (ABI). A reference data group and project template was created for each gene using the seqscape manager software. The reference data group is the sequence against which the analysis programme compares the sample sequence. In essence this involves importing and accurately numbering the coding sequence flanked by a small portion of intron for each exon.
If a sequence variant was identified that predicted an amino acid change it was compared to the reference gene sequence in Ensemble for any known single nucleotide polymorphisms. A second database, dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/, was additionally searched. If neither database identified the sequence variant as a polymorphism it was entered into a SIFT (sorting intolerant from tolerant) prediction programme: http://sift.jcvi.org/www/SIFT_BLink_submit.html to prioritise which changes were most likely to be deleterious and therefore of a pathological nature. A more laborious but similar approach was employed by also checking isoform sequences of other animal species to determine the conservation of the amino acid change. If these combined efforts indicated the change was likely to be pathological control samples were analysed (minimum of 166 chromosomes) for the presence of the amino acid substitution.

2.3 ZNF9 anlaysis

One of the aims of this work was to assess whether known disease genes accounted for all cases examined with EMG proven myotonia. A minority of samples were identified in whom testing of the CLCN1 gene (performed by the diagnostic lab) and SCN4A revealed no pathogenic changes. These samples were analysed for the presence of the quadruplet nucleotide repeat expansion in intron 1 of the ZNF9 gene associated with myotonic

dystrophy type 2 (DM2). These primers and methods had been optimized by my predecessor Dr Doreen Fialho.

A 2-step fluorescent PCR based method was used. In the initial step primers flanking the repeat sequence (ZNF9F and ZNF9R) were used for amplification. Samples which showed only one allele size (apparent homozygous) were amplified in the second step using a quadruplet primed PCR.

PCR step 1 (ZNF9 PCR with primers flanking the repeat sequence):

A 25µl PCR mixture contained: 10x Amplitaq Gold Buffer (2.5µl), $MgCl_2$ (1.5µl), 10x dNTPs (2.5µl), 10 pmol/µl forward/reverse primer (0.5 µl each), Amplitaq Gold Polymerase (0.15µl), ddnH_2O (16.85µl), DNA (1µl). The PCR conditions were: 95˚C 10 min (94˚C 45 sec, 57˚C 45 sec, 72˚C 1 min) x 30 cycles, 72˚C 10 min.

Primer sequences for step 1:

ZNF9F: 5'- 6 – FAM – GCCTAGGGGACAAAGTGAGA - 3'

ZNF9R: 5'- GGCCTTATAACCATGCAAATG - 3'

PCR step 2 (ZNF9 quadruplet primed PCR):

A 20µl PCR mixture contained: 10x FastStart Taq Buffer (2µl), $MgCl_2$ (1.2µl), 10x dNTPs (1µl), primer mix (1µl), 5U/µl FastStart Taq DNA polymerase (0.2µl) (Roche), 5M Betaine (8µl), np H_2O (5.6µl), DNA (1µl). The primer mix contained 5 µM ZNF9F + 5µM ZNF9_Tail_R + 0.5 µM ZNF9_Tail_CCTG_R.

Primer sequences for step 2:

ZNF9F as above

ZNF9_Tail_R: 5'- TACGCATCCCAGTTTGAGACG - 3'

ZNF9_Tail_CCTG_R: 5' –

TACGCATCCCAGTTTGAGACGCCTGCCTGCCTGCCTG - 3'

An aliquot of the PCR product (2-3µl) from PCR step 1 and subsequently step 2 was added to 12µl HiDi Formamide and 0.2 µl LIZ500 size standard (ABI) and heated to 95˚C

for 5 min and then immediately transferred onto ice for a minimum of 5 min. The resulting denatured single stranded DNA was processed with an automated DNA analyser 3730 (ABI).

2.4 Mutagenesis experiments

The human SCN4A clone was supplied within a pCDH vector as a gift from H Lerche. Site directed mutagenesis was performed using the Quick Change II Site Directed Mutagenesis Kit (Stratagene) to create a construct with the R675G mutation.

Design of primers for mutagenesis of the SCN4A clone

The reference SCN4A sequence was provided with the clone. The relevant segment was highlighted and then altered to introduce the nucleotide change that would produce the desired mutation. This mutant sequence was used to design the primers such that the desired mutation was in the middle of the primer with 10-15 base pairs of normal sequence either side.

R675G

F R L L **G** V F K

CTCCTTCCGTCTGCT**G**GGAGTCTTCAAGC Forward primer

GCTTGAAGACTCC**C**AGCAGACGGAAGGAG Reverse primer

Primers were reconstituted to a concentration of 10pmol per μL. The mutagenesis reaction required 125ng of each primer. This was calculated using the formula:

$$\frac{125\text{ng of oligonucleotide}}{330 \times 29\text{(no of base pairs in primer)}} \times 1000 = 1.3\text{microL}$$

Synthesis of mutant DNA

The mutagenesis PCR reaction was prepared with the following reagents to a total volume of 50µL: 5µL of 10x buffer, 1µL (11.78ng) of DNA, 1.3µL of forward primer, 1.3µL of reverse primer, 1µL of DNTPs, 3µL of Quick solution and 37.4µL of ddnH2O. Once the constituents were mixed, 1µL of PfuUltra HF DNA polymerase (2.5U/µL) was added. Cycling conditions were as follows: 95°C for 1min, followed by 18 cycles of 95°C for 50secs. 60°C for 50secs and 68°C for 7mins(1min/kb of plasmid) with a final extension step of 68°C for 7mins.

Dpn I digestion of the amplification product

1µl of the *Dpn* I restriction enzyme (10U/µl) was added directly to the amplification product. The reaction mixture was mixed by gently pipetting the solution up and down several times. The reaction mixture was spun in a microcentrifuge for 1 minute and immediately incubated at 37°C for 1 hour to digest the parental (i.e., the nonmutated) supercoiled dsDNA.

Transformation of TOP – 10 Cells

Aliquots of the TOP 10 bacterial cells were removed from the freezer and placed immediately on ice to thaw. 1µl of the sample DNA was pipetted into the cell vial and mixed gently by pipetting up and down. The mixture was left on ice for a further 30mins. It was then heat pulsed at 42°C for 30secs and placed back in ice. 250µl of SOC (Super Optimal broth with Catabolite repression) medium was added and the vial incubated at 37°C for l hour. Sterile inoculating loops were then used to spread the cell mixture over a prepared agar plate and incubated overnight at 37°C. Agar plates were prepared using LB (lysogeny broth) agar at a concentration of 35g/L with the addition of ampicillin 1µl/ml.

Plates were inspected the following day for growth of any cell colonies. If colonies were present they were individually "picked" using a sterile pipette and the pipette placed into a 15ml falcon tube containing 4ml of LB Broth with ampicillin 1µl/ml. The falcon tube was incubated overnight in an incubator with shaking tray at 37°C.

Plasmid DNA purification

Bacteria that had multiplied overnight and were suspended in the falcon tubes were transferred to a 1.5 mL tube and spun in a microcentifuge for 5mins. This produced a pellet of bacterial cells and the remaining supernatant was discarded. Plasmid DNA purification was performed using a Qiaprep mini-prep kit (Qiagen) and according to the kit manual.

The bacterial cells were resuspended by adding 250µl of Buffer P1 (resuspension buffer) and mixed by gently pipetting up and down. An additional 250µl of Buffer P2 (lysis buffer) was then added and the solution gently mixed by inverting the eppendorf. The lysis reaction was left for 5mins then neutralized by adding 350µl of Buffer P3 (neutralization buffer) and the tube immediately mixed by inverting 5 times. The solution was then spun in a microcentrifuge for 10mins at 13 000rpm.

While the lysed bacteria were spinning new tubes were labeled and binding columns placed inside. The binding columns were washed by adding 500µl of Buffer PB and centrifuged for 60secs. The flow through was discarded.

The original tubes were removed from the centrifuge and a white pellet could be observed. The supernatant was transferred to the pre-prepared binding columns and the columns spun for 60secs. The flow through was discarded. 750µl of Buffer PE (wash buffer) was added to the binding column which was spun again for 30-60secs. The flow through was discarded and the tube spun for a further 60secs. Any additional flow through was discarded. Columns were placed in clean labeled tubes. The DNA was eluted by adding 50µl of Buffer EB. Columns were left to stand for 1min and then spun for 1min. The flow through comprised of purified DNA which was frozen.

Confirming successful mutagenesis of the SCN4A clone

Purified DNA was digested using the following reagents: 1.5µl DNA, 1.5µl 10 x buffer, 0.15µl 100x BSA, 0.3µl Enzyme ACC 65I, 12µl of H20 and incubated at 37°C for 60mins. A 1% agarose gel was prepared as previously detailed and digestion samples run on the gel with a lkb DNA ladder. The presence of 2 fragments of 4.5kb and 7.0kb indicated successful DNA mutagenesis. To further confirm, DNA was also directly

sequenced using the methods outlined above as this also ensured no unintentional additional mutations had been introduced.

Maximising the purified DNA sample

Once it was confirmed the DNA sample contained only the intended mutant sequence, maxipreps were prepared using a Qiagen Maxiprep kit and protocol. TOP-10 cells were transformed using the methods described above with 1µl of purified mutant DNA. Agar plates were incubated overnight. If there was successful growth of colonies the colonies were "picked" using a sterile pipette and the pipette placed in a 1L flask containing 200ml of LB broth (with 1mg/ml of ampicillin). The flask was incubated overnight at 37°C on a shaking tray.

Maxipreps were prepared using the Qiagen maxiprep protocol as follows:

1. Broth was poured into a plastic centrifuge bottle and spun for 15mins at 5000 rpm.
2. Supernatant was poured off and bacterial pellet resuspended in 10 ml Buffer P1**.** Resuspended pellet solution was transferred to a plastic centrifuge tube.
3.10 ml of Buffer P2 was added, mixed gently, and incubated at room temperature for 5 minutes.
4.10 ml of cold Buffer P3 was added, mixed immediately but gently by inversion, and incubated on ice for 20 minutes.
5. The tube was then spun for 30mins at 12,000 rpm at 4°C . During the last 10mins of the spin, Qiagen-tip 500 columns were equilibrated by adding 10 ml Buffer QBT and allowing the column to drain.
6. The supernatant was decanted into 2 Falcon 50ml tubes.
7. Decanted supernatant was added to the column and allowed to flow through.
8. The column was washed twice with 30 ml Buffer QC.
9. DNA was eluted by adding 2 x 5 ml Buffer QF and the eluate caught in a fresh plastic centrifuge tube.
10. DNA was precipitated with 7 ml room-temperature isopropanol and the tube spun for 30secs at 12,000 rpm at 4°C.

11. The supernatant was poured off, 5ml 70% ethanol added and the tube spun for 5mins at 12, 000 rpm at 4°C.
12. Supernatant was poured off; the tube respun for 5mins at 12, 000 rpm at 4°C.
13. DNA was allowed to air-dry for 5mins; resuspended in 500µl dH_20 and, transferred to an eppendorf.

Confirmation of the correct DNA sequence was again performed by restriction digest and direct DNA sequencing.

2.5 Cell culture methods

All cell work was performed under sterile technique in sterile tissue culture cabinets within a dedicated tissue culture facility.

Thawing human embryonic kidney (HEK) cells

Stocks of HEK cells were stored in a liquid nitrogen facility. Prior to removing cells from storage flasks for cell culture were prepared in the fume hood. Growth medium constituted DMEM, 10% FBS and 1% glutamax (Invitrogen). 50ml aliquots were stored in the fridge and removed to warm to room temperature. 5ml were transferred using bulb pipettes to a pre-labeled sterile cell culture flask. A further 5mls were pipetted to a 10ml falcon tube. At this stage cell vials were removed from the liquid nitrogen tank and immediately transferred to the water tank (37°C) in the cell culture laboratory until thawed. The cells in solution were transferred to the falcon tube and mixed gently with the fresh medium. The falcon tube was spun down at 12 000rpm for 5mins to create a cell pellet. The supernatant was discarded and 5mls of fresh warm growth medium gently mixed with the cells using a bulb pipette. This solution was transferred to the prepared culture flask and the solution gently washed repeatedly over the flask base 20 times in an attempt to create an even distribution of cells and avoid “clumps”. The flask was placed in the incubator at 37°C.

Splitting HEK cells

HEK cells were split when approximately 80% confluent. This was guided by direct observation of the flask under a microscope on a daily basis but as a general guide cells were split three times a week. A new flask was prepared by labeling and loading with 5mls of fresh growth medium warmed to room temperature. Growth medium was removed from the flask containing the HEK cell culture with a bulb pipette and discarded. 1ml of trypsin was added to the flask and the flask rolled gently to coat the whole cell layer. Effectiveness of the trypsin was guided by sight as the cell layer could be seen to detach from the flask base but as a general guide this took approximately 3-5mins. The reaction was terminated at this point by the addition of 5mls of fresh growth medium. The cells now in trypsin/growth medium solution were transferred to a 10ml falcon tube and the tube spun at 12 000rpm for 5mins. A cell pellet was now visible and the medium was discarded. 5mls of fresh media was added to the pellet and mixed gently by pipetting up and down. Approximately 1ml of this solution (volume transferred depended on confluency of cells and size of the pellet) was transferred to the prepared flask containing fresh growth medium and washed twenty times over the bottom of the flask to achieve an even distribution of cells. The new flask was returned to the incubator at 37°C.

Myoblast cultures

Aliquots of a healthy culture of normal myoblasts were given to me by Dr Carl Adkin at the Institute of Child Health. These had been cultured from healthy muscle donated by a teenager undergoing scoliosis surgery. Thawing, culture and splitting of cells was performed along similar methods to the HEK cells but a different growth medium was used comprising of: 500ml Skeletal Muscle Cell Growth Medium(Promocell C-23060), 25ml supplement mix(Promocell), 50ml FBS and 7.5ml L-glutamine. Myoblasts were also split at approx. 50% confluence to prevent fusion and formation of myotubes.

Reverse transcription PCR

RNA was extracted from robust myoblast cell culture using an RNAeasy kit (Quiagen). Reverse transcription PCR was performed using an ABI high capacity cDNA RT kit. Each 10µl reaction contained: 2µl 10xbuffer, 0.8µl dNTPs, 2µl primer, 1µl RT and 4.2µl of H20.

2.6 Transfection of human embryonic kidney cells

HEK cells were split in the manner described above 24hours prior to cell transfection. When the cell pellet was reconstituted with 5mls of fresh growth media one drop of the solution was added to six 5ml petri dishes which each contained three 1cm circular glass coverslips that had been pre-coated with poly-D-lysine, and 2-3mls of growth media. Petri dishes were incubated overnight at 37°C to allow for early growth of the cells. Human Embryonic Kidney cells (HEK cells) were transfected using the calcium phosphate precipitation method. The purified mutant SCN4A DNA, WT SCN4A DNA, SCN1B DNA samples and GFP aliquots were analysed with a nanodrop to assess the DNA concentration in each sample. This was to allow calculation for the transfection reaction such that each mixture contained a 1:1:1 ratio of SCN1A, SCN1B and GFP (green fluorescent protein). As such 12.7µl of calcium chloride, 4µl of GFP, 4µl of WT SCN4A DNA, 1.5µl of SCN1B DNA and 77.8 µl of H2O were pipetted into a sterile eppendorf to a total volume of 100µl. 100µl of BES (bis-hydroxyethylaminoethansulfonate) was added to the mixture and gently pipetted up and down. Constituents for the mutant DNA transfection were mixed in a second sterile eppendorf: 12.7µl of calcium chloride, 4µl of GFP, 20µl of mutant SCN4A DNA, 1.5µl of SCN1B DNA and 61.8µl of H2O to a total volume of 100µl and mixed gently with 100µl of BES. Each reaction was left to stand at room temperature for 10mins. The mixture was then distributed evenly between three of the prepared petri dishes containing glass cover slips and the dish gently swirled before being placed back in the incubator for 8-10hours. After this time the media was changed to replenish the dish with fresh growth

media. Transfected cells were incubated for 48hrs before being used in patch clamp experiments.

Coating cover slips with poly-D-lysine

50ml of poly-D-lysine was poured into a sterile autoclaved glass bottle and made up to 100ml with autoclaved water. This was then divided into 10ml aliquots in falcon tubes for freezing. 200 cover slips were placed in a 50ml falcon tube with one 10ml aliquot of poly-D-lysine. This was placed on a rotator for 1hour to evenly coat all the cover slips. After one hour the poly-D-lysine was poured off and the cover slips rinsed several times with sterile water. The cover slips were then autoclaved to sterilise prior to use in patch clamp experiments.

2.7 Patch clamp experiments

Patch clamp experiments were performed on HEK cells 48-72 hours post-transfection. Standard whole-cell patch clamp recordings were performed at room temperature. The external bath solution used to bathe the coverslips and cells was prepared as a 1L solution containing:

mM		1L
145	NaCl	8.47g
4	KCl	2ml(2M)
1.8	$CaCl_2$	1.8ml(1M)
1	$MgCl_2$	1ml(1M)
10	HEPES	2.383g

The external solution was pH tested and brought to a pH of 7.35 with NaOH(1M), usually requiring approximately 3-5ml.

Several pipette solutions were trialed but the internal pipette solution used for all cells in the final analysis contained CsCl (150mM); Hepes (10mM) and EGTA (10mM) at a pH of 7.3. Pipettes were fabricated using capillary glass and a pipette puller using pipettes with resistances of between 2.5-5MΩ. Cells with series resistance above 10MΩ were discarded. To control variability, recordings from WT SCN4A cells were routinely intercalated with recordings from mutant SCN4A cells. Leak subtracted currents were recorded using –*P*/4 protocol with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data was sampled at 20kHz and filtered at 5kHz, and analysed using LabView software (v. 8.0, National Instruments, Austin, TX, USA) with programs written by Prof D Kullman.

Results - Phenotyping

Phenotype can be markedly diverse in the skeletal muscle channelopathies even among individuals with the same genotype. An example of the phenotypic variability that can be observed is provided by the SCN4A G1306E mutation associated with sodium channel myotonia. The original phenotype reported with this mutation was so severe the individual suffered permanent myotonia that included the respiratory muscles and led to hypoxia and acidosis requiring ventilatory support(Lerche *et al*., 1993). In contrast, a more recent report observed that although affected individuals had relatively severe myotonia they did not exhibit respiratory involvement and were able to carry out daily activities including work without treatment.(Colding-Jorgensen *et al*., 2006) As a consequence of this wide variability there are few clinically useful genotype-phenotype correlations. The channelopathy database was updated and reviewed regularly to identify potentially informative phenotypes and any possible genotype-phenotype correlations that emerged were explored further.

3.1 Observation of neonatal hypotonia in paramyotonia congenita due to the I693T SCN4A mutation

A female patient, BWL, referred to the clinical service for skeletal muscle channelopathies with PMC and seen by me described her mother reporting that she had been "paralysed" and floppy for the first four days after birth with an inability to breastfeed. The lady's mother was deceased so further details could not be sought. Attempts to obtain the birth records proved unsuccessful as they had been destroyed. This lady was known to have genetically confirmed paramyotonia congenita with the point mutation I693T having been identified in exon 13 of the SCN4A gene by the diagnostic clinical service. Due to the unusual birth history and a rather severe phenotype causing biopsy proven evidence of myopathy at the age of 15 with subsequent use of a wheelchair from her late twenties this lady was presented as an "interesting case" at an international meeting of the Non-Dystrophic Myotonias. This led to collaboration with

Bertrand Fontaine's group who attended the meeting and also reported neonatal hypotonia occurring in families they had seen with the same SCN4A mutation (I693T). Details of the four French children from two unrelated families were sent to me by Damien Sternberg.

Phenotype of neonates carrying the I693T SCN4A mutation

Four unrelated families in total (2 in the UK, 2 in France) were identified who all carried the I693T mutation and reported cases of neonatal hypotonia with variable difficulties in feeding and respiratory compromise (see Table 9). Symptoms were self-limiting within 10 days in each case. All affected individuals later went on to develop the typical symptoms of cold and exercise exacerbated myotonia seen in PMC. The mutation was inherited from the mother in two cases, the father in one and was apparently sporadic in the other although parental DNA was not available to confirm this.

The proband identified in one of the UK families became pregnant shortly after the recognition of neonatal hypotonia with this mutation. Advice was given to her and to the obstetric and paediatric teams involved in her care regarding the possible development of neonatal hypotonia, feeding or respiratory difficulties along with reassurances that these symptoms were most likely to be self-limiting and would require only supportive care. She went on to deliver a healthy baby boy who did display hypotonia and feeding difficulties requiring a short term nasogastric tube. Symptoms were self limiting and he was later able to breast feed and to date has had normal development. Subsequent genetic testing confirmed he had inherited the I693T mutation from his mother.

	Sex	Year of birth	APGAR at birth	Onset of Hypotonia	Impairment of sucking-swallowing	Desaturation	Kalemia (mmol/l)	Motor Development
Family 1 (English)	Female 1-1	1950	NA	Birth	Day1-bottle fed,unable to breast feed	No	NA	Walking delayed (19/12)
Family 2 (English)	Female 2-1	1975	NA	Birth	No (hypotonia limited to limbs)	No	NA	Walking delayed (22/12)
Family 3 (French)	Female 3-1	1987	10	Day1, some hours after birth	Day1-3 some hours after birth, nasogastric tube	Day1, some hours after birth (cyanosis) ; no reported treatment	5.1 then normal	Normal
	Male 3-2	1997	10	Day1, some hours after birth when bathed	Day1-3 onset in bath	No	Normal	NA
Family 4 (French)	Male 4-1	2004	NA	Day1	Day1-3 no treatment reported	No	NA	Normal
	Male 4-2	2007	10	Day1,some hours after birth	Day1-6, some hours after birth nasogastric tube	Day2-6, nasal oxygen required	Normal	NA

NA-Not Available

Table 9: Features of hypotonia and motor development in six neonates carrying the Nav1.4 I693T mutation.

