Understanding the Pathophysiology of Skeletal Muscle Channelopathies

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Submitted for PhD
Declaration

I, Karen Suetterlin 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

Skeletal muscle channelopathies are rare inherited neuromuscular conditions caused by ion channel gene mutations. They are grouped into the non-dystrophic myotonias and the periodic paralyses (PP). An intriguing but as yet unexplained phenomenon is that phenotype severity varies significantly with species, gender and age.

The first part of my thesis explores my hypothesis that this phenotype variability in skeletal muscle channelopathies reflects physiological differences in skeletal muscle excitability. To do this, I reverse translated Muscle Velocity Recovery Cycles (MVRCs), an *in vivo* technique for assessing ion channel function, from humans to mice.

My data suggest that murine skeletal muscle has increased chloride conductance compared to human skeletal muscle. This could explain the species difference seen between mouse models of Myotonia Congenita and humans with clinical disease. I also found gender differences in healthy murine muscle excitability that vary by muscle and are associated with gender difference in the skeletal muscle channelome. On the background of these physiological gender differences in murine muscle excitability, the effect of gain-of-function sodium channel mutation is more severe in male mice.

Finally, I found that the phenotype change with age seen in patients with PP also occurs in a mouse model. Surprisingly, an increased resistance to potassium-induced weakness was most pronounced in old wild-type mice, suggesting it is a phenomenon of normal aging. In contrast, the onset of permanent progressive weakness was specific to PP mouse muscle. My experiments suggest this may be due to acquired ryanodine receptor dysfunction resulting in sarcoplasmic reticulum calcium leak and impaired mitochondrial oxidative capacity.

The aim of the second part of my thesis was to extend our knowledge of ClC-1 structure-function and improve genetic counselling for patients with Myotonia Congenita (MC). I identified a novel molecular pathomechanism for MC and found that dominant mutations cluster in the first half of the channel sequence.
Combining variant location with functional characterisation significantly improves the accuracy of genetic counselling we can provide patients.
Impact Statement

This work is the first description of Muscle Velocity Recovery Cycles (MVRCs) in mice. MVRCs were developed in humans and enable in vivo assessment of muscle excitability properties and ion channel function. My work demonstrates that, in mice, MVRCs can be performed both in vivo and ex vivo and can be combined with pharmacological blockade to selectively probe ion channel function. My work also provides normal data for both male and female mice at a range of ages. MVRCs are a powerful new tool to characterise transgenic mouse models and to explore skeletal muscle excitability and specific ion channel function. They will benefit researchers investigating ion channel function, skeletal muscle physiology and mouse models of neuromuscular disease.

The direct comparison and identification of differences between human and murine muscle excitability helps explain some of the phenotype differences between mice and humans with skeletal muscle channelopathies. An improved understanding of differences between model organisms and human physiology is critical to improve translation of findings from animal models into humans and will benefit both patients and researchers in the field of neuromuscular disease.

An understanding of gender differences in phenotype is also key to ensure gender parity in investigation and treatment of these disorders. My findings suggest that the long exercise test, one of the key diagnostic tests in the work-up of periodic paralysis, is more likely to be negative in young women with genetically confirmed periodic paralysis. Therefore, a long exercise test may be of less diagnostic value in this subgroup of patients. This is important to highlight as awareness of this may reduce young women with periodic paralysis’ time to diagnosis. A second key point is that this gender difference was only apparent when young and old groups were compared separately. This has important implications when considering trial design. The identified gender differences in murine muscle excitability and the skeletal muscle channelome also reinforce the importance of preclinical studies being performed in animals of both sexes and suggest gender differences in the response to therapeutics may exist. These findings are relevant to academics or
pharmaceutical companies involved in basic research and reinforce the need for
gender parity in research subjects at both clinical and pre-clinical level.

The work investigating change in phenotype with age provides the first description
of the pathophysiology of permanent progressive weakness in periodic paralysis.
The mechanism will need to be confirmed in patients with Hyperkalaemic Periodic
Paralysis and then for other types of Periodic Paralysis but represents an important
first step for both patients and researchers in this field. It also provides a replicable
example whereby studying a rare monogenic disease can provide insight that is
relevant and applicable for the broader population e.g. on how normal muscle
physiology changes with age.

Finally, the correlation of our large service-level clinical, genetic, and functional
data set provides an important step forward for patients with Myotonia Congenita.
This work means that an initial estimation of the risk of an associated dominant
inheritance pattern can be given based on the variant location in the protein alone.
Once functional characterisation is available this risk can be refined substantially so
that the accuracy of genetic counselling, we are able to provide patients is much
greater and has a more defined evidence base. This framework will benefit both
clinicians looking after and the people with Myotonia Congenita.
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Finally, I would like to thank Professor Avan Sayer, my mentor on the MRC Fellowship, for her time, care, and wisdom.

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List of Abbreviations

9AC = 9 Anthracene Carboxylic Acid

ATS = Andersen Tawil Syndrome

CaV1.1 = Skeletal Muscle Voltage Gated Calcium Channel

CIC-1 = Skeletal Muscle Voltage Gated Chloride Channel

CMAP = Compound Muscle Action Potential

CRU = Calcium Release Unit

$E_K$ = Equilibrium potential (for potassium)

Hyper PP = Hyperkalaemic Periodic Paralysis

Hypo PP = Hypokalaemic Periodic Paralysis

Kir2.1 = Inward Rectifier Potassium Channel 1

$K_o$ = Extracellular potassium

MC = Myotonia Congenita

$Na_v1.4$ = Skeletal Muscle Voltage Gated Sodium Channel

NDM = Non-dystrophic Myotonia

PMC = Paramyotonia Congenita

PP = Periodic Paralysis

RMP = Resting Membrane Potential

RyR1 = Ryanodine Receptor 1

SCM = Sodium Channel Myotonia

SERCA = SarcoEndoplasmic Reticulum Calcium ATPase

SOCE = Store Operated Calcium Entry
SR = Sarcoplasmic Reticulum

TEVC = Two Electrode Voltage Clamp

$V_m = \text{Membrane potential}$
1 Introduction

The skeletal muscle channelopathies are a group of genetic disorders whose manifestations range from flaccid paralysis to myotonia. They are broadly divided into the periodic paralyses (PP) and the non-dystrophic myotonias (NDM) (Figure 1-1). The periodic paralyses include Hyperkalaemic Periodic Paralysis (Hyper PP), Hypokalaemic Periodic Paralysis (Hypo PP) and Andersen-Tawil Syndrome (ATS). The non-dystrophic myotonias (NDM) include Myotonia Congenita (MC), Paramyotonia Congenita (PMC) and Sodium Channel Myotonia (SCM). All these conditions are caused by mutations in skeletal muscle ion channels that alter muscle excitability. In the case of PP, ion channel mutation predisposes to episodes of sarcolemmal inexcitability which manifest as muscle weakness or paralysis. These episodes of weakness are typically associated with high (Hyper PP) or low (Hypo PP) serum potassium levels. In NDM, ion channel mutations predispose to episodes of sarcolemmal hyperexcitability that manifest as myotonia, and patients experience as muscle stiffness or pain.

![Figure 1-1 Clinical and genetic spectrum of the primary skeletal muscle channelopathies. Adapted from (Suetterlin, Männikkö and Hanna, 2014). Non-dystrophic myotonias and periodic paralyses with the associated genes (second row) are placed on the spectrum of clinical presentations. ADMC= Autosomal Dominant Myotonia Congenita, ARMC = Autosomal Recessive Myotonia Congenita.](image)

There is high phenotypic variability in skeletal muscle channelopathies. This is partly due to mutation-specific effects (Sternberg et al., 2001; Miller et al., 2004) but significant variability also occurs within families with the same mutation (Sternberg et al., 2001; Colding-Jorgensen, 2005). This is particularly true for MC.
Gender is one of the factors known to influence channelopathy phenotype. Men have been reported to be more severely affected than their female relatives in Hypo PP (Links et al., 1990; Ke et al., 2013), Hyper PP (Ptacek et al., 1991) and MC (MailAnder et al., 1996; Deymeer et al., 1999). Age also affects phenotype in PP. A consistent observation is that around middle-age the severity of paralytic attacks may reduce and fixed, permanent weakness develop (Biemond and Daniels, 1934; McManis, Lambert and Daube, 1986; Links et al., 1990; Sternberg et al., 2001). The mechanisms underlying the increased severity in males and the change from an episodic to a degenerative phenotype with age are not known.

Our understanding of the pathophysiology of skeletal muscle channelopathies has been greatly advanced by transgenic mouse models (Hayward et al., 2008; Wu et al., 2011, 2012; Corrochano et al., 2014). PP mouse models recapitulate many of the features of PP. Weakness can be reliably induced by artificially raising (Hayward et al., 2008; Corrochano et al., 2014) or lowering (Wu et al., 2011, 2012) potassium levels. The gender difference in phenotype severity is also present (Wu et al., 2012; Corrochano et al., 2014) as is the onset of permanent weakness with age in Hyper PP mice (Corrochano et al., 2014). However, mice and humans clearly have significant differences in their physiology and therefore, not surprisingly, mice are not a phenocopy of humans with periodic paralysis. The most striking difference is that mice with Hypo PP do not have spontaneous episodes of weakness. In fact, the locomotor behaviour of Hypo PP mice is indistinguishable from their wild-type (WT) littermates (Wu et al., 2011, 2012). The reason for this is not known but it suggests that something intrinsic to murine muscle excitability may protect against spontaneous episodes of weakness in Hypo PP.

This thesis investigates the effect of physiological and pathophysiological variation on skeletal muscle channelopathy phenotype. The underlying tenet of my thesis is that phenotype difference provides an opportunity to better understand both the physiology and pathophysiology of skeletal muscle.
1.1 **Skeletal Muscle Physiology**

The processes involved in skeletal muscle contraction can be broadly divided into those relating to excitability, excitation-contraction coupling, calcium-induced muscle contraction and ionic homeostasis (Figure 1-2). All these processes require energy, usually in the form of ATP.

![Figure 1-2 The Processes Involved in Skeletal Muscle Contraction.](image)

Skeletal muscle excitability involves the initiation of an action potential at the neuromuscular junction (not shown) and subsequent propagation of an action potential along the sarcolemma and into the t-tubules. Muscle Velocity Recovery Cycles (MVRCs) and Frequency ramp response provide an indirect measurement of muscle excitability by examining changes in conduction velocity and CMAP amplitude. Excitation-contraction coupling links muscle excitability to muscle contraction and requires depolarisation of the t-tubule voltage-gated calcium channel (CaV1.1, yellow). Activation of CaV1.1 physically activates the sarcoplasmic reticulum (SR) calcium release channel (RyR1, grey) a process known as excitation-contraction coupling. Caffeine application bypasses excitability to act on RyR1 directly. Calcium released through RyR1 triggers calcium-induced muscle contraction but also stimulates mitochondrial ATP synthesis. The energy charge is the ratio of ATP to ADP and AMP and reflects the metabolic status of the tissue. ATP is critical for ionic homeostasis e.g. to maintain skeletal muscle resting membrane potential via the Na⁺ K⁺ ATPase.
(green & yellow stripe) and SR calcium stores via the SR Ca2+ ATPase (grey & yellow stripe). ATP is also critical for myosin-actin cross bridge cycling.

1.1.1 Skeletal Muscle Excitability

Skeletal muscle action potential initiation and propagation involves 3 contiguous electrophysiological processes in different surface membrane regions (Fraser, Huang and Pedersen, 2011). Firstly, transmitter release from nerve terminals at the end plate activates Ach receptors at the motor endplate. This triggers Na\(^+\) influx which depolarises the adjacent sarcolemma activating skeletal muscle voltage-gated sodium channels (NaV1.4). Ongoing Na\(^+\) influx through NaV1.4 depolarises adjacent sarcolemma, activating NaV1.4 channels and allowing the action potential to be propagated along the sarcolemma and into specialised invaginations of the sarcolemma known as t-tubules.

Once the upstroke of the action potential exceeds approximately -60mV, voltage gated K\(^+\) channels are opened. This facilitates K\(^+\) efflux and sarcolemmal and t-tubular repolarisation. However, as t-tubules represent a confined space where the tortuosity factor is increased and potassium diffusion restricted, repeated K\(^+\) efflux following an action potential can rapidly increase t-tubular K\(^+\) concentration. If unchecked, this activity-induced t-tubule K\(^+\) increase would lead to membrane depolarisation (Frank, 1957; Gage and Eisenberg, 1967). Therefore, t-tubule proteins are specialised to provide rapid potassium reuptake and buffering. The two key proteins involved in t-tubule K\(^+\) reuptake are Kir2.1 channels and the Na\(^+\) K\(^+\) ATPase t-tubule isoform (α2) (DiFranco, Hakimjavadi, et al., 2015; DiFranco, Yu, et al., 2015). The key protein for t-tubule K\(^+\) buffering is the skeletal muscle voltage-gated chloride channel (CIC-1) (DiFranco, Herrera and Vergara, 2011).

Action potential initiation and propagation is an all-or-nothing phenomenon and is followed by an absolute refractory period where further action potentials cannot be triggered no matter how large the stimulus. However, once the absolute refractory period is over, subthreshold changes in excitability can modulate the likelihood of action potential initiation and/or speed of its propagation (Farmer, Buchthal and Rosenfalck, 1960; Juel, 1988; Z’Graggen and Bostock, 2009; Fraser, Huang and Pedersen, 2011; Pedersen, L.-H. Huang and Fraser, 2011). This is demonstrated by the observation that multiple subthreshold stimuli can eventually
evoke a response despite the stimulus intensity remaining constant and that 15 to 20% less current is needed to trigger a second action potential 20 to 30ms after the first (Farmer, Buchthal and Rosenfalck, 1960). This reduction in threshold current is a result of charge accumulation in the t-tubule membrane (Frank, 1957; Gage and Eisenberg, 1969). The passive dissipation of t-tubule membrane charge gradually returns the membrane to baseline and can be observed following an action potential in skeletal muscle (Figure 1-3A). This phenomenon has been referred to as the negative or early ‘Depolarising after Potential’ (DAP). The DAP is known to arise from the t-tubule membrane as detubulated skeletal muscle fibres have no DAP (Freygang, Goldstein and Hellam, 1964; Gage and Eisenberg, 1967) (Figure 1-3B).

Figure 1-3 After Depolarisations in Skeletal Muscle. A. The presence of an after depolarisation following an action potential. B. Detubulation removes after depolarisation. Taken from (Gage and Eisenberg, 1969).

The more action potentials that are triggered the longer the membrane takes to recover to baseline. For example, recovery takes around 50 times longer following a pair of stimuli than following a single action potential (Farmer, Buchthal and Rosenfalck, 1960). This is because the depolarisation that remains after decay of the early after potential, known as the late DAP, shows significant summation (Freygang, Goldstein and Hellam, 1964). Peak amplitude of the late DAP increases by approximately 1mV per impulse for the first 8 impulses delivered at 10ms intervals (Freygang, Goldstein and Hellam, 1964). Moreover, as trains of action
potentials become longer, resting membrane potential gradually depolarises (Freygang, Goldstein and Hellam, 1964; Gage and Eisenberg, 1969). The late DAP has been attributed to potassium accumulation in the t-tubules during muscle activity. Consistent with this hypothesis, de-tubulation removes the late DAP and prevents the gradual depolarisation of RMP in response to trains of action potentials (Gage and Eisenberg, 1969).

Figure 1-4 Muscle Velocity Recovery Cycles of my TA muscle. A. Varying the inter-stimulus interval detects a period of early supernormality and late supernormality. B. The effect of increasing the number of conditioning stimuli on early and late supernormality. The point referred to as the Muscle Relative Refractory Period is also shown.
Both early and late DAP are associated with a period of increased speed of conduction known as supernormality. In frog Sartorius fibres the early supernormal period begins between 4-5msec after the first stimulus and continues for up to 30msec (Farmer, Buchthal and Rosenfalck, 1960). The late supernormal period beings around 100ms after the first stimulus and its duration may be prolonged with an increasing number of conditioning stimuli (Z’Graggen and Bostock, 2009). Muscle Velocity Recovery Cycles (MVRCs) exploit this relationship between excitability and conduction velocity in order to provide an indirect measure of skeletal muscle excitability (Z’Graggen and Bostock, 2009). Direct measurement of excitability is not possible as uniform polarisation of long, thin muscle fibres would require large injections of current that would likely damage the muscle fibre (Z’Graggen and Bostock, 2009).

In human MVRCs, early supernormaility which reflects the early DAP, peaks with an inter stimulus interval of less than 15ms (Z’Graggen and Bostock, 2009) (Figure 1-4). Late supernormality, which reflects the late DAP, peaks with an interstimulus interval of between 50 and 150ms (Z’Graggen and Bostock, 2009) (Figure 1-4). Early and late supernormality can be distinguished by time frame and by the effect of additional conditioning stimuli (Figure 1-4). Increasing the number of stimuli has a greater effect on late supernormality (Z’Graggen and Bostock, 2009). This is expected given that the early DAP is associated with a passive dissipation of charge from the t-tubule membrane whilst the late DAP is associated with the active accumulation of potassium in the t-tubules (Gage and Eisenberg, 1969). The point at which there is no difference in conduction velocity between conditioned and unconditioned stimuli is known as the Muscle Relative Refractory Period and represents the end of the relative refractory period in the excitability recovery cycle (Farmer, Buchthal and Rosenfalck, 1960; Juel, 1988; Z’Graggen and Bostock, 2009).

1.1.2 Excitation-contraction coupling

In order for muscle to contract in response to an electrical stimulus the t-tubule network must be intact (Gage and Eisenberg, 1967). Detubulation of skeletal muscle with glycerol means that high frequency stimulation of the muscle can be
performed without any evidence of movement (Gage and Eisenberg, 1967). However, glycerol treated fibres can still be made to contract via application of caffeine (Gage and Eisenberg, 1969). Caffeine acts directly on ryanodine receptors (RyR1) to trigger Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR).

The point at which excitation and contraction are physically linked is the triad or calcium release unit (CRU). This is a structure that links t-tubules with SR terminal cisternae. The CRU includes voltage gated calcium channels (CaV1.1) situated within the t-tubule membrane that physically interact with RyR1 channels situated within the SR membrane (Figure 1-2). CaV1.1 acts as a t-tubule voltage sensor. Depolarisation of the t-tubule membrane activates CaV1.1 which physically activates RyR1 causing Ca\(^{2+}\) efflux from the SR (Figure 1-2).

### 1.1.3 Calcium-induced muscle contraction

Excitation-contraction coupling facilitates near synchronous opening of RyR1 channels throughout the length of the muscle fibre in response to a propagated action potential. Each RyR1 opening creates a local Ca\(^{2+}\) spark which then diffuses into the bulk cytoplasm. Multiple synchronised RyR1 openings result in a rapid cell-wide increase in calcium up to 100-fold its baseline concentration with each tetanic contraction (Periasamy et al., 2017). This rapid, massive increase in cytosolic calcium triggers muscle contraction. Crossbridge cycling and hence muscle contraction can continue as long as there are sufficient amounts of ATP and Ca\(^{2+}\) in the cytoplasm.

### 1.1.4 Ionic Homeostasis

The initiation and propagation of an action potential and the release of Ca\(^{2+}\) via RyR1 trigger huge changes in the ionic milieu of the cell. Interstitial potassium can reach values as high as 10mM during fatiguing exercise (Sejersted and Sjøgaard, 2000). Intracellular Na\(^{+}\) is reported to double (Clausen, 2010) and intracellular Ca\(^{2+}\) can increase 100-fold with each tetanic contraction (Periasamy et al., 2017). Ionic homeostasis, particularly of Na\(^{+}\), K\(^{+}\) and Ca\(^{2+}\) is critical for repolarisation and muscle relaxation to occur.
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Ionic Homeostasis

There is one active transport system for Na\(^+\) and K\(^+\), the Na\(^+\) K\(^+\) ATPase (Figure 1-2). This pump is responsible for generating the transmembrane Na\(^+\) and K\(^+\) gradients upon which action potential generation and propagation rely. As well as being central to muscle excitability, the transmembrane Na\(^+\) gradient drives cotransport of amino acids, chloride and the antiport of hydrogen and calcium (Clausen, 2003) thus it is also important for wider ionic homeostasis and cell nutrition.

The Na\(^+\) K\(^+\) pump extrudes 3 Na\(^+\) out of the cell for every 2 K\(^+\) it pumps in. It therefore has a hyperpolarising effect. Two isoforms exist of the Na\(^+\) K\(^+\) ATPase in muscle, the α1 and α2 isoforms. The α2 isoforms is found in the t-tubules, whilst the α1 isoform is predominantly in the sarcolemma. Whilst the α1 isoform is primarily concerned with maintenance of resting membrane potential, the α2 isoform is primarily concerned with activity-induced t-tubule K\(^+\) reuptake and has little effect on the resting membrane potential (DiFranco, Hakimjavadi, et al., 2015). The activity of the pump requires ATP and in skeletal muscle is estimated to account for 7% of total energy turnover (Clausen, 2003; Barclay, Woledge and Curtin, 2007).

The Na\(^+\) K\(^+\) pump is stimulated acutely by depolarisation, increase in intracellular sodium and/or increase in extracellular potassium concentration. Repetitive tetanic stimulation of rat soleus in vivo has demonstrated hyperpolarisation of resting membrane potential that is secondary to the electrogenic pump. This starts almost immediately, reaches a peak around 9 minutes and then starts to decline (Hicks and McComas, 1989).

Hormones such as epinephrine, norepinephrine, insulin, IGF-1 can also stimulate Na\(^+\) K\(^+\) pump activity acutely, as can calcitonin gene related peptide released from nerve terminals (Clausen, 2003). Thyroid hormones, adrenal steroids, exercise training or inactivity, fasting, growth, hypoxia and potassium deficiency can all exert an effect on long term regulation of the pump, usually via up or down regulation of gene expression.

Whilst Na\(^+\) and K\(^+\) homeostasis are critical for the initiation and propagation of action potential, Ca\(^{2+}\) homeostasis is critical for muscle contraction and relaxation.
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Ionic Homeostasis

(Figure 1-2). In the resting state Ca\(^{2+}\) concentration in the cytosol is maintained between 50 and 100nmol/L (Periasamy et al., 2017). On RyR1 opening, cytosolic Ca\(^{2+}\) is increased around 100-fold (Periasamy et al., 2017). For skeletal muscle to relax, this cytosolic Ca\(^{2+}\) flood must be cleared and actively pumped back into the SR. The key protein for maintaining the enormous concentration gradient between SR and cytoplasm is the SR Ca\(^{2+}\) ATPase or SERCA pump. The isoforms expressed in skeletal muscle vary with fibre type. SERCA 1 is expressed in fast twitch fibres whilst SERCA 2 is expressed in both fast and slow twitch fibres. The dynamics of Ca\(^{2+}\) release and reuptake vary with fibre type and the different SERCA isoforms contribute to the differences in Ca\(^{2+}\) dynamics with fibre type (Schiaffino and Reggiani, 2011).

Although the majority of Ca\(^{2+}\) homeostasis is performed via SERCA reuptake into the SR, ‘forward mode’ of the Na\(^+\)/Ca\(^{2+}\) exchanger and activity of the plasma membrane Ca\(^{2+}\) ATPase can also contribute. These proteins are situated within the t-tubule membrane or sarcolemma respectively and therefore result in Ca\(^{2+}\) extrusion to the extracellular space (Periasamy et al., 2017). This has the potential to deplete the SR Ca\(^{2+}\) store with repetitive stimulation.

Depletion of SR Ca\(^{2+}\) stores with repetitive stimulation is prevented by a process called Store Operated Calcium Entry (SOCE). SOCE involves the SR Ca\(^{2+}\) sensor STIM1 and the calcium release-activated calcium channel ORAI1. STIM1 senses local depletion of Ca\(^{2+}\) immediately behind RyR1 channels. This triggers STIM1 to cluster and translocate from the SR to the t-tubule membrane where it activates ORAI1, facilitating extracellular Ca\(^{2+}\) entry (Koenig, Choi and Launikonis, 2018). Thus the SOCE transient is shaped by both action potential frequency and SR Ca\(^{2+}\) pump activity (Koenig, Choi and Launikonis, 2018). This helps prevent contraction failure by ensuring that SR Ca\(^{2+}\) stores are maintained during high frequency stimulation.

Mitochondria also have a key role in skeletal muscle calcium dynamics. They are tethered to the SR in the vicinity of the CRU (Franzini-armstrong and Boncompagni, 2011) and act as dynamic calcium sinks (Rossi et al., 2011). Tetanic stimulation of skeletal muscle results in robust and sustained Ca\(^{2+}\) accumulation in mitochondria.
1.1.5 Energetic requirements

Skeletal muscle accounts for around 40% of body mass, consumes nearly 80% of available glucose and regulates basal metabolic rate and whole body expenditure (Barclay, Woledge and Curtin, 2007; Periasamy et al., 2017). Muscle can increase its energy expenditure 20 to 30-fold during intense exercise and continued exercise can exhaust fat reserve (Periasamy et al., 2017). This energy expenditure is primarily in the form of ATP. As described above, ATP production is coupled with muscle contraction via Ca\(^{2+}\) stimulation of oxidative phosphorylation in mitochondria (Jouaville et al., 1999).

Ionic homeostasis is responsible for nearly 50% of skeletal muscle energy expenditure and the two key consumers are the Na\(^+\)/K\(^+\) ATPase and SERCA pump. The Na\(^+\)/K\(^+\) ATPase accounts for approximately 7% of total skeletal muscle energy turnover (Barclay, Woledge and Curtin, 2007). SERCA activity accounts for 30 to 40% of the energetic cost of isometric contraction (Barclay, Woledge and Curtin, 2007). The majority of the remaining energetic cost is cross-bridge cycling (Barclay, Woledge and Curtin, 2007).

1.1.6 Skeletal Muscle response to Hypokalaemia

The membrane response of skeletal muscle to reduction in serum potassium is bistable (Siegenbeek van Heukelom, 1991). This bistability occurs because of the properties of the inward rectifier potassium channels. In skeletal muscle this is predominantly Kir2.1 (DiFranco, Hakimjavadi, et al., 2015). Kir 2.1 channels exhibit a strong dependence on resting membrane potential (V\(_m\)) and extracellular K\(^+\) concentration (K\(_o\)). Kir 2.1 are open when V\(_m\) is equal to, or more negative than, the potassium equilibrium potential (E\(_K\)). E\(_K\) is the potential at which net flow of potassium through any open channels is 0. E\(_K\) thus reflects a combination of electrical and chemical gradients acting on the ion. Thus, if extracellular K\(^+\) is reduced sufficiently, or V\(_m\) rises sufficiently, Kir2.1 will close. When Kir2.1 channels
close, the membrane depolarises until delayed rectifier channels open at around -60mV and stabilise the membrane potential. Thus, the bistability of the sarcolemma results in a bimodal distribution of RMP (Jurkat-Rott et al., 2009). These two states of sarcolemmal RMP are referred to as P1 or P2. In P1, Kir2.1 channels are open and the sarcolemmal RMP is around -80 to -90mV. In P2 Kir2.1 channels have closed and the sarcolemmal RMP is around -60mV (Jurkat-Rott et al., 2009).

In response to mild or moderate hypokalaemia, Kir 2.1 channels remain open and healthy skeletal muscle hyperpolarises (Geukes Foppen, van Mil and van Heukelom, 2002; Jurkat-Rott et al., 2009). However, if hypokalaemia is severe, Kir 2.1 channels will close and the membrane will depolarise to P2 (Siegenbeek van Heukelom, 1991; Geukes Foppen, van Mil and van Heukelom, 2002; Jurkat-Rott et al., 2009). The exact potassium concentration at which this occurs varies between muscles but in healthy muscle is usually less than 1.5mM K⁺ (Geukes Foppen, van Mil and van Heukelom, 2002).

However, Kir2.1 closure occurs in milliseconds whilst hypokalaemia-induced sarcolemmal depolarisation takes place over minutes (Siegenbeek van Heukelom, 1991; Geukes Foppen, van Mil and van Heukelom, 2002). Barium, a Kir 2.1 blocker, also depolarises the sarcolemma in minutes (Gallant, 1983; Geukes Foppen, van Mil and van Heukelom, 2002). It takes 5 to 10 minutes for barium-induced depolarisation of the sarcolemma, despite the fact that barium binds to Kir2.1 channels within milliseconds (20-200ms) and increases membrane resistance within seconds (30s) (Geukes Foppen, 2004). Thus, the time scale of the depolarisation response to hypokalaemia or indeed barium is far longer than would be predicted if sarcolemmal response were due to the closure of Kir2.1 alone (Siegenbeek van Heukelom, 1991; Geukes Foppen, van Mil and van Heukelom, 2002; Geukes Foppen, 2004). This suggests the involvement of other factors in determining the response of $V_m$ to changes in $K_o$.

Several pieces of experimental data suggest that chloride movement is a major factor in determining the response of $V_m$ to changes in $K_o$ (van Mil, Geukes Foppen and Siegenbeek van Heukelom, 1997; Geukes Foppen, van Mil and van Heukelom,
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1.1.7 Skeletal Muscle response to Hyperkalaemia

In contrast to hypokalaemia, the response to hyperkalaemia in skeletal muscle is not bistable and involves progressive depolarisation of $V_m$ with increasing $K_o$. Initially the depolarisation caused by an increase in extracellular $K^+$, takes skeletal muscle closer to the action potential firing threshold so reducing the current required to reach threshold (Juel, 1988). However, if the rise in potassium is excessive or prolonged, it will result in greater depolarisation, sodium channel inactivation and eventually loss of force (MacIntosh, Holash and Renaud, 2012). This is one mechanism that is implicated in the development of fatigue in skeletal muscle (MacIntosh, Holash and Renaud, 2012).

During and after high intensity exercise there are rapid changes in potassium along the surface of the muscle fibre. Skeletal muscle contains 75% of the body’s potassium (Sejersted and Sjøgaard, 2000). Potassium is released with each action potential into the interstitial space which acts as a reservoir. Thus whilst a single action potential may increase the muscle fibre surface potassium from 2.5mM to 4mM a train of 6 action potentials at 10Hz can increase it from 2.5 to 10mM (Sejersted and Sjøgaard, 2000). This is particularly true in the t-tubules where, due to constrained space, the effective $K^+$ concentration reached is much higher than at the sarcolemma (Sejersted and Sjøgaard, 2000; DiFranco, Hakimjavadi, et al., 2015). A significant increase in $K_o$ causes $E_K$ to become positive relative to $V_m$. This
triggers Kir channels to open. Inward Kir current has a major role in clearing K⁺ from t-tubules (DiFranco, Hakimjavadi, et al., 2015). An increase in K₀ also triggers Na⁺ K⁺ pump activation. Hyperpolarisation of Vₘ secondary to Na⁺ K⁺ pump activation further augments uptake of K⁺ through Kir channels as they are stimulated by hyperpolarisation (Hicks and McComas, 1989; Sejersted and Sjøgaard, 2000; DiFranco, Hakimjavadi, et al., 2015).

The effects of t-tubule potassium accumulation are also modified by Cl⁻ redistribution in skeletal muscle. Chloride redistribution means that despite the rapid accumulation of K⁺ in the interstitial space, cells may remain polarised for several seconds or minutes due to the stabilising effect of Cl⁻ on Vₘ. If the stabilising effect of Cl⁻ on membrane potential is removed during hyperkalaemia (e.g. by reduction of extracellular Cl⁻) than depolarisation and subsequent loss of force is rapidly accelerated (van Emst et al., 2004). However, the relationship of hyperkalaemia, force and Cl⁻ in skeletal muscle is dynamic and complex as reduction of extracellular Cl⁻ also leads to a slow recovery of force following its accelerated loss (van Emst et al., 2004). This recovery of force in high potassium was never observed in standard extracellular Cl⁻ concentrations (van Emst et al., 2004)

1.1.8 Species Differences in Mammalian Skeletal Muscle Physiology
There are obvious differences in geometry of skeletal muscles between different mammals. Given these geometric differences, it is not surprising there is an associated difference in gait speed: smaller animals generally have much faster leg cycle speeds than larger mammals (Heglund, Taylor and McMahon, 1974). This difference in gait speed is also associated with a difference in the percentage of fast or slow twitch muscle fibres. For example, human skeletal muscle is made up of predominantly slow twitch fibres and does not contain type IIb fibres, a type of fast twitch glycolytic fibre. In contrast IIb fibres make up most fibres in mouse muscle (Augusto, Padovani and Campos, 2004). A study comparing transcriptomics of mouse and human muscle found that of 6 mouse muscles studied, soleus, the only mouse muscle without type IIb fibres and with predominantly type I or IIA oxidative fibres, was the most similar on a molecular level to human muscle (Kho et
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Species Differences in Mammalian Skeletal Muscle Physiology (al., 2006). This was independent of the anatomic location, muscle type, age or sampling method.

In addition to differences in fibre type proportions there is a marked difference in skeletal muscle ATP usage between mammals (Figure 1-5). Rates of ATP splitting by activation (sarcolemmal Na$^+$ extrusion, sarcolemmal K$^+$ reuptake and SR Ca$^{2+}$ reuptake) as well as cross-bridge processes are shown in Figure 1-5. The difference in fibre type partly explains the much higher activation and cross-bridge ATPase splitting per gram of rodent muscle compared to human skeletal muscle tissue (Barclay, Woledge and Curtin, 2007). However, it does not account for it entirely as the rate of ATP splitting per gram of slow twitch rodent muscle is still 5-10 times higher than for fast twitch human muscle (Figure 1-5). This suggests mammalian differences in ionic homeostasis and/or cross-bridge cycling exist.

Consistent with this, there is some evidence that species difference in skeletal muscle excitability exist. One such example is the observation that 9-Anthracene Carboxylic acid (9AC), a CIC-1 inhibitor, produces myotonia resembling MC in human and goat muscle fibres but has no myotonic effects on frog muscle fibres (Bryant and Morales-Aguilera, 1971).

![Figure 1-5 Absolute Rates of ATP Hydrolysis for Activation and Cross-Bridge Processes in Skeletal Muscle of Different Species. Bars show rates of ATP splitting by activation processes (black section of bars) and cross bridge cycling (grey section of bars). Data are for isometric contraction.' (Barclay, Woledge and Curtin, 2007)]
1.1.9 Gender Differences in Human Skeletal Muscle Physiology

Male skeletal muscle has been reported to have more type IIA fibres whilst female skeletal muscle has more type I fibres (Roepstorff et al., 2006). The IIA fibres are also larger in male compared to female muscle (Brooke, 1969; Roepstorff et al., 2006) and the relationship between the size of different fibre types is distinct in males and females. In male skeletal muscle, type I fibres that are larger than type II fibres are pathological, however in females this may be normal (Brooke, 1969).

While male skeletal muscle is stronger than female and this strength difference may be related to an increased number and size of type II fibres (Hunter, 2014), female skeletal muscle is more fatigue resistant than male (Roepstorff et al., 2006; Hunter, 2014). An increased number of type I fibres in women may help explain this observation but factors associated with smaller IIa fibres are also likely to be involved. For example, smaller type IIa fibres are associated with an increased mean number of capillaries per muscle fibre and an increased capillary density which, when combined results in increased femoral venous blood flow per kg body weight in female skeletal muscle (Roepstorff et al., 2006).

The extent of the gender difference in fatigue varies according to muscle group (Hunter, 2014) and may also vary with the type of activity e.g. isometric vs dynamic muscle activity. Repeated bouts of high intensity exercise induce smaller ATP reduction in women than men (Esbjörnsson-Liljedahl, Bodin and Jansson, 2002) and it has been shown that absolute energy expenditure is lower for females relative to males at the same exercise intensity (Montero et al., 2018). Moreover there is a gender difference in energy substrate utilisation: female skeletal muscle relies more on fat during exercise and male on carbohydrate (Montero et al., 2018).

In addition, recent work using mouse skeletal muscle has suggested that mitochondrial volume density is higher in female than male TA and that female mitochondria exhibit greater calcium uptake than male (Watanabe et al., 2020). These metabolic differences may be associated with fibre type differences and are also likely to be involved in the differential susceptibility to fatigue observed between males and females.
Relatively little work has been done examining the effects of gender on skeletal muscle ion channels. One of the few studies performed is on sex hormone modulation of ClC-1 expressed in *xenopus* oocytes (Fialho et al., 2008). This study found that whilst 17β oestradiol had a minor inhibitory effect on ClC-1 channel activity, application of testosterone or progesterone had more profound inhibitory effects that were dose dependent (Fialho et al., 2008). Progesterone has also been shown to cause near complete abolition of ClC-1 currents in murine muscle fibres (Burge, Hanna and Schorge, 2013). The time course of inhibition following application in both muscle fibres and heterologously expressed *xenopus* oocyte channels was rapid suggesting that the effect is non-genomic. However, as the dose of progesterone applied to murine muscle fibres was in excess of that reported physiologically, and the effect of testosterone has only been reported in heterologously expressed ClC-1 channels, *in vivo* confirmation of these results is required.

In contrast to skeletal muscle, a gender difference in the functional expression of cardiac ion channels is well recognised. The basis for this is not completely understood, however, several studies have revealed smaller calcium transients and reduced functional expression of repolarising potassium channels in female cardiac tissue compared to male (Drici Milou D. et al., 1996; Pham and Rosen, 2002; Fülöp et al., 2006; Kurokawa and Furukawa, 2013; Parks and Howlett, 2013). This is compatible with the observation of multiple gender differences on ECG that predominantly affect repolarisation (Pham and Rosen, 2002; Fülöp et al., 2006; Kurokawa et al., 2016). This gender difference in cardiac physiology is clinically significant as female sex is an independent risk factor for syncope and sudden death in congenital long QT syndromes (Pham and Rosen, 2002) and 65-75% of drug-induced long QT arrhythmias occur in women (Kurokawa et al., 2016). However, it should be noted that the gender difference only emerges after puberty when the risk of syncope or death for males with congenital long QT syndrome is drastically reduced as their QT interval shortens (Drici Milou D. et al., 1996; Fülöp et al., 2006; Kurokawa and Furukawa, 2013). In contrast, the risk of drug induced long QT arrhythmia is increased in men after middle age as QT interval prolongs...
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Age-related Change in Skeletal Muscle Physiology

with age (Drici Milou D. et al., 1996; Fülöp et al., 2006; Kurokawa et al., 2016). This change in the QT interval with age in men has been attributed to increase in testosterone at puberty and reduction after middle age (Vink et al., 2018). It remains to be seen whether gender and more specifically sex hormones have a similar effect in skeletal muscle.

1.1.10 Age-related Change in Skeletal Muscle Physiology

Life course studies have shown that skeletal muscle mass and strength increase in early life, plateau and then decline from middle into old age (Sayer et al., 2013). Around the age of 40, optimal motor performance begins to decline (Baker and Tang, 2010; Lehto, 2016), muscle mass decreases (Smith and Mittendorfer, 2016) and mitochondrial abnormalities begin to be accepted as within the normal range for age on muscle biopsy (Pesce et al., 2001)(Figure 1-6). However, a reduction in motor ability from the age of 40 is only apparent at the extremes of motor performance e.g. master athletes or maximal grip force (Figure 1-6), for other activities that are within normal physiological capacity, a decline is not seen until later, around the age of 65 Figure 1-6). This suggests that if physiological capacity is stretched to the limit, a deficit in muscle function appears from around age 40 but otherwise clinical deficits may not be seen for another 20 years.

Sarcopenia is the loss of muscle mass and function with age. The definition was recently updated to include loss of muscle function as well as mass (Cruz-Jentoft et al., 2019). This stemmed from the repeated observation that the loss of muscle strength with age precedes and exceeds the loss of muscle mass. Perhaps more importantly, loss of muscle strength is more closely associated with morbidity and mortality than loss of muscle mass (Sayer et al., 2013). Given the paucity of structural change in sarcopenia, change in skeletal muscle excitability and/or ionic homeostasis are good candidates to help explain the decline in strength with age. In support of this, when the specific tension of aged muscle fibres was measured using a technique that bypasses action potential initiation, action potential propagation and excitation-contraction coupling, no age related decline was found (Murgia et al., 2017). This suggests that the deficit in aged skeletal muscle may lie
with skeletal muscle excitability, excitation-contraction coupling or ionic homeostasis.

Figure 1-6 The decline in skeletal muscle function with age. A. Cross-cohort centile curves for grip strength (Dodds et al., 2014). B. Effect of age on marathon finishing times among male amateur runners in Stockholm marathon 1979 - 2014 (Lehto, 2016). C. Muscle mass throughout life in men and women (Smith and Mittendorfer, 2016). D. Usual walking speed by age (Schott, 2017). E. Age-related occurrence and
There have been relatively few studies examining changes in skeletal muscle excitability or ion channel function with age. However, a decline in chloride conductance (Pierno et al., 1999), a decline in the alpha subunit of the calcium channel (CaV1.1) leading to excitation-contraction uncoupling (Renganathan, Messi and Delbono, 1997), an increased dependence on extracellular calcium to propagate action potentials (Weisleder et al., 2006), a reduction in SR Ca\(^{2+}\) stores (Lamboley et al., 2015, 2016) and a reduction in SOCE (Thornton et al., 2011) have all been reported in aged muscle. Some of these changes could reflect the selective atrophy of fast twitch glycolytic fibres that occurs with age (Deschenes, 2004; Wang and Pessin, 2013; Cheema et al., 2015; Kramer et al., 2017) as the excitability profile and consequently skeletal muscle ion channel functional expression is different in fast and slow twitch fibres (Schiaffino and Reggiani, 2011). For example, ClC-1 and CaV1.1 protein expression is much higher in fast twitch compared to slow twitch fibres (Schiaffino and Reggiani, 2011) and thus a relative decrease in fast twitch fibre volume could contribute to a reduction in these proteins.

The relative atrophy of fast twitch glycolytic fibres with increasing age is intriguing given that disuse, immobilisation and denervation, factors commonly implicated in the sarcopenic process, classically cause slow twitch fibre atrophy. Fast twitch glycolytic fibres are more sensitive to states of nutrient scarcity such as starvation, cancer and diabetes (Matsakas, 2009). Metabolic factors also form 8 out of the 9 hallmarks of ageing (Aunan et al., 2016). This suggests that the preferential atrophy of type II fibres in aged muscle reflects nutrient depletion or perceived nutrient depletion and indicates a key role for metabolic factors in the aetiology of sarcopenia.

This suggestion is supported by the observation that anabolism is impaired in elderly muscle (Moore, 2014; Wall et al., 2015; Bosaeus and Rothenberg, 2016). Impaired anabolism is not due to a deficit in the capacity for protein synthesis per se, as if protein or essential amino acids are given in excess then older muscle can
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match younger rates of synthesis (Rieu et al., 2009; Moore, 2014). It is also not due to differences in the circulating and interstitial amino acid concentrations as these were found to be equal or higher in older individuals than in the young (Durham et al., 2010). Instead the deficit appears to be in nutrient delivery into the cell (Chevalier et al., 2011) because of age-related microvasculature dysfunction that affects delivery and impairs amino acid transport (Dillon et al., 2011). As with many other systems, an increase in capillary basement membrane thickness is reported in skeletal muscle with increasing age (Groen et al., 2014; Scioli et al., 2014). The microvasculature of fast twitch muscle appears to be selectively more vulnerable to these age-related changes. (Scioli et al., 2014)

Ionic homeostasis is one of the major energetic requirements of skeletal muscle accounting for approximately 50% of energy requirement (1.1.5). An increase in skeletal muscle sodium content in men with age has been suggested from MRI spectroscopy (Kopp et al., 2013). This suggests that old muscle and perhaps particularly old male muscle may be less able to meet the energy requirements of ionic homeostasis (predominantly Na⁺ extrusion and Ca²⁺ reuptake). The energy cost of intermittent contraction is also specifically increased in old muscle (Layec et al., 2015). Intermittent contraction requires repolarisation and calcium reuptake and therefore in contrast to continuous contraction represents Na⁺ K⁺ pump and SERCA pump activity in addition to myosin ATPases (Layec et al., 2015). Elderly subjects had similar ATP synthesis rates to younger subjects during continuous and intermittent contraction. However, the ATP cost of intermittent (but not continuous) contractions was much greater in older subjects. The ATP cost of intermittent contraction was also significantly correlated with dynamic single leg extensor peak power. Thus, there is evidence to suggest ionic dysregulation occurs with age in skeletal muscle.

These findings could have implications for skeletal muscle disorders that affect excitability such as skeletal muscle channelopathies. The available data on how the clinical phenotype of Hyper PP, Hypo PP and MC changes with age are discussed in the relevant sections below.
1.2  Hypokalaemic periodic paralysis (Hypo PP)

1.2.1  Hypo PP Genetics

In 1994 Jurkat-Rott et al identified mutations in CACNA1S as the cause of Hypo PP (Jurkat-Rott et al., 1994). Genetic heterogeneity was then confirmed by linkage to a locus away from CACNA1S in a family with a clear Hypo PP phenotype (Plassart et al., 1994). Subsequently mutations in SCN4A were identified as an alternative genetic cause of Hypo PP (Jurkat-Rott et al., 2000).

Hypo PP due to mutations in CACNA1S is referred to as Hypo PP I and Hypo PP due to mutations in SCN4A as Hypo PP II. It was noted that the identified mutations in both CACNA1S and SCN4A all affected S4 arginines and in a cohort of clinically diagnosed Hypo PP patients, S4 arginine mutations account for 90% of cases (Matthews et al., 2009). Since then a third gene, ATP1A2 has been identified as causing Hypo PP (Sampedro Castañeda et al., 2018). ATP1A2 encodes the t-tubule Na+ K+ ATPase. Hypo PP due to mutations in ATP1A2 could be distinguished from classical PP as the proband also had CNS features of epilepsy and learning difficulties (Sampedro Castañeda et al., 2018).

1.2.2  Hypo PP Clinical Features

Paralytic attacks in Hypo PP, as the name suggests, are associated with low serum potassium (Biemond and Daniels, 1934). The average intra-attack serum potassium level is 2.4 (Miller et al., 2004). Attacks tend to occur at night or in the early morning (Pearson, 1964) and usually last several hours to days. Attacks frequently involve paralysis of all 4 limbs but usually spare muscles above the neck (Biemond and Daniels, 1934; Miller et al., 2004), and tend to occur at intervals of weeks or months unless provoked by some ‘indiscretion’ (Pearson, 1964). Such ‘indiscretions’ include a large carbohydrate meal, excess alcohol, stress, and rest after intensive exercise (Bickerstaff, 1953). There is usually a delay of several hours between the trigger and the onset of weakness although severe cold can trigger immediate weakness (Bickerstaff, 1953).

The frequency and severity of attacks are very variable even within the same family (Sternberg et al., 2001) such that ‘nothing can be predicted from the severity of the
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Hypo PP (Helweg-Larsen, Hauge and Sagild, 1955). In the pre-genetic and pre-intensive care era it was said that fatal attacks occurred in up to 10% of cases (Pearson, 1964) and at least one fatal attack was reported by all the large kindreds in Denmark (Helweg-Larsen, Hauge and Sagild, 1955). All of these patients were between the ages of 15-34 at death (Helweg-Larsen, Hauge and Sagild, 1955). However, it may be that these families actually had a variant of ATS and died from sudden cardiac death which is a known association with ATS.

