Modeling of Excitation in Skeletal Muscle

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MODELING OF EXCITATION IN SKELETAL MUSCLE

A Dissertation submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy

By

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Abstract

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Recent experimental findings in the Rich lab suggest there are important gaps in our understanding of muscle excitability in various disease states. To generate and test hypotheses as well as to determine whether our current understanding of various aspects of muscle excitation can fully explain experimental findings, an accurate model of muscle excitation was needed. Previous studies have modeled excitation of muscle, but in each case, important aspects were omitted. One reason for this is that little effort has been made to accurately simulate muscle action potentials. In this thesis I present progress made towards generation of a model of muscle excitation that more accurately simulates experimental data than any model to date. I began by accurately simulating the spatial arrangement of t-tubules based on recent detailed imaging studies of t-tubules performed in the Voss lab. This allowed examination of whether the reduction in t-tubule diameter in muscle from a mouse model of Huntington’s disease could account for the reduction in muscle capacitance. My simulations indicate the reduction in t-tubule diameter is insufficient to explain the reduction in capacitance and suggest there is an alteration of muscle membrane itself in Huntington’s disease. I next derived parameters used to simulate the behavior of ion channels involved in generation of action potentials. I did this by reverse engineering the parameters from action potentials recorded in the
Rich lab. The derived parameters led to more accurate modeling of action potentials than previously possible. In addition, sensitivity analysis was performed to identify the key parameters that govern action potential characteristics. Finally, I combined t-tubule geometry with the accurately simulated action potentials to explore the currently accepted idea that action potential propagation into t-tubules is necessary for the process of excitation contraction coupling. My simulations suggest action potential-induced depolarization may spread to the center of fibers intracellularly such that action potential propagation into t-tubules is not necessary for excitation contraction coupling. If true, this would be a significant departure from the current understanding of the role of t-tubules in excitation contraction coupling. My model opens the way for future studies of dysregulation of muscle excitability in a number of different muscle diseases.
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To Caleb
Chapter I: Purpose and Specific Aims

Purpose

The process of excitation contraction coupling converts electrical excitation of muscle into the contraction that underlies movement. In the current view of excitation contraction coupling, action potentials invade into the center of muscle via t-tubules and this invasion, which is thought to occur in an all-or-none fashion, triggers release of Ca$^{2+}$ from intracellular stores to trigger all-or-none muscle contraction. Recent studies in the Rich lab suggest this view may not be accurate. Failure of excitation contraction coupling occurs in a more graded fashion and is accompanied by graded failure of action potentials. This finding was unexpected and suggests our understanding of muscle excitability and action potential invasion into t-tubules may not be complete.

Surprisingly, to our knowledge, no quantitatively accurate model of action potentials and their invasion into t-tubules has ever been generated. Thus, it is not presently possible to determine whether our current understanding of muscle excitation is accurate or whether there are gaps. We thus set out to create a model of skeletal muscle, which is both structurally and electrically accurate. Use of this model will allow us to generate and test hypotheses in a number of different disorders of muscle excitability.

Specific Aims

My first step was to generate a structurally accurate model of t-tubules in skeletal muscle. Accurately modeling t-tubules allowed me to explore the physiologic consequences of t-tubule abnormalities in skeletal muscle of a mouse model of Huntington’s Disease.
Specific Aim I: To test the hypothesis that reduction in t-tubule diameter in Huntington’s Disease muscle accounts for the decrease in electrical estimates of capacitance in Huntington’s Disease muscle.


Although classically recognized as a neurodegenerative disorder, there is increasing evidence of cell autonomous toxicity in skeletal muscle in Huntington’s Disease. The Voss laboratory recently demonstrated that skeletal muscle fibers from the R6/2 model mouse of Huntington’s Disease have a decrease in specific membrane capacitance, suggesting a loss of transverse tubule (t-tubule) membrane. This suggested that a loss and/or disruption of the skeletal muscle t-tubule system contributes to changes in EC coupling. Further work in the Voss lab found that the cross-sectional area of t-tubules at the triad were 25% smaller in R6/2 compared to age-matched control skeletal muscle. A reduction in t-tubule diameter could explain the reduction in measured capacitance and might also contribute to defects in EC coupling. Using computer simulation of reduced t-tubule diameter I explored whether this could account for the reduction of electrical estimates of membrane area derived from measuring cellular capacitance. I discovered that there is a voltage gradient in t-tubules due to current flow in the t-tubules that can account for disparities in optical and electrical measures of
skeletal muscle membrane. I further discovered that the reduction in t-tubule diameter cannot fully account for the reduction in capacitance in Huntington’s Disease muscle. I conclude that there must be an abnormality in the membrane itself that causes the reduction in capacitance. My work suggests further experiments should be performed to study the composition of muscle membrane in muscle from the mouse model of Huntington's Disease.

**Specific Aim II: Derivation of Hodgkin-Huxley parameters from action potentials in skeletal muscle.**

Previous models of skeletal muscle action potentials have used parameters derived from voltage clamp studies of Na\(^+\) and K\(^+\) channels. Surprisingly, only one study we are aware of made any attempt to verify that the Hodgkin-Huxley (H-H) parameters used accurately simulate recorded action potentials. This is of concern because there are several studies suggesting that the recording techniques used during voltage clamp studies may alter the behavior of Na\(^+\) channels. Unless action potentials are accurately simulated they are of little use in testing and generating hypotheses. I set out to accurately model skeletal muscle action potentials such that the Rich lab could generate and test hypotheses. I took the novel approach of directly deriving H-H parameters from action potentials recorded in the Rich lab. One reason this approach may not have been previously taken is that there are many parameters to fit such that arriving at a solution is difficult. I developed a series of steps to model different groups of parameters to break the problem down into manageable parts. My derived parameter set accurately simulated
recorded action potentials. A number of the parameters I derived differ from parameters previously used. To explore the importance of parameters in shaping action potentials I performed sensitivity analysis. I conclude several parameters previously used should be modified in modeling studies moving forward. My accurate simulation of action potentials will allow for future studies of action potential failure in a number of situations.

**Specific Aim III: Modeling of action potential-induced depolarization into the center of muscle fibers**

It is currently widely accepted in the field of excitation contraction coupling that the function of t-tubules in skeletal muscle is to allow for action potential propagation into the interior of the fiber (Calderón, Bolaños, and Caputo 2014; Allard 2018; Hernández-Ochoa and Schneider 2018). This propagation of depolarization via the t-tubule is thought to trigger charge movement in Cav1.1 channels, which are located within the t-tubules, to trigger Ca$^{2+}$ release from the sarcoplasmic reticulum via the ryanodine receptor. Modeling of the t-tubule response to current injection performed in Aim 1 caused us to call this sequence of events into question. My simulation of the spread of depolarization following current injection suggests propagation of depolarization into the interior of the fiber may not require activation of Na$^+$ channels in the t-tubules. To test this hypothesis, I eliminated Na$^+$ channels from t-tubules. Depending on the estimate of conductance of extracellular saline, depolarization in the center of the fiber occurred very well in the absence of Na$^+$ channel activation in t-
tubules. My modeling suggests t-tubules may not function to conduct action potentials into the center of the fiber. This conclusion, if substantiated, leads to the corollary that failure of action potential conduction into t-tubules cannot be a contributor to depolarization-induced failure of excitation contraction coupling.
Chapter II: Significance and Background

Why model skeletal muscle action potentials?

The goal of my thesis is to accurately model skeletal muscle t-tubule structure and action potentials in order to advance our understanding of skeletal muscle function. The first modeling of skeletal muscle action potentials was performed close to 50 years ago (Adrian and Peachey 1973) and has been followed by many other studies. The primary focus of these studies has been exploration of the effect of K+ accumulation in the t-tubule system on action potential shape during repetitive firing in the setting of disease (S. C. Cannon, Brown, and Corey 1993) and normal muscle function (Wallinga et al. 1999; Fortune and Lowery 2009). What is to be gained by performing yet another modeling study of skeletal muscle? We believe there are several reasons to improve on previous models of skeletal muscle action potentials.

One goal of modeling is to determine whether experimental data can be explained by our current understanding. For modeling to perform this function, the model has to generate simulations that closely mimic the response of real muscle. Surprisingly, the field has not made the effort to determine whether simulated responses generated agree with recorded responses. In my first Aim, I constructed a spatially accurate model of muscle t-tubules based on optical measures of membrane. I show that the passive response of skeletal muscle to current injection in Huntington’s Disease and wild type muscle cannot both be modeled using the same value for specific capacitance per unit area of the membrane. This answer identifies a gap in our knowledge and suggests
further experiments looking for a difference in specific membrane capacitance are needed.

Another situation in which we wish to determine whether there are gaps in our current understanding relates to the muscle disease hyperkalemic periodic paralysis. In this disease, paralysis occurs due to depolarization of skeletal muscle. Recent studies performed in the Rich lab suggest failure of excitation during depolarization is more complicated than previously suspected. Action potentials appear to fail gradually, rather than in all or none fashion. While it is beyond the scope of my thesis, the hope is to eventually model depolarization-induced action potential failure to determine whether a relatively simple Hodgkin-Huxley model of muscle excitability is sufficient to explain experimental findings or whether the lab needs to continue to search for novel currents and/or ion channel behaviors to explain the development of paralysis in this disorder.

A second goal of modeling is to generate new hypotheses. As will be shown in the final aim of this thesis, generation of my spatially accurate model of t-tubule structure has led to generation of a novel hypothesis regarding the function of t-tubules in skeletal muscle. Currently it is thought that the function of t-tubules is to allow for action potential propagation into the center of the fiber. I proposed that this may not be the case: depolarization may spread to the center of muscle via the intracellular compartment independent of action potential propagation in t-tubules. This novel hypothesis has important implications for diseases in which the is failure of excitation contraction coupling due to hypoexcitability. In the conclusion of the aim I will discuss our novel hypothesis regarding the function of t-tubules.
**Muscle Overview**

Skeletal muscle has been called the “organ of motion” (Szent-Györgyi 2004) and its importance to normal daily life as we know it, while often taken for granted, cannot be overstated. Contracting skeletal muscle provides the means by which we act on the world around us. It is muscle that converts thought to action via action potentials (APs), the electrical signals initiated by motoneurons and propagated through individual muscle fibers (Brownstone 2006).

Normal movement is accomplished through repeated, voluntary contraction and relaxation of skeletal muscle. Contraction at the cellular level is initiated through a process known as excitation-contraction coupling in which APs are converted to mechanical force via muscle fiber shortening (Allen, Lamb, and Westerblad 2008). Relaxation occurs when APs stop firing and muscle fibers return to resting length. Normal movement depends on the ability to regulate muscle activation by regulating firing of APs.

Dysregulation of AP generation or propagation leads to problems with muscle movement. Too many APs, or APs fired at the wrong time, impairs muscle relaxation resulting in the muscle stiffness of myotonia. Too few APs, or APs failing to fire when needed, results in weakness or paralysis (Metzger et al. 2019). In order to develop novel therapies for disorders of muscle excitability, an enhanced understanding of the underlying pathophysiology is required.