3.2 Stridor expands the neonatal presentations of skeletal muscle sodium channelopathy

Following on from the observation of this neonatal presentation of paramyotonia congenita a patient seen by me in clinic and enrolled in the natural history trial with paramyotonia congenita described her second son as having severe stridor with some respiratory and feeding difficulties at birth. She queried whether his stridor was related to PMC. Initially this was uncertain as although the defective Nav1.4 channel would be present in all skeletal muscles including the larynx, neonatal stridor is common for numerous other reasons and it was not possible to draw conclusions from a single case. His medical records were reviewed with permission. Discussion once again with Bertrand Fontaine and Damien Sternberg's group in France identified two additional French cases however which suggested the association was not co-incidental and the stridor may be a direct result of the myotonic disorder.

Features of neonatal stridor

The lady's four year old son was referred to paediatric services following the diagnosis of paramyotonia congenita (PMC) in her. The boy was the mother's second child and was born by ventouse delivery at 39 weeks following an uncomplicated pregnancy. APGAR scores were normal and post natal examination was unremarkable. Within 24 hours of delivery he was transferred to the neonatal intensive care due to inspiratory stridor and poor feeding. Over the following week he was unable to take sufficient oral feeds and required supplemental nasogastric feeding. Stridor persisted and he required intermittent oxygen therapy for desaturations that occurred while attempting to bottle feed or when crying. Laryngoscopy showed findings consistent with laryngomalacia.

The infant continued to have persistent inspiratory stridor for the first six months of life. Feeds were prolonged but he gained weight appropriately and had no further apnoeic episodes. Motor milestones were mildly delayed, sitting independently at nine months and walking at 19 months. From the age of one year exotropia was noted. This is

currently under investigation by an opthalmologist and possibly reflects myotonia of the extraocular muscles.

At the age of 23 months he fell in the garden on a cold day and complained of leg weakness, refusing to stand or walk. The weakness recovered spontaneously within five hours. A second similar episode occurred at age 27 months after playing in the garden in winter. In addition to these two episodes of muscle weakness his mother noted muscle stiffness occurring on an almost daily basis from age two. Both muscle stiffness and weakness were exacerbated by cold weather and exertion.

At the age of four he continues to have episodes of inspiratory stridor exacerbated by viral illness, cold weather, and prolonged laughter or crying. A humidifier is helpful in aborting such episodes.

He has recently been treated with the sodium channel blocker mexiletine which has had some beneficial effect on his degree of myotonia and exotropia.

The child's mother, grandfather and great uncle all reported similar episodes of muscle stiffness and weakness exacerbated by cold and exercise. There was no prior family history of stridor. All affected family members including the child described had DNA sent to the diagnostic genetic lab at the NHNN under the NCG scheme for channelopathies by the specialist responsible for their clinical care and were shown to have the SCN4A mutation T1313M associated with PMC(McClatchey *et al*., 1992b).

In addition to this case two further adult cases of PMC in France in whom neonatal stridor was documented in their medical records were identified by Damien Sternberg although no further details were available. The first carried an E1702K familial mutation, and the other an A444D de novo mutation.

Neonatal myotonia was recorded in Eulenburg's first description of PMC but distinct neonatal phenotypes of sodium channelopathy have not previously been recognised. A case of fatal infantile myotonia with severe respiratory compromise was recently reported in a child carrying a new SCN4A mutation N1297K(Gay *et al*., 2008). Since describing the case of stridor another series has reported more severe laryngospasm in three infants with sodium channel mutations, one of whom also died(Lion-Francois *et al*., 2010).

This emerging evidence of neonatal presentations is of paramount importance when counselling expectant families with a history of sodium channel disorder and is important to bear in mind in children presenting with these features even if family history is lacking as de novo mutations occur. In the milder phenotypes I have described symptoms generally only required supportive care and invasive investigations are not required. The recently published more severe cases generally responded to mexiletine therapy. Ultimately the delivery of neonates at risk of inheriting sodium channel disorders should be considered relatively high risk and appropriate obstetric, anaesthetic and paediatric services must be available. The recognition of these phenotypes will limit unnecessary investigation and prompt early appropriate therapy.

3.3 Observation of inflammatory infiltrates in skeletal muscle channelopathies

Advances in specialised electrophysiological tests and genetic analysis although not infallible have significantly improved the ability to diagnose skeletal muscle channelopathies. Many of the reported histopathological findings in the channelopathies predate the availability of genetic testing and commonly include a vacuolar myopathy or tubular aggregates in the periodic paralyses although it must be noted that these occur in all of the sub groups. Biopsy findings in the non-dystrophic myotonias are non-specific myopathic changes. Morphological analysis is ultimately not diagnostic but prior to the availability of less invasive investigations it was used to support a diagnosis of channelopathy and much of the data available is from typical cases without severe or unusual symptoms. It is rare in the UK that a diagnostic biopsy is now performed in the channelopathies.

Regular searches of the database of patients referred to the channelopathy service identified three cases of genetically confirmed skeletal muscle channelopathies who all underwent muscle biopsies due to reports of either proximal weakness or severe muscle pain in conjunction with a raised creatine kinase (CK). In each case the biopsies were reported as showing inflammatory infiltrates which are not a recognised or described finding in channelopathies. The referring clinicians were contacted in each case to request

further clinical details and any available biopsy slides. All three kindly responded with clinical details and in two cases biopsy material was sent for review by Dr Janice Holton. In the remaining case biopsy material had unfortunately been destroyed but detailed biopsy reports were obtained.

Histopathology

Each patient had undergone a muscle biopsy in another centre and tissue sections stained for haematoxylin and eosin (H&E), Gomori's trichrome method, lipid and glycogen in addition to enzyme histochemical preparations for succinic dehydrogenase, cytochrome oxidase and nicotinamide adenine dinucleotide dehydrogenase tetrazolium reductase were available for review. Immunohistochemical preparations submitted for review were: Case 1: CD3 and MHC class I; Case 2: immunohistochemical staining for CD3, CD20, spectrin, dystrophin, merosin, dysferlin, sarcoglycans, emerin and desmin; and Case 3: MHC Class I, CD3, CD68 and CD20.

Clinical features of channelopathy patients with inflammatory biopsies

Case One

An 80 year old man complained of episodes of muscle weakness after periods of prolonged rest from the age of four. Initially only the lower limbs were affected but by his late teens the upper limbs were also involved. The episodes usually lasted hours and occasionally days. A clinical diagnosis of familial periodic paralysis was made when he was aged 21 following reports of similar episodes of weakness in his mother and sister. From his early 50s mild proximal muscle weakness was noted on examination. At the age of 56, following a positive potassium challenge his diagnosis was revised to hyperkalaemic periodic paralysis and he was treated with salbutamol. Creatine Kinase (CK) at this time was noted to be 436U/L. At the age of 65 he began complaining of difficulty rising from a chair and climbing stairs. Examination confirmed limb weakness greater proximally than distally. Clear difficulty rising from a chair without using his

hands for leverage was observed and a degree of thigh atrophy was noted. EMG showed myopathic changes and minimal myotonic discharges. His CK was 2729U/L. Muscle biopsy showed internal nuclei, fibre atrophy, fibre hypertrophy with occasional split fibres, some regenerating fibres and an increase in endomysial connective tissue. In addition there was a dense inflammatory cell infiltrate focused around small capillaries and necrotic fibres which was demonstrated by immunohistochemistry to consist mainly of T-lymphocytes with restricted MHC 1 expression. Diagnoses of vasculitis and polymyositis were considered from the biopsy findings but the overall clinical picture was felt most consistent with a diagnosis of polymyositis and he was started on prednisone 60mg once daily. A few hours after the initial dose of steroids he suffered a severe attack of paralysis lasting 48 hours. Following recovery from this he was restarted on prednisone 10mg once daily with gradual improvement of his muscle weakness.
Ten years later he re-presented with further complaints of leg weakness and difficulties in mobilisation. At this time he was on 5mg of prednisone although it is not clear if this had been continuous since commencement 10 years earlier. Azathioprine 50mg bd was added to his therapy but he continued to deteriorate and was admitted to hospital where proximal lower limb power was documented as ranging from 3/5 to 4/5. The azathioprine dose was further increased to 100mg bd and he was discharged from hospital. When he was reviewed in clinic 2 months later he reported no real change in his mobility difficulties although only "mild" proximal leg weakness was recorded. Genetic analysis by the diagnostic channelopathy service confirmed the presence of the common T704M point mutation in the SCN4A gene associated with hyperkalaemic periodic paralysis(Ptacek *et al*., 1991).

Case Two

A 42 year old lady with the F167L mutation in the CLCN1 gene associated with myotonia congenita(George, Jr. *et al*., 1994) complained of daily muscle cramps affecting all muscle groups but predominantly the limbs from early childhood. This was confirmed to be myotonia on EMG. Family history revealed similar symptoms in her mother and two maternal aunts. The patient complained of severe cramps with increasing age and began to experience episodes of muscle weakness after exertion although in between

episodes reported normal muscle power. Examination was generally unremarkable but a CK was raised at 1109U/L.

Muscle biopsy was performed and demonstrated increased variation in fibre size, evidence of fibre regeneration and 1 necrotic fibre. Two small foci of inflammation composed of CD3 immunoreactive T lymphocytes were noted in perimysium and endomysium (Fig 16). Invasion of intact myofibres by T cells was not evident and MHC Class I immunohistochemistry was not available for review. There was no evidence of a vacuolar myopathy and tubular aggregates were not present. She was started on prednisone with a resultant reduction in CK to 3-400U/L. All attempts to reduce prednisone were unsuccessful and the patient was unable to tolerate other immunosuppressants.

Case Three

A 38 year old man complained of episodes of muscles stiffness when exposed to the cold affecting predominantly his tongue, hands and feet from early childhood. This was confirmed by electrophysiological testing to be myotonia. There was no family history of note. Myotonia occurred daily but symptoms remained fairly static until his late teens when he additionally complained of episodes of acute muscle swelling predominantly affecting his forearms and thighs. Initially these were non-painful, and CK and muscle power were normal. Later however he complained of myalgia accompanying the episodes of swelling which could occur 2-3 times a month and last up to a week. An ultrasound scan confirmed hypoechoic regions within the muscles but normal subcutaneous tissue. An intermittent peripheral eosinophilia was noted in conjunction with the episodes of myalgia. A muscle biopsy showed no evidence of regeneration, necrosis, vacuolation of fibres or tubular aggregates. Inflammatory infiltrates composed of a mixture of T lymphocytes and macrophages were present in the perimysium with a single endomysial cluster (Fig 16B and 16C). Invasion of intact muscle fibres by T cells was not apparent. Immunohistochemical staining for MHC Class I demonstrated an increase in sarcolemmal and sarcoplasmic expression (Fig 16D). The biopsy findings were interpreted as being compatible with polymyositis although the clinical history was not typical.

He had an excellent response to prednisone, 20mg od reduced to 5mg od which reduced the frequency and severity of the muscle swelling and myalgia although he unfortunately developed osteopenia. Attempts to introduce other steroid sparing immunosupressive agents were unsuccessful as he did not respond to methotrexate, cyclosporine, imatinib or hydroxyurea and did not tolerate azathioprine. Genetic analysis by the diagnostic channelopathy service confirmed the presence of the L1436P mutation in the SCN4A gene associated with sodium channel myotonia(Matthews *et al*., 2008b).

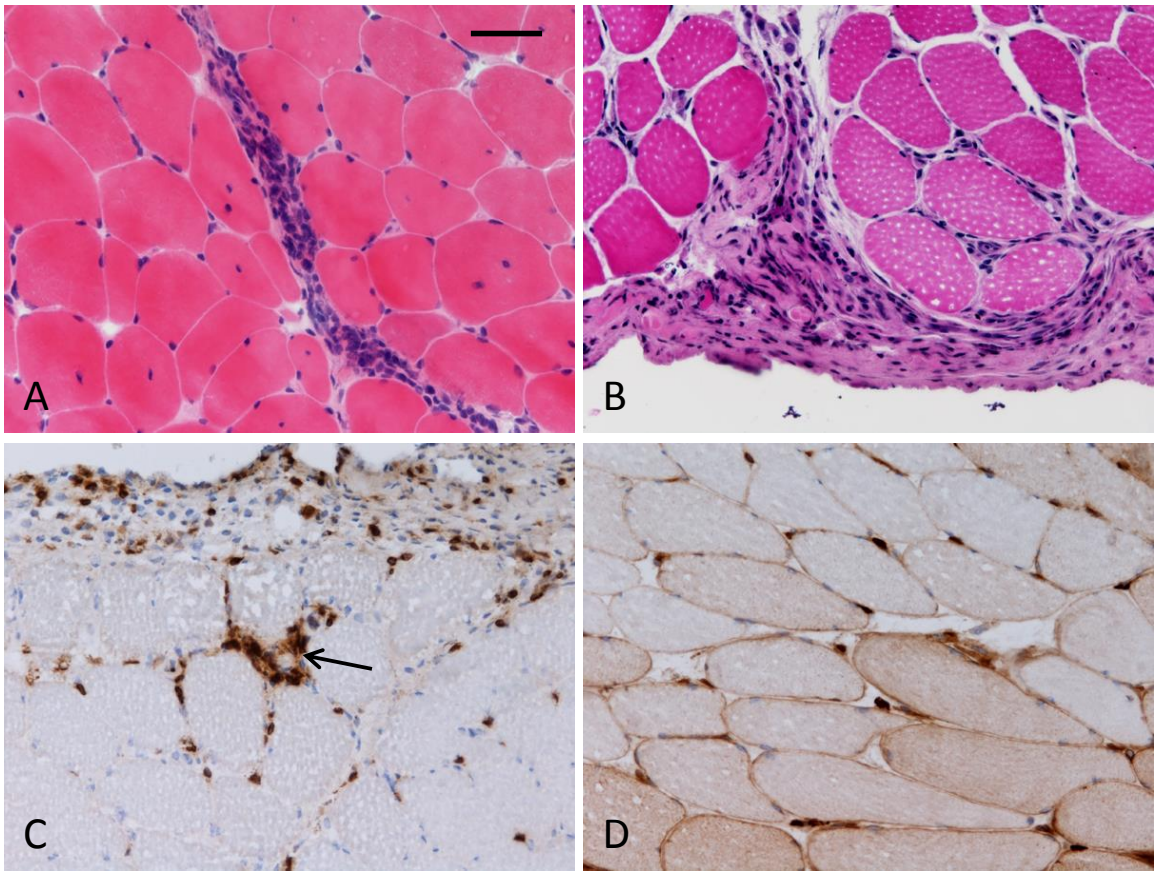


Fig 16: In case 2 a prominent focus of perimysial inflammation was found (A). Similarly the inflammation in case 3 was predominantly perimysial (B) with focal extension into the endomysium (arrow in C). The inflammatory cells included abundant T lymphocytes (C) and increased expression of MHC Class I at the sarcolemma and within the sarcoplasm of fibres was demonstrated (D). A: case 2; B – D: case 3. A & B: haematoxylin and eosin, C: CD3 immunohistochemistry; D: MHC Class I immunohistochemistry. Bar in A represents 50µm in A – D.

Case	Predominant clinical features	Examination findings	Investigations	Biopsy Findings	Response to steroid therapy
1	Episodic muscle paralysis Progressive proximal limb weakness	Proximal limb weakness, MRC grade 3/5 to 4/5	CK 2729U/L EMG: polyphasic units + short duration units, fibrillations, fasciculations and myotonic discharges	Internal nuclei, fibre atrophy, fibre hypertrophy with occasional split fibres, some regenerating fibres and an increase in endomysial connective tissue. Dense inflammatory cell infiltrate focused around small capillaries and necrotic fibres composed mainly of T-lymphocytes. MHC I upregulation on occasional muscle fibres.	High dose prednisone (60mg) led to severe paralytic attack lasting 48hrs 10mg better tolerated with improved muscle strength
2	Muscle cramps and severe myalgia	Myotonia Normal muscle power	CK 1109U/L EMG: massive myotonic discharges at every insertion point that precluded analysis of MUAP configuration, voluntary recruitment or interference pattern.	Increased variation in fibre size, evidence of fibre regeneration and 1 necrotic fibre. Two foci of inflammation composed of CD3 immunoreactive T lymphocytes noted in perimysium and endomysium. Immunohistochemistry unavailable for review.	Reduction in CK to 3-400.
3	Muscle cramps and episodic limb oedema with myalgia	Myotonia Normal muscle power	CK normal EMG: myotonic discharges	Inflammatory infiltrates composed of a mixture of T lymphocytes and macrophages were present in the perimysium with a single endomysial cluster (Fig 16). Immunohistochemical staining for MHC Class I demonstrated an increase in sarcolemmal and sarcoplasmic expression	Excellent response with significant reduction in symptoms of oedema and myalgia

Table 10: Summary of clinical, biochemical, neurophysiologic and histological findings in each case

Channelopathies are rare disorders and for the reasons outlined biopsy samples are scarce. It is impossible to be certain that the observation of inflammatory infiltrates in these three cases is not merely co-incidental. To try to ascertain the likelihood of this however I consulted Dr Gareth Ambler, statistician at UCL who reviewed the data. The prevalence rates of both skeletal muscle channelopathies and idiopathic inflammatory myopathy (IIM) have each been estimated to be 1 in 100,000(Emery., 1991; Fontaine., 1994; Hilton-Jones., 2001). A null hypothesis was taken that the development of both diseases in these three cases was coincidental. Considered statistically, if the likelihood of developing a skeletal muscle channelopathy and an IIM is independent then the chance of having both is one in 10^{-10}. The probability of observing UK patients with both diseases (if this association were by chance alone) can be calculated using the Poisson distribution since p is small and the UK population is large; Poisson lambda = 10^{-10} (independent probability of two events in an individual) x 6.5 x 10^{7} (UK population) = 0.0065, and the probability of observing three or more patients with both diseases (assuming independence) is 4.5 x 10^{-8}. This small p value indicates that the occurrence of both diseases in the same individual is highly unlikely to be independent.
Whether such inflammatory changes are present in a greater number of channelopathy patients will be difficult to establish but it is reasonable to suggest that a muscle biopsy be considered in those in whom a CK>1000IU/L is observed in conjunction with significant myalgia or weakness. Steroid use has to be interpreted cautiously as in one case high dose steroids caused significant exacerbation of the periodic paralysis but lower doses may be beneficial.

3.4 Pain and morbidity in the non-dystrophic myotonias

There is little in the literature that gives consideration to the morbidity of the skeletal muscle channelopathies. Multiple case reports and one recent series(Trip *et al*., 2009a) do suggest pain may be an overlooked feature in the non-dystrophic myotonias. It was a personal observation that many patients who attended clinic or participated in the natural history trial of non dystrophic myotonia often volunteered pain as a troubling symptom.

To look at this more subjectively all participants enrolled in the natural history study were asked about their symptoms. In the first year of enrollment of UK based participants the majority (16/19) reported stiffness as their most prominent symptom which would be as predicted for these myotonic disorders. All 19 had EMG evidence of myotonia and in18/19 pathogenic mutations associated with myotonic disorders have been identified. However 3/19 (16%) reported pain was their most prominent symptom (see Fig 17). Of these 3, 1 carried a sodium channel mutation and 2 chloride channel mutations.

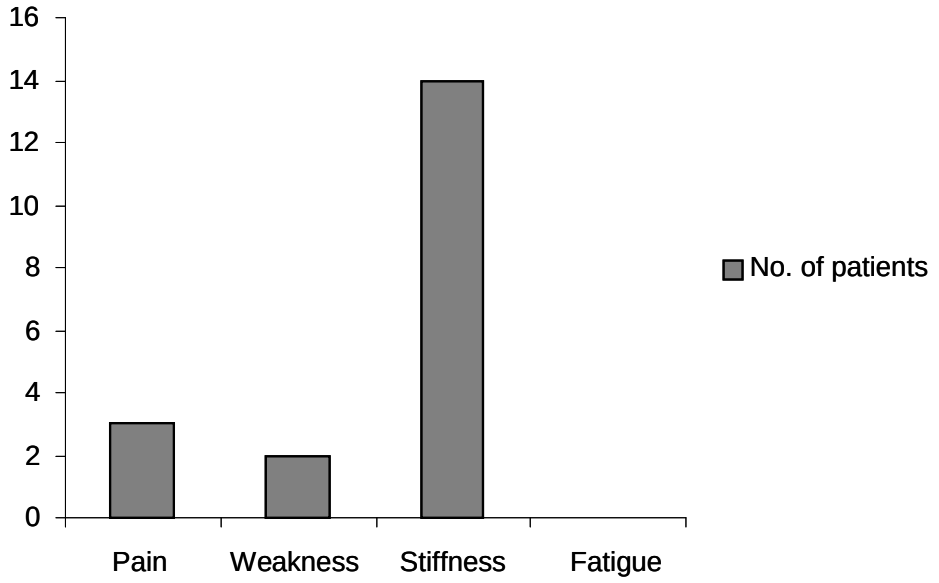


Fig 17: NDM patient reports of their most prominent clinical symptom

Participants were then asked about specific symptoms in detail including whether they experienced pain in association with muscle stiffness or even in the absence of muscle stiffness. If they answered yes they were asked to rate the severity of their maximum level of pain and their average level of pain on a pain scale of 0 – 9, 9 being the most severe pain imaginable and 0 being no pain. Of 19 patients 16/19 (84%) reported some pain, 7 of these associated only with stiffness, 7 both with and without stiffness and 2 without any stiffness exacerbation. Mean maximum and average severities are detailed in fig 18. There was no discernible difference in severity of pain whether it occurred with stiffness i.e. painful myotonia or in the absence of stiffness.