During paralytic attacks tendon reflexes are reduced (Bickerstaff, 1953). There is some suggestion that muscle swells as an increase in limb circumference is also reported (Bickerstaff, 1953). Muscles of articulation, mastication and respiration are usually spared (Bickerstaff, 1953; Miller et al., 2004). Electrocardiographic changes are reported in association with attacks and tend to reflect hypokalaemia e.g. flattening of t-wave and appearance of U-wave (Bickerstaff, 1953).

In between attacks 72% describe some residual weakness (Miller et al., 2004) and myalgia has also been reported for some patients, particularly those with Hypo PP II (Sternberg et al., 2001). Occasionally patients present with permanent muscle weakness without history of antecedent paralytic attack (Buruma and Bots, 1978).

In terms of treatment up to 85% find Acetazolamide very helpful (Miller et al., 2004; Suetterlin et al., 2019) but its exact mechanism of action remains unknown. From our personal experience in the NHS Highly Specialised Muscle Channelopathy Clinic at the National Hospital for Neurology and Neurosurgery in London, Spironolactone is also effective at reducing the attacks.

1.2.3 Hypo PP Neurophysiology

In contrast to Hyper PP and NDM, myotonia is not a feature of Hypo PP (McManis, Lambert and Daube, 1986; Fournier et al., 2004; Miller et al., 2004; Tan et al., 2011) so in between attacks, unless myopathy has developed, needle EMG and NCS should be normal. Therefore, as rest after exercise is a trigger for both Hyper and Hypo PP, tests measuring the neurophysiological response to rest after exercise, were developed to aid the investigation and diagnosis, of skeletal muscle channelopathies (McManis, Lambert and Daube, 1986). In the Long Exercise Test
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(LET) also known as the McManis test, the Compound Muscle Action Potential (CMAP) of abductor digiti minimi (ADM) is recorded every 30-60 seconds for 1 to 5 minutes to establish a baseline. Patients are then asked to perform intermittent maximal contractions of ADM for 5 minutes with brief periods of rest to prevent muscle ischaemia (McManis, Lambert and Daube, 1986). The CMAP is recorded after each minute of exercise. At the end of 5 minutes exercise the patient is instructed to relax completely while CMAP responses to single shocks are recorded every 2 minutes for 50 minutes (Tan et al., 2011). Consistent with rest after exercise triggering attacks in Hypo PP, patients exhibit a progressive decline in their CMAP amplitude in the 50 minutes rest period. A 40% reduction from peak CMAP amplitude or 30% reduction from baseline CMAP amplitude is considered significant and represents a positive test (Tan et al., 2011).

Whilst the LET induces a localised, partial PP attack that is more suited to routine clinical practice (and current ethical guidelines) previous electrophysiological studies investigated more generalised attacks. During an attack of generalised Hypo PP there is almost complete electrical silence (Troni, Doriguzzi and Mongini, 1983). On recovery, once stimulation induces a recordable action potential, conduction velocity is reduced compared to the baseline interictal value and action potential duration is prolonged (Troni, Doriguzzi and Mongini, 1983; Links, van der Hoeven and Zwarts, 1994). However, baseline muscle fibre conduction velocity is also reduced compared to controls (Troni, Doriguzzi and Mongini, 1983; Links, van der Hoeven and Zwarts, 1994) providing evidence of an alteration in skeletal muscle excitability that persists in the interictal period. The reduction in conduction velocity both during and between attacks in patients with Hypo PP would be consistent with an increased membrane resistance secondary to increased membrane permeability (Zwarts et al., 1988).

The muscle fibre conduction velocity technique described above requires single fibre stimulation and measurement of conduction velocity in the same fibre at a specified distance (Troni, Doriguzzi and Mongini, 1983; Zwarts et al., 1988; Links et al., 1990). This is technically challenging and would be difficult to apply in a clinical setting. However, in 2009 Muscle Velocity Recovery Cycles (MVRCs) using the
response from multiple fibres were described (Z’Graggen and Bostock, 2009).
MVRCs greatly reduce the technical difficulty whilst increasing reliability (Z’Graggen and Bostock, 2009; Z’graggen et al., 2016) compared to single fibre conduction velocity studies. MVRCs use changes in muscle conduction velocity as an indirect measure of muscle excitability (see Figure 1-4, 1.1.1) and have been used to investigate muscle membrane properties in different genetic channelopathies (SV Tan et al., 2012; Tan et al., 2014, 2018, 2020) as well as other conditions including critical illness myopathy (W. J. Z’Graggen et al., 2011) and uraemic myopathy (Z’Graggen et al., 2010).

When MVRCs were used to investigate membrane properties of patients with periodic paralysis there was no significant difference in MVRCs from patients with Hypo PP I or II (Tan et al., 2020) but compared to controls, patients with Hypo PP (I & II combined) exhibited an increased Muscle Relative Refractory Period (MRRP) and reduced Early Supernormality (ESN). An increased MRRP and reduced ESN would be consistent with partial depolarisation and sodium channel inactivation in Hypo PP muscle compared to control (Tan et al., 2020).

1.2.4 Hypo PP Imaging

There is differential muscle involvement in Hypo PP. Evidence of variable muscle degeneration in Hypo PP has been reported using Computed Tomography (CT) imaging of muscles (Links et al., 1990). Full body CT scans of a large family with periodic paralysis were performed and 18 different muscle groups graded according to the severity of changes seen (Links et al., 1990). In this study, the muscles of the leg and pelvic girdle were more affected than arm and shoulder girdle. Some muscles e.g., Sartorius, Gracilis and Rectus Femoris were relatively spared and some distal muscles e.g. soleus and gastrocnemius were found to be affected to the same extent as the proximal leg muscles. There was no difference in the number or severity of muscles affected (CT sum score) in patients with or without history of paralytic attack but CT sum score did increase significantly with age.

An increase in fatty degeneration with age has also been reported using MRI to look at Hypo PP patient muscle (Jurkat-Rott et al., 2009). Fatty degeneration was
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Hypokalaemic periodic paralysis (Hypo PP) is more prevalent in Hypo PP patients with permanent weakness (Jurkat-Rott et al., 2009). Fat is associated with lower Na\(^+\) concentration than muscle but despite the association of fatty degeneration with permanent weakness, myoplasmic Na\(^+\) seen on \(^{23}\)Na MRI was higher in weak Hypo PP muscles (Jurkat-Rott et al., 2009). \(^{1}\)H - MRI demonstrated that Na\(^+\) overload was accompanied by water overload in the form of oedema. Local cooling increased \(^{23}\)Na and \(^{1}\)H signal intensities and weakness in all Hypo PP patients but not controls. This observation may explain the swelling of muscles associated with an attack that was observed clinically by Bickerstaff (Bickerstaff, 1953). Treatment with acetazolamide reduced myoplasmic \(^{23}\)Na signal intensities and increased strength in the 6 severely affected Hypo PP patients in whom it was trialled (Jurkat-Rott et al., 2009).

1.2.5 Hypo PP Pathology

The classical features of periodic paralysis myopathy are vacuoles and tubular aggregates (Miller et al., 2004). Goldflam first reported the characteristic vacuoles of Hypo PP myopathy in 1897. Since then some authors report that vacuoles are formed during the course of an induced attack (Shy et al., 1961; Hofmann and Smith, 1970) whilst others state they are unrelated to paralytic episodes (Gordon, Green and Lagunoff, 1970).

A preferential reduction in size of type II fibres has also been noted (Shy et al., 1961). In 4 of 8 men examined the mean type II fibre diameter was less than the type I diameter, a finding which is considered to be pathological in men (Brooke, 1969) (see 1.1.8).

1.2.6 Hypo PP Life course

Hypo PP symptom onset is usually after puberty and the attack frequency and severity are at their peak during puberty and late adolescence (Bickerstaff, 1953; Miller et al., 2004). The reason for this e.g., whether this is due to changes in sex hormones or perhaps the number of ‘indiscretions’ triggering attacks is not known.

A reduction in attack frequency and severity around the age of 40 has also been reported (Biemond and Daniels, 1934; Bickerstaff, 1953; Rudel et al., 1984; Sternberg et al., 2001) and coincides with the onset of permanent progressive
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weakness (Rudel et al., 1984; Sternberg et al., 2001). However, it is not clear that
the two are causally related as patients with no antecedent history of paralytic
attack can also develop permanent progressive weakness (Buruma and Bots, 1978;
Zwarts et al., 1988; Links et al., 1990) and the age of onset of permanent weakness
can be very variable or may not even occur.

It has generally been assumed that Hypo PP is not life limiting (Miller et al., 2004),
however I am not aware of studies specifically assessing this. However, significant
morbidity has been reported. For example, severe disability (e.g. wheel chair
bound) was reported for all members of the large family studied by Links who were
above the age of 70 (Links et al., 1990).

1.2.7 Hypo PP Gender differences

Gender difference in the severity and manifestation of Hypo PP are reported
(Jurkat-Rott et al., 1994; Sternberg et al., 2001; Miller et al., 2004). These gender
differences include reduced penetrance and reduced attack severity in females
that are affected. A gender difference in the penetrance and/or phenotype
severity of Hypo PP is supported by the male predominance in patients that attend
our UK national skeletal muscle channelopathies referral centre (male/female ratio
3.52) (Horga et al., 2013) and in the periodic paralysis genotype phenotype study
(62% male Vs 38% female) (Miller et al., 2004).

As the diagnosis of periodic paralysis has typically relied heavily on the presence
and description of paralytic attacks, so has the definition of gene penetrance (Ke et
al., 2013). However, it is known that some patients present with permanent
progressive weakness without any history of antecedent attacks (Buruma and Bots,
1978; Sternberg et al., 2001). Therefore, it may be that females have a similar rate
of permanent progressive weakness but a lower rate of paralytic attack. It is
striking therefore that when subdivided, the gender difference in the penetrance
of episodic attack was reduced in females whilst the penetrance of progressive
myopathy was no different from males (Sternberg et al., 2001; Miller et al., 2004).

Intriguingly, the magnitude of gender difference in penetrance varies with different
mutations in the same gene. There is a definite gender difference in penetrance
Introduction Hypokalaemic periodic paralysis (Hypo PP) Genotype-phenotype correlations observed for Hypo PP I due to R528H mutations but not R1239H mutations (Fouad et al., 1997; Miller et al., 2004). The severity and penetrance seems to broadly correlate with size of gating pore current (Sokolov, Scheuer and Catterall, 2008; Jurkat-Rott et al., 2009; Wu et al., 2018). R1239H has a larger gating pore current than R528H and thus a greater percentage of fibres in the depolarised P2 state (Jurkat-Rott et al., 2009). R1239H is associated with complete penetrance of symptoms in both sexes as well as a more severe myopathy (Fouad et al., 1997; Miller et al., 2004).

An awareness of gender difference in attack severity may be particularly important for the diagnosis of periodic paralysis. If females have less severe attacks, you might expect them to be less likely to have a positive LET result as this induces a localised attack. In keeping with this a significant gender difference in long exercise test response was described in a Chinese population (Jin et al., 2017). In this study, decrement from peak CMAP amplitude was significantly greater for both male controls and males with periodic paralysis compared to respective female groups. The authors propose gender-specific diagnostic cut offs for decrement from peak CMAP amplitude during the long exercise test of 46.8% for men and 26.9% for women. However, a gender difference was not reported in previous studies examining the long exercise test (McManis, Lambert and Daube, 1986; Fournier et al., 2004; Tan et al., 2011). The reason for this discrepancy remains to be determined but possibilities include differences in the age, the ethnicity or the pathogenic mutation of subjects. However, if such a discrepancy is also apparent in the UK population, it could increase the number of false negative tests in females with periodic paralysis and thus prolong their diagnostic journey.

1.2.8 Hypo PP Genotype-phenotype correlations

There are clinical and histopathological differences reported for Hypo PP caused by CACNA1S mutations and Hypo PP II caused by SCN4A mutations. Hypo PP II patients report more interictal myalgia and are less likely to find treatment with acetazolamide effective (Sternberg et al., 2001). In terms of biopsies, 80% of Hypo PP I patient biopsies have evidence of vacuolar myopathy, 13% other myopathic changes, and only 7% tubular aggregates (Miller et al., 2004). In contrast only 50%
of Hypo PP II patient biopsies had evidence of vacuolar myopathy whilst 50% had tubular aggregates (Miller et al., 2004).

There are also phenotype differences for patients with different mutations in the same gene. For example, in addition to the variation in gender difference according to genotype, patients with an S4 arginine substitution to a glycine are less sensitive to acetazolamide treatment than patients with other S4 arginine substitutions (Matthews 2009).

1.2.9 Hypo PP Pathophysiology

1.2.9.1 Human studies

Given the association with carbohydrate meals and low serum potassium levels, for a long time the primary deficit in Hypo PP was believed to be metabolic (Shy et al., 1961; Engel et al., 1965). However, as the change in potassium was variable and inconsistent Bickerstaff and others proposed that the fundamental defect was within muscle itself (Bickerstaff, 1953; Bradley, 1969; Gordon, Green and Lagunoff, 1970). This suggestion was supported by the observation that paralytic attacks are provoked by serum potassium levels that cause no weakness in normal healthy individuals and that affected muscle fibres do not respond to electrical stimulation whether direct or via the innervating nerve, during an attack of paralysis (Bickerstaff, 1953; Engel et al., 1965; Engel and Lambert, 1969).

A deficit in excitability was suggested when Hoffmann demonstrated that during episodes of spontaneous or induced paralysis Hypo PP muscle fibres depolarise (Hofmann and Smith, 1970). Rudel extended this work to confirm that isolated fibres from Hypo PP patients depolarise in response to low extracellular potassium (Rudel et al., 1984). This was termed paradoxical depolarisation as in response to the same extracellular potassium concentration, healthy fibres hyperpolarise (Rudel et al., 1984; Jurkat-Rott et al., 2009). Further evidence to support a specific deficit in excitability, is that direct application of caffeine during an attack causes contracture despite the muscle fibre remaining inexcitable to electrical stimuli (Engel and Lambert, 1969). Caffeine bypasses excitability to directly activate RyR1 receptors (Figure 1-2, 1.1.2).
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The fact that the RMP of Hypo PP fibres relative to control fibres was also depolarised interictally (Hofmann and Smith, 1970; Rudel et al., 1984) provided further evidence for an intrinsic muscle problem. This was further delineated by Jurkat-Rott et al who demonstrated that the bimodal distribution of resting membrane potential was right-shifted in Hypo PP patient muscle fibres (Jurkat-Rott et al., 2009). In normal muscle fibres, 95% of fibres are in a hyperpolarised ‘P1’ state and only 5% of fibres are in a depolarised ‘P2’ state (see 1.1.6). In Hypo PP fibres there is an increased proportion of muscle fibres in the P2 state (9 to 24% depending on mutation) resulting in RMP that is depolarised (-74 to -77mV depending on mutation) (Jurkat-Rott et al., 2009). Lowering extracellular potassium to 1.5mM K⁺ increased the proportion of fibres in P2 for both healthy controls and Hypo PP, but to a much greater degree for Hypo PP: 13% of healthy control fibres in 1.5mM potassium were in P2 compared to 83 to 99% (depending on mutation) of Hypo PP fibres (Jurkat-Rott et al., 2009).

Later work that explored the bistability of skeletal muscle membranes, demonstrated hysteresis e.g. a higher K⁺ concentration was required to repolarise the membrane than had been required to depolarise the membrane (Geukes Foppen, van Mil and van Heukelom, 2002). This phenomenon was also seen by Rudel in Hypo PP muscle fibres: fibres did not always repolarise on return to normokalaemia (Rudel et al., 1984). These observations may help explain the clinical observation that although weakness is triggered by hypokalaemia, return of normokalaemia does not always return strength, indeed peak weakness is sometimes found when serum potassium levels are normalising (Bickerstaff, 1953; Gordon, Green and Lagunoff, 1970) (Figure 1-7).
In terms of the molecular basis for the paradoxical depolarisation of Hypo PP fibres in response to moderate hypokalaemia, a major clue came when Hoffmann demonstrated a role for sodium flux (Hofmann and Smith, 1970). Whilst Hypo PP fibres were depolarised at rest, removal of sodium from the bathing solution repolarised them to control levels (Hofmann and Smith, 1970). This was not due to movement through the NaV1.4 alpha pore as in subsequent experiments TTX, a NaV1.4 alpha pore blocker, had no effect on the RMP of Hypo PP patient fibres (Rudel et al., 1984). Thus, these data suggested that an anomalous inward sodium current was present although its relation to attacks of paralysis was not delineated.

In 1984, a role for chloride conductance in Hypo PP paradoxical depolarisation was also suggested as fibres could be repolarised and force and excitability returned by
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A transient shift of chloride equilibrium potential to a highly negative value (Rudel et al., 1984). These observations are not due to a primary deficit in CIC-1 as there is no significant difference in either specific membrane conductance or capacitance between Hypo PP patients and controls (Rudel et al., 1984). Moreover, a similar beneficial effect could also be precipitated by Cromokalin, a KATP channel activator. Cromokalin increased baseline twitch force in Hypo PP but not control fibres and repolarised Hypo PP fibres in 1mM K⁺ to control values (Grafe et al., 1990; Jurkat-Rott et al., 2000).

Thus, studies in human fibres were able to replicate the association of weakness with low potassium and reveal this was due to paradoxical depolarisation of the Hypo PP muscle fibre. They demonstrated a bimodal distribution of resting membrane potential that is left shifted in Hypo PP and exacerbated by low serum potassium levels. Removal of sodium from the extracellular solution corrected baseline depolarisation suggesting an anomalous inward sodium current, however TTX had no effect suggesting this current’s passage was not via the NaV1.4 alpha pore. The recovery from paradoxical depolarisation is also complex. Experiments revealed repolarisation of Hypo PP fibres requires more than just the return of normokalaemia and can be accelerated by changes in chloride conductance and/or KATP channel activity. However, the exact molecular mechanism of paradoxical depolarisation required heterologous expression and animal studies to determine.

1.2.9.2 Heterologous expression

Prior to the discovery of mutations in SCN4A as a cause of Hypo PP, heterologous expression of Hypo PP variants was limited by the technical difficulty of obtaining adequate expression levels of CaV1.1 (Wu et al., 2018). Some studies were performed using the muscular dysgenesis mouse cell line that lacks the alpha subunit of CaV1.1 (Jurkat-Rott et al., 1998) and others using xenopus oocyte (Morrill and Cannon, 1999). These studies demonstrated reduced expression of Hypo PP CaV1.1 variants compared to control. The mutations expressed in xenopus oocytes altered the voltage dependence and kinetics of activation but these effects were in opposite directions for different mutations and did not explain the paradoxical depolarisation that occurs in response to low potassium.
Hypokalaemic periodic paralysis (Hypo PP) (Morrill and Cannon, 1999). NaV1.4 is much more amenable to heterologous expression. However, similar to CaV1.1, initial studies demonstrated varied, non-specific effects on channel function that did not fully explain the paradoxical depolarisation in response to low serum K (Jurkat-Rott et al., 2000; Kuzmenkin et al., 2002).

Thus, for a long time it was not clear how mutations in two different ion channels with very different roles - one fundamental for the initiation and propagation of an action potential and the other for excitation-contraction coupling - could manifest with the same phenotype. The clue was that 90% of Hypo PP mutations are situated in the S4 voltage sensor of either NaV1.4 or CaV1.1 (Matthews et al., 2009) and the breakthrough in understanding came after it was found, during a structural study of potassium channels, that mutation of an S4 arginine to histidine creates an anomalous pore, the ‘gating pore’ running through the voltage sensor region of the channel (Starace and Bezanilla, 2004). Subsequently gating pore currents were demonstrated in mutations causing Hypo PP II (Sokolov, Scheuer and Catterall, 2008). Depending on the mutation the gating pore may conduct Na\(^+\) (Sokolov, Scheuer and Catterall, 2008) or H\(^+\) (Struyk et al., 2008) and be activated at depolarised or hyperpolarised voltages (Sokolov, Scheuer and Catterall, 2008; Struyk et al., 2008; Wu et al., 2018). The amplitude of gating pore current also varies with mutation (Struyk et al., 2008; Jurkat-Rott et al., 2009; Wu et al., 2018). Gating pore currents have been demonstrated using heterologous expression for all identified NaV1.4 Hypo PP variants. However, it is only very recently that gating pore currents have been demonstrated in heterologously expressed CaV1.1 variants. This was made possible by the finding that co-expression of CaV1.1 with STAC3 in *xenopus* oocytes greatly increased CaV1.1 channel expression levels such that gating pore currents and their selectivity could be determined (Wu et al., 2018).

Thus, anomalous gating pore currents have been reported for all the *SCN4A* variants associated with Hypo PP and all the *CACNA1S* variants that have been studied. An anomalous gating pore current was also identified in association with a pathogenic mutation in ATP1A2 recently described as a novel Hypo PP gene, (1.2.1)
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Hypokalaemic periodic paralysis (Hypo PP) (Sampedro Castañeda et al., 2018). Thus, anomalous gating pore currents provide a unifying mechanism for the pathogenesis of Hypo PP.

1.2.9.3 Animal models

I found no report of Hypo PP occurring spontaneously in animals other than humans. However, transgenic mouse models of both Hypo PP 1 (CaV1.1) and Hypo PP II (NaV1.4) have been created.

1.2.9.3.1 R528H CaV1.1 Transgenic mouse

A knock in mouse model of Hypo PP I was generated using the mouse homolog of one of the most common PP mutations, R528H in CaV1.1 (Wu et al., 2012).

Clinical Features

R528H mice are viable and exhibit no weight deficit compared to wild-type (WT) (Wu et al., 2012). Somewhat surprisingly, R528H mice also exhibit no spontaneous attacks of weakness and locomotor activity is indistinguishable from WT. However, weakness is consistently induced when potassium is sufficiently lowered in vivo or ex vivo. In vivo, this required 48 hours pre-treatment with an oral potassium binding resin followed by an insulin and glucose infusion. The serum potassium after 48 hours of potassium binding resin was 2.7mM and caused no observable change in locomotor behaviour. The potassium during insulin and glucose infusion that followed the 48-hour pre-treatment with a potassium binding resin was around 2.0mM, the same concentration as was used for ex vivo testing. This is lower than the average intra-attack potassium levels reported for human muscle (Rudel et al., 1984; Miller et al., 2004).

Heterozygous male R528H mice had mild hindlimb weakness (15% reduction compared to male WT mice) but no forelimb weakness (Wu et al., 2012). Homozygous grip strength was not reported but the reduction in Extensor Digitorum Longus (EDL) peak force was double that seen in heterozygotes (Wu et al., 2012). A gene dosage effect was also suggested by the histology. Structural change was minimal in all muscles from heterozygous animals. In homozygous animals, whilst TA was relatively unaffected, there were marked vacuolar changes in gastrocnemius. This is in keeping with features of periodic paralysis myopathy.
Introduction

Hypokalaemic periodic paralysis (Hypo PP) found in humans (1.2.5). Ultrastructural studies showed disruption of the triad with massive dilatation of the transverse tubules and terminal cisternae of the sarcoplasmic reticulum (Wu et al., 2012).

During insulin and glucose infusion, CMAP and twitch force were recorded simultaneously from gastrocnemius and soleus. As occurs during the LET in humans (1.2.3), there was a marked decrease in CMAP amplitude which corresponded to a decrease in peak twitch force within minutes of infusion for both heterozygous and homozygous R528H mice (Wu et al., 2012). However, homozygous R528H mice also had an unexplained, but consistent, transient 10% increase in muscle force 10 minutes into the infusion without any concomitant detectable change in CMAP amplitude. This phenomenon was never observed for heterozygous mice. (Wu et al., 2012).

Life course, Gender Differences and Genotype-Phenotype Correlation

In terms of changes across the life course it is not possible to comment on changes in old age as animals were only examined up until 8 months of age however up until that point hind limb grip weakness was not progressive (Wu et al., 2012).

As for R528H human patients (1.2.7) (Fouad et al., 1997; Miller et al., 2004) there were significant gender differences in the phenotype severity of R528H heterozygous mice (Wu et al., 2012). In contrast to males, heterozygous R528H females had normal hindlimb grip strength, isolated EDL peak force and potassium response (Wu et al., 2012). However, there was no gender difference for homozygous animals and, when subjected to the glucose plus insulin challenge, the gender difference for heterozygous animals was removed (Wu et al., 2012). This suggests that if the trigger is sufficient (e.g. gene dosage or severe enough hypokalaemia), the protective effect of female gender on attack severity is removed.

Pathophysiology

The anomalous gating pore current as the pathomechanism for Hypo PP was confirmed in R528H mouse fibres using two-electrode-voltage-clamp (TEVC) to
Introduction

Hypokalaemic periodic paralysis (Hypo PP)

Pathophysiology

demonstrate a gating pore current (Wu et al., 2012). This was possible as a very large number of fibres could be examined, and results averaged out to reveal the small amplitude gating pore current. In addition to confirming the presence of a gating pore current, authors demonstrated that the magnitude of gating pore current increased with gene dosage (Wu et al., 2012). This suggests that the magnitude of gating pore current explains the more severe phenotype and complete penetrance in homozygous compared to heterozygous animals (see also 1.2.7).

1.2.9.3.2 R669H NaV1.4 Transgenic Mouse

A mouse model of Hypo PP I was generated by introducing the mouse ortholog (mNa_v.4-R663H) of the human Hypo PP I NaV1.4 mutation R669H using homologous recombination (Wu et al., 2011).

Clinical Features

As with the R528H mice, and in stark contrast to humans with the condition, mutant mice did not exhibit any spontaneous episodes of weakness (Wu et al., 2011) and required K⁺ levels of around 2mM to induce episodes of weakness (Wu et al., 2012). However, distinct from the R528H mice, R669H mice show slow, large amplitude oscillations in tetanic force for K⁺ levels at the transition zone between normal and susceptibility to marked weakness (Wu et al., 2011). This was 3mM K⁺ for heterozygous muscles and 4mM K⁺ for homozygous muscles. Micromolar ouabain supressed these oscillations such that intermittent recovery of force did not occur and revealed an increased dependency of R669H mouse muscle on pump activity to maintain excitability even in normal K⁺ (Wu et al., 2011). The effect of ouabain on R528H CaV1.1 mouse muscle was not assessed (Wu et al., 2012).

In contrast to the CaV1.1 R528H mouse strain, there was no significant difference between R669H and WT mice in fore or hindlimb grip strength. There was also minimal histological change reported. The only abnormality was some subsarcolemmal red structures on trichrome, ‘consistent with tubular aggregates’ (Wu et al., 2011). However, as electron microscopy was normal their origin was unclear.
Introduction

Hypokalaemic periodic paralysis (Hypo PP) The role of chloride conductance in paradoxical depolarisation

Life course, Gender Differences and Genotype-Phenotype Correlation

As only young adult mice (3-6 months) were used not much comment on life course changes can be made. However, Hypo PP II mice did exhibit some of the phenotype differences reported between humans with Hypo PP I and II. Firstly, as for humans with Hypo PP II acetazolamide exacerbated the severity of potassium-induced weakness in the R669H mice and secondly, as for humans with Hypo PP II there was no gender difference in phenotype observed in R669H mice (Sternberg et al., 2001; Wu et al., 2011).

Pathophysiology

TEVC on fibres from R669H muscle revealed the presence of a gating pore current activated at hyperpolarised potentials (Wu 2012). Like the CaV1.1 R528H mouse, a larger gating pore current was observed in fibres from homozygous animals and this was associated with a more depolarised RMP (Wu 2011, 2012). As for the CaV1.1 mouse, and in contrast to studies on humans with Hypo PP, heterozygous R669H mice were not depolarised at baseline compared to WT (Wu 2011).

1.2.10 The role of chloride conductance in paradoxical depolarisation

A role for chloride conductance in the aetiology of paradoxical depolarisation was first suggested by Rudel in 1984 based on observations from Hypo PP patient muscle fibres (Rudel et al., 1984)(1.2.9.1). Work on healthy muscle has shown that an increase in myoplasmic chloride increases the potassium concentration at which hypokalaemia-induced depolarisation will occur (Geukes Foppen, 2004; Wu, Mi and Cannon, 2013a, 2013b) (1.1.6).

The transgenic mouse models of Hypo PP have enabled the role of chloride conductance in Hypo PP to be further explored and better characterised. In both CaV1.1 and NaV1.4 heterozygous Hypo PP mice, bumetanide, a Na K 2Cl exchanger blocker, prevents (NaV1.4) or massively attenuates (CaV1.1) loss of force due to due to low potassium (Wu, Mi and Cannon, 2013a, 2013b). The effect of bumetanide is chloride dependent. In chloride free conditions, the NaV1.4 R669H muscle was less susceptible to hypokalaemia induced loss of force, exhibiting only
Introduction

Hypokalaemic periodic paralysis (Hypo PP) The role of chloride conductance in paradoxical depolarisation

A 10% decrease in heterozygous muscle compared to 60% in normal chloride conditions (Wu, Mi and Cannon, 2013b).

Figure 1-8 Slow kinetics of acidosis-induced loss of force in HypoKPP muscle. (A) The time course for the development of susceptibility to post-acidosis loss of force was determined by varying the duration of a 25% CO2 challenge (bars, left panel). Plots show average responses from CaV1.1-R528H soleus (6 min: n = 9, 30 min: n = 6; 80 min: n = 4). (B) Data were corrected for the decline in force during the 25% CO2 exposure (dashed line, panel A) to more clearly show the exponential kinetics for the onset of susceptibility with a time constant of 33 min. Plots in the top row show a pairwise comparison for the transient loss of force induced by a rapid transition of bath pH to 7.4 (blue) versus a slower pH recovery for the soleus from the other hind leg of the same CaV1.1-R528H mouse in a separate tissue bath (purple). The bottom row shows the bath pH monitored with a reference electrode. Exemplary comparisons for the two soleus muscles from a single mouse (left and middle panels) show an attenuation for the loss of force when the pH transition occurred slowly over 20 min. A slower recovery of bath pH over 50 min prevented the post-acidosis loss of force (right, n = 5). Figure and legend from (Mi et al., 2019)

One of the key triggers for attacks in Hypo PP is rest after exercise (1.2.2) and the aetiology of this was a longstanding mystery in the field. To investigate the phenomenon, Professor Steve Cannon’s group looked at the effect of acidosis and recovery from acidosis, as occurs during and after exercise, on hypokalaemia-induced loss of force. They found that whilst acidosis protects against
Hypokalaemic periodic paralysis (Hypo PP) The role of chloride conductance in paradoxical depolarisation

hypokalaemia-induced loss of force, recovery from acidosis causes a consistent, transient loss of force in isolated Hypo PP mouse muscle (Figure 1-8). This even occurred in normokalaemia. The phenomenon of transient loss of force on recovery from acidosis was more robust than the loss of force observed on exposure to low potassium solutions (Mi et al., 2019).

Acidosis inhibits CIC-1 channels (Pedersen, de Paoli and Nielsen, 2005) but does not inhibit the Na K 2 Cl exchanger (Aickin, Betz and Harris, 1989). This means that when CIC-1 channels are inhibited, whether by acidosis or 9AC, chloride accumulates within the muscle (Pedersen et al., 2016). It has previously been reported that alkalinisation of frog skeletal muscle following acidosis results in efflux of the accumulated chloride causing membrane depolarisation (Hutter and Warner, 1967). A similar phenomenon was observed in Hypo PP mice on recovery from acidosis (Mi et al., 2019). The evidence that this transient loss of force on recovery from acidosis was due to changes in chloride conductance was a) it did not occur in chloride free conditions b) it was blocked by pre-treatment with bumetanide and c) it was attenuated by pre-treatment with 8µm 9AC (higher doses to completely prevent loss of force were not possible due to increasing myotonia with more complete CIC-1 block (Mi et al., 2019). Moreover, the time frame of loss of force on recovery from acidosis coincided exactly with the time frame of amelioration of myotonia in the M1592V Hyper PP mouse exposed to the same conditions. This amelioration in myotonia was taken as suggestive of CIC-1 inhibition being released.

Thus, there is evidence that the post-acidosis loss of force in Hypo PP mice is chloride dependent and triggered by a rapid increase in chloride conductance via CIC-1 when pH is quickly returned to normal range. This dramatic depolarising Cl\(^{-}\) efflux occurs because, in contrast to CIC-1, the Na\(^{+}\) K\(^{+}\) 2Cl\(^{-}\) exchanger is not inhibited by acidosis and therefore continues to accumulate myoplasmic Cl\(^{-}\) during exercise. It is sudden release of CIC-1 inhibition that causes depolarisation sufficient to lose force as when pH recovers more slowly, loss of force can be prevented (Figure 1-8) (Mi et al., 2019).
1.3 Hyperkalaemic periodic paralysis (Hyper PP)

1.3.1 Hyper PP Genetics
The skeletal muscle voltage gated sodium channel gene was identified as the causative gene for Hyper PP in 1991 (Ptacek et al., 1991). Hyper PP is therefore allelic with paramyotonia congenita (Ptacek et al., 1991), sodium channel myotonia (Ptacek et al., 1992), sodium channel congenital myopathy (Zaharieva et al., 2016) and sodium channel myasthenia (Arnold et al., 2015; Habbout et al., 2016). To date no other causative gene has been identified for Hyper PP.

1.3.2 Hyper PP Clinical Features
In 1951, Tyler described the clinical manifestations and inheritance of a type of periodic paralysis without hypokalaemia (Tyler et al., 1951). The association of paralytic attack with raised potassium was given the name Familial Periodic Adynamia by Helweg-larsen (Helweg-Larsen, Hauge and Sagild, 1955) and Adynamia Episodica Hereditaria by Gamstorp (Gamstorp, 1957) and is now referred to as Hyper PP. The prevalence of Hyper PP is estimated to be $0.13 \times 10^{-5}$ of the UK population (Horga et al., 2013).

Gamstorp described Hyper PP as an autosomal dominant condition in which attacks of paralysis of the extremities and trunk often, but not always, occur in association with raised serum potassium levels (Gamstorp, 1957). ECG changes associated with hyperkalaemia may be seen during an attack (Gamstorp, 1957). Attacks are most frequently precipitated by rest of a few minutes up to hours after exertion (Helweg-Larsen, Hauge and Sagild, 1955; Gamstorp, 1957; Poskanzer and Kerr, 1961; Miller et al., 2004). Helweg reported “practically all patients have experienced difficulty in rising when having rested for a while after a period of hard muscular work” (Helweg-Larsen, Hauge and Sagild, 1955). The more severe the exertion, the more severe the subsequent attack (Gamstorp, 1957). However, intriguingly the limb is never paralysed during exercise (Gamstorp, 1957; Ricker et al., 1989). In fact, attacks can sometimes be prevented by patients performing moderate muscular exercise (Walter and Auböck, 1980).
The weakness generally begins in legs and ascends upwards and recovery occurs in the opposite direction (Helweg-Larsen, Hauge and Sagild, 1955; Gamstorp, 1957; Walter and Auböck, 1980). Attacks are typically shorter than Hypo PP attacks and usually last minutes to hours (Helweg-Larsen, Hauge and Sagild, 1955; Gamstorp, 1957) although they can last days (Miller et al., 2004). They may be localised or generalised (Helweg-Larsen, Hauge and Sagild, 1955; Gamstorp, 1957; Miller et al., 2004). Attacks usually affect the limbs, but patients may also report facial paralysis, difficulties with speech and swallowing and respiratory impairment. Attacks typically occur during the day (Gamstorp, 1957). Ingested potassium may trigger the attacks (Gamstorp, 1957) but a heavy meal or carbohydrate load does not (Poskanzer and Kerr, 1961). Some patients describe pain associated with attacks of weakness (Bradley, 1969). Most patients feel well in between attacks; however, some describe dull pain, tenderness and stiffness which may persist for weeks (Gamstorp, 1957).

In most, but not all attacks, tendon reflexes are weakened but pupillary and plantar reflexes are normal (Gamstorp, 1957). Whether the attack is triggered by rest after intense exercise or potassium ingestion the clinical characteristic are the same (Gamstorp, 1957). In half of the attacks precipitated by rest after exertion, the potassium level was elevated but in half it remained normal (Gamstorp, 1957). Thus serum potassium is not always elevated with paralytic attacks and in some patients, during some attacks it has even been reported to be low (Pearson, 1964). This confusion over associated potassium levels led to a distinct condition of normokalaemic periodic paralysis being described. However, this is likely a clinical variant of hyper PP, as one of the descendants of the family in which it was described was found to have a common Hyper PP mutation (Chinnery et al., 2002).

Some patients have a good therapeutic response to acetazolamide (Suetterlin et al., 2019). Salbutamol has also been reported to prevent attacks (Hanna et al., 1998). During an attack, infusion of NaCl has been reported to abort the attack (Poskanzer and Kerr, 1961) as has treatment with Calcium chloride or calcium carbonate (Gamstorp, 1957; Pearson, 1964).
1.3.3 Hyper PP Neurophysiology

As for Hypo PP (see 1.2.3), the majority, but not all, patients with hyper PP have a positive LET (Fournier et al., 2004; Tan et al., 2011). However, other neurophysiological findings distinguish Hyper PP from Hypo PP both during and between attacks.

During an attack of Hypo PP there is electrical silence, increased action potential duration and conduction velocity slowing (Troni, Doriguzzi and Mongini, 1983; Zwarts et al., 1988) (see 1.2.3). However, in Hyper PP increased spontaneous activity (Gamstorp, 1957), reduced action potential duration (Gamstorp, 1957; Buchthal, Engbaek and Gamstorp, 1958; Morrison, 1960) and stable muscle fibre conduction velocity (Buchthal, Engbaek and Gamstorp, 1958; Morrison, 1960) are reported. In between attacks interictal myotonia is present in 74% of genetically confirmed patients with Hyper PP (Miller et al., 2004) but has only recently been identified in handful of patients with Hypo PP (Luo et al., 2018; Poulin et al., 2018).

Hyper PP patients also have changes in MVRCs that were in the opposite direction from Hypo PP patients (Tan et al., 2020). In contrast to Hypo PP, the early supernormality and muscle relative refractory period were within normal range for most Hyper PP patients. However, Hyper PP patients had an increase in the area under the curve after 2 conditioning stimuli (2XMSN, p = 0.0017), and an increased extra residual supernormality after 5 conditioning stimuli. The increased extra residual supernormality is also seen in patients with Myotonia Congenita (Tan et al., 2014) and is likely a reflection of susceptibility to myotonia (Tan et al., 2020). The increased area under the curve may represent increased inward Na⁺ flux that reduces threshold to depolarisation and thus increases supernormality (see 1.1.1).

Hyper PP has been described as in continuum with Paramyotonia Congenita. Patients with both conditions complain of myotonia and episodic weakness and they are caused by mutations in the same gene. However, patients with Hyper PP can be completely distinguished from those with Paramyotonia Congenita based on their response to a 30Hz frequency ramp (Tan et al., 2020). This suggests that although clinical features overlap there are distinct molecular pathomechanisms involved.
1.3.4 Hyper PP Imaging

Whole body MRI of a family of 7 Hyper PP (T704M) patients demonstrated a predilection for the posterior compartment of the lower leg with sparing of the upper limbs, medial and posterior thigh and anterior and lateral compartments of lower leg (Lee et al., 2015). In fact, all atrophic muscles were situated in the posterior compartment of lower leg or anterior compartment of thigh. This is strikingly similar to that reported on a CT study of Hypo PP patients (Links et al., 1990) (see 1.2.4). A follow up study 3 years later demonstrated progression of fat infiltration in the lower leg in these patients providing evidence of chronic progressive myopathy in Hyper PP (Jeong et al., 2018).

MRI spectroscopy has also shown that myoplasmic 23Na$^+$ signal increases during an attack of Hyper PP (Weber et al., 2006) and that interictal myoplasmic 23Na$^+$ signal correlates with the presence of permanent weakness (Amarteifio et al., 2012).

1.3.5 Hyper PP Pathology

Similar to Hypo PP the predominant features of Hyper PP myopathy are vacuoles and tubular aggregates (Walter and Auböck, 1980; Bradley et al., 1990; Miller et al., 2004). An increase in sarcoplasmic glycogen, vacuolation of mitochondria and changes in the I band region of myofibrils are all consistent features considered secondary (Bradley et al., 1990). Muscle structure may also be normal in patients with Hyper PP (Bradley et al., 1990; Miller et al., 2004) especially young ones (Bradley et al., 1990).

1.3.6 Hyper PP Life course

The onset of Hyper PP tends to be in the first decade (Helweg-Larsen, Hauge and Sagild, 1955; Gamstorp, 1957; Miller et al., 2004). The frequency and severity of attacks change with age (Gamstorp, 1957; Bradley, 1969; Charles et al., 2013). Gamstorp described that attacks were short and frequent in childhood but became longer and more severe around puberty. This has also been observed by other authors (Charles et al., 2013). In approximately half the large kindred Gamstorp described, the attacks reduced in frequency and severity and after age fifty or sixty the attacks sometimes ceased. Similar findings were reported in a retrospective
Introduction

Hyperkalaemic periodic paralysis (Hyper PP) patient questionnaire (Charles et al., 2013) and in other case reports (Bradley, 1969).

1.3.7 Hyper PP Gender differences

In contrast to Hypo PP no gender difference in penetrance has been reported for Hyper PP (Helweg-Larsen, Hauge and Sagild, 1955; Gamstorp, 1957). However, it has been observed that fixed weakness is more common in affected male relatives (Ptacek et al., 1991) and that attack severity is increased in men (Poskanzer and Kerr, 1961; Ptacek et al., 1991). This is also supported by the fact that the male/female gender ratio of Hyper PP patients attending our NHS Highly specialised muscle channelopathy clinic is 2.08 suggesting affected males may be more likely to seek treatment.

1.3.8 Hyper PP Genotype-phenotype correlations

Most of the genotype-phenotype correlation has been performed by comparing patients with the two most common mutations in Hyper PP: T704M and M1592V (Miller et al., 2004). Patients with Hyper PP due to T704M mutation have a younger age of onset (0.8 Vs 5 years) and more frequent attacks (28 Vs 3/month) that are shorter in duration (8 Vs 89 hours) compared to M1592V Hyper PP (Miller et al., 2004). T704M patients are also less likely to report an attack triggered by illness (0 Vs 38%) and are less likely to respond to acetazolamide: all of the Hyper PP patients who did not respond to acetazolamide (approximately 25%) had the T704M mutation (Miller et al., 2004).

1.3.9 Hyper PP Pathophysiology

1.3.9.1 Human studies

Gamstorp induced attacks of Hyper PP in her patients either with rest after exertion or potassium ingestion. In 45 of 49 attacks the peak serum potassium was within 15 minutes of peak weakness (Gamstorp, 1957). The association of weakness with potassium was confirmed in vitro using isolated muscle fibres from Hyper PP patients: raising potassium to 7mM decreased force in Hyper PP but not healthy control fibres (Lehmann-Horn et al., 1987; Ricker et al., 1989).
As mentioned in 1.3.2 patients do not develop weakness during exercise but instead it occurs during a period of rest after exercise or with cold exposure (Ricker et al., 1989). In keeping with this the initial potassium rise during exercise in patients with Hyper PP is no different than for controls. However, in the Hyper PP patients there is a second rise approximately 10 minutes after exercise. It is during this second rise that an attack of paralysis develops (Ricker et al., 1989) Figure.1-9. The reason for this delayed rise in serum potassium is not known. However, it appears to correlate with the time point at which Na K ATPase induced hyperpolarisation starts to decline following repetitive stimulation of skeletal muscle (Hicks and McComas, 1989) (1.1.4).

Figure 1-9 Loss of force in Hyper PP is due to a second rise in serum $K^+$ that occurs during rest after exercise. A. Hyper PP patient (B) healthy individual. Figure and figure legend from (Ricker et al., 1989)

In 1963, by measuring membrane potentials from exposed Vastus medialis muscle of Hyper PP patients, Creutzfeld et al demonstrated that the resting membrane potential depolarised during attacks of weakness (Creutzfeldt et al., 1963). This was true for both a spontaneous attack and an attack provoked by a potassium chloride infusion(Creutzfeldt et al., 1963). Later studies on isolated muscle fibres from patients with Hyper PP revealed a persistent inward $Na^+$ current that could be blocked by tetrodotoxin (TTX). NaV1.4 blockade with TTX hyperpolarised Hyper PP patient fibres to such an extent that there was no longer a significant difference in resting membrane potential between patient and healthy control fibres (Ricker et
Hyperkalaemic periodic paralysis (Hyper PP) was first described by al., 1989). Thus, an anomalous inward Na+ current was identified as the primary pathological feature of Hyper PP. Intriguingly, whilst TTX application consistently repolarised the fibres, returning the fibres to 3.5mM K+ did not (Ricker et al., 1989).

In 1991, Cannon et al performed single channel recording on patient and control myotubes (Cannon, H. Brown and Corey, 1991). There was no difference in recordings obtained at 3.5mM K+, however, at 10mM K+ a proportion of the channels in the Hyper PP patient’s myotubes switch to a non-inactivating mode. Ongoing Na+ influx through these non-inactivating channels was sufficient to depolarise the sarcolemma and inactivate both WT and normally functioning mutant channels. This also explained why whole cell voltage clamp experiments on patient myotubes (Rudel, Ruppersberg and Spittelmeister, 1989) failed to detect a deficit: the voltage clamp held the membrane at a potential that restored function of WT and inactivating mutant channels. The small non-inactivating current through mutant channels was therefore obscured by the current through normal channels.

Intriguingly, several in vitro studies have demonstrated what appears to be a dissociation between force and excitability in Hyper PP muscle. Firstly, when the bath solution temperature around hyper PP fibres was reduced to 24°C there was no reduction in resting membrane potential nor change in action potential appearance, however, the fibre did not contract (Ricker et al., 1989). At 26 °C a contraction was barely visible, at 28 and 30 °C the contraction became more visible and at 32 °C it was strong enough to throw the electrode out of the fibre (Ricker et al., 1989). The converse situation where force was maintained but excitability lost has also been observed with changes in pH (Lehmann-Horn et al., 1987). Acidosis prevented loss of force in Hyper PP fibres exposed to raised potassium, it also helped to recover force in Hyper PP fibres that had been exposed to raised potassium, however, this effect was not due to repolarisation as there was no change in the RMP (Lehmann-Horn et al., 1987). A similar phenomenon of dissociated force and excitability was noted in the transgenic CaV1.1 Hypo PP mouse where homozygous animals consistently showed an unexplained 10%
increase in force 10 minutes into the glucose and insulin infusion without any observable change in CMAP amplitude (Wu et al., 2012) (1.2.9.3.1).