**Excitability of skeletal muscle**
The normal resting potential of skeletal muscle is close to \(-85\) mV (M. M. Rich and Pinter 2001; Novak et al. 2015). Membrane resting potential in skeletal muscle is set by transmembrane K\(^+\), Cl\(^-\) and Na\(^+\) ion gradients and their relative conductances. At rest, net transmembrane current is zero meaning K\(^+\) and Cl\(^-\) current out equals Na\(^+\) current in. Membrane potential will be closest to the reversal potential of the ion with the largest relative conductance. The reversal (Nernst) potential for a given ion, can be calculated by the Nernst equation \(E_{ion} = \frac{RT}{zF} \ln \left( \frac{[i_{ion_out}]}{[i_{ion_in}]} \right)\) if the ion gradients are known. Resting membrane potential is very near the K\(^+\) Nernst because K\(^+\) has much higher conductance than Na\(^+\) at rest (Jurkat-Rott and Lehmann-Horn 2004). ClC-1 chloride channels are responsible for 60 to 80% of resting conductance (Palade and Barchi 1977), but do not contribute to resting potential because Cl\(^-\) is passively distributed across the cell membrane (Adrian 1961). This means that any prolonged change in steady-state membrane potential will cause Cl\(^-\) to redistribute to accommodate this new equilibrium (Sejersted and Sjøgaard 2000). Because Cl\(^-\) reversal is very near resting membrane potential, ClC-1 channels help stabilize membrane potential to prevent spontaneous AP generation (Jurkat-Rott and Lehmann-Horn 2004).

Voltage-insensitive K\(^+\) channels are responsible for most of the background “leak” conductance responsible for setting resting potential (Jurkat-Rott and Lehmann-Horn 2004). The K\(^+\) channel open at rest is the classic inward-rectifying Kir2.1 channel which conducts inward K\(^+\) currents when membrane potential is more negative than the K\(^+\) reversal, but conducts less outward K\(^+\) current due to intracellular pore block by Mg (Hibino et al. 2010). Despite being less effective in passing outward K\(^+\) current, Kir channels pass enough current to set the resting potential near \(-85\) mV.
There are some voltage-gated $K^+$ channels that contribute to regulation of resting potential in muscle. These include both fast-inactivating (Kv3.4, Kv1.4) and slowly-inactivating (Kv7.4, Kv7.5) “KCNQ” Kv channels (DiFranco, Quinonez, and Vergara 2012). These $K^+$ channels open below the threshold of APs to repolarize the membrane and are important modulators of electrical excitability (Jurkat-Rott and Lehmann-Horn 2004).

Finally, electrogenic pumps also directly contribute to resting potential via electrogenic current. The Na/K-ATPase produces a small outward hyperpolarizing current by exchanging three Na$^+$ out for two K$^+$ in, and contributes ~3 mV hyperpolarization to resting potential (Torben Clausen 2003).

Like nerve and cardiac tissue, skeletal muscle is electrically excitable, having the ability to generate and propagate APs. Muscle APs are initiated by trains of APs from lower motoneurons. As APs arrive at the neuromuscular junction, acetylcholine is released into the synaptic cleft. Binding of acetylcholine to post-synaptic receptors causes endplate potentials. In healthy muscle each endplate potential triggers a single AP (Metzger et al. 2019).

The ability to generate action potentials comes from expression of ion channels that open and close in response to changes in cell membrane potential (Jurkat-Rott and Lehmann-Horn 2004; Jurkat-Rott, Fauler, and Lehmann-Horn 2006). APs have distinct phases: 1) an initial resting or baseline potential, 2) depolarization to threshold, 3) a sudden spike, and 4) repolarization back to baseline (Bean 2007). AP phases are dominated by distinct ionic current flows that are both time- and voltage-dependent (Jurkat-Rott and Lehmann-Horn 2004). At rest, skeletal muscle membrane is permeable...
to K⁺ and Cl⁻, but relatively impermeable to Na⁺ (Jurkat-Rott and Lehmann-Horn 2004). During depolarization, Na⁺ begins flowing in until threshold is reached (defined by the point at which inward flow of Na⁺ is equal and opposite outward flow of K⁺ and Cl⁻) (FitzHugh 1960; Noble and Stein 1966). An AP spike is initiated the moment outward K⁺ and Cl⁻ are overcome by a sudden increase in inward Na⁺ current. The AP peaks as inward Na⁺ current quickly inactivates (Jurkat-Rott and Lehmann-Horn 2004). Repolarization to baseline is driven by a large outward K⁺ current (Bean 2007; Metzger et al. 2019).

The fast depolarization responsible for producing an AP spike is due to a large inward current carried by the Nav1.4 channels (Jurkat-Rott and Lehmann-Horn 2004). Nav1.4 channels have three distinct states: closed (ready to be activated), open (passing current), and inactivated (closed and refractory to opening). These channels have very fast kinetics meaning they have the ability to open and close very quickly. They also quickly inactivate, limiting the amount of Na⁺ current passed and the length of time the membrane remains depolarized (J. Patlak 1991). Activation is both time- and voltage-dependent. Local membrane depolarization (such as near the neuromuscular junction (NMJ)) causes opening of local Nav1.4 channels. This causes further depolarization that activates more Nav1.4 channels downstream (Jurkat-Rott and Lehmann-Horn 2004). Recovery from fast inactivation is voltage-dependent, requiring brief hyperpolarization of membrane potential over a few milliseconds (Jurkat-Rott and Lehmann-Horn 2004). Nav1.4 channels also display slow inactivation that occurs when average membrane potential remains relatively depolarized over seconds to minutes, such as during AP
trains. Recovery from slow inactivation requires longer membrane hyperpolarization that lasts seconds to minutes (Ruff 1996b, 1996a; Mark M. Rich and Pinter 2003).

Membrane repolarization following the AP peak is mainly driven by opening of slowly-inactivating Kv channels, particularly Kv1.1, with some contribution from Kv1.7 and Kv1.8 (Jurkat-Rott and Lehmann-Horn 2004). These channels have slower kinetics than the Nav1.4 channels and pass an outward hyperpolarizing current with a delayed onset compared with AP initiation. Thus, they are called “delayed rectifier” K\(^+\) channels.

**T-tubules in skeletal muscle**

Muscle fibers are unique among cell types having most of their cell membrane contained within the cell in the form of t-tubules (L. D. Peachey 1966; Andersson-Cedergren 1959). T-tubules are formed from invaginations of the surface membrane into the core of the fiber during embryonic development (Clara Franzini-Armstrong and Jorgensen 1994; Chal and Pourquié 2017). Although the t-tubules are “inside” the muscle fiber, their lumens are actually continuous with the extracellular space (Lee D. Peachey and Franzini-Armstrong 2010), meaning the luminal fluid is in quasi-equilibrium with the extracellular fluid at rest (L. D. Peachey 1966). T-tubules add a lot of membrane surface area, about 80% of total cell membrane, and are therefore responsible for most of the capacitance of skeletal muscle (Lee D. Peachey and Franzini-Armstrong 2010). Due to their long, narrow, branching geometry, t-tubules have a large surface area-to-volume ratio (L. D. Peachey 1965; Lee D. Peachey and Franzini-Armstrong 2010). This means there is a large amount of surface area for ion conductance, and a very small volume for changes in ion concentrations to occur (Jurkat-
This becomes important during intense muscle activity when large ionic fluxes are occurring (Sejersted and Sjøgaard 2000; Allen, Lamb, and Westerblad 2008). T-tubules are thought to perform the critical role of carrying APs from the fiber surface to the center for synchronized muscle activation and contraction (Jurkat-Rott, Fauler, and Lehmann-Horn 2006). T-tubules are the site of excitation-contraction coupling, in which electrical impulses are converted to mechanical force. The narrow t-tubules structure may ensure AP spread occurs unidirectionally along fiber radius by imposing an access resistance at the fiber surface (Jurkat-Rott, Fauler, and Lehmann-Horn 2006). As APs propagate from the fiber surface down into the t-tubules, they cause voltage-sensing Cav1.1 channels in the t-tubule membrane to change conformation (Catterall 2011; Bannister and Beam 2013; Hernández-Ochoa and Schneider 2018). This conformation change opens ryanodine receptors (RyRs) in the sarcoplasmic reticulum, to which Cav1.1 are physically coupled, causing Ca\(^{2+}\) to be released into the cytoplasm (Allen, Lamb, and Westerblad 2008; Allard 2018; Hernández-Ochoa and Schneider 2018). A transient Ca\(^{2+}\) spike in the cytoplasm allows cross-bridge cycling to occur, causing the fiber to shorten in contraction (Dulhunty 2006; Allard 2018). Ca\(^{2+}\) is then quickly removed by SERCA pumps, also located in the SR, to stop cross-bridge cycling and allow relaxation to occur (Dulhunty 2006; Allard 2018).

It has long been assumed that ion channel densities in the t-tubules and the surface membrane are not identical (Jaimovich et al. 1976; Palade and Barchi 1977). While the exact distribution of ion channels between the surface and t-tubule membranes is currently unknown, techniques ranging from immunofluorescence to detubulation to ion
withdrawal have been applied to estimate the range of likely densities based on known behavior (Jurkat-Rott, Fauler, and Lehmann-Horn 2006). T-tubules are believed to contain anywhere from half the total Nav1.4 channels (Jaimovich et al. 1976; DiFranco and Vergara 2011) to an equivalent density with the surface membrane (Moore and Tsai 1983). This relatively high density allows active spread of depolarization from the fiber surface to the interior of the fiber along the t-tubule membrane (Jurkat-Rott and Lehmann-Horn 2004; Jurkat-Rott, Fauler, and Lehmann-Horn 2006).

Kir2.1 are highly expressed in the t-tubules and are the predominant K\(^+\) channel in the t-tubule (Jurkat-Rott, Fauler, and Lehmann-Horn 2006; Kristensen, Hansen, and Juel 2006; Allen, Lamb, and Westerblad 2008). In this location, these channels, as strong inward rectifiers, (Hibino et al. 2010) confer unique advantages. During depolarization to threshold, they essentially become blocked allowing the Na\(^+\) current to quickly reach its maximum. Because of their inward rectification, they prevent large K\(^+\) losses from active muscle, reducing K\(^+\) accumulation in t-tubules and improving muscle endurance during intense activity (Sejersted and Sjøgaard 2000; Jurkat-Rott, Fauler, and Lehmann-Horn 2006; Kristensen, Hansen, and Juel 2006).

ClC-1 channel density is also thought to be high in the t-tubules, based on data from experiments using diverse techniques to estimate their relative distribution (Jurkat-Rott, Fauler, and Lehmann-Horn 2006; Palade and Barchi 1977; Dulhunty 1979; Coonan and Lamb 1998; Thomas H. Pedersen et al. 2004). However, this has yet to be confirmed by immunofluorescence (Gurnett et al. 1995; Jurkat-Rott, Fauler, and Lehmann-Horn 2006). One recent modeling study estimated their density to be at least 60% based on data from fluorescence signals using voltage-sensitive dyes (Di Franco, Herrera, and
Vergara 2011). It is thought that Cl\(^{-}\) does not contribute much to single APs, having most of its effect at rest. However, recent experiments suggest Cl\(^{-}\) may redistribute much more rapidly than previously supposed and may indirectly dampen firing rate during AP trains by reducing the rate of repolarization (Voss lab, unpublished data; also see (Bækgaard Nielsen et al. 2017)).