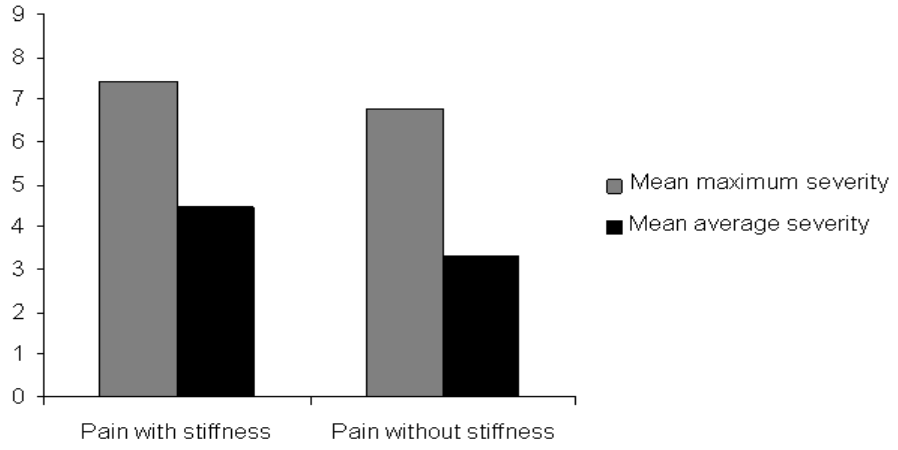


Fig 18: Mean maximum and average severity of pain scores in NDM patients

When considered by genotype, 100% (11/11) of patients with sodium channel mutations reported some pain, and 50% (3/6) of those with chloride channel mutations. This indicates that pain is significantly more likely to be reported by patients carrying sodium channel mutations than patients carrying chloride channel mutations ($p = 0.029$; Fisher's exact test). Of the remaining two patients who reported pain, one was diagnosed with DM2 and in the other no mutation has yet been identified. This data contributes to the emerging evidence that pain and its severity is a common and significant feature of non-dystrophic myotonia which may increase morbidity. It also suggests it may be more prominent or severe in those carrying sodium channel mutations but this would require further exploration in larger groups.

Results – Genotyping

4.1 Genotyping the sodium channel myotonic disorders

Patient identification

Using the channelopathy database, patients were identified with a clinical history consistent with myotonic sodium channel disease, either paramyotonia congenita, sodium channel myotonia or hyperkalaemic periodic paralysis. All patients are screened for the most common SCN4A mutations in exons 13, 22 and 24 by the diagnostic genetic lab as part of the NCG channelopathy service. I identified 17 patients in whom no such mutations were found for further study and performed direct DNA sequencing of all 24 exons of SCN4A (analysis of exons 13, 22 and 24 was repeated) and quantitative PCR for the trinucleotide repeat of ZNF9 known to cause myotonic dystrophy type 2 (DM2). All those with a phenotype of PMC or SCM had EMG proven myotonia. Cases of hyperkalaemic periodic paralysis included some with atypical features. If no abnormality was detected in SCN4A or ZNF9, the CLCN1 gene was kindly sequenced by geneticists Dr Robyn Labrum or Dr Richa Sud in the diagnostic lab. Selected patients had additional gene sequencing performed by other centres.

Sodium channel myotonia – new mutations

Of the 17 patients identified, I considered 13 to have PMC or SCM and 4 to have features of hyerPP. Amino acid substitutions were identified in 9/13 myotonic patients including 3 previously unreported, V445L, L128P and M135L. Controls were run for all of these novel changes including analysis of conservation across species and use of analysis websites to predict the likelihood of these changes being pathogenic. V445L and L128P were highly conserved, absent in controls and predicted to be deleterious to channel function. M135L was not highly conserved, was present in one control sample and predicted to be a tolerable amino acid substitution. It was not however listed on any SNP database as a known polymorphism. Both the patient and the positive control were

believed to be of Jewish origin. Taken together these findings suggest that this substitution may be a polymorphism that is more prevalent in certain ethnic groups.

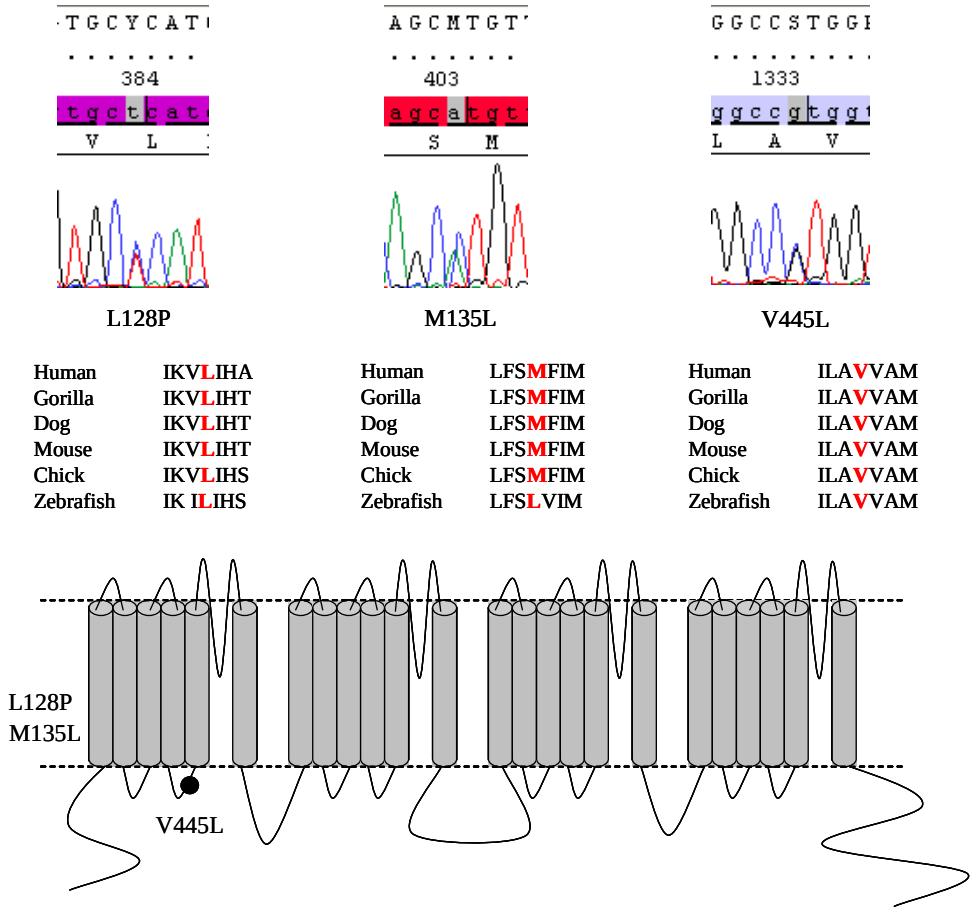


Fig 19: Electropherograms, conservation across species and position in Nav1.4 channel of new SCN4A mutations

	Genotyped	SCN4A	CLCN1	DM 2	Other Genes	Phenotype	EMG Myotonia
1MZ	Yes	V1293I				Sodium channel myotonia	Yes
2LG	Yes	V445L*				Sodium channel myotonia	Yes
3LF	No	Negative	Negative	Negative	DM1 negative	Sodium channel myotonia	Yes
4EC	No	Negative	Awaited – more DNA requested	Negative	DM1 negative	Infantile myotonia	Yes
5BC	Yes	Negative			CRYAB gene c.343delT (p.Ser115ProfsX14) + c.343delT (p.Ser115ProfsX14).	Infantile onset severe limb and respiratory muscle stiffness	First EMG ?myotonia Second EMG Pseudomyotonia
6CM	No	Negative	Negative	Negative	DM1 negative	Sodium channel myotonia	Yes
7MH	Yes	Negative	Mutation detected	Negative		Sodium channel myotonia	Yes
8BB	Yes	Q270K				Paramyotonia Congenita	Yes
9EL	Yes	E1702K				Paramyotonia Congenita	Yes
10CO	Yes	L128P*				Sodium Channel Myotonia	Yes
11DL	Yes	V1293I				Sodium Channel Myotonia	Yes
12JSC	No	M135L**				Paramyotonia Congenita	Yes
13RT	No	Negative	Negative	Failed		Paramyotonia Congenita	Yes
14SR	No	Negative	Negative	ND	RYR1 silent changes only Brother congenital myasthenic syndrome	Atypical hyperkalaemic periodic paralysis	No
15JV	Yes	Negative	Negative	Positive		Hyperkalaemic Periodic Paralysis	Myotonia – not clear if clinical or EMG
16LD	No	Negative	Negative	Negative	Congenital myasthenia genes awaited	Atypical periodic paralysis/myasthenia	No
17EP	No	Negative	ND	ND	KCNA1 Negative	Hyperkalaemic periodic paralysis	No

*denotes new gene mutations not previously reported. **denotes a new single amino acid substitution, not reported as a mutation or polymorphism but probably benign

Table 11: Summary of genetic results for 17 patients considered clinically to have PMC or hyperPP

Sodium channel myotonia – non-genotyped cases

The remaining 4 myotonic cases in whom no mutation was identified are considered in detail and are referred to by their order in table 11.

Patient No. 3 (LF)

This lady is from a large Sicilian family living in Australia. She complained of muscle stiffness and paralysis from infancy. She reported the first time she was taken for a swimming lesson at age 3 she nearly drowned as the cool water caused her muscles to become stiff. When she was rescued from the bottom of the pool she was found in a flexed position which required rewarming over about an hour before her muscles could be relaxed and began contracting normally again. If she exerts herself she can be in bed with muscle weakness and muscle discomfort for days. Recently she ran to catch a plane (the first time she had run in 20 years) and ended up paralysed in her airplane seat for the flight back from Florida to Australia. Cold temperature was a powerful exacerbator of her myotonic symptoms especially the peripheries, the eyelids and extraocular muscles. Her neurologist in Australia had ice cooled her eyes for 1 minute after which she could not open them very easily, had diplopia for several minutes and a mild facial droop. She spent most of her time "paralysed" in the hotel room after a recent trip to Canada, where she got cold on the first day. Cold also produced a tension-like headache and cervical muscle "tightness". She avoids some foods like olives and some fruits which she thinks can precipitate attacks. She had no dysmorphic or systemic features suggestive of myotonic dystrophy.

The long exercise (5minutes) and cooling repetitive stimulation testing were only mildly positive. In contrast muscle cooling down to 20^{o}C with needle EMG was very suggestive of the transition of changes that have previously been reported in PMC and she had associated clinical symptoms which took several hours to resolve.

She responded well to low dose mexiletine reporting after 3 days that it was the first time in the last 20 years she had not woken in the morning with muscle cramp and pain.

In terms of family history it was known her son and daughter had similar symptoms. They were from a large Sicilian family and her Australian neurologist was making attempts to identify affected members of preceding generations.

Patient No. 4 (EC)

A male infant referred to Great Ormond Street Hospital at the age of 15 months with hypotonia. No specific cause had been identified and there was no family history of note. EMG had shown unexpected but clear myotonic discharges. Myotonic dystrophy had been excluded by the referring paediatric team.

Patient No. 6 (CM)

A forty-nine year old lady who was first symptomatic during her 3rd pregnancy at the age of 42 with muscle stiffness that was worse with stress and after exercise. EMG confirmed myotonia and profound CMAP drop after short exercise test. Her parents were unaffected, her sister reported vague symptoms but had normal neurophysiology, and 3 sons were asymptomatic.

Patient No. 13 (RT)

A forty-three year old Italian lady who experienced her first symptoms at the age of twenty. There was evidence of clinical and EMG myotonia, affecting mainly her limbs but also face, neck and abdominal muscles, triggered by cold. She reported episodes of muscle paralysis triggered by exercise and cold lasting hours. There was proximal and distal limb muscle wasting with moderate to severe proximal and mild to moderate distal muscle weakness. Repetitive stimulation caused a 33% CMAP reduction on electrophysiological testing. There was no family history of note.

Hyperkalaemic periodic paralysis – non-genotyped cases

Of the remaining four/seventeen patients (see genotyping section 4.1 and table 11) categorized as having a phenotype consistent with possible hyperkalaemic periodic paralysis SCN4A mutations were not identified in any. In one a pathological expansion of the ZNF9 gene associated with DM2 was present. The remaining three had some atypical

clinical features and additional genes were or are being sequenced by other laboratories. Their clinical features are considered in detail.

Patient No 14 (SR)

A 24 year old man previously under the care of paediatric colleagues until the age of 18. He had been investigated extensively for myopathy but no specific cause was found and he was given a diagnosis of unspecified congenital myopathy. He described brief episodes of lower limb weakness that occurred mainly in the cold including one episode where he fell down some stairs due to his legs giving way. There was some myalgia but no particular history to suggest myotonia. Symptom severity did seem to be fluctuant and from the ages of approx 12-14 his limb weakness had been so severe he had required a wheelchair but this had improved and at the time of his review in clinic he was independently ambulant and attending college.

On EMG no myotonic discharges were demonstrated. After muscle cooling a reduction in CMAP amplitude with increase in CMAP area was observed. At the time the significance of this was unclear and it was thought to possibly be pathological. Later studies by Dr Veronica Tan (consultant neurophysiologist at the NHNN) of large numbers of patients with skeletal muscle channelopathies and healthy volunteers showed this change to actually be a normal variant. This man had no prior family history of myopathy. He was one of four children and his younger brother was affected with a very similar clinical course to his own.

During the write up of this thesis I was advised a congenital myasthenia mutation has been identified in this patient's brother by another lab but I do not have access to the formal report. Confirmation of the same mutation in this patient is apparently pending but is very likely to be the explanation for his symptoms.

Patient No 16 (LD)

A 51 year old male born 7 weeks prematurely. Delayed walking (not until approx 2 years) was observed and he was never able to run or keep up with peers physically due to a feeling of weakness in his arms and legs. From the age of twenty he avoided stairs due to limb weakness. There was very slow progression in symptoms until age thirty four

following a flu like illness when he complained of worsening muscle weakness. Since this episode he has continued to complain of episodes of worsening limb weakness 2-3 times a year. Clinical examination at age 35 showed ptosis, external opthalmoplegia and proximal limb weakness with significantly depressed reflexes. At age 38 he presented with an episode of bulbar weakness requiring a nasogastric tube and ultimately a percutaneous endoscopic gastrostomy(PEG) tube for feeding. However there was spontaneous resolution after 6 months with eventual removal of the PEG. He continues to have episodes of limb weakness, with some fatigue and dysphagia in addition to permanent proximal myopathy.

Electrophysiological tests showed myopathic potentials and repetitive nerve stimulation showed some decrement in CMAP. Stimulation single fibre EMG showed abnormal jitter and blocking. A McMannis test for periodic paralysis was positive. Muscle biopsy showed non-specific myopathic changes only.

There is no family history of similar illness or symptoms. He is one of four children (2 brothers and one sister).

Patient No 17 (EP)

A forty eight year old woman who complained of episodes of paralysis from early childhood (at least age five). In addition she suffered from exercise intolerance, pain and stiffness especially in the cold, which rendered her unable to walk. She had been given a previous diagnosis of fibromyalgia and investigations at another centre had included testing voltage gated potassium channel antibodies which were positive. Two EMGs performed at our centre on separate occasions did not show any evidence of neuromyotonia or myotonia. A Mcmannis test was positive on the first occasion and negative on the second.

This lady extensively researched her family history and provided the following details (as quoted from her notes):

Her son – now 11 yrs

- "At a few hours old, possibly on the 2nd day, was taken away as he was not breathing properly."

- "Many floppy episodes, but also has Congenital Adrenal Hyperplasia (CAH), therefore cannot distinguish what may have been PP or CAH."
- "Complains of pains in legs after sport and on waking day after exercise, can't walk properly. Sometimes, pains in legs apparent w/o exercise (like football, swimming etc), although walking, stairclimbing will have been done."
- "Complains of pains in legs and feet on walking distance, though none at outset."
- "On holiday Cyprus, Dec 03, aged 6, was swimming in a freezing swimming pool. He swam for a short while and then just seized up and sank to the bottom and lay there unmoving. I had to dive in and drag him out."

Her daughter – now 16 yrs:

- "As a toddler, had many floppy episodes following play. Eventually went into hospital for week, but attacks did not occur. No diagnosis made. Tested for narcolepsy, revealed unusual brainwave pattern, but not connected to episodes."
- "Complains of pains in limbs after sport, especially in the arms."
- "Had disabling pain in legs as 8 yr old ?osteomyelitis, kept in hospital."
- "Age 15 had ?viral illness – couldn't walk, pain in back too severe to move. MRI scan done, revealed Parr's (sic) Process in formation of spine, but not responsible for inability to walk."
- "Age 13 -16 suffered intermittently with chronic fatigue type disorder, floppy aching limbs, nausea, headaches, GI disturbances. Missed a lot of school. Coincided with a passion for eating tomatoes."

Her mother – died in 1997, aged 79, cause of death: myocardial infarction

- "My mother had persistent weakness, mostly I recall of her arms. She also was very fatigued, and needed to sleep during the day. I had little contact with her after the age of 18."

Her brother – now 63 yrs:

- "Left home as I was born, joined the Royal Navy, and settled in Scotland where he still lives. I do not know him very well."

- "He is my half brother – we share a mother."
- "Admits he has suffered from a disabling pain and weakness condition for over 30 years. Does not like to talk about it at present, but admits he has seen many doctors with no positive outcome. He is also very intolerant of illness, but spends his life having sporadic episodes of intense pain with weakness, which then 'clear up'. He suffers daily now."

Her maternal grandmother – died aged 36 yrs in 1930

- "Was described as 'very weak"

Overall the majority of cases studied with a PMC or SCM phenotype could be genotyped by analysing known disease causing genes. A small minority however could not. Cases 3 and 6 (Table 11) particularly case 3 have phenotypes very suggestive of a sodium channel myotonic disorder with EMG confirmation of myotonia but no mutation identified. Other genetic mechanisms e.g. large scale re-arrangements have not been excluded by the direct sequencing I performed and remain a plausible explanation. Such a large family however with several living generations of both affected and unaffected individuals offers an ideal opportunity to consider the possibility of a new disease causing gene.

The four cases classed as a phenotype of hyperkalaemic periodic paralysis did have some atypical features and the majority of typical cases were again accounted for by mutations in SCN4A (and hence not selected for further study or described here). In one case a diagnosis of DM2 was made which was somewhat unexpected from the predominant complaint of episodes of severe muscle weakness although the description of myotonia would be consistent with this diagnosis. The remaining three cases were selected as other atypical phenotypes are beginning to be described including congenital myasthenia with periodic paralysis(Tsujino *et al*., 2003) and more recently an atypical periodic paralysis phenotype with a ryanodine receptor mutation(Zhou *et al*., 2010). The more of these atypical cases described and genotyped the clearer a picture of their phenotype will hopefully emerge, providing future guidance both in terms of diagnosis and understanding pathogenesis.

4.2 Genotyping hypokalaemic periodic paralysis

All the mutations known to cause hypoPP had been noted to occur exclusively in the S4 segments of either Cav1.1 or Nav1.4. The pathophysiology of hypoPP is incompletely understood but in 2007 an aberrant cation pathway was identified in two of the hypoPP SCN4A mutations by two independent groups(Sokolov *et al*., 2007; Struyk *et al*., 2007). It was proposed that this pathway was integral to the pathomechanism of disease and offered a possible explanation as to why the mutations seemed to exclusively affect voltage sensors. At this time only four of the eight voltage sensors of both channels had been implicated by pathogenic mutations.

I hypothesized that if the aberrant cation pathway was integral to the disease mechanism then all voltage sensors could potentially be implicated in disease. I therefore sequenced the coding areas of CACNA1S and SCN4A that corresponded to all the voltage sensors in selected hypoPP cases in whom none of the common mutations had been found.

By searching the channelopathy database I identified and screened a group of 83 patients with a clinical diagnosis of hypoPP. In 64 of these, previously reported common mutations, either R528G/H (25 cases) or R1239G/H (39 cases) in CACNA1S (DII/S4 and DIV/S4), were identified via the clinical diagnostic service. I analysed DNA from the remaining 19 cases. After confirming common mutations were negative I looked for the less common mutations in exons 12, 13 and 18 of SCN4A(DII/S4 and DIII/S4) known to also be associated with hypokalaemic periodic paralysis, hypoPP 2. Six of these were positive for mutations that have been reported previously, namely the substitutions R672C/H/S and R1132Q in SCN4A. This left 13/83 patients in whom the causative genotype was unknown.

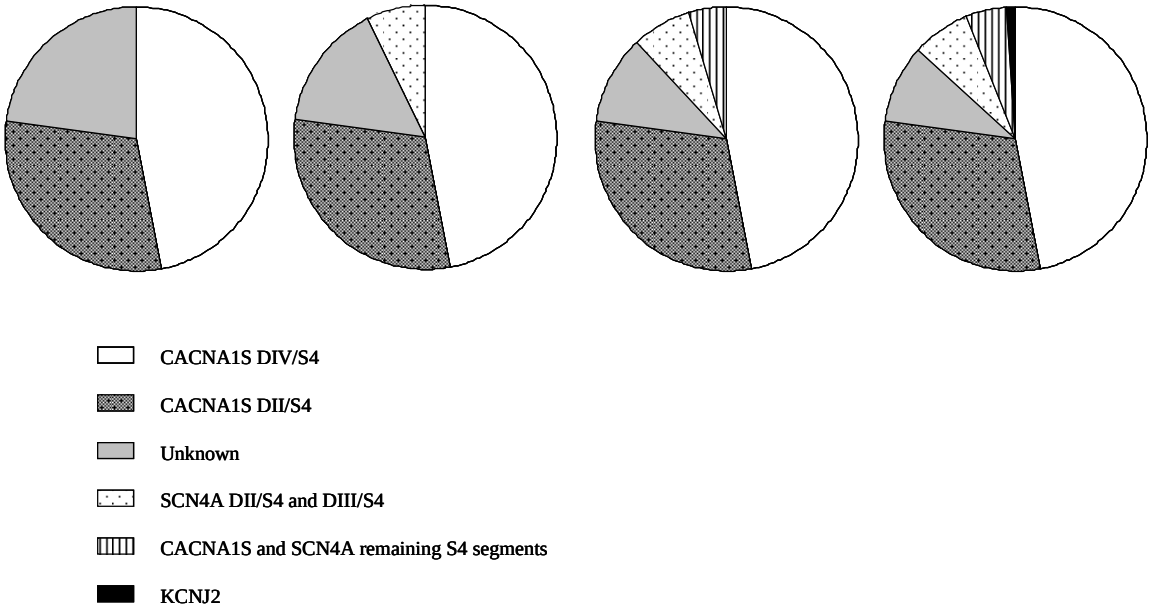


Fig 20: Illustration of diminishing number of patients with a phenotype of hypoPP in whom no genetic diagnosis could be made with escalation of genetic analysis

Hypokalaemic periodic paralysis – new mutations

In 4/13 cases three novel mutations were found, all of which neutralized arginine residues in S4 segments. These mutations were absent from 240 control chromosomes. One mutation was in the S4 segment of domain III (DIII/S4) of CACNA1S: c.2700G>T; p.R900S. The other two mutations neutralized arginine residues in S4 segments of SCN4A; the first in DI c.664C>T;p.R222W and the second in DIII c.3404G>A;p.R1135H. R222W was found in two apparently unrelated kindreds. All four individuals with the new mutations had a typical HypoPP phenotype. Age at onset of attacks of muscle paralysis was in the second decade, with attacks usually occurring at night or in the early morning, and associated with low serum potassium levels or with provocative factors that would induce low serum potassium. The frequency and severity of attacks were not different from those in individuals with previously reported

mutations. To my knowledge, these individuals received no pharmacologic therapy or potassium supplementation only.