1.3.9.2 Heterologous expression
In 1993, using heterologous expression of rat NaV1.4 channels in a mammalian cell line, it was shown that Hyper PP mutations resulted in macroscopic NaV1.4 currents that failed to inactivate properly (Cannon and Strittmatter, 1993). However, in this heterologous expression system only 3 of 13 T698M and 3 of 10 M1592V transfected cells had a clear increase in steady state Na⁺ current as the potassium was increased from 1 to 40mM (Cannon and Strittmatter, 1993). This contrasted with the result obtained from patient myoblasts, where increasing the potassium to 10mM increased the number of inactivating mutant channels. The raised K⁺ does not appear to act on the mutant channel directly.

The proof of principle that disruption of NaV1.4 inactivation could cause myotonia was provided by ATXII an anemone toxin that selectively disrupts NaV inactivation in a way similar to Hyper PP mutations (Cannon and Corey, 1993). Application of ATXII to rat skeletal muscle made it myotonic as evidenced by myotonia on EMG, after-depolarisations, repetitive firing persisting beyond the duration of the stimulus and a prolonged twitch time. Detubulating the fibres with glycerol abolished the afterdepolarisation and the post-stimulus firing suggesting myotonia is due to K⁺ accumulation in the t-tubules and that vulnerability to this K⁺ accumulation may be what renders Hyper PP muscle more sensitive to increases in serum K⁺. This work was subsequently extended to show that some, but not all, hyper PP mutations exhibited defects in slow sodium channel inactivation (Hayward, Brown and Cannon, 1997).

1.3.9.3 Animal models
1.3.9.3.1 Quarter Racehorse
A disorder that appeared similar to Hyper PP was described in quarter horses and subsequently confirmed to be due to mutations in NaV1.4 (Rudolph et al., 1992). Similar to the findings in human muscle, horses with Hyper PP are depolarised relative to controls but addition of TTX hyperpolarised their muscle to control
values (Pickar et al., 1991). This provided further evidence that an anomalous inward Na\(^+\) current through the NaV1.4 alpha pore was the cause of Hyper PP. Interestingly, as for human Hyper PP myotubes, Hyper PP horse myotubes exhibited an 8 fold increase in steady state open probability at the end of a long depolarising pulse compared to wild-type (Cannon et al., 1995). As with the human channels, this behaviour was infrequent but tended to cluster and there was also a trend for more frequent bursts of non-inactivating behaviour from mutant channels when potassium concentration was increased.

1.3.9.3.2 M1592V Hyper PP mice

**Clinical Features**

The first transgenic mouse model of PP was developed in 2008. This mouse had one of the two common Hyper PP mutations M1592V (Hayward et al., 2008). Heterozygous mice are viable without reduction in longevity. Homozygous mice occurred with reduced frequency and were usually found dead at birth. Mice were initially reported to appear normal without any spontaneous episodes of weakness (Hayward et al., 2008), however, in subsequent studies heterozygous M1592V mice were found to have very brief (<60 seconds) episodes of immobility or hind-limb dragging (Corrochano et al., 2014; Khogali et al., 2015).

Isolated M1592V mouse muscle recapitulate the loss of force on exposure to high potassium and accelerated recovery in high calcium (Hayward et al., 2008; Lucas et al., 2014; Ammar et al., 2015; Khogali et al., 2015) that has been reported for humans with Hyper PP (Gamstorp, 1957). In addition, simulating rest after exercise triggered weakness in Hyper PP but not WT mouse muscle. After fatiguing stimulation in 10mM K\(^+\), muscle force recovery in M1592V, but not WT EDL aborted after 10 minutes and sustained weakness ensued (Clausen et al., 2011). The time course of this is remarkably similar to that reported in human muscle fibres (Ricker et al., 1989) (1.3.8.1) and also to the time course of Na K pump activity slowing down after an increase induced by exercise (Hicks and McComas, 1989).
Like Hypo PP transgenic mice (1.2.9.3) heterozygous M1592V mice showed minimal histological change on muscle biopsy, whilst homozygous animals showed marked change in gastrocnemius but not TA. At 4 months, TA from homozygous animals had mild nonspecific changes whilst gastrocnemius showed marked vacuolar changes with centrally located clear vesicles (Hayward et al., 2008). Ultrastructural studies of gastrocnemius showed disruption of the CRU with massive dilatation of the transverse tubules and SR terminal cisternae (Hayward et al., 2008).

Neurophysiology

All M1592V mice had electrical myotonia on EMG but a provocative challenge e.g. inducing acidosis to inhibit CIC-1 channels was needed to cause clinical myotonia (Hayward et al., 2008). In freely moving mice with implanted EMG electrodes periods of reduced EMG activity occurred (Khogali et al., 2015) suggesting possible paralytic-like attacks. These tended to follow EMG bursts of activity but only 29% of EMG activity bursts were followed by a period of reduced activity. EMG bursts of activity that were followed by reduced activity were, on average, 2.5 times longer than those that weren’t (Khogali et al., 2015).

Life course, gender differences and genotype-phenotype correlation

Gender differences in M1592V mice have not been specifically studied. However, life course studies of M1592V mice up to the age of 12 months (human equivalent of approximately 40 years old) have been performed. As for humans with Hyper PP (1.3.6), the frequency of immobility attacks is maximal around puberty or late adolescence (1 month) (Khogali et al., 2015). However, in contrast to humans, attacks were rarely seen outside of this age and were not seen at all in 3 to 6 month old animals which may explain why the initial study did not observe any immobility attacks (Hayward et al., 2008). The periods of reduced EMG activity that were observed in 2-month old M1592V mice also became shorter and less frequent and were barely seen by 6 months of age (Khogali et al., 2015) suggesting they may correlate with periods of immobility. There was no significant difference in baseline tetanic force or ex vivo potassium-induced weakness between EDL or soleus muscles from mice that were 1, 4 or 12 months old.
Introduction

Hyperkalaemic periodic paralysis (Hyper PP)

Hyper PP

Pathophysiology

Similar to the human (Ricker et al., 1989) and horse (Pickar et al., 1991) studies, M1592V mouse soleus had an anomalous Na\(^+\) influx that was 5.7 fold larger than WT and was TTX-suppressible (Clausen et al., 2011; Ammar et al., 2015). Addition of TTX also repolarised M1592V mouse soleus RMP to WT values (Clausen et al., 2011). This anomalous inward Na\(^+\) current makes M1592V soleus more dependent on the Na\(^+\) K\(^+\) pump and thus more sensitive to 1µm ouabain (a Na\(^+\)K\(^+\) ATPase blocker) (Ammar et al., 2015). Intriguingly, although the addition of ouabain depolarised WT and Hyper PP RMP to a similar degree (8mV and 7mV respectively) it reduced Hyper PP soleus force by a much greater degree (42% Vs 7% for WT soleus). This was suggested to be because the RMP of Hyper PP soleus is on the steepest part of the tetanic force to RMP curve (Figure 1-10) thus further depolarisation has a disproportionate effect on force (Ammar et al., 2015).

![Figure 1-10](image)

**Figure 1-10** The differential effect of elevated K\(^+\) on different muscles from WT and Hyper PP mice (Ammar et al., 2015).

However, although depolarisation is TTX suppressible, the susceptibility to potassium-induced weakness is not simply related to NaV1.4 protein expression or Na\(^+\) conductance (Khogali et al., 2015). Diaphragm expresses NaV1.4 to levels 2 fold greater than soleus, has twice the TTX sensitive Na\(^+\) influx of EDL & Soleus and yet, in contrast to EDL and soleus, there was no significant difference in RMP.
Introduction

Hyperkalaemic periodic paralysis (Hyper PP)

Pathophysiology

between WT and Hyper PP diaphragm at any potassium concentration (Ammar et al., 2015). The reason for this is not yet known.

1.3.9.3.3 I582V (Draggen) Hyper PP mouse

These mice were found through an unbiased ENU mutagenesis screen. They were called Draggen because of unprovoked intermittent attacks of hind-limb dragging or immobility. A missense mutation in SCN4A was found (c.1744AA>G, p.I582V) this is the mouse equivalent to I588V a mutation identified in a patient with Hyper PP. Functional characterisation of human I588V and mouse I582V NaV1.4 in HEK293 cells revealed a 6mV shift in the hyperpolarising direction for the voltage dependence of channel activation and a 3mV shift in the hyperpolarising direction for the voltage dependence of fast inactivation (Corrochano et al., 2014).

Clinical Features

As for M1592V mice, the Draggen mutation is homozygous lethal. Homozygous pups died perinatally with respiratory difficulties evidenced by collapsed lungs but otherwise morphologically normal organs. The diaphragm was also morphologically normal, so it seems that the cause of death was functional impairment of respiratory muscles. Heterozygous Draggen mice are viable with a normal lifespan (Corrochano et al., 2014).

Draggen hind-limb immobility attacks usually last a few seconds and, as for humans with Hyper PP (1.3.3), are accompanied by increased EMG activity. Isolated EDL from Draggen mice reacts similarly to WT EDL in response to hypokalaemia but loses more force than WT in response to hyperkalaemia. Draggen mice also develop reduced grip strength compared to WT starting at around 50 to 60 weeks of age. In keeping with this and in contrast to the M1592V mice, heterozygote Draggen mice exhibit the classic histological features of PP myopathy on muscle biopsy (Corrochano et al., 2014) (1.3.5).

Life course, gender difference and genotype-phenotype correlation

There was a clear gender difference in phenotype for Draggen mice. Male, but not female Draggen mice have reduced body weight compared to WT. This is despite
similar lean mass and was shown to be linked with an increased energy expenditure and reduced fat compared to WT (Corrochano et al., 2014). These metabolic changes are associated with abnormal activation of AMPK, the key intracellular energy sensor: there was increased basal activation blunted skeletal muscle contraction-induced activation of AMPK in male Draggen mice (Corrochano et al., 2014).

There is also a gender difference in the penetrance of hind-limb dragging attacks. Whilst 100% of male Draggen mice had an immobility attack by the time they were 60 weeks old, only 38% of females did. In addition, the frequency of hind-limb dragging attacks observed over the lifetime of individual mice was increased in males (8±0.9 episodes in males Vs 2.6±0.4 episodes in females up to 60 weeks). This is despite 100% penetrance of EMG myotonia for both genders and a similar degree of fibre type transformation of TA at 12 weeks (Corrochano et al., 2014). This suggests physiological factors inherent to male gender may lower the threshold for a hind-limb dragging attack in Draggen mice.

Heterozygote Draggen mice exhibit a progressive decline in grip strength from middle age (Corrochano et al., 2014). As is reported for humans with PP (Sternberg et al., 2001; Miller et al., 2004), there was no apparent gender difference in the onset, progression or penetrance of permanent weakness (Corrochano et al., 2014).

1.4 Myotonia Congenita (MC)

1.4.1 MC Genetics

MC is caused by loss of function mutations in CLCN1 (Koch et al., 1992). Mutations can be nonsense, missense or splicing. Missense mutations may cause dominant or recessive MC whilst nonsense and splice site mutations are overwhelmingly associated with recessive MC (Suetterlin, Männikkö and Hanna, 2014). Although mutation hotspots have been described (Fialho et al., 2007), both dominant and recessive mutations are found throughout the length of the channel sequence and in some cases both dominant and recessive inheritance is reported for the same variant (Koch et al., 1992; Zhang et al., 1996). This ambiguity is compounded by
the fact that until recently, relatively little was known about ClC-1 channel structure. In contrast to the major voltage-gated cation channels, it has no clear voltage sensor, and the structure-function relationship is complex and not well understood. Therefore, it is currently extremely difficult to accurately predict the pathogenicity and likely inheritance pattern of a novel ClC-1 variant based on genetic data alone.

1.4.2 MC Clinical Features
MC, as the name suggests is characterised by myotonia. Myotonia is hyperexcitability of the muscle which manifests as repetitive after discharges on electromyogram (EMG) and patients tend to describe as muscle stiffness. MC symptoms are limited to skeletal muscle as extra-muscular CLCN1 transcript expression is minimal.

MC is classically associated with the warmup phenomenon where patients describe that their muscle stiffness improves with activity. In contrast to sodium channel myotonia, the limbs are relatively more affected than the eyes (J Trip et al., 2009). Cold can exacerbate myotonia in both MC and PMC and thus is not a good discriminator (J Trip et al., 2009).

Recessive myotonia differs from dominant myotonia as it has a later onset, is more severe and is usually associated with transient weakness (Deymeer et al., 1999). Transient weakness can be distinguished from the weakness associated with PMC as it occurs at the onset of activity and improves with repetition. In contrast the weakness associated with PMC is exacerbated by repetition.

1.4.3 MC Neurophysiology
Nerve conduction studies should be normal in patients with MC. EMG demonstrates myotonia. Myotonia due to ClC-1 mutations may be distinguished from Myotonia due to NaV1.4 mutations by measuring the first inter discharge interval (Drost et al., 2015). This was longer than 30ms in 31 of 32 chloride channel myotonia patients and shorter than 30ms in 34 of 34 sodium channel myotonia patients. The sound of myotonia on the EMG machine is also reported to differ between sodium and chloride channel myotonia, with sodium channel myotonia
Introduction

Myotonia Congenita (MC) Neurophysiology

sounding like dolphins and chloride channel myotonia the more classical motorcycle revving (Tan et al., 2011).

Figure 1-11 Short Exercise Test Patterns. Figure adapted from two separate figures in (Tan et al., 2011). AR MC = autosomal recessive myotonia congenita; AD MC = autosomal recessive myotonia congenita; PMC = paramyotonia congenita; SCM = sodium channel myotonia; RT = room temperature; CL = after cooling (plotted as % of post cooling baseline); RW = after rewarming (plotted as % of precooling baseline). Symbols and error bars represent mean ± standard error of the mean.”

However, a more established way of distinguishing between Na⁺ and Cl⁻ channel myotonia is using the short exercise tests (SET) (Fournier et al., 2004; Tan et al., 2011) (Figure 1-11). The SET uses differences in the response to exercise and cooling to help differentiate MC, SCM and PMC. Patients are asked to perform maximal voluntary contraction of abductor digiti minimi for 10 to 12 seconds. CMAPS are then recorded 2 seconds immediately after the end of exercise and
Introduction

Myotonia Congenita (MC)

MC Imaging

every 10 seconds for 50 seconds. The short exercise test is repeated three times with 60 seconds between the beginning of each trial. An icepack is then applied to the abductor digiti minimi and the test repeated after cooling and again after rewarming the muscle (Figure 1-11).

The classic SET pattern for recessive MC (type 1) reflects transient weakness (Figure 1-11). Typically, the CMAP amplitude as a percentage of baseline increases with repetition in recessive MC. In PMC the converse occurs (type II pattern) and the CMAP amplitude reduces with successive exercise trials and is further reduced by cooling and after rewarming (Figure 1-11). For SCM there is no change in CMAP amplitude with repetition (Type III or normal pattern) (Fournier et al., 2004; Tan et al., 2011). Dominant MC usually follows this pattern too.

Muscle Velocity Recovery cycles have also been performed in patients with MC. Compared to controls, people with MC have increased early supernormality that is particularly prominent around a 20ms interstimulus interval and enhanced late supernormality that is amplified after a train of impulses (Tan et al., 2014). The enhanced supernormality requires 2 or 5 impulses to become apparent. This contradicts the notion that ClC-1 channels provide the major component of resting membrane conductance and strongly suggests that depolarisation is required to activate ClC-1 channels in skeletal muscle (Tan et al., 2014). Rapid trains of impulses of >15Hz were associated with fall in the amplitude of CMAP response in recessive MC patients. This is analogous to the fall in CMAP response in the SET (Tan et al., 2014). Thus, frequency ramp testing may be useful for patients who are unable to comply with the voluntary activation required for SET.

1.4.4 MC Imaging

A hyperintense stripe on MRI with STIR has been associated with MC. The stripe extended by more than one third of the muscle on an axial image and was found more frequently in association with chloride channel rather than sodium channel myotonia (Morrow et al., 2013).
1.4.5 **MC Pathology**

Changes observed on muscle biopsies from patients with MC are characteristically quite mild and may include a reduction in type IIb fibres (Crews, Kaiser and Brooke, 1976). If there is gross abnormality on biopsy than another disorder should be considered and excluded.

1.4.6 **MC Gender differences**

There is evidence of a gender difference in the penetrance of MC with reduced penetrance or lower clinical expressivity in females (Mailander et al., 1996; Deymeer et al., 1999; Horga et al., 2013). In studies examining the asymptomatic parents of recessive MC patients the majority (74-100%) of fathers had myotonic discharges on EMG whilst a minority (0-24%) of the mothers did (Mailander et al., 1996; Deymeer et al., 1999). It was even suggested that presence of abundant myotonic discharges in the mother, should prompt the clinician to search more carefully to historical clues pointing to dominant inheritance. The increased penetrance in males is reflected by a male/female ratio of 2.0 in recessive MC patients attending our national skeletal muscle channelopathy clinic (Horga et al., 2013).

1.4.7 **MC Genotype-phenotype correlations**

Genotype-phenotype correlation in MC has been particularly difficult to do as there are so many different mutations combined with huge heterogeneity in phenotype (Colding-Jorgensen, 2005). However, certain features have been reported to help differentiate between recessive and dominant MC. It is said that in dominant MC, myotonia is more pronounced in the upper limb and in recessive MC it is more pronounced in the lower limb. Somewhat surprisingly, recessive MC is reported to have a later age of onset (around 4 years old) compared to dominant MC (around 2 years old). As discussed in 1.4.2 and 1.4.3, recessive MC is also associated with the phenomenon of transient weakness which is not reported in dominant MC (Colding-Jorgensen, 2005).

1.4.8 **MC Pathophysiology**

In contrast to Hypo PP, for which I could find no reports of the condition occurring spontaneously outside of Humans, MC is reported to have occurred spontaneously
Introduction

Myotonia Congenita (MC) in goats, cattle, horses, dogs and mice (Bryant and Morales-Aguilera, 1971; Amann, Tomlinson and Hankison, 1985). Therefore, much of the seminal work investigating the pathophysiology of MC was performed in animal rather than human tissue. The order of this section is changed to reflect this.

1.4.8.1 Animal models of MC

1.4.8.1.1 Myotonic Goat

The classical studies on MC were performed on the myotonic or ‘Tennessee’ fainting goat. These goats are an American breed of meat goat that stiffens or falls when startled. In addition to being very muscular, they were desirable as they were unable to jump over normal fences. They were first described in the scientific literature in 1904 and described as MC in 1939 (Brown and Harvey, 1939).

When intercostal muscle fibres from these goats were examined in vitro they were found to discharge repetitively (Bryant, 1969). Once the number of action potentials in an evoked train exceeded 10-15, stopping the current did not stop the repetitive firing of action potentials (Adrian and Bryant, 1974). Although it took several action potentials to trigger repetitive activity there was an approximately linear relation such that one action potential contributes approximately 1mV to a subsequent after-depolarisation. However, this after depolarisation was abolished in myotonic fibres detubulated with glycerol (Figure 1-12) (Adrian and Bryant, 1974). This was not due to excitation-contraction uncoupling as the after depolarisations persisted in the presence of dantrolene, an excitation-contraction uncoupler (Adrian and Bryant, 1974). Instead, it suggested firstly that myotonic discharge is due to normal muscle activity producing cumulative after-depolarisation which, when large enough can initiate repetitive discharges and secondly that myotonic activity requires the integrity of the t-tubules system. This is in keeping with the finding that detubulation of rat skeletal muscle prevented myotonia induced by anemone toxin (Cannon and Corey, 1993).
Studies in normal and myotonic goat muscle fibres provided overwhelming evidence that the myotonia was due to reduced chloride conductance. Firstly, normal goat muscle fibres were found to behave in the same way as myotonic goat fibres when exposed to chloride free solution (Bryant, 1969; Adrian and Bryant, 1974). Secondly, in addition to repetitive firing, chloride free solution increases membrane resistance 3 to 5 fold (Bryant, 1969) and when cable properties of skeletal muscle fibres from myotonic goats were examined they had a 3-fold higher membrane resistance than normal goat skeletal muscle fibre in standard solution (Lipicky and Bryant, 1966; Bryant, 1969). Thirdly, blocking chloride channels with 9AC rendered normal goat muscle myotonic (Bryant and Morales-Aguilera, 1971).

Thus, experimental evidence suggested chloride channel dysfunction in the t-tubules as the underlying cause of myotonia in Tennessee myotonic goats. Chloride dysfunction was subsequently confirmed in humans with MC with the identification of pathogenic mutations in CLCN1 (Koch et al., 1992).

1.4.8.1.2 Mto Mouse

The Mto mouse occurred due to spontaneous mutation in Jackson Lab Bar harbour SWR/J inbred mouse strain (Heller et al., 1982).

Figure 1-12 Detubulation prevents after depolarisation in myotonic fibres. Figure and legend from (Adrian and Bryant, 1974).
Introduction

Myotonia Congenita (MC)

MC Pathophysiology

Heterozygote mice were unaffected. Homozygote mice exhibited extensor posturing of the hind limbs, consistent with clinical myotonia, from 2 weeks of age. This was reliably triggered by shaking the cage or dropping the animals from a height of 10cm (heller). The myotonia reduced with repetition suggestive of the warmup phenomenon described in patients but was exacerbated with exposure to cold. As with humans (1.4.5) histopathological appearances were not significantly different from WT animals. However, both male and female Mto mice exhibited a weight deficit compared to WT or heterozygote littermates which hasn’t been reported for humans.

In terms of life course changes, by 14 days clinical myotonia was apparent, by 30 days homozygous mto mice weighed 10% less than heterozygous or WT littermates, by 70 days they weighed 40% less had increased muscle bulk in neck and shoulder girdles as well as pectus excavatum deformity, by 80 days there was thoracic kyphosis around T3 to T5. Homozygous mice could live up to 1 year. There was no apparent gender difference in phenotype for these mice.

1.4.8.1.3 Adr Mouse

Around the same time as the mto mouse, another myotonic mouse strain arose spontaneously from an independent laboratory (Watkins, WJ and Watts, DC., 1984). As for the mto mice, heterozygous mice are unaffected. Homozygous mice exhibit a deficit in weight gain from 10 days and progressive weight loss from 12 weeks (Watkins, WJ and Watts, DC., 1984). As for the mto mice and in contrast to Draggen Hyper PP mice (1.3.9.3) weight loss occurred in both sexes and was also associated with reduced muscle mass. Homozygous adr muscle weight was reduced by 2 weeks of age and was 62% of control values by 6 weeks of age (Watkins, WJ and Watts, DC., 1984). Similar to human studies, histology indicates that type II muscle fibres are more affected in adr mice.

The life course of these animals is remarkably similar to that described for the homozygous mto animals (Heller 1982). The first clinical signs of myotonia were noted at 10-12 days of age. This is the mouse equivalent of childhood (sexual maturity occurs at 6 to 8 weeks). By 3 weeks animals were slower than unaffected
littermates. Life span for adr mice is reduced to as little as 12 weeks up to 1 year depending on the genetic background (Watkins, WJ and Watts, DC., 1984).

1.4.8.2 Heterologous expression

ClC-1 structure-function relationship is complex, dynamic and not fully understood. In contrast to the cation channels, ClC-1 is a homodimer (Miller and White, 1984; Dutzler et al., 2002) and each subunit contains its own chloride selective pore. The two chloride selective pores can be gated individually or concurrently in processes known as fast- and slow gating respectively. The voltage sensitivity of the channel is believed to arise from its voltage dependent interaction with chloride ions (Jentsch, Günther and Pusch, 1995; Rychkov et al., 1996). There is no known voltage sensor. Although the exact mechanism of gating has not been elucidated, ClC-1 gating is known to be modulated by many different physiological signals. These include changes in pH (Rychkov et al., 1996), ATP, NADP (Bennetts et al., 2005), redox status (Zhang, Tseng and Chen, 2008) and PKC phosphorylation (Camerino et al., 2014). All these factors appear to shift the voltage dependence of modulation to a similar degree – in the region of 40mV. There is also evidence that differential modulation of ClC-1 according to the muscle’s metabolic state is possible. For example, ATP inhibition of ClC-1 currents is enhanced in low pH (Tseng, Bennetts, and Chen 2007) and oxidation renders ClC-1 insensitive to ATP inhibition (Tseng, Bennetts, and Chen 2007; X.-D. Zhang, Tseng, and Chen 2008b). This diversity of regulation may help account for the heterogeneity in phenotype of patients with MC (Colding-Jorgensen, 2005).

The structural and functional complexity of ClC-1 means that despite a number of crystal and cryo-electron structures of members of the ClC super family (Dutzler, 2003; Meyer and Dutzler, 2006; Feng et al., 2010; Park, Campbell and MacKinnon, 2017) our current understanding of CLC-1 structure-function does not allow accurate prediction of the functional or clinical impact of an identified ClC-1 variant. Therefore, variants are further investigated by studying their functional properties in heterologous expression systems. Functionally recessive mutations exert their effect by loss-of-function of the mutated subunit only (Wollnik, Kubisch
Examples include frameshift and nonsense mutations that do not form subunits that could impair the function of co-expressed wild-type (WT) subunits and missense mutations that do not affect the functional properties of the wild-type subunits in the channel dimer (Kubisch et al., 1998). In contrast, in dominant MC mutant subunits reduce the currents of the CIC-1 dimer at physiological voltages (George et al., 1993; Pusch et al., 1995).

Despite these well-established principles a systematic correlation between the functional properties of the channels and their clinical consequences has not been performed. Functional expression data comes from various systems and conditions, genetic data are at times incomplete and the inheritance pattern of clinical symptoms is not always systematically categorised. Thus, currently, the value of functional expression in determining the likely mode of inheritance is suggestive.

1.4.8.3 Human studies

There have been few studies on fibres from patients with MC. One key study was performed by Rudel in 1988 (Rüdel, Ricker and Lehmann-Horn, 1988). This confirmed reduced chloride conductance in humans with MC and demonstrated that RMP was not different from healthy control muscle fibres. More recently, work has highlighted the dynamic regulation of CIC-1 in active human (Riisager et al., 2016) and rodent (Pedersen et al., 2009; Pedersen, Paoli and Flatman, 2009) skeletal muscle. This demonstrated that muscle activity is associated with rapid, protein kinase C dependent CIC-1 inhibition in both rodent and human muscle fibres and this activity-induced CIC-1 inhibition facilitates maintenance of muscle fibre excitability during sustained activity.

1.5 Summary and Aims

There is significant variability in skeletal muscle channelopathy phenotype with age, gender and species. It is not known why this variation occurs. One possibility is that physiological differences alter how ion channel dysfunction manifests clinically. If this is true, understanding the extent of normal physiological variation
in skeletal muscle excitability should extend our understanding of both skeletal muscle physiology and phenotype variability in skeletal muscle channelopathies. It may also identify novel pathways that could be exploited for therapeutic benefit.

Whilst examining physiological variation might help explain pathological variation in phenotype. Examining pathological variation may also provide useful information on physiological function that can be used to the benefit of patients. Our current understanding of ClC-1 structure-function does not allow us to accurately predict a mutation’s functional effect based on its location alone. As a result, accurate genetic counselling for patients with a CLCN1 variant has been incredibly difficult. This combined with the phenotypic heterogeneity that can exist within the same family with the same mutation and the heterogeneity in inheritance patterns makes genetic counselling for people with MC extremely difficult. Our centre has one of the largest MC data sets in the world and provides functional characterisation for identified CLCN1 variants. Correlation of these data sets could significantly improve our understanding of ClC-1 structure-function and physiology and in so doing greatly improve the accuracy of genetic counselling we are able to provide patients with suspected MC.

This thesis aims to address the following perceived gaps in our current knowledge.

1. Why do Hypo PP mice not have spontaneous attacks of weakness; is this due to species’ differences in muscle excitability?
2. Why are males with muscle channelopathies more severely affected than their female relatives; is this due to gender differences in muscle excitability?
3. Why does the PP phenotype change with age; is this due to ‘normal’ aging or the consequence of chronic ion channel dysfunction?

Can the accuracy of genetic counselling for people with MC be improved?
2 Methods

2.1 Mice
Male heterozygous Draggen mice were obtained from MRC Harwell and bred with C57/Bl J6 female mice obtained from Charles River Laboratories. Successive generations were bred with different breeding female C57/BlJ6 mice to maintain the colony. Mice were fed *ad libitum* and housed according to home office guidelines. All experimental procedures were carried out under licence from the UK Home Office (Scientific Procedures Act 1986) and following approval by the UCL Institute of Neurology Animal Welfare Ethical Review Panel.

2.1.1 MC Life course
Although described as non-dystrophic, there is evidence that fat infiltration occurs with age in MC as T1 weighted changes on MRI and echo intensity on muscle ultrasound increase significantly with age in MC patients compared to controls. Both muscle echo intensity and T1 weighted change were also inversely correlated with force (J. Trip *et al.*, 2009; Morrow *et al.*, 2013).

2.1.2 Genotyping
Mice had ear biopsies taken at time of weaning (21 days) for genotyping. Genotyping was performed by Stuart Martin at UCL genotyping facility using the following *SCN4A* Primers: *SCN4A_UPS_EX.F* CTG GAA GAG GCC CAT CAGA; *SCN4A_UPSInt.R* CCC ACG GTG TCC TGT and PCR conditions of:

1. 95°C for 2 Min
2. 95°C for 30 Sec
3. 56°C for 30 Sec
4. 72°C for 40 Sec
5. 73°C 4 Min
6. 10°C Hold

Steps 2-4 were repeated for 35 cycles. Samples were run on a gel to check successful PCR. HpyCH4V digest (New England BioLabs, Cat No R0620S) was used. A 2x mix was prepared and 5µl aliquoted into PCR tubes. 5 µl of PCR product was
then added to each tube and tubes incubated at 37°C for 3 hours. The product was resolved on a 3.0% TBE gel. WT alleles (120 bp + 100 bp), tdTomato allele (200 bp).

### 2.1.3 Procedures

All experimental procedures on the mice were performed under terminal anaesthesia. Initially I used intraperitoneal anaesthesia (1ml/100g bodyweight 4.5% chloral hydrate). However, as I developed the *in vivo* recording techniques (2.2) the anaesthetic time increased and therefore to avoid the need for intraperitoneal injection mid procedure I changed to inhaled isoflurane as the anaesthetic. Isoflurane flow rate was titrated according to depth of anaesthesia and respiratory rate.

Tissue was dissected under terminal anaesthesia and either flash frozen in liquid nitrogen or stored as described for subsequent histology (see 5.3.3) or electron microscopic examination (see 5.3.4). Cervical dislocation was performed at the end of the experiment.

### 2.2 Muscle Velocity Recovery Cycles

Muscle velocity recovery cycles (MVRC) measure changes in conduction velocity to give an indirect measure of muscle excitability and thus ion channel function *in vivo*. Muscle excitability cannot be measured directly, as the currents necessary to attempt uniform depolarisation of a long, thin, unmyelinated muscle fibre would likely destroy it. However, changes in conduction velocity closely parallel changes in excitability and can therefore be used as a proxy for excitability (Z’Graggen and Bostock, 2009).

Muscle velocity recovery cycles look at changes in conduction velocity after an action potential. Therefore, one or more conditioning stimuli must precede a test stimulus to examine the recovery cycle. The test stimulus is constant stimulus intensity. It is always the response to the test stimulus that is assessed. The variables are the number of conditioning stimuli and the time between conditioning and test stimulus.

Muscle velocity recovery cycles are calculated by measuring the time to peak of a CMAP in response to a test stimulus. This is referred to as the latency. The velocity
Methods

Muscle Velocity Recovery Cycles

A recovery cycle is obtained by comparing the conditioned and unconditioned response at a specific inter-stimulus interval (Z'Graggen and Bostock, 2009). Varying the inter-stimulus interval allows the different phases of the velocity recovery cycle to be mapped out as the test stimulus will then be triggered during different parts of the excitability recovery cycle (e.g., absolute refractory period, relative refractory period, early or late supernormality). The changes in the pattern of supernormality of the recovery cycle after a single conditioning response compared with multiple conditioning stimuli helps to distinguish early supernormality, attributed to the passive accumulation of charge, from late supernormality attributed to the active accumulation of potassium in the t-tubules (Z'Graggen and Bostock, 2009).

MVRCs have been reported in humans with Na\(^+\), K\(^+\) and Cl\(^-\) channel channelopathies as well as in patients with hyperkalaemia secondary to renal failure and with critical illness myopathy (Z’Graggen and Bostock, 2009; W. J. Z’Graggen et al., 2011; S V Tan et al., 2012; Tan et al., 2014, 2017). In these patients it has been demonstrated that MVRCs are sensitive to changes in membrane permeability, resting membrane potential and alteration in ionic concentrations (S V Tan et al., 2012; Tan et al., 2014, 2017). MVRCs have not previously been reported in rodents.

2.2.1 MVRC protocol

MVRCs were recorded with 1, 2 and 5 conditioning stimuli all separated by 10ms intervals as described previously for humans (Tan et al., 2014). The inter-stimulus interval between the last conditioning stimulus and the test stimulus varied from 1000 to 1.4ms in 34 steps in an approximately geometric series (specifically 1000, 900, 800, 700, 600, 500, 450, 400, 350, 300, 260, 220, 180, 140, 110, 89, 71, 56, 45, 35, 28, 22, 18, 14, 11, 8.9, 7.1, 5.6, 4.5, 3.5, 2.8, 2.2, 1.8 and 1.4ms).

2.2.2 Frequency ramp protocol

These have not been performed before in rodents. The protocol used was as described previously for humans (Tan et al., 2014). “To characterise the effects of progressive muscle activation, the test stimulus was preceded by a 1 second train of stimuli at a frequency that was increased by 1Hz on successive 2 second cycles
Methods

Muscle Velocity Recovery Cycles from 1 to 30Hz. The average stimulation rate was therefore increased from 0.5 to 15.5Hz over 1 min. Stimulus cycles with the test stimulus alone were recorded before (10 cycles at 0.5Hz), during the ramp, and for a further 30 seconds after the end of the ramp.” (Tan et al., 2014)

2.2.3 Muscle Velocity Recovery Cycles in Humans

I recruited healthy male (n=3) and female (n=9) volunteers aged between 20 and 30 for MVRCs on their TA under ethics (10/H0802/6) “An investigation into Membrane Potential Changes in the Muscle Ion Channelopathies”. MVRCs were performed on the TA of these subjects by Dr Veronica Tan as she has previously described (S. Veronica Tan et al., 2012; Tan et al., 2014, 2017). MVRCs were also carried out on my frontalis and, as shown in Figure 2-1, my orbicularis occuli.

![Figure 2-1 Experimental setup for recording MVRCs from human orbicularis occuli](image.png)

To my knowledge there are not any studies that look specifically at interrater variability in MVRCs. However, it has been shown that Human MVRC parameters show no correlation with baseline latency, inter-electrode distance or apparent conduction velocity (distance/latency) (Werner J. Z'Graggen et al., 2011). This provides some evidence that intra and inter subject variability in MVRC parameters is predominantly secondary to biological variables as opposed to technical variables that may occur with different operators (e.g. electrode position).
2.2.4 Mouse Muscle Velocity Recovery Cycles Experimental setup in mice

Unlike for humans an anaesthetic is required to perform MVRCs in mice. For the first few recordings, when developing the MVRC technique chloral hydrate administered via intraperitoneal injection was used. However, I changed to inhaled anaesthesia as this can be adjusted and maintained without requiring additional intraperitoneal injection, which on occasion disturbed recording electrodes. However, inhalational anaesthetics have been shown to modulate NaV channels in vitro and isoflurane is known to reduce cortical excitability (Pelosi et al., 2001). There is no such report for chloral hydrate. I found no difference in the morphology or parameters of the initial mouse MVRCs recorded using intraperitoneal chloral hydrate or the subsequent MVRCs recording using inhaled isoflurane anaesthesia. This is in keeping with the finding that there was no difference in mouse peripheral nerve excitability measurements recorded under the influence of inhaled isoflurane or intraperitoneal injection of chloral hydrate (Boërio, Greensmith and Bostock, 2011) and the finding that sevoflurane had no effect on the recovery cycle of human cortical neurons (Burke et al., 2000).

Recordings were performed on mice aged between 3 and 6 months (human equivalent age 18 to 30 years) unless otherwise stated. The mouse was anaesthetised using isoflurane. Anaesthesia was induced in an anaesthetic chamber. After induction, the mouse was placed on its back on a heat mat and anaesthesia maintained via a nose cone (see Figure 2-2). Isoflurane was set according to respiratory level and depth of anaesthesia and usually maintained with a flow rate around 2.5 litres/minute with Oxygen flow at 1 litre/minute. Fur was shaved from skin over the muscles to be examined (Figure 2-2).
A monopolar stimulating needle electrode (28G TECA, Viasys Healthcare Madison, Wisconsin) was inserted into the distal muscle (Figure 2-3). A reference anode was inserted above and lateral to the monopolar stimulating electrode on the lateral edge of the muscle. The reference anode consisted of a 27G hollow bore disposable steel needle attached to reference anode lead with crocodile clip. Stimuli consisting of 0.05ms rectangular current pulses were delivered. Muscle activity was recorded with a concentric needle electrode (disposable 30G concentric EMG needle, TECA) inserted into the proximal end of the muscle. A ground electrode was inserted under the skin in the axilla. The ground electrode consisted of a 27G hollow bore disposable steel needle that was bent to make it easier to insert under the skin and attached to crocodile clip on the ground cable. Surface temperature over the muscle was recorded at the end of the recording.
Figure 2-3 Experimental setup for recording of MVRCs from A. mouse Tibialis Anterior (TA) and B. mouse triceps in vivo.

Recordings were performed on either Tibialis Anterior (TA, Figure 2-3A) or Triceps (Figure 2-3B) in vivo. A subset of recordings was performed on soleus in situ. For these recordings the gastrocnemius was dissected and retracted enabling direct stimulation and recording from the soleus (Figure 2-4).

Figure 2-4 Experimental setup for recording of MVRCs from mouse soleus in situ

The signal was amplified (gain 1000, bandwidth 50Hz to 2 kHz) and digitised (NI DAQ) using a sampling rate of 20 kHz. The electrodes were adjusted to obtain a stable negative peak response with a stimulus of 3-10mA. Stimulation and recording were controlled by QTRAC software using the M3REC3.QRP protocol.
Methods

Muscle Velocity Recovery Cycles

Surface temperature over the muscle was measured at the end of the recording either using an infra-red thermometer or by applying a glass thermometer to the skin overlying the muscle.

It took approximately 10 minutes to anaesthetise, position and prepare the mouse for MVRCs (including electrode placement for the first muscle). The MVRC recording itself takes 4 minutes 20 seconds. The frequency ramp recording takes 2 minutes 30 seconds. Performing MVRC recordings on both TA and both triceps of a single mouse took approximately 50 minutes from initiating the anaesthetic.

2.2.5 Muscle Velocity Recovery Cycles in Rats

Surplus female adult wild-type rats were used (weight 320 to 600g). MVRCs were performed as described above for mice with the exception that a larger anaesthetic chamber and nose cone were used.

2.2.6 Muscle Velocity Recovery Cycles in Isolated Muscle

![Image](image_url)

_Figure 2-5 Experimental setup for recording MVRCs ex vivo_

Ex vivo muscle recordings were performed as above except the EDL muscle was first removed from the mouse with tendons intact. EDL was placed in solution (NaCl 118mmol; KCl 2mmol; MgSO$_4$ 1.18mmol; CaCl$_2$ 2.54mmol; NaH$_2$PO$_4$)
Methods

Pharmacological Modulation

1.18mmol; Glucose 10mmol; NaHCO_3) in a warmed tissue chamber (Figure 2-5). The muscle chamber was maintained at 30 degrees Celsius as this was the temperature measured from the surface of EDL in situ prior to dissection using an infra-red thermometer. The solution was constantly bubbled with 95% oxygen 5% carbon dioxide. Needles were inserted as described above: monopolar needle at distal end of muscle, reference anode above and lateral and concentric recording electrode at proximal end (Figure 2-5). I found that it was easier to use a monopolar needle as the anode due to space constraints and the longer length of needle. The ground was attached to a metal screw that was also exposed to solution within the chamber (Figure 2-5). The rest of the equipment setup was unchanged.

2.3 EMG recording from mouse TA

EMG recordings were made using a Viking EMG machine from mouse TA.

2.4 Pharmacological Modulation

Baseline MVRCs and frequency ramp was performed (as described in 2.2) on TA and triceps on one side. After this, intraperitoneal injection of the relevant drug was given with recording needles still in situ. Following injection MVRCs were repeated on the muscle with recording needles in situ before being performed on the contralateral TA and triceps. For this reason, when all recordings were successful there could be more recording post injection than pre injection from the same animal.

2.4.1 9-Anthracene Carboxylic Acid (9AC)

9AC is a ClC-1 channel blocker. I used a dose that has been previously described for in vivo dosing of 9AC in a rat model of myotonia (Desaphy et al., 2013). As per the published paper, a solution equivalent to 2.4 g/l 9AC was prepared fresh each day in distilled water containing 0.3% bicarbonate. The volume of intraperitoneal injection was adjusted to get either 30 mg/kg or 5mg/kg body weight. Recordings were made 10 to 60 minutes post injection.
2.4.2 Barium

Barium is known to block Kir 2.1 channels and has been used to model HypoPP (Gallant, 1983). To investigate the effect of Kir2.1 blockade 250µl of 100μm Barium in 0.9% saline was injected IP. Recordings were made 2 to 30 minutes post dose.

2.4.3 Dantrolene

Dantrolene prevents RyR1 channels opening to release calcium and cause muscle contraction in response to muscle depolarisation. At extremely high doses dantrolene can cause CNS effects such as incoordination. However, the ED$_{50}$ for these effects was 153mg/kg whilst the ED$_{50}$ for muscle relaxation was 10-20mg/kg by intraperitoneal injection (Ellis et al., 1973). To ensure relaxation whilst remaining well below the ED$_{50}$ for muscle incoordination, I applied a dose of 30mg/kg of dantrolene by intraperitoneal injection. Recordings were made 30 to 60 minutes post dose.

2.4.4 Iberiotoxin

Iberiotoxin is a toxin that acts as a BK channel blocker. I used a dose of 1µg in 250µl of 0.9% Saline for intraperitoneal injection recordings were made approximately 2 minutes post intraperitoneal injection.

2.5 Data Analysis

All data was analysed using QtracP software and the M3ANAL8.QPP analysis file. This automates analysis of both MVRCs and 30Hz frequency ramp. As described by Tan et al. after filtering the responses, latencies were measured from the start of the test stimulus to the negative peak of the muscle action potential (S. Veronica Tan et al., 2012; Tan et al., 2014, 2016, 2017). The effects of 1, 2 and 5 conditioning pulses on the latency of the test response were calculated as percentage differences compared with the responses to the test stimulus alone. Only recordings that were clean and where multifibre response was stable e.g., did not become too small to accurately record and did not significantly change shape were included. Where there were multiple recordings from the same muscle the one with the least artefact was chosen.
Statistics

In some cases, MVRCs were recorded where there was no supernormality (Figure 2-6). This pattern occurred in association with clear trauma to the muscle e.g., blood at point of electrode insertion or after contracture or severe clinical myotonia in a Draggen muscle. Usually, repositioning the electrodes away from the site of trauma resulted in a typical MVRC appearance with clear supernormality. When this was not the case, perhaps due to excessive damage of that muscle, the contralateral muscle had the typical MVRC pattern with supernormality. Therefore, MVRCs with no supernormality and increasing subnormality were considered artefactual and excluded from analysis.

Figure 2-6 The two types of MVRC pattern. In this case both are from a young Draggen Mouse. The one on the left from the left TA and the one on the right from the right TA. The recording on the left has the normal MVRC appearance. The recording on the right has no supernormality but increasing subnormality.

2.6 Statistics

Unless otherwise specified the n number refers to the number of muscles on which the assay was performed. To determine statistical significance Welch or Welch rank test was performed depending on normality (Liliefors’s test). When Welch test was performed data are presented as bar graphs of mean ± SEM. When Welch rank test was performed, data are presented as box plots. As MVRCs and frequency ramp analysis involves examining multiple parameters simultaneously an increased threshold for statistical significance of p≤0.01 was applied.

A normality test was first performed on data to check how to proceed. Where there were two groups for comparison that passed the normality test a student’s t-test was performed. This was usually with Welch correction as there were often small differences in group size. When there were two groups, and normality was
Statistics

rejected for one or more either Welch Rank test (MVRC or frequency ramp data) or Mann Whitney U test (other data) was performed.

For three or more groups, when the primary question was to look for difference between groups and not interaction between factors, either a one-way ANOVA or a Kruskal Wallis ANOVA was performed depending on normality. Post hoc Tukey testing was performed when the ANOVA result was significantly different ($p<0.05$ unless otherwise specified). However, if the primary question was whether or not there was interaction between factors a two-way ANOVA was performed.

Finally, when categorical data was classified in two different ways (e.g., specific histological feature observed on histology of either Draggen or WT muscle) a two tailed Fisher’s exact test was used (http://vassarstats.net/) to examine the significance of any association between the two kinds of classification.

All data are represented as mean ± standard error of the mean (SEM) unless otherwise stated. As MVRC and frequency ramp analysis involves examining multiple parameters simultaneously an increased threshold for statistical significance of $p<0.01$ was applied.
3 Species Difference in Muscle Excitability

3.1 Motivation

The primary skeletal muscle channelopathies are rare conditions with an incidence of less than 1 per 100,000 of the population. The gold standard for diagnosis is now genetic testing so muscle biopsy is no longer part of the routine diagnostic work up. This means human tissue is very scarce.

Studying channel biophysics in patient myotubes and heterologous expression of mutated ion channels has enabled significant advances in understanding the pathophysiology of paralytic attacks in skeletal muscle channelopathies (Cannon, H. Brown and Corey, 1991; Cannon and Corey, 1993; Cannon and Strittmatter, 1993; Sokolov, Scheuer and Catterall, 2008; Struyk et al., 2008). However, the exploration of physiological functions and systemic interactions between organs requires a whole organism (Barré-Sinoussi and Montagutelli, 2015). Although genetic PP is caused by single ion channel gene mutation whose expression (apart from in ATS) is limited to skeletal muscle, the PP phenotype shows large intra and inter family variability in severity and changes with age suggesting potential systemic modulation (Gamstorp, 1957; Sternberg et al., 2001; Miller et al., 2004; Chalissery et al., 2018). Moreover, triggering an attack involves changes in serum potassium concentration and therefore is not limited to skeletal muscle alone. Therefore, to start to understand the mechanisms behind phenotype heterogeneity with age or gender a whole organism is necessary in the first instance.

However, translation of findings from animal models for the benefit of human patients has been notoriously poor (Wells, 2015). However, in this same paper, Wells goes on to say that although “...critics have argued that animal experiments are misleading or even of no value. The alternative view is that animal experiments are informative in the majority of cases but only if we conduct them appropriately and analyse them critically with care.” (Wells, 2015) I would agree with this alternative view and suggest that in order to be able to analyse animal derived data ‘critically with care’ we need to understand how the physiology of the model
Species Difference in Muscle Excitability

Motivation

Organism differs from our patients. This understanding is crucial in order to improve data interpretation and translation but also because if we identify pathways that ameliorate or exacerbate the phenotype in model organisms, we may also be discovering novel pathways amenable to therapeutic manipulation in humans or animals with the condition.