Cav1.1 channels are highly expressed in t-tubular membrane, but are absent from the sarcolemma (Jorgensen et al. 1989). Despite their crucial role as the voltage sensor in excitation contraction coupling, Cav1.1 calcium channels do not pass significant current under physiologic conditions due to a slow activation time constant and relatively depolarized half-maximum activation (Jurkat-Rott and Lehmann-Horn 2004; Dayal et al. 2017). Because of this, it is thought that these channels play little to no role in APs (Dayal et al. 2017).

APs in intact organisms rarely occur as single events, but rather as trains of multiple spikes. During AP trains, large transmembrane fluxes of Na\(^{+}\) and K\(^{+}\) occur, potentially changing resting ion gradients if activity is intense or prolonged. Given the large surface area and small volume of the t-tubules, changes in ion gradients across the t-tubular membrane may be particularly pronounced (Simeon P. Cairns et al. 2003; S. P. Cairns et al. 1997; S. P. Cairns and Lindinger 2008). Relatively small ionic fluxes can result in disproportionately large changes in ion concentrations in the narrow t-tubular space. Because the t-tubules have such a long, narrow branching geometry, the accumulated K\(^{+}\) cannot readily diffuse out of the t-tubular space. As K\(^{+}\) concentration increases in the t-tubules, E\(_{K}\) within the t-tubules becomes progressively depolarized. Because E\(_{K}\) sets the lower bound of the resting potential, membrane potential within the
t-tubules also becomes depolarized (Allen, Lamb, and Westerblad 2008; Sejersted and Sjøgaard 2000).

**Depolarization-induced failure of excitation contraction coupling**

For normal movement, muscle must be able to generate adequate force via contraction. Weakness is failure to generate normal force at a given level of stimulus or effort. Weakness based on a failure of excitability is usually accompanied by a depolarized resting potential. A depolarized resting potential often results from depolarization of the K\(^+\) Nernst due to increased extracellular K.

For example, during intense exercise, K\(^+\) is dumped into the extracellular space with the repolarization of each AP by the delayed rectifier Kv channels. Interstitial K\(^+\) averages 11-13 mM in human muscle during exercise (McKenna, Bangsbo, and Renaud 2008; Green et al. 2000), approximately two times greater than is typically measured in plasma (Sjøgaard, Adams, and Saltin 1985). Restoration of K\(^+\) equilibrium is mostly dependent on Na/K-ATPase and Kir (Sejersted and Sjøgaard 2000; Allen, Lamb, and Westerblad 2008), which can become overwhelmed at high rates of muscle stimulation (T. Clausen et al. 1987; Overgaard et al. 1999; Juel 1986).

The weakness and loss of excitability from changes in extracellular K\(^+\) are even more dramatic in hyperkalemic periodic paralysis (HPP). HPP is an autosomal dominant inherited muscle disorder caused by gain-of-function mutations of Nav1.4 which is triggered by intense exercise following rest or a carbohydrate-rich meal (Stephen C. Cannon 2015). These events temporarily raise extracellular K\(^+\) and depolarize the membrane potential enough to activate mutated Nav1.4. Because the mutations decrease
inactivation, they effectively result in a Na\(^+\) persistent inward current. A persistently activated Nav current will continue to depolarize muscle until all the normal Nav1.4 channels are inactivated. With extensive (fast and slow) inactivation of Nav1.4 channels, muscle becomes inexcitable and is paralyzed until the K\(^+\) Nernst and resting potential return to normal to allow Nav channels to recover. Shifts in extracellular K\(^+\) concentration that have little to no effect in normal muscle, cause sustained membrane depolarization in HPP with attacks of weakness or even paralysis lasting hours to days.

In addition to the above disorders, there is an acquired muscle channelopathy known as intensive care unit acquired weakness, in which there is an electrical component of weakness due to a combination of depolarization of the resting potential and an acquired sodium channelopathy (Teener and Rich 2006; Friedrich et al. 2015). This weakness often persists months after discharge from the ICU.

In each of these cases, failure to generate and propagate APs results in muscle force failure because depolarization of the t-tubules is insufficient to trigger Ca\(^{2+}\) release for the sarcoplasmic reticulum. What is not clear is how failure of APs actually happens and how this contributes to decreased excitability and reduced muscle force.

APs have long been established as all-or-none phenomena. Studies of AP generation undertaken in the early twentieth century lead to formulation of the “all-or-none principle” of APs (Pratt 1917; Cole and Curtis 1939). The all-or-none principle of APs states that if the magnitude of a given stimulus is large enough to bring membrane potential to threshold, an AP will be fired. Any stimulus less than this will only produce a graded potential (Fauler, Jurkat-Rott, and Lehmann-Horn 2012). This all-or-none phenomenon is so unquestioned it is taught in standard textbooks as a basic property of
excitable tissue (Boron and Boulpaep 2017; Koeppen and Stanton 2017). However, it must be noted that resting potential was normal in these studies. Much less is known about how AP failure proceeds under pathologic conditions in which there is prolonged membrane depolarization.

Prolonged depolarization of membrane potential caused by build-up of K\(^+\) in t-tubules or build-up of extracellular K\(^+\) can cause progressive slow inactivation of Nav channels (Ruff 1996b, 1996a; Mark M. Rich and Pinter 2003). Inactivating Nav channels causes AP threshold to become increasingly higher (S. P. Cairns et al. 1997; Krnjevic and Miledi 1958), peaks become lower, and rate of rise is less steep. Because the availability of Nav channels is decreasing, increasing stimulus is required for AP generation and propagation. Complete AP failure can result if enough Nav channels become inactivated such that the magnitude of the Na\(^+\) current required to reach threshold exceeds the available Nav conductance (Teener and Rich 2006; Allen, Lamb, and Westerblad 2008).

Previous work has hinted at graded failure of APs and their potential contribution to fatigue or weakness (S. P. Cairns et al. 1997; M. M. Rich and Pinter 2001; Simeon P. Cairns et al. 2003; Mark M. Rich and Pinter 2003). Lannergren and Westerblad reported ‘action potential fatigue’ in repetitively stimulated muscle, noting decreased AP amplitude, increased duration, and depolarized resting potential (Lännergren and Westerblad 1987). They suggested excitation contraction coupling failure as a mechanism for force reduction due to changes in AP shape, especially noting the weak early after potential, which is thought to indicate reduced regenerative activity in the t-tubules (Westerblad and Lannergren 1986).
In experiments in whole muscle, artificial elevation of K⁺ in the bath solution resulted in progressive membrane depolarization and gradual decrease of AP amplitude. There was no sudden AP failure (Mark M. Rich and Pinter 2003; Ammar et al. 2015). Contractile force showed a similar progressive decline in elevated extracellular K⁺, with effects beginning at 7 mM and total force failure at 14 mM, as resting potential depolarized from -80 to -55 mV (S. P. Cairns et al. 1997; Ammar et al. 2015). In a related study, extracellular Na⁺ was progressively decreased to mimic the Na⁺ depletion that may occur in t-tubules during high frequency stimulation. As Na⁺ was decreased, AP amplitude and force progressively decreased while resting potential remained normal (~-78 to -79 mV) (Simeon P. Cairns et al. 2003). This suggests that a reduction in AP amplitude alone may be sufficient to cause loss of force.

There thus exists in the literature a disconnect between data suggesting graded failure of excitation and the currently accepted idea of all or none failure of action potentials. One question that can be explored using computer simulation of action potentials is whether a model producing all or none action potentials at normal resting potential produces graded failure of excitation as the membrane potential is depolarized. Such a finding would resolve the discrepancy between data showing graded failure and the currently accepted view of all or none action potential failure. In order to address this question, it is necessary to generate an accurate model of APs in skeletal muscle. Such a model must accurately represent t-tubules to allow for investigation of action potential invasion into t-tubules as this is a critical step in excitation contraction coupling.
Generation of a computer model to address questions regarding disorders of muscle excitability:

Early models of skeletal muscle APs were based on data from amphibians. However, gating of ion channels in mammalian muscle differs from amphibian, especially Nav channels (Adrian and Marshall 1977). Later, as data became available from mammalian studies (Beam and Donaldson 1983; Pappone 1980; Adrian and Marshall 1977), models began incorporating parameters from those analyses (S. C. Cannon, Brown, and Corey 1993). Parameters for ion conductances in a single model were derived from experiments in multiple species (e.g., combining data from frog and rat), and were often taken verbatim from one model and used in another (S. C. Cannon, Brown, and Corey 1993; Wallinga et al. 1999; Fortune and Lowery 2009; Fraser, Huang, and Pedersen 2011). Most of the model parameters for ion conductances were chosen empirically to achieve outputs that were qualitatively similar to experimental data, rather than by fitting the data itself.

In contrast to previous models, I will be using a quantitative approach to match specific features of intracellular recordings of APs, specifically, rate of rise, peak height, and rate of repolarization. These characteristics are important indicators of excitability (Novak et al. 2015; Filatov, Pinter, and Rich 2005) and should not be glossed over when studying AP failure. Careful matching of these characteristics in simulated APs has been omitted in previous models (Adrian and Peachey 1973; S. C. Cannon, Brown, and Corey 1993; Wallinga et al. 1999; Fortune and Lowery 2009; Fraser, Huang, and Pedersen 2011) with the exception of one from the Rich lab that made a cursory effort to match action potential peak and half-width (Novak et al. 2009). In all other cases, if the model
produced simulated APs that were qualitatively similar to experimental APs, it was considered sufficient and no further analysis was done.
Chapter III: General Methods

Model implementation

I have implemented the model in MATLAB, (The MathWorks Inc. 2019) a well-known language for scientific computing that allows a lot of flexibility in how the model is specified. I had originally explored NEURON (Hines and Carnevale 2018), a modeling software tailored to modeling neurons, and found it could not accommodate the very different geometry of skeletal muscle. Because of MATLAB’s flexibility, it allows complete control over the geometry specification, as well as any other features of the model. In addition, MATLAB has available several types of ordinary differential equation (ODE) solvers that can perform numerical integrations efficiently, making it an excellent choice for modeling and simulations (Shampine and Reichelt 1997).

Modularization of code

The model code is written in modules. Each module is a group of files containing scripts for model parameters, simulation control, geometry specification, the differential equations for the mathematical model, and the solver being applied to them. Having each of these in separate code files makes it simple to swap out different sets of parameters or styles of equations. This also makes it simple to test model behavior under different starting conditions or to use alternative styles of channels instead of Hodgkin-Huxley.