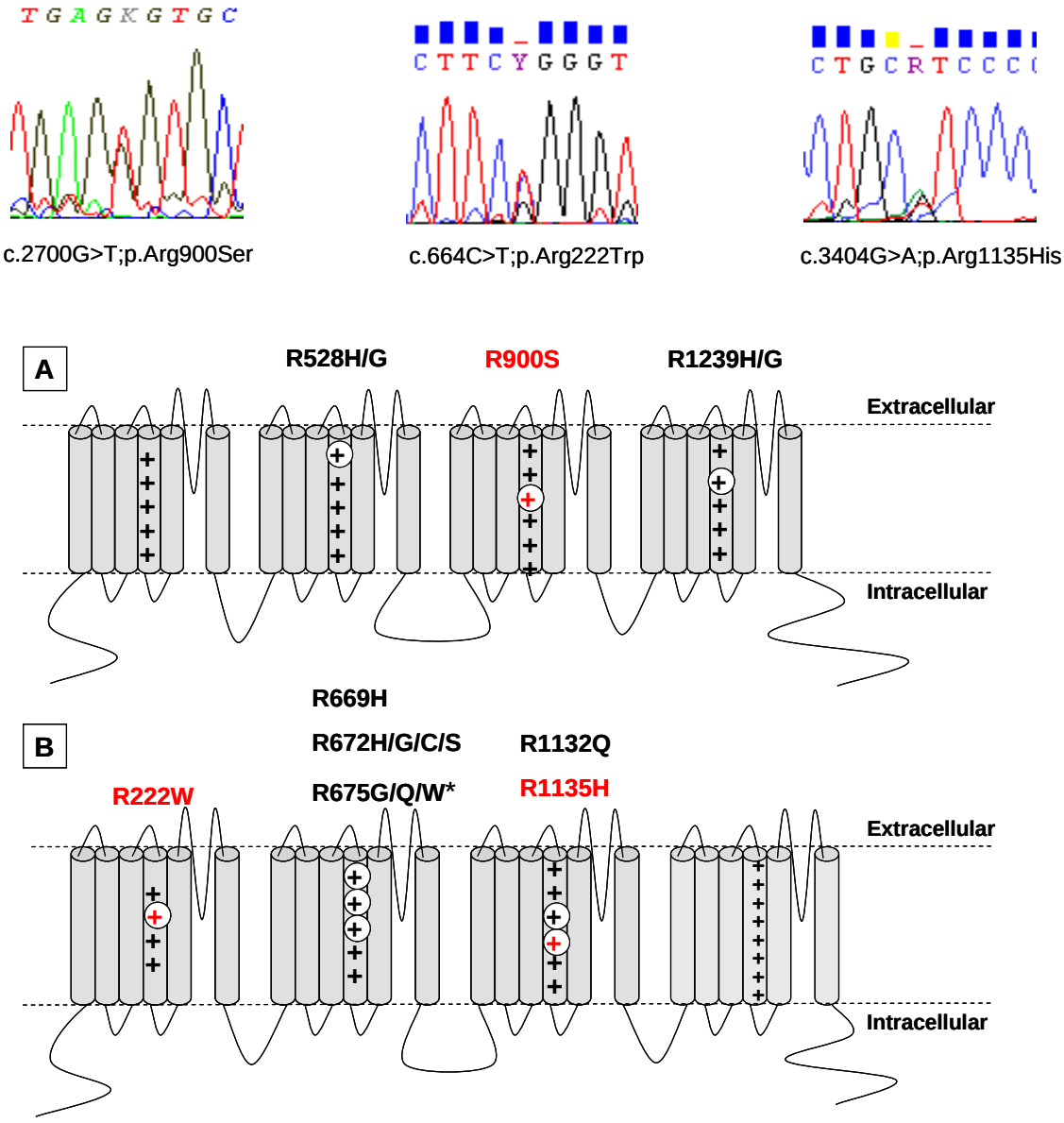


Fig 21: Electropherograms of new mutations with their relative positions in A: Cav1.1 and B: Nav1.4 shown alongside previously described hypoPP mutations. *mutations associated with an atypical phenotype of potassium sensitive normokalaemic periodic paralysis

I then analysed DNA from the remaining 9/13 cases for the entire coding region of CACNA1S and the coding regions of SCN4A that correspond to the S1-3 segments of Nav1.4. The incomplete sequencing of SCN4A was due to limited available DNA. I took a directed approach to the sequencing hypothesizing that if the voltage sensors themselves were not implicated it was possible that the S1-3 segments would be as theoretically this may produce a similar cation leak. I found no further mutations with this analysis.

Finally I analysed the entire coding region of KCNJ2 associated with ATS. A novel mutation was identified in 1/9 cases. Control chromosomes were sequenced but none carried the mutation which was highly conserved and predicted to be a deleterious amino acid substitution. Eight cases remained in whom no mutation was identified.

Gene	Mutation	No of Cases
CACNA1S	R528G/H	25
CACNA1S	R1239G/H	39
CACNA1S	R900S*	1
SCN4A	R672C/H/S	4
SCN4A	R1132Q	2
SCN4A	R222W*	2
SCN4A	R1135H*	1
KCNJ2	V223E*	1
Unidentified		8

*denotes new gene mutations, not previously reported.

Table 12: Summary of genetic results for 83 patients with a phenotype of hypoPP

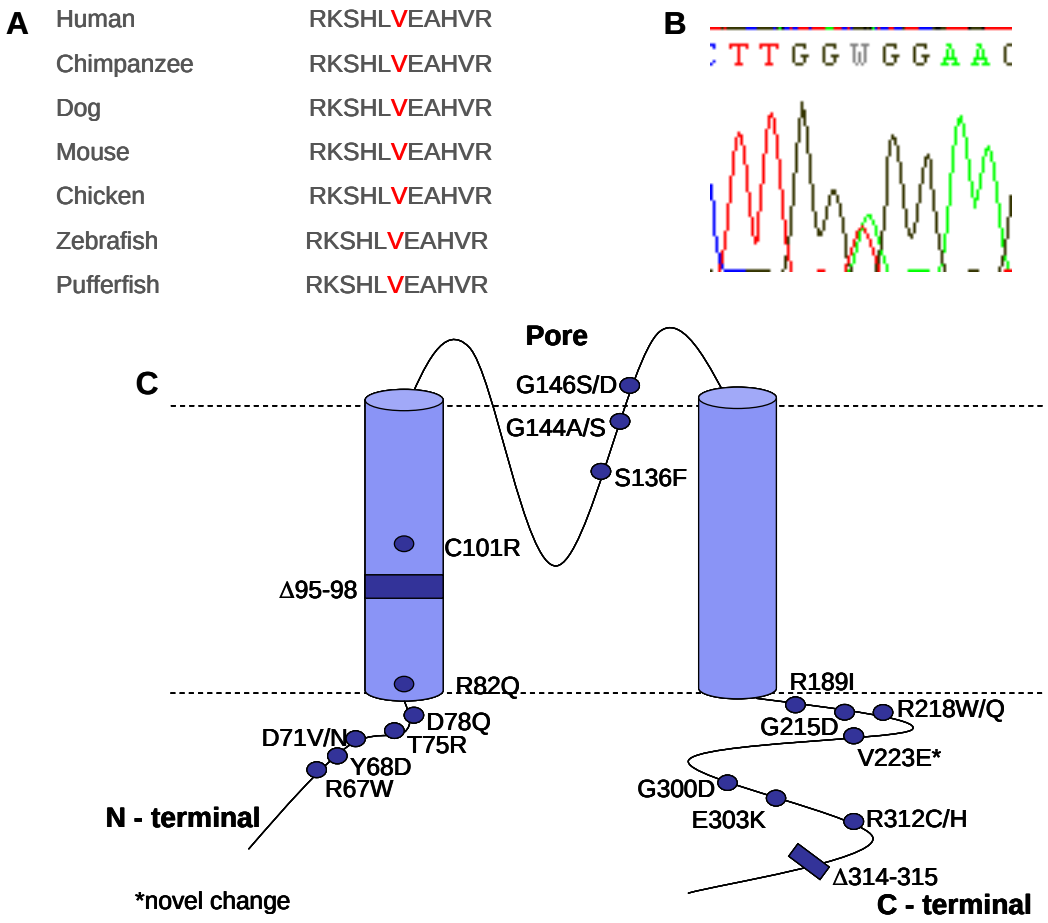


Fig 22: Conservation across species, electropherogram and diagrammatic representation of Kir2.1 illustrating the novel sequence change V223E.

Ninety percent of this cohort of hypoPP carried voltage sensor mutations that neutralized a key arginine residue. Not only does this data emphasise that there is a greater spread of residues (both intra and extracellular) and domains implicated in the pathogenesis of hypoPP but provides clear genetic support for the gating pore hypothesis. The remaining 10% also display typical phenotypes however and while alternative genetic mechanisms remain unexplored this small but significant minority of patients is a tempting pointer to other disease causing genes.

Results – Treatment Response

5.1 Genotype influences treatment response in hypokalaemic periodic paralysis

The mechanism of action of acetazolamide in hypoPP is not understood although it is generally used as the first line therapeutic agent. Despite this there has never been a randomized controlled trial to determine its efficacy, partly due to logistical difficulties given the relative rareness of the disease. There have been several reports of patients with SCN4A mutations (hypoPP2) responding deleteriously to acetazolamide therapy. However other reports have disputed this. With the additional discovery of the aberrant cation leak (including protons) with certain Nav1.4 mutations it is feasible that disturbed pH homeostasis may offer a mechanism to explain why acetazolamide, a carbonic anhydrase inhibitor could be beneficial. However histidine substitutions were shown to produce a proton selective gating pore whereas glycine substitutions allowed a larger and less selective cation leak, suggesting any disruption in pH that occurs and therefore any amelioration offered by acetazolamide, may not be equal for all mutations. Whether a similar hypothesis applies to mutations of Cav1.1 is unclear as no functional work has yet been carried out to determine the presence or absence of a gating pore.

Literature and patient review

As my aim was to determine if genotype influenced the response to acetazolamide only cases of genetically confirmed hypokalaemic periodic paralysis were selected (cohort 1) from the published literature. Given the relative rarity of hypoPP, I conducted a literature search on Pubmed (www.ncbi.nlm.nih.gov) using the key terms “hypokalaemic periodic paralysis”, “periodic paralysis”, “acetazolamide” and “carbonic anhydrase inhibitors”. Only articles published in English in or after 1994 (when the genetic basis of hypoPP was identified) were considered. Articles meeting these criteria were reviewed and any reported treatment and treatment response were recorded (Table 13). The data in table 13 was then used to summarise the response to acetazolamide by genotype (Table 14). Other

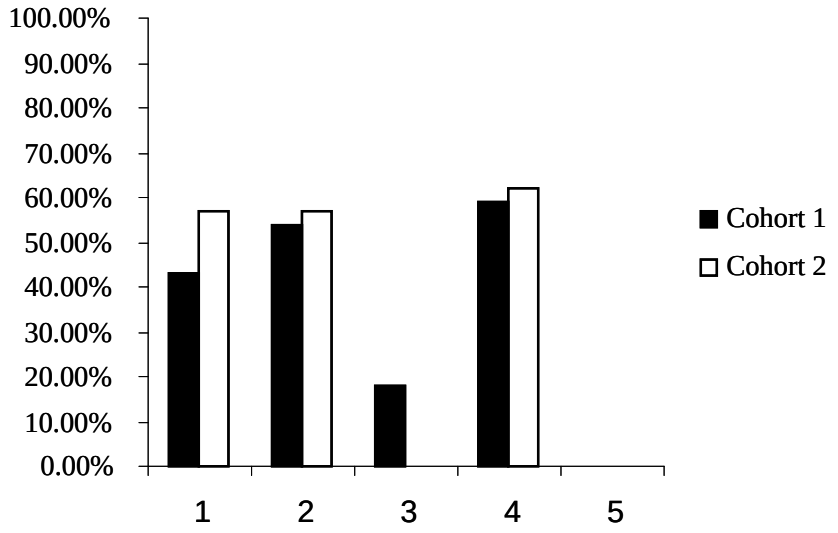
treatments were reported but only the response to acetazolamide was summarised as this was the most frequently used and the numbers treated with alternatives were very small. In order to create a comparison group to test the validity of the findings and to attempt to avoid introducing any bias, case records of patients with genetically confirmed hypoPP attending the neuromuscular clinic at NHNN under the care of Professor Hanna were reviewed (cohort 2) by a visiting fellow Dr Simona Portaro. Patients were reviewed at clinic on a six monthly or once yearly basis. Documented frequency, severity and duration of paralytic attacks before and after therapy with acetazolamide was analysed to ascertain response as either beneficial, detrimental or no change. I used this data to summarise the response to acetazolamide by genotype in the same way as for cohort 1 (table 15).

Patients with SCN4A substitutions at the R675 position who had received acetazolamide therapy were identified in each cohort. The published phenotype of these mutations however has been described as potassium sensitive, normokalaemic periodic paralysis and not hypoPP. I met patients from a single kindred in cohort 2 carrying the R675G SCN4A mutation many times and they did also demonstrate potassium sensitivity and normal serum potassium levels during an attack of paralysis. As such they did not display a typical hypoPP phenotype and after consideration all patients carrying SCN4A R675 substitutions were excluded from analysis as the aim was to determine the efficacy of acetazolamide in hypoPP as accurately as possible within the limitations of a retrospective analysis.

Response to acetazolamide by genotype

In cohort 1, 54% (21/39) of patients with a CACNA1S mutation treated with acetazolamide reported a beneficial response, compared with only 18% (3/17) of those with an SCN4A mutation. In the combined cohort this was a benefit for only 43% (24/56) of patients. However, if only patients with the most common HypoPP mutations (R528H, R1239H) were considered, the benefit improved to 59% (20/34). In cohort 2 the findings were similar; with 57% (8/14) of patients with CACNA1S mutations which also

represented the cohort as a whole, reporting a beneficial response. The positive response rate increased to 62% (8/13) when the R528H and R1239H group were considered alone. Closer evaluation of the relationship between precise genotype and treatment response showed that in both cohorts when one of the arginine residues located at the extracellular side of the sarcolemma (see Fig 21) was substituted for a glycine residue (R528G, R1239G, R672G), there was never a beneficial response to acetazolamide, and there was often a deleterious effect reported.



1. All patients in cohort
2. CACNA1S mutations only
3. SCN4A mutations only
4. Common CACNA1S mutations only
5. Extracellular glycine substitutions

Fig 23: Histogram depicting the response rate to acetazolamide by genotype for each cohort

This data is retrospective and imperfect, but it does provide some indication of response rates to acetazolamide and tentatively suggests a correlation with genotype i.e. R to H substitutions respond the best and R to G substitutions respond poorly. It is likely the poor response rate in the SCN4A group also reflects this as the amino acid substitutions are much more varied compared to the CACNA1S group where they are predominantly R to H. Overall however only approximately 50% of hypoPP patients are adequately treated by the current first line therapeutic agent, acetazolamide. This represents a significant unmet need in terms of treatment. Both randomized controlled trials and development of alternative therapies are required.

Gene	Mutation	No. of genotyped patients in series who received treatment	Treatment Given	Response to Treatment	Ref
CACNA1S	R528G	NS	KCL	Aborted attacks of paralysis	(Wang *et al*., 2005)
CACNA1S	R528G	1	Acetazolamide 250mg b.d + potassium salts Combination of spirinolactone 25mg b.d, amiloride 5mg b.d and potassium salts	No response to acetazolamide Combination of diuretics and potassium reduced frequency and severity of attacks	(Kil *et al*., 2010)
CACNA1S	R528H	1	Low carbohydrate diet, potassium 40meq od and acetazolamide 250mg tds.	Reduced frequency and severity of attacks.	(Hecht *et al*., 1997)
CACNA1S	R528H	4 (1 kindred)	Acetazolamide discontinued for a trial of verapamil versus placebo.	Baseline response to acetazolamide not reported. Two patients reported significant improvement with verapamil, and two no response.	(Links *et al*., 1998)
CACNA1S	R528H	5 (3 kindreds)	Dietary modification(3pts) OR Acetazolamide(1pt) OR Spirinolactone(1pt)	All patients attack free following treatment	(Kim *et al*., 2001)
CACNA1S	R528H	8	Acetazolamide	Beneficial for 5 pts No effect on symptoms for 3 pts	(Sternberg *et al*., 2001)
CACNA1S	R528H	1	Acetazolamide, Spirinolactone, Potassium Aspartate and Potassium Chloride	Little effect on symptoms from any of the listed medications.	(Kawamura *et al*., 2004)
CACNA1S	R528H	NS	Acetazolamide	Beneficial for most pts treated. One reported worsening of symptoms	(Miller *et al*., 2004)
CACNA1S	R528H	5 (no of kindreds NS)	Acetazolamide	Beneficial for 2 pts No effect for 3 pts	(Kim *et al*., 2007)
CACNA1S	R1239H	5 (1 kindred)	Acetazolamide and potassium supplements continued in 4/5 pts consenting to a trial of verapamil versus placebo	Baseline response to acetazolamide not reported but no additional response to verapamil experienced.	(Links *et al*., 1998)
CACNA1S	R1239H	13	Acetazolamide	Beneficial for 8 pts No effect on symptoms for 4 pts Deleterious effect on symptoms for 1 pt	(Sternberg *et al*., 2001)
CACNA1S	R1239H	4 (1 kindred)	KCL and Acetazolamide	Beneficial for 3 pts. No effect for 1 pt but medication taken irregularly	(Kusumi *et al*., 2001)
CACNA1S	R1239H	NS	Acetazolamide	Beneficial effect for most pts treated	(Miller *et al*., 2004)
CACNA1S	R1239H	1	Acetazolamide	No effect	(Kim *et al*.,

					2007)
CACNA1S	R1239G	1	Spirinolactone 25mg bd	Improved symptoms Note: 2 clinically affected cousins reported no effect with ACZ treatment but an improvement with spirinolactone. Genetic analysis was not performed on these 2 pts.	(Kim *et al*., 2005)
CACNA1S	R1239G	3 (1 kindred)	Acetazolamide OR a combination of spirinolactone, amiloride and potassium supplements	No effect of ACZ for 2 pts Deleterious effect of ACZ for 1 pt but then treated successfully with a combination of diuretics and potassium supplements	(Kim *et al*., 2007)
CACNA1S	R897S	1	Potassium supplements 2-3mmol/kg/day and acetazolamide 5-10mg/kg/day	Treated from age 2-6years. Frequency and severity of attacks reduced but duration of attacks increased.	(Chabrier *et al*., 2008)
SCN4A	"Sodium channel mutations" NO	4	Acetazolamide	Deleterious effect	(Miller *et al*., 2004)
SCN4A	R669H	2	Pt 1: Dietary regime, potassium supplements and a carbonic anhydrase inhibitor (not specified)	Reduced frequency of attacks	(Bulman *et al*., 1999)
			Pt 2: acetazolamide 250mg bd	Attack free for 2 months then one severe attack responding to potassium infusion. No long-term response documented	
SCN4A	R669H	1	Acetazolamide	No response	(Kim *et al*., 2007)
SCN4A	R669H	1	Potassium supplements	No attacks during 12 month follow up period	(Maffe *et al*., 2009)
SCN4A	R672G	3 (1 kindred)	Acetazolamide	Deleterious effect	(Sternberg *et al*., 2001)
SCN4A	R672G	1	Acetazolamide OR Combination of spirinolactone, amiloride and potassium supplements	Deleterious effect of ACZ but then treated successfully with a combination of diuretics and potassium supplements	(Kim *et al*., 2007)
SCN4A	R672C	NS	Either acetazolamide 500mg a day OR dichlorphenamide 100mg a day	Attack free for at least 6 months on average	(Kim *et al*., 2004)

SCN4A	R672C	1	Acetazolamide	No response	
SCN4A	R672S	3 (1 kindred)	acetazolamide triamterene	Severe quadriparesis within 12 - 60 hours of acetazolamide. Reduction in frequency and severity of attacks with Triamterene	(Bendahhou *et al*., 2001)
SCN4A	R672S	1	Acetazolamide 250mg bd and spirinolactone 100mg per day	Reduced frequency of attacks	(Davies *et al*., 2001)
SCN4A	R672S	1	Acetazolamide 250mg per day and potassium taken at onset of attack or before strenuous exercise	Reduced frequency of attacks	(Venance *et al*., 2004)
SCN4A	R675G	5 (1 kindred)	Acetazolamide 250mg per day	1 pt no response to 10 years continuous treatment 4 pts reported a reduced duration of attacks with intermittent ACZ treatment	(Vicart *et al*., 2004)
SCN4A	R675Q	2 (2 kindreds)	Acetazolamide 500mg per day	Pt 1: no benefit Pt 2: attack free since taking ACZ (length of treatment not documented)	(Vicart *et al*., 2004)
SCN4A	R675W	1	Acetazolamide 250mg per day	Reduced frequency and severity of attacks	(Vicart *et al*., 2004)
SCN4A	R1132Q	1	Acetazolamide Potassium peri-attack	No response to acetazolamide Potassium ingestion reduced duration of attack	(Carle *et al*., 2006)

NS: Not Specified

Table 13: Details of response to therapy for genetically confirmed cases of hypokalaemic periodic paralysis reported in the literature.