This is certainly pertinent to skeletal muscle channelopathies because as discussed in 1.2.9.3, rodents with acquired or genetic models of Hypo PP do not have spontaneous episodes of weakness (1.4.8.1.2, Table 3-1) whilst rodents with Myotonia Congenita (1.4.8.1.3) are more severely affected than humans with the condition. This suggests that species differences in muscle excitability and/or specific ion channel function may protect against spontaneous attacks of paralysis in rodents with periodic paralysis but may exacerbate the phenotype of mice with Myotonia Congenita.

Table 3-1 Paralytic Attacks in the Genetic Mouse Models of Periodic Paralysis

<table>
<thead>
<tr>
<th></th>
<th>Hypo PP I (R528H, CACNA1S)</th>
<th>Hypo PP II (R669H, SCN4A)</th>
<th>Hyper PP (M1592V, SCN4A)</th>
<th>Hyper PP (“Draggen” I582V, SCN4A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous episodes detectable from observing motor behaviour</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Conditions for induced attack in vivo</td>
<td>48 hours oral K binding resin followed by Insulin + Glucose</td>
<td>48 hours oral K binding resin followed by Insulin + Glucose infusion via</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
</tbody>
</table>
### Species Difference in Muscle Excitability

**Aim of the Chapter**

Experiments in this chapter aim to determine if there is a species difference in muscle excitability between humans and mice that might contribute to species differences in skeletal muscle channelopathy phenotype.

<table>
<thead>
<tr>
<th>Conditions for induced attack</th>
<th>central venous catheter</th>
<th>2mM K</th>
<th>2mM K</th>
<th>10mM K &amp; 1.3mM Ca</th>
<th>12mM K</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>in vitro</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Effect of micromolar ouabain</strong></td>
<td>Not tested</td>
<td></td>
<td></td>
<td>Exacerbated loss of force and suppressed recovery from <em>in vitro attack</em> (homozygotes tested)</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Exacerbated loss of force and suppressed recovery from <em>in vitro attack</em> (heterozygotes tested)</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

3.2 **Aim of the Chapter**

Experiments in this chapter aim to determine if there is a species difference in muscle excitability between humans and mice that might contribute to species differences in skeletal muscle channelopathy phenotype.
3.3 Results 1: Comparing Murine and Human MVRCs

3.3.1 Mouse Vs Human MVRCs

The Muscle Velocity Recovery Cycle technique is established in humans (Z’Graggen and Bostock, 2009; Z’Graggen et al., 2010; Werner J. Z’Graggen et al., 2011; W. J. Z’Graggen et al., 2011; Boërio et al., 2012; S. Veronica Tan et al., 2012; Tan et al., 2014, 2017) (see 1.1.1) and has been performed in pigs (Ackermann et al., 2014; Boërio et al., 2018) but has not been reported previously for rodents. Therefore, in collaboration with Dr Veronica Tan and Professor Hugh Bostock I developed this technique in mice to look for species differences in muscle excitability that might explain the phenotype discrepancies in mice with skeletal muscle channelopathies. Dr Tan taught me how to perform MVRCs in mice based on her experience in humans. I performed all the mouse experiments. Dr Tan performed the MVRCs in humans. Professor Bostock wrote the additional protocol for a prolonged MVRC in humans (3.4.4). Dr Tan performed the additional human MVRCs on my muscles (frontalis (3.4.1), orbicularis oculi (3.4.2), prolonged TA recording (3.4.4)).

![Figure 3-1 Comparison of MVRCs from Human and Mouse TA.](image)

A. **Comparison of MVRCs from 1, 2 and 5 conditioning stimuli in human TA (red) and mouse TA (blue)**. B. **Comparison of MVRCs from 1, 2 and 5 conditioning stimuli from human TA (triangles) and mouse TA (circles) in response to 1 (red), 2 (green) and 5 (blue) conditioning stimuli. Human TA n=26 muscles, 10 male, 16 female. Mouse TA n=70 muscles, 34 male, 36 female. Data are mean ± SEM**

The morphology of MVRCs is profoundly different in rodent muscle compared to humans (Figure 3-1). In Humans and pigs, the two species on which MVRCs have been reported
Species Difference in Muscle Excitability

previously, there are two clear phases of supernormality known as early and late supernormality (Z’Graggen and Bostock, 2009; Ackermann et al., 2014; Boërio et al., 2018). Increasing the number of conditioning stimuli predominantly affects the second phase, late supernormality (Z’Graggen and Bostock, 2009; Ackermann et al., 2014; Boërio et al., 2018). In contrast, mouse TA MVRCs have only one distinct phase of supernormality followed by a phase of subnormality and Increasing the number of conditioning stimuli has a small but uniform effect across the MVRC (Figure 3-1).

**Figure 3-2 Differences in Mouse and Human Late Supernormality.**

The late supernormality was defined for human MVRCs as the maximal decrease in latency between 50 and 150ms interstimulus interval. The dot plots values are of the percentage reduction in latency in response to one conditioning stimulus (A) or five conditioning stimuli (B) compared to a test stimulus alone. The red values are for healthy human TA, the blue values are for WT mouse TA. In contrast to the MVRC plots shown in figure 1, for the dot plots, a value greater than 0 reflects an increase in conduction velocity (supernormality), whilst a value less than 0 reflects a reduction in conduction velocity (subnormality). Human TA n=26 muscles, 10 male, 16 female. Mouse TA n=70 muscles, 34 male, 36 female.

There was a significant difference (p=1.782⁻²³) between mouse and human TA latency change during the MVRC interstimulus interval of 50 to 150ms, defined as late supernormality in human MVRCs (Figure 3-2). Similarly, at an inter-stimulus interval of 950ms, mouse TA exhibited slowing in conduction or residual subnormality in response to 1 conditioning stimulus (-0.295 (-0.438, 0.0125 n=64) whereas Human TA exhibits residual
supernormality (0.08 (0.0175, 0.16 n=26)) (figure 3-1, 3-3). Increasing the number of conditioning stimuli increased the significance of difference between mouse and human TA at the 950ms interstimulus interval (p=1.206$^{-8}$ for 1, p=8.481$^{-21}$ for 5).

![Human Vs Mouse TA](image)

**Figure 3-3.** Residual Supernormality in Response to 5 Conditioning Stimuli.

The Residual supernormality was defined for human MVRC as the decrease in latency at 950ms interstimulus interval. The dot plots values are of the percentage reduction in latency in response to 5 conditioning stimuli compared to a test stimulus alone. Like in figure 3-2, a value greater than 0 reflects an increase in conduction velocity (supernormality), whilst a value less than 0 reflects a reduction in conduction velocity (subnormality). The red values are for healthy human TA (data courtesy of Dr Veronika Tan) and the blue values are for WT mouse TA. Human TA n=26 muscles, 10 male, 16 female. Mouse TA n=70 muscles, 34 male, 36 female.

Mouse early supernormality was smaller (p=7.162$^{-20}$) and peaked later (p=1.437$^{-8}$) than human early supernormality (Figure 3-1, 3-4). In human TA, peak early supernormality to a single conditioning stimulus occurred at an inter-stimulus interval of 7.87ms (6.97, 8.67 n=26) and started to decrease between 10 and 15ms. In Mouse TA, peak early supernormality to a single conditioning stimulus occurred at an interstimulus interval of 11ms (8.9, 14 n=63) and remained largely unchanged until 20ms (Figure 3-4). The relative
refractory period was significantly longer in mouse compared to human TA (figure 3-4, p=0.001).

**Human Vs Mouse TA**

Muscle Relative Refractory Period (1CS)

*Figure 3-4 Differences in early supernormality and muscle relative refractory period (MRRP) between human and mouse TA.*

*Human (red) and Mouse (blue) MVRCs in response to 1 conditioning stimulus the dotted line marks the MRRP, defined as the inter-stimulus interval at which conditioned and unconditioned responses have the same latency i.e., 0% change. Human TA n=26 muscles, 10 male, 16 female. Mouse TA n=59 muscles, 29 male, 30 female. Data are mean ± SEM*

### 3.3.2 Mouse Vs Human Frequency Ramp

The murine TA response to a 30Hz frequency ramp was also vastly different from human TA (Figure 3-5). Instead of the increase in conduction velocity (reduction in latency) and increase in response amplitude that is seen in human TA, murine TA exhibited an immediate slowing of conduction velocity that progressed with increasing frequency of stimulation and was associated with a progressive reduction in amplitude of response.
3.4 Results 2: The effect of muscle dimension and fibre type on MVRCs and 30Hz frequency Ramp

In order to try to investigate whether these species’ differences in muscle excitability are the result of the obvious differences in mouse / human muscle geometry I focussed on MVRCs and compared MVRCs from selected mouse and human muscles (see Table 3-2), where relevant I also compared 30 Hz frequency ramp morphology.

Muscles had to be accessible and clearly identifiable to be sure the recording was from the correct muscle. I then chose suitable muscles that represented the extremes of the categories in table 3-2.
Species Difference in Muscle Excitability  

Muscle depth or physiological cross-sectional area

Table 3-2 Dimensions of muscles selected for MVRC recording (Henriksson-Larsen, 1985; Freilinger et al., 1990; Augusto, Padovani and Campos, 2004; Eng et al., 2008; Mathewson et al., 2012; Kammoun et al., 2014; Charles et al., 2016). TA = Tibialis Anterior. PCSA = the area of the cross section of a muscle perpendicular to its fibres at its largest point. nk = not known (no published data found).

<table>
<thead>
<tr>
<th></th>
<th>Human TA</th>
<th>Human Frontalis</th>
<th>Human Orbicularis Occuli</th>
<th>Mouse TA</th>
<th>Mouse Triceps (long head)</th>
<th>Mouse Soleus</th>
<th>Mouse EDL</th>
<th>Rat TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Fibre diameter (µm)</td>
<td>60.2</td>
<td>39.8</td>
<td>31.8</td>
<td>55</td>
<td>nk</td>
<td>45.47</td>
<td>nk</td>
<td>nk</td>
</tr>
<tr>
<td>Fibre length (cm)</td>
<td>7.25</td>
<td>4.7</td>
<td>6.5</td>
<td>0.79</td>
<td>0.532</td>
<td>0.443</td>
<td>0.504</td>
<td>1.64</td>
</tr>
<tr>
<td>Muscle length (cm)</td>
<td>28.4</td>
<td>4.7</td>
<td>6.5</td>
<td>1.29</td>
<td>1.093</td>
<td>0.923</td>
<td>1.11</td>
<td>2.91</td>
</tr>
<tr>
<td>Muscle depth (cm)</td>
<td>2.6</td>
<td>0.5</td>
<td>nk</td>
<td>nk</td>
<td>nk</td>
<td>nk</td>
<td>nk</td>
<td>nk</td>
</tr>
<tr>
<td>Muscle mass (mg)</td>
<td>nk</td>
<td>nk</td>
<td>nk</td>
<td>45.2</td>
<td>103.65</td>
<td>7.89</td>
<td>8.23</td>
<td>662</td>
</tr>
<tr>
<td>Physiological Cross-Sectional Area (PCSA, mm²)</td>
<td>1900</td>
<td>nk</td>
<td>nk</td>
<td>5.3</td>
<td>15.49</td>
<td>1.42</td>
<td>0.181</td>
<td>380</td>
</tr>
</tbody>
</table>

3.4.1 Muscle depth or physiological cross-sectional area

Muscle fibre diameter is reported to be relatively constant across species (Paul, 2001). In keeping with this, the fibre diameter in mouse and human TA is similar. However, the dimensions of the muscles are obviously very different (see table 3-2). The thinnest accessible human muscle I identified was frontalis at 0.5 cm thick (Costin et al., 2015). To see if depth of muscle might play a role in the mouse human differences, Dr Veronica Tan performed muscle excitability recordings on my frontalis (2.2.3).

The MVRC recording from my frontalis was similar to my TA in morphology but the amplitude of early and late supernormality was reduced (Figure 3-6). However, although supernormality was smaller, human frontalis MVRCs still has two distinct phases of supernormality and no subnormality (Figure 3-6).
Species Difference in Muscle Excitability  Muscle depth or physiological cross-sectional area

A. Human TA Vs Frontalis (1CS)

B. Human TA Vs Frontalis (5CS)

Figure 3-6  Comparison of MVRCs recorded from my TA and Frontalis muscle.

A. MVRCs in response to 1 conditioning stimulus.  Red is the response from my TA and grey is the response from my frontalis. B. MVRCs in response to 5 conditioning stimuli.  Red is the response from my TA and grey is the response from my frontalis.

It is difficult to identify accessible muscles that differ in one dimension only. Hence, as well as being thinner, human frontalis is also shorter and has a greater percentage of fast twitch fibres than human TA (see table 3-2). Therefore, to try to see if alteration in the extent of early supernormality was associated with muscle depth, rather than fibre-type or length, I identified two accessible muscles (mouse TA and mouse Triceps) of similar length and fibre-type composition but a threefold difference in physiological cross-sectional area and mass (see table 3-2).

Physiological cross-sectional area is defined as the cross section of a muscle perpendicular to its fibres at its largest point. The reason for the discrepancy in measurement unit was that I could not find a published value for physiological-cross sectional area in human frontalis, but it is commonly used in studies of mouse muscle architecture where depth is usually not recorded (Eng et al., 2008; Mathewson et al., 2012; Charles et al., 2016).

Therefore, I developed a technique to perform MVRC on mouse triceps brachi (methods 2.2.2) and compared this to recordings from mouse TA (Figure 3-7).
Species Difference in Muscle Excitability

Fibre-type Differences

A. Mouse TA Vs Triceps (1CS)

B. Mouse TA Vs Triceps (5CS)

Figure 3-7. Comparison of MVRCs recorded from mouse TA and mouse Triceps.

A. MVRCs of mouse triceps (grey) and TA (blue) in response to 1 conditioning stimulus. B. MVRCs of mouse triceps (grey) and TA (blue) in response to 5 conditioning stimuli. Triceps, n=30 muscles, 20 male, 10 female. TA, n=70 muscles, 34 male, 36 female. Data are mean ± SEM.

Mouse triceps MVRCs were significantly different from mouse TA MVRCs. Differences included the inter-stimulus interval at which maximal early supernormality occurred (p=5.125⁻⁶, figure 3-7), the MRRP (p=0.00125, figure 3-7) and early supernormality in response to 5 conditioning stimuli (p=0.00364). However, there was no significant difference between ‘late supernormality’ measurements (actually measuring degree of subnormality) to either 1 (p=0.02618) or 5 (p=0.8339, figure 3-7) conditioning stimuli.

Tripling physiological cross-sectional area and mass did not result in the appearance of late supernormality nor reduce late subnormality on mouse MVRCs, in fact, the onset and extent of subnormality was remarkably similar for mouse TA and Triceps (Figure 3-7).

3.4.2 Fibre-type Differences

Table 3-3 Fibre-type proportions of muscles examined using MVRCs

<table>
<thead>
<tr>
<th></th>
<th>Human Tibialis Anterior 121314</th>
<th>Human Frontalis 15</th>
<th>Human Orbicularis Occuli 16</th>
<th>Mouse Tibialis Anterior 1718</th>
<th>Mouse Triceps (long head) 19</th>
<th>Mouse Soleus 18 20</th>
<th>Mouse EDL</th>
<th>Rat Tibialis Anterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Slow oxidative (I)</td>
<td>50-75% (depending on depth)</td>
<td>57%</td>
<td>15%</td>
<td>0</td>
<td>5</td>
<td>37</td>
<td>0.44</td>
<td>0.43</td>
</tr>
<tr>
<td>% Fast Oxidative (IIa)</td>
<td>nk</td>
<td>nk</td>
<td>nk</td>
<td>9(4)</td>
<td>15</td>
<td>54</td>
<td>4.01</td>
<td>10.13</td>
</tr>
<tr>
<td>% Fast Glycolytic (IIb)</td>
<td>nk (0)</td>
<td>nk (0)</td>
<td>nk (0)</td>
<td>91(56)</td>
<td>80 (58)</td>
<td>9 (0)</td>
<td>95.52 (66.01)</td>
<td>89.44 (69.75)</td>
</tr>
</tbody>
</table>
Other than the obvious difference in muscle geometry, there is a stark difference in fibre type proportions between mice and humans (Table 3-3). Mice have a far higher proportion of fast twitch glycolytic fibres than humans and they also have myosin heavy chain isoform type IIb fibres which are not present in human muscle. Therefore, to see if fibre type differences could explain differences in MVRC morphology, I compared MVRC recordings from human orbicularis oculi, which is 85% fast twitch, with human TA, whose fibre type proportion depends on the depth of recording and ranges from 10 to 50% fast twitch (See Table 3-3 and Figure 3-8A, B). I then compared mouse soleus which is 63% fast twitch oxidative and has no type IIb fibres, with mouse TA which is 99% fast twitch and predominantly type IIb (see Table 3-3 and Figure 3-8C, D).

A. Human TA Vs Orbicularis Oculi (1CS)

B. Human TA Vs Orbicularis Oculi (5CS)

C. Mouse TA Vs Soleus (1CS)

D. Mouse TA Vs Soleus (5CS)

Figure 3-8 Comparison of MVRCs recorded from fast and slow twitch human and murine muscles

A. MVRCs in response to 1 conditioning stimulus from my orbicularis oculi (grey, n=1) and my TA (red, n=1). B. MVRCs in response to 5 conditioning stimuli from my orbicularis oculi (grey, n=1) and my TA (red, n=1). C. Mouse MVRCs in response to 1 conditioning stimulus recorded in situ from the soleus(grey, n=4 female muscles) and in vivo from TA(blue, n=3 female muscles) of the same animal. D. Mouse MVRCs in response to 5 conditioning stimuli recorded in situ from the soleus(grey, n=4 female muscles) and in vivo from TA(blue, n=3 female muscles) of the same animal. In C & D data are mean ± SEM
Species Difference in Muscle Excitability  
Fibre-type Differences

As mouse soleus muscle is deep to the gastrocnemius and does not induce a specific, easily identifiable movement on contraction, it was difficult to be certain I was recording from soleus in vivo. For this reason, the soleus recording was performed by dissecting back the gastrocnemius and directly recording and stimulating from the soleus muscle in situ. The soleus recordings are compared to the TA recordings from the same animal (n=3). To ensure any differences were physiological rather than result of dissecting and exposing the muscle, soleus in situ recordings are compared with Mouse Extensor Digitorum Longus (EDL) ex vivo recordings (Figure. 3-9). In contrast to mouse soleus, mouse EDL is 99.5% fast twitch and predominantly type IIb fibres (table 3-3).

My orbicularis oculi and my TA had a similar muscle relative refractory period (Figure. 3-8A). The orbicularis oculi supernormality is smaller; however, like human TA it has two distinct phases of supernormality and no subnormality (Figure. 3-8A, B).

![Figure 3-9 Comparison of MVRCs recorded from mouse EDL ex vivo and mouse soleus in situ.](image)

A. Mouse EDL Vs Soleus (1CS)  
B. Mouse EDL Vs Soleus (5CS)

The mouse soleus recordings had a longer muscle relative refractory period than TA of the same animal (Figure. 3-8C). The alteration in relative refractory period was not seen in mouse EDL that was dissected and recorded from ex vivo (figure 3-9A). This suggests the difference reflects the reported physiological difference in resting membrane potential (Ruff
and Whittlesey, 1993b, 1993a; Ruff, 1996) between slow and fast twitch muscles, rather than the fact that the soleus muscle was directly exposed to record in situ. Despite the difference in muscle relative refractory period, the overall morphology of the MVRC recordings was similar. Mouse soleus, like mouse TA has only one phase of supernormality and exhibits late subnormality. This suggests that the lack of late supernormality and presence of late subnormality on mouse MVRC is not due to species’ differences in fibre type.

**Figure 3-10** Comparison of 30Hz frequency ramp recordings from mouse TA and soleus.

*Soleus in situ (grey, n=3 muscles) and TA (blue, n=3 muscles) in vivo recordings from the same animal. Data are mean ± SEM*

Mouse soleus frequency ramp latency was similar to mouse TA and distinct from human muscle. There was a progressive increase in latency with increasing frequencies of stimulation and in 2 out of the three animals this was associated with a reduction in response amplitude (Figure. 3-10). Therefore, the progressive increase in latency during the frequency ramp cannot be attributed to the presence of type IIb fibres in rat and mouse muscle.
3.4.3 Gait speed

The speed and magnitude of murine muscle movements are obviously different from human ones. The stride frequency of a mouse at the ‘trot-gallop’ transition is over 400 strides per minute (Heglund, Taylor and McMahon, 1974). This is in contrast with larger mammals such as dogs (approximately 200 strides per minute), horses (approximately 100 strides per minute) (Heglund, Taylor and McMahon, 1974) and human walk-run transition at 70 strides per minute (Hansen et al., 2017). Rats have a trot-gallop transition of approximately 270 strides per minute (Heglund, Taylor and McMahon, 1974), this is much slower than mouse but still faster than dog and much faster than human. Therefore, the difference between human and murine MVRCs may reflect a necessary functional adaption to a higher stride frequency. If so rat MVRCs, despite a muscle length approaching frontalis (table 2) and a mass 8 times greater than mouse TA might have a similar morphology to mouse TA MVRCs because of the functional requirements of rodent muscle.

![Figure 3-11 Comparison of MVRCs recorded from mouse TA and rat TA.](image)

**A. Mouse TA Vs Rat TA (1CS)**  
**B. Mouse TA Vs Rat TA (5CS)**

Rat TA, exhibited greater supernormality that was slightly more prolonged, however, like mouse TA it had only one clear phase of supernormality (Figure 3-11). Rat TA also had late subnormality in response to 5 conditioning stimuli although this was much smaller than that seen in mouse TA (Figure 3-11B). Rat TA frequency ramp resembled mouse TA (Figure 3-12).
Species Difference in Muscle Excitability

Twitch speed and speed of Muscle Velocity Recovery Cycle

### Figure 3-12 Comparison of Rat Vs Mouse Frequency Ramp.

*Rat TA (grey, n=4 female muscles) and mouse TA (blue, n=59 muscles (29 male, 30 female).*

*Data are mean ± SEM*

However, the MRRP of Rat TA was shorter than mouse TA (figure 3-11A) and in fact was similar to human TA. This suggests that the factors behind the increased MRRP in mouse are unlikely to be responsible for either the lack of clear late supernormality or the progressive increase in latency during 30Hz frequency ramp.

### 3.4.4 Twitch speed and speed of Muscle Velocity Recovery Cycle

Mouse twitch speed is approximately twice as fast as rat and 6 times as fast as human twitch speed (Marsden and Meadows, 1970; Wetzel and Gros, 1998). In contrast to human TA, Rat and mouse TA MVRCs have only one clear phase of supernormality, however it appears prolonged compared to human early supernormality (Figure. 3-1, 3-4). Therefore, I hypothesised, that rodent MVRCs may simply be faster such that early and late supernormality merge and are more difficult to differentiate. If this were the case human MVRCs may also exhibit some subnormality if the recording was prolonged. To test this Dr Veronica Tan did a prolonged MVRC recording on my TA using a specific protocol kindly
Species Difference in Muscle Excitability    
Twitch speed and speed of Muscle Velocity
Recovery Cycle

provided by Professor Hugh Bostock. The response to a test stimulus was measured for up to 3 seconds after the conditioning stimuli (as opposed to the standard 1 second protocol). Assuming morphology remained in proportion, at this point subnormality would have started in mouse muscle. However, even in extended recording no late subnormality was seen on human MVRCs (Figure 3-13).

Prolonged Human TA MVRC recording

![Prolonged Human TA MVRC recording](image)

Figure 3-13 Prolonged MVRC Recording from human TA.

In this recording the response to a test stimulus was measured for up to 3 seconds after the conditioning stimulus (increased from 1 second in the standard MVRC protocol). The traces shown are from the raw recording. A. Latency (ms) from test stimulus to peak of Compound Muscle Action Potential (CMAP) response. Black is for test stimulus alone, burgundy for 1 conditioning stimulus, green for 2 conditioning stimuli and bright red for 5 conditioning stimuli. The decrease in interstimulus interval is shown in B as the delay between conditioning and test stimulus. In the raw recording, the interstimulus intervals are plotted from the longest on the left to shortest on the right. Thus, early supernormality (rectangle) and muscle relative refractory period (arrow) are seen on the right of the trace. At no point do the conditioned responses (burgundy, green, red) have a longer latency than the unconditioned response (black). Therefore, there is no subnormality.
Species Difference in Muscle Excitability

However, if you plot the duration of supernormality against the stride frequency at the trot-gallop transition for those species in which MVRCs have been performed, there is a significant correlation.

3.4.5 Ex vivo muscle excitability

A paralytic attack can be easily induced in muscle from mice with periodic paralysis ex vivo but spontaneous attacks of paralysis have not been observed in vivo (table 3-1). It seems likely that this is because more extreme changes in serum potassium are necessary to trigger an attack of paralysis in mice compared to humans. However, it could also be that muscle excitability is altered in mice ex vivo making the muscle more susceptible to paralysis.

The traces for EDL recordings performed ex vivo were already shown as a comparison to soleus in situ in Figure. 3-9. The morphology of EDL ex vivo looks similar to mouse TA and mouse Triceps. There was no significant difference in MRRP (p=0.015, 4), but early supernormality to 1 and 5 conditioning stimuli were significantly greater in EDL ex vivo compared to TA in vivo (p=8.830^-6 and p=0.0002 respectively ). However, there was no significant difference in late supernormality to 1 or 5 conditioning stimuli (p=0.25851).

In several of the published periodic paralysis mouse papers, muscle is left to acclimatise ex vivo for up to 1 hour before the potassium assay is started. Therefore, I looked to see if the MVRCs changed over this time period. The MVRCs remained stable for over 90 minutes post dissection (Figure. 3-14). It is also possible that oxidative capability is compromised in vitro, and therefore I hypothesised that repeated MVRCs may cause metabolic stress which might manifest in the MVRCs by the development of late supernormality suggesting impaired t-tubule potassium clearance and/or alteration of the muscle relative refractory period suggesting depolarisation of resting membrane potential. However, repeating MVRCs up to 4 times in a row had no discernible effect on the MVRCs either immediately after dissection or at 90 minutes post dissection (Figure. 3-14 B).
Species Difference in Muscle Excitability

Chloride Buffering: ClC-1 conductance

Figure 3-14 Ex vivo MVRCs remain stable for up to 90 minutes post dissection.

A. MVRCs in response to 1, 2 and 5 conditioning stimuli of same EDL muscle immediately following dissection (black) and 90 minutes following dissection (red). B. Repeated MVRCs from the same isolated EDL muscle started 90 minutes after dissection.

3.5 Results 3: MVRCs and Selective Pharmacological Blockade

Given the similar appearances of rat and mouse muscle MVRCs and frequency ramp, despite the difference in their dimensions, it raises the question as to whether the species’ difference in muscle excitability could be due to differences in the functional expression of ion channels and pumps to facilitate different functional requirements associated with different stride frequencies. As described above, late supernormality is attributed to progressive potassium accumulation in the t-tubules following muscle activity (Z’Graggen and Bostock, 2009). Therefore, the apparent lack of LSN in mouse muscle could be because, less potassium is released, potassium is cleared more rapidly from the t-tubules, or potassium is buffered more effectively by an increased chloride conductance.

3.5.1 Chloride Buffering: CIC-1 conductance

Assessing the contribution of CIC-1 has the advantage that an in vivo model of Myotonia Congenita (Desaphy et al., 2013) using 9 Anthracene Carboxylic Acid (9AC) to block CIC-1 has been developed. This means there is an established intraperitoneal dose of 9AC for CIC-1 blockade according to weight. Moreover, CIC-1 blockade would not be predicted to
Species Difference in Muscle Excitability

Chloride Buffering: ClC-

1 conductance depolarise the muscle. This combination made ClC-1 an attractive channel to start with to determine whether MVRCs could be used in combination with specific ion channel blockade to investigate ion channel function in vivo.

The in vivo model of Myotonia Congenita was developed in rats. Therefore, I performed a species dose conversion for use in mice (Nair and Jacob, 2016), this resulted in doses of approximately 5mg/kg. This dose did not create two clear phases of supernormality as is seen in humans; however, it did postpone the onset of subnormality (Figure 3-15A) and was sufficient to induce myotonia in the mouse (Figure 3-15B).

![Figure 3-15](image)

**A.** 5mg/kg 9AC response to 1 conditioning stimulus

**B.** Myotonia induced by 9AC

To see if more complete blockade of ClC-1 could induce late supernormality, I used the rat dose of 9AC on mouse muscle. Using 30mg/kg 9AC in mouse TA removed subnormality and induced late supernormality (Figure 3-16). It also augmented the effect of increased conditioning stimuli (figure 3-16B). Whilst 9AC had no significant effect on MRRP (Figure 3-16A), it did increase early supernormality to 5 conditioning stimuli (p=0.0001, Figure 3-16B). More importantly, as hypothesised, it reduced and delayed late subnormality and instead induced late supernormality to one conditioning stimulus (p=0.002) as well as to 5 conditioning stimuli (p=0.007, Figure 3-16A,B) compared to the same muscles prior to 9AC exposure.

Thus, whilst partial chloride channel blockade was sufficient to induce electrical myotonia and postpone the onset of subnormality it did not result in two phases of supernormality (Figure 3-15). In contrast, more complete chloride channel blockade by increased doses of
Species Difference in Muscle Excitability  Chloride Buffering: CIC-1 conductance

9AC resulted in clear late supernormality that made mouse TA MVRC look human-like (Figure 3-16).

However, increased CIC-1 activity does not account for species’ differences in frequency ramp as blockade of CIC-1 by 9AC exacerbated the increase in latency and drop in response amplitude seen in mouse TA (Figure 3-16C). This is consistent with the report that the amplitude of response during the 30Hz frequency ramp fell quicker in humans with recessive Myotonia Congenita than healthy controls (Tan et al., 2014). Therefore, increased CIC-1 conductance does not explain the appearances of the murine 30Hz frequency ramp.

A. Mouse TA pre 9AC Vs post 9AC (1CS)

B. Mouse TA pre 9AC Vs post 9AC (5CS)

C. Mouse TA pre 9AC Vs post 9AC

Figure 3-16 A: The effect of 30mg/kg 9 Anthracene carboxylic acid (9AC) a skeletal muscle voltage gated chloride channel (CIC-1) blocker on mouse TA MVRCs and 30Hz Frequency ramp.
Species Difference in Muscle Excitability

3.5.2 Potassium clearance from the t-tubules

The fact that induction of clear late supernormality on MVRC required doses of 9AC approximately 6 times greater than that required to induce myotonia suggests that other channels may have compensated for impaired ClC-1 function at lower doses of 9AC. In addition to increased chloride channel activity, more rapid and complete K$^+$ reuptake from mouse t-tubules would be predicted to reduce or prevent late supernormality. There are two proteins that are known to play a key role in t-tubule potassium reuptake. These are the t-tubule Na K ATPase and the inwardly rectifying potassium channel Kir2.1 (DiFranco, Hakimjavadi, et al., 2015; DiFranco, Yu, et al., 2015).

3.5.2.1 The t-tubule Na K ATPase

There are two isoforms of the sodium potassium ATPase in skeletal muscle. These are encoded by the genes ATP1A1 and ATP1A2. ATP1A1 is located at the plasmalemma and is critical for maintaining resting membrane potential (Radzyukevich et al., 2013). ATP1A2 is located in the t-tubules, its activity is triggered by high extracellular potassium and it contributes to reuptake of t-tubule potassium following muscle activity (DiFranco, Hakimjavadi, et al., 2015). It is not active at rest and provides a minimal contribution to maintenance of resting membrane potential (DiFranco, Hakimjavadi, et al., 2015). Isoform specific block of the t-tubule Na K ATPase can be achieved with micromolar ouabain (DiFranco, Hakimjavadi, et al., 2015). Micromolar ouabain has also been shown to exacerbate induced attacks in Hypo PP II and M1592V Hyper PP mouse models (Table 3-1) (Hayward et al., 2008; Wu et al., 2011). This made it an attractive target to see if it might also contribute to the lack of supernormality in mouse MVRC.

There is no established in vivo dose for blockade of the t-tubule Na K ATPase. Therefore, I tried intraperitoneal injection of 250µl of 10µm Ouabain as this was the dose that was reported to block at least 95% of pumps in vitro/ex vivo without causing membrane depolarisation (DiFranco, Hakimjavadi, et al., 2015). In three of fourteen muscles (8 male, 6
female), this resulted in the disappearance of late subnormality and the appearance of clear late supernormality (see 4.9.3.3). I never observed this pattern in the absence of pharmacological blockade.

### A. Repeated MVRCs pre ouabain

![Graph showing MVRCs before ouabain injection.]

### B. Repeated MVRCs post ouabain

![Graph showing MVRCs after ouabain injection.]

**Figure 3-17** The effect of 10µm ouabain injected intraperitoneally on mouse TA MVRCs.

**A.** Raw recording demonstrating repeated MVRC in normal mouse TA without any evident change in MVRC morphology. **B.** After 250µl of 10µm IP ouabain the first MVRC had the normal mouse TA morphology with late subnormality (filled arrow), however, in the second MVRC subnormality was reduced and by the third MVRC there was clear late supernormality (empty arrow).

On the first occasion that ouabain induced late supernormality the muscle had continued baseline stimulation for approximately 10 minutes post dose. When the effect was not replicated in the four subsequent experiments, I reasoned it might have been due to ongoing activity unmasking the pump impairment. Therefore, I looked to see if repetitive MVRC would bring out the deficit.

In muscle that had not been exposed to ouabain, MVRC could be repeated continuously - I performed 9 MVRC in a row with minimal change in its morphology (Figure. 3-17A). On the
first occasion I tried repetitive MVRC in muscle exposed to ouabain, clear late supernormality appeared on the third MVRC (Figure. 3-17 B unfilled arrow). However, the ouabain effect was not consistent. In approximately 60% of the animals there was no clear effect on late supernormality after ouabain and in approximately 15%, ouabain appeared to induce some depolarisation as evidenced by reduced supernormality. This variation is most likely because I did not have an established dose by weight for in vivo administration of ouabain to selectively block the t-tubule Na K pump. Therefore, in some of the animals, the ouabain dose may have been sufficient to block the sarcolemmal Na K pump causing some membrane depolarisation whilst in others it was more selective for the t-tubule Na K pump. This may be due to differences in body weight between animals and the fact that animals of both genders were used (see 4.9.1.3).

However, although not observed in all animals tested, the appearance of two clear phases of supernormality in 3 out of 14 WT mouse TA that had been exposed to ouabain compared to 0 out of 70 WT mouse TA in the absence of ouabain was significant (p= 0.007, two-tailed fisher exact test) and suggests the effect may be real. That ouabain-induced late supernormality required multiple consecutive MVRCs to appear provides some evidence that late supernormality is due to activity induced potassium accumulation in the t-tubules.

**Frequency ramp pre and post ouabain**
Species Difference in Muscle Excitability Potassium clearance from the t-tubules: The Inward Rectifier Potassium Channel (Kir2.1)

* muscles). Red = response to mouse TA to 30Hz frequency ramp after single MVRC cycle all in the presence of micromolar ouabain (n=3 muscles). Data are mean ± SEM

The evidence from MVRC work of increased K⁺ reuptake and chloride conductance in mouse muscle compared to humans is perhaps surprising given the appearance of the mouse TA frequency ramp (Figure. 3-5). Given the published observations that ouabain exacerbates induced paralytic attacks in mouse models of periodic paralysis (Hayward et al., 2008; Wu et al., 2011) and my observations of its effects on multiple but not single MVRCs, I chose to explore the effect of ouabain on mouse frequency ramp. In the presence of ouabain, the amplitude of response dropped more quickly and to a greater extent (Figure. 3-18). The amplitude of response at 15Hz was significantly smaller for mouse TA in the presence of ouabain (p=0.01, n=3).

Therefore, increased t-tubule Na K ATPase activity could help explain the species’ difference in appearance of MVRCs but does not explain the species difference in 30Hz frequency ramp. The fact that ouabain did not induce clear late supernormality in all animals tested, suggests that either mice have other compensatory mechanisms that are able to clear potassium when t-tubule Na K ATPase function is impaired and/or the dose of ouabain was not sufficient for each animal. The frequency ramp findings would argue against the latter.

In order to try to confirm this, I attempted dose titration of ouabain on ex vivo MVRCs by attempting to expose the same muscle to 0, 1, 3, 10, 30 and 100 µM ouabain and repeating MVRCs at each concentration after 2 minutes equilibration. Unfortunately, the change of solution often caused muscles to move and be damaged by the electrodes and therefore, it was not possible to confidently distinguish technical artefact from physiological results. To obtain valid data, I would have required additional muscles, ideally from older animals with larger muscles and I was not able to pursue this due to limits on time and older animals.

3.5.3 Potassium clearance from the t-tubules: The Inward Rectifier Potassium Channel (Kir2.1)

The inward rectifier potassium channel Kir2.1 is known to contribute to potassium reuptake from skeletal muscle t-tubules during muscle activity (Difranco et al., 2015). Kir 2.1 channel behaviour is responsible for the bistable nature of skeletal muscle membrane potential in response to hypokalaemia (1.1.6). Mutations in Kir2.1 channels cause Andersen Tawil
Species Difference in Muscle Excitability Potassium clearance from the t-tubules: The Inward Rectifier Potassium Channel (Kir2.1)

Syndrome a form of periodic paralysis and Kir2.1 blockade as a result of barium toxicity causes a Hypo PP-like attack. Therefore, species’ differences in the functional expression of Kir2.1 channels would be an excellent candidate to explain mouse-human differences in MVRCs and potentially the resistance to spontaneous attacks in transgenic mice with Hypo PP.

Barium is a commonly used blocker of Kir 2.1. It has been used extensively in heterologously expressed cells as well as on muscle ex vivo. However, as for ouabain, I could find no report of an in vivo dose of Barium that would block Kir2.1 without depolarising the muscle. Therefore, I started by injecting a dose (100µm) reported to block Kir2.1 in whole muscle ex vivo without causing muscle depolarisation (Gallant, 1983) and injected 250µl intraperitoneally. This dose did not result in depolarisation as indicated by the lack of prolongation of MRRP (Figure. 3-19).

Barium had minimal effect on MVRCs in response to one conditioning stimulus (Figure. 3-19A), however, it did appear to increase supernormality as well as delaying the onset and reducing the amplitude of subnormality in response to 5 conditioning stimuli (Figure. 3-19B). On one occasion IP injection of 6µg Barium in 2 divided doses 30 minutes apart in a 19-week-old male WT mouse of 27g resulted in the appearance of clear late supernormality, absence of subnormality and some residual supernormality when the recording was performed within 2 to 3 minutes of the second IP injection. However, on further experiments, this was not replicated. This was despite increasing the dose to an equivalent dose per gram of body weight.

It was not possible to simply increase the dose of barium further for three reasons. Firstly, Kir 2.1 contributes to resting membrane potential and therefore excess block would result in muscle depolarisation which would itself affect MVRCs. Secondly, Kir2.1 channels are also present in cardiac muscle and excessive blockade could have caused fatal cardiac arrhythmia and thirdly in higher doses Barium loses its Kir2.1 specificity. In an attempt to bypass cardiac toxicity, I tried to look at the effect of Barium ex vivo, but as for ouabain was hampered by technical difficulty. The fact that Barium did induce clear late supernormality in one of the animals tested suggests that Kir2.1 blockade may play a role in t-tubule potassium clearance in the mouse. However, as for ouabain, it was difficult to get
consistent results as I did not have an established dose/weight to administer.

A. Mouse TA _pre_ Vs _post_ Barium (1CS)  
B. Mouse TA _pre_ Vs _post_ Barium (5CS)  

C. Mouse TA _pre_ Vs _post_ Barium

*Figure 3-19 The effect of Barium on mouse TA MVRC*  
A. MVRCs in response to 1 conditioning stimulus in mouse TA _pre_ (blue) and 2 to 15 minutes post 250µl of 100µm Barium IP (grey).  
B. MVRCs in response to 5 conditioning stimuli in mouse TA _pre_ (blue) and 2-15 minutes post 250µl of 100µm Barium IP (grey).  
C. 30 Hz frequency ramp in mouse TA _pre_ (blue) and 5 to 18 minutes post 250µl of 100µm Barium IP (grey). Blue, _n=4_ male muscles, grey, _n=5_ male muscles. Data are mean ± SEM
As for ClC-1 and the t-tubule Na K ATPase an increase in Kir2.1 activity does not seem to explain the species difference in frequency ramp as blocking Kir2.1 exacerbated the drop in amplitude whilst the progressive increase in latency was unaltered (Figure. 3-19C).

### 3.6 Summary of findings

Mouse MVRCs exhibit a single, prolonged phase of supernormality. Moreover, and in contrast to human and pig MVRCs, mouse MVRCs exhibit pronounced late subnormality. The response of mouse muscle to 30Hz frequency ramp is one of progressive increase in latency and reduction in the amplitude of response. This contrasts with human muscle’s response to 30Hz frequency ramp.

My data suggests that increased activity of ClC-1, Kir2.1 and the t-tubule Na K ATPase may contribute to the species’ difference in the appearance of MVRCs. However, the reason for the differences on frequency ramp remains to be determined.

### 3.7 Discussion

#### 3.7.1 MVRCs are technically feasible in rodent muscle

MVRCs can be performed *in vivo* in mice and rats (Figure. 3-1, 3-11). They can also be performed *ex vivo* on explanted mouse muscle (Figure. 3-9, 3-14). Combining MVRC with pharmacological blockade of ion channels offers a mechanism by which the effect of ion channel dysfunction on muscle excitability can be examined. This makes it a unique tool in the investigation of ion channel function and exceptionally suited to investigate species difference in muscle excitability as the technique has already been established in humans and pigs. Developing the MVRC technique for use in mice is a particularly useful experimental tool given the large number of transgenic mice lacking specific ion channels.

#### 3.7.2 Mouse muscle excitability is distinct from humans and pigs.

##### 3.7.2.1 MVRCs

The factors contributing to early and late supernormality in humans and pigs – the depolarising after potential and potassium accumulation in the t-tubule – appear to merge into one period of supernormality which is of shorter duration in mice. This is followed by a period of subnormality that is not seen in humans (Figure. 3-1) or pigs (Ackermann *et al.*, 2014; Boërio *et al.*, 2018).
The depolarising after potential which is believed to underlie supernormality is present in mouse muscle (Juel, 1988). The ‘late depolarising after potential’ is caused by potassium accumulation in t-tubules after trains of action potentials (Freygang, Goldstein and Hellam, 1964). The lack of clear late supernormality in mouse muscle suggests potassium may not accumulate in the same way, to the same extent and/or is more effectively buffered in the t-tubules than in humans or pig skeletal muscle.

This observation is supported by our pharmacological studies. Complete ClC-1 blockade with 30mg/kg doses of 9AC removes subnormality and induces late supernormality making mouse TA look more like human TA MVRCs (Figure. 3-16). However, this dose is more than that required to produce myotonia – a phenomenon associated with the late depolarising after potential and potassium accumulation in t-tubules (Adrian and Bryant, 1974; Cannon and Corey, 1993). In fact, 5mg/kg 9AC is enough to induce myotonia in mouse muscle. MVRCs performed after an IP dose of 5mg/kg dose 9AC do not exhibit any late supernormality but do have delayed onset of subnormality (Figure 3-15A). This suggests that the timely onset of subnormality in mouse MVRCs is a key physiological requirement for normal muscle relaxation. The appearance of subnormality on MVRCs appears to be specific to rodents as it was not observed in human muscle even with prolonged recordings (Figure 3-13). Given its association with myotonia, the delayed onset of subnormality may be the mouse equivalent to increased residual supernormality (950ms) that is seen on MVRCs in humans with myotonia (Tan et al., 2014, 2018, 2020).

Kir 2.1 and Na K pump blockade produced human-like MVRCs with clear late supernormality on occasion, but this effect was not consistent. Kir 2.1 activity is a particularly good candidate to contribute to subnormality as Kir channels are reported to ‘amplify hyperpolarisation induced by other ion channels and transporters’ in other tissues such as the vascular endothelium (Jackson, 2017). It is likely therefore that they amplify the hyperpolarisation induced by the t-tubule Na K pump.

The inconsistency in inducing late supernormality with barium and ouabain is likely due to a) a lack of an established in vivo dose for skeletal muscle effects b) possibility of systemic toxicity and c) the fact that in contrast to 9AC, both Barium and ouabain will cause muscle depolarisation if dose is sufficient. The ex vivo technique would facilitate accurate dose
titration to maximise channel block without excess membrane depolarisation. Unfortunately, this was not possible to complete within the time scale available to me.

Thus, the appearance of mouse MVRCs and the results from MVRCs combined with specific ion channel blockade provide evidence that potassium is more effectively buffered in mouse t-tubules by an increased chloride conductance. There is also a suggestion that K\(^+\) is more rapidly cleared by both increased Kir2.1 and t-tubule Na K ATPase activity. However, this requires more definitive evidence to confirm it.

### 3.7.2.2 30Hz Frequency Ramp

I did not identify a reason for the species differences in frequency ramp. The morphology of mouse frequency ramp would be consistent with progressive depolarisation and sodium channel inactivation. This could have been attributed to the observation that mouse muscle MRRP is prolonged compared to human suggesting relative depolarisation. However, rat TA had the same frequency ramp morphology as mouse TA but an MRRP that was not significantly different from human TA (Figure 3-11,3-12). Moreover, the findings on MVRCs with selective pharmacological blockade suggest that mouse TA is more protected than human against activity-induced potassium accumulation and t-tubule depolarisation.

Another possibility is that there is increased sodium channel inactivation at the same membrane potential in rodent compared to human TA. Discrepancies between the behaviour of rodent and human NaV1.4 have been suggested by heterologous studies of the same Hyper PP mutations using either the rat or the human NaV1.4 clone. Rat Nav1.4 containing mutations corresponding to the human T704M and M1592V reported an increase in persistent non-inactivating sodium currents which allowed Na current to flow even after tens of milliseconds (Cannon and Strittmatter, 1993). However, subsequent studies of the same mutations heterologously expressed in human Nav1.4 channels found no impairment of fast inactivation (Yang et al., 1994; Bendahhou et al., 1999; Hayward, Sandoval and Cannon, 1999; Rojas et al., 1999), but did find a shift of activation in the hyperpolarised direction by 5-10 mV, and a shift of the midpoint of the inactivation curve in the depolarised direction. On the other hand, a comparison of the kinetics of heterologously expressed mouse and human NaV1.4 reported no significant differences
(Corrochano et al., 2014). Therefore, the exact mechanism underlying the differences in frequency ramp appearance between mouse and human skeletal muscle is not yet clear.

3.7.3 Relevance of findings to mouse models of skeletal muscle channelopathies
The differences in muscle excitability do however provide some insights into the phenotype differences between mice and human with skeletal muscle channelopathies. An increased reliance on chloride conductance in mouse muscle may explain why adr and mto mice (1.4.8.1) are so much more severely affected than humans with recessive Myotonia Congenita.