ODE solver (ode15s)
The model equations are a set of ODEs and all model simulations require simultaneous solution of these equations by integration. To accomplish this, I am using the ‘ode15s’ numerical integration algorithm. This ODE solver is made specifically for handling ‘stiff’ problems. Stiff problems are characterized by sudden changes in the solution values, such as when an AP spike is occurring or during the step up or down in membrane potential during voltage clamp (The MathWorks Inc. 2020b). When regular ODE solvers encounter stiff problems, they start taking smaller and smaller step sizes. Because of this, the time needed to reach a solution may become impractical or the solver may fail to reach a solution altogether. Ode15s uses the backward Euler method, an implicit numerical method that can take larger step sizes, and will handle stiff problems efficiently without risking numerical instability (The MathWorks Inc. 2020a).

The model equations are a type of initial value problem that starts at some initial state. Parameters are supplied for the initial conditions including (to name just a few) the resting membrane potential, ion reversal potentials, and the state of the channels (closed). At each time step, the solver integrates the set of differential equations to find the solution for that time step. The results of the previous step become the ‘initial conditions’ for the next step. When the solver is finished, the result is a matrix of solutions for each parameter at each time step (The MathWorks Inc. 2020a).

Curve Fitting

For curve fitting, I am using the ‘lsqcurvefit’ function from MATLAB’s Optimization Toolbox package (The MathWorks, Inc. 2021). Lsqcurvefit is a data fitting algorithm that efficiently applies least squares fitting to a nonlinear function. Because the
functions that describe the time course of ionic currents are nonlinear functions, this fitting algorithm is the appropriate choice for fitting Nav current traces from voltage clamp experiments.

To run the fitting algorithm, the data to be fit, a user-specified function to be fit to the data, and initial parameter values (and their bounds) are supplied to the lsqcurvefit function. Over many iterations, the parameters that best fit the data are found. The fitting result is given as the final parameter values and the goodness of fit as the sum of squared residuals.

**Statistical analyses**

In my work I use both qualitative and quantitative comparisons to evaluate the effectiveness of my models. For the cases in which my modeling output was simulating specific capacitance measurements (Aim 1) or changes in Na⁺ conductance in the t-tubules (Aim 3), I had a modeling sample size of one, so statistics could not be used. In Aim 2, after fitting experimental AP data, I obtained 8 parameter sets (one for each trace fitted). To evaluate the quality of the simulated APs from each parameter set, I compare AP peak height, half-width, rate of rise, and rate of repolarization of the simulated APs with the experimental APs using a paired student’s t-test with Bonferroni correction to account for multiple comparisons. Statistical comparisons were made with OriginPro (OriginLab Corporation 2019b).

**Data Used**
Aim 1

Capacitance values from (Waters et al. 2013; Miranda et al. 2017)

Imaging data from (Romer et al. 2021)

Aim 2

Specific Aim I: To test the hypothesis that reduction in t-tubule diameter in Huntington’s Disease muscle accounts for the decrease in electrical estimates of capacitance in Huntington’s Disease muscle.

Prior to attempting to model action potentials, it was necessary to develop a spatially accurate model of t-tubules. The generation of an accurate model of t-tubules was used to explore the physiologic consequences of a t-tubule defect in muscle from Huntington’s disease discovered by the Voss lab. This work has now been published and I am co-first author (Romer et al. 2021). The results presented in this chapter represent my portion of the published work.

Introduction

Huntington’s disease (HD) is a progressive, fatal and incurable degenerative disorder. The disease is caused by a heritable expansion of trinucleotide (CAG) repeats within the huntingtin (Htt) gene (MacDonald et al. 1993) and is estimated to afflict approximately 5.7 per 100,000 individuals of European descent with devastating cognitive and motor defects (Pringsheim et al. 2012). Specifically, motor symptoms include chorea, rigidity, dystonia, bradykinesia and muscle weakness. HD is largely characterized as a neurodegenerative disorder and the motor symptoms are generally thought to be a consequence of striatal defects (Lo and Hughes 2010; Strand et al. 2005;
Wells and Ashizawa 2011; MacDonald et al. 1993). However, the Htt gene is expressed in many tissues, including skeletal muscle (MacDonald et al. 1993; Strand et al. 2005; Miranda et al. 2017; McCourt et al. 2016; Hoogeveen et al. 1993). Significant skeletal muscle pathology has been reported for both human patients and rodent models (Busse et al. 2008; Djoussé et al. 2002; Gizatullina et al. 2006; Kosinski et al. 2007; Julien et al. 2007). Pathological changes in HD skeletal muscle include metabolic and mitochondrial defects (Lodi et al. 2000; Mielcarek and Isalan 2015; Turner, Cooper, and Schapira 2007), atrophy (Ehrnhoefer et al. 2014; Ribchester et al. 2004; She et al. 2011), weakness (Busse et al. 2008; Hering et al. 2016), and altered expression of genes needed for muscle differentiation (Luthi-Carter et al. 2002; Strand et al. 2005). Moreover, in an HD human case study, reduced muscle performance was reported prior to the presentation of neurological symptoms (Kosinski et al. 2007). These studies support the hypothesis that muscle autonomous effects of the mutant htt gene contribute to the HD pathology.

Other groups have reported dysregulation of Ca\textsuperscript{2+} homeostasis and weakness in the skeletal muscle of R6/2 HD mice (Braubach et al. 2014; Hering et al. 2016), suggesting defects in excitation-contraction (EC) coupling, the process whereby an action potential is converted into mechanical force generation. Skeletal muscle EC coupling involves a tightly regulated functional and structural interaction between Cav1.1, the voltage-sensing L-type Ca\textsuperscript{2+} channel in the transverse tubule (t-tubule) membrane, and ryanodine receptor type 1 (RyR1), the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release channel (C. Franzini-Armstrong, Protasi, and Ramesh 1998; Eltit, Franzini-Armstrong, and Perez 2015; DiFranco et al. 2011; Meza et al. 2013). Disruptions in EC coupling have been shown to underlie multiple disease states that affect skeletal muscle (Andronache et al.
Previously, the Voss lab found an early-onset and progressive reduction in R6/2 muscle fiber capacitance normalized to surface area ($C_{m,s}$, $\mu$F/cm$^2$) compared to controls beyond that expected for the disease-related reduction in muscle fiber size (Miranda et al. 2017). A decrease in capacitance would reduce the amount of current required to depolarize the fiber, which could contribute to the increased excitability observed in diseased muscle (Waters et al. 2013). This decrease in $C_{m,s}$ was likely due to a partial loss or disruption of the t-tubule system. Because the proper arrangement of t-tubules next to the sarcoplasmic reticulum in triads is essential for the process of EC coupling, a structural defect in the t-tubules could cause muscle weakness and disruption in Ca$^{2+}$ homeostasis (Hong et al. 2014; Al-Qusairi et al. 2009; Ibrahim et al. 2011). For example, altered t-tubule networks in cardiomyocytes underlie heart failure in both animal models and humans (Cannell, Crossman, and Soeller 2006; He et al. 2001; Kaprielian et al. 2000; Kostin et al. 1998; Louch et al. 2004; Hong et al. 2014; Ibrahim et al. 2011), which likely influences EC coupling (Louch et al. 2006). Detubulation could also help explain weakness in HD muscle (Hering et al. 2016; Busse et al. 2008) and the dysregulated Ca$^{2+}$ signaling found in R6/2 skeletal muscle (Braubach et al. 2014). Thus, the Voss lab hypothesize that the t-tubule system and cell signaling mechanisms that underlie t-tubule development or maintenance are disrupted in R6/2 skeletal muscle fibers. They measured the whole cell density and ultrastructure of t-tubules in control and late-stage R6/2 muscle and found no obvious change (Romer et al. 2021). They did, however, find a 25% decrease in t-tubule diameter using EM.
The goal of this aim was to determine the mechanism underlying a discrepancy between optical measures and electrical measures of the amount of membrane in muscle from mice with Huntington’s Disease. We simulated voltage clamp measurements of wild type and HD muscle capacitance measurements, which probe the amount of membrane in t-tubules to determine whether the reduction in t-tubule diameter could explain the difference in estimates of membrane area. We used a computational model of skeletal muscle with the t-tubules represented by a radial cable. The model utilized our optical measures of the mammalian t-tubule system. Early radial cable models were based on studies in amphibian skeletal muscle (Adrian, Costantin, and Peachey 1969; Adrian, Chandler, and Hodgkin 1970). However, amphibian skeletal muscle has only one triad per sarcomere, whereas mammalian has two (L. D. Peachey 1966; Andersson-Cedergren 1959). More triads per sarcomere means greater t-tubule density in mammalian muscle, which could impact measures of capacitance. Others have applied the radial cable structure to mammalian models (Kim and Vergara 1998; Wallinga et al. 1999; Thomas H. Pedersen, L-H Huang, and Fraser 2011), but we are the first to develop a model directly from measurements of the t-system in mammalian skeletal muscle using high-powered optical methods. Our simulations suggest the discrepancy between optical and electrophysiologic estimates of the amount of membrane is due to a change in the membrane of Huntington’s Disease muscle that reduces its intrinsic capacitance.

**Methods**

*Capacitance Calculations from Optical Data*
Calculations of t-tubule membrane surface area and their contribution to total capacitance were made under the following assumptions: 1) muscle fiber shape may be approximated by a cylinder, 2) t-tubules were also roughly cylindrical and uniformly spaced throughout the fiber, 3) there was no difference in gross cellular t-tubule density and 3-dimensional arrangement between control and HD fibers (see Table 1, (Romer et al. 2021), 4) skeletal muscle membrane has a specific capacitance normalized to total membrane surface area (C_m,S+TT) of 0.9 μF/cm^2 (Hodgkin and Nakajima 1972).

A unit volume was defined as a cube of 10 μm per side (equal to 1 × 10^{-9}cm^3) for which the amount of t-tubule membrane contained could be calculated from measured t-tubule density and spacing (see Table 1, (Romer et al. 2021). The number of t-tubules, \( n \), in the unit volume was found by

\[
 n = \frac{10 \mu m}{TT_x} \times \frac{10 \mu m}{TT_y}
\]

(1)

where \( TT_x \) and \( TT_y \) are the measured axial and orthoaxial spacing, respectively.