Gene	Mutation	Total number of genotyped pts treated with ACZ	Beneficial effect	Deleterious effect	No response
CACNA1S	All mutations	39	21	2	16
CACNA1S	R528G	1	0	0	1
CACNA1S	R528H	16	9	0	7
CACNA1S	R1239H	18	11	1	6
CACNA1S	R1239G	3	0	1	2
CACNA1S	R897S	1	1	0	0
SCN4A	All mutations	17	3	11	3
SCN4A	Mutations not specified	4	0	4	0
SCN4A	R669H	2	1	0	1
SCN4A	R672G	4	0	4	0
SCN4A	R672C	1	0	0	1
SCN4A	R672S	5	2	3	0
SCN4A	R1132Q	1	0	0	1

Table 14: Summary of response to acetazolamide by genotype for cohort 1 (published cases)

Gene	Mutation	Total number of genotyped pts treated with ACZ	Beneficial effect	Deleterious effect	No response
CACNA1S	All mutations	14	8	2	4
CACNA1S	R528H	6	4	2	0
CACNA1S	R1239H	7	4	0	3
CACNA1S	R1239G	1	0	0	1

Table 15: Summary of response to acetazolamide by genotype for cohort 2 (UK cases)

Results - Pathophysiology

6.1 How does neutralisation of an intracellular voltage sensor arginine of Nav1.4 influence the pathomechanism of hypoPP?

It is known that the common mutations in CACNA1S and SCN4A associated with hypoPP produce "loss of function" effects on the alpha pore of Cav1.1 and Nav1.4 although this has failed to explain the mechanism of paradoxical membrane depolarisation of the muscle membrane in the presence of hypokalaemia. The gating pore hypothesis has offered some new insights into the pathophysiology of hypoPP. It is noteworthy however that not all the voltage sensors have equal numbers of positive charge and it is likely that each voltage sensor has additional specificities. As the voltage sensor moves across the membrane in response to changes in membrane potential it has been observed that mutations of variably placed arginines determine whether the aberrant gating pore occurs at depolarised or hyperpolarised potentials.

Mutations of one arginine, R675G/Q/W in DII of Nav1.4 produce a unique phenotype of potassium sensitive normokalaemic periodic paralysis. Mutations of this arginine, which is placed more toward the intracellular side of the membrane, have recently been shown to create a cation leak at depolarised potentials(Sokolov *et al*., 2008). What is unknown is whether mutations of these inwardly placed arginine residues also produce a loss of function effect on the alpha pore.

To investigate the function of the alpha pore, I carried out whole-cell patch clamp recording in cultured mammalian HEK293 cells. Patch clamp recordings were made from HEK cells co-transfected with human wild type SCN4A or a mutant construct containing the R675G mutation and cDNA encoding human SCN1B, the sodium channel beta subunit, which although not contributing directly to the main pore of the Nav1.4 channel, has been shown to be important for regulating sodium channel function(Isom., 2001).

Effects of R675G on the Nav1.4 alpha pore

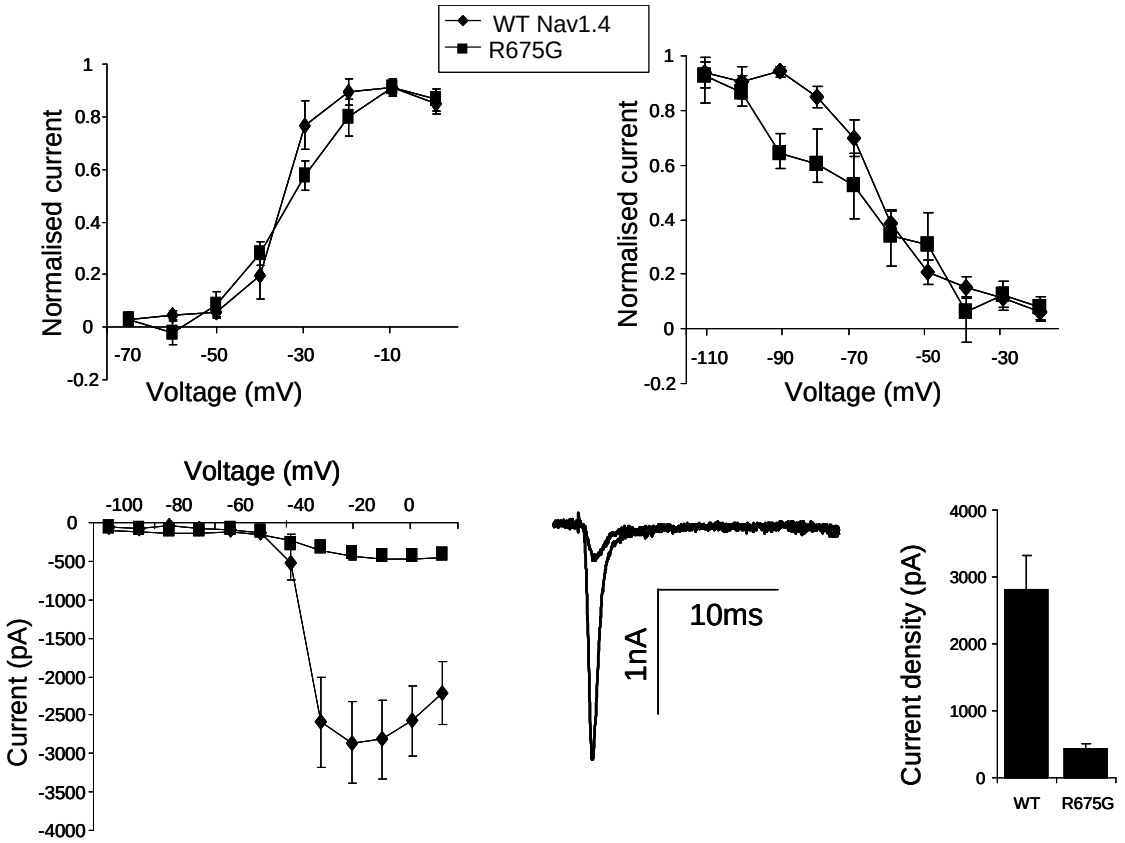


Fig 24: Comparison of activation, inactivation, peak current and current density observed for the R675G mutation and WT Nav1.4

A significant reduction (6 fold) in peak current and current density was observed for the mutant in comparison to the wild type (Figure 24). There was no difference in the voltage dependence of activation. There was a suggestion of enhanced inactivation for the mutant but this was not significant, partly because the small currents produced by SCN4A-R675G were too close to the noise threshold for accurate measurements of inactivating currents.

The large reduction in peak transient currents is consistent with the effects on the alpha pore observed for extracellular voltage sensor mutations (R669H, R672G/H/S). All of these mutations give reduced current density accompanied by small or non-significant effects on inactivation. The 6-fold reduction in current density for R675G was greater than that reported for the other mutants (2 to 4 fold reduction).The small differences in the kinetics of inactivation introduced by these mutants have been discussed in the literature, and there is a consensus that these kinetic changes cannot explain the significant reduction in current density that is observed(Struyk *et al*., 2000; Kuzmenkin *et al*., 2002). Although alternative hypotheses have been postulated, such as altered protein folding or trafficking, no work has been carried out to date to address this specific question.

In channels containing mutations affecting external arginines, the gating pore current has been estimated to be between 1% and 0.02% of the current through the main alpha pore(Cannon., 2010). Given the significantly reduced current density seen in R675G of approximately 500pA, any leak current of this size would not have been detectable in these recordings, as the noise inherent to whole cell currents is greater than 5 pA rms (1% of 500 pA).For this reason although my data show a robust loss of peak current through the alpha pore, I was unable to assess whether the R675G mutation also introduced a leak current.

6.2 Developing a human myoblast expression system

All published work examining gating pore currents from SCN4A mutants has been in xenopus oocytes where very large currents can be obtained. I carried out recordings in HEK cells, which allow accurate clamping of peak alpha pore currents, but the total current amplitudes were not sufficient for investigation of leak currents. While both of these model systems have advantages, they may not replicate the physiology of muscle cells sufficiently to recapitulate the mechanism of muscle channelopathies. For example, mutations in SCN4A may be modified by endogenous accessory subunits present only in muscle cells, or muscle cells may have specialized trafficking or expression machinery for regulating highly-expressed channels like SCN4A. In addition the pathology of

SCN4A mutations may involve buffering (possibly via carbonic anyhydrases inhibited by acetazolamide). Neither HEK cells or xenopus oocytes would test these sorts of muscle-specific mechanisms. Therefore, I investigated whether it was possible to culture human myoblasts and record currents in patch clamp configuration.

Preliminary recordings were made using a myoblast culture from a healthy control teenager that was kindly donated by Dr Carl Adkin working at the Dubowitz Neuromuscular Centre. It was established that robust sodium currents can be recorded in these cells. Recordings made from isolated myoblasts plated on coverslips for only 48-72 hours allowed identification of modest sodium currents (Fig 25). When the voltage dependence of activation and inactivation of this current was compared with current obtained from HEK cells transfected with wild type Nav1.4 there were no detectable significant differences (see figure 25).

In addition to determine whether SCN4A was expressed endogenously in these cells I extracted RNA from an aliquot of the same myoblast culture and performed reverse transcription PCR. The cDNA obtained from this procedure underwent further PCR using primer pairs specific for SCN4A cDNA. An SCN4A clone was used as a positive control and genomic DNA as a negative control. Fragments of anticipated size for SCN4A cDNA were amplified for the SCN4A clone and cDNA obtained from the myoblasts. No product was obtained from the genomic DNA confirming the SCN4A gene was expressed in the cell culture which is consistent with Nav1.4 channels having contributed to the sodium current recorded

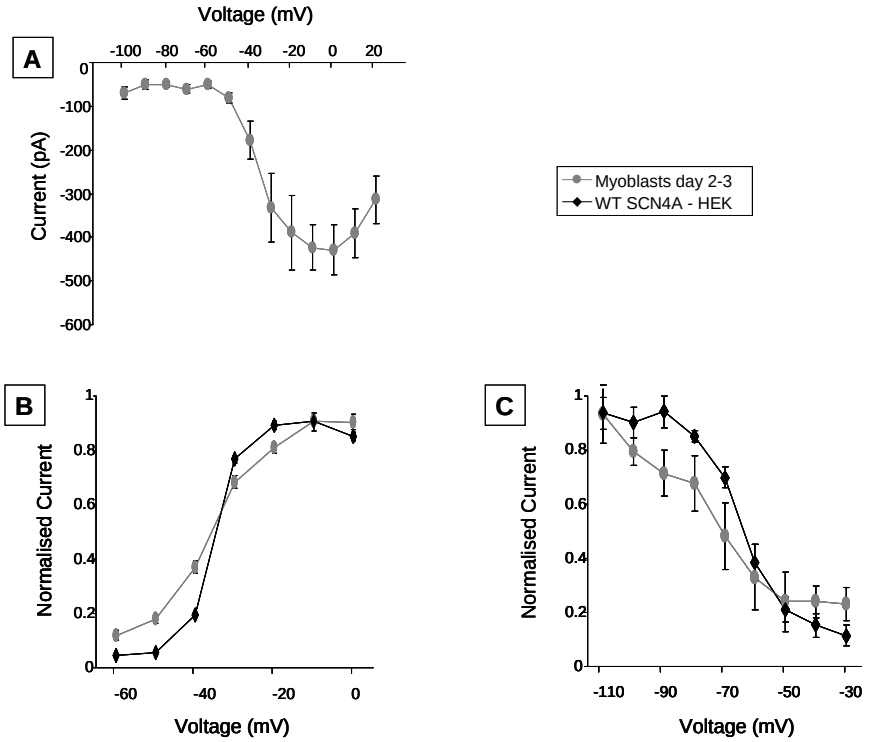


Fig 25: Current density and voltage dependence of activation and inactivation of sodium currents obtained after 48-72hours of human myoblasts in culture compared with HEK cells transfected with WT Nav1.4.

Culture time influenced the density of sodium current and also the voltage dependence of activation. There is a much larger sodium current (average 6nA) and a left shift in activation of sodium current recorded from more mature myoblasts (day 10) which is likely to reflect a contribution to the overall current from both Nav1.4 and Nav1.5 (which has a left shift of activation of approx 10-15mv compared to Nav1.4). These preliminary experiments have indicated low sodium extracellular solutions will be required to reduce sodium currents and to provide better clamp of the sodium current seen in mature myoblasts. In addition, given the likely contribution of the Nav1.5 channels, it may be necessary to apply tetrodotoxin to quantify the relative contributions of Nav1.4 (TTX sensitive) and Nav1.5 (TTX insensitive) to the overall sodium current.

Although myoblasts potentially offer a system for recapitulating much more of the muscle cell physiology, there are many technical considerations to be made and optimized before a myoblast system may be used to study the gating pore in voltage sensor mutations of Nav1.4. A major hurdle will be the need for obtaining muscle biopsies from willing patients who carry these mutations. However the early recordings made suggest endogenous Nav1.4 is expressed, and consequently characterization of sodium currents in these cells may be a viable albeit technically challenging future project.

Discussion - Phenotype

Aim

To examine the phenotype of the skeletal muscle channelopathies in patients referred to the NCG service for channelopathies at the NHNN to determine

c. if there were any clinically useful phenotype-genotype correlations that could be made and
d. if there were more extensive aspects to the phenotypes than is currently recognised.

Meeting patients through both the specialist muscle clinic and the natural history trial of non-dystrophic myotonia combined with analysis of the existing channelopathy database was an enormously valuable and rewarding experience. Using this approach I was able to identify several unexplored aspects of the phenotype that had clinically relevant correlations.

7.1 Observation of neonatal hypotonia in paramyotonia congenita due to the I693T SCN4A mutation

Neonatal paramyotonia congenita often presents with myotonic phenomenon when an infant cries or is washed, especially with cool water(Eulenberg A., 1886). Despite the recognition that myotonic symptoms may present at birth, this was not generally considered to be particularly detrimental to the neonate or to require any specific consideration in counseling expectant mothers. Recently a rare lethal neonatal paramyotonia case with extreme sensitivity to cold was reported and attributed to an SCN4A mutation (N1297K)(Gay *et al*., 2008). However, neonatal hypotonia had not been recognised as a clinical manifestation of muscle sodium channel dysfunction. The observations I gathered in the six individuals studied from four unrelated French and

English families indicate that neonatal hypotonia with additional symptoms of poor feeding and respiratory distress can be a manifestation of muscle sodium channel dysfunction common to individuals with the I693T sodium channel mutation. Pathological muscle hypotonia is an important neonatal presentation and can be a sign of either a peripheral or central abnormality. Careful diagnostic assessment and often multiple investigations are required(Laugel *et al*., 2007). Recognition of a sodium channel mutation as a cause of neonatal hypotonia may prevent unnecessary and invasive investigations. For example, a muscle biopsy is very unlikely to be contributory. EMG assessment has been shown to be beneficial in adults if performed according to specific protocols(Fournier *et al*., 2006; Fournier *et al*., 2004) but these are not easily applicable in neonates.

Treatment in all the cases studied was supportive as symptoms were self-limiting but it is important to recognize that symptoms may be cold-triggered and temperature dependent, indicating, a constant warm ambient temperature is essential in such cases.

All cases studied carried the I693T mutation. The detrimental effects of the I693T mutation on sodium channel gating are similar to other PMC mutations i.e. the overall effect is one of impaired inactivation(Plassart-Schiess *et al*., 1998). These detrimental effects were exacerbated in vitro by cold temperature but this is not an exclusive finding. Whether the association of neonatal hypotonia is entirely specific to the I693T mutation is not yet clear. Paramyotonia congenita is rare and it may be that neonatal hypotonia has occurred in infants carrying other mutations but the direct association with PMC not been made or reported.

7.2 Stridor expands the neonatal presentations of skeletal muscle sodium channelopathy

Initially it was hard to determine from a single case if the occurrence of stridor in the child with PMC was related to PMC itself. However discussion with Dr Damien Sternberg in France and his observations of stridor in two other cases was very suggestive and hypothetically it seemed likely that stridor could reflect laryngeal myotonia.

Hyperkalaemic periodic paralysis (HyperPP) is a dominantly inherited disorder which is allelic to PMC and is characterised by episodes of muscle weakness and myotonia, although weakness is the predominant symptom(Venance *et al*., 2006). In both these disorders gene mutation results in a "gain of function" sodium channel defect. It has been proposed that the two disorders reflect ends of a spectrum of the same disease(Cannon., 2006).

Stridor has been described in patients with myotonic dystrophy, in both neonates and adults.(Heuschkel *et al*., 2003; Ahmadian *et al*., 2002) In addition there are several reports of stridor in American Quarter horses affected by the equine form of hyperkalaemic periodic paralysis (HyperPP). In one series 63/68 affected horses suffered stridor in association with exertion, muscle weakness or excitement(Carr *et al*., 1996). There is also one case report of an adult with PMC in whom provocative testing with muscle cooling induced severe laryngeal myotonia with stridor(Ferriby *et al*., 2006). Overall it is likely the stridor reflects myotonia of the laryngeal muscles. In addition, the child described experienced feeding and respiratory difficulties which were also noted in the cases of neonatal hypotonia suggesting these are common neonatal features of PMC. Dysphagia and respiratory compromise are rarely reported in adult cases(Lerche *et al*., 1993).

Following on from the observations here, another report has recently been published of severe neonatal laryngospasm occurring in three neonates carrying de novo SCN4A mutations (G1306E and A799S). These infants had life threatening episodes of stridor with apnoea and feeding difficulties. Symptoms responded to sodium channel blockers although in one infant the diagnosis was made posthumously(Lion-Francois *et al*., 2010). Taken together these observations of hypotonia and stridor with resultant feeding and respiratory difficulty indicate that offspring of parents with sodium channel mutations are at risk of neonatal complications. This is an important development in understanding the phenotypes of skeletal muscle sodium channelopathies and should be reflected accordingly in the advice given to patients considering having children and the relevant clinicians involved in their delivery and early post-natal care.

7.3 Observation of inflammatory infiltrates in skeletal muscle channelopathies

The natural history of the skeletal muscle channelopathies is not extensively documented but development of a proximal myopathy is established particularly in the periodic paralyses(Becker PE., 1977; Nagamitsu *et al*., 2000; Miller *et al*., 2004; Links *et al*., 1990; Bradley *et al*., 1990; Plassart *et al*., 1996; Vicart *et al*., 2004). Whether the myopathy is related to the severity and frequency of paralytic attacks is unclear(Buruma *et al*., 1978; Links *et al*., 1990) but there is some evidence that its development may be associated with increasing age(Links *et al*., 1990; Plassart *et al*., 1994). In addition it is common for moderate to severe myalgia to be reported(Rosenfeld *et al*., 1997; Ptacek *et al*., 1994b; Colding-Jorgensen *et al*., 2006; Vicart *et al*., 2004; Fialho *et al*., 2007) and moderately raised creatine kinase(1-2000U/L)(Links *et al*., 1990; Colding-Jorgensen *et al*., 2006; Plassart *et al*., 1996; Fialho *et al*., 2007) to be noted in the skeletal muscle channelopathies although there is no evidence to link these to the presence or absence of myopathy.

A characteristic history in conjunction with specialised neurophysiologic techniques(Fournier *et al*., 2004) allows for a diagnosis in the majority of cases of skeletal muscle channelopathy and it is rare that a diagnostic muscle biopsy is now performed in the UK.

In the three cases I studied a clinical diagnosis of skeletal muscle channelopathy had been made prior to the muscle biopsy and was later genetically confirmed. Biopsy was performed due to additional clinical features that were deemed unusual or severe for a muscle channelopathy. In case one the proximal myopathy could have been attributed to the myopathy that is reported in periodic paralysis but the very high CK (2729U/L) was atypical for this. The severe myalgia and moderate elevation of CK (1109U/L) reported in case two are also described features of the channelopathies and it is possible that at least some of the myalgia is due to myotonia. However as the myalgia became severe enough to be the patient's primary complaint a biopsy was performed. The initial complaint of muscle swelling in case three is not a typical feature of the muscle

channelopathies and with the later development of significant myalgia a biopsy was felt to be warranted. Collectively the clinical features of weakness, myalgia and significantly elevated CK in these three cases suggest that these symptoms are secondary, at least in part, to the observed inflammatory process.

The biopsy findings in these cases did not fulfil all morphological criteria for IIM, particularly the lack of invasion of intact fibres. However upregulation of MHC class I expression (case I and III) is supportive and it is not unreasonable that a diagnosis of IIM had been made in these cases, especially when each was considered in isolation. There are numerous examples of other genetic muscle disorders in which inflammatory infiltrates have been observed and even diagnosed as IIM(Tidball *et al*., 2005). It is impossible to exclude the possibility that the observed inflammatory process is a co-incidental finding and two independent pathologies (channelopathy and IIM) are present in each of the three individuals, but statistically this seems unlikely.

Whether this inflammatory response plays a potential pathogenic role in the development of the myopathy is not known. Morphology data is reported for the skeletal muscle channelopathies, especially the periodic paralyses, but in many cases pre-dates the availability of genetic testing. A vacuolar myopathy and or tubular aggregates support the diagnosis of periodic paralysis. With improved electrophysiological and genetic diagnostic methods muscle biopsy is now less common. Without a larger number of biopsy samples from similar cases it is not possible to know how accurately inflammatory muscle infiltrates may correlate to clinical presentation.

Although there was some improvement in symptoms and a reduction in CK with prednisone in two cases, in one case high dose prednisone induced a severe paralytic attack, although low dose steroids were better tolerated. Worsening of the paralytic symptoms of periodic paralysis with glucocorticoids has been reported(rzel-Hezode *et al*., 2009a). Other immunosuppressive agents had no clear additional benefit in any of the three cases.