It is uncertain to what extent an increased resting chloride conductance in mouse muscle explains the resistance to spontaneous attacks of weakness in rodent models of Hypo PP. It has recently been demonstrated that rapid release of CIC-1 inhibition on recovery from acidosis triggers a transient loss of force in Hypo PP transgenic mice (Wu, Mi and Cannon, 2013a, 2013b; Mi et al., 2019). This is because of a sudden efflux of accumulated chloride that depolarises the sarcolemma. Myoplasmic accumulation of chloride is increased during acidosis because of ongoing activity of the Na K 2Cl cotransporter (Aickin, Betz and Harris, 1989) combined with inhibition of CIC-1 channels(Mi et al., 2019). Given this role for chloride conductance in the aetiology of attacks, it is possible that an increased chloride conductance in rodents may protect against spontaneous attacks of weakness in rodent models of Hypo PP. However, it is also likely that other differences in muscle excitability exist, for example with the potassium clearance via Kir and/or t-tubule α2-NaK ATPase.

Thus, more work is required to fully document the differences in rodent and human skeletal muscle physiology.

3.7.4 Implications for the mouse as a model of neuromuscular disease
This study does not mean that mice cannot or should not be used as a model organism for neuromuscular diseases. It should not be surprising that mouse muscle excitability is different from human as mice are very different from humans! To date, papers describing new animal models tend to focus on the similarities in phenotype between the animal model and humans with the condition to reinforce that it is a valid model. However, phenotype differences do not negate the model validity. A model is, by definition, not the real thing. A model represents the real thing. It is therefore important for researchers to
understand which aspects are accurately reflected and which are not. Improved understanding of our model organisms means we can improve animal study design, interpretation, and effective translation.

However, in addition to improved translation, understanding phenotype differences between humans and model organisms also provides an opportunity. For example, a better understanding of why rodents are resistant to spontaneous episodes of weakness in Hypo PP may identify new protective pathways that can be manipulated to prevent attacks in humans too.
4 Gender Difference in Muscle Excitability

4.1 Motivation

In MC, it has been reported that myotonic symptoms are more pronounced in men (Colding-Jorgensen, 2005), that pregnancy worsens myotonia (Wu et al., 2002; Colding-Jorgensen, 2005) and in some cases that females are only symptomatic during pregnancy (Lacomis, Gonzales and Giuliani, 1999; Colding-Jorgensen, 2005) (see 1.4.7). The Periodic paralysis phenotype is also reported to be more severe in males (1.2.7 and 1.3.7). Affected females often have reduced attack frequency compared to their affected male relatives (Troni, Doriguzzi and Mongini, 1983; Miller et al., 2004; Li et al., 2012; Ke et al., 2013) and in several kindreds a complete absence of paralytic attacks in female carriers (Li et al., 2012; Ke et al., 2013) has been reported. However, despite the observation that paralytic attacks are more severe in males with periodic paralysis, the accepted diagnostic cut offs for LET decrement to date have been the same for males and females. (Tan et al., 2011) Although, a significant gender difference in long exercise test response has been reported in a Chinese population (Jin et al., 2017). In this study, decrement from peak CMAP amplitude was 70% greater for both male Vs female healthy controls and males Vs females with periodic paralysis. The authors propose gender-specific cut offs for decrement from peak CMAP amplitude during the long exercise test of 46.8% for men and 26.9% for women.

Gender specific cut offs for the duration of cardiac depolarisation and repolarisation, reflected in an electrocardiogram by the QT interval, are well established in cardiology having first been noted in the 1920s (Bazett, 1920). As described in 1.1.9, there is also a significant body of evidence that gender differences in the cardiac channelome exist and are clinically significant (1.1.9). For example, the risk of sudden death or syncope in males with congenital long QTc syndrome, a cardiac channelopathy predisposing to arrhythmia, is massively reduced after puberty whilst in females it is not (Drici Milou D. et al., 1996; Vink et al., 2018).

In contrast to the cardiac literature, relatively little work has focussed on the mechanisms underlying gender differences in the severity of skeletal muscle channelopathy phenotypes. Understanding the physiological basis of this gender difference is necessary to a) improve the sensitivity and specificity of investigations for both male and female patients undergoing
the diagnostic work up for periodic paralysis and b) recognise protective factors in female physiology that may help to identify novel approaches to symptomatic treatment for both males and females with the condition.

4.2 Aims

Experiments in this chapter aim to determine

1) Is there a gender difference in long exercise test results for genetically confirmed patients with periodic paralysis and if so, is it affected by age?
2) Is there a gender difference in healthy human or mouse muscle excitability as assessed by Muscle Velocity Recovery Cycles and Frequency Ramp measurements?
3) Is there a gender difference in the effect of SCN4A mutation on muscle excitability as assessed by Muscle Velocity Recovery Cycles and Frequency Ramp measurements in Hyper PP (Draggen) mice?
4) How do differences in the functional expression of the skeletal muscle channelome contribute to these murine gender differences?

4.3 Specific Methods

4.3.1 Retrospective review of long exercise test data

Patients who had long exercise testing at the National Hospital for Neurology and Neurosurgery between 2009 and 2017 were included. Demographic, clinical and genetic data were collected from patient records as part of a service evaluation and all tests were performed as part of routine clinical care. Data was collected by Ana Ribeiro as part of an MSc project for which I was the secondary supervisor. I performed the data analysis described in this thesis (4.4).

The long exercise test was performed according to the original protocol described by McManis et al., 1986. The CMAPs were monitored for 5 minutes before exercise, during the 5 minutes of exercise, every minute during the first 6 minutes of rest and every 2 minutes after this point. The post-exercise period lasted for 50 minutes. The percentage CMAP increment from baseline amplitude and the percentage decrement from both peak and baseline amplitude were recorded. There are no studies that I am aware of looking at intra and inter-rater variability in long exercise test. Different groups have reported different
sensitivities and specificites of the Long Exercise Test, however, these studies were also performed on different patient cohorts, making the significance of that for interrater variability difficult to determine (McManis, Lambert and Daube, 1986; Fournier et al., 2004; Tan et al., 2011; Jin et al., 2017) However, we do have evidence to suggest intraindividual variability. In our data set 16 patients had a repeat Long Exercise test performed. The mean %difference in decrement from peak CMAP amplitude between the two Long Exercise Tests in these patients was 13.3±7% (Ana Ribeiro). Next generation RNA sequencing I performed RNA extraction and data analysis whilst UCL genomics performed library preparation, sequencing and bioinformatics pipeline as described below.

\subsection*{4.3.1.1 RNA extraction}
Mouse MVRCs were performed on TA and triceps as described (section 2.2.2). After this the contralateral muscle which had not had MVRCs performed was dissected whilst the animal was under terminal anaesthesia and, after removing the tendons, immediately flash frozen in liquid nitrogen. Less than 30mg of muscle was added to 300µl Buffer RLT (Qiagen RNeasy Fibrous Tissue Mini Kit) in Lysing Matrix Tubes M (MP Bio). Samples were disrupted and homogenised using 2 x 40 second runs at 4.0m/s on the MP Fastprep 24 system. RNA was then extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen) as per the supplied protocol.

\subsection*{4.3.1.2 Library preparation}
Samples were processed using the KAPA RNA HyperPrep Kit with RiboErase (Roche p/n KK8560) according to manufacturer’s instructions.

Before library prep, 4µL of a 1:10,000 dilution of either Mix1 or Mix2 of the ERCC spike-in control (ThermoFisher p/n 4456740) was added to each sample.

Briefly, complementary probes were hybridised to ribosomal RNA sequences. RNase-H was then used to selectively degrade DNA-RNA hybrids, whilst leaving non-hybridised RNA intact. DNA probes were then degraded by DNaseI before undergoing bead-based clean-up to remove degraded RNA and DNA fragments, leaving just non ribosomal sequences behind.

The purified RNA was fragmented using chemical fragmentation (heat and divalent metal cation) and primed with random hexamers. Strand-specific first strand cDNA was generated
using Reverse Transcriptase in the presence of Actinomycin D. This allows for RNA dependent synthesis while preventing spurious DNA-dependent synthesis. The second cDNA strand was synthesised using dUTP in place of dTTP, in order to mark the second strand.

The resultant cDNA is then end repaired and “A-tailed” at the 3’ end to prevent self-igation and adapter dimerisation.

Truncated adaptors, containing a T overhang are ligated to the A-Tailed cDNA. Successfully ligated cDNA molecules were then enriched with limited cycle PCR (200ng total RNA input material - 13 PCR cycles). The high-fidelity polymerase used in the PCR is unable to extend through uracil. This means only the first strand is amplified for sequencing, thus making the library strand specific. The primers used extend the adaptor to full length and contain sequences that allow each library to be uniquely identified by way of a sample-specific 6bp index sequence.

4.3.1.3 Sequencing

Libraries to be multiplexed in the same run are pooled in equimolar quantities, calculated from Qubit and Bioanalyser fragment analysis.

Samples were sequenced on the NextSeq 500 instrument (Illumina, San Diego, US) using a 76bp single read run with a corresponding 8bp UMI read.

4.3.1.4 Bioinformatic pipeline

Run data were demultiplexed and converted to fastq files using Illumina’s bcl2fastq Conversion Software v2.19. Fastq files were then aligned to the human genome UCSC hg38 using RNA-STAR 2.5.2b then UMI deduplicated using Je-suite (1.2.1). Reads per transcript were counted using FeatureCounts and differential gene expression was estimated using the BioConductor package SARTools, a DESeq2 wrapper.

All annotation and sequences were obtained from Illumina iGenomes (http://emea.support.illumina.com/sequencing/sequencing_software/igenome.html)
4.3.1.5 Data analysis

Differential gene transcript expression lists were first filtered using a predefined list of proteins that could account for MVRC differences (see table 4-1). To ensure all proteins of these functional classes were identified a second pass analysis was performed using EnrichR (Kuleshov et al., 2016) which generated GO Biological function tables and GO Molecular function tables for all differentially expressed gene lists. These tables were reviewed for any genes with functional terms related to ion channels, ion pumps, ion transporters, membrane conduction, membrane repolarisation, muscle contraction ion channel, pump or transporter modulation.

Table 4-1 List of proteins used to prioritise analysis of differentially expressed genes grouped according to functional rationale for their prioritisation.

<table>
<thead>
<tr>
<th>NaV inactivation</th>
<th>Resting membrane potential</th>
<th>Ion channel modulators</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaV alpha subunit</td>
<td>Na/K ATPase</td>
<td>Protein kinases (PKA, PKC etc)</td>
</tr>
<tr>
<td>NaV beta subunit</td>
<td>Na/K/2Cl pump</td>
<td>Calmodulin kinases</td>
</tr>
<tr>
<td>Repolarisation</td>
<td>Inward rectifying potassium channels</td>
<td>Calcium release and reuptake</td>
</tr>
<tr>
<td>Delayed rectifier potassium channels</td>
<td>T-tubule K+ reuptake</td>
<td>SR Ca2+ ATPase</td>
</tr>
<tr>
<td>Ca2+ activated potassium channels</td>
<td>t-tubule Na/K ATPase</td>
<td>Store Operated Calcium Entry</td>
</tr>
<tr>
<td>Voltage gated chloride channel</td>
<td>Inward rectifying potassium channels</td>
<td>ORAI1 &amp; STIM1</td>
</tr>
<tr>
<td>Na/Ca exchanger (reverse mode)</td>
<td></td>
<td>RyR1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na/Ca exchanger</td>
</tr>
</tbody>
</table>
4.4 Results 1: Gender difference in the Long Exercise Test

4.4.1 Comparison of Long Exercise Test results from males and females with genetically confirmed periodic paralysis

Figure 4-1 Comparison of long exercise test parameters in males and females with genetically confirmed periodic paralysis.

A. Percentage compound muscle action potential (CMAP) decrement from baseline.  B. Percentage CMAP decrement from peak. Horizontal black lines represent current diagnostic cut off for a positive long exercise test

A positive LET is defined as a 30% decrement from baseline (Figure 4-1A) or a 40% decrement from peak (Figure 4-1B). In this study the mean ± SD decrement from baseline in genetically confirmed males was 36% ± 15 whilst for females it was 33% ± 17. The mean decrement from peak CMAP amplitude in genetically confirmed males with periodic paralysis was 47.8% ±15 whilst for genetically confirmed females it was 40.0% ± 18. These differences were not significant.

A higher percentage of females with genetically confirmed periodic paralysis had a negative LET (42% (5/12) of females Vs 24% (7/29) males) although this was not statistically significant. As the long exercise test is essentially a locally induced attack of paralysis this is in keeping with the observation of reduced attack severity in females.
Results 1: Gender difference in the Long Exercise Test

4.4.2 There is a significant gender difference in decrement from peak CMAP amplitude in patients under age 40

When the long exercise test results were divided according to whether the patient was under or over 40 at the time of the test, there was a significant difference between decrement from peak in males and females under 40 (p=0.01) but not in those over age 40 (p=0.97, Figure.4-2A).

Figure 4-2 Comparison of long exercise test parameters in male and female patients with genetically confirmed periodic paralysis divided by age over or under 40.

A. Patients with all forms of primary periodic paralysis (Hypo PP, Hyper PP, ATS). B. Patients with ATS and Hypo PP only. Horizontal lines represent current diagnostic cut off for decrement from peak CMAP amplitude (solid black line) and from baseline CMAP amplitude (dashed grey line) for positive long exercise test. Data are mean ± SEM *student’s t-test with welch correction.

Hyper PP patients have been reported to have significantly greater CMAP increment during the long exercise test (Fournier et al., 2004) and so might reasonably expected to have a greater subsequent CMAP decrement potentially contributing disproportionately to these results. CMAP increment during long exercise test is not reported to be significant a feature for ATS or Hypo PP patients (Fournier et al., 2004). Therefore, to make sure the apparent gender difference was not due to the fact there were 6 times more males than females with Hyper PP (table 4-2), I repeated the analysis excluding Hyper PP patients (Figure 4-2B). The
significance of the gender difference in CMAP decrement was increased (p=0.00005) and therefore not simply due to a disproportionate number of male patients with Hyper PP.

Table 4-2 Genetic diagnoses, gender and age of patients that underwent long exercise test

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th>M/F</th>
<th>Mean Age ± SD Males</th>
<th>Mean Age ± SD Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATS</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>40±17</td>
<td>34±16</td>
</tr>
<tr>
<td>Hypo PP</td>
<td>13</td>
<td>5</td>
<td>2.6</td>
<td>34±13</td>
<td>44±13</td>
</tr>
<tr>
<td>Hyper PP</td>
<td>6</td>
<td>1</td>
<td>6</td>
<td>47±17</td>
<td>35±0</td>
</tr>
</tbody>
</table>

4.5 Gender difference in human muscle excitability

CMAP amplitude decrement is due to a reduced availability of NaV1.4 channels (Asawa, Shindo and Momoi, 2004). In periodic paralysis this is due to depolarisation of the muscle membrane and consequent inactivation of NaV 1.4 (Cannon, H. Brown and Corey, 1991; Hayward, Sandoval and Cannon, 1999; Struyk et al., 2008). CMAP amplitude increment with voluntary contraction or repetitive stimulation is attributed to an increase in muscle fibre conduction velocity that results in greater synchronisation of muscle electrical events (Hoeven, Weerden and Zwarts, 1993; Asawa, Shindo and Momoi, 2004).

Alterations in muscle conduction velocity following conditioning stimuli are the basis of the Muscle Velocity Recovery Cycle technique (Z’Graggen and Bostock, 2009) (see 1.1.1 and 2.2). I used this technique to explore the mechanisms underlying the gender differences in LET reported in healthy volunteers (Jin et al., 2017) and that I observed in patients under 40 with genetically confirmed periodic paralysis (Figure 4-2).

4.5.1 Healthy Young Adult Human Muscle Excitability

Dr Veronica Tan performed MVRCs in healthy volunteers and patients with periodic paralysis as (S V Tan et al., 2012; Tan et al., 2020). The age range of healthy controls was 27 to 66, mean age 44.2. There were no significant gender differences when these MVRCs were compared. However, as the gender difference on long exercise testing was only significant in patients under 40, we felt that gender difference may have been obscured. Therefore, I recruited healthy young (aged 20 to 30 years old) adult volunteers for MVRCs. I sent a generic email to all research students at the Institute of Neurology, as well as departmental
email to those who worked in the laboratory of Professor Linda Greensmith and to the Department of Experimental Epilepsy. There was no payment for taking part, but travel and lunch were compensated up to a maximum of £10. Our target for recruitment was 10 females and 10 males under the age of 30. To date 9 females and 3 males aged between 20 and 30 have been recruited. Dr Veronica Tan performed MVRCs on Tibialis Anterior (TA).

A comparison of healthy young adult male and female MVRCs is shown in Figure 4-3A and frequency ramps in Figure 4-3B. None of the MVRC parameters reached our criteria for statistical significance (p≤0.01). There was a significantly greater % decrement from baseline CMAP amplitude in response to the last stimulus in the train at 30Hz in males compared to females (57.3% males Vs 83.4% females, p=0.007) (Figure 4-3B), however, there was no concurrent significant difference in latency change, suggesting this may have been artefact. A greater number of male participants are needed before making any definitive conclusions. Recruitment will continue to try to address this but will be outside the time frame of my PhD.

A. Comparison of MVRCs from the TA of healthy male (blue, n=3) and female (red, n=9) volunteers aged between 20 and 30 years old. B. Comparison of 30Hz frequency ramp from the TA of healthy male (blue, n=3) and female (red, n=9) volunteers aged between 20 and 30 years old. Data are mean ± SEM
4.6 Gender difference in mouse muscle excitability

The gender difference in periodic paralysis phenotype severity is also reported in transgenic mice with periodic paralysis (Wu et al., 2012; Corrochano et al., 2014). It is not possible to perform long exercise testing on mice as it requires voluntary muscle activation; however, as described in chapter 3 and methods (section 2.2.2), MVRCs and 30Hz frequency ramps are possible. Therefore, I used the MVRC technique on WT and Draggen mice to investigate gender differences in healthy muscle excitability and gender differences in the effect of an SCN4A mutation (I582V) on muscle excitability.

Mice have the advantage that environmental and genetic confounders can be reduced by using mice of the same genetic background that are housed in the same environment. Moreover, in the case of Draggen Hyper PP mice, heterozygous mutant mice can be compared to their WT siblings. This increases the power to detect an effect of gender or genotype alone. MVRCs, combined with pharmacological manipulation and next generation RNA sequencing data, are a powerful tool to identify mechanisms behind potential gender differences. These results can then be investigated and confirmed in humans.

I performed recordings on WT and Draggen male and female TA and triceps brachii muscles. A summary of all the MVRC and frequency ramp data are given in table 4-3.
## Gender difference in mouse muscle excitability

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>WT Male TA (25)</th>
<th>WT Female TA (27)</th>
<th>WT Female Tri (15)</th>
<th>Draggen male TA (12)</th>
<th>Draggen male Tri (17)</th>
<th>Draggen female TA (17)</th>
<th>Draggen female Tri (17)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MVRC (n)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRRP</td>
<td>4.1 ± 1.2</td>
<td>6.9 ± 2.9†</td>
<td>5.1 ± 2.1</td>
<td>6 ± 2</td>
<td>5.6 ± 1.5*‡</td>
<td>7.8 ± 1.6†</td>
<td>4.4 ± 1.9†</td>
<td>6.5 ± 2.2‡</td>
</tr>
<tr>
<td>M2RRP</td>
<td>3.9 ± 1.6</td>
<td>5.5 ± 2.4†</td>
<td>4.3 ± 1.5</td>
<td>5 ± 1.8</td>
<td>6.3 ± 4.4*‡</td>
<td>7.4 ± 2.4†</td>
<td>4 ± 2*†</td>
<td>5.9 ± 2.4‡</td>
</tr>
<tr>
<td>M5RRP</td>
<td>3.8 ± 1.7</td>
<td>4.9 ± 2.5†</td>
<td>4.8 ± 1.7</td>
<td>5 ± 2.4</td>
<td>4.9 ± 2.5</td>
<td>6.8 ± 2.1†</td>
<td>4.3 ± 2.4</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>MSuperN@(ms)</td>
<td>11.4 ± 4</td>
<td>13.9 ± 1.9</td>
<td>12.8 ± 2.6</td>
<td>14.1 ± 1.8</td>
<td>12.8 ± 2.6</td>
<td>15 ± 0</td>
<td>10.7 ± 3.8‡</td>
<td>14.2 ± 1.7‡</td>
</tr>
<tr>
<td>MSuperN(&lt;15)%</td>
<td>3.6 ± 2.3</td>
<td>4.3 ± 3</td>
<td>2.7 ± 1.5‡</td>
<td>5.5 ± 3†</td>
<td>2.3 ± 0.9*†</td>
<td>4.3 ± 2†</td>
<td>3.8 ± 1.4*‡</td>
<td>4.8 ± 3.1</td>
</tr>
<tr>
<td>M5SuperN(20)%</td>
<td>5.5 ± 4.2‡</td>
<td>8.3 ± 3.9‡</td>
<td>3.4 ± 2.2*‡</td>
<td>8.7 ± 6.9†</td>
<td>5.3 ± 3.5</td>
<td>6.6 ± 4.5</td>
<td>6 ± 4</td>
<td>7.7 ± 5.4</td>
</tr>
<tr>
<td>M5SuperN(50-150)%</td>
<td>3.1 ± 2.6</td>
<td>4.3 ± 3</td>
<td>1.7 ± 1.1†</td>
<td>5.4 ± 3.9†</td>
<td>1.4 ± 1.2†</td>
<td>5.3 ± 2.4†</td>
<td>2.6 ± 1.7†</td>
<td>4.8 ± 2.8‡</td>
</tr>
<tr>
<td>M5SuperN(50-150)%</td>
<td>4.7 ± 4.1†</td>
<td>7.6 ± 4.2†</td>
<td>2.8 ± 2.3†</td>
<td>8.3 ± 7.2†</td>
<td>4.3 ± 3.4</td>
<td>5.7 ± 5.4</td>
<td>5.7 ± 4</td>
<td>7.7 ± 5.2</td>
</tr>
<tr>
<td>MLSuperN(50-150)%</td>
<td>0.1 ± 0.6</td>
<td>-0.1 ± 0.8</td>
<td>-0.1 ± 0.4</td>
<td>0.3 ± 0.8</td>
<td>0 ± 0.3</td>
<td>-0.1 ± 0.6</td>
<td>0.2 ± 0.8</td>
<td>0.1 ± 0.6</td>
</tr>
<tr>
<td>MXSuperN(50-150)%</td>
<td>-0.2 ± 0.5</td>
<td>-0.1 ± 0.6</td>
<td>-0.2 ± 0.3</td>
<td>-0.3 ± 0.4</td>
<td>0 ± 0.8</td>
<td>0 ± 0.5†</td>
<td>0 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>MX5SuperN(50-150)%</td>
<td>-0.5 ± 1.2*</td>
<td>-0.2 ± 2.3</td>
<td>-1.3 ± 0.8*</td>
<td>-0.3 ± 2.3</td>
<td>-0.9 ± 1</td>
<td>-1 ± 1.7</td>
<td>-0.6 ± 1.3</td>
<td>0 ± 1.5</td>
</tr>
<tr>
<td>MRSuperN(950)%</td>
<td>-0.3 ± 0.4</td>
<td>-0.3 ± 0.3</td>
<td>-0.2 ± 0.6</td>
<td>-0.3 ± 0.3</td>
<td>-0.3 ± 0.4</td>
<td>-0.3 ± 0.3</td>
<td>-0.3 ± 0.8</td>
<td>-0.1 ± 0.3</td>
</tr>
<tr>
<td>MX5RSuperN(950)%</td>
<td>-0.4 ± 0.7</td>
<td>-0.5 ± 0.4</td>
<td>-0.6 ± 1</td>
<td>-0.6 ± 0.5</td>
<td>-0.8 ± 0.4</td>
<td>-0.5 ± 1.1</td>
<td>-0.8 ± 0.6</td>
<td></td>
</tr>
<tr>
<td><strong>Frequency Ramp (n)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLatency(ms)</td>
<td>21 ± 0.4†</td>
<td>2.8 ± 0.4‡</td>
<td>2.1 ± 0.4†</td>
<td>2.5 ± 0.4†</td>
<td>2.2 ± 0.4†</td>
<td>3.1 ± 0.3*‡</td>
<td>2 ± 0.5†</td>
<td>2.7 ± 0.3*‡</td>
</tr>
<tr>
<td>MPeak(mV)</td>
<td>0.4 ± 0.4</td>
<td>0.5 ± 0.3</td>
<td>0.4 ± 0.3</td>
<td>0.6 ± 0.4</td>
<td>0.4 ± 0.2</td>
<td>0.4 ± 0.3</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>ML(15Hz)%</td>
<td>105.2 ± 3.4</td>
<td>105.3 ± 10.8</td>
<td>105.5 ± 4.5</td>
<td>105.7 ± 5</td>
<td>104.4 ± 3.8</td>
<td>108.7 ± 16.2</td>
<td>102.9 ± 4.6</td>
<td>104.8 ± 8.6</td>
</tr>
<tr>
<td>ML(15Hz)%</td>
<td>103.5 ± 1.7</td>
<td>104.7 ± 3.9</td>
<td>103.2 ± 3.3</td>
<td>103.7 ± 4.5</td>
<td>102.6 ± 2.6†</td>
<td>108.7 ± 6*‡</td>
<td>102 ± 3.6</td>
<td>101.8 ± 6.7*</td>
</tr>
<tr>
<td>MPk(15Hz)%</td>
<td>87.8 ± 17.5†</td>
<td>69.5 ± 23.2‡</td>
<td>83.1 ± 14.9</td>
<td>85.1 ± 22.2</td>
<td>90.4 ± 19.7*</td>
<td>65.2 ± 22.9</td>
<td>97.7 ± 22.2</td>
<td>83 ± 13.9</td>
</tr>
<tr>
<td>MPk(15Hz)%</td>
<td>96.6 ± 12.7‡</td>
<td>82.7 ± 17.5*</td>
<td>91.2 ± 10.7</td>
<td>89.1 ± 11.2</td>
<td>96.2 ± 12.8</td>
<td>76.4 ± 14.1†</td>
<td>99.9 ± 15.2*</td>
<td>84.7 ± 16.1‡</td>
</tr>
<tr>
<td>MLat(30Hz)%</td>
<td>110.3 ± 3.9</td>
<td>113.5 ± 14.4</td>
<td>112.2 ± 7.5</td>
<td>116.5 ± 18</td>
<td>108.9 ± 6.8†</td>
<td>125 ± 10.8</td>
<td>105.3 ± 14</td>
<td>117.7 ± 15.4</td>
</tr>
<tr>
<td>MPk(30Hz)%</td>
<td>58.4 ± 26†</td>
<td>33.5 ± 26.3†</td>
<td>56.6 ± 21.7</td>
<td>46 ± 24.8</td>
<td>69.4 ± 31.2†</td>
<td>28.8 ± 19.1*‡</td>
<td>73.7 ± 35.1*</td>
<td>64.7 ± 42.5†</td>
</tr>
<tr>
<td>MPk(30Hz)%</td>
<td>85.5 ± 27.8‡</td>
<td>61.6 ± 20.7†</td>
<td>72.2 ± 18.3</td>
<td>65 ± 36.8</td>
<td>85.7 ± 23.1†</td>
<td>38.7 ± 19.8‡</td>
<td>91.8 ± 35.2</td>
<td>61.8 ± 38</td>
</tr>
<tr>
<td>MPk(30-15Hz)%</td>
<td>-6.6 ± 22.3*</td>
<td>-21.1 ± 16.3*</td>
<td>-19 ± 13.7*</td>
<td>-24.1 ± 33.5</td>
<td>-10.3 ± 17.4†</td>
<td>-37.7 ± 20.7†</td>
<td>-8.2 ± 23.9</td>
<td>-22.8 ± 32.1</td>
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<tr>
<td>MLat(30Hz+30s)%</td>
<td>104.9 ± 2.4†</td>
<td>104.5 ± 11†</td>
<td>105.9 ± 4.5‡</td>
<td>111.1 ± 5.9‡</td>
<td>104.6 ± 3.5</td>
<td>111.2 ± 10.7</td>
<td>105 ± 7.1</td>
<td>111.4 ± 10</td>
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Gender difference in mouse muscle excitability
<table>
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<tr>
<th>Muscle</th>
<th>Gender Difference</th>
<th>30Hz + 30s%</th>
<th>30-15Hz%</th>
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<td>MPk</td>
<td></td>
<td>96.4 ± 19.7</td>
<td>7.1 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>†</td>
<td>89.3 ± 16.1†</td>
<td>8.8 ± 15.9</td>
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<td></td>
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<td>85 ± 15</td>
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<tr>
<td></td>
<td>+</td>
<td>74.2 ± 24.9</td>
<td>12.8 ± 14.2</td>
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<tr>
<td></td>
<td>‡</td>
<td>86.3 ± 19.1‡</td>
<td>6.3 ± 5.5‡</td>
</tr>
<tr>
<td></td>
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<td>16.29 ± 9.9‡</td>
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<tr>
<td>MLatf</td>
<td></td>
<td>92.9 ± 31.2</td>
<td>3.4 ± 13.5‡</td>
</tr>
<tr>
<td></td>
<td>†</td>
<td>69.9 ± 22.5</td>
<td>15.9 ± 11.3</td>
</tr>
</tbody>
</table>

*significant gender difference (compared to same muscle from animal of the same genotype) † significant genotype difference (compared to same muscle from animal of the same gender) ‡ significant TA/Triceps difference (compared to different muscle from animal of the same gender and genotype)
4.6.1 TA Muscle Excitability

4.6.1.1 Differences in MVRCs from Male and Female WT TA

There was no significant gender difference in WT TA MVRCs in response to 1 conditioning stimulus (Figure 4-4A). However, in response to 5 conditioning stimuli supernormality was increased and the onset of subnormality delayed in males compared to females (Figure 4-4B-D).

Increasing the number of conditioning stimuli helps to distinguish the effect of activity-induced potassium accumulation from the passive depolarising after potential held by the high capacitance of the t-tubule membrane (Z’Graggen and Bostock, 2009). The fact that the significant gender differences were in response to 5 conditioning stimuli suggests that they were related to activity-dependent t-tubule potassium accumulation.

Modest increases in t-tubule potassium (or reductions in chloride buffering of potassium) during muscle activity augment excitability (Sejersted and Sjøgaard, 2000). However, if excessive, increased t-tubule potassium will cause depolarisation of the membrane triggering sodium channel inactivation and reducing excitability (Sejersted and Sjøgaard, 2000). Depolarisation sufficient to cause significant sodium channel inactivation would manifest on MVRCs as an increased MRRP due to prolonged absolute refractory period, and on 30Hz frequency ramp as a reduction in the amplitude of response with increasing frequency of stimulation.

WT male TA did not show evidence of excessive depolarisation. The MRRP in male TA was shorter (Figure 4-4 A, E) than for female and, there were no significant differences between WT male and female TA on the 30Hz frequency ramp (Figure 4-4F). This suggests that any increase in activity-induced t-tubule potassium accumulation in WT male TA was compensated for by increased potassium clearance and/or buffering in the applied 30 Hz frequency range.
Gender difference in mouse muscle excitability

Figure 4-4 Comparison of MVRCs and 30Hz frequency ramp from male WT TA (red) and female WT TA (green).
**A. MVRCs in response to 1 conditioning stimulus.** B. MVRCs in response to 5 conditioning stimuli. C. Early supernormality in response to 5 conditioning stimuli* p=0.01 D. Late supernormality in response to 5 conditioning stimuli **p=0.008** E. Muscle relative refractory period. F. Response to 30Hz frequency ramp. Male WT TA (n=25 for MVRCs, n=22 for frequency ramp); female WT TA (n=27 for MVRCs, n=23 for frequency ramp). Data are mean ± SEM

### 4.6.2 Triceps’ muscle excitability

It is known that some muscles are more susceptible to acute attacks of paralysis in mice and to fat infiltration observed on muscle imaging in patients (Links *et al.*, 1990; Ammar *et al.*, 2015). For example, isolated mouse soleus requires lower potassium concentrations than extensor digitorum longus (EDL) to induce a paralytic attack in Hyper PP M1592V mice (Khogali *et al.*, 2015) and the upper limbs are relatively spared from the fatty infiltration observed on CT imaging of muscle from patients with periodic paralysis (Links *et al.*, 1990).

In Draggen mice, dragging/immobility episodes were observed in the hind limbs but not reported for the forelimbs (Corrochano *et al.*, 2014). In the CaV1.1 R528H Hypo PP mouse model there was a genotype difference in male hind, but not forelimb grip strength, whilst for Hypo PP female mice neither hind nor forelimb were significantly different from WT (Wu *et al.*, 2012). This suggests that a) forelimb muscles may be more resistant to ion channel dysfunction and b) they may not show the same gender difference in MVRCs as observed in the TA (Figure 4-4).

As described in section 2.2.2 I developed a technique to perform MVRCs in mouse triceps brachii. I used this to examine male and female WT triceps to determine firstly whether different muscles have distinct excitability patterns and secondly whether gender differences in MVRCs are ubiquitous or muscle specific.

### 4.6.3 Differences in TA and Triceps’ MVRCs

Mouse triceps muscle excitability was different to TA. Compared to TA, Triceps of both genders had greater supernormality on MVRCs (Figure 4-5 A&B, table 4-3). In male triceps MRRP was prolonged compared to TA (Figure 4-5A). In female triceps the onset of subnormality was delayed compared to TA (Figure 4-5B).
Gender difference in mouse muscle excitability

![Figure 4-5 Comparison of MVRCs from male and Female WT triceps and TA in response to 1, 2 and 5 conditioning stimuli.](image)

**A.** Male WT triceps (light red, n=23) Vs male WT TA (black, n=25).  **B.** Female WT triceps (light green, n=15) Vs female WT TA (black, n=27).  Data are mean ± SEM

On frequency ramp in male triceps there was a larger increase in latency and reduction in amplitude (Figure 4-6A).  In female triceps, latency was slower to return to baseline after 30 Hz frequency ramp (Figure 4-6B).

![Figure 4-6 Comparison of response to 30Hz frequency ramp for male and female WT triceps and TA.](image)

**A.** WT male triceps (red, n=17) Vs TA (black, n=22).  **B.** WT female triceps (green, n=13) Vs TA (black, n=23).  Data are mean ± SEM

There was no significant gender difference for triceps’ MVRCs or 30Hz frequency ramp (table 4-3, Figure 4-7).  The only parameter that came close to significance was the unconditioned latency (time to peak of an unconditioned test stimulus p=0.03).  Although
supernormality was much greater in WT triceps compared to TA for both genders, the relative increase in supernormality was larger for female triceps compared to TA than for male triceps compared to TA (table 4-3, Figure 4-5). This large increase in female supernormality effectively removed the gender difference in supernormality that was observed in TA (Figure 4-7 D-E).

These findings provide evidence that gender difference in mouse muscle excitability varies between muscles and that different muscles have distinct excitability profiles. As only one human muscle was examined, it is not known if in the same is true for human muscle gender differences.
Gender difference in mouse muscle excitability

A. MVRCs in response to 1 conditioning stimuli. B. MVRCs in response to 5 conditioning stimuli. C. 30Hz frequency ramp. D. Comparison of early supernormality in response to 5 conditioning stimulus for male (red) and female (green) WT TA (dark) and triceps (light). E. Comparison of late supernormality in response to 5 conditioning stimulus for male (red) and female (green) WT TA (dark) and triceps (light). (WT M TA = WT male TA, n=25; WT F TA = WT female TA, n=27; WT M Tri = WT male triceps, n=25; WT F Tri = WT female triceps, n=15). Data are mean ± SEM

Figure 4-7 Comparison of MVRCs and 30Hz frequency ramp recording from male (light red) and female (light green) WT triceps.
4.7 Gender difference in the effect of SCN4A mutation on muscle excitability

Given the finding that there is a physiological gender difference in muscle excitability in TA I wanted to see if the effect of ion channel mutation is different in male and female TA. To do this, I used Draggen mice (Corrochano et al., 2014). Draggen mice have a mutation in SCN4A (c.1762 A>G p.I582V) that is reported to cause Hyper PP in human patients (see 1.3.9.3.3). Mice of both genders have full penetrance of EMG myotonia. However, as described in 4.1 there is a significant gender difference in the penetrance and/or severity of clinically evident hind limb dragging episodes.

4.7.1 Male TA

Figure 4-8 Comparison of MVRCs from male WT and Draggen TA.

A. MVRCs from male WT and Draggen TA in response to 1 conditioning stimulus  
B. MVRCs from male WT and Draggen TA in response to 5 conditioning stimuli.  
C. Muscle relative refractory period (MRRP) after one conditioning stimulus  
D. Muscle relative refractory period (MRRP) after five conditioning stimuli

Draggen male TA had a significantly longer MRRP (p=0.003) in response to one conditioning stimulus and a trend towards reduced supernormality (p=0.02) compared to WT male TA.
Gender difference in the effect of SCN4A mutation on muscle excitability

(table 4-3, Figure 4-8A,B). This would be consistent with baseline depolarisation and consequent sodium channel inactivation in Draggen TA relative to WT. Depolarisation relative to WT muscle has been reported for the other Hyper PP mouse model (M1592V) (Ammar et al., 2015) the gender of the animals used for these experiments was not specified.

4.7.2 Female TA

![Figure 4-9 Genotype differences in female TA and triceps' MVRCs and response to 30Hz frequency ramp.](image)

**A. TA MVRCs in response to 1 conditioning stimulus in female Draggen (grey, n=17) Vs WT (green, n=27)**  
**B. TA MVRCs in response to 5 conditioning stimuli in female Draggen (grey, n=17) Vs WT (green, n=27)**  
**C. TA 30Hz frequency ramp in WT (green, n=23) and Draggen (n=17) females. Data are mean ± SEM**

In contrast to male muscle, the effect of the Draggen SCN4A mutation on female muscle was to increase supernormality. MRRP was not prolonged in Draggen female TA compared to WT female (Figure 4-9 A & B, table 4-3) and Draggen female TA had significantly greater early supernormality in response to 1 conditioning stimulus (p=0.007, Figure 4-9A, table 4-3) and significantly greater late supernormality in response to 2 conditioning stimuli (p=0.009, table 4-3).
Gender difference in the effect of SCN4A mutation on muscle excitability

The onset of subnormality was delayed in Draggen female compared to WT TA (Figure 4-9B). After 5 conditioning stimuli in WT female TA, subnormality began at around 40ms inter-stimulus interval. In Draggen female TA the onset of subnormality was between a 50 and 60ms inter-stimulus interval. This is likely a reflection of myotonia as the onset of subnormality was also delayed when WT muscle was made myotonic by exposure to 9AC (Figure 3-15). These findings suggest that delayed onset of subnormality is the mouse equivalent to the increased residual supernormality that is observed on MVRCs in humans with myotonia (Tan et al., 2014, 2018).

The increased excitability of Draggen female TA was not associated with greater decrement on frequency ramp (Figure 4-9C, table 4-3). The amplitude of response as a percentage of baseline at 15Hz was 83.1 ± 3.1% for WT female TA compared with 97.7 ± 5.4% for Draggen female TA (p=0.03, table 4-3). The amplitude of response as a percentage of baseline for the first action potential in the train at 30Hz was 72.18 ± 3.83% for WT female TA compared with 91.78 ± 8.54% for Draggen female TA (p= 0.05).

4.7.3 Male Triceps

In male triceps there was a significant genotype difference in MRRP in response to 5 but not 1 conditioning stimuli (table 4-3, Figure 4-10A-D). Draggen male triceps also displayed greater late subnormality in response to 5 conditioning stimuli when compared against WT male (Figure 4-10C).

The amplitude in response to the first stimulus in a train at 30Hz (p=0.01) and in response to 0.5Hz stimulation for 30 seconds after the frequency ramp had finished was significantly smaller in Draggen compared to WT male triceps (p=0.005, Figure 4-11, table 4-3). The fall in amplitude and increase in latency in Draggen triceps during the frequency ramp suggests that the membrane depolarises, or more sodium channels inactivate at the same membrane potential with trains of impulses. The failure of amplitude to recover after the frequency ramp suggests some fibres become depolarised to inexcitability and remain inexcitable after the end of the ramp. This pattern is similar to that observed in humans with PMC (Tan et al., 2017).
Gender difference in the effect of SCN4A mutation on muscle excitability

Figure 4-10 Comparison of MVRCs from male WT and Draggen triceps.

A. MVRCs in response to 1 conditioning stimulus. B. Muscle Relative Refractory Period after 1 conditioning stimulus. C. MVRCs in response to 5 conditioning stimuli. D. Muscle Relative Refractory Period after 5 conditioning stimuli. Where data are normally distributed plots are mean ±SE when data are not normally distributed box and whisker plot is shown. WT triceps = red, n=23. Draggen triceps = blue, n=12.

Thus, in male TA whilst MRRP in response to 1 conditioning stimulus was prolonged, MRRP in response to 5 conditioning stimuli and the 30Hz frequency ramp were not significantly different between Draggen and WT males (see section 4.7.1). In male triceps there was no difference in MRRP in response to 1 conditioning stimulus, but MRRP was significantly prolonged in response to 5 conditioning stimuli and there was a significantly greater decrement in the amplitude of response in Draggen male triceps compared to WT during 30Hz frequency ramp (Figure 4-11).
Figure 4-11 Genotype differences in male triceps and TA response to 30Hz frequency ramp

A. Response to 30Hz frequency ramp in male WT and Draggen triceps  
B. CMAP amplitude as percentage of baseline in response to the first stimulus in the train at 30Hz (MPkf(30Hz)%)  
**p=0.096.  
C. CMAP amplitude as percentage of baseline in response to 0.5Hz stimulation 30 seconds after the end of the frequency ramp (MPk(30Hz+30s)%) **p=0.005.  
(WT M TA= WT male TA, n=23; D M TA= Draggen male TA, n=15, WT M Tri = WT male triceps, n=17; D M Tri= Draggen male triceps, n=10).
4.7.4 Female Triceps

**A** Female WT Vs Draggen triceps 1CS

**B** Female WT Vs Draggen triceps 5CS

C  Female WT Vs Draggen triceps

*Figure 4-12 Genotype differences in female triceps’ MVRCs and response to 30Hz frequency ramp.*

**A. Triceps MVRCs in response to 1 conditioning stimulus in female Draggen (magenta, n= 17) Vs WT (green, n=15)**. **B. Triceps MVRCs in response to 5 conditioning stimuli in female Draggen (magenta, n=17) Vs WT (green, n=15)**. **C. Triceps 30Hz frequency ramp in Draggen (magenta, n=16) Vs WT (green, n=13) females.**

In contrast to male triceps, there were no significant genotype differences between Draggen and WT female triceps MVRCs or frequency ramp (table 4-3, figure 4-12).

4.8 Summary of Muscle Excitability Results

Wild type male mouse TA had increased supernormality compared to WT female TA. On this background, gain of function sodium channel mutation appeared to have more drastic effects. MVRCs from male Draggen TA suggested excess depolarisation and sodium channel
inactivation whereas MVRCs from female Draggen TA suggested hyperexcitability compared to WT. The former would be in keeping with depolarisation induced weakness and the latter with myotonia alone. This suggests that the hind-limb dragging episodes observed in male mice are a combination of myotonia and weakness.

However, the effect of ion channel mutation also depends on the excitability profile of a particular muscle. Supernormality was increased in amplitude and duration in healthy triceps compared to TA. On this background, male Draggen triceps showed a greater decrement and impaired recovery following 30 Hz frequency ramp compared to male WT triceps whilst there was no genotype difference for female triceps.

Thus, the effect of SCN4A mutation on muscle excitability differs according to the specific muscle and gender of the animal. My data suggest that male muscles exhibit greater supernormality than female muscles and Draggen muscles greater supernormality than WT muscles. Muscle supernormality follows a U shape curve (Z'Graggen and Bostock, 2009): with small membrane depolarisation towards threshold, muscle velocity increases, however, with larger membrane depolarisation moving beyond threshold, muscle velocity will slow as some sodium channels inactivate. The data in this section suggest that on a background of increased supernormality, the NaV1.4 gain-of-function in male muscle appears sufficient to tip the muscle into depolarisation and sodium channel inactivation. However, in female muscle NaV1.4 gain of function can increase supernormality without resulting in excess membrane depolarisation and sodium channel inactivation.

### 4.9 Gender differences in the functional expression of the skeletal muscle channelome

To investigate the physiological basis of the distinct excitability patterns of male and female muscle, I examined differences in the gene transcript expression of ion channels and related proteins using high depth RNA sequencing. RNA sequencing was performed on male and female Draggen and WT TA, and male and female WT triceps. Four muscles were included for each group from age-matched young animals (range 15 to 20 weeks old). The list of group comparisons performed, and corresponding number of differentially expressed genes are provided in table 4-4.

I prioritised gene analysis using a predefined list of proteins that could account for the observed changes in muscle excitability (see table 4-1 in section 4.3.2). Genes encoding
proteins from this list that were differentially expressed are given for each group comparison in table 4-5. I will discuss the differentially expressed genes for male and female WT mice first, then for Draggen and WT mice.

Table 4-4 Number of differentially expressed genes for each group comparison

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<th>Group comparison</th>
<th>Up</th>
<th>Down</th>
<th>Total</th>
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<td>Draggen female TA Vs Draggen male TA</td>
<td>88</td>
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<td>464</td>
<td>889</td>
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<tr>
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<td>Genotype (WT Draggen)</td>
<td>WT Muscle (TA Triceps)</td>
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<td>-----------------------</td>
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<td>WT Tri</td>
<td>Draggen TA</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>♂ WT</td>
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<tr>
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<td>SCN2B; SCN4B; CAMK1D; CAMK1G; CAMK2D</td>
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<td>ATP1B1; KCNK3; ATP1A3</td>
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<td>KCNJ11</td>
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<td>ABCC8; ATP1B1; LRRC52</td>
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<td>ARPP19; PKIG; PRKG1</td>
<td>PRKCA</td>
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<td>CASQ2; CACNA1C; SLC8A3; ORAI1; STIM1; ATP2A2; CACNG6</td>
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**Table 4-5** Differentially expressed genes that encode proteins with functions predicted to affect MVRC parameters.
4.9.1  WT TA Gender difference

4.9.1.1  Increased expression of repolarising potassium channel transcripts in male muscle

Compared to female TA, male TA had increased supernormality in response to 5 conditioning stimuli and a delayed onset of subnormality (Figure 4-4). This suggests an increased effect of activity dependent t-tubule potassium accumulation on MVRCs of male mouse TA. This could be due to an increased K+ efflux per action potential, reduced K+ reuptake following an action potential and/or reduced chloride buffering of potassium released following an action potential.