Next, the average surface area of a single t-tubule from this unit volume was calculated using the t-tubule perimeter measurements from electron micrographs (see Figure 3, (Romer et al. 2021). The inner t-tubule perimeter, \( p_i \), was defined as the measured perimeter (see Table 2, (Romer et al. 2021) minus an outer margin of 5 nm to account for the inner leaflet thickness (Andersson-Cedergren 1959). The average surface area of a single t-tubule in the unit volume, with side length, \( l \), of 10 μm, was equal to \( p_i l \). From this value, surface area of the transverse t-tubular elements (TTS_{trans}) per unit volume was calculated as
The total surface area of the t-tubule system ($SA_{TTS}$) per unit volume is found by dividing the surface area of the transverse t-tubules ($TTS_{trans}$) per unit volume by the fraction of the transverse pixel elements (Table 1, (Romer et al. 2021)).

\[
\frac{SA_{TTS}}{unit\ vol} = \left(\frac{TTS_{trans}}{unit\ vol}\right) \cdot \left(\frac{\%\ transverse\ pixels}{\%\ total\ pixels}\right)
\]

The surface area of the longitudinal t-tubular elements is difference of the $SA_{TTS}$ and the $TTS_{trans}$.

\[
\frac{TTS_{long}}{unit\ vol} = \frac{SA_{TTS}}{unit\ vol} - \frac{TTS_{trans}}{unit\ vol}
\]

To obtain total membrane surface area of the t-tubules, it was necessary to calculate fiber surface area and volume. Surface area ($SA = \pi dl$) and volume ($Vol = \frac{\pi}{4}d^2l$) of a fiber may be estimated from the diameter and length, assuming the fiber shape is approximated by a cylinder. Average fiber surface area was calculated from microscopy measurements taken during electrophysiological recordings (Waters et al. 2013). Fiber volume was calculated from the relationship total fiber volume = $\frac{d}{4}(SA)$, where $d$ is fiber diameter and $SA$ is fiber sarcolemma surface area. Because R6/2 nuclei have a greater density and therefore occupy a larger percent volume in R6/2 fibers
than in control, intracellular space available for TTS membrane would be reduced in these fibers. To account for this difference, it was necessary to adjust for the volume occupied by the nuclei in each fiber type. Nuclei reside at the periphery of mature muscle fibers in control and R6/2 fibers, such that the nuclei appear to lie half in and half out of the fiber volume (Video 1 and 2, (Romer et al. 2021)). The available volume becomes

\[ \text{adjusted fiber volume} = \text{total fiber volume} \times \left(1 - \frac{1}{2} \left(\% \text{ nuclei volume}\right)\right) \]  

(5)

Total t-tubule surface area was

\[ S_{ATTS} = \text{adjusted fiber volume} \times \left(\frac{S_{ATTS}}{\text{unit vol}}\right) \]  

(6)

and total membrane surface area of a fiber was

\[ \text{total } S_{Afiber} = S_{A_{surface}} + S_{ATTS} \]  

(7)

Assuming skeletal muscle membrane has a \( C_{m,S+TT} \) of 0.9 F/cm\(^2\) (Hodgkin and Nakajima 1972), total fiber capacitance (\( C_{\text{total}} \)) is obtained from total membrane surface area by

\[ C_{\text{total}} = 0.9 \, \mu F/cm^2 \times \text{total } S_{Afiber} \]  

(8)

Finally, by dividing total capacitance by sarcolemma surface area, we obtain the specific capacitance normalized to sarcolemmal surface area (\( C_{m,S} \)).
\[
C_{m,S} = \frac{C_{\text{total}}}{SA_{\text{surface}}}
\]  

Note, \( C_{m,S} \) is commonly obtained in electrophysiology studies by dividing the total fiber capacitance by the fiber surface area estimated from a brightfield image and is often referred to as simply the fiber specific capacitance, \( C_m \).

**Model Structure**

We followed the general approach of Wallinga et al. (Wallinga et al. 1999) for the radial cable equations. Total membrane current density \( I_m, \frac{\mu A}{cm^2} \) was the sum of a capacitive current \( I_c, \frac{\mu A}{cm^2} \) and ionic current \( I_{\text{ionic}}, \frac{\mu A}{cm^2} \) between the intracellular and extracellular compartments, and a t-tubular current \( I_T, \frac{\mu A}{cm^2} \): \( I_m = I_c + I_{\text{ionic}} + I_T \), such that \( I_c = C_{m,S+T}(\frac{dV_m}{dt}) \), \( I_{\text{ionic}} = g_{\text{leak}}(V_m - E_{\text{leak}}) \), and \( I_T = \frac{V_m - V^n}{R_a} \). In these equations, \( g_{\text{leak}} \) was the leak conductance in \( \frac{mS}{cm^2} \), \( V_m \) was the membrane potential in \( mV \). \( E_{\text{leak}} \) was the reversal potential of the leak conductance in \( mV \), and \( V^n \) was the membrane potential across the t-tubule membrane for the outermost shell where \( n \) is the total number of shells. The t-tubular current flows from the lumen of the outermost shell of the t-tubule compartment to the extracellular space across the access resistance \( R_a, k\Omega \cdot cm^2 \). To define current flow involving the t-tubule shells, several geometric factors were used. Rho \( (\rho) \) was the unitless ratio of t-tubule volume to fiber volume, zeta \( (\zeta) \) was the ratio of t-tubule volume to surface area measured in cm, and the unitless tortuosity factor sigma \( (\sigma) \) specified the fraction of t-tubule branches oriented radially. To account
for the effects of altered t-tubular luminal resistance and radius on capacitance measurements, values of rho and zeta for the model were obtained using empirical microscopy data. Rho (ρ) was calculated by dividing total tubule volume by fiber volume. Total tubule volume was divided by total tubule surface area to obtain zeta (ζ).

For the $i^{th}$ shell, the membrane surface area in cm$^2$ shared between the t-tubule shell “$i$” and the intracellular compartment was $A^i_t = \frac{\rho Vol_i}{\zeta}$, where $Vol_i$ was the volume of the t-tubule shell “$i$” in cm$^3$, $Vol_1 = \pi x (r_1^2 - r_{i-1}^2)$ and $r_i$ was the radius of shell “$i$” in cm.

The shells were connected by a luminal conductance of shell “$i$” in mS, $g^i_{L,t} = \frac{2\pi r_i x \tilde{G}_L}{\Delta r}$, where $\tilde{G}_L$ was the effective t-tubular cable conductivity in mS/cm and $\Delta r$ was the radial thickness of the t-tubule lumen in cm. $\tilde{G}_L = \rho \sigma G_L$, where $G_L$ was the conductivity of fluid in the t-tubule lumen in mS/cm. $V^i_t$ was defined as the potential of the intracellular space relative to the potential of the lumen of the $i^{th}$ t-tubule shell in mV. The capacitive current for each shell “$i$” was $I^i_{C,t} = C_{m,S+TT} \left( \frac{dV^i_t}{dt} \right)$ in $\mu A/cm^2$. The ionic current of each shell “$i$” was $I^i_{ionic,t} = g_{leak} (V^i_t - E_{leak,t})$ in $\mu A/cm^2$, where $E_{leak,t}$ was the reversal potential of the t-tubule leak conductance in mV. The currents flowing into each shell were equal to the currents flowing out of each shell: $I^i_{C,t} + I^i_{ionic,t} = g^i_{L,t} \left( V^{i+1}_t + V^{i-1}_t - 2V^i_t \right) / A^i_t$. The t-tubule leak channel density was assumed to equal that of the sarcolemma because the electrophysiologic data was recorded with all known channels blocked (Waters et al. 2013; Miranda et al. 2017).

Model Simulations and Output
Model simulations of two-electrode voltage clamp were run in MATLAB using three model fiber types: normal control (normal fiber size with normal t-tubules), small control (small fiber size with normal t-tubules), and R6/2 (small fiber size with R6/2 t-tubules). Model output includes steady-state t-tubule membrane potential versus radial location within the fiber for the three fiber types. The model obtains $C_{m,s}$ from the integration of capacitive currents produced during a voltage clamp step (analogous to the method used with empirical voltage clamp data) combined with estimates of total membrane area and fiber surface area based on imaging data acquired for this study. Our model of two-electrode voltage clamp was based on the approach of Katz and Schwartz (Katz and Schwartz 1974) with the following five parameters: gain $K = 23,000$; $t = 0.0001$ ms; $R_S = 10,000$ k$\Omega$; $R = 1$ k$\Omega$; and $a = 9,990$.

Results

*Calculation of Fiber Capacitance Using Only Optical Measurements of T-tubule System*

Calculations of total t-tubule system (TTS) surface area were made from electron and confocal microscopy data (Romer et al. 2021) to determine if the reduced t-tubule diameters can explain the reduced specific capacitance in R6/2 muscle compared to control (Waters et al. 2013; Miranda et al. 2017). Three scenarios were simulated: a control fiber with t-tubule dimensions based on measurements from normal controls, an R6/2 fiber with t-tubule dimensions based on measurements from R6/2 fibers, and a fiber the same size as R6/2 but with normal t-tubule dimensions designated “small control”.

35
We simulated the amount of t-tubule membrane in a unit volume of $1 \times 10^{-9}$ cm$^3$ (Figure 1) based on the density of t-tubules (Table 1, (Romer et al. 2021) and the size of the t-tubules (Table 2, (Romer et al. 2021). Sample sizes for all measures of gross t-tubule morphology were 13 fibers from 5 control mice and 13 fibers from 5 R6/2 mice, with the exception of orthoaxial spacing. Sample sizes for this measure were 7 control fibers and 4 R6/2 fibers. For ultrastructural measurements, sample sizes were 765 t-tubules from 18 fibers in 6 control mice and 872 t-tubules from 18 fibers in 6 R6/2 mice. The total amount of t-tubular membrane per fiber was extrapolated from the unit volume. This use of the unit volume requires a uniform t-tubule density throughout the muscle fibers. The confocal stacks of representative control and R6/2 fibers in Videos 1 and 2 illustrate the uniform distribution of t-tubules in both genotypes.

Figure 1.
Figure 1: **Calculation of total t-tubule surface area.** A representative two-photon image of a FDB fiber stained with Di-8-ANEPPS is shown on the left. The inset of a 10 μm per side unit volume on the right was used to calculate the total t-tubule surface area. T-tubules are shown as rods in the unit volume. Values of t-tubule density obtained from the AutoTT analysis of muscle fibers stained with Di-8-ANEPPS (Table 1, (Romer et al. 2021)) were used to for the x-axis (axial) and y-axis (orthoaxial) spacing in the unit volume. Ultrastructure Measurements (Table 2, (Romer et al. 2021)) were used for the t-tubule diameters.
For the unit volume, we calculated the average surface area of the transverse t-tubule system (TTS\textsubscript{trans}), the longitudinal t-tubule system (TTS\textsubscript{long}), and total t-tubule surface area (SA\textsubscript{TTS}). The average surface area of the TTS\textsubscript{trans} per unit volume was 2.85 \times 10^{-6} \text{cm}^2 for normal and small control fibers, and 2.42 \times 10^{-6} \text{cm}^2 for R6/2 fibers, which was determined by multiplying the number of transverse t-tubules in the unit volume (131.3 for control and 131.4 for R6/2) by the surface area of a single transverse t-tubule with a length of 10 \text{μm} (2.17 \times 10^{-8} \text{cm}^2 for control and 1.84 \times 10^{-8} \text{cm}^2 for R6/2). To determine the SA\textsubscript{TTS} of the unit volume, we divided the surface area of the TTS\textsubscript{trans} by the fraction of transverse pixel elements (Table 1, (Romer et al. 2021)). The SA\textsubscript{TTS} per unit volume was 4.09 \times 10^{-6} \text{cm}^2 in the two control fibers and 3.70 \times 10^{-6} \text{cm}^2 in the R6/2 fiber. It follows that the surface area of the TTS\textsubscript{long} per unit volume was the SA\textsubscript{TTS} surface area minus the TTS\textsubscript{trans} surface area, which was 1.24 \times 10^{-8} \text{cm}^2 for the control fibers and 1.28 \times 10^{-8} \text{cm}^2 for the R6/2.