Based on these observations it is reasonable to propose that a diagnostic muscle biopsy should be considered in cases of skeletal muscle channelopathy presenting with the symptoms of severe myalgia and/or sub acute weakness and an accompanying elevated CK (>1000U/L) to ascertain the presence of any inflammatory infiltrates. The therapeutic

benefit of immunosuppressants is not established by this small number of cases but they do tentatively indicate steroids could have a role. The optimal dose however is undetermined and higher doses may be detrimental.
The statistical evidence indicates that the co-existence of an inflammatory process and skeletal muscle channelopathy is very unlikely to be co-incidental. The possible implications this may have for understanding of the mechanisms of muscle damage in the skeletal muscle channelopathies will require future exploration.

7.4 Pain and morbidity in the non-dystrophic myotonias

There are almost no studies reporting on the morbidity or quality of life in any of the skeletal muscle channelopathies. It was a personal observation that many of the patients I met however did report pain as a prominent feature of their illness. Data gathered from the patients I studied in the UK arm of the natural history study in the NDMs has illustrated pain is a common and prominent aspect of the clinical presentation. It also seems to be significantly more common in sodium channelopathies compared with chloride channelopathies. These findings support those recently published in one of the very few studies that has attempted to analyse morbidity in the channelopathies(Trip *et al*., 2009a). This is very suggestive that pain has been an under-recognised and potentially undertreated feature of the muscle channelopathies. These illnesses have often been considered to be benign but this data together with the increasing evidence for development of myopathy in the literature(Links *et al*., 1990; Buruma *et al*., 1978; Miller *et al*., 2004; Nagamitsu *et al*., 2000) would indicate quality of life deserves more consideration. Pain was not exclusively related to symptoms of muscle stiffness (myotonia) so it is not entirely clear if treating the myotonia would abolish all pain. However this early data does suggest the presence of pain should be a considered factor when assessing the benefits or initiation of drug therapy for the NDMs. Analysis of the combined cohort of patients across all centres participating in the natural history study will be important to further address this issue.

Conclusions: Phenotype

1. Neonatal phenotypes of the skeletal muscle sodium channel myotonic disorders exist. Severity is variable. Symptoms include hypotonia with feeding and respiratory difficulties (including stridor) that is transitory and requires supportive care only. Others support the recognition of neonatal phenotypes and have additionally reported severe presentations of myotonia, feeding and respiratory difficulties (including stridor) that can be fatal.
2. Inflammatory changes occur in the muscle biopsies of some channelopathy patients that statistically seem unlikely to be co-incidental although the exact significance and any influence this may have on the development of myopathy requires further exploration.
3. Pain is an under-recognised feature of the skeletal muscle channelopathies and morbidity associated with these diseases deserves more consideration.

Discussion - Genotype

Aim

To genetically characterize patients to assess if new mutations in known genes existed and if these genes could account for 100% of a carefully phenotyped cohort or if new genes may be implicated.

8.1 Genotyping the sodium channel myotonic disorders

Paramyotonia congenita and sodium channel myotonia

Of the 17 patients identified with a phenotype consistent with a sodium channel myotonic disorder, I considered 13 to have PMC or SCM. Of this group 9/13 was genotyped. Previously reported SCN4A mutations (V1293I, Q270K and E1702K) were identified in four(Koch *et al*., 1995; Fournier *et al*., 2006; Miller *et al*., 2004) and another three had novel amino acid substitutions (L128P, V445L and M135L) although it is probable only L128P and V445L are pathogenic and possible that M135L is a rare polymorphism. Mutations in SCN4A responsible for myotonic phenotypes appear to affect amino acids distributed randomly across the protein with no discernible pattern. The novel changes found here illustrate it is likely that new mutations will continue to be reported throughout SCN4A. Although exons 13, 22 and 24 represent mutation hotspots screening of the entire coding region of SCN4A is still required to make a genetic diagnosis in a significant number of patients.

Of the remaining two patients who were genotyped one carried a CLCN1 mutation. This illustrates the difficulty that remains, despite more advanced electrophysiological tests, in differentiating some cases of sodium channel myotonia from chloride channel myotonia clinically. One group genotyped 93% of their cohort of patients with NDM by employing in tandem analysis of SCN4A and CLCN1(Trip *et al*., 2008). This highlights a real need for both genes to be analysed if the first is negative.

The final patient was an atypical case under the care of paediatric colleagues, consultant paediatrician Dr Elizabeth Wraige. The patient was an infant who had been well until approximately four months of age. They were then admitted to intensive care in respiratory distress that required long-term tracheostomy. The child was noted to be "stiff" and a first EMG suggested difficulty in differentiating between myotonic potentials and pseudomyotonic potentials. A trial of mexiletine was initially felt to be helpful but later withdrawn as any benefits seemed to plateau. As a result SCN4A analysis was undertaken by me but no pathogenic changes were found. A second EMG later confirmed pseudomyotonic potentials. An initial biopsy at 5 months was non-specific but a second biopsy at 6 months of age showed changes of myofibrillar myopathy. Many parallels were noted between this child and a group of Indian-Canadian children who had been described years earlier as having autosomal recessive fatal hypertonic muscular dystrophy although no genetic diagnosis had been reached(Lacson *et al*., 1994).

As muscle biopsy suggested a myofibrillar myopathy analysis of associated genes was undertaken by the NCG genetic laboratory at Newcastle. A homozygous mutation was identified in the CRYAB gene(Forrest *et al*., 2011). Attempts had been made to contact the authors of the original hypertonic muscular dystrophy paper but were unsuccessful. Shortly after the identification of the CRYAB mutation in this infant the same homozygous CRYAB mutation was identified in the Canadian cohort(Del Bigio *et al*., 2011).

From the work done in this thesis and reports in the literature neonatal channelopathy phenotypes of varying severity are emerging. This includes fatal cases characterised by myotonia, respiratory and feeding difficulties(Gay *et al*., 2008). Although the infant above ultimately displayed evidence of a myofibrillar myopathy the initial presentation was potentially in keeping with the described sodium channel neonatal phenotypes. It suggests SCN4A analysis should still be considered in similar cases as the number of reported neonatal cases and extent of the neonatal phenotype may continue to expand.

The 4/13 patients with a myotonic phenotype in whom no mutation was identified all had EMG confirmed myotonia. Of these four, one was an infant being investigated for

hypotonia and the EMG myotonia was an unexpected finding. Brief bursts of myotonia are seen in other myopathies(Milone *et al*., 2012) including congenital myopathies and the myotonia in this case may ultimately be accounted for in this way, although no definite diagnosis has yet been reached.

The other three cases however, had phenotypes that can be considered strongly consistent with non-dystrophic myotonia. Only direct automated sequencing of the coding regions of SCN4A and CLCN1 was undertaken. It is possible gene deletions or duplications may occur and would not have been picked up by this method. Recently a pathogenic intron change has been described in SCN4A in a family with PMC(Kubota *et al*., 2011). This particular change would have been detected as it was close to the intron/exon border and the primers I designed overlapped these regions. However other deeper intron changes have not been excluded nor have changes in the promoter region of the gene. A final possibility is that other myotonia genes exist. One of the non-genotyped cases came from a large Sicilian family and a future project could examine linkage in this family to assess the possibility.

Hyperkalaemic periodic paralysis

In the group of 17 patients with a sodium channel myotonic phenotype I identified four patients with a phenotype consistent with hyperkalaemic periodic paralysis. In 1/4 I identified a pathological expansion of the ZNF9 gene consistent with a diagnosis of DM2. This patient was described as having episodes of muscle paralysis since childhood, triggered by cold, antihistamines, and corticosteroids. She also complained of paralysis of the respiratory muscles and bladder, with fasciculations and myotonia noted during episodes. Her mother had the same symptoms, her father was unaffected. Her brother was noted to have muscle hypertrophy and an elevated CK.

The other three cases had some atypical features for hyperkalaemic periodic paralysis and it is perhaps not surprising that they do not carry any mutations in SCN4A. During my thesis I was asked to sequence another patient with atypical hyperkalaemic periodic paralysis by Prof Francesco Muntoni (consultant paediatrician) in whom his team had identified a ryanodine receptor gene mutation. This patient had no pathogenic SCN4A mutations and functional expression work carried out by Prof Muntoni's team confirmed

the pathogenicity of the RYR1 gene mutation(Zhou *et al*., 2010). This case illustrates however that atypical cases of hyperkalaemic periodic paralysis and potentially other muscle channelopathies may reflect new disease causing genes. Additionally the identification of an abnormal expansion of the ZNF9 gene in the fourth case of hyperkalaemic periodic paralysis I studied was unexpected and emphasises the need to reflect on these atypical cases. As more are identified, common phenotypic features may help to group some together and aid in the future identification of additional disease causing genes.

8.2 Genotyping hypokalaemic periodic paralysis

The new mutations found in CACNA1S and SCN4A in patients with a phenotype of hypokalaemic periodic paralysis significantly expands the spectrum of S4 segment arginine mutations which cause HypoPP and adds new genetic evidence to support the hypothesis that loss of positive charge in S4 voltage sensors is important in the molecular pathogenesis of this muscle channelopathy. Overall, the 74/83 HypoPP cases genetically characterized by voltage sensor charge loss argue that arginine mutations in the voltage sensors of both channels are the overwhelmingly most important cause of HypoPP. These results, furthermore, suggest that screening for arginine mutations in S4 segments should yield a genetic diagnosis in approximately 90% of cases of HypoPP.
It remains unclear how S4 mutations lead to the HypoPP phenotype. Previous functional studies of the effect of the CACNA1S/SCN4A S4 mutations on the gating properties of the main pore all pointed to a loss of function defect(Lapie *et al*., 1996; Morrill *et al*., 1999; Struyk *et al*., 2000; Kuzmenkin *et al*., 2002; Carle *et al*., 2006). However, such a loss of function mechanism does not readily explain the prolonged depolarization of the sarcolemmal membrane associated with attacks of paralysis or the episodic occurrence of hypokalaemia(Cannon., 2006). The individual contribution of each S4 segment to channel gating is not yet fully understood. Current evidence suggests that the S4 segments of DIII and IV of Nav1.4 play a more significant role in fast inactivation(Cha *et al*., 1999). It is therefore of interest to note that replacement of the outermost arginine in

DIV/S4 of SCN4A does not produce a HypoPP phenotype but rather one of paramyotonia congenita(Ptacek *et al*., 1992). In contrast, replacement of the third outermost arginine in DII/S4 of SCN4A produces a potassium-sensitive periodic paralysis(Vicart *et al*., 2004). Only one non-arginine substitution has been described in an S4 segment of either channel, G1456E[SCN4A] also in domain IV, which resulted in a phenotype of paramyotonia congenita(Sasaki *et al*., 1999).

Two recent studies have suggested that a gating pore current may be important in the pathogenesis of HypoPP(Struyk *et al*., 2007; Sokolov *et al*., 2007), and this data is fully consistent with this hypothesis.

In response to depolarizing voltages, S4 segments undergo a conformational change that moves these segments outwards through a gating pore (omega pore) which leads to the opening of the central pore of the channel (alpha pore). The outermost arginines in S4 occupy and occlude the narrowest part of the gating pore at the resting membrane potential while internal arginine residues occlude it at depolarized potentials. Recently, it was shown that in addition to disrupting the gating of the main pore (alpha pore), mutations that neutralize the two outermost arginines (R669/R672) in DII/S4 of Nav1.4 also generate a monovalent cation leak through the gating pore at hyperpolarized potentials(Sokolov *et al*., 2007). A histidine substitution at R672 produces a proton specific pore leak whereas other amino acid substitutions at this position cause a nonselective cation leak(Struyk *et al*., 2007). The leak current is thought to be mainly mediated by protons and could contribute to the pathophysiology of HypoPP, possibly by an accumulation of intracellular protons and disruption of the intracellular homeostasis of pH. Consistent with the role of arginines in occluding the gating pore, a 10-fold larger leak current occurs with a glycine substitution compared with the histidine substitution at R663 in the rat isoform of Nav1.4 (comparable to R669 in the human isoform)(Struyk *et al*., 2007).

The finding of additional mutations that affect S4 arginines adds to the possibility these arginines play a central role in HypoPP. Furthermore, it expands the number of channel domains and the range of arginines affected, notably including arginines buried more deeply in the channel. In the Shaker potassium channel, replacing more intracellular arginine residues with histidine residues has been shown to produce proton leak currents

at depolarized potentials in contrast to substitutions of outer arginines that generate leak currents at hyperpolarized potentials(Starace *et al*., 2001). If the mutation of intracellular arginines associated with hypoPP leads to similar changes in SCN4A, then patients with mutations affecting these residues may have leak currents at depolarized potentials rather than at rest. Functional characterization of these new mutations will be important for determining the pathophysiological effect of these more intracellular arginine residues. In summary, the results obtained by sequencing all S4 segments of SCN4A and CACNA1S has shown that new mutations do exist, additional S4 segments are implicated in disease pathogenesis, and enabled the identification of a mutation in 90% of patients, adding significant genetic evidence to support the gating pore hypothesis.

Sequencing the entire coding region of CACNA1S and the remaining sequence of SCN4A coding for all S1-3 segments in the remaining nine cases did not add any new information. I did hypothesise that neutralisation of key negative charges in the S1-3 segments that interact with the positive charges in the voltage sensors to maintain the integrity of the omega pore could also produce an aberrant leak current that may be responsible for some cases of hypoPP. No such mutations were found but only a small number of genetically unexplained samples remained (nine cases) and it is still possible such mutations will be found if larger numbers of similar cases are sequenced. Analysis of the coding region of KCNJ2 identified a novel change in 1/9 remaining cases. Mutations of KCNJ2 are associated with Andersen-Tawil Syndrome. This is the only skeletal muscle channelopathy that also has systemic features and is characterised by the triad of periodic paralysis, dysmorphic features and cardiac conduction defects although two out of three is considered acceptable for diagnosis(Venance *et al*., 2006). The dysmorphic features can be very subtle and the cardiac conduction defects may be asymptomatic so it is not surprising that occasionally, as in this case, patients are diagnosed clinically as hypokalaemic periodic paralysis only. This has important implications however as hypoPP is not associated with conduction defects whereas ATS is. These conduction defects are often significant and in some cases fatal so it is essential this diagnosis is not missed. The conduction defects can be asymptomatic but the resting ECG may still show abnormalities(Venance *et al*., 2006). Overall this data suggests that

ATS should be considered in all otherwise unexplained cases of hypoPP, prompting performance of a resting ECG and consideration of genetic testing.

Eight cases of hypoPP remain in whom no genetic diagnosis was made. The same genetic mechanisms that could explain the unidentified myotonic cases, i.e. gene re-arrangements, intron or promoter region changes are also relevant to these hypoPP cases. The possibility of new genes however is tantalising. A shared observation between all the known hypoPP genes (CACNA1S, SCN4A, KCNJ2) is that they all impair inward rectifying potassium channel current. Mutations of KCNJ18 have recently been identified as the cause of some cases of thyrotoxic periodic paralysis and more recently sporadic cases of hypoPP. Future projects could analyse the remaining 8 cases identified here for this and other potassium channel gene mutations.

Conclusions: Genotype

1. The number of new mutations in known causative genes associated with sodium channel myotonic disorders, hypoPP and ATS continues to expand.
2. Most typical cases of each phenotype are accounted for by known genes but a small minority are still genetically unexplained. This may represent a. unexamined genetic mechanisms or b. new genes.
3. More atypical phenotypes tend not to be accounted for by known genes. These cases deserve further consideration.
4. The large proportion of hypoPP cases accounted for by voltage sensor mutations supports the recently proposed "gating pore hypothesis" disease mechanism.

Discussion – Treatment

Aim

To assess if genotype has any influence on response to treatment with acetazolamide in hypokalaemic periodic paralysis.

9.1 Genotype influences treatment response in hypokalaemic periodic paralysis

There are no consensus guidelines for the treatment of hypoPP. Current pharmacological agents commonly used include potassium supplements, potassium sparing diuretics and carbonic anhydrase inhibitors (acetazolamide and dichlorphenamide). Dichlorphenamide is the only therapy for hypoPP to have undergone a randomised double blind placebo controlled cross over trial. This trial showed a significant efficacy of dichlorphenamide in reducing attack frequency but the inclusion criteria were based on clinical diagnosis of hypoPP and not genetic confirmation(Tawil *et al*., 2000). Aside from this there is very little trial evidence to support the use of any treatment in hypoPP and no randomised controlled trial evidence supporting the most common choice; acetazolamide (Sansone *et al*., 2008).

The data gathered in this thesis from a literature review of cases and retrospective analysis of our own patients suggest that at least half the patients treated with acetazolamide do not get a satisfactory response. A tentative correlation between detrimental response and genotype does emerge and those patients with substitutions to glycine of arginine residues situated towards the extracellular side of the voltage sensors of either Cav1.1 or Nav1.4 may be predicted not to respond to acetazolamide. The highest response rates were seen in the common CACNA1S arginine(R) to Histidine(H) substitutions. These observations are particularly noteworthy in light of experimental data indicating that the deleterious effects of the R672G substitution on channel gating are insensitive to reductions in pH in vitro. This is in contrast to the deleterious effects of the R669H mutation which were ameliorated by an acidic pH. These in vitro observations

predict that such patients would not respond to acetazolamide which produces a metabolic acidosis(Kuzmenkin *et al*., 2002). The deleterious effects studied relate to the ion selective alpha pore. More recent studies have identified an anomalous proton selective gating pore due to R669H and R672H SCN4A mutations and a less selective cation conducting pore in the R672G SCN4A mutation(Struyk *et al*., 2007; Sokolov *et al*., 2007). Similar gating pores have yet to be identified in the CACNA1S mutations but are proposed as a likely pathomechanism. This may additionally suggest that the R to H substitutions are likely to have a greater effect on pH concentration gradients and as a result more likelihood of a beneficial response to acetazolamide therapy compared to the R to G substitutions.

Overall these findings suggest genotype is an important factor in determining treatment response to acetazolamide in hypoPP and illustrate an additional benefit of achieving a genetic diagnosis where possible. This form of retrospective analysis is imperfect however and no correction could be made for dose or duration of treatment or influence of co-existing treatments. Additionally only the effect of acetazolamide on episodic muscle weakness was studied. Any beneficial effect of acetazolamide on preventing myopathy was not considered. Without randomised controlled trials the exact efficacy of acetazolamide is unknown. In addition its mechanism of action is unclear although it has become first line therapy and suggested to have benefit for the majority of patients for almost half a century(Venance *et al*., 2006). This data suggest however that the response rate may be more modest than currently generally considered. This highlights a need for randomised controlled trials but also suggests development of new treatments is warranted.

Conclusions: Treatment response HypoPP

1. Retrospective analysis suggests acetazolamide is efficacious for only approximately 50% of patients with hypoPP.
2. Those with R to H amino acid substitutions have the most favourable response.
3. Those with R to G amino acid substitutions have the poorest response.
4. Genotypic differences in response may reflect the specific effects of amino acid substitution on the aberrant channel gating pore current produced.

Discussion – Pathophysiology

Aim

To functionally characterize voltage sensor mutations of SCN4A in light of the recently described gating pore seen in some of the Nav1.4 mutations.

10.1 Functional consequences of the R675G SCN4A mutation

The identification of an aberrant current via the gating pore in some SCN4A mutations associated with hypoPP has led to significant steps forward in understanding the pathophysiology of this disorder. Much remains unexplained however. Although all S4 segments act generically as voltage sensors each undoubtedly has its own specificities and it is likely this even applies to each individual key positively charged residue. The phenotype of the Nav1.4 mutant I studied, (R675G) is not entirely in keeping with hypoPP(Vicart *et al*., 2004). It has many shared features, mainly long attacks of paralysis lasting hours to days to even weeks. Potassium levels are normal or reduced but oddly attacks can be precipitated by increased potassium levels. It has been shown however to introduce a gating pore current as was demonstrated for substitutions of the adjacent R669 and R672 residues(Sokolov *et al*., 2008). The difference with R675G is that the current occurred at depolarising potentials. Clearly R675G shares some phenotypic and pathological features of other hypoPP causing mutations but has other exclusive aspects. Expression of the SCN4A R675G mutation in HEK cells shows a significant (6 fold) reduction in current density through the alpha pore compared with wild type. This is similar to the reduced current density seen with substitutions of the adjacent arginines R672 and R669.

These functional experiments confirm that an arginine placed nearer the intracellular surface of the S4 segment has a similar effect on the gating of the main pore to one on the extracellular surface in that they all produce a loss of function defect. This is most severe

however for R675G in that a 6 fold reduction in current density is seen as opposed to the 2-4 fold reduction described for R669 and R672 substitutions(Struyk *et al*., 2000; Kuzmenkin *et al*., 2002).

The reason for the reduction in current density however is not clear. It cannot be explained by an impairment of channel opening as the voltage dependence of activation is indistinguishable from the wild type. Similarly channel inactivation did not significantly differ between WT and mutant. Other possibilities are that the mutation interferes with protein production or trafficking such that a reduced number of sodium channels are expressed at the sarcolemma. Alternatively it may be that the same number of channels is physically present in the membrane but that a significant proportion is non-functional.

These hypotheses warrant further exploration by single channel recordings and by immunofluorescent staining using a Nav1.4 antibody to give an estimate of channel expression at the sarcolemma.

10.2 Establishing a myoblast system

Preliminary studies suggested it would not be possible to study gating pore currents in an HEK cell model. Although a system for doing so is established in oocytes, these are far removed from human skeletal muscle. Early attempts were made to establish if human muscle cell cultures could provide a viable and more physiologically relevant alternative. Robust sodium currents were obtained but several limitations were identified even in this early work including very large currents that are difficult to stabilise and contribution to the sodium current from Nav1.5 channels. Ultimately human tissue is also a relatively rare commodity that requires careful handling, storage and culture. However despite the challenges, a myoblast system has the attraction of being a more physiologically relevant tissue in which to study disease pathomechanisms, making the establishment of such a system a worthy future project.

Conclusions: Pathophysiology

1. A more intracellularly placed arginine (R675G) in the voltage sensor of domain II, Nav1.4, has similar although more severe detrimental effects on the alpha pore to extracellularly placed basic residues studied in this domain.
2. Human muscle cell cultures have potential to be a viable physiologically relevant model in which to study gating pore currents but there are many technical challenges to be considered.