The identity of repolarising K+-channels in skeletal muscle is not fully established but is likely to consist of voltage gated K+-channels (delayed rectifiers). The expression of 5 gene transcripts (\(KCNG4; SLC8A3; DPP6; AMIGO1, LRRC38\)) that code for delayed rectifier potassium channels or their auxiliary subunits were significantly increased in male WT TA compared to female WT TA (Table 4-5). In contrast there was no significant increase in transcript expression for any genes related to potassium channels or their auxiliary subunits in female WT TA (Figure 4-13, table 4-5).

![Male Vs Female Wild-type TA](image)

**Figure 4-13** Gender differences in gene transcript expression of WT TA.

*The number of genes from each functional group of proteins listed in table 5-5 is shown according to whether they were significantly upregulated in WT male TA (blue, n=4) or WT female TA (red, n=4).*
4.9.1.2 Gender difference in ClC-1 activity

**A.** WT female TA MVRCs in response to 5 conditioning stimuli pre 30mg/kg 9AC (green, n=3) and post 30mg/kg 9AC (grey, n=4). **B.** Male WT TA pre 30mg/kg 9AC (red, n=2) and post 30mg/kg 9AC (blue, n=3). **C.** Comparison of early supernormality in response to 5 conditioning stimuli for male and female TA pre and post 30mg/kg 9AC. **D.** Comparison of late supernormality in response to 5 conditioning stimuli for male and female TA pre and post 30mg/kg 9AC. *same animals, however as in some cases MVRCs performed pre and post 9AC on one side, MVRCs post 9AC are higher.*

Increased K⁺ efflux would be exacerbated by reduced t-tubule chloride buffering. There was no significant difference in CLCN1 transcript expression between male and female TA. To try to ascertain if differences in the functional expression of ClC-1 contribute to the observed
gender difference in TA MVRC, I reviewed my 9AC data from the previous chapter (3.5.1). I hypothesised that if ClC-1 activity is higher in female TA, blockade with 9AC should increase female TA supernormality towards male values. 

In keeping with this, following maximal ClC-1 block with 30mg/kg 9AC, gender difference in the amplitude of *early* supernormality (Figure 4-14 A,B,C) was removed. However, gender difference in the amplitude of *late* supernormality was increased (Figure 4-14D). Although numbers were small (n=4 for female TA, n=3 for male TA) subnormality was removed in male TA (figure 4-14B) but only delayed and reduced in female TA (figure 4-14A). The suggestion that 9AC had a disproportionate effect on late supernormality in male TA suggests that male TA either releases more potassium per action potential or may rely more heavily on chloride buffering of activity-induced t-tubule potassium accumulation than female TA. This needs confirmation with additional experiments.

4.9.1.3 *Late supernormality was only observed in male WT TA following micromolar ouabain*

If male mice have greater K⁺ efflux per action potential and reduced ClC-1 activity, they may rely more heavily on t-tubule K⁺ reuptake via the t-tubule Na K ATPase. It was interesting therefore that on reviewing the data all 3 out of the 14 TA muscles that exhibited clear late supernormality on MVRCs post ouabain (3.5.2.1) were male. This is despite 6 out of the 14 recordings coming from female TAs.

4.9.1.4 *Summary of male WT Vs female TA*

The data suggest that increased expression of transcripts that code for either potassium channels or their auxiliary subunits result in greater K⁺ efflux per action potential in WT male TA compared to WT female. This combined with reduced ClC-1 activity in male TA would explain the significant differences in early and late supernormality in response to 5 conditioning stimuli. The suggestions that male muscle might rely more heavily on t-tubule Na K reuptake to limit excessive depolarisation would fit with these observations but needs to be confirmed with further experiments.
4.9.2 WT Triceps Gender difference

![Figure 4-15](image)

**Figure 4-15  Gender differences in gene transcript expression of WT Triceps.**

The number of genes from each functional group of proteins listed in table 4-3 is shown according to whether they were significantly upregulated in WT male triceps (blue, n=4) or WT female triceps (red, n=4).

There was no significant difference between male and female wild type triceps MVRC or 30 Hz frequency ramp. The Parameter that came closest to being significantly different was the unconditioned latency (table 4-3). However, there were significant differences in gene transcript expression between male and female WT triceps. (Figure 4-15, table 4-4, table 4-5).

As described for TA and reported previously for cardiac muscle (Drici Milou D. et al., 1996; Fülöp et al., 2006; Parks and Howlett, 2013), male WT triceps had increased expression of gene transcripts encoding repolarising potassium channels and proteins involved with calcium release and reuptake (Figure 4-16). In addition, and in contrast to TA, $CLCN1$ and $SCN4A$ transcript expression was significantly higher in male compared to female triceps.

Therefore, despite the lack of significant gender difference on WT triceps MVRCs and frequency ramp, there were significant differences in ion channel transcript expression. This suggests the increased supernormality in male and female triceps may have a different molecular basis.
4.9.3 WT TA Vs Triceps muscle differences

Triceps of both sexes display larger supernormality than TA in response to 1, 2 and 5 conditioning stimuli (section 4.6.2.1). Supernormality was smaller in female compared to male TA but of similar amplitude in triceps. Thus, the difference in the amplitude of supernormality between TA and triceps is larger for female than male mice.

In order to try to understand the mechanism behind the distinct excitability profiles of male and female TA and triceps, I compared TA and triceps transcript expression profiles for each gender and combined this with data from pharmacological modulation of channels pre and post MVRCs.

4.9.3.1 Changes in the expression of repolarising potassium channel gene transcripts

*KCNMA1*, the gene encoding the large conductance Ca\(^{2+}\) activated potassium channel (BK channel) alpha subunit was significantly increased in in both male and female WT triceps compared to TA (table 4-5, male =1.6 fold, \(p_{adj}=3^{-7}\), female 16-fold \(p_{adj}=8^{-32}\)). In both male and female triceps this increase in BK channel transcript expression was associated with a decrease in the expression of smaller conductance voltage-gated potassium channel transcripts. *KCNB1* which encodes the Kv2.1 delayed rectifier potassium channel was reduced 0.5-fold (i.e. 50% less) in male triceps Vs TA (\(p_{adj}=1.1^{-8}\)) and 0.5 fold in female triceps Vs TA (\(p_{adj}=0.015\)). *KCNG4* encodes the Kv6.4 subunit that forms heteromers with Kv2.1 to cause a hyperpolarising shift in its voltage dependence of inactivation was reduced 0.7-fold in male (\(p_{adj}=0.047\)) and 0.5 fold in female (\(p_{adj}= 0.0009\)) triceps compared to TA. Reduction in expression of *KCNN3* mRNA which encodes the small conductance Ca activated potassium channel was also seen (male fold change 0.5, \(p_{adj}= 0.0002\), fold change female 0.2, \(p_{adj} =2^{-15}\)).

The increase in *KCNMA1* mRNA expression was 10-fold greater for female triceps Vs female TA than for male triceps Vs male TA. BK channels have a unitary conductance of approximately 260pS (Yan and Aldrich, 2012). This compares to approximately 10pS for the small conductance Ca activated potassium channel (SK) and 7.1pS for the voltage-dependent delayed rectifier potassium channel Kv2.1. Thus, increased expression and/or activity of BK channel transcripts have the potential to greatly increase activity induced K\(^+\) efflux.
Gender differences in the functional expression of the skeletal muscle channelome

To investigate whether increased BK channel transcript expression could be responsible for the large increase in supernormality in female triceps compared to TA I used iberiotoxin a selective BK channel blocker and compared the effect on triceps and TA of the same WT female animal. One microgram intraperitoneal iberiotoxin reduced supernormality in both TA and triceps (Figure 4-16A&B). However, the extent of supernormality reduction was far greater in triceps (Figure 4-16B). The results were from one animal and therefore should be treated with caution until confirmed with greater numbers. However, the result suggests that the increased expression of BK channel transcripts in triceps may play a significant role in the greater supernormality of female triceps compared to TA.

**Figure 4-16 The effect of iberiotoxin, a selective BK channel blocker, on female triceps and TA MVRCs.**

A. Female TA pre (black) and post (red) 1µg intraperitoneal iberiotoxin. B. Female triceps pre (black) and post (red) 1µg intraperitoneal iberiotoxin. All recordings are from the same animal.

**4.9.3.2 A reduction in ClC-1 activity in female triceps compared to TA**

An additional potential cause of increased supernormality is reduced chloride buffering of potassium via ClC-1 channels. There was no significant difference in CLCN1 transcript expression between female WT triceps and TA. This suggests that any difference in ClC-1 buffering of potassium would be due to post-translational modification, modulation of protein expression or direct modulation of ClC-1 channel function. In order to investigate this, I compared the effect of 30mg/kg 9AC on supernormality in female triceps compared to TA.
In WT female TA 30mg/kg 9AC increased *early* supernormality by approximately 500% (Figure 4-17A). In WT female triceps 30mg/kg 9AC increased *early* supernormality by around 50% (Figure 4-17B). However, in female triceps, 9AC seemed to have a much larger effect on *late* supernormality. This was similar to what was observed for male TA compared to female TA (4.9.1.2) and also suggests that female triceps either releases more K+ per action potential or relies more heavily on ClC-1 for buffering of potassium than female TA.

**Figure 4-17** The effect of 9 Anthracene Carboxylic Acid, a ClC-1 blocker on female TA and triceps.

**A.** MVRCs pre (n=3) and post 9AC (n=4) in female WT TA. **B.** MVRCs pre (n=6) and post (n=5) 9AC in female WT triceps.

### 4.9.3.3 T-tubule potassium reuptake

In keeping with this, transcript expression of *ATP1A2* (the t-tubule Na⁺K⁺ ATPase isoform) and *ATP1B1* (the Na K pump β subunit) were significantly reduced in female triceps compared to TA (0.8-fold reduction in triceps Vs TA for *ATP1A2* padj=0.049; 0.4 fold for *ATP1B1* padj=0.0004). The combination of increased BK channel and reduced t-tubule Na⁺/K⁺ ATPase transcript expression would increase t-tubule potassium efflux per action potential. This combined with reduced ClC-1 activity could explain the significantly higher supernormality in female triceps MVRCs compared to TA and disproportionate effect of 9AC on late supernormality in female triceps.

If large enough, an increase in t-tubule potassium concentration could result in excess depolarisation and sodium channel inactivation. Female WT triceps’ MRRP was not
Gender differences in the functional expression of the skeletal muscle channelome

significantly different from TA and there was no significant decrement on frequency ramp compared to TA (table4-3). Therefore, there was no evidence that increased supernormality in female triceps resulted in excessive depolarisation, sodium channel inactivation or prolonged repolarisation compared to TA.

The resistance to excessive depolarisation despite the likely increase in potassium efflux into the t-tubule and reduction in chloride buffering could be explained by an increased transcript expression of the alpha 3 isoform of the Na K ATPase in female WT triceps compared to TA (ATP1A3, 15 fold, padj=5.22) This isoform is specialised in intracellular Na clearance (Clausen, Hilbers and Poulsen, 2017) and therefore would be well suited to limiting activity induced depolarisation.

4.9.3.4 Changes in calcium release genes

In cardiac tissue males exhibit greater calcium transients in response to the same depolarising stimulus(Parks and Howlett, 2013). It was striking therefore that both male triceps and male TA had increased expression of transcripts from genes predicted to augment cytosolic calcium release compared to both female triceps and female TA (Figure 4-13, 4-15).

Gene transcript expression changes also suggest that calcium transients may be greater in male triceps compared to TA. This needs to be confirmed experimentally. However, consistent with the idea that Ca\(^{2+}\) regulation is altered in male WT triceps compared to TA, the expression of ATP2A2 mRNA that encodes the slow twitch muscle sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) 2 isoform was significantly increased (5.6-fold, padj1.6). A switch to this isoform can be associated with increased basal intracellular Ca\(^{2+}\) levels as are found in slow twitch muscle (Schiaffino and Reggiani, 2011).

As well as potentially increasing contraction force, an increase in cytosolic calcium would augment BK channel activity and thus potassium efflux via a left shift in BK channel voltage dependence of activation (Yan and Aldrich, 2012). Expression of Orai1 and Stim1, gene transcripts were significantly increased in male, but not female WT triceps compared to TA (Stim1 1.2 fold, padj=0.04, Orai1, 1.4 fold, padj=0.04). Stim1 is the SR calcium sensor that interacts with Orai1 to trigger calcium influx from t-tubules into the cytoplasm when SR stores are depleted (Koenig, Choi and Launikonis, 2018). Stim1 and Orai1 mediated Ca\(^{2+}\)
influx was recently shown to be triggered by each action potential induced Sr Ca\textsuperscript{2+} release (Koenig, Choi and Launikonis, 2018). Thus, an increase in SOCE would augment action-potential induced rise in intracellular calcium and result in greater BK channel activation in response to the same depolarising stimulus.

![WT Male Triceps pre and post Dantrolene](image)

*Figure 4-18 The effect of 10mg intraperitoneal Dantrolene on male WT triceps MVRCs (n=4 animals, black = pre dantrolene, red=post dantrolene in the same animal’s triceps). The filled circles are for responses to 1, 2 and 5 conditioning stimuli. The dotted lines are SEM.*

To assess whether action potential-induced calcium release was augmenting supernormality in male triceps, I examined the effect of dantrolene, an excitation-contraction uncoupler on MVRCs from male WT triceps. Uncoupling Sr Ca\textsuperscript{2+} release from membrane depolarisation prevents action potential induced SOCE (Koenig, Choi and Launikonis, 2018). Dantrolene reduced early supernormality, increased subnormality and prolonged MRRP (Figure 4-18).

This suggests that augmented calcium release may be important. However, Dantrolene has also been shown to reduce PKC mediated ClC-1 inhibition (Pedersen et al., 2009). In order to differentiate an effect on BK channels from one on ClC-1 channels additional experiments are needed comparing the effect of iberiotoxin (BK channel blocker) and 9AC (ClC-1 blocker) on male WT TA and triceps. Unfortunately, I was not able to complete these in the time frame available to me.
4.9.4 WT TA Vs triceps 30Hz Frequency Ramp

There was a significant decrement in amplitude of response on frequency ramp for male WT triceps compared to TA (Figure 4-6). The decrease in amplitude on 30Hz frequency ramp may reflect selectively increased inhibitory potassium or chloride currents that effectively short-circuit the sodium influx, increasing depolarisation of membrane potential and consequent sodium channel inactivation or a left-shifted voltage dependence of inactivation for sodium channels.

It is unlikely that the increased expression of BK channel transcripts explains male triceps’ greater decrement in amplitude on frequency ramp because female WT triceps had no significant decrement in CMAP amplitude on frequency ramp compared to TA (Figure 4-6) despite a 10-fold greater increase in BK channel transcript expression. Secondly there was no significant change in latency on male WT triceps frequency ramp (Figure 4-6) as would be expected with a significant increase in potassium conductance and thirdly, selective blockade of BK channels with iberiotoxin had no effect on the frequency ramp in WT triceps (Figure 4-19A).

Calcium induced inactivation of NaV1.4 has been reported (Yoder et al., 2019). Given the increased expression of mRNA transcripts of proteins involved with store operated calcium entry in WT male triceps, greater calcium induced inactivation of NaV1.4 channels could explain the greater decrement in amplitude response during the 30Hz frequency ramp. To assess if calcium dependent sodium channel inactivation was contributing to the observed decrement in amplitude on 30Hz frequency ramp I used dantrolene. As described above, dantrolene is an excitation-contraction uncoupler such that RyR1 does not open and release SR Ca\(^{2+}\) in response to membrane depolarisation. This should attenuate any Ca\(^{2+}\) induced inactivation of NaV1.4.

Dantrolene did not prevent or significantly alter the decrement in CMAP amplitude in WT male triceps’ frequency ramp (Figure 4-19B). Therefore, the drop in CMAP during the 30Hz frequency ramp appears to be due to electrical activity but not Ca\(^{2+}\) dependent.
Gender differences in the functional expression of the skeletal muscle channelome

Figure 4-19 Effect of Iberiotoxin and Dantrolene on male WT triceps frequency ramp.

A. 30 Hz frequency ramp in male WT triceps pre (black) and post (red) selective BK channel blockade using an intraperitoneal injection of 1µg iberiotoxin (n=1). B. 30 Hz frequency ramp in male WT triceps (n=4) pre (black) and post (red) intraperitoneal injection of 10mg dantrolene. Pre and post recordings were performed on the same animals.

Left shift in the voltage dependence of NaV1.4 activation could also explain the frequency ramp observations. The NaV1.4 beta subunit is known to modulate the voltage dependence of the alpha subunit. It causes a left shift in the voltage dependence of activation and inactivation (Ilas et al., 2013). Thus, an increase in expression of the SCN1B gene or activity of the NaV1.4 beta subunit protein could explain the greater decrement in amplitude on frequency ramp. In keeping with this, triceps from WT males had a significantly increased SCN1B mRNA (1.3-fold, padj=0.02) compared to TA. There was no significant difference in SCN1B expression between WT female triceps and TA.

Thus, it seems likely that the greater decrement in amplitude of response during the 30Hz frequency ramp in male WT triceps is related to the increased expression of the NaV1.4 beta subunit mRNA transcript. In female triceps, my data suggests, that the disproportionate increase in supernormality is due to the combined effect of increased potassium efflux via BK channels, reduced potassium reuptake and reduced chloride buffering. This may be compensated for by a concomitant increase in transcripts encoding the alpha 3 isoform of the Na K Pump.
The effect of the Draggen mutation on the skeletal muscle ion channelome varies with gender

4.10 The effect of the Draggen mutation on the skeletal muscle ion channelome varies with gender

4.10.1 Male TA

![Male wild-type Vs male Draggen TA](chart)

**Figure 4-20 Differences in gene transcript expression between WT and Draggen male TA.**

The number of genes from each functional group of proteins listed in table 5-3 is shown according to whether they were significantly upregulated in WT male TA (black, n=4) or Draggen male TA (red, n=4).

Male Draggen TA and male WT TA had 1469 differentially expressed genes (table 4-4). This was by far the greatest number of differentially expressed genes for any group comparison. The significant fibre-type transformation that is reported to occur in Draggen TA by 12 weeks is likely to account for at least some of the large number of differentially expressed genes. (Corrochano et al., 2014). One example of this may be the significant reduction in CLCN1 transcript expression (table 4-5) as this is one of the first gene expression changes associated with fibre type transformation to slower twitch muscle (Camerino et al., 2014).

Draggen male TA had MVRC changes consistent with depolarisation and sodium channel inactivation rendering it relatively inexcitable compared to WT (see section 4.7.1). Proteins
The effect of the Draggen mutation on the skeletal muscle ion channelome varies with gender that maintain resting membrane potential or increase t-tubule potassium reuptake were upregulated in Draggen male compared to WT male TA (table 4-5, fig4-20). These include inward rectifying potassium channels (KCNJ2, KCNJ8), inward rectifying potassium channel subunits (ABCC8), the sarcolemmal and t-tubule Na/K ATPase pumps (ATP1A1, ATP1A2) and their auxiliary subunit (ATP1B1). Large (BK) and small (SK) conductance Ca\textsuperscript{2+} activated potassium channels (KCNMA1, KCNN3) were also increased in Draggen male TA as was a BK auxiliary subunit that allows BK channel activity independent of calcium (LRRC52 (Yan and Aldrich, 2012)) and genes associated with store operated calcium entry (ORAI1, STIM1).

Many of these proteins typically have lower levels in slower twitch muscle (Schiaffino and Reggiani, 2011) and therefore the fibre-type change cannot account for the increase in transcript expression. However, increase in expression of these proteins would be predicted to limit depolarisation of male Draggen TA and link K\textsuperscript{+} efflux more tightly to action-potential induced SR Ca\textsuperscript{2+} release. This suggests that Draggen male TA gene expression reflected secondary changes to try to cope with excessive depolarisation and sodium channel inactivation compared to WT.

4.10.2 Female TA

Draggen female TA had increased supernormality compared to WT (section 4.7.2). Only 74 genes were differentially regulated between Draggen and WT female TA. This was the smallest of all the group comparisons. There were no genes related to proteins from my predefined list that were upregulated in WT female TA. Five genes were significantly upregulated in Draggen TA (figure 4-21, table 4-5).

Of these 5, 3 transcripts related to BK channels or their modulation. These were KCNMA1 that encodes the BK channel alpha subunit (1.6 fold increase, padj=0.002), PRKG1 (1.6 fold increase, padj=0.002) a protein kinase which has been reported to increase BK channel activity (Zhou et al., 2010) and LRRC52 (4.5 fold increase, padj =0.01) an auxiliary subunit of BK channels (Yan and Aldrich, 2012).

KCNMA1 and LRRC52 were also significantly upregulated in Draggen male compared to WT male. LRRC52 shifts BK channel voltage dependence in the hyperpolarising direction by approximately -100mV in the absence of Ca and by approximately -70mV in the presence of calcium (Yan and Aldrich, 2012). According to heterologous expression studies, the co-
expression of BK channels with LRRCS2 would be predicted to significantly increase BK channel activity even in the absence of significant increases in Ca\(^{2+}\) (Yan and Aldrich, 2012).

![Female wild-type Vs female Draggen TA](image)

**Figure 4-21** Differences in gene transcript expression between WT and Draggen female TA.

The number of genes from each functional group of proteins listed in table 5-3 is shown according to whether they were significantly upregulated in WT female TA (black, n=4) or Draggen female TA (red, n=4).

As described in section 4.9.3.1 (Figure 4-16) iberiotoxin, a selective BK channel blocker, reduced supernormality. This provides preliminary evidence that greater BK channel activity or expression may augment supernormality and could explain the increased supernormality seen in Draggen female TA compared to WT female MVRCs. The significant increase in BK channel transcript expression in WT female triceps may also account for the lack of genotype difference on female triceps MVRCs. This needs to be confirmed with additional experiments looking at the effect of iberiotoxin on WT as well as Draggen female TA and triceps.

### 4.11 Summary of Functional Expression Data

**Male Vs Female WT TA**
Male WT TA had increased expression of repolarising potassium channel gene transcripts (Figure 4-13) and in contrast to female TA, the effect of CIC-1 blockade was predominantly on late supernormality (Figure 4-14). An increased K+ efflux per action potential could explain the increased supernormality in response to 5 conditioning stimuli that was observed for male WT TA MVRCs compared to female WT TA (Figure 4-4). An increased K+ efflux per action potential would also explain why blocking CIC-1 in male TA had a larger effect on late supernormality as late supernormality is believed to represent the effects of activity-induced potassium accumulation.

**Male Draggen TA**

In male Draggen TA compared to male WT TA, transcripts encoding proteins that maintain resting membrane potential, increase t-tubule potassium reuptake and/or increase the activity of calcium activated potassium channels were upregulated (Figure 4-20). An increase in expression of these proteins would be predicted to limit depolarisation of male Draggen TA and link K+ efflux more tightly to depolarisation induced SR Ca2+ release. Thus it appears that Draggen male TA gene transcript expression reflected compensatory gene expression changes to try to limit the excessive depolarisation indicated on MVRCs.

**Female Draggen TA**

In female Draggen TA compared to female WT TA, 3 out of the 5 significantly upregulated channel genes related to BK channel function. Preliminary experiments with Iberiotoxin, a BK channel blocker, suggest it reduces supernormality on MVRCs. Therefore, an increase in BK channel transcript expression may underlie the increased supernormality of female Draggen TA compared to WT. However, this needs to be confirmed with greater experimental numbers.

**Triceps Vs TA**

Gene transcript expression and pharmacological manipulation suggest the molecular mechanisms underlying the greater supernormality in triceps compared to TA MVRCs vary with gender. In WT female triceps, compared to TA there was massively increased expression of BK channel transcripts, potentially reduced CIC-1 activity and moderately reduced expression of the t-tubule Na K pump transcript.
In male triceps the increase in BK channel transcript expression was 10-fold smaller but was associated with an increase in expression of store operated calcium entry protein transcripts. An increase in store operated calcium entry would augment BK channel activity by increasing cytosolic calcium. In addition, in male triceps, increased expression of the NaV1.4 beta subunit transcript, if translated to an increase in protein levels would be expected to cause a left shift in NaV1.4 inactivation potentially explaining the greater decrement in amplitude of response on the 30Hz frequency ramp.
4.12 Discussion

4.12.1 There is a gender difference in long exercise test results for genetically confirmed patients under 40.

There is a significant gender difference in decrement from peak CMAP amplitude on long exercise test in patients under the age of 40 with genetically confirmed periodic paralysis (Figure 4-1). This is in line with the gender difference in long exercise test reported for a Chinese population (Jin et al., 2017). The observation that in our cohort the gender difference is only significant in those under 40 may explain why previous studies looking at the long exercise test reported no significant gender difference (Tan et al., 2011). The finding of gender difference in excitability limited to those under 40 has also been described in a study of changes in nerve excitability with age and gender (Yerdelen et al., 2006).

The lack, or relative reduction, of acute paralytic attacks in female patients with periodic paralysis has been reported to delay diagnosis, even in families where a diagnosis of periodic paralysis is established (Chalissery et al., 2018). It is highly likely a negative long exercise test would add to that delay. Therefore, this work has important implications for the diagnosis of periodic paralysis and suggests either a lower cut off for a positive long exercise test should be applied to females under 40 as suggested by Jin et al (Jin et al., 2017) or that clinicians should be aware that the long exercise test is more likely to be negative in young females and ascribe less weight to a negative result. In short, a negative test does not exclude PP and might even be expected in a young female.

The lack of significant difference in long exercise test parameters over the age of 40 is intriguing given that it has been frequently reported that in patients with periodic paralysis, the frequency and severity of paralytic attacks decline with age (Links et al., 1990; Sternberg et al., 2001; Miller et al., 2004). This has been reported to occur around the age of 40 and is associated with the onset of permanent progressive weakness (Links et al., 1990; Sternberg et al., 2001). The pathomechanism of this phenotype alteration is currently not known but will be discussed in the next chapter. However, the fact that there are case reports of patients, usually female, presenting at a later age with features of periodic paralysis myopathy without antecedent history of paralytic attacks (Basali, 2015; Chalissery et al., 2018) indicates that the myopathy and occurrence of paralytic attacks may have distinct
pathomechanisms. My personal view is that chronic subclinical electrical disturbance – most likely subclinical depolarisation in the case of Hypo PP and myotonia or hyperexcitability in the case of Hyper PP, predisposes to the development of myopathy. However, regardless of the mechanism, the observation implies that whilst female physiology protects against acute attacks of paralysis it does not prevent the development of permanent progressive weakness. This is in keeping with the observation that whilst the penetrance of paralytic attacks was significantly higher for men with CaV1.1 R528H and R1239H mutations (Sternberg et al., 2001) there was no gender difference in the penetrance of permanent muscle weakness (Sternberg et al., 2001). Similar observations on the gender dependent presentation of acute attack and permanent weakness were also reported for the Draggen mouse model used in this study. Penetrance of the myotonic hind limb dragging episodes was reduced in female Draggen mice; however, they exhibited the same decline in grip strength after middle age observed in their male siblings (Corrochano et al., 2014).

4.12.2 Relevance of physiological gender differences in muscle excitability to pathophysiology of skeletal muscle channelopathies

MVRC and gene transcript expression data reported in this chapter suggest that gender difference in healthy young murine TA muscle excitability may be due to a greater expression of repolarising potassium channels in male muscle and a reduced activity of ClC-1. It remains to be established if the same is true for human skeletal muscle. However, it is reassuring that similar gender differences in gene expression as I found independently in both murine TA and triceps are already published in the cardiac literature from studies on humans and that these have been demonstrated to have clinical significance (Drici Milou D. et al., 1996; Gaborit et al., 2010; Kurokawa et al., 2016; Vink et al., 2018).

Increased expression of repolarising potassium channels is reported in the cardiac literature for human males compared to females. (Pham and Rosen, 2002; Fülöp et al., 2006; Parks and Howlett, 2013; Kurokawa et al., 2016) In cardiac tissue, this increase in repolarising potassium channels in males is protective and is believed to underlie the differential gender susceptibility to long QT syndrome (Drici Milou D. et al., 1996). It is interesting therefore that for one large kindred of 41 members with Andersen Tawil Syndrome, the only skeletal muscle channelopathy with cardiac manifestations, ventricular arrhythmias segregated in
female members and periodic paralysis segregated in male members as autosomal dominant traits (Andelfinger et al., 2002).

4.12.2.1 Hyper PP
Draggen mice carry a mutation that is associated with Hyper PP. All male mice display episodes of hind-limb dragging while less than 50% of females show similar episodes. Moreover, males exhibit a weight deficit relative to WT whereas females do not. The reason for this phenotype discrepancy had not been clear as EMG penetrance of myotonia was 100% for both males and females (Corrochano et al., 2014).

My finding of gender difference in the effect of the Draggen mutation on muscle excitability helps explain the phenotype discrepancy. On the male TA background of increased supernormality, the Draggen gain-of-function NaV1.4 mutation was sufficient to prolong MRRP and reduce supernormality. However, on the female TA background, the Draggen gain of function NaV1.4 mutation increased supernormality and delayed onset of subnormality. Whilst the male Draggen MVRCs would be consistent with a degree of weakness, the increased supernormality and delayed onset of subnormality of female Draggen MVRCs would be consistent with myotonia. As discussed in chapter (3 3.7), delayed onset of subnormality seems to be the murine equivalent of residual supernormality in humans with myotonia (Tan et al., 2014, 2016, 2018). This suggests that male Draggen hind limb dragging attacks reflect the combination of myotonia and weakness whilst female Draggen is predominantly myotonia.

The next step would be to confirm that a similar difference exists in humans with Hyper PP. This is hampered by the condition’s rarity and the increased variability of MVRCs in humans exposed to different environments and with increased genetic heterogeneity. However, if similar differences do exist in humans, an increased K⁺ efflux per action potential combined with reduced ClC-1 activity in male muscle would help explain the gender difference in Hyper PP attack severity as it would effectively increase the concentration of t-tubular potassium to which working muscle is exposed.

4.12.2.2 Hypo PP
Differences in dynamic ClC-1 control provide a possible explanation for the gender difference in acute attacks that has been reported for Hypo PP. As discussed in chapter
(3.7.3) sudden release of ClC-1 inhibition following acidosis causes a transient loss of force in Hypo PP mice (Figure 4-22). The release has to be abrupt to trigger weakness as when pH is gradually returned to baseline over a period of 50 minutes no weakness occurs.

My 9AC data suggests that ClC-1 activity may be greater in female TA compared to male (Figure 4-14). However, there was no significant difference in CLCN1 gene transcript expression. As testosterone has been shown to inhibit ClC-1 to a much greater extent than oestrogen, one potential explanation for the gender difference in acute attacks of Hypo PP is that ClC-1 is under greater dynamic inhibition in working male muscle. This could facilitate greater myoplasmic Cl⁻ accumulation and consequently greater Cl⁻ efflux and sarcolemmal depolarisation if ClC-1 inhibition is abruptly released.

4.12.2.3 Myotonia Congenita

Reduced ClC-1 activity in male TA would certainly help explain gender difference in phenotype severity in patients with dominant Myotonia Congenita. However, the largest gender difference in prevalence in our channelopathy clinic was reported for patients with autosomal recessive MC (Horga et al., 2013). Some of these patients have two mutations
predicted to result in nonsense-mediated decay and therefore it seems unlikely that active CIC-1 inhibition could account for the observed gender difference. However, physiologically increased $K^+$ efflux in male muscle would exacerbate any pathological reduction in chloride conductance as the need for effective chloride buffering of potassium is increased. This was suggested by the increased gender difference in late supernormality on MVRCs when CIC-1 is blocked by 9AC (4.9.1.2). Thus, an increased $K^+$ efflux per action potential as a result of increased expression of delayed rectifier $K^+$ channel transcripts in male muscle could help account for the gender difference in MC severity.

In summary, my data suggests that an exacerbation of physiological gender difference in muscle excitability may explain gender differences in the phenotype of skeletal muscle channelopathies.

4.12.3 Up-regulated genes encode proteins targeted by first line periodic paralysis therapies

BK channel transcript expression was upregulated in Draggen compared to WT muscle and in triceps compared to TA. Acetazolamide, the first line therapy for periodic paralysis and an adjunctive therapy for myotonia, amongst other actions, activates BK channels (Dinardo et al., 2012). However, the exact mechanism of acetazolamide’s therapeutic effect in channelopathies remains unknown. It is generally believed its therapeutic effect in periodic paralysis is because of inducing systemic acidosis rather than any local effects on muscle excitability. However, Acetazolamide reduced the severity of induced attacks on isolated CaV1.1 muscle in vitro by 50% (Wu et al., 2012; Mi et al., 2019) and therefore, in addition to systemic metabolic effect must have a direct effect on muscle.

Murine MVRCs would be a powerful tool to investigate acetazolamide’s effect on skeletal muscle excitability further and help clarify its mechanism of action in periodic paralysis. Firstly, comparing MVRCs after acetazolamide administration in vivo and ex vivo would help distinguish acetazolamide’s direct effect on muscle excitability from an indirect effect as a result of systemic acidosis. Secondly, the effect of acetazolamide on specific ion channels could be delineated by combining acetazolamide with specific ion channel blockers.

Activation of BK channels is one interesting possibility raised from this gene expression data
and by other groups previously (Dinardo et al., 2012) and could be tested using iberiotoxin (Figure 4-16).

The RNA expression data suggest that 1. physiological adaptions in Draggen muscle may mirror the pharmacological treatments we give patients to try to reduce symptoms and 2. differences in gene transcript expression between muscles may also determine which muscles are most susceptible to NaV1.4 gain-of-function mutation.

4.12.4 Importance of these findings.

Neuroscience is one of the areas where there is the greatest sex bias in research with single sex studies on male animals outnumbering those on female animals by 5.5 to 1 (Beery and Zucker, 2011). However, if the ionic basis of skeletal muscle excitability and the effect of the same pathogenic mutation differ between males and females, it is likely that treatments will also vary in their efficacy and diagnostic tests in their sensitivity (0). Therefore it is crucial that preclinical and clinical data are obtained from both genders and the data examined for any potential sex difference before combining (Shansky, 2019).

Based on my findings, data from young and older adults need to be examined separately for any potential sex difference. This is crucial for the design of both pre-clinical and clinical studies in the future. If not, as for the long exercise test for the diagnosis of periodic paralysis, this may lead to the development of investigations and medications that are less sensitive or effective for one gender.
Motivation

5 Change in Periodic Paralysis Phenotype with Age

5.1 Motivation

The pathophysiology of acute attacks of periodic paralysis is reasonably well understood and much work has shown that, for all types of PP, partial muscle membrane depolarisation triggered because of dysfunctional channels results in the attacks of weakness (Hayward et al., 2008; Struyk and Cannon, 2008; Jurkat-Rott et al., 2009; Wu et al., 2011, 2012; Suetterlin, Männikkö and Hanna, 2014). Initially muscle strength is normal in between attacks of paralysis. However, an unexplained but consistent clinical feature is that with increasing age, a second clinical phase is observed in which attack frequency declines and a severe, often disabling myopathy develops (Links et al., 1990; Miller et al., 2004). It is unclear why dysfunction of implicated ion channels exhibits this biphasic natural history. However, in my personal experience from seeing patients in the NHS Highly Specialised Service Skeletal muscle channelopathy clinic, patients often say the onset of permanent progressive weakness is the most distressing part of their condition and the one they are most concerned about for their children.

There is 1 published study, that has attempted to systematically examine the change in phenotype with age (Links et al., 1990). It was before the genetic era and looked at increase in hypodense regions in muscle with age on CT in a family with periodic paralysis (Figure 5-1A). Unpublished work by Dr Jasper Morrow at our centre demonstrates a significant increase in periodic paralysis patients’ MRI muscle fat fraction over the age of 40 (Figure 5-1B). I am not aware of any published work investigating the mechanisms behind a reduction in attack severity with age.
Motivation

Figure 5-1  Studies on age related change in periodic paralysis

A. Whole body CT was performed on affected members of a family with periodic paralysis. CT was graded according to hypodense regions in muscle (score of 0 - 4) neck, abdomen, pelvis, upper thorax, thigh, lower leg and the score from different muscles summed to give a whole body score that is presented according to age. (Links et al., 1990) B. Mean MRI muscle fat fraction from 12 patients with genetically confirmed Hypo PP compared to 12 age-matched healthy controls (Dr Jasper Morrow, unpublished).

This change from an episodic to a degenerative phenotype is a phenomenon not limited to periodic paralysis. It has also been observed in CNS channelopathies e.g., episodic ataxia. This suggests it is not a muscle-specific phenomenon and a more systemic change may be the cause. It is striking therefore that, 40 is also the age at which optimal motor performance declines (Schott, 2017), muscle mass starts to decline ((Smith and Mittendorfer, 2016) and certain abnormalities start to be accepted as within the normal range for age on muscle biopsy (e.g. up to 5 cox negative fibres (Pesce et al., 2001)) (Figure 1-6). This suggests the possibility that ‘normal’ age-related change may contribute to the phenotype transition in periodic paralysis.

Skeletal muscle channelopathies are rare, phenotypically heterogeneous and the attacks are often triggered by a range of environmental factors. This makes systemic examination of phenotype change with age, especially in relation to attack severity, extremely challenging. There are four published mouse models of periodic paralysis (see 3.2.1). Although there is
difference in phenotype and, as discussed in chapter 3, muscle excitability, between mice and humans, using a mouse model for preliminary investigations into age related change is still an attractive experimental approach. Firstly, because PP is a dominant condition and therefore heterozygous mice with periodic paralysis mutations can be studied. Comparing heterozygote mice with their WT siblings means that changes due to ‘normal’ ageing (e.g., phenomenon also observed in WT) can be distinguished from changes due to ageing in the presence of single ion channel dysfunction (phenomenon observed in mutant mice only). Secondly, inducing a paralytic attack in a mouse model of periodic paralysis is an attractive way of looking to see if a reduction in attack severity with age is a genuine physiological phenomenon as environmental variables can be controlled, and the assay performed on mice of the same sex and genetic background thus increasing the power to detect any difference due to age alone. Thirdly, mice can go from a human equivalent age of young adult to elderly within 2 years whereas the corresponding time frame in a human would be at least 50 years. Finally, ageing research has demonstrated that many of the key features of aging are conserved across mammalian species.

Heterozygote Draggen mice exhibit episodic attacks of hind-limb dragging, the number and severity of which can be very variable. However, an attack of weakness can be reliably induced ex vivo by exposure of Draggen muscle to a high potassium solution. The heterozygote Draggen mice also reproduce onset of fixed weakness with a progressive decline in grip strength from middle age as well as classic histological features of PP myopathy on muscle biopsy (Corrochano et al., 2014). Therefore, in this study, Draggen mice were used to characterise changes in ageing muscle and compared with ‘normal’ ageing observed in muscle from wild-type (WT) littermates.

5.2 Aims

The aims of the experiments described in this chapter are:

1. Confirm whether a reduction in the severity of potassium-induced weakness and the onset of permanent progressive weakness occurs with age in a transgenic mouse model of periodic paralysis (as is observed in humans with periodic paralysis).
2. If so, determine whether this phenotype change is due to ‘normal’ age related change or ageing in the presence of single ion channel dysfunction.
3. Explore the pathophysiology behind any observed phenotype change with age.

5.3 Specific Methods

5.3.1 Mice

5.3.1.1 Wheel

Some animals had a voluntary running wheel added to their cage. This was a standard plastic (flying saucer) running wheel. For the 24 to 48 hours preceding an experiment a subset of these had a modified version of the same wheel that could measure the number of wheel rotations. This was designed by Dr Marco Leite who kindly lent the equipment to me.

5.3.2 Isolated Muscle Tension testing.

5.3.2.1 Experimental setup

The EDL or soleus muscle was dissected and mounted horizontally in a bath containing physiological solution (NaCl 118mmol; KCl 2mmol; MgSO₄ 1.18mmol; CaCl₂ 2.54mmol; NaH₂PO₄ 1.18mmol; Glucose 10mmol; NaHCO₃). The bath was the centre well of a three-well custom-made chamber designed by Scientific Systems Design Inc and purchased via Digitimer (Figure.5-2). The proximal muscle tendon was tied to a steel bar within the muscle chamber. The distal tendon was tied to an isometric force transducer (Dynamometer UFI Devices). Two muscles could be mounted simultaneously in the central chamber. Fluid in all three chambers was maintained at 30 degrees Celsius and continuously bubbled with 95% O₂, 5% CO₂. Temperature was measured using an infrared thermometer.

Tetanic stimuli were delivered using square wave pulses of 0.02 milliseconds duration at supra-maximal intensity and applied via platinum wires positioned either side of the muscle. Muscles were adjusted to their optimal preload length to produce maximal tetanic contraction. Isometric contractile responses were recorded using a pen recorder (Lectromed Multitrace 2) and digitized with PicoScope PC Oscilloscope 4424.
Figure 5-2 Experimental setup for muscle tension testing ex vivo

5.3.2.2 Caffeine contracture force

To measure caffeine contracture force baseline twitch and tetanic force measurements were first taken as described above (5.3.2.1). Caffeine (50mM) solution was made by adding caffeine powder to the baseline muscle chamber solution (NaCl 118mmol; KCl 2mmol; MgSO$_4$ 1.18mmol; CaCl$_2$ 2.54mmol; NaH$_2$PO$_4$ 1.18mmol; Glucose 10mmol; NaHCO$_3$) and mixing on a roller at room temperature for 20 minutes. The caffeine solution was pre-oxygenated and warmed in one of the side chambers to the same temperature as the main chamber (30°C) (Figure 5-2). Solution was removed from the main chamber using a 5ml syringe and the side chamber solution plug was removed so the 50mM caffeine solution could flow into the main chamber. The subsequent caffeine contracture was recorded via the force transducer as described in 5.3.2.1.

5.3.2.3 Induced paralytic attack assay

Isolated soleus muscles from young (13 to 26 week), middle aged (55 to 75 week) and old (95 to 104 week) Draggen mice and their WT littermates were used. The human age equivalents are approximately 20 to 30 years, 43 to 50 years and 65 to 70 years respectively (The Jackson Laboratory, 2020).
I used the same ionic conditions as described by previous groups examining the M1592V hyperkalaemic periodic paralysis mouse model (Hayward et al., 2008; Lucas et al., 2014; Ammar et al., 2015; Khogali et al., 2015). This consists of 10 minutes of stable (within 1g) baseline force measurements in 4.75mM K+ and 2.34mM Ca2+, followed by 20 minutes in 10mM K+ and 1.3mM Ca2+ followed by 20 minutes in 4.75mM K+ and 4mM Ca2+. The concurrent changes in calcium were included as they were found to exaggerate the differences between Hyper PP mutant and WT mice. Muscles were maintained at 30˚C, solutions were bubbled with 95% O2 and 5% CO2 and tetanic force measured by 50mV stimulation at 100Hz every 2 minutes during baseline and high potassium solutions and at 2, 5, 10, 15 and 20 mins during recovery solution.

5.3.2.4 Analysis

Picoscope software was used to store and analyse the data. Muscles that had a baseline maximal tetanic force that was significantly weaker (>35% difference) compared to the contralateral muscle exposed to the same conditions were excluded as likely artefactual recordings secondary to injury. Similarly, muscles that had a caffeine contracture force / tetanic force ratio of >40% were excluded as this is associated with eccentric injury (Ingalls et al., 1998).

5.3.3 Histology

Soleus muscles from young, middle aged and old (age range 13 – 103 weeks) adult male WT and Draggen mice were dissected in combination with gastrocnemius under isoflurane anaesthesia, snap-frozen in isopentane and 10µm sections were cut in a cryostat. Sections were stained using Haemotoxylin and Eosin (H&E), Cytochrome Oxidase (COX) and Succinate dehydrogenase (SDH) using standard protocols (Muscle Biopsy - 5th Edition, no date). Semi-quantitative analysis was performed, assessing structural pathology, COX-negative fibres and fibre typing, whilst blinded to animal age and genotype.

5.3.4 Electron Microscopy

Soleus muscles were pinned on cork board at approximately resting length before fixation in 3% EM grade glutaraldehyde in 0.1M Sodium cacodylate buffer and 5mM CaCl2 pH7.4. Secondary fixation in 1% aqueous OsO4 was followed by dehydration through graded ethanol followed by propylene oxide, and then embedding in Araldite resin. Ultrathin
sections (60nm) were cut and placed on copper mesh grids, stained with 25% uranyl acetate in methanol, and Reynold’s lead citrate. All reagents were purchased from Agar Scientific (Essex, UK). The fixation and preparation of tissues for electron Microscopy was performed by Kerry Venner, EM department, Institute of Neurology, UCL.

Tissues were examined using a Philips CM12 TEM with Kodak MegaView G3 imaging system and Emsis (www.emsis.eu) Radius software. Images were taken and analysis was performed whilst blinded to animal age and genotype.

To ensure random selection of areas for analysis, a standardised procedure was used to select the region from which to take images. The edge of the sample was first found using low magnification (e.g.x620) and to avoid damage from processing and to try to ensure internal fibre area were selected, moved in from the edge by one diagonal grid square. An image was then taken of the centre of each diagonal grid square at x11500 magnification. This process was continued until we encountered the sample edge and then repeated while moving back at 90 degrees from the initial direction so as to zig zag across the sample.

If there was a region of clear artefact or the presence of a large blood vessel, then the image was taken of the closest unaffected region or, if that was not possible, the affected grid was skipped entirely. A minimum of 7 different fibres were included for each muscle.

5.3.4.1 Stereological analysis

Stereology is based on geometrical and statistical considerations and allows unbiased estimates of volumes, surfaces, lengths and numbers of the structures of interest (Weibel, 1969; Mobley and Eisenberg, 1975; Tschanz, Burri and Weibel, 2011). Images are analysed by superimposing ‘test systems’ on an image of known area. All images were taken at x10500 magnification with an imprinted scalebar on the digitally acquired image. I used STEPanizer, a stereology counting system that is freely available to perform stereology assessments (Tschanz, Burri and Weibel, 2011). STEPANIZER is able to scale the image to the pixel resolution of a monitor by manually measuring the scale bar and calibrating the image appropriately. Test systems can then be created to enable volume, length, surface area or number estimation. There is a counting module, so that different features can be counted and labelled on the same image (Figure 5-3) and an export function for the transfer of data to an excel spread sheet” (Tschanz, Burri and Weibel, 2011).
As for histological analysis, I was blinded to all demographic data (e.g. genotype and age of muscle) during the analysis.

5.3.4.1.1 Volume assessment
A structure’s volume within a tissue can be estimated using a Point counting test system (Figure 5-3) (Weibel, 1969; Tschanz, Burri and Weibel, 2011). Points are defined as corners of the crossing of horizontal or vertical lines as this defines an infinite small point (Figure 5-3). For mitochondrial volume analysis 0.5µm spacing between points was used as is recommended for structures with over 1% density(Mobley and Eisenberg, 1975). Therefore, a points test system with 81 tiles was used on STEPanizer (Figure 5-3). Severely altered mitochondria were excluded from the volume analysis. Severely altered mitochondria were defined as mitochondria with any or several of: clear disruption of the external membrane, severe vacuolisation and disruption of the mitochondria internal cristae, mitochondria containing myelin figures as per previous published study (Pietrangelo et al., 2015).