Completing our calculations required estimates of sarcolemma surface (non-t-tubular outer membrane) and muscle fiber volume. For consistency with data used to show the reduced specific capacitance in R6/2 muscle, we estimated the average sarcolemma surface area and fiber volume from standard bright-field microscopy measurements taken during electrophysiological recordings (Waters et al. 2013; Miranda et al. 2017). Additionally, R6/2 fibers have a greater density of cell nuclei than controls (Figure 2, (Romer et al. 2021)), which may help explain some of the loss of R6/2 t-tubule membrane suggested by the reduced specific capacitance. Therefore, we adjusted the fiber volumes by subtracting the fractional volume occupied by the nuclei from total fiber volume of each type. The adjusted fiber volumes were 1.15 \times 10^{-6} \text{cm}^3 for normal control,
0.763\times 10^{-6} \text{ cm}^3 \text{ for small control}, \text{ and } 0.756\times 10^{-6} \text{ cm}^3 \text{ for R6/2}. \text{ Multiplying these volumes by the TTS surface area per unit volume (SA}_{TTS}) give an effective total t-tubular surface area per fiber; 47.0\times 10^{-4} \text{ cm}^2 \text{ for normal control}, 31.2\times 10^{-4} \text{ cm}^2 \text{ for small control}, \text{ and } 28.0\times 10^{-4} \text{ cm}^2 \text{ for R6/2}. \text{ The sum of the surface area of the sarcolemma and the total t-tubular surface area give total membrane surface areas: normal control } = 56.1\times 10^{-4} \text{ cm}^2, \text{ small control } = 38.5\times 10^{-4} \text{ cm}^2, \text{ and R6/2 } = 35.3\times 10^{-4} \text{ cm}^2.\text{ Finally, specific capacitance normalized to sarcolemma surface area (C}_{m,S}) was calculated from the total capacitance and sarcolemma surface area for each case. Assuming skeletal muscle membrane has a specific capacitance normalized to total membrane surface area, including the sarcolemma and t-tubules, (C}_{m,S+TT}) of 0.9 \mu F/cm^2 (Hodgkin and Nakajima 1972), total capacitance was calculated from total membrane surface area by C_{total} = 0.9 \mu F/cm^2 \times total\ SA_{fiber}. \text{ For normal control, this was } 50.5\times 10^{-4} \mu F; \text{ for small control, } 34.6\times 10^{-4} \mu F; \text{ and for R6/2, } 31.8\times 10^{-4} \mu F. \text{ By dividing total capacitance by sarcolemma surface area, the C}_{m,S} for the normal control, small control, and R6/2 fibers were 5.5, 4.7, and 4.4 \mu F/cm^2, respectively. We also estimated C}_{m,S} without accounting for the volume of nuclei; the values for the normal control, small control, and R6/2 fibers were 5.7, 4.9, and 4.5 \mu F/cm^2, respectively. Thus, the volume lost to nuclei appears to have little effect on fiber capacitance. The estimate of control C}_{m,S} using only optical data was slightly higher than the empirically obtained value of 5.1 \mu F/cm^2, whereas the calculated R6/2 C}_{m,S} was significantly higher than the measured value of 3.4 \mu F/cm^2 (Waters et al. 2013; DiFranco et al. 2013). These calculations account for the loss of membrane due to the reduced t-tubule diameter in R6/2 fibers. However, a change in current flow because of the altered t-tubule geometry were not
reflected in these estimates.

*Model Simulations of Voltage Clamp*

We hypothesized the smaller diameter of the R6/2 t-tubules may restrict current flow in the t-tubule lumen and therefore help explain the lower specific capacitance values obtained using voltage clamp data relative to the value estimated using only optical data. To test this hypothesis, we simulated two-electrode voltage clamp of skeletal muscle with the t-tubules modeled as a radial cable to represent their spatial relationship with the sarcolemma (Figure 2). To account for membrane morphology, the following geometric factors were used based on our confocal and electron microscopy data: rho (\(\rho\)) was the ratio of t-tubule volume to fiber volume, zeta (\(\zeta\)) was the ratio of t-tubule volume to surface area, and the tortuosity factor sigma (\(\sigma\)) specified the fraction of t-tubule branches oriented radially. The geometric parameters used in our model are shown in Table 1.
Figure 2.
Figure 2: **Luminal t-tubule voltage gradients.** A) Circuit diagram of skeletal muscle model with the t-tubule compartment represented by a radial cable. Shown is the luminal t-tubule voltage gradient during a step to -65 mV from a resting potential of -85 mV. B) T-tubule membrane potential (intracellular relative to lumen) vs. time at selected locations within a normal control model fiber. Toward the center of the fiber, steady state potential is less depolarized than the sarcolemma. C) Effective step size as a function of distance from the surface during a step to -65 mV from a resting potential of -85 mV in control (black), R6/2 (magenta), a small control (gray), and control with high t-tubule luminal conductance (black, open circle).
Table 1.

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Normal control</th>
<th>R6/2</th>
<th>Small control</th>
</tr>
</thead>
<tbody>
<tr>
<td>length (cm)</td>
<td>527x10^-4</td>
<td>519x10^-4</td>
<td>519 x10^-4</td>
</tr>
<tr>
<td>diameter (cm)</td>
<td>52 x10^-4</td>
<td>43 x10^-4</td>
<td>43 x10^-4</td>
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<tr>
<td>$\rho$ (unitless)</td>
<td>1.17</td>
<td>0.98</td>
<td>1.17</td>
</tr>
<tr>
<td>$\zeta$ (cm)</td>
<td>2.87x10^-6</td>
<td>2.65 x10^-6</td>
<td>2.87 x10^-6</td>
</tr>
<tr>
<td>$\sigma$ (unitless)</td>
<td>0.34</td>
<td>0.34</td>
<td>0.34</td>
</tr>
</tbody>
</table>
Table 1: **Summary of the geometrical parameters used for modeling.** The length and diameter values are from Waters et al., 2013. Rho (ρ) is the unitless ratio of t-tubule volume to fiber volume, zeta (ζ) is the ratio of t-tubule volume to surface area in cm, and tortuosity factor sigma (σ) is a unitless value that specifies the fraction of t-tubules branches oriented radially.
The model consists of a series of 50 concentric cylindrical shells that subdivide the t-tubule structure into multiple concentric compartments (Fig. 2A). The outer t-tubule shell was connected to the extracellular space via an access resistance \( R_a \) and each shell was connected to the next via a luminal t-tubular conductance \( G_L \). Each t-tubule shell was connected to the intracellular space through a common node. This arrangement allowed spatial tracking of changes in membrane potential radially within the t-tubules from the outer to the center shell. At rest, each shell of the t-tubular compartment was assumed to be isopotential with the sarcolemma and the ion concentrations in the t-tubular lumen were assumed to be homogeneous in the radial direction and equal to the ion concentrations in the extracellular fluid.

The model predicted that the finite luminal conductance caused a voltage gradient in the t-tubules at steady state if the membrane was held at any voltage other than resting potential in control and R6/2 fibers. This effect was not a voltage clamp error and was most pronounced at the center of the model fiber (Figure 2B, 2C). Thus, during a voltage step, the change in t-tubular membrane potential near the center of the fiber was smaller than it was near the sarcolemma. As a result, a 20 mV step at the sarcolemma corresponds to a 15 mV step in the t-tubules near the center of the fiber. Because the effective step size was reduced near the center of the fiber, capacitive current from that region was reduced. This led to an underestimation of specific capacitance by electrophysiological methods. This was because in the calculation of capacitance via electrophysiological methods, all of the membrane in the fiber, including t-tubule membrane, was assumed to have experienced the same voltage step as the sarcolemma. When luminal conductance in the normal size control fiber was made very large, the
voltage gradient within the t-tubules was eliminated and capacitance by electrophysiological methods approached capacitance by membrane surface area. (Figure 2C)

As expected, the voltage gradient in the t-tubules from the sarcolemma to the interior of the fiber was greater in the R6/2 model compared to the small control because the reduced diameter of the R6/2 t-tubules increased the luminal resistance. This contributes to the reduced estimate of $C_{m,S}$ by electrophysiological methods in R6/2 fibers compared to small control fibers. Unexpectedly, the model of a normal control fiber had a greater voltage gradient in the t-tubules than either the small control or the R6/2 models, which was due to differences in fiber radius. The higher current required for voltage clamp caused the larger t-tubule voltage gradient in normal control compared to R6/2 or small control fibers. Therefore, two geometric factors affected estimates of $C_{m,S}$. A decrease in t-tubule radius caused an underestimation of $C_{m,S}$. Also, a larger fiber radius increased the underestimation of $C_{m,S}$ due to the t-tubule luminal resistance. The simulations suggest that for the geometries considered here, the fiber radius effect was stronger than the t-tubule radius effect (Figure 2C).

Table 2 summarizes the $C_{m,S}$ values obtained by the three measurement methods described in this study for R6/2 fibers and normal controls. Empirical electrophysiology values underestimate $C_{m,S}$ relative to the optical only estimates for the reasons discussed above. Using the geometrical parameters of the model (rho, zeta, and sigma) to estimate $C_{m,S}$, and assuming that $C_{m,S+TT} = 0.9 \text{ mF/cm}^2$; the values for control (5.67 mF/cm$^2$) and R6/2 (4.48 mF/cm$^2$) were nearly identical to the optical only estimates. The model, by including the effects of current flow through the finite t-tubule conductivity, predicts
lower $C_{m,S}$ values for both control and R6/2 fibers relative to the optical only method. For control fibers, the model estimate of $C_{m,S}$ was nearly identical to that obtained with experimental electrophysiology. Despite the success of model in accurately describing t-tubular voltage changes in control fibers, it did not explain the reduced capacitance of R6/2 muscle. This suggests that factors independent of the t-tubule structure cause the reduced capacitance in R6/2 muscle.
Table 2.

<table>
<thead>
<tr>
<th>Measurement method</th>
<th>Control (µF/cm$^2$)</th>
<th>R6/2 (µF/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophysiology</td>
<td>5.1 ± 0.2</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>n = 26</td>
<td>n = 20</td>
</tr>
<tr>
<td>Optical Only</td>
<td>5.7</td>
<td>4.5</td>
</tr>
<tr>
<td>Model Electrophysiology</td>
<td>5.0</td>
<td>4.1</td>
</tr>
</tbody>
</table>
Table 2: Summary of capacitance values normalized to fiber surface area ($C_{m,S}$).