Reference List

Ahmadian JL, Heller SL, Nishida T, Altman KW. Myotonic dystrophy type 1 (DM1) presenting with laryngeal stridor and vocal fold paresis. Muscle Nerve 2002; 25: 616-618.

Aichele R, Paik H, Heller AH. Efficacy of phenytoin, procainamide, and tocainide in murine genetic myotonia. Exp Neurol 1985; 87: 377-381.

Alfonsi E, Merlo IM, Tonini M, Ravaglia S, Brugnoni R, Gozzini A *et al*. Efficacy of propafenone in paramyotonia congenita. Neurology 2007; 68: 1080-1081.

Andersen ED, Krasilnikoff PA, Overvad H. Intermittent muscular weakness, extrasystoles, and multiple developmental anomalies. A new syndrome? Acta Paediatr Scand 1971; 60: 559-564.

Aoike F, Takahashi MP, Sakoda S. Class Ic antiarrhythmics block human skeletal muscle Na channel during myotonia-like stimulation. Eur J Pharmacol 2006; 532: 24-31.

Becker PE. Myotonia congenita and syndromes associated with myotonia. Topics in Human Genetics. Stuttgart, Germany: Georg Thieme; 1977.

Bendahhou S, Cummins TR, Griggs RC, Fu YH, Ptacek LJ. Sodium channel inactivation defects are associated with acetazolamide-exacerbated hypokalemic periodic paralysis. Ann Neurol 2001; 50: 417-420.

Bendahhou S, Cummins TR, Hahn AF, Langlois S, Waxman SG, Ptacek LJ. A double mutation in families with periodic paralysis defines new aspects of sodium channel slow inactivation. J Clin Invest 2000; 106: 431-438.

Bendahhou S, Cummins TR, Kula RW, Fu YH, Ptacek LJ. Impairment of slow inactivation as a common mechanism for periodic paralysis in DIIS4-S5. Neurology 2002; 58: 1266-1272.

Bendahhou S, Cummins TR, Tawil R, Waxman SG, Ptacek LJ. Activation and inactivation of the voltage-gated sodium channel: role of segment S5 revealed by a novel hyperkalaemic periodic paralysis mutation. J Neurosci 1999a; 19: 4762-4771.

Bendahhou S, Cummins TR, Kwiecinski H, Waxman SG, Ptacek LJ. Characterization of a new sodium channel mutation at arginine 1448 associated with moderate paramyotonia congenita in humans. J Physiol (Lond) 1999b; 518: 337-344.

Biemond A, Daniels A.P. Familial Periodic Paralysis And Its Transition Into Spinal Muscular Atrophy. Brain 1934; 57: 91-108.

Bouhours M, Luce S, Sternberg D, Willer JC, Fontaine B, Tabti N. A1152D mutation of the Na+ channel causes paramyotonia congenita and emphasizes the role of DIII/S4-S5 linker in fast inactivation. J Physiol 2005; 565: 415-427.

Bouhours M, Sternberg D, Davoine CS, Ferrer X, Willer JC, Fontaine B *et al*. Functional characterization and cold sensitivity of T1313A, a new mutation of the skeletal muscle sodium channel causing paramyotonia congenita in humans. J Physiol 2004; 554: 635-647.

Bradley WG, Taylor R, Rice DR, Hausmanowa-Petruzewicz I, Adelman LS, Jenkison M *et al*. Progressive myopathy in hyperkalemic periodic paralysis. Arch Neurol 1990; 47: 1013-1017.

Brini M. Ryanodine receptor defects in muscle genetic diseases. Biochem Biophys Res Commun 2004; 322: 1245-1255.

Bulman DE, Scoggan KA, van O, Nicolle MW, Hahn AF, Tollar LL *et al*. A novel sodium channel mutation in a family with hypokalemic periodic paralysis. Neurology 1999; 53: 1932-1936.

Buruma OJ, Bots GT. Myopathy in familial hypokalaemic periodic paralysis independent of paralytic attacks. Acta Neurol Scand 1978; 57: 171-179.

Cannon SC. Physiologic principles underlying ion channelopathies. Neurotherapeutics 2007; 4: 174-183.

Cannon SC. Spectrum of sodium channel disturbances in the nondystrophic myotonias and periodic paralyses. Kidney Int 2000; 57: 772-779.

Cannon SC. Voltage-sensor mutations in channelopathies of skeletal muscle. J Physiol 2010; 588: 1887-1895.

Cannon SC. Pathomechanisms in channelopathies of skeletal muscle and brain. Annu Rev Neurosci 2006; 29: 387-415.

Carle T, Lhuillier L, Luce S, Sternberg D, Devuyst O, Fontaine B *et al*. Gating defects of a novel Na+ channel mutant causing hypokalemic periodic paralysis. Biochem Biophys Res Commun 2006; 348: 653-661.

Carr EA, Spier SJ, Kortz GD, Hoffman EP. Laryngeal and pharyngeal dysfunction in horses homozygous for hyperkalemic periodic paralysis. J Am Vet Med Assoc 1996; 209: 798-803.

Catalano A, Carocci A, Corbo F, Franchini C, Muraglia M, Scilimati A *et al*. Constrained analogues of tocainide as potent skeletal muscle sodium channel blockers towards the development of antimyotonic agents. Eur J Med Chem 2008; 43: 2535-2540.

Catterall WA. Structure and function of voltage-gated ion channels. Annu Rev Biochem 1995; 64: 493-531.

Catterall WA. Ion channel voltage sensors: structure, function, and pathophysiology. Neuron 2010; 67: 915-928.

Ceccarelli M, Rossi B, Siciliano G, Calevro L, Tarantino E. Clinical and electrophysiological reports in a case of early onset myotonia congenita (Thomsen's disease) successfully treated with mexiletine. Acta Paediatr 1992; 81: 453-455.

Cha A, Ruben PC, George AL, Jr., Fujimoto E, Bezanilla F. Voltage sensors in domains III and IV, but not I and II, are immobilized by Na+ channel fast inactivation. Neuron 1999; 22: 73-87.

Chabrier S, Monnier N, Lunardi J. Early onset of hypokalaemic periodic paralysis caused by a novel mutation of the CACNA1S gene. J Med Genet 2008; 45: 686-688.

Charlet B, Savkur RS, Singh G, Philips AV, Grice EA, Cooper TA. Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing. Mol Cell 2002; 10: 45-53.

Clare BW, Supuran CT. A perspective on quantitative structure-activity relationships and carbonic anhydrase inhibitors. Expert Opin Drug Metab Toxicol 2006; 2: 113-137.

Cleland JC, Griggs RC. Treatment of neuromuscular channelopathies: current concepts and future prospects. Neurotherapeutics 2008; 5: 607-612.

Colding-Jorgensen E, Duno M, Vissing J. Autosomal dominant monosymptomatic myotonia permanens. Neurology 2006; 67: 153-155.

Conn JW, Fajans SS, Louis LH, Streeten DH, Johnson RD. Intermittent aldosteronism in periodic paralysis; dependence of attacks on retention of sodium, and failure to induce attacks by restriction of dietary sodium. Lancet 1957; 272: 802-805.

Dalakas MC, Engel WK. Treatment of "permanent" muscle weakness in familial Hypokalemic Periodic Paralysis. Muscle Nerve 1983; 6: 182-186.

Davies NP, Eunson LH, Samuel M, Hanna MG. Sodium channel gene mutations in hypokalemic periodic paralysis: an uncommon cause in the UK. Neurology 2001; 57: 1323-1325.

Davies NP, Imbrici P, Fialho D, Herd C, Bilsland LG, Weber A *et al*. Andersen-Tawil syndrome: new potassium channel mutations and possible phenotypic variation. Neurology 2005; 65: 1083-1089.

Del Bigio MR, Chudley AE, Sarnat HB, Campbell C, Goobie S, Chodirker BN *et al*. Infantile muscular dystrophy in Canadian aboriginals is an alphaB-crystallinopathy. Ann Neurol 2011; 69: 866-871.

Dupre N, Chrestian N, Bouchard JP, Rossignol E, Brunet D, Sternberg D *et al*. Clinical, electrophysiologic, and genetic study of non-dystrophic myotonia in French-Canadians. Neuromuscul Disord 2009; 19: 330-334.

Emery AE. Population frequencies of inherited neuromuscular diseases--a world survey. Neuromuscul Disord 1991; 1: 19-29.

Eulenberg A. Ueber eine familiäre, durch 6 generationen verfolbare form congenitaler paramyotonie. Neurologisches Centralblatt 1886; 12: 265-272.

Favre I, Moczydlowski E, Schild L. On the structural basis for ionic selectivity among Na+, K+, and Ca2+ in the voltage-gated sodium channel. Biophys J 1996; 71: 3110-3125.

Featherstone DE, Fujimoto E, Ruben PC. A defect in skeletal muscle sodium channel deactivation exacerbates hyperexcitability in human paramyotonia congenita. J Physiol 1998; 506 (Pt 3): 627-638.

Ferriby D, Stojkovic T, Sternberg D, Hurtevent JF, Hurtevent JP, Vermersch P. A new case of autosomal dominant myotonia associated with the V1589M missense mutation in the muscle sodium channel gene and its phenotypic classification. Neuromuscul Disord 2006; 16: 321-324.

Fialho D, Kullmann DM, Hanna MG, Schorge S. Non-genomic effects of sex hormones on CLC-1 may contribute to gender differences in myotonia congenita. Neuromuscul Disord 2008; 18: 869-872.

Fialho D, Schorge S, Pucovska U, Davies NP, Labrum R, Haworth A *et al*. Chloride channel myotonia: exon 8 hot-spot for dominant-negative interactions. Brain 2007; 130: 3265-3274.

Fleischhauer R, Mitrovic N, Deymeer F, Lehmann-Horn F, Lerche H. Effects of temperature and mexiletine on the F1473S Na+ channel mutation causing paramyotonia congenita. Pflugers Arch 1998; 436: 757-765.

Fontaine B. Primary periodic paralysis and muscle sodium channel. Adv Nephrol Necker Hosp 1994; 23: 191-197.

Forrest KM, Al-Sarraj S, Sewry C, Buk S, Tan SV, Pitt M *et al*. Infantile onset myofibrillar myopathy due to recessive CRYAB mutations. Neuromuscul Disord 2011; 21: 37-40.

Fouad G, Dalakas M, Servidei S, Mendell JR, Van den BP, Angelini C *et al*. Genotype-phenotype correlations of DHP receptor alpha 1-subunit gene mutations causing hypokalemic periodic paralysis. Neuromuscul Disord 1997; 7: 33-38.

Fournier E, Arzel M, Sternberg D, Vicart S, Laforet P, Eymard B *et al*. Electromyography guides toward subgroups of mutations in muscle channelopathies. Ann Neurol 2004; 56: 650-661.

Fournier E, Viala K, Gervais H, Sternberg D, rzel-Hezode M, Laforet P *et al*. Cold extends electromyography distinction between ion channel mutations causing myotonia. Ann Neurol 2006; 60: 356-365.

Gallant EM. Barium-treated mammalian skeletal muscle: similarities to hypokalaemic periodic paralysis. J Physiol 1983; 335: 577-590.

Gay S, Dupuis D, Faivre L, Masurel-Paulet A, Labenne M, Colombani M *et al*. Severe neonatal non-dystrophic myotonia secondary to a novel mutation of the voltage-gated sodium channel (SCN4A) gene. Am J Med Genet A 2008; 146: 380-383.

George AL, Jr., Sloan-Brown K, Fenichel GM, Mitchell GA, Spiegel R, Pascuzzi RM. Nonsense and missense mutations of the muscle chloride channel gene in patients with myotonia congenita. Hum Mol Genet 1994; 3: 2071-2072.

Green DS, George AL, Jr., Cannon SC. Human sodium channel gating defects caused by missense mutations in S6 segments associated with myotonia: S804F and V1293I. J Physiol 1998; 510 (Pt 3): 685-694.

Griggs RC, Engel WK, Resnick JS. Acetazolamide treatment of hypokalemic periodic paralysis. Prevention of attacks and improvement of persistent weakness. Ann Intern Med 1970; 73: 39-48.

Griggs RC, Moxley RT, III, Riggs JE, Engel WK. Effects of acetazolamide on myotonia. Ann Neurol 1978; 3: 531-537.

Hammaren E, Kjellby-Wendt G, Lindberg C. Quantification of mobility impairment and self-assessment of stiffness in patients with myotonia congenita by the physiotherapist. Neuromuscul Disord 2005; 15: 610-617.

Haruna Y, Kobori A, Makiyama T, Yoshida H, Akao M, Doi T *et al*. Genotype-phenotype correlations of KCNJ2 mutations in Japanese patients with Andersen-Tawil syndrome. Hum Mutat 2007; 28: 208.

Hayward LJ, Brown RH, Jr., Cannon SC. Inactivation defects caused by myotonia-associated mutations in the sodium channel III-IV linker. J Gen Physiol 1996; 107: 559-576.

Hayward LJ, Kim JS, Lee MY, Zhou H, Kim JW, Misra K *et al*. Targeted mutation of mouse skeletal muscle sodium channel produces myotonia and potassium-sensitive weakness. J Clin Invest 2008; 118: 1437-1449.

Hayward LJ, Sandoval GM, Cannon SC. Defective slow inactivation of sodium channels contributes to familial periodic paralysis. Neurology 1999; 52: 1447-1453.

Hecht ML, Valtysson B, Hogan K. Spinal anesthesia for a patient with a calcium channel mutation causing hypokalemic periodic paralysis. Anesth Analg 1997; 84: 461-464.

Heine R, Pika U, Lehmann-Horn F. A novel SCN4A mutation causing myotonia aggravated by cold and potassium. Hum Mol Genet 1993; 2: 1349-1353.

Heinemann SH, Terlau H, Stuhmer W, Imoto K, Numa S. Calcium channel characteristics conferred on the sodium channel by single mutations. Nature 1992; 356: 441-443.

Heuschkel RB, Fletcher K, Hill A, Buonomo C, Bousvaros A, Nurko S. Isolated neonatal swallowing dysfunction: a case series and review of the literature. Dig Dis Sci 2003; 48: 30-35.

Hilton-Jones D. Inflammatory muscle diseases. Curr Opin Neurol 2001; 14: 591-596.

Hirn C, Shapovalov G, Petermann O, Roulet E, Ruegg UT. Nav1.4 deregulation in dystrophic skeletal muscle leads to Na+ overload and enhanced cell death. J Gen Physiol 2008; 132: 199-208.

Hofmann WW, Smith RA. Hypokalemic periodic paralysis studies in vitro. Brain 1970; 93: 445-474.

Hogrel JY. Quantitative myotonia assessment using force relaxation curve modelling. Physiol Meas 2009; 30: 719-727.

Horlings CG, Drost G, Bloem BR, Trip J, Pieterse AJ, van Engelen BG *et al*. Trunk sway analysis to quantify the warm-up phenomenon in myotonia congenita patients. J Neurol Neurosurg Psychiatry 2009; 80: 207-212.

Isom LL. Sodium channel beta subunits: anything but auxiliary. Neuroscientist 2001; 7: 42-54.

Jackson CE, Barohn RJ, Ptacek LJ. Paramyotonia congenita: abnormal short exercise test, and improvement after mexiletine therapy. Muscle Nerve 1994; 17: 763-768.

Jurkat-Rott K, Lehmann-Horn F, Elbaz A, Heine R, Gregg RG, Hogan K *et al*. A calcium channel mutation causing hypokalemic periodic paralysis. Hum Mol Genet 1994; 3: 1415-1419.

Jurkat-Rott K, Mitrovic N, Hang C, Kouzmekine A, Iaizzo P, Herzog J *et al*. Voltage-sensor sodium channel mutations cause hypokalemic periodic paralysis type 2 by enhanced inactivation and reduced current. Proc Natl Acad Sci U S A 2000; 97: 9549-9554.

Jurkat-Rott K, Weber MA, Fauler M, Guo XH, Holzherr BD, Paczulla A *et al*. K+-dependent paradoxical membrane depolarization and Na+ overload, major and reversible contributors to weakness by ion channel leaks. Proc Natl Acad Sci U S A 2009; 106: 4036-4041.

Kawamura S, Ikeda Y, Tomita K, Watanabe N, Seki K. A family of hypokalemic periodic paralysis with CACNA1S gene mutation showing incomplete penetrance in women. Intern Med 2004; 43: 218-222.

Kelly P, Yang WS, Costigan D, Farrell MA, Murphy S, Hardiman O. Paramyotonia congenita and hyperkalemic periodic paralysis associated with a Met 1592 Val substitution in the skeletal muscle sodium channel alpha subunit--a large kindred with a novel phenotype. Neuromuscul Disord 1997; 7: 105-111.

Kil TH, Kim JB. Severe respiratory phenotype caused by a de novo Arg528Gly mutation in the CACNA1S gene in a patient with hypokalemic periodic paralysis. Eur J Paediatr Neurol 2009b.

Kim JB, Kim MH, Lee SJ, Kim DJ, Lee BC. The genotype and clinical phenotype of Korean patients with familial hypokalemic periodic paralysis. J Korean Med Sci 2007; 22: 946-951.

Kim JB, Lee KY, Hur JK. A Korean family of hypokalemic periodic paralysis with mutation in a voltage-gated calcium channel (R1239G). J Korean Med Sci 2005; 20: 162-165.

Kim MK, Lee SH, Park MS, Kim BC, Cho KH, Lee MC *et al*. Mutation screening in Korean hypokalemic periodic paralysis patients: a novel SCN4A Arg672Cys mutation. Neuromuscul Disord 2004; 14: 727-731.

Kim SH, Kim UK, Chae JJ, Kim DJ, Oh HY, Kim BJ *et al*. Identification of mutations including de novo mutations in Korean patients with hypokalaemic periodic paralysis. Nephrol Dial Transplant 2001; 16: 939-944.

Koch MC, Baumbach K, George AL, Ricker K. Paramyotonia congenita without paralysis on exposure to cold: a novel mutation in the SCN4A gene (Val1293Ile). Neuroreport 1995; 6: 2001-2004.

Kubota T, Kinoshita M, Sasaki R, Aoike F, Takahashi MP, Sakoda S *et al*. New mutation of the Na channel in the severe form of potassium-aggravated myotonia. Muscle Nerve 2009; 39: 666-673.

Kubota T, Roca X, Kimura T, Kokunai Y, Nishino I, Sakoda S *et al*. A mutation in a rare type of intron in a sodium-channel gene results in aberrant splicing and causes myotonia. Hum Mutat 2011; 32: 773-782.

Kusumi M, Kumada H, Adachi Y, Nakashima K. Muscle weakness in a Japanese family of Arg1239His mutation hypokalemic periodic paralysis. Psychiatry Clin Neurosci 2001; 55: 539-541.

Kuzmenkin A, Muncan V, Jurkat-Rott K, Hang C, Lerche H, Lehmann-Horn F *et al*. Enhanced inactivation and pH sensitivity of Na(+) channel mutations causing hypokalaemic periodic paralysis type II. Brain 2002; 125: 835-843.

Lacson AG, Seshia SS, Sarnat HB, Anderson J, DeGroot WR, Chudley A *et al*. Autosomal recessive, fatal infantile hypertonic muscular dystrophy among Canadian Natives. Can J Neurol Sci 1994; 21: 203-212.

Lapie P, Goudet C, Nargeot J, Fontaine B, Lory P. Electrophysiological properties of the hypokalaemic periodic paralysis mutation (R528H) of the skeletal muscle alpha 1s subunit as expressed in mouse L cells. FEBS Lett 1996; 382: 244-248.

Laugel V, Cossee M, Matis J, de Saint-Martin A, Echaniz-Laguna A, Mandel JL *et al*. Diagnostic approach to neonatal hypotonia: retrospective study on 144 neonates. Eur J Pediatr. 2008 May;167(5):517-23.

Lennox G, Purves A, Marsden D. Myotonia fluctuans. Arch Neurol 1992; 49: 1010-1011.

Lerche H, Heine R, Pika U, George AL, Jr., Mitrovic N, Browatzki M *et al*. Human sodium channel myotonia: slowed channel inactivation due to substitutions for a glycine within the III-IV linker. J Physiol 1993; 470: 13-22.

Leyburn P, Walton JN. The treatment of myotonia: a controlled clinical trial. Brain 1959; 82: 81-91.

Links TP, Arnoldus EP, Wintzen AR, van der HJ, Gerritsen JJ, Brandenburg HC. The calcium channel blocker verapamil in hypokalemic periodic paralysis. Muscle Nerve 1998; 21: 1564-1565.

Links TP, Zwarts MJ, Wilmink JT, Molenaar WM, Oosterhuis HJ. Permanent muscle weakness in familial hypokalaemic periodic paralysis. Clinical, radiological and pathological aspects. Brain 1990; 113 (Pt 6): 1873-1889.

Lion-Francois L, Mignot C, Vicart S, Manel V, Sternberg D, Landrieu P *et al*. Severe neonatal episodic laryngospasm due to de novo SCN4A mutations: a new treatable disorder. Neurology 2010; 75: 641-645.

Logigian EL, Blood CL, Dilek N, Martens WB, Moxley RT, Wiegner AW *et al*. Quantitative analysis of the "warm-up" phenomenon in myotonic dystrophy type 1. Muscle Nerve 2005; 32: 35-42.

Maffe S, Signorotti F, Perucca A, Bielli M, Hladnik U, Ragazzoni E *et al*. Atypical arrhythmic complications in familial hypokalemic periodic paralysis. J Cardiovasc Med (Hagerstown) 2009; 10: 68-71.

Matthews E, Guet A, Mayer M, Vicart S, Pemble S, Sternberg D *et al*. Neonatal hypotonia can be a sodium channelopathy: recognition of a new phenotype. Neurology 2008a; 71: 1740-1742.

Matthews E, Tan SV, Fialho D, Sweeney MG, Sud R, Haworth A *et al*. What causes paramyotonia in the United Kingdom? Common and new SCN4A mutations revealed. Neurology 2008b; 70: 50-53.

McArdle B. ADYNAMIA EPISODICA HERED1TARIA AND ITS TREATMENT. Brain 1962; 85: 121-148.