![Figure 5-3 STEPanizer stereology counting software to estimate the volume of mitochondria per field a grid of defined area is used and every time a mitochondrion falls on a point it is counted](image)

5.3.4.1.2 Structure Counting
To estimate the number of a certain structure within a tissue, the number of that structure that are observed within a defined area are counted (Figure 5-4)(Weibel, 1969; Mobley and Eisenberg, 1975; Boncompagni et al., 2006; Tschanz, Burri and Weibel, 2011; Pietrangelo et al., 2015).
For mitochondrial number, morphology and location, mitochondria were counted as described by Boncompagni et al previously (Pietrangelo et al., 2015). Namely, if an individual mitochondrion extended from I to A band it was counted for both. If it extended all the way across to the adjacent I band then it would be counted for two I bands and one A band (Figure 5-4). Results are presented as numbers /100 µm². Only those images where Z bands, I bands and A bands were clearly discernible were used (Figure 5-4).

For CRU number counting structures formed by the association of SR and transverse tubules were identified and counted as CRUs (Figure 5-4).

![STEPanizer stereology counting software to estimate the number of structures per field of defined area. In this case it is mitochondria.](image)

5.3.4.2 Statistical Analysis

To compare groups, rather than pool all images, the mean value for each animal was used to calculate a group mean. This was done as some samples had more images than others. Therefore, I felt using individual means to calculate the group (e.g. old WT) mean was more
Specific Methods

representative as it avoided unduly weighting the group mean towards the muscle with the most images.

5.3.5 Protein Quantification

5.3.5.1 Protein extraction

Sample buffer (Tris HCl 75mM, pH 6.8, SDS 1%) was prepared. Lysis buffer was made by adding 1 tablet of Roche Complete Mini Protease inhibitor (14583920) to 7mls of sample buffer and kept on ice. Samples were placed in a lysing matrix M tube (MPBio) and 70 to 150µl lysis buffer added (depending on the size of the sample). Samples were homogenised with either 2 x 60 second runs at 4.0m/s or 1x50 second run at 6.0m/s using the MP fast prep 24 homogeniser. To begin with, samples were spun at 4°C for 10 minutes at 10,000 g following lysis. However, as several of the tubes cracked and the samples were lost, this was changed to a 30 second spin at room temperature. Supernatant was extracted with a pipette.

Protein was quantified using Bradford reagent, the Bio Rad DC Protein quantification assay or the Pierce protein quantification assay. These processes were very similar and the appropriate manufacturer instructions were followed. To ensure sample concentration in the working range of the assay, samples were diluted 1 in 20 in sample buffer. Standards were also diluted in sample buffer. Standards were of Bovine Serum Albumin (BSA) and contained 2µg/µl, 1.5µg/µl, 1 µg/µl, 0.75 µg/µl, 0.5 µg/µl, 0.125µg/µl, 0.025µg/µl and 0 µg/µl. Standards and samples were measured in triplicate for each dilution, unless yield had been very low, in which case they were measured in duplicate.

5.3.5.2 Western Blotting

For each sample, 5µl NuPAGE LDS Sample Buffer (4X) was first mixed with 2µl of NuPAGE Sample Reducing Agent (10X) before adding 20µg sample protein that was diluted to make a total volume of 20µl. Samples were loaded onto NuPAGE Tris-Acetate 3-8% 15 well mini gels. 10µl Himark (Thermo fisher) and/or 5µl Seeblue (Thermo fisher) pre-stained protein standards were used.

A ThermoFisher mini gel tank was used for gel electrophoresis. Electrophoresis was performed using NuPAGE Tris-Acetate SDS Running Buffer (20X) with 2.5ml NuPAGE
Antioxidant per litre of running buffer. The gel was run for 2 hours either in the cold room (4°C) or with the tank surrounded by ice.

Wet transfer was performed using BioRad tanks at 30V for 5-6 hours either in the cold room (4°C) or with the tank surrounded by ice. The gel was transferred onto a nitrocellulose membrane using 7.5 x 10 cm thick Blot Paper (BioRad). Following transfer, most membranes were stained with Ponceau to measure total protein and a photo taken. They were then washed with TBS prior to incubation in TBS with 10% milk for 1 hour at room temperature or overnight at 4°C. Membranes were then incubated with anti-RyR1 (abcam 2868) 1:1000 in TBST for a minimum of 1 hour at room temperature on a shaker. After primary antibody incubation membranes were washed 3 times for 10 minutes in TBST before incubation with an HRP conjugated secondary antibody at a dilution of 1:4000 (sc 2005#A2216 goat anti-mouse) for a minimum of 1 hour at room temperature. After secondary antibody incubation membranes were washed 3 times for 10 minutes in TBST. Luminata crescendo forte was applied to the membrane and images taken using Chemidoc MP imager in chemiluminescence blot mode. Membranes that were not stained with ponceau immediately after transfer were stained after chemiluminescence was performed and a photo taken using the Chemidoc MP imager on colorimetric mode.

5.3.6 Metabolic Assays (Methods provided by Dr Mike Orford)

5.3.6.1 Energy Charge

The energy charge assay was performed by Dr Michael Orford at the Institute of Child Health, UCL. To extract the adenine nucleotides, TA muscle samples which had been immediately flash frozen in liquid nitrogen following dissection were weighed and homogenized in 0.5ml ice cold 1.0 M perchloric acid. 250μl of the homogenate was neutralized with 200μl 0.5M KHCO3 in 1M KOH. The precipitated proteins and potassium perchlorate produced were removed by centrifugation at 13.000x g for 5 min and the clear supernatants stored at -20°C until derivatization. 100 μl of 1.0 M sodium acetate (pH 4.5) and 20 μl 4M chloracetaldehyde were mixed with 100 μl of the neutralized extract, and heated at 60°C for 40 min. After the incubation, samples were placed on ice for 5 min to cool and halt the reaction. Subsequently, 20 μl of the cooled derivatised sample was analysed by HPLC using a C18 reversed phase column (Hypersil 5 ODS 4.6 x 150 mm, 3 μm) at a flow rate of 0.8ml/min using a gradient from 100% 0.2 M KH2PO4, pH 5.0 to 98.9% 0.2
M KH2PO4, pH 5.0 1.1% acetonitrile over 31 minutes. Etheno-adenine nucleotides were determined by fluorescence detection at excitation/emission spectra pairs of 290ex/415em nm. Peaks corresponding to the retention times for ATP, ADP and AMP were integrated and peak areas obtained were used to calculate AEC using the equation:

\[
AEC = \frac{[\text{ATP}]}{[\text{ATP}]+[\text{ADP}]} + 0.5 \frac{[\text{ADP}]}{[\text{ATP}]+[\text{ADP}]+[\text{AMP}]}
\]
Results 1: Is the change from an episodic to a degenerative phenotype with age replicated in an animal model of periodic paralysis?

5.4 Results 1: Is the change from an episodic to a degenerative phenotype with age replicated in an animal model of periodic paralysis?

5.4.1 There is increased resistance to the induced paralytic attack with age in both Draggen and WT mice

Soleus and EDL are the two muscles that are most suited for studying the induced ex vivo attack. This is because they can be dissected in their entirety, with tendons attached, and oxygenation can be maintained relatively well ex vivo. I chose to focus on slow-twitch soleus rather than fast-twitch EDL during these experiments for several reasons. Firstly, because soleus is the mouse muscle that is most similar to human skeletal muscle in terms of gene expression and fibre-typing (Kho et al., 2006). Secondly, as Draggen TA has been shown to switch towards a more oxidative fibre type as early as 12 weeks of age (Corrochano et al., 2014), it seemed sensible to choose a slow twitch oxidative muscle. This was to avoid simply measuring the effect of fibre type transformation in the functional assays and to increase likelihood of translation as human muscle is already predominantly oxidative. Thirdly, soleus is more susceptible to induced attacks of weakness than EDL (Ammar et al., 2015; Khogali et al., 2015). Therefore, it is likely that age-related resistance to induced attacks of weakness would be easier to detect in soleus.

Isolated soleus muscles from young (13 to 26 week), middle aged (55 to 75 week) and old (95 to 104 week) male Draggen mice and their male WT littermates were used. The human age equivalents are approximately 20 to 30 years, 43 to 50 years and 65 to 70 years respectively. The study was limited to males in the first instance as penetrance is incomplete in females.

I used the same ionic conditions as described by previous groups examining the M1592V hyperkalaemic periodic paralysis mouse model (Hayward et al., 2008; Lucas et al., 2014; Ammar et al., 2015; Khogali et al., 2015). This consists of 10 minutes of stable (within 1g) baseline force measurements in 4.75mM K+ and 2.34mM Ca2+, followed by 20 minutes in 10mM K+ and 1.3mM Ca2+ followed by recovery in 4.75mM K+ and 4mM Ca2+. The concurrent changes in calcium were included as they were found to exaggerate the differences between Hyper PP mutant and WT mice (Hayward et al., 2008; Clausen et al.,
Results 1: Is the change from an episodic to a degenerative phenotype with age replicated in an animal model of periodic paralysis? (2011; Khogali et al., 2015). Muscles were maintained at 30°C, solutions were bubbled with 95% O2 and 5% CO2 and tetanic force measured every 2 minutes by 50mV stimulation at 100Hz.

![Figure 5-5](image)

**Figure 5-5** Changes in potassium-induced weakness with age in WT and Draggen soleus. A. Potassium-induced weakness assay for young (black, n=5) Vs middle aged (blue, n=4) Vs old (red, n=5) wild-type (WT) soleus muscles. B. Potassium-induced weakness assay for young (grey, n=5), Vs middle aged (light blue, n=5) Vs old (light red, n=8) Draggen soleus muscles. All force measurements are normalised to the mean force from the 10-minute baseline period and shown +/- SEM. *One-way ANOVA used to compare the force after 20 minutes of hyperkalaemic, hypocalcaemic conditions for all groups. **p<0.01

Old WT mice were significantly more resistant to potassium induced loss of force than either young or middle-aged ones (Figure 5-5A, one-way ANOVA at 30 minutes p=0.009, post hoc Tukey test p=0.01 Old vs young, p=0.02 Old vs middle aged). Old Draggen mice were also significantly more resistant to potassium induced loss of force than either young or middle-aged ones (Figure 5-5B, one-way ANOVA at 30 minutes p=0.007, post hoc Tukey p=0.02 vs young, 0.01 vs mid age). No significant differences were found between young and middle-aged groups in WT or Draggen mice.

Changes in baseline force with age were not responsible for the apparent resistance to induced paralytic attack as resistance to loss of force in high potassium, low calcium solution was found in old muscles (95-104 weeks) with both low and high baseline force (see fig5-6 A&B). In two cases old WT soleus’ tetanic force actually increased during exposure to the
Results 1: Is the change from an episodic to a degenerative phenotype with age replicated in an animal model of periodic paralysis?

high potassium low calcium solution (Figure 5-6A). This was never observed in young muscles (Figure 5-6A).

Figure 5-6 Increased resistance to the induced paralytic attack assay with age is not due to differences in absolute force. A Tetanic force at baseline and after 20 minutes exposure to high potassium solution. Results are from individual young (YW), middle-aged (MW) and old (OW) WT soleus. D. Tetanic force at baseline and after 20 minutes exposure to high potassium solution. Results are from individual young (YD), middle-aged (MD) and old (OD) Draggen soleus.

There was also a difference in response to the hypercalcaemic, normokalaemic recovery solution with age and genotype. While force recovered fully in the Draggen mice following return to the recovery solution, the recovery was incomplete in WT mice (end of assay force – baseline force= -4.3 ±1.7g WT Vs -0.1 ±1.5g Draggen, p=0.01 (Figure 5-5). In fact, old WT soleus actually lost force during exposure to the recovery solution while young and middle aged WT muscle showed incomplete recovery as has been observed previously (Hayward et al., 2008).

In summary, reduction in paralytic attack severity with age also occurs in a transgenic mouse model of periodic paralysis. The fact that resistance to potassium-induced muscle weakness was observed in old WT mice implies reduction in attack severity in Draggen mice is at least in part, a phenomenon of ‘normal’ ageing rather than the chronic consequence of ion channel dysfunction. This suggests that in humans, like Draggen mice, the reduced severity of paralytic attacks with age may be due to features of normal muscle ageing that are conserved across a broad evolutionary distance.
Results 1: Is the change from an episodic to a degenerative phenotype with age replicated in an animal model of periodic paralysis?

5.4.2 Draggen mouse soleus develops permanent muscle weakness with age

A decline in grip strength from middle age has already been demonstrated in male and female Draggen mice using in vivo measures (Corrochano et al., 2014). Soleus muscle tetanic force was not measured. Therefore, I wanted to confirm that, in addition to the reduction in attack severity, male Draggen soleus also exhibited the onset of permanent progressive weakness with age that has been observed in periodic paralysis patients.

Figure 5-7 Soleus baseline tetanic force according to age. There is no significant change in the baseline tetanic force of male WT soleus with age up to 104 weeks (ANOVA of linear fit, p=0.14). There is a significant reduction in male Draggen soleus baseline tetanic force with age up to 104 weeks (ANOVA of linear fit, p=0.03). There is no significant difference in rate of decline between Draggen and WT soleus (p=0.8), but the intercept was significantly different (p=0.005)

There was a significant reduction in baseline tetanic strength in old male Draggen but not WT soleus with age (p= 0.03Vs p=0.9, ANOVA of linear fit, Figure 5-7) although the slope of decline did not differ between WT and Draggen (p=0.8). However, the intercept of linear fits
5.5 Results 2: Exploring the pathophysiology behind the increased resistance to K+ induced muscle weakness with age

5.5.1 Age dependent changes in muscle excitability

Loss of force during high potassium-induced weakness is due to depolarisation of the muscle membrane and consequent inactivation of Nav1.4 sodium channels (Hayward et al., 2008). Sarcolemmal membrane potential is determined by membrane permeability, intracellular and extracellular ionic concentrations. The extracellular ionic concentrations in this assay were constant; therefore, variation in extracellular K+, Cl−, and Na+ can be excluded as a potential cause of the change in induced attack severity with age.

However, a significant change in intramuscular ionic concentration would be expected to affect membrane permeability and/or resting membrane potential. Therefore, to look for changes in muscle excitability with age, in collaboration with Dr Veronica Tan and Professor Hugh Bostock, I used MVRCs.

I was not able to perform MVRC on soleus as it is not possible to be certain in vivo that you are recording from soleus and additionally significantly more animals would be required to do both MVRC and the induced paralytic attack assay on soleus. I chose TA as an alternative, as it is the muscle used for MVRC in humans and it has been shown to exhibit permanent weakness and features of periodic paralysis myopathy in 60 week old Draggen mice (Corrochano et al., 2014). I chose triceps because in contrast to TA, it is reported to be resistant to sarcopenia (Pannerec et al., 2016); therefore any differences in excitability may be of interest for normal muscle ageing. I chose two muscles as I wanted to be able to distinguish an effect specific to an individual muscle from a more systemic one.

I performed MVRCs on TA and triceps from 13- to 26-week-old, 55 to 75 week old and 95 to 104 week old, wild type and Draggen mice using the technique as described for humans.
Results 2: Exploring the pathophysiology behind the increased resistance to K+ induced muscle weakness with age

There were no significant differences in MVRCs in response to 1, 2 or 5 conditioning stimuli with age in either Draggen or WT TA or triceps (Figure 5-8).

The lack of significant change in any of the MVRC parameters with age suggests that an alteration in baseline muscle excitability is not behind the reduction in attack severity or onset of permanent progressive weakness with age. However, a resistance to K+ induced depolarisation may not have been detected as MVRCs were performed on resting muscle without exposure to a high potassium solution.
Results 2: Exploring the pathophysiology behind the increased resistance to K+ induced muscle weakness with age.

Figure 5-8 Muscle Velocity Recovery Cycles (MVRCs) in TA and Triceps muscles for young, middle-aged and old WT and Draggen mice.  A. WT TA MVRCs in response to 1 conditioning stimulus  B. WT TA MVRCs in response to 5 conditioning stimuli C. Draggen TA MVRCs in response to 1 conditioning stimulus D. Draggen TA MVRCs in response to 5 conditioning stimuli E. WT triceps MVRCs in response to 1 conditioning stimulus F. WT triceps MVRCs in response to 5 conditioning stimuli G. Draggen Triceps MVRCs in response to 1 conditioning stimulus. H. Draggen Triceps MVRCs in response to 5 conditioning stimuli. WT TA young n=25; middle-aged, n=17, old, n=17. Draggen TA young, n=15; middle-aged, n=12 old, n=12. WT Triceps young, n=23; middle-aged, n=6, old, n=13. Draggen triceps young, n=14; middle-aged, n=3, old, n=10. Black= young, Blue = middle-aged, Red= old. Values are mean ± SEM, n=number of individual muscles per group.
Results 2: Exploring the pathophysiology behind the increased resistance to K+ induced muscle weakness with age

As MVRCs are done *in vivo* it is not possible to directly control the K\(^+\) concentration surrounding the muscle. However, a single action potential can increase [K\(^+\)] on the muscle fibre surface from 2.5 to 4mM and a train of 6 action potentials at 10Hz can increase it from 2.5 to 10mM (Sejersted and Sjøgaard, 2000). Therefore, potassium can be increased, as would occur with exercise, by increasing the frequency of stimulation.

The loss of force in the induced attack assay is accompanied by a reduction in CMAP amplitude that reflects depolarisation of the membrane and inactivation of NaV1.4 channels (Hayward *et al.*, 2008). Therefore, to look for evidence of a change in response to activity-induced potassium accumulation with age I examined the effect of increasing frequency of muscle stimulation on CMAP amplitude of TA *in vivo*. To do this I used the 30Hz frequency ramp (See 2.2.1.2) as described for humans previously (Tan *et al.*, 2014, 2017).

![Figure 5-9 Response of WT and Draggen TA to in vivo 30Hz frequency ramp by age. A. Response of WT TA to 30 Hz frequency ramp. Young (13-26 weeks, black n=22), middle aged (55-75 weeks blue, n=13) and old (95-104 weeks red n=14) mice. B. Response of Draggen TA to 30 Hz frequency ramp. Young (13-26 weeks, grey n=15), middle aged (55-75 weeks light blue, n=8) and old (95-104 weeks light red n=9) mice. The change in latency (time from stimulus to peak of response) is plotted on the top row as a percentage change from baseline latency. The change in amplitude of response is plotted on the middle row as a percentage change from baseline amplitude of response. During the frequency ramp (shown on bottom row) the percentage change in response to both the first and last stimulus in the train are shown. The baseline is the mean latency and amplitude of response during the period of 0.5Hz stimulation that precedes the frequency ramp. Data are mean ± SEM. (Kruskal Wallis ANOVA and post hoc t-test with welch correction <0.005 <0.0001)
30 Hz frequency ramp recordings were performed on the TA of young, middle-aged and old WT and Draggen mice (Figure 5-9). Surprisingly, rather than showing increased resistance to CMAP amplitude decrement, Old WT TA had a significantly greater decrement to both the first and the last stimulus in the train at both 15 and 30Hz than young or middle-aged TA (Figure 5-9A). The CMAP amplitude in old WT TA also failed to recover to baseline after 30s rest (Figure 5-9A). For Draggen TA there was no significant change in response to the 30Hz frequency ramp with age (Figure 5-9B).

This evidence suggests that the maintenance of tetanic force in old muscle subject to the K$^+$ induced paralytic assay is not due to a resistance to K$^+$ induced sarcolemmal depolarisation. Instead, the data are consistent with old muscle developing mechanisms to maintain force despite sodium channel (NaV) inactivation and sarcolemmal depolarisation.

5.6 Results 3: Exploring the pathophysiology behind the onset of a degenerative phenotype with age in Draggen mice

5.6.1 Caffeine contracture force is significantly reduced in old Draggen soleus

Muscle contraction is composed of three major stages: the triggering and propagation of an action potential (muscle excitability), the conversion of an action potential into calcium release (excitation-contraction coupling) and calcium-induced muscle contraction. Underpinning these three stages is the need for sufficient ATP to maintain ionic homeostasis (Figure 1-2).

I found no significant changes in WT or Draggen muscle velocity recovery cycles with age (Figure 5-8) and although, there was a decline in amplitude of response during a 30Hz frequency train in old compared to young or middle-aged WT TA the force deficit was specific to Draggen TA (Figure 5-9). This suggests that significant changes in muscle excitability are not the cause for the permanent progressive weakness in Draggen muscle.

Therefore, the aetiology of the permanent progressive weakness is likely to lie with a failure of excitation-contraction (EC) coupling, calcium-induced muscle contraction or with an impairment of energy homeostasis (Figure 1-2). To investigate excitation-contraction coupling I compared caffeine contracture force with tetanic force from supramaximal electrical stimulation. Caffeine acts directly on RyR1 and bypasses muscle excitability and...
Results 3: Exploring the pathophysiology behind the onset of a degenerative phenotype with age in Draggen mice.

Excitation-contraction coupling to release Ca$^{2+}$ from intracellular stores. Therefore, if caffeine contracture is maintained in old mice but tetanic force is reduced it suggests impairment in muscle excitability or excitation-contraction coupling rather than impaired calcium-induced muscle contraction. (Ingalls et al., 1998) However, if caffeine contracture force is also reduced it suggests an impairment at the level of the ryanodine receptor or beyond e.g. with sarcoplasmic reticulum (SR) calcium release or calcium-induced muscle contraction.

Caffeine contracture force was significantly reduced with age in Draggen but not WT soleus (Figure 5-10A). This strongly suggests that the pathophysiology of permanent progressive weakness in Draggen muscle lies at the level of SR Ca$^{2+}$ release via RyR1 or beyond. The ratio of caffeine contracture force to tetanic force was decreased with age in both WT and Draggen soleus (Figure 5-10B). If there was a deficit in excitation-contraction coupling an increase in this ratio would be expected as caffeine contracture force would be maintained whilst tetanic force would be reduced.

![Graph A: Caffeine Contracture Force](image1)

**Figure 5-10** Caffeine contracture force is significantly reduced in old Draggen soleus.

**A.** There is a significant reduction in old Draggen soleus caffeine contracture force. One-way ANOVA $p=0.004$ *post hoc Tukey test significantly different from Old Draggen.**

**B.** The ratio of caffeine contracture force to tetanic force declines with age in both WT and Draggen soleus. Data Mean ±SEM are shown for each group, post hoc Tukey testing ****<0.001.
Results 3: Exploring the pathophysiology behind the onset of a degenerative phenotype with age in Draggen mice

The fact that the reduction in caffeine contracture force was specific to old Draggen muscle is consistent with it being due to the chronic consequence of single ion channel function rather than a phenomenon of normal ageing.

5.6.2 RyR1 protein expression and Calcium Release Unit number are maintained in old Draggen soleus

Figure 5-11 RyR1 protein expression and Calcium Release Unit (CRU) number are maintained in old Draggen soleus A. Ponceau to compare total protein and western blot to measure RyR1 protein expression in adult and old Draggen soleus (Abcam 2868 1:1000) AD= Adult Draggen (18-68 weeks), OD = Old Draggen (99-100 weeks) B. Longitudinal Transmission Electron Micrograph of Old WT Soleus at 11,500 magnification. A calcium release unit is marked by the yellow arrow. C. Number of calcium release units (CRU) per 100µm of soleus. Individual data points represent mean values from multiple fibres (≥7) for each animal. Data Mean ±SEM are shown for each group, post hoc Tukey testing *<0.05, **<0.01, ***<0.005, ****<0.001.
A reduction in RyR1 protein expression is one possible cause of reduced caffeine contracture force. However, western blot comparing young and old Draggen soleus RyR1 protein expression showed no significant difference in band density (Figure 5-11A). It is possible that RyR1 protein expression could be maintained but RyR1 mislocalised at a site away from the triad. Therefore, I wanted to check that the number of calcium release units, defined as the SR-TT tubule junctions where RyR1 and CaV1.1 physically interact (Figure 5-11B), were maintained in old Draggen soleus. Using electron microscopy I counted the number of calcium release units in soleus muscles as described previously (Boncompagni et al., 2006). There was no reduction in calcium release unit number in old Draggen soleus (Figure 5-11C). Instead, there was a trend towards an increased number of calcium release units in Draggen compared to WT of the same age (p=0.2). This suggests that a functional impairment in RyR1 calcium release rather than reduced protein expression accounts for the reduced caffeine contracture force.

5.6.3 Weight gain is impaired in old Draggen mice

Male Draggen mice have reduced body mass and increased energy expenditure compared to their WT littermates (Corrochano et al., 2014). Male Draggen mice who have had an ‘immobility’ attack show increased basal AMPK activation whilst AMPK activation in response to muscle stimulation is reduced. AMPK is the key regulator of the cell’s metabolic status and is sensitive to ATP concentrations within the cell. If ATP is depleted, AMPK will activate catabolic pathways, inhibit anabolic pathways and stimulate mitochondrial biogenesis to try to maintain cellular ATP levels in the short and long-term (Roepstorff et al., 2006; Corrochano et al., 2014).

Skeletal muscle mitochondria are located adjacent to the calcium release units (Boncompagni, Protasi and Franzini-Armstrong, 2012). Mitochondrial ATP production is stimulated by increases in cytosolic calcium (Jouaville et al., 1999). Mouse models with acquired RyR1 leak or impaired RyR1 calcium release due to RyR1 mutation have been shown to have impaired ATP production (Hanson et al., 2015) as a result of depolarised mitochondrial membranes (Umanskaya et al., 2014; Hanson et al., 2015). The reduced caffeine contracture force in old Draggen soleus, suggests reduced RyR1 calcium release. This implies that the ATP production in aged Draggen muscle may be impaired. Given their
Results 3: Exploring the pathophysiology behind the onset of a degenerative phenotype with age in Draggen mice increased energy expenditure Draggen mice may be particularly sensitive to a disruption of ATP production.

Figure 5-12 The weight difference between Draggen males and their WT siblings increases into old age.

A) The body weights of male Draggen mice (red) and male WT siblings (black) from weaning to time of terminal experiment (62 to 730 days). B) Left, comparison of mean Draggen and WT (WT) weight around weaning (17 to 33 days), young adulthood (62 to 208 days) middle age (266 to 551 days), and old age (663 to 725 days). Right, the difference in mean body weight between Draggen males and their WT male siblings increases with age up to 2 years (730 days).
Results 3: Exploring the pathophysiology behind the onset of a degenerative phenotype with age in Draggen mice

If catabolism is increased with age in Draggen mice due to a failure to meet ATP requirements, the weight difference between Draggen and WT mice should also increase with age. The published differences in weight between Draggen males and WT littermates were only reported on up until 54 weeks of age. I investigated whether weight difference increased with age up to 104 weeks (730 days). Consistent with this hypothesis, the mean weight difference was greatest in old mice (Figure 5-12).

Access to a voluntary running wheel from middle age has been shown to prevent age-related loss of muscle mass in WT animals (White et al., 2017). Given the evidence of AMPK dysregulation and potential impairment of mitochondrial ATP production with age in Draggen males, I wanted to see if Draggen mice were able to activate anabolic pathways in the face of increased activity and hence increased ATP requirements from greater muscle activity. Therefore, I examined the effect of access to a voluntary running wheel on the muscle mass of both WT and Draggen mice.

Figure 5-13 Weight of dissected TA muscle at time of terminal experiment in mice with and without access to a voluntary running wheel. Middle aged wheel = voluntary running wheel added to cage when mice aged 51-57 weeks and TA muscle dissected and weighed after 9-18 weeks (WT, n=6 muscles) or after 24 weeks (Draggen, n=6 muscles). Old wheel = voluntary running wheel added to the cage when mice aged 72-74 weeks and TA muscle was dissected and weighed after 25 weeks (n=6 muscles for WT and Draggen).

In old WT mice, access to a voluntary running wheel from middle age was associated with an increase in muscle weight (Figure 5-13). The mean TA weight for old WT males with access
Results 3: Exploring the pathophysiology behind the onset of a degenerative phenotype with age in Draggen mice

to a running wheel from middle age was 66mg Vs 56mg with no wheel (p=0.00002, t-test with Welch correction). In contrast, in old Draggen mice, access to a voluntary running wheel from middle age led to a trend towards reduction in TA muscle weight (mean TA weight wheel 54mg Vs no wheel 57mg, p=0.09, t-test with welch correction).

This was not because Draggen mice did not use the wheel. For the 24 to 48 hours preceding the terminal experiment a subset of these mice (two WT and two Draggen) were in an individual cage and the distance they ran on the wheel was measured. In this time both Draggen mice that were measured were active on the wheel to a similar or greater extent than their WT siblings. This suggests that whilst old WT mice were able to activate anabolic pathways to build muscle mass, old Draggen mice were not.

5.6.4 Adenylate energy charge is reduced in old Draggen mice

![Figure 5-14](image)

**Figure 5-14** The adenylate energy charge is significantly reduced in old Draggen TA. Bars represent mean ±SEM. Post hoc Tukey testing**<0.01

One possible explanation for the failure to increase Draggen muscle weight with exercise is that Draggen mice activated catabolic instead of anabolic pathways. The activation of catabolic or anabolic pathway is determined by the cell’s metabolic status. This metabolic status is reflected by the adenylate energy charge which measures the ratio of ATP to ADP and AMP. Therefore, if this hypothesis were true, you would expect a reduction in the adenylate energy charge as it reflects a reduction in the ratio of ATP to ADP and AMP.
Results 3: Exploring the pathophysiology behind the onset of a degenerative phenotype with age in Draggen mice

Consistent with this notion, the energy charge on TA muscles dissected and immediately flash frozen following muscle velocity recovery cycles and frequency ramp testing (Figure 5-14) was significantly reduced in old Draggen mice compared to both old WT (p=0.004, t-test with Welch correction) and middle aged Draggen (one-way ANOVA to compare young, middle aged and old Draggen TA p=0.004, post hoc Tukey middle-aged vs old p=0.006).

![Figure 5-15 Mitochondrial Volume and Function in Adult and Old WT and Draggen Soleus. A. Mitochondrial volume was assessed using STEPaniser (Tschanz, Burri and Weibel, 2011). Data points represent the mean mitochondrial volume for individual muscles (each muscle had at least 7 different fibres analysed). Bars represent mean ±SEM of each group. There was no significant difference between groups. B. The number of WT and Draggen soleus muscles with one or more COX negative fibre on COX/SDH staining were counted and]
compared for each age group and are represented as a percentage of the total number of samples. The total number of samples is given in brackets for each group on the x axis.

A reduction in energy charge reflects a reduction in the concentration of ATP relative to AMP. This could be because of impaired ATP production and/or increased utilisation. There was no evidence of reduced mitochondrial volume in Draggen muscle (Figure 5-15), the volume of mitochondria per muscle fibre was in keeping with number reported previously for old WT mice (Pietrangelo et al., 2015). There was also no evidence of mitochondrial complex I or IV deficiency as, if anything, Draggen mice had fewer COX negative fibres than WT mice (Figure 5-15B).

5.6.5 Structural Core Pathology Is Present in the Muscles of Draggen Mice

However, on reviewing the COX/SDH stains, I noticed multiple biopsies that had areas with no or reduced COX and SDH stain (Figure 5-16). These samples appeared normal on H&E staining. A lack of oxidative staining with normal appearing cytoplasm on H&E stain is consistent with cores or core-like regions. A core is defined as central clear zone in fibres stained for cytoplasmic or mitochondrial membranes. As SDH and COX stain for mitochondrial membrane the cores I observed represent an absence of mitochondrial membrane in the muscle.

One of 10 WT soleus examined (age range 27 to 103 weeks, mean age 78 weeks) and 11 of the 16 Draggen soleus examined (age range 13 to 103 weeks, mean age 66 weeks) had cores and core-like regions (p=0.005, two-tailed fisher exact test) (Figure.5-16). This included animals with (n=7 Draggen, n=5 WT) and without (n=8 WT, n= 9 Draggen) access to a voluntary running wheel.

The only WT animal with core pathology was a 62-week-old animal that did not have access to a voluntary running wheel. Six of 9 Draggen mice, that did not have access to a voluntary running wheel had cores compared to 5 of 7 Draggen mice with access to a voluntary running wheel. In those without access to a voluntary running wheel cores were not seen in the youngest two animals (13 and 14 weeks) but were seen in a 27-week-old animal. In those with access to a voluntary running wheel two of the three middle-aged animals did not have cores, whilst all old animals did. When combined, the average age of Draggen soleus with cores was 80.6±23.3 weeks (n=11) versus an average age of 36.4±32 weeks
Results 3: Exploring the pathophysiology behind the onset of a degenerative phenotype with age in Draggen mice without cores (n=5). Cores were found in 6 out of the 7 old (>97 weeks) Draggen soleus muscles vs 0 out of 4 old WT.

Figure 5-16 Soleus muscle pathology in young and ageing wild-type and Draggen mice. Young wild-type mouse (A-C). Young Draggen mouse (D-F). Ageing wild-type mouse (G-I). Ageing Draggen mice (J-L and M-O). Sections stained with NADH-TR (A, B, D, E, G, H, J, K, M, N) and COX-SDH (G, F, I, L, O). Scanning magnification view (A, D, G, J, M) in all animals shows a
transverse section through the mid-belly of the gastrocnemius-soleus muscle. The smaller oxidative, type I fibre predominant soleus (circle) is present under the larger mixed-fibre type gastrocnemius. In the young wild-type (B, C) and young Draggen (E,F) the muscle architecture is normal. In the ageing wild-type mouse (H, I), there is subtle uneven oxidative staining in a proportion of fibres, but no overt pathology. In contrast, two ageing Draggen mice (K, L and N, O), both show florid core pathology affecting several fibres, ranging from marked unevenness of oxidative staining, multicores, discrete small cores and occasionally well-defined larger cores (N, O, arrow). Scale bar: A, D, G, J, M = 1 mm; N = 50 µm; B, C, E, F, H, I, K, L, O = 100 µm.

Cores and mini-cores are seen in patients with myopathies due to mutation of RyR1 (Phadke, 2019). Impaired RyR1 function is associated with depolarised mitochondrial membrane potential and impaired mitochondrial ATP production (Umanskaya et al., 2014; Hanson et al., 2015). The finding of cores in many (6/7) old Draggen but not WT soleus suggests that the reduced caffeine contracture force in old Draggen is associated with acquired RyR1 dysfunction. The observation of cores in a 27-week-old animal, suggests that core formation precedes the reduction in caffeine contracture force in Draggen muscle.

Apart from the structural core pathology described above, there were no other overt myopathic or dystrophic changes, and no significant mitochondrial pathology in the ageing wild-type and Draggen mice.

5.7 Summary of findings
The change from an episodic to a degenerative phenotype with age in periodic paralysis is conserved across species and appears to be a phenomenon of normal ageing. My definition of normal ageing was ageing in the absence of overt pathology. In contrast, the age-related muscle pathology was specific to Draggen muscle indicating that it is caused, or accelerated by, chronic genetic ion channel dysfunction.

My data suggests that the reduction in induced attack severity with age is not due to a resistance to K+ induced sarcolemmal depolarisation. Instead, the data are consistent with old muscle (from mice over 95 weeks old) developing mechanisms to maintain force despite sodium channel (NaV) inactivation and sarcolemmal depolarisation.

The appearance of permanent weakness and features of periodic paralysis myopathy in Draggen mice with age is associated with a reduction in caffeine contracture force, a reduction in the adenylate energy charge and the appearance of core-like regions. Cores
have been associated with impaired Ca release because of RyR1 dysfunction in both mice and humans with RyR1 mutations. Thus, the finding of core-like regions in older Draggen muscle links the reduced caffeine contracture force to the reduced adenylate energy charge. My data suggest that acquired RyR1 dysfunction resulting in impaired mitochondrial ATP production is behind the onset of permanent progressive weakness with age in Draggen muscle.
5.8 Discussion

There is no published study on the pathophysiology behind the observed phenotype change in periodic paralysis. Thus, the experiments described herein address a major gap in our knowledge of this condition. My data confirms that the change in phenotype with age is conserved across species as evidence by its presence in a transgenic mouse model of paralysis. The fact that the reduction in episodic attack, but not the onset, of permanent progressive weakness was also observed in WT animals suggest whilst the former is a phenomenon of ‘normal ageing’, the latter is the chronic consequence of single ion channel dysfunction.

The lack of significant change in mouse MVRCs with age implies that increased resistance to potassium-induced weakness is not due to changes in baseline muscle excitability. The fact that decrement in amplitude of response after a train of 30 stimuli was similar (Draggen TA, WT triceps) or more pronounced (WT TA) in old compared to young or middle-aged mouse TA suggests that the maintenance of tetanic force in old (mouse ≥95 weeks) muscle subject to the potassium-induced weakness assay is also not due to a resistance to potassium-induced sarcolemmal depolarisation. Variation in extracellular K+, Cl−, and Na+ cannot underlie the increased resistance of old muscle to potassium-induced weakness as extracellular ionic concentrations were the same in the ex vivo assay for all ages. Therefore, the data are consistent with old muscle developing intrinsic mechanisms to maintain force, despite normal (or for old WT TA apparently increased) sodium channel inactivation and sarcolemmal depolarisation.

The exact mechanism for the apparent resistance to potassium-induced weakness with age remains to be determined. However, our data suggest that force and excitability can be differentially regulated in old muscle and more specifically that reduced excitability may not translate into the expected reduction in force. Reviewing the literature on PP, previous studies have also provided evidence that force and excitability can be dissociated (Engel and Lambert, 1969; Troni, Doriguzzi and Mongini, 1983; Lehmann-Horn et al., 1987; Wu, Mi and Cannon, 2013b, 2013a). In isolated human myofibres from a patient with Hyper PP, reduction of pH restored force but not resting membrane potential during potassium-induced weakness (Lehmann-Horn et al., 1987). In the Hypo PP mouse models, acetazolamide was effective at preventing CMAP decrement, but did not prevent loss of
Discussion

muscle force during induced attacks (Wu, Mi and Cannon, 2013a, 2013b). In humans with Hypo PP, action potential initiation and propagation failed after muscle twitch tension during an induced attack of paralysis (Engel and Lambert, 1969) and in another study the patient had regained sufficient muscle strength to flex their arm against gravity before direct electrical stimulation could elicit a muscle twitch (Troni, Doriguzzi and Mongini, 1983). How force is maintained despite a depolarised resting membrane potential as observed in the Hyper PP muscle fibres, and why the maintenance of CMAP does not translate into maintenance of force in mice or humans with Hypo PP, remains unclear, but may shed light on how healthy and PP old muscle can maintain tetanic force despite depolarisation.

Figure 5-17. Summary of Findings in Aged Draggen Muscle  In aged Draggen muscle there was no significant change in muscle excitability with age. However, caffeine contracture force and energy charge were significantly lower than in old WT muscle and core-like regions were prevalent. Core-like regions are typical of RyR1 myopathies and associated with impaired ATP production. This links the reduced caffeine contracture force with the lower energy charge in old Draggen muscle. Experiments described in this chapter suggest that progressive acquired RyR1 dysfunction is responsible for the onset of fixed weakness in old Draggen mice.
Baseline soleus tetanic force of Draggen mice showed significant decline with age (p=0.03). The intercept (p=0.006), but not the slope of a linear fit to force-age data was different between Draggen and wild-type mice, suggesting lower baseline ex vivo tetanic force in Draggen mice (Figure 5-7). A lower baseline force for Draggen muscle was not detected in grip strength experiments in vivo, but grip strength did decline in Draggen mice after 60 weeks of age (Corrochano et al., 2014). As the baseline force decline was not detected in vivo it is possible that the reduced baseline force in Draggen may be the result of increased sensitivity of Draggen muscle to the ex vivo conditions. A depolarisation of resting membrane potential by approximately 20mv has been reported when comparing the same mouse muscle resting membrane potential measured in vivo or in situ versus ex vivo (Kleeman, Partridge and Glaser, 1961). As our MVRC data suggest male Draggen muscle is relatively depolarised compared to WT muscle in vivo (figure 5-8, 4.7.1), an additional depolarisation of this scale would potentially have a disproportionate effect on Draggen, compared to WT muscle force measured ex vivo.

Age over 95 weeks in Draggen muscle was associated with reduced caffeine contracture force, decreased Energy charge, and structural core pathology (Figure 5-17). Pathogenic mutations in at least 8 genes have been associated with core myopathies in humans and occur most frequently due to mutations in RYR1, TTN and MYH7 44. Core-like regions have also been reported in mouse models with impaired ATP production secondary to RyR1 mutation (Umanskaya et al., 2014; Hanson et al., 2015). Thus, acquired RyR1 dysfunction seems the most likely cause for the reduced caffeine contracture force, decreased energy charge and the structural core pathology observed in aged Draggen muscle.

Acquired RyR1 dysfunction resulting in Ca\(^{2+}\) leak and consequent SR Ca\(^{2+}\) store depletion has been reported in normal aged mouse and human muscle (Andersson et al., 2011; Umanskaya et al., 2014; Lamboley et al., 2015, 2016). In these studies, SR calcium concentration and release rather than caffeine contracture were measured. This difference in technique may explain why I failed to see any significant deficit in WT muscle caffeine contracture. However, given these observations in normal ageing, it seems likely that fixed progressive weakness in Draggen mice represents a form of accelerated ageing rather than a process distinct to Draggen muscle.
Pertinent to these observations are conclusions from a detailed time course study of molecular changes across the life span in rats for various ageing tissue (Shavlakadze et al., 2019) that showed the most prominent common pathway down-regulated with aging was related to mitochondrial oxidative phosphorylation and respiratory electron transport (in skeletal muscle, liver and kidney), consistent with the proposal that the mitochondria become less competent with age, depriving cells of critical supplies of ATP. Calcium release through RyR1 is known to stimulate mitochondrial ATP production (Jouaville et al., 1999). Thus, in normal aging, the combination of impaired calcium release reducing the stimulus for mitochondrial ATP production and decreased mitochondrial oxidative capacity with age could form a double hit for ageing skeletal muscle. This would be expected to manifest earlier in male Draggen mice because of their increased energy requirements (Corrochano et al., 2014). As exercise would exacerbate this energy deficit by increasing energy requirement further, the observation that old Draggen mice were unable to mount an anabolic response to exercise is in keeping with this hypothesis (Figure 5-13).

This study reinforces the close links between membrane excitability, RyR1 and mitochondrial function. The interconnected nature of RyR1 and mitochondrial function with membrane excitability is apparent in both humans and mice as evidenced by reports of humans with RyR1 mutations (Matthews et al., 2018) and mitochondrial DNA mutations (Auré et al., 2013) that have a periodic paralysis-like phenotype; a patient with PP due to mutation in NaV1.4 responding to treatment with coenzyme Q10 (da et al., 2016, p. 10) and mice with an RyR1 mutation and core-like regions on muscle biopsy that exhibit potassium responsive weakness similar to periodic paralysis (Hanson et al., 2015).

One of the limitations of this study is that we did not measure the frequency of spontaneous attacks in Draggen mice and therefore cannot comment on whether reduction in the severity of potassium-induced weakness is associated with a reduction in the frequency of spontaneous attacks. In addition, we limited the study to male mice because, as for humans with the condition, some female carriers are asymptomatic (Ke et al., 2013; Corrochano et al., 2014). It is not known if a gender difference in the PP phenotype change with age exists and so for the reasons detailed in Chapter 4, future work should confirm that a similar phenomenon is observed in females and is associated with the same pathophysiology.
An increased energy expenditure and/or deficit in body weight have not, to my knowledge, been reported in humans with PP. However, PP is a rare disease, and it is likely that unless specifically looked for subtle difference in body weight may have been missed. A decrease in the ATP to ADP ratio has been observed for patients with Myotonic Dystrophy and associated with a small but significant reduction in mitochondrial function (Barnes, 1997). This suggests that the increased energetic requirement of ion channel dysfunction may also affect human muscle but may not be associated with clear weight discrepancies. Like the study in Myotonic Dystrophy, magnetic resonance spectroscopy could be used to confirm an ATP deficit in older humans with periodic paralysis. If present, muscle biopsies to specifically look for core pathology should be considered.

Structural pathology consistent with cores has been reported in one kindred with normokalaemic PP (Shi et al., 2019) which is believed to be within the spectrum of Hyper PP (Chinnery et al., 2002). However, cores have not been a common finding in PP. In the reported case, the gastrocnemius was biopsied, and the biopsy was taken after a very prolonged attack after which the patient had not fully recovered force. Gastrocnemius is one of the most commonly and severely affected muscles on MRI of Hyper PP patients (Lee et al., 2015). However, our clinical practice, as for many other centres in Europe and the US, is to perform biopsies of quadriceps, deltoids or biceps. This, along with the fact that patients tend to be biopsied early in the course of the disease, may explain why cores have not to date been a prominent feature reported for PP patients.

In summary, experiments described in this chapter demonstrate that phenotype transition with age also occurs in the Draggen mouse model of Hyperkalaemic periodic paralysis. The data suggest that whilst intrinsic muscle ageing protects against potassium-induced weakness in Draggen mice, it is also associated with impaired SR Ca\(^{2+}\) release, a process that seems to be accelerated in Draggen muscle. Thus, aging can modify the clinical effect of genetic mutation and studying phenotype change with age in monogenic disease can yield novel insight into both disease physiology and the ageing process itself.
6 Can MC inheritance pattern be more accurately predicted?

6.1 Motivation
The diagnosis of MC is based on the identification of mutations in CLCN1 and a compatible phenotype. However, mutations can be dominant or recessive and are found throughout the length of the CLC-1 channel sequence (1.4.1). Moreover, our current understanding of CLC-1 structure-function does not allow us to accurately predict if and how a mutation reduces channel function or what the clinical impact of an identified ClC-1 variant may be. This combined with the significant heterogeneity in phenotype severity makes genetic counselling for people with an identified CLCN1 variant extremely difficult.

Heterologous expression of identified CLCN1 variants has been used to help determine pathogenicity and likely inheritance pattern (1.4.8.2). However, a systematic correlation between the functional properties of the variant channels and their clinical consequences has not been performed. This is needed as an evidence base to inform genetic counselling. Functional expression data comes from various systems and conditions, genetic data are at times incomplete and the inheritance pattern of clinical symptoms is not always systematically categorized. Thus, currently, the value of functional expression to genetic counselling is suggestive. A thorough analysis of the correlation of functional properties of the channel and the inheritance pattern of clinical symptoms is required to validate reporting of the pathogenicity and inheritance pattern of novel CLCN1 variants.

6.2 Aims
Therefore, the aims of the experiments described in this chapter were to:

(1) Validate the use of functional expression in MC diagnosis
(2) Extend understanding of ClC-1 structure-function
(3) Improve the interpretation of the pathogenicity and inheritance pattern of identified ClC-1 variants
6.3 Specific Methods

6.3.1 Ethics

The correlation of ClC-1 variant functional characteristics with reported inheritance pattern of myotonic symptoms was conducted as part of a service evaluation of the NHS Highly Specialised Muscle Channelopathy Service at the National Hospital for Neurology and Neurosurgery. No procedures were performed outside of routine clinical care.