Measurements obtained using experimental electrophysiology (Electrophysiology) are from Waters et al., 2013. The Optical Only data shows calculations of $C_{m,S}$ using only confocal and electron microscopy data from this study. The Model Electrophysiology data shows estimates of $C_{m,S}$ from the mathematical model of voltage changes in the t-tubules using optical data from this study.
Discussion

The capacitance of skeletal muscle determines the amount of current required to depolarize muscle. Previous studies of membrane excitability defects in the skeletal muscle of R6/2 Huntington’s disease mice revealed that the specific membrane capacitance normalized to fiber surface area ($C_{m,S}$) progressively decreased in parallel to the increasing disease symptoms (Waters et al. 2013; Miranda et al. 2017). This loss of capacitance could contribute to increased excitability in diseased muscle, particularly when combined with decreases in resting $\text{Cl}^-$ and $\text{K}^+$ currents (Waters et al. 2013). Total fiber capacitance is proportional to the amount of cell membrane and the capacitance normalize to surface and t-tubule membrane $C_{m,S+TT}$ would be approximately 0.9 $\mu$F/cm$^2$ if all of the extracellular membrane resides at the surface of the cell. The $C_{m,S}$ of skeletal muscle (~5 $\mu$F/cm$^2$) and cardiac muscle greatly exceeds 0.9 $\mu$F/cm$^2$ because of the transverse tubules (t-tubules), which are narrow invaginations of the surface membrane. The 33% reduction found using electrophysiology in R6/2 skeletal muscle $C_{m,S}$ compared to age-matched controls suggests a partial loss of R6/2 t-tubule membrane (Waters et al. 2013; Miranda et al. 2017). This decrease could be explained by a reduction in t-tubule membrane or a change in electrophysiological properties in R6/2 muscle.

Images of live disassociated muscle fibers indicated that the t-tubule system measured above the diffraction limit (~250 nm) was unaffected in R6/2 skeletal muscle (Romer et al. 2021). Although there was not a change in R6/2 t-tubules using confocal microscopy, skeletal muscle t-tubules have a mean diameter ~20-40 nm, which is well below the diffraction limit of light microscopy (C. Franzini-Armstrong 1975; Sandow
Furthermore, with live imaging, t-tubule integrity and density decreases with time (Guo and Song 2014). T-tubule ultrastructure in fixed dissociated fibers using transmission electron microscopy (TEM), which had a resolution of <1nm revealed intact triads with t-tubules in apposition to terminal cisternae of the sarcoplasmic reticulum. However, relative to controls, the R6/2 t-tubules were reduced in perimeter (13%), mean diameter (12%), and cross-sectional area (26%).

To assess whether smaller t-tubules could account for the reduced $C_m$ in R6/2 fibers, we calculated the expected $C_{m,S}$ based on the t-tubule structural properties found in our microscopy data, which predicts the decrease in capacitance based on only the loss of membrane. We also built a mathematical model of current flow through the t-tubules that utilized our microscopy data, which would predict the functional changes due to reduced R6/2 t-tubule radius. Our calculated and modeled $C_{m,S}$ values were compared with experimental $C_{m,S}$ measurements obtained using two-electrode voltage-clamp. Because muscle fibers closely approximate an ellipsoid with a rough surface, the assumption of cylindrical fibers could lead to underestimation of surface-to-volume ratio and an overestimation of the actual capacitance (Kim, DiFranco, and Vergara 1996; Hodgkin and Nakajima 1972). To minimize the impact of this assumption on our comparison, we assumed cylindrical fibers to obtain $C_{m,S}$ for both experimental and modeled simulations. The calculated $C_{m,S}$ using only optical data for normal control fibers of 5.5 µF/cm$^2$ (or 5.7 µF/cm$^2$ without accounting for volume lost to nuclei) was greater than the experimental value of 5.1 µF/cm$^2$ (Miranda et al. 2017; Waters et al. 2013). However, our mathematical model predicted a control $C_{m,S}$ of 5.01 µF/cm$^2$, which was in high agreement with the empirical electrophysiology value. Thus, the voltage gradient
generated by current flow through the t-tubule luminal resistance likely explains the discrepancy between the optical only and experimental electrophysiology estimates of control $C_{m,S}$. The voltage gradient due to current flow along the t-tubule luminal resistance has been described previously (Adrian, Costantin, and Peachey 1969; Adrian and Almers 1974). Another implication of the model correctly predicting the empirical $C_{m,S}$ value for control fibers (Waters et al. 2013) is that electrophysiology experiments with FDB fibers were completed with good voltage control.

The ability of our mathematical model to predict the empirical electrophysiology results for control fibers implies that we did not miss an important structural element, such as t-tubular microdomains. Microdomains have been identified in cardiac muscle and consist of membrane microfolds in the t-tubule sculpted by the cardiac isoform of BIN1 protein (Hong and Shaw 2017). In cardiac myocytes, these microfolds appear to improve contact between RyR1 and voltage sensing $\text{Ca}^{2+}$ channels, creating a $\text{Ca}^{2+}$ signaling microdomain (Hong et al. 2014). Exquisite detail of cardiac microdomain nanostructure has been revealed through a combination of imaging approaches including super-resolution light microscopy, three-dimensional tomography reconstruction TEM and stimulated emission depletion imaging (STED) (Hayashi et al. 2009; Wagner et al. 2012; Hong et al. 2014; Jayasinghe et al. 2014). High resolution electron microscopy would be needed to ultimately confirm the presence or absence of microdomains in the much narrower t-tubules of skeletal muscles. However, the ability of our model, which did not include microdomains, to explain the capacitance of control fibers suggests that microdomains are minimally present or absent in skeletal muscle. Altogether, this suggests that microdomains may be a unique structure in much larger cardiac t-tubules.
and do not likely contribute to the loss of t-tubule membrane in R6/2 skeletal muscle fibers.

Our estimates of R6/2 $C_{m,S}$ based on optical data only (4.4 µF/cm$^2$, or 4.5 µF/cm$^2$ without considering nuclei) or with the mathematical model (4.1 µF/cm$^2$) were considerably higher than the experimental value (3.4 µF/cm$^2$). Voltage clamp issues do not seem likely because the smaller R6/2 fibers would be expected to have less space clamp issues than control fibers. It is possible that access to the longitudinal t-tubule elements was reduced in R6/2 skeletal muscle. Excluding the longitudinal elements would result in a calculated $C_{m,S}$ of 3.2 µF/cm$^2$. Thus, partially decreased access to the longitudinal t-tubules could have the reduced experimental $C_{m,S}$ of R6/2 muscle. Such a defect could cause fatigue in R6/2 muscle because t-tubule extensions have been proposed to help replenish sarcoplasmic reticulum Ca$^{2+}$ via STIM1 and Orai1 after exercise (Boncompagni et al. 2017).

Another change that would affect electrophysiological measurements is the amount of tortuosity present in the t-tubules, which we did not measure in this study. Tortuosity represents the branching of the t-system, specifically the percentage of tubules oriented in the radial direction. Changes in tortuosity would impact luminal resistance, which could change electrophysiological measures of capacitance. If tortuosity increases, fewer branches of the tubular system will be oriented radially and luminal resistance will increase. However, an exploration using our mathematical model revealed that tortuosity would have to increase by nearly four times to reproduce our average experimental electrophysiological measurement of $C_{m,S}$ of 3.4 µF/cm$^2$ for R6/2 muscle. Given the
conserved gross tubular structure of R6/2 fibers, a four-fold increase in tortuosity seems unlikely.

Another possibility is that specific capacitance normalized to total membrane surface area ($C_{m,S+TT}$) in R6/2 fibers is not the same as in normal controls. If membrane composition has been altered in the R6/2 muscle such that membrane thickness is increased, $C_{m,S+TT}$ could be lower than the typical 0.9 uF/cm$^2$ for muscle. This could happen if there was a decrease in membrane protein density in the R6/2 muscle membrane. It has been shown that the presence of proteins in biological membranes compresses the lipid bilayer, which reduces membrane thickness and thereby increases membrane capacitance (Hanai, Haydon, and Taylor 1965; Fettiplace, Andrews, and Haydon 1971). Indeed, our previous studies have shown decreases in current through muscle chloride (CLIC-1) and inwardly rectifying potassium (Kir) as well as reduced expression of $K_{v}3.4$ and $K_{v}1.5$ (Waters et al. 2013; Miranda et al. 2017, 2020). These changes in the membrane would not show up in our optical measurements and could only be inferred from electrophysiological data. By reducing $C_{m,S+TT}$ in our R6/2 model to 0.75 uF/cm$^2$ (a change of 17%), we could reproduce our electrophysiological value of 3.4 uF/cm$^2$. This is well within the physiological range for measured $C_{m,S}$ in multiple cell types (0.5 - 1.0 uF/cm$^2$) (Gentet, Stuart, and Clements 2000; Golowasch et al. 2009).

Lastly, actual changes in R6/2 t-tubule structure and membrane composition may well be multifactorial, including effects of both increased tortuosity and decreased $C_{m,S+TT}$.

The smaller R6/2 t-tubules suggests that the cell signaling mechanisms controlling t-tubule development or maintenance are disrupted in R6/2 muscle. Because we found no differences in Z-band thickness between control and R6/2 fibers or in t-tubule size
between soleus (slow-twitch) and FDB (fast-twitch) muscles, the t-tubules pathology is likely independent of fiber type switching. Previous work has linked chloride channel defects in R6/2 muscle to mRNA splicing defects (Waters et al. 2013; Miranda et al. 2017). Thus, the Voss lab examined the splicing of amphiphysin 2 protein (Bin1), which bends and curves membranes into tubes (Al-Qusairi and Laporte 2011). Moreover, muscleblind-like protein 1 (Mbnl1) is a splicing factor for Bin1 and forms abnormal aggregates in R6/2 skeletal muscle (Miranda et al. 2017; Fugier et al. 2011). They found normal levels of total Bin1 mRNA in R6/2 muscle with the inclusion of exon 11, which encodes a polybasic amino acid sequence that helps Bin1 bind to the t-tubule membrane (Al-Qusairi and Laporte 2011). However, the relative inclusion of exon 17 was increased in R6/2 gastrocnemius and TA muscle. This change was not detected in the soleus muscle, suggesting that HD preferentially targets fast-twitch over slow-twitch muscle, which is consistent with changes in fiber type specific mRNA and proteins (Miranda et al. 2017). The inclusion of exon 17 in Bin1 is characteristic of cardiac muscle (Hong et al. 2014). In cardiac muscle, Bin1 causes the formation of t-tubules and the clustering of Cav1.2 channels (De La Mata et al. 2019). The possibility that Bin1 with exon 17 imparts a cardiac phenotype on R6/2 skeletal muscle is intriguing and consistent with the Voss lab finding that the distance between the t-tubule and SR membranes is increased in R6/2 muscle. Such a defect could help explain the altered Ca$^{2+}$ release events that have been described in R6/2 skeletal muscle (Braubach et al. 2014). Future experiments are required to determine if Bin1 and cardiac specific exons can be used to rescue t-tubules in R6/2 skeletal muscle fibers similar to what has been demonstrated in cardiac tissue by Hong et al., 2014.
The Voss lab finding of a significant decrease in t-tubule size at the triad that is associated with Bin1 missplicing that may explain the altered EC coupling reported in R6/2 mice (Braubach et al. 2014). The decreased t-tubule size may also affect membrane excitability in R6/2 muscle. K⁺ accumulation in the t-tubules during repetitive stimulation causes a depolarization that is known to decrease action potential amplitude and, in severe cases, lead to full muscle inexcitability (Stephen C. Cannon 2015; Nielsen, Ørtenblad, and Lamb 2004; Renaud and Light 1992; Mark M. Rich and Pinter 2003; Yensen, Matar, and Renaud 2002; S. P. Cairns et al. 1997). Because the decrease in R6/2 t-tubule diameter would cause a greater reduction in t-tubule volume, K⁺ buildup may occur at an increased rate in R6/2 muscle. Thus, the reduced t-tubule radius may help explain the more rapid depolarization that has recently been shown to occurs in R6/2 muscle during high frequency stimulation (Miranda et al. 2020), perhaps causing increased fatigue or motor impersistance. Future modeling of K⁺ build-up using our model of t-tubules will allow us to determine whether this is the case.