McClatchey AI, Kenna-Yasek D, Cros D, Worthen HG, Kuncl RW, DeSilva SM *et al*. Novel mutations in families with unusual and variable disorders of the skeletal muscle sodium channel. Nat Genet 1992a; 2: 148-152.

McClatchey AI, Van den BP, Pericak-Vance MA, Raskind W, Verellen C, Kenna-Yasek D *et al*. Temperature-sensitive mutations in the III-IV cytoplasmic loop region of the skeletal muscle sodium channel gene in paramyotonia congenita. Cell 1992b; 68: 769-774.

McManis PG, Lambert EH, Daube JR. The exercise test in periodic paralysis. Muscle Nerve 1986; 9: 704-710.

McPhee JC, Ragsdale DS, Scheuer T, Catterall WA. A mutation in segment IVS6 disrupts fast inactivation of sodium channels. Proc Natl Acad Sci U S A 1994; 91: 12346-12350.

Michel P, Sternberg D, Jeannet PY, Dunand M, Thonney F, Kress W *et al*. Comparative efficacy of repetitive nerve stimulation, exercise, and cold in differentiating myotonic disorders. Muscle Nerve 2007; 36: 643-650.

Mikala G, Bahinski A, Yatani A, Tang S, Schwartz A. Differential contribution by conserved glutamate residues to an ion-selectivity site in the L-type Ca2+ channel pore. FEBS Lett 1993; 335: 265-269.

Miller TM, as da Silva MR, Miller HA, Kwiecinski H, Mendell JR, Tawil R *et al*. Correlating phenotype and genotype in the periodic paralyses. Neurology 2004; 63: 1647-1655.

Milone M, McEvoy KM, Sorenson EJ, Daube JR. Myotonia associated with caveolin-3 mutation. Muscle Nerve 2012; 45: 897-900.

Mitrovic N, George AL, Jr., Heine R, Wagner S, Pika U, Hartlaub U *et al*. K(+)-aggravated myotonia: destabilization of the inactivated state of the human muscle Na+ channel by the V1589M mutation. J Physiol 1994; 478 Pt 3: 395-402.

Mitrovic N, George AL, Jr., Lerche H, Wagner S, Fahlke C, Lehmann-Horn F. Different effects on gating of three myotonia-causing mutations in the inactivation gate of the human muscle sodium channel. J Physiol 1995; 487 (Pt 1): 107-114.

Mitrovic N, Lerche H, Heine R, Fleischhauer R, Pika-Hartlaub U, Hartlaub U *et al*. Role in fast inactivation of conserved amino acids in the IV/S4-S5 loop of the human muscle Na+ channel. Neurosci Lett 1996; 214: 9-12.

Morrill JA, Cannon SC. Effects of mutations causing hypokalaemic periodic paralysis on the skeletal muscle L-type Ca2+ channel expressed in Xenopus laevis oocytes. J Physiol 1999; 520 Pt 2: 321-336.

Moxley RT, III, Logigian EL, Martens WB, Annis CL, Pandya S, Moxley RT *et al*. Computerized hand grip myometry reliably measures myotonia and muscle strength in myotonic dystrophy (DM1). Muscle Nerve 2007; 36: 320-328.

Nagamitsu S, Matsuura T, Khajavi M, Armstrong R, Gooch C, Harati Y *et al*. A "dystrophic" variant of autosomal recessive myotonia congenita caused by novel mutations in the CLCN1 gene. Neurology 2000; 55: 1697-1703.

Okuda S, Kanda F, Nishimoto K, Sasaki R, Chihara K. Hyperkalemic periodic paralysis and paramyotonia congenita--a novel sodium channel mutation. J Neurol 2001; 248: 1003-1004.

Patton DE, West JW, Catterall WA, Goldin AL. Amino acid residues required for fast Na(+)-channel inactivation: charge neutralizations and deletions in the III-IV linker. Proc Natl Acad Sci U S A 1992; 89: 10905-10909.

Petitprez S, Tiab L, Chen L, Kappeler L, Rosler KM, Schorderet D *et al*. A novel dominant mutation of the Nav1.4 alpha-subunit domain I leading to sodium channel myotonia. Neurology 2008; 71: 1669-1675.

Plassart E, Eymard B, Maurs L, Hauw JJ, Lyon-Caen O, Fardeau M *et al*. Paramyotonia congenita: genotype to phenotype correlations in two families and report of a new mutation in the sodium channel gene. J Neurol Sci 1996; 142: 126-133.

Plassart E, Reboul J, Rime CS, Recan D, Millasseau P, Eymard B *et al*. Mutations in the muscle sodium channel gene (SCN4A) in 13 French families with hyperkalemic periodic paralysis and paramyotonia congenita: phenotype to genotype correlations and demonstration of the predominance of two mutations. Eur J Hum Genet 1994; 2: 110-124.

Plassart-Schiess E, Lhuillier L, George AL, Jr., Fontaine B, Tabti N. Functional expression of the Ile693Thr Na+ channel mutation associated with paramyotonia congenita in a human cell line. J Physiol 1998; 507 (Pt 3): 721-727.

Plaster NM, Tawil R, Tristani-Firouzi M, Canun S, Bendahhou S, Tsunoda A *et al*. Mutations in Kir2.1 cause the developmental and episodic electrical phenotypes of Andersen's syndrome. Cell 2001; 105: 511-519.

Poskanzer DC, Kerr DN. Periodic paralysis with response to spironolactone. Lancet 1961; 2: 511-513.

Ptacek LJ, George AL, Jr., Barchi RL, Griggs RC, Riggs JE, Robertson M *et al*. Mutations in an S4 segment of the adult skeletal muscle sodium channel cause paramyotonia congenita. Neuron 1992; 8: 891-897.

Ptacek LJ, George AL, Jr., Griggs RC, Tawil R, Kallen RG, Barchi RL *et al*. Identification of a mutation in the gene causing hyperkalemic periodic paralysis. Cell 1991; 67: 1021-1027.

Ptacek LJ, Gouw L, Kwiecinski H, McManis P, Mendell JR, Barohn RJ *et al*. Sodium channel mutations in paramyotonia congenita and hyperkalemic periodic paralysis. Ann Neurol 1993; 33: 300-307.

Ptacek LJ, Tawil R, Griggs RC, Engel AG, Layzer RB, Kwiecinski H *et al*. Dihydropyridine receptor mutations cause hypokalemic periodic paralysis. Cell 1994a; 77: 863-868.

Ptacek LJ, Tawil R, Griggs RC, Meola G, McManis P, Barohn RJ *et al*. Sodium channel mutations in acetazolamide-responsive myotonia congenita, paramyotonia congenita, and hyperkalemic periodic paralysis. Neurology 1994b; 44: 1500-1503.

Resnick JS, Engel WK, Griggs RC, Stam AC. Acetazolamide prophylaxis in hypokalemic periodic paralysis. N Engl J Med 1968; 278: 582-586.

Richmond JE, VanDeCarr D, Featherstone DE, George AL, Jr., Ruben PC. Defective fast inactivation recovery and deactivation account for sodium channel myotonia in the I1160V mutant. Biophys J 1997; 73: 1896-1903.

Ricker K, Haass A, Hertel G, Mertens HG. Transient muscular weakness in severe recessive myotonia congenita. Improvement of isometric muscle force by drugs relieving myotomic stiffness. J Neurol 1978; 218: 253-262.

Ricker K, Lehmann-Horn F, Moxley RT, III. Myotonia fluctuans. Arch Neurol 1990; 47: 268-272.

Ricker K, Moxley RT, III, Heine R, Lehmann-Horn F. Myotonia fluctuans. A third type of muscle sodium channel disease. Arch Neurol 1994; 51: 1095-1102.

Rojas CV, Wang JZ, Schwartz LS, Hoffman EP, Powell BR, Brown RH, Jr. A Met-to-Val mutation in the skeletal muscle Na+ channel alpha-subunit in hyperkalaemic periodic paralysis. Nature 1991; 354: 387-389.

Rosenfeld J, Sloan-Brown K, George AL, Jr. A novel muscle sodium channel mutation causes painful congenital myotonia. Ann Neurol 1997; 42: 811-814.

Rossignol E, Mathieu J, Thiffault I, Tetreault M, Dicaire MJ, Chrestian N *et al*. A novel founder SCN4A mutation causes painful cold-induced myotonia in French-Canadians. Neurology 2007; 69: 1937-1941.

Rudel R, Dengler R, Ricker K, Haass A, Emser W. Improved therapy of myotonia with the lidocaine derivative tocainide. J Neurol 1980; 222: 275-278.

Rudel R, Lehmann-Horn F, Ricker K, Kuther G. Hypokalemic periodic paralysis: in vitro investigation of muscle fiber membrane parameters. Muscle Nerve 1984; 7: 110-120.

Ruff RL. Insulin acts in hypokalemic periodic paralysis by reducing inward rectifier K+ current. Neurology 1999; 53: 1556-1563.

Arzel-Hezode M, McGoey S, Sternberg D, Vicart S, Eymard B, Fontaine B. Glucocorticoids may trigger attacks in several types of periodic paralysis. Neuromuscul Disord 2009a; 19: 217-219.

Arzel-Hezode M, Sternberg D, Tabti N, Vicart S, Goizet C, Eymard B *et al*. Homozygosity for dominant mutations increases severity of muscle channelopathies. Muscle Nerve 2010 Apr;41(4):470-7.

Sacconi S, Simkin D, Arrighi N, Chapon F, Larroque MM, Vicart S *et al*. Mechanisms underlying Andersen's syndrome pathology in skeletal muscle are revealed in human myotubes. Am J Physiol Cell Physiol 2009; 297: C876-C885.

Sansone V, Meola G, Links TP, Panzeri M, Rose MR. Treatment for periodic paralysis. Cochrane Database Syst Rev 2008; CD005045.

Sasaki R, Takano H, Kamakura K, Kaida K, Hirata A, Saito M *et al*. A novel mutation in the gene for the adult skeletal muscle sodium channel alpha-subunit (SCN4A) that causes paramyotonia congenita of von Eulenburg. Arch Neurol 1999; 56: 692-696.

Scheibe RJ, Mundhenk K, Becker T, Hallerdei J, Waheed A, Shah GN *et al*. Carbonic anhydrases IV and IX: subcellular localization and functional role in mouse skeletal muscle. Am J Physiol Cell Physiol 2008; 294: C402-C412.

Schoser BG, Schroder JM, Grimm T, Sternberg D, Kress W. A large German kindred with cold-aggravated myotonia and a heterozygous A1481D mutation in the SCN4A gene. Muscle Nerve 2007; 35: 599-606.

Sokolov S, Scheuer T, Catterall WA. Gating pore current in an inherited ion channelopathy. Nature 2007; 446: 76-78.

Sokolov S, Scheuer T, Catterall WA. Depolarization-activated gating pore current conducted by mutant sodium channels in potassium-sensitive normokalemic periodic paralysis. Proc Natl Acad Sci U S A 2008; 105: 19980-19985.

Standen NB, Stanfield PR. A potential- and time-dependent blockade of inward rectification in frog skeletal muscle fibres by barium and strontium ions. J Physiol 1978; 280: 169-191.

Starace DM, Bezanilla F. Histidine scanning mutagenesis of basic residues of the S4 segment of the shaker k+ channel. J Gen Physiol 2001; 117: 469-490.

Sternberg D, Maisonobe T, Jurkat-Rott K, Nicole S, Launay E, Chauveau D *et al*. Hypokalaemic periodic paralysis type 2 caused by mutations at codon 672 in the muscle sodium channel gene SCN4A. Brain 2001; 124: 1091-1099.

Streib EW SSYT. Transient Paresis in Myotonic Syndromes: a simplified electrophysiologic approach. Muscle & Nerve 1982; 5: 719-723.

Streib EW. Paramyotonia congenita: successful treatment with tocainide. Clinical and electrophysiologic findings in seven patients. Muscle Nerve 1987; 10: 155-162.

Struyk AF, Cannon SC. A Na+ channel mutation linked to hypokalemic periodic paralysis exposes a proton-selective gating pore. J Gen Physiol 2007; 130: 11-20.

Struyk AF, Cannon SC. Paradoxical depolarization of BA2+- treated muscle exposed to low extracellular K+: insights into resting potential abnormalities in hypokalemic paralysis. Muscle Nerve 2008a; 37: 326-337.

Struyk AF, Markin VS, Francis D, Cannon SC. Gating pore currents in DIIS4 mutations of NaV1.4 associated with periodic paralysis: saturation of ion flux and implications for disease pathogenesis. J Gen Physiol 2008b; 132: 447-464.

Struyk AF, Scoggan KA, Bulman DE, Cannon SC. The human skeletal muscle Na channel mutation R669H associated with hypokalemic periodic paralysis enhances slow inactivation. J Neurosci 2000; 20: 8610-8617.

Supuran CT, Scozzafava A. Carbonic anhydrase inhibitors and their therapeutic potential. Expert Opinion on Therapeutic Patents 2000; 10: 575-600.

Takahashi MP, Cannon SC. Enhanced slow inactivation by V445M: a sodium channel mutation associated with myotonia. Biophys J 1999; 76: 861-868.

Tang S, Mikala G, Bahinski A, Yatani A, Varadi G, Schwartz A. Molecular localization of ion selectivity sites within the pore of a human L-type cardiac calcium channel. J Biol Chem 1993; 268: 13026-13029.

Tawil R, McDermott MP, Brown R, Jr., Shapiro BC, Ptacek LJ, McManis PG *et al*. Randomized trials of dichlorphenamide in the periodic paralyses. Working Group on Periodic Paralysis. Ann Neurol 2000; 47: 46-53.

Tawil R, Ptacek LJ, Pavlakis SG, DeVivo DC, Penn AS, Ozdemir C *et al*. Andersen's syndrome: potassium-sensitive periodic paralysis, ventricular ectopy, and dysmorphic features. Ann Neurol 1994; 35: 326-330.

Tidball JG, Wehling-Henricks M. Damage and inflammation in muscular dystrophy: potential implications and relationships with autoimmune myositis. Curr Opin Rheumatol 2005; 17: 707-713.

Torres C, Moxley RT, Griggs RC. Quantitative testing of handgrip strength, myotonia, and fatigue in myotonic dystrophy. J Neurol Sci 1983; 60: 157-168.

Tricarico D, Barbieri M, Camerino DC. Acetazolamide opens the muscular KCa2+ channel: a novel mechanism of action that may explain the therapeutic effect of the drug in hypokalemic periodic paralysis. Ann Neurol 2000; 48: 304-312.

Tricarico D, Barbieri M, Mele A, Carbonara G, Camerino DC. Carbonic anhydrase inhibitors are specific openers of skeletal muscle BK channel of K+-deficient rats. FASEB J 2004; 18: 760-761.

Tricarico D, Lovaglio S, Mele A, Rotondo G, Mancinelli E, Meola G *et al*. Acetazolamide prevents vacuolar myopathy in skeletal muscle of K(+) -depleted rats. Br J Pharmacol 2008; 154: 183-190.

Tricarico D, Servidei S, Tonali P, Jurkat-Rott K, Camerino DC. Impairment of skeletal muscle adenosine triphosphate-sensitive K+ channels in patients with hypokalemic periodic paralysis. J Clin Invest 1999; 103: 675-682.

Trip J, de VJ, Drost G, Ginjaar HB, van Engelen BG, Faber CG. Health status in non-dystrophic myotonias: close relation with pain and fatigue. J Neurol 2009a; 256: 939-947.

Trip J, Drost G, van Engelen BG, Faber CG. Drug treatment for myotonia. Cochrane Database Syst Rev 2006; CD004762.

Trip J, Drost G, Verbove DJ, van der Kooi AJ, Kuks JB, Notermans NC *et al*. In tandem analysis of CLCN1 and SCN4A greatly enhances mutation detection in families with non-dystrophic myotonia. Eur J Hum Genet 2008; 16: 921-929.

Trip J, Pillen S, Faber CG, van Engelen BG, Zwarts MJ, Drost G. Muscle ultrasound measurements and functional muscle parameters in non-dystrophic myotonias suggest structural muscle changes. Neuromuscul Disord 2009b. Jul;19(7):462-7

Trudell RG, Kaiser KK, Griggs RC. Acetazolamide-responsive myotonia congenita. Neurology 1987; 37: 488-491.

Tsujino A, Maertens C, Ohno K, Shen XM, Fukuda T, Harper CM *et al*. Myasthenic syndrome caused by mutation of the SCN4A sodium channel. Proc Natl Acad Sci U S A 2003; 100: 7377-7382.

Van den Bergh P, Van den Wyngaert F, J M Brucher. K+ sensitivity in pure paramyotonia congenita. Neurology 1991;41(Suppl 1); 419-20.

Varadi G, Strobeck M, Koch S, Caglioti L, Zucchi C, Palyi G. Molecular elements of ion permeation and selectivity within calcium channels. Crit Rev Biochem Mol Biol 1999; 34: 181-214.

Vassilev PM, Scheuer T, Catterall WA. Identification of an intracellular peptide segment involved in sodium channel inactivation. Science 1988; 241: 1658-1661.

Vedantham V, Cannon SC. Slow inactivation does not affect movement of the fast inactivation gate in voltage-gated Na+ channels. J Gen Physiol 1998; 111: 83-93.

Venance SL, Cannon SC, Fialho D, Fontaine B, Hanna MG, Ptacek LJ *et al*. The primary periodic paralyses: diagnosis, pathogenesis and treatment. Brain 2006; 129: 8-17.

Venance SL, Jurkat-Rott K, Lehmann-Horn F, Tawil R. SCN4A-associated hypokalemic periodic paralysis merits a trial of acetazolamide. Neurology 2004; 63: 1977.

Vicart S, Sternberg D, Fontaine B, Meola G. Human skeletal muscle sodium channelopathies. Neurol Sci 2005; 26: 194-202.

Vicart S, Sternberg D, Fournier E, Ochsner F, Laforet P, Kuntzer T *et al*. New mutations of SCN4A cause a potassium-sensitive normokalemic periodic paralysis. Neurology 2004; 63: 2120-2127.

Volosin K, Greenberg RM, Greenspon AJ. Tocainide associated agranulocytosis. Am Heart J 1985; 109: 1392-1393.

Wagner S, Lerche H, Mitrovic N, Heine R, George AL, Lehmann-Horn F. A novel sodium channel mutation causing a hyperkalemic paralytic and paramyotonic syndrome with variable clinical expressivity. Neurology 1997; 49: 1018-1025.

Walsh R, Wang Y, Statland J, Bundy B, Barohn RJ, CINCH study group. The nondystrophic myotonias: genotype-phenotype correlation and longitudinal study. Clinical phenotype characterization [abstract]. Neurology 2007; 68(Suppl 1):A297.

Wang GK, Calderon J, Wang SY. State- and use-dependent block of muscle Nav1.4 and neuronal Nav1.7 voltage-gated Na+ channel isoforms by ranolazine. Mol Pharmacol 2008a; 73: 940-948.

Wang GK, Mitchell J, Wang SY. Block of persistent late Na+ currents by antidepressant sertraline and paroxetine. J Membr Biol 2008b; 222: 79-90.

Wang J, Dubowitz V, Lehmann-Horn F, Ricker K, Ptacek L, Hoffman EP. In vivo sodium channel structure/function studies: consecutive Arg1448 changes to Cys, His, and Pro at the extracellular surface of IVS4. Soc Gen Physiol Ser 1995; 50: 77-88.

Wang Q, Liu M, Xu C, Tang Z, Liao Y, Du R *et al*. Novel CACNA1S mutation causes autosomal dominant hypokalemic periodic paralysis in a Chinese family. J Mol Med 2005; 83: 203-208.

Wang Y, Statland J, Walsh RJ, Brundy B, Barohn RJ, Herbelin L *et al*. Clinical phenotype characterization of nondystrophic myotonias[abstract]. J Clin Neuromusc Dis 2008; 9(1): 367.

Weber MA, Nielles-Vallespin S, Huttner HB, Wohrle JC, Jurkat-Rott K, Lehmann-Horn F *et al*. Evaluation of patients with paramyotonia at 23Na MR imaging during cold-induced weakness. Radiology 2006; 240: 489-500.

Wetzel P, Gros G. Inhibition and kinetic properties of membrane-bound carbonic anhydrases in rabbit skeletal muscles. Arch Biochem Biophys 1998; 356: 151-158.

Wetzel P, Scheibe RJ, Hellmann B, Hallerdei J, Shah GN, Waheed A *et al*. Carbonic anhydrase XIV in skeletal muscle: subcellular localization and function from wild-type and knockout mice. Am J Physiol Cell Physiol 2007; 293: C358-C366.

Wu FF, Gordon E, Hoffman EP, Cannon SC. A C-terminal skeletal muscle sodium channel mutation associated with myotonia disrupts fast inactivation. J Physiol 2005; 565: 371-380.

Wu FF, Takahashi MP, Pegoraro E, Angelini C, Colleselli P, Cannon SC *et al*. A new mutation in a family with cold-aggravated myotonia disrupts Na(+) channel inactivation. Neurology 2001; 56: 878-884.

Yang N, George AL, Jr., Horn R. Molecular basis of charge movement in voltage-gated sodium channels. Neuron 1996a; 16: 113-122.

Yang N, Ji S, Zhou M, Ptacek LJ, Barchi RL, Horn R *et al*. Sodium channel mutations in paramyotonia congenita exhibit similar biophysical phenotypes in vitro. Proc Natl Acad Sci U S A 1994; 91: 12785-12789.

Zhang L, Benson DW, Tristani-Firouzi M, Ptacek LJ, Tawil R, Schwartz PJ *et al*. Electrocardiographic features in Andersen-Tawil syndrome patients with KCNJ2 mutations: characteristic T-U-wave patterns predict the KCNJ2 genotype. Circulation 2005; 111: 2720-2726.

Zhou H, Lillis S, Loy RE, Ghassemi F, Rose MR, Norwood F *et al*. Multi-minicore disease and atypical periodic paralysis associated with novel mutations in the skeletal muscle ryanodine receptor (RYR1) gene. Neuromuscul Disord 2010; 20: 166-173.