6.3.2 Genotyping

CLCN1 genetic testing was performed by the diagnostic molecular genetics laboratory at the National Hospital for Neurology & Neurosurgery. This is the national centre for Myotonia Congenita genetic testing. Since 2007 this consists of Sanger sequencing all 23 exons of CLCN1 plus flanking intronic regions, or targeted sequencing of specific exons for relatives of individuals in whom sequence variants have already been identified. For samples processed prior to 2007, at least the proband subsequently underwent full sequencing of all CLCN1 exons unless it was not possible to obtain DNA to do so. Where no mutations are found, a single recessive mutation is identified or a homozygous mutation is identified, Multiplex Ligation-dependent Probe Amplification (MLPA) is performed to assess for exon deletions or duplications. Whenever possible, the inheritance and allelic distribution of the variants is investigated by sequencing CLCN1 in family members.

6.3.3 Categorising inheritance pattern using the clinical and genetic data available

Patients referred to our diagnostic service for Myotonia Congenita testing and in whom CLCN1 missense variants were identified and functionally characterised were included. Demographic, clinical, electrophysiological and genetic data were collected from referral forms or clinic notes when seen internally. However, as the clinical information available, especially with regards phenotyping and genotyping of family members was not always complete, the inheritance of the ClC-1 variant could not be confirmed in all cases. In these cases, the reported inheritance pattern of myotonic symptoms was used. Inheritance patterns were divided into five categories as defined below.

6.3.3.1 Dominant inheritance of myotonic symptoms

- Dominant: Patients with one CLCN1 variant and reported parent to child transmission.
• Dominant with variable penetrance: Parents self-reported asymptomatic but found to have variant and clinical or EMG myotonia on examination or, in families with no known history of consanguinity, parents reported asymptomatic (clinical or neurophysiological examination details not known) and uncle, aunt, nephew, niece or half sibling affected.

6.3.3.2  **Recessive inheritance of myotonic symptoms**

- Recessive: Homozygous patient with asymptomatic parents or compound heterozygous patient with asymptomatic parents both of which were genotyped.
- Probable recessive: Patients with two or more CLCN1 variants, parents asymptomatic but not both genotyped.

6.3.3.3  **Sporadic inheritance of myotonic symptoms**

- Sporadic: Patients with a lone CLCN1 variant whose parents were asymptomatic and did not have the variant.
- Probable sporadic: Patients with a lone CLCN1 variant whose parents were reported asymptomatic but not genotyped and there was no other family history of condition.

6.3.3.4  **Insufficient data to categorise**

- Family history not given on referral form or some history given but was insufficient to categorise patient e.g., known that mother not affected but not known whether father affected or not.

6.3.3.5  **Likely benign**

- Variant did not segregate with reported myotonic symptoms (asymptomatic carriers had normal clinical and/or neurophysiological examination).
- Allele frequency greater than 1% in Gnomad

6.3.3.6  **Variants’ whose contribution to myotonic symptoms cannot be determined:**

- Double Trouble e.g., patients with at least one CLCN1 variant who also had a mutation in another myotonia causing gene that could independently account for the phenotype e.g. an SCN4A mutation causing Paramyotonia Congenita
- Multiple CLCN1 variants: Patients with multiple CLCN1 variants where relative contribution of each variant to symptoms is not possible to determine e.g., co-allelic
Specific Methods

inheritance of variant or dominant inheritance of myotonic symptoms reported and DNA only available from one individual with two variants.

6.3.4 Xenopus Oocytes

_Xenopus laevis_ toads were sacrificed in accordance with the Animals (Scientific Procedures) Act 1986 and ovarian follicles removed by colleagues at UCL main campus.

6.3.4.1 Xenopus oocyte preparation

Mature oocytes were isolated and defolliculated after incubation with 2 mg/ml Collagenase Type A (Roche) in OR-2 (in mM): NaCl 82.5, KCl 2, MgCl₂ 1, HEPES 5 (pH 7.6). The oocytes were incubated in Modified Barth’s solution (in mM): NaCl 88, KCl 1, MgSO₄ 1.68, HEPES 10, Ca(NO₃)₂ 0.47, NaHCO₃ 2.4, CaCl₂ 0.41, supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml) and amikacin (100 µg/ml) (pH 7.4).

6.3.5 Molecular biology

6.3.5.1 Site directed mutagenesis

The mutations were introduced into WT _CLCN1_ cDNA in the pTLN vector by Quikchange site directed mutagenesis (Stratagene) (Fialho et al., 2007). Successful mutagenesis was confirmed for each clone by sequencing the entire gene.

6.3.5.2 In vitro transcription

The ClC-1 mRNA was transcribed from MluI linearized vector using mMessagemMachine SP6 kit (Ambion). For the recessive mutations with no functional expression a second mRNA preparation was performed, and gel electrophoresis was used to confirm its integrity.

6.3.6 Two-electrode Voltage Clamp

Two-electrode voltage clamp experiments were performed using GeneClamp 500B, DigiData 1200, and Clampex software (all Axon Instruments) at room temperature in ND96 extracellular media (in mM): NaCl 96, KCl 2, MgCl₂ 1, HEPES 5, CaCl₂ 1.8 (pH 7.4 with NaOH). Recording electrodes were filled with 3 M KCl and had a tip resistance <1 MΩ.

The standard voltage protocol consisted of holding voltage of -80 mV, a pre-pulse step to +60mV for 250ms, test voltage family ranging from -150mV to +190mV in 10mV increments for 250ms and a tail voltage step to -100mV. The effects of preconditioning voltages on channel activity were analysed using holding voltages of -40mV or -80mV, and pre-pulse
voltages of -140mV or +60mV. Other voltage protocols used are described in the results and in the legends.

6.3.7 Homology Modelling of CIC-1

I aligned the CIC-1 protein sequence (Uniprot P35523) using NCBI BLAST against available structures in the protein data bank (http://www.rcsb.org). I first made the model in 2014 and refined the model in January 2017 as the CIC-K cryostructure was published (Park, Campbell and MacKinnon, 2017). The CIC-K cryostructure and Cm-CIC structure (Feng et al., 2010) were used as templates for the model as these proteins had the largest query cover and the highest sequence identity. Sequence alignment was performed using COBALT (Papadopoulos and Agarwala, 2007) followed by manual adjustment based on structural data and associated published sequence alignments (Dutzler, 2003; Feng et al., 2010). I used MODELLER (Sali, 2008) to generate the CIC-1 model. The region linking CBS 1 and 2 (670-797), the N-terminal region (1-110) and the large cytoplasmic C-terminal region (867-988) are disordered regions of the protein structure (Meyer and Dutzler, 2006). There were therefore unresolved in the template structures, were not possible to model and thus are not presented. PyMOL (Schrodinger, Inc) was used for graphical representation of the model.

6.3.8 Data analysis

Data analysis and presentation was prepared using Clampfit (Axon instruments), Origin (OriginLab) and Excel (Microsoft) software.

The tail currents were measured routinely 4ms after the test pulse to avoid contamination by the capacitance artefact. To assess the voltage dependence of activation the current-voltage relationship was fitted with a Boltzmann equation: \( I(V) = \frac{I_{\text{max}}}{1+\exp((V_{\text{mid}}-V)/V_{c})} + C \), where \( I_{\text{max}} \) is the amplitude of the fit, \( C \) the offset current, \( V_{\text{mid}} \) the voltage at which the current is \( (I_{\text{max}}+C)/2 \) and \( V_{c} \) the slope factor. We fixed the value of \( C \) in the fitting process to the baseline current level derived from the current-voltage plot at the most hyperpolarized voltages.

Some variants did not produce currents of amplitude large enough to assess the voltage dependence of activation. The cells with tail current amplitude <1 µA after a voltage step to +80 mV were considered void of functional CIC-1 channel expression and excluded from
analysis of voltage dependence of activation. At voltages >+60 mV a clear endogenous current component develops in the oocytes. However, this has very little effect on the tail current amplitude recorded at -100mV but may have a small effect on estimation of the voltage dependence of activation for mutant channels that activate at extreme positive voltages.

The tail current amplitude of the WT channels was variable. Due to the large number of oocyte batches with variable expression level and varying time range after injection we refrained from systematically comparing the current amplitude levels of the cells with tail current amplitude > 1 µA.
6.4 Results 1: The use of functional expression in MC diagnosis

6.4.1 Genetic and clinical overview of the cohort

In total 221 probands with 89 different CLCN1 missense variants that were detected by our diagnostic laboratory and referred for functional testing were included.

Based on the clinical description of the patient, family history and genetic data available I categorised the inheritance patterns of myotonic symptoms (6.3.3). Out of the 221 probands, 74 were classified as having a dominant family history and 9 as dominant with variable penetrance. Twenty-six patients were classed as recessive, 30 as probable recessive and 19 as probable sporadic. For 42 patients there was insufficient clinical information to classify the inheritance pattern of myotonic symptoms. Five patients had an alternative genetic diagnosis explaining symptoms, in 4 families the CLCN1 variant did not segregate with symptoms and for 12 the relation of the CLCN1 variant to the inheritance of myotonic symptoms was uncertain.

6.4.2 Functional analysis of CIC-1 variants

The effect on CIC-1 channel function of the 89 CLCN1 missense variants identified in these patients was tested in the *Xenopus laevis* oocyte expression system. This is performed as part of our routine diagnostic work up. This functional characterisation service is run by Dr Roope Männikkö. Variants also included a previously reported MC mutation absent in our cohort (P480L) and 4 variants assigned as benign polymorphisms (W118G, A437T, K614N, P697L). CIC-1 variants were expressed alone and the variants with loss-of-function effects were also co-expressed with WT CIC-1 channels to simulate the heterozygous state.

Of the 89 missense variants identified in our patient cohort, 87 clustered into 3 distinct functional groups. We called these WT-like, reduced functional expression and shift in the voltage dependence of activation. The latter two groups could be further subdivided according to the presence or absence of a dominant negative effect of mutant subunits on WT subunits in simulated heterozygous conditions. Approximately 80 of the variants were characterised by Dr Männikkö prior to my starting at the MRC centre. I present representative data from the variants that I categorised for each of the different functional groups below.
Results 1: The use of functional expression in MC diagnosis

6.4.2.1 WT like variants

Twenty-six variants were classified as ‘WT like’, because the $V_{\text{mid}}$ of activation was within 1.5 SDs of WT ($V_{\text{mid}}$(WT) = -34.7 ± 0.9 mV, n=141, SD= 10.7 mV) (Figure 6-1). One variant H664P, activated at more hyperpolarized voltages than the WT channels ($V_{\text{mid}}$ = -54.0 ± 2.5 mV, n=7). In the absence of any loss-of-function effect it was grouped with the 27 WT-like variants.

Figure 6-1 Representative data for the WT like functional group.

A. Current traces for WT homomers. B. Current traces for R105C homomers. (C) Current voltage plot for WT and R105C. The tail current amplitudes were normalized to the maximum current derived from the fit of Boltzmann equation to the data. Scale bars are 100ms (x-axis), 2 µA (y-axis). In A and B, for voltage pulse positive to +60 mV only the currents during the tail pulse are shown. Standard voltage protocol was used in A,B and C (Holding voltage -80mV, prepulse +60mV see 6.3.6). Reduced Functional Expression

No currents were detected in 22 variants when expressed alone (Figure 6-2). In heterozygous form 15 of these 22 expressed tail currents with WT-like voltage dependence (Figure 6-2). The absence of dominant negative effects on the functional properties of these 15 led us to classify them as ‘Functionally Recessive’.

Figure 6-2 Representative data for the Functionally Recessive Variants in the Reduced Functional Expression Group.
6.4.2.3 Variants with shifted voltage dependence in simulated heterozygous form

The remaining 7 variants with reduced functional expression (no currents as homomers) yielded currents with right shifted voltage dependence of activation in the simulated heterozygous form (Figure 6-3). This reflects a dominant negative effect of the mutant subunit on the co-expressed WT subunit. I will refer to this group as “Shift in voltage dependence of activation in simulated heterozygous form” or VS\textsubscript{het} to differentiate them from variants that showed currents with shifted voltage dependence in homomeric form (VS\textsubscript{hom}, see below). It remains to be determined if currents were undetectable in homomeric channels because the voltage dependence of activation was so far right shifted it was outside our range of test voltages (+200mV) or because of direct effects on channel expression or conductance.

Figure 6-3 Representative data for the shift in voltage dependence in simulated heterozygous form group.

- A. Current trace for P480H homomers.
- B. Current trace for P480H in simulated heterozygous conditions.
- C. Normalised current voltage plot of P480H in simulated heterozygous conditions compared to WT. Scale bars are 100ms (x-axis), 2 µA (y-axis). Current traces are as described in Figure 6-1.

6.4.2.4 Variants with shifted voltage dependence in homomeric form

Thirty-seven variants produced robust currents as homomers, but their voltage dependence of activation was shifted to more depolarized voltages (Figure 6-4). The \( V_{\text{mid}} \) was considered shifted if it was more than 1.5 standard deviations positive compared to WT \( V_{\text{mid}} \) (-34.3...
Results 1: The use of functional expression in MC diagnosis

±0.6mV, SD=10.4). The $V_{\text{mid}}$ of these variants ranged from -17.7 mV (F167L) to +126 mV (G551D). The shift in voltage dependence of activation reduces the current amplitude at physiological voltages and confirms loss-of-function effects for these variants.

Figure 6-4 Representative Data for the shift in voltage dependence in homomeric form group.

A. Current trace for C179Y, an example of a variant in this group with a dominant negative effect. B. Normalised current voltage plot of WT (filled black circles), C179Y homomeric (filled red circles) and C179Y in simulated heterozygous conditions (empty red circles). C. Current trace for D822N an example of a functionally recessive variant in this group (no dominant negative effect). D. Normalised current voltage plot of WT (filled black circles), D822N homomeric (filled red circles) and D822N in simulated heterozygous conditions (empty red circles). The vertical line in B and D represents the upper cut off point for a WT-like voltage dependence of activation ($V_{\text{mid}}$ = the voltage at which half maximal current is reached). Scale bar is 250ms (x) and 5 µA (y) Current traces are as described in Figure 6-1.

When co-expressed with WT channels, 36 out of 37 exhibited a $V_{\text{mid}}$ closer to WT $V_{\text{mid}}$ than the homomorphic channels. Only the F297S variant displayed a greater shift in the voltage of
Results 1: The use of functional expression in MC diagnosis

half-maximal activation in simulated heterozygous condition (3.1 ± 3.6mV, n=8) than in homomeric form (-6.2 ± 3.2mV, n=11, p= 0.070). For 24 of the 37 variants, the voltage dependence in simulated heterozygous conditions was right shifted consistent with a dominant negative effect on the co-expressed WT subunit (Figure 6-4 A&B). For the remaining 13 variants the voltage dependence was WT-like in simulated heterozygous conditions (Figure 6-4 C & D). As described above (6.4.2.2) these 11 were considered to be functionally recessive variants.

6.4.2.5 Variants with unusual properties

One variant I characterised, M485K, in addition to shifting the voltage dependence of activation did not seem to close properly, showing increased baseline currents (Figure 6-5). Even at -150mV there was residual current in M485K channels (normalised current 0.25, red line Figure 6-5) Vs 0.08 for WT (black line, Figure 6-5). M485K het was intermediate to the M485K homomeric and WT ClC-1 Channels (Figure 6-5). Loss-of-function by a depolarising shift in the voltage dependence of activation is consistent with MC. The impact of increased baseline activity, a gain-of-function feature, on clinical phenotype is not known.

Figure 6-5 Current voltage plot for WT (black filled circle), M485K homomer (red filled circle) and M485K in simulated heterozygous conditions (red empty circle).

Tail current amplitudes were normalized to the maximum current derived from the fit of Boltzmann equation to the data.
In addition to M485K, three other variants, R421C, L332R and P342L showed properties that could not be described only with reduced current amplitude or shifted voltage dependence. However, whilst R421C and M485K could be included in the voltage shifting group, L332R and P342L did not fit into one of the 3 functional groups. As I was not involved in the characterization of these variants, I have not included a description of these variants within my thesis.

6.4.2.6 Alternative pathomechanism for variants with WT-like voltage dependence of activation

Although 63% of the WT-like variants are categorised as of Uncertain or Unlikely Clinical Significance (6.3.3), 29% of variants with WT-like properties were categorised as recessive and 8% as sporadic. I performed further functional characterisation on one of these WT variants and identified a potentially novel molecular pathomechanism of MC.

6.4.2.6.1 Absence of hysteresis in the voltage dependence of activation

In *Xenopus* oocytes the voltage dependence of activation of the WT ClC-1 channel is left shifted 40mV by a depolarising prepulse (holding voltage -40 mV, pre-pulse voltage +60 mV; Vmid= -43.7 ± 1.1 mV, n=161) compared to a hyperpolarising prepulse (holding voltage -80 mV, pre-pulse voltage -140 mV; Vmid=-4.4 ± 0.9 mV, n=112 p<<0.001) (figure 6-6A). The dependence of the state of a system on its history is known as hysteresis. The presence and role of ClC-1 hysteresis has not been previously described. However, ClC-1 hysteresis would facilitate a larger activity-induced increase in Cl⁻ current at physiological voltages compared to a model where ClC-1 activation exhibits no hysteresis.

One of the WT-like variants in our cohort, L587V, was not sensitive to preconditioning voltages (Figure 6-6B). One of the predicted consequences of the lack of hysteresis in this variant is that it will not shift to a ‘high activity’ state following prior depolarisation. Thus, following a hyperpolarising prepulse the relative activity of the channel is ~0.2 at -60mV for both WT and the L587V channel (Figure 6-6), however, following a depolarising prepulse whilst the relative activity of WT channels more than doubles to 0.5 the relative activity of L587V mutant channels remains around 0.2 (see arrows in figure 6-6 A and B). Upon repetitive stimulation (10ms at + 50 mV followed by 5ms tail pulse at -100 mV at frequencies faster than 5Hz) the increase in tail current amplitude was much larger for WT
than L587V channels (Figure 6-6C and D). A lack of increase in channel activity upon repetitive stimulation would confer a loss-of-function and may constitute the pathomechanism of MC. L587V was found as a homozygous mutation in 2 pedigrees.

Figure 6-6 Lack of Hysteresis in the voltage dependence of activation of L587V channel.

A & B Current voltage trace for WT (A) n=143 and L587V B (n=7). From holding voltage (Vh) of either -80 or -40 mV, a 250ms pre-pulse (VPP) was applied to either -180mV or +60 mV followed by test voltage steps ranging from -150 mV to +190 mV in 10 mV increments (only traces in response to pulses up to +150 mV are shown) and a 250ms tail voltage step to -100 mV. Pink = Vh -40 mV, VPP +60mV; Red = Vh -80mV VPP =+60mV; Blue = Vh-40mV, VPP -180mV; Black = Vh -80mV, VPP-180mV. Tail current amplitudes were normalized to the maximum current derived from the fit of Boltzmann equation to the data. C. WT (n= 6) and D.L587V (n=5) Channels were stimulated for 10ms at +40mV followed by 5ms tail pulse at -100mV at 5, 10, 20, 50 and 66 Hz. Current normalised to tail current value at trace 1. WT ClC-1 channels show a 175% increase following 66Hz stimulation vs ~60% for L587V).

6.4.3 Correlation of functional data with the inheritance patterns of clinical symptoms

The frequency of inheritance patterns of clinical symptoms was distinct within each functional group (Figure 6-7). The clinical and functional assessment of dominance correlated well.
Results 1: The use of functional expression in MC diagnosis

Figure 6-7 Inheritance Pattern of Clinical Symptoms According to The Presence or Absence of a Dominant Negative Effect in Simulated Heterozygous Conditions.

The number of CIC-1 variants rather than the number of families within each group are counted. When there was more than one family with the same CIC-1 variant the number in each classification of inheritance was divided by the total number so that results were not unduly weighted towards common mutations. Families with unknown family history and CIC-1 variants belonging to the outlier group were not included. Percentage of CIC-1 variants is shown on the y-axis (see 6.3.3 for definitions of clinical categories).

6.4.3.1 WT like variants

No pathogenicity could be assigned to these 26 variants based on our functional expression experiments with the standard voltage protocol (-80mV holding voltage +60mV preconditioning pulse). The absence of loss-of-function effects for these variants suggests that at least some of them may be benign polymorphisms. Accordingly, the properties of four SNPs (W118G, A437T, P697L and K614N) included in the study were WT like. These variants have no known associated pathogenicity, although W118G may act as modifier of pathogenicity (Raheem et al., 2012).

Consistent with the absence of pathogenicity of the WT-like group, 63% of WT-like variants were categorised as of ‘Unlikely or Uncertain Clinical Significance’. This was significantly more than other functional groups (p=0.00007) (Figure 6-7). WT-like variants also had the highest mean frequency in ExAC. None of the WT-like variants were associated with a dominant inheritance pattern of myotonic symptoms.
6.4.3.2 *Functionally Recessive Variants*

The 28 variants that yielded currents with WT like voltage dependence of activation in simulated heterozygous conditions but either no currents (15) or currents with shifted voltage dependence of activation as homomers (13) were considered functionally recessive. Consistent with recessive effects on CLC-1 channel function, 89% of these were found in probands categorised as having a ‘recessive’ or ‘probably recessive’ inheritance of myotonic symptoms (Figure 6-7).

However, 1 of these variants, G285V was found in a proband with a dominant inheritance pattern of myotonic symptoms. Intriguingly another variant at the same residue, G285E, was initially thought to be functionally recessive but additional experiments and analysis by Dr Roope Männikkö after I had completed my project, revealed it had evidence of a mild dominant negative effect. G285E was found in 26 probands four of which were classified as dominant (2 with reduced penetrance), 3 as sporadic, 10 as recessive or probably recessive, 2 as Uncertain or unlikely pathogenicity and 6 as unknown.

6.4.3.3 *Functionally Dominant Variants*

Thirty-one variants exhibited dominant negative effects on co-expressed WT subunits. Consistent with a dominant effect on CLC-1 channel function 59% of the functionally dominant variants were categorised as dominant, 12% as sporadic and 22% as a recessive inheritance pattern of clinical symptoms (Figure 6-7). Seven percent of the variants were in the uncertain or unlikely pathogenicity group. This was because one of the 40 probands with the G230E variant had a second CLCN1 variant (L198V) and both probands with the P480H variant had a second CLCN1 variant (M485V and R894X). Unfortunately, in these cases segregation data was not sufficient to determine the effect of each variant in isolation.

6.4.3.4 *Outlier variants*

The two outlier variants P342L and L332R were both found in isolation in a proband without family history of myotonic symptoms.
6.4.3.5 The Magnitude of Shift in Voltage Dependence and Associated Dominant Inheritance

Figure 6-8 The Voltage Dependence of Activation Under Simulated Heterozygous Conditions According to Whether the Associated Clinical Inheritance Pattern of Symptoms was Dominant, Recessive or Both Modes of inheritance had Been Reported.

These data are from recordings using the standard voltage protocol (-80holding voltage, +60mV prepulse, see Figure 6-1).

The magnitude of the shift in voltage dependence of activation under simulated heterozygous conditions seems to reflect the risk of dominant inheritance of clinical symptoms (Figure 6-8). The variants with the most right shifted voltage dependence in simulated heterozygous conditions were associated with dominant inheritance pattern whilst the variants with voltage dependence closest to WT were associated with recessive inheritance. However, there was significant overlap for variants in the middle with no clear cut off point at which associated inheritance turned from dominant to recessive (Figure 6-8). Considering the phenotypic variability of the patients it is perhaps not surprising that the cut-off voltage in defining the risk of dominant inheritance of MC is not absolute. It is of note though that all variants with \( V_{\text{mid}} \) negative to the cut-off voltage we used to assess...
pathogenicity in homomeric condition were only found in recessive pedigrees. This suggests that these variants can be reported as recessive.

F297S was the only variant for which the voltage of half maximal activation was more positive in simulated heterozygous than in homomeric form. All 7 families with F297S were classified as having dominant inheritance of myotonic symptoms. The increased shift in the voltage dependence of activation in the heterozygous form for F297S is unique among our variants and suggests a pathomechanism for the dominant inheritance pattern.

The inheritance pattern of clinical symptoms for the kindred carrying M485K was recessive despite its large shift in voltage dependence. It may be that failure of M485K variant channels to close properly allows some additional Cl− flow which reduces severity of phenotype. This remains to be confirmed.

6.5 Results 2: ClC-1 structure-function

6.5.1 Mapping the functional groups to ClC-1 model
I next mapped the variants onto my homology model of ClC-1 and asked whether the variants in different functional groups would cluster in separate regions of the channel structure. All variants with a shift in the voltage dependence of activation were combined into a single group. Since creating my model, a ClC crystal structure has been published (Park, Campbell and MacKinnon, 2017). Mapping the data onto the crystal structure provided similar results to those described below.

6.5.2 Clustering of functional groups on the CLC-1 homology model
Variants that shift the voltage dependence of activation are significantly more likely to be located in the first of the structurally similar halves of the ClC-1 subunit (helices B-I and the IJ-loop residues 111-347) than variants from other functional groups (p=0.00001) (Figure 6-9). Seventy percent of the voltage shifting variants affected residues in this region. Other residues that show shift in voltage dependence of activation when mutated are located directly adjacent to this region in the selectivity filter (P480S, P480H, G483, M485, V536), the subunit interface (G551, M560), helix O (G523) or the second CBS domain (D822, P883).
Results 2: CIC-1 structure-function

Figure 6-9 Characterised CIC-1 Variants Cluster on Homology Model According to their Functional Group. CIC-1 is a dimer. Helices B to the end of the U linker are shown in wheat, helices J to R in light blue and the cytoplasmic regions in in pale green. The key residues for the Cl selectivity filter are shown as red sticks (S179, E232, Y578). Unstructured regions are not shown (residues 1-110, 670-797 and 867-988). Images are from transmembrane front view (left), transmembrane side view (middle) and extracellular view (right). A. Residues corresponding to the voltage shifting variants are shown in yellow for those whose voltage dependence of activation (Vm) in simulated heterozygous conditions is not within our defined WT-like range (within 1.5 SD of WT Vm) and orange for those whose Vm was within our defined WT range. B. Residues corresponding to the functionally recessive variants which exhibit no currents as homomers are shown in blue. C. Residues corresponding to the functionally WT-like variants are shown in green.

Functionally recessive variants which exhibit no currents as homomers were significantly more likely to be located in the second half of the CIC-1 subunit (p= 0.001) than variants from other groups. There was a large cluster of these functionally recessive variants towards the extracellular end above the selectivity filter (Figure 6-9B). Functionally recessive variants outside this region were found in the protein-membrane interface (A221E) in
Results 3: Interpretation of the pathogenicity and inheritance pattern of identified ClC-1 variants

regions important for channel gating (G276 (Fialho et al., 2007), C277 (Weinberger et al., 2012), G285 (Kubisch et al., 1998) and one variant, V640F, was in the CBS domain.

WT like variants were significantly more likely to be found in the cytoplasmic regions (N- and C- termini and CBS domains, residues 1-111 and 586-988) than variants from other functional groups (p=0.0000006) (Figure 6-9C). Eighty three percent of cytoplasmic variants were WT like. Only two variants in the WT like group (A402V and F494V) affected residues in the intra-membrane α-helices (Figure 6-9C).

6.5.3 Location of variants with unusual properties

The variant that I characterised that was insensitive to preconditioning voltages (absence of hysteresis), L587V, is located on the intracellular side underneath the Cl⁻ selectivity filter in a cytoplasmic loop that links the second half of the ClC transmembrane region with cytoplasmic region. The variant that prevented normal channel closure at hyperpolarized voltages (M485K) was located within 5 Angstroms of E232 the protopore fast gate. It is in the region of D136G another variant that has been reported to exhibit standing current (Schmidt-Rose and Jentsch, 1997). F297S the only variant that exhibited a greater shift in its voltage dependence of activation in simulated heterozygous conditions lies in a predicted beta sheet at the subunit interface.

6.6 Results 3: Interpretation of the pathogenicity and inheritance pattern of identified ClC-1 variants

6.6.1 Combining clinical, functional, structural and genetic information enables estimation of the likely clinical significance and associated inheritance pattern of ClC-1 variants

A disproportionate number of variants were located in helices B to J (Figure 6-10). In fact, 82% of patients had at least one variant in this region. In contrast, only 20% of variants were found in the cytoplasmic regions and only 9% of patients had one or more variants in this region. 50% of variants in the cytoplasmic regions were categorised as of ‘Unlikely or Uncertain Clinical Significance’ this was significantly more than variants from other regions (p =0.002). The likelihood of being categorised as of ‘Unlikely or Uncertain Clinical Significance’ could be refined with knowledge of functional group: 60% of cytoplasmic WT-
Results 3: Interpretation of the pathogenicity and inheritance pattern of identified ClC-1 variants

like variants versus 0% of cytoplasmic variants belonging to other functional groups were
categorised as of Unlikely or Uncertain Clinical Significance (see table 6-1).

Figure 6-10 The associated inheritance pattern of clinical symptoms plotted according to
variant location (see 6.3.3 for definition of categories).

Helices B to J: total variants = 41 of which 38 had probands with sufficient data to categorise
inheritance pattern. Helices J to R: total variants = 30, of which 21 had probands with
sufficient data to categorise inheritance pattern. Cytoplasmic regions: total variants = 18, of
which 15 had probands with sufficient data to categorise inheritance pattern.

In contrast, all but one of the variants with sufficient clinical or genetic data to categorise as
a dominant inheritance pattern of myotonic symptoms (6.3.3), were in helices B to I and the
IJ loop (Figure 6-10, Table 6-1). For variants located in this region, the likelihood of an
associated dominant inheritance pattern of myotonic symptoms was 45% (table 6-1). This
risk estimate can be stratified further with functional characterisation. For example, if the
variant was in this region and of the VS \text{het} functional group (no current from homomers but
currents with a shift in the voltage dependence of activation under simulated heterozygous
conditions), the likelihood of an associated dominant inheritance pattern of myotonic
symptoms increased to 81% whereas if in the WT-like functional group it reduced to 0%
(table 6-1). There were significantly more variants associated with a dominant inheritance
pattern of myotonic symptoms in this region compared to all other regions combined
(p<0.0001). In fact, in our cohort, the only variant outside this region with sufficient data to
categorise as being associated with a dominant inheritance pattern was P480S. However, it should be noted there were also variants with functional evidence of a dominant negative effect but for whom family history was either unknown or clinical data was insufficient to categorise the associated inheritance pattern (see 6.3.3).

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<th>Total Patients</th>
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<th>Sporadic</th>
<th>Recessive</th>
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<th>Total Patients</th>
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Table 6-1. The numbers from this data set provide guidance for clinical decision making and genetic counselling by providing an indication of the likely clinical significance and associated inheritance pattern of a ClC-1 missense variant according to location in the ClC-1 protein and functional group. The numbers given should be considered within the limitations specified in the text. Definitions of inheritance pattern of clinical symptoms given in 6.3.3’ and functional groups given in 6.4.2. Patients with 2 or more functionally characterised missense mutations were included under each variant, probands with unknown inheritance pattern of myotonic symptoms and those with ClC-1 variants belonging to the outlier group were not included. Therefore, for some variants it was not possible to categorise the associated inheritance pattern of clinical symptoms.
6.7 Summary

By correlating the functional properties of 89 CLCN1 variants with available clinical and genetic data from the 221 probands carrying these variants we have validated the use of functional expression in genetic counselling of MC. The analysis clarifies the interpretation of functional properties of novel variants and improves the accuracy of genetic counselling we can provide for patients and their relatives. In addition, the data provides novel insights into the molecular and cellular physiology of CLC-1.
6.8 Discussion

6.8.1 Improving genetic counselling for Myotonia Congenita

This dataset represents the largest MC patient cohort and the largest group of functionally characterised CIC-1 variants reported. The systematic correlation of functional data from a large number of variants in standardised conditions with clinical, structural and genetic data has allowed us to construct an evidence-based framework (table 6-1) to guide genetic counselling for people with MC. The key questions in any genetic counselling are whether the variant is causative and whether the variant will be dominantly inherited. These questions have been particularly hard to address in MC and consequently the aim of my analysis was to provide an evidence-based estimate of pathogenicity and inheritance pattern using functional expression data. Using the framework guided by our analysis an initial estimation of the clinical significance and likely associated inheritance pattern can be given based on variant location alone. Where functional expression is available, this risk estimate can be significantly improved once the variant’s functional group is known (table 6-1).

The framework is yet to be verified outside of our large cohort and has its limitations. For example, the nature of the mutation, not only the location, contributes to the functional profile of the variant as exemplified by cases affecting residues G276, M485 and P480 in our cohort. However, it forms an important step in providing improved prediction of the pathogenicity and inheritance of novel CIC-1 missense variants. In addition, it stratifies the interpretation of functional data according to the following principles.

1. For many variants with WT-like properties a differential diagnosis or non-association with MC is found. This implies that for novel variants with WT-like properties an alternative cause for MC should be sought. This is particularly true if the WT-like variant is found in association with a dominant inheritance pattern (0% in our cohort). However, once differential diagnoses are excluded, and for WT-like variants with strong clinical or genetic evidence of association with MC, more detailed characterization of functional properties and/or expression levels of the mutant CLC-1 channel should be pursued. My data suggests that reduced hysteresis in voltage dependence of activation constitutes a novel pathomechanism of recessive MC.
Can MC inheritance pattern be more accurately predicted? Discussion

However, not all changes will be detected in a heterologous system. For example, P408A has a high frequency in our MC cohort. The cDNA change c.1222A>G creates an AG dinucleotide which in silico prediction software suggests could create a mutant splice acceptor site. The effect of the mutation would not be detected in our expression system as the in vitro transcribed mRNA does not undergo splicing events in the Xenopus oocyte. A minigene splice assay may be used to assess erroneous splicing effects in CLCN1 (Ulzi et al., 2014).

2. The behaviour of variants that show WT-like behaviour in the simulated heterozygous condition is recessive regardless of the magnitude of voltage shift in homomeric channels. The only variant with recessive functional properties in the simulated heterozygous condition and reports of dominant inheritance was G285V. It is unclear why this mutation should be associated with dominant MC despite a recessive effect on channel function.

3. The voltage dependence of activation of functionally dominant variants in simulated heterozygous condition is shifted to more depolarized voltages regardless of the properties of the homomeric channel. Eighty-seven percent of probands with these variants reported a dominant or sporadic inheritance pattern.

6.8.2 Missense variants associated with both dominant and recessive modes of inheritance

Some ClC-1 variants have been associated with both dominant and recessive inheritance patterns (Koch et al., 1992; Zhang et al., 1996; Kubisch et al., 1998). In our large dataset, the only variant that was associated with both dominant and recessive inheritance patterns in families with sufficient segregation data was G285E. In the majority of cases (10 out of 26), this variant was associated with a recessive inheritance pattern. However, in 2 it was categorised as dominant, in 2 it was dominant with variable penetrance and in 3 probably sporadic.

G285 is located on the inner aspect of helix H at the subunit interface. The residue appears to line the subunit protopore. Mutation from glycine to any other amino acid increases the length of the amino acid side chain. Mutagenesis on the model shows that mutation to valine or indeed glutamate mean that the G285 residue is then within 4 angstroms of hydrophobic residues on helix G. Thus, it is plausible that increased side chain length could
Can MC inheritance pattern be more accurately predicted? Discussion

lead to conformational changes that affect the dimer interface and/or access to the chloride selectivity filter. Whether this is the case and why this results in variable inheritance patterns remains to be determined.

6.8.3 Novel insights into ClC-1 structure-function

In addition to validating the use of functional expression in genetic counselling combining the functional characterization of 89 mutant channels with location on a homology model has provided significant insight into the molecular physiology of CLC-1. Voltage shifting mutations are significantly more likely than variants from other functional groups to be in the first of the structurally similar transmembrane halves of the CLC-1 subunit (p=0.00001). This suggests that this region is particularly important for the common gating of the channel. Functionally recessive variants are significantly more likely than variants from other functional groups to be in the second of the structurally similar transmembrane halves of the ClC-1 subunit (p=0.001) suggesting that this region is perhaps more associated with protopore gating. Finally, WT-like variants are significantly more likely than variants from other functional groups to be located in the cytoplasmic regions (p=<0.00001).

Only one of the variants showed a larger shift in the voltage dependence of activation in simulated heterozygous rather than in homomeric condition. The affected residue, F297, lies in an intracellular beta sheet at the subunit interface with its backbone interacting with the backbone of F297 of the neighbouring subunit (Figure 6-9B). It is feasible that the F297S mutation in a single subunit causes larger disruption to channel gating than a symmetrical mutation in both subunits.

Finally, this work provides the first description of hysteresis in the voltage dependence of activation of the CLC-1 channel. Hysteresis is “a phenomenon in which the energy required for a system to transition between two states is different for the forward versus the backward reaction” (Tilegenova, Cortes and Cuello, 2017). Hysteresis has been shown to be present in variety of voltage gated channels (Männikkö, Pandey and Larsson, 2005; Tilegenova, Cortes and Cuello, 2017) and to have physiological importance (Männikkö, Pandey and Larsson, 2005), in particular for channels involved in repolarization (Männikkö, Pandey and Larsson, 2005). The voltage sensitivity of CLC-1 is dependent on Cl⁻ concentration and it is likely that hysteresis occurs because of chloride concentration on
both sides of the membrane. This has not been shown in muscle, but the finding of an MC variant lacking hysteresis raises the hypothesis that this occurs in muscle and is important for electrical stability of the muscle. This would constitute a novel pathomechanism for MC.

6.8.4 Potential mechanism for ClC-1 Hysteresis

L587 lies in the loop that connects the last transmembrane helix with cytoplasmic regions of the ClC-1 channel. The biophysical difference between leucine and valine is minimal (they only differ by one additional carbonyl group in the side chain) and therefore it is somewhat surprising that the substitution exhibits such a profound effect.

Given the speed at which it occurs, it seems unlikely the voltage dependent hysteresis in WT ClC-1 is the result of any second messenger process. Potential triggers that would fit with the observed timescale include charge effects on membrane lipids or change in local ionic strength secondary to voltage dependent channel activity. As mentioned above, it is known that the voltage dependence of ClC-1 is affected by chloride concentration and thus ionic strength. Intriguingly, a role for the CBS domain in sensing ionic strength has been described in the ABC transporter OpuA (Biemans-Oldehinkel, Mahmood and Poolman, 2006; Mahmood, Biemans-Oldehinkel and Poolman, 2009).

In OpuA ionic strength is sensed by a surface exposed cationic region in the CBS domain. Below a threshold ionic strength value the transporter is kept in an electrostatically locked position by interaction of this cationic region with the anionic membrane lipids (Biemans-Oldehinkel, Mahmood and Poolman, 2006; Mahmood, Biemans-Oldehinkel and Poolman, 2009). This interaction is modulated by the concentration of anionic membrane lipids as well as two distinct stretches of 7 anionic amino acids found within the disordered CBS domain loops (Figure 6-11). Thus, an electrostatic switch mechanism helps keep the channel closed until a threshold ionic strength is reached. Without these regions the channel is either not sensitive to ionic strength or the threshold for activation is altered (Figure 6-11).

In ClC-1 I have identified an equivalent cationic region of three consecutive lysine residues in the disordered region of the second CBS loop as well as equivalent anionic regions, each 7 amino acids long, one in the first CBS loop and one in the C-terminus as has been described for OpuA.
The role of an electrostatic switch in ClC-1 activation and/or chloride sensitivity has not been explored. However, given the similarity in sequence in the CBS domains of OpuA and ClC-1, it seems possible a similar mechanism for ion sensing may exist in ClC-1 and a response to change in ionic strength (Cl⁻ concentration) could account for the channel’s apparently voltage-dependent hysteresis. This will need to be confirmed experimentally and could be done by functional characterisation of ClC-1 mutants that lack the cationic or anionic regions of the CBS domain.

![Schematic representation of the activity of OpuA](image)

**Figure 6-11** Taken from (Mahmood, Biemans-Oldehinkel and Poolman, 2009).
# Conclusion

This work aimed to investigate aspects of phenotype heterogeneity in skeletal muscle channelopathies. Using phenotype differences as a window to predict physiological variability in skeletal muscle excitability has proved an effective approach. I have identified species’ differences in excitability that help explain phenotype differences between mice and humans with skeletal muscle channelopathies. I have also identified gender differences in WT mouse skeletal muscle excitability that helps to explain the increased phenotype severity in male Draggen Hyper PP mice. In terms of the change from an episodic to a degenerative phenotype with age in periodic paralysis, I have confirmed a similar phenomenon occurs in Draggen Hyper PP mice and identified the likely aetiology for permanent progressive weakness. Somewhat surprisingly, it was changes associated with *normal* aging that accounted for the increased resistance to potassium-induced weakness in old mice. Thus, physiological variation clearly accounts for a proportion of pathological variation.

In addition to physiological variation the work on CIC-1 variants in chapter 6 addressed more typical genotype-phenotype correlation in MC. This study focussed on correlating inheritance pattern with genotype. The findings improve the guidance we can provide to patients with myotonia in whom a *CLCN1* variant is identified. The CIC-1 variants identified in the IJ linker region that affect response to pre-pulse voltages suggest a potential region that is involved in CIC-1 voltage sensing. The variant that prevents hysteresis in channel activation suggests CIC-1 hysteresis is physiologically relevant. Hysteresis is a new observation of CIC-1 channel behaviour. CIC-1 hysteresis may contribute to the dynamic nature of CIC-1 regulation in working muscle. Thus, studying the pathogenicity of CIC-1 variants has facilitated new observation with regards to CIC-1 structure/function and how this relates to inheritance for people with MC.

However, this work also demonstrates that exploring the mechanisms behind pathological variation (e.g., in skeletal muscle channelopathy phenotype) can extend our understanding of normal physiology. For example, it was particularly striking that similar gender differences in ion channel related gene expression were present in both TA *and* triceps muscle *and* have previously been reported for cardiac muscle from various species including humans (Drici
Milou D. et al., 1996; Pham and Rosen, 2002; Parks and Howlett, 2013) (4.11). This consistency suggests the differential expression may be a systemic effect due to hormonal modulation.

These findings are not just academic. Gender difference in physiology, particularly with regards cardiac excitability can have profound consequences. For example as a result of pre-clinical and clinical trials historically having been completed predominantly in males, females experience more frequent and more severe adverse drug events than male subjects (Carey et al., 2017). My data suggests gender difference in physiology also influences the sensitivity of diagnostic tests (Figure 4-2). It is important to note that these differences were only apparent when gender was compared in patients under the age of 40. This reflects what has been reported for congenital long QT syndrome, where the gender difference in phenotype starts after puberty and diminishes after middle age. Therefore, in order to identify these important gender differences, young adult and older subjects should not be grouped together but need to be analysed separately (4.4.2). This will be difficult to achieve in rare diseases as subjects for trials are already limited. However, alternative trial designs such as the recent example of Bayesian aggregated n-of-1 trials (Stunnenberg et al., 2015, 2018) could be considered and would facilitate more targeted analysis. Aggregated n-of-1 trials are especially suited to conditions such as skeletal muscle channelopathies where therapies are often quick in onset and offset.

Like the insights on gender difference in skeletal muscle physiology, exploring the mechanism behind phenotype change with age in periodic paralysis brought new insight into normal ageing physiology. Experiments in Chapter 5 suggest that healthy old murine muscle develops mechanisms to maintain force during prolonged hyperkalaemia despite depolarisation and sodium channel inactivation. This implies that force and excitability can somehow become dissociated in old skeletal muscle exposed to hyperkalaemia. This is intriguing and I am hopeful that it is a phenomenon that may also be observed in aged humans as dissociation of force and excitability has been reported several times and in independent groups performing human studies on muscle from patients with periodic paralysis (see 5.8). If this finding does translate to humans, it would be tempting to speculate that this phenomenon could be exploited to temper the more severe potassium-induced weakness that occurs in younger patients with periodic paralysis. However, the concern would be that the onset of a reduction in attack severity coincides with the onset of progressive permanent weakness and the
mechanisms may somehow be linked. Therefore, further work is needed to delineate these mechanisms and better understand the relationship of reduced attack severity with onset of permanent progressive weakness with age.

The obvious next step for this work is to confirm that similar gender and age-related differences occur in humans with skeletal muscle channelopathies. An appreciation of differences between mouse and human skeletal muscle excitability should also help predict areas where findings may be less likely to translate or be more difficult to identify with sufficient power in humans. For example an increased chloride conductance in mouse compared to human skeletal muscle may make gender differences in skeletal muscle excitability easier to appreciate in mice as chloride channels are reported to be hormone responsive (Fialho et al., 2008; Burge, Hanna and Schorge, 2013). However, the preliminary work in an animal model will mean that we can predict and focus on specific parameters where we would expect change thus significantly increasing our power to detect that change. This is crucial in conditions like skeletal muscle channelopathies that are rare and phenotypically heterogenous.

One key aspect of my thesis is that it demonstrates for the first time that MVRCs are technically feasible in mice both in vivo and ex vivo and can be combined with selective pharmacological blockade (2.2). This combination makes MVRCs an extremely useful tool to selectively probe ion channel function. The next stage to maximise their utility would be to develop a model so that the effect of specific ion channel dysfunction can be better predicted. This would help to further define species differences in ion channel expression and better characterise mouse models of neuromuscular disease. The development of such a model would likely be an iterative process with selective pharmacological blockade and MVRCs in transgenic mice being used to refine the model further. The combination of a model with the fact that MVRCs can be performed in both humans and animal models would make MVRCs an extremely powerful tool to help interpret genetic variants of uncertain significance identified using next generation sequencing techniques. Such a tool will be in high demand in the future given the explosion in availability of omics data and the ongoing difficulty in its timely and accurate interpretation.
8 References


References


References


References


References


References


Appendix

9 Appendices

9.1 List of Publications

9.1.1 First Author:


9.1.2 Second Author:


9.1.3 Middle Author:


4. ‘Loss-of-function mutations in SCN4A cause severe foetal hypokinesia or “classical” congenital myopathy (2016), Brain, 139(3): 674–691.

9.2 Manuscripts Under review:

1. Submitted to Journal Cachexia Sarcopenia Muscle Communications: Ageing contributes to phenotype transition in a mouse model of periodic paralysis. K.J. Suetterlin, S. V. Tan,


### 9.3 Manuscript in preparation:


2. Species difference in muscle excitability and its implications for mouse models of neuromuscular disease. K. J. Suetterlin, R. Männikkö, E. Matthews, L. Greensmith, M.G.

### 9.4 Published Abstracts


5. Measuring activity and sedentary behaviour in people with muscle ion channelopathy and myotonic dystrophy type 1 and assessing the use of individual goal setting to support increasing habitual physical activity. Journal of Neuromuscular Disorders Vol 28 Supplement 1, S29 2018


11. Functional characterisation of the novel ClC-1 variants C179Y and A529V using Two-Electrode Voltage-Clamp and review of ClC-1 structure-function. K Suetterlin, M.G.