Conclusion

In collaboration with Dr. Romer from the Voss lab, my modeling of t-tubules suggests that the t-tubule network in R6/2 skeletal muscle is intact but the individual t-tubules are reduced in diameter, which contributes to the reduced $C_{m,S}$ reported previously. My model shows that empirical estimates of $C_{m,S}$ will be underestimates because of the voltage gradient caused by current flow through the t-tubule lumen. The magnitude of the underestimated $C_{m,S}$ will be greater in larger diameter fibers and smaller if t-tubule luminal conductance is increased. The altered architecture of R6/2 t-tubules
and Bin1 splicing reported in our collaborative study likely helps explain the
dysregulated Ca^{2+} signaling in R6/2 skeletal muscle (Braubach et al. 2014) and may lead
to weakness and fatigue in Huntington’s disease. The goal of this aim was to determine
the underlying cause for loss of specific capacitance in HD muscle. Two approaches
were used: 1) estimation of capacitance by membrane surface area from optical data, and
2) simulations of two-electrode voltage clamp capacitance measurements. The first
approach led us to conclude that loss of membrane was not the true cause of reduced
capacitance because capacitance by surface area was higher than capacitance measured
electrophysiologically. The second approach led to the insight that smaller diameter t-
tubules may increase resistance in the TTS, thereby altering the electrophysiologic
properties and providing a partial explanation of lower measured capacitance in the
disease fibers. While it does not prove the mechanism underlying the difference in
measurements of capacitance by optical data versus electrophysiology, this work
provides a theoretical framework to explain the discrepancy in the data because model
electrophysiology capacitance was very near experimental.
Chapter V

Specific Aim II: Derivation of Hodgkin-Huxley parameters for single action potentials in skeletal muscle.

Introduction

In 1952, Hodgkin and Huxley published their model, the first to quantitatively describe the electrical properties of an excitable membrane (Hodgkin and Huxley 1952d; Noble 1966), and which still shapes the thought of the entire field of electrophysiology today (Daly et al. 2015; Beeman 2014). The experimental preparation Hodgkin and Huxley used was the squid giant axon. A portion of the axonal membrane was clamped to specified potentials using two fine silver wires threaded axially through the center of the axon via a glass capillary (Hodgkin, Huxley, and Katz 1952). Using this system, they recorded the time-course of electrical currents in the axon in order to better understand the mechanisms regulating changes in membrane permeability to Na\(^+\) and K\(^+\) ions in response to changes in membrane potential (Hodgkin and Huxley 1952a, 1952b, 1952c). The channels carrying these ions had yet to be discovered, so the permeability changes were described as voltage-dependent reactions.

The Hodgkin-Huxley model consists of a set of equations that describe the currents flowing across an excitable membrane. Total membrane current is the sum of a capacitive current and an ionic current: 

\[ I_m = C_m \frac{dV}{dt} + I_i, \]

where \( V \) is the instantaneous membrane voltage. The ionic current is a sum of a Na\(^+\) current, a K\(^+\)...
current, and a non-specific leak current: \( I_l = I_{Na} + I_K + I_{\text{leak}} \). The Na\(^+\) current is a product of the theoretical maximum Na\(^+\) conductance \( g_{Na} \), the driving force for Na\(^+\) ions \( (V - E_{Na}) \), and the activation gating variable \( m \) and inactivation gating variable \( h \): \( I_{Na} = g_{Na} m^3 h (V - E_{Na}) \). Similarly, the K\(^+\) current has a theoretical maximum K\(^+\) conductance \( g_K \), a driving force for K\(^+\) ions \( (V - E_K) \), and an activation gating variable \( n \): \( I_K = g_K n^4 (V - E_K) \). The non-specific leak current, \( I_{\text{leak}} = g_{\text{leak}} (V - E_{\text{leak}}) \), has a constant conductance \( g_{\text{leak}} \) and associated driving force \( (V - E_{\text{leak}}) \).

Each gating variable \( (m, h, \text{ and } n) \) is governed by a rate equation:

\[
\begin{align*}
\frac{dm}{dt} &= \alpha_m (1 - m) - \beta_m m \\
\frac{dh}{dt} &= \alpha_h (1 - h) - \beta_h h \\
\frac{dn}{dt} &= \alpha_n (1 - n) - \beta_n n
\end{align*}
\]

For each rate equation, there is a pair of equations for the rate coefficients \( \alpha \) and \( \beta \). For gating variables \( m \) and \( n \), \( \alpha \) is the forward rate of activation and \( \beta \) is the reverse rate, or deactivation. For gating variable \( h \), \( \beta \) is the forward rate of inactivation and \( \alpha \) is the reverse rate, or relief of inactivation.

\[
\begin{align*}
\alpha_m &= \bar{\alpha}_m (V - \bar{V}_m) / (1 - \exp(-\frac{(V - \bar{V}_m)}{k_\alpha m})) \\
\beta_m &= \bar{\beta}_m \exp(-\frac{(V - \bar{V}_m)}{k_\beta m}) \\
\alpha_h &= \bar{\alpha}_h \exp\left(\frac{(V - \bar{V}_h)}{k_\alpha h}\right) \\
\beta_h &= \bar{\beta}_h / (1 + \exp(-\frac{(V - \bar{V}_h)}{k_\beta h}))
\end{align*}
\]
\[ \alpha_n = \bar{\alpha}_n \left( V - \bar{V}_n \right) / \left( 1 - \exp\left( -\frac{V - \bar{V}_n}{k\alpha n} \right) \right) \]

\[ \beta_n = \bar{\beta}_n \exp\left( -\frac{V - \bar{V}_n}{k\beta n} \right) \]

Table 3 lists the parameters of the rate coefficients and their effects with respect to voltage.
Table 3: Parameters of rate coefficients ($\alpha$ and $\beta$) of gating variables $m, h, n$

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Effect on rate coefficients with respect to voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\bar{V}_m, \bar{V}_h, \bar{V}_n$</td>
<td>Set voltage dependence</td>
</tr>
<tr>
<td>$k_\alpha m, k_\beta m, k_\alpha h, k_\beta h, k_\alpha n, k_\beta n$</td>
<td>Set steepness of rate of change</td>
</tr>
<tr>
<td>$\bar{\alpha}_m, \bar{\beta}_m, \bar{\alpha}_h, \bar{\beta}_h, \bar{\alpha}_n, \bar{\beta}_n$</td>
<td>Scale rate of change</td>
</tr>
</tbody>
</table>
The Hodgkin-Huxley equations have been applied to models of different types of excitable tissue in different species (Noble 1962, 1966; Jack, Noble, and Tsien 1983; Daly et al. 2015). In models of skeletal muscle, parameters have been obtained from studies in species such as frog (Adrian and Peachey 1973), goat (Adrian and Marshall 1976), and a combination of frog and rat (S. C. Cannon, Brown, and Corey 1993). Several models have simply re-used previously published parameters from other models, presumably due to the technical difficulties inherent in a classical Hodgkin-Huxley analysis (S. C. Cannon, Brown, and Corey 1993; Wallinga et al. 1999; Fortune and Lowery 2009). We are unaware of any studies that directly compared their simulated APs with experimentally recorded traces, save one preliminary study from the Rich lab (Novak et al. 2015). To our knowledge, no one has quantitatively compared the simulated APs with those recorded experimentally to check the accuracy of their APs.

The enduring utility of the Hodgkin-Huxley model may be largely attributed to its simplicity. The model is relatively easy to understand conceptually and convincing-looking APs can be produced using only three ionic conductances. Because the equations involved are relatively few, there are fewer parameters that must be optimized and less computational power is required for simulations. However, fewer equations may mean oversimplification and inadequate means to model real behavior.

Our own attempts at AP simulation using parameters from the literature (S. C. Cannon, Brown, and Corey 1993; Filatov, Pinter, and Rich 2005) were unsatisfactory when compared with APs we had recorded from muscle. Simulated APs had a noticeably sharper peak and the repolarization phase was either too steep or included an after-hyperpolarization that is often seen in nerve but not muscle. Thus, we were left with the
question: Were other models using incompletely optimized parameters? Given that optimization is a difficult and time-consuming task, perhaps the extra effort wasn’t justified for those studies when a basic AP shape would do. Or, perhaps a more fundamental question: Is the basic Hodgkin-Huxley (H-H) model missing some element crucial for simulating APs in skeletal muscle? The original H-H model was constructed from data recorded from the giant axons of squid. Our studies are done in hindlimb muscles of mice. In addition to the obvious differences between mammalian and cephalopod species, there are well-known differences in electrical properties of muscle and nerve, not to mention very different experimental conditions (temperature, bath solution, voltage clamp setup, to name a few).

As far as we are aware, all studies using H-H parameters, have used parameters derived from voltage clamp studies. Derivation of parameters from voltage clamp recordings involves several steps, starting with plotting the steady-state activation (m, n) and inactivation (h) curves from peak current magnitudes. Next, time constants are derived from fitting the current traces for each voltage step to exponential functions. The rate coefficients, α and β, are calculated from the time constants and their corresponding steady-state values, and plotted versus voltage. Finally, these are fitted to exponential functions to obtain the voltage dependence of the time constants (Fu et al. 2011). While using voltage clamp recordings of isolated currents has the advantage that parameters for each current can be derived independently and accurately, if the current of interest has been altered by the process of recording the current, the enhanced ability to accurately derive parameters would be of little benefit.
The use of voltage clamp recordings of currents for derivation of H-H parameters for modeling studies may be problematic. There are several studies suggesting that gating properties of Na\(^+\) channels are significantly altered by the methods used to record voltage clamped currents. One of the first steps in voltage clamp using patch clamping is the formation of a tight seal between the glass of the pipette and the cell membrane.

There is a study that demonstrated in heart muscle that this process significantly alters the voltage dependence of gating of cardiac Na\(^+\) channels (Eickhorn, Drägert, and Antoni 1994). After formation of a seal, in whole cell patch clamp studies, the membrane under the pipette is ruptured and the solution in the pipettes is dialyzed into the cell. Dialysis of the intracellular solution in muscle fibers is also performed during two electrode voltage clamp studies (Waters et al. 2013; Hawash, Voss, and Rich 2017). There is a study suggesting that as the intracellular milieu is replaced with solution in the pipette, hyperpolarizing shifts occur in the voltage dependence of gating of the muscle Na\(^+\) channel isoform Nav1.4 (D. W. Wang, George, and Bennett 1996). Finally, even in loose patch recordings in which a tight seal is not formed and the intracellular milieu is left unperturbed, the Rich lab has found that prolonged alteration of the holding potential also causes shifts in the voltage dependence of Na\(^+\) channel gating in skeletal muscle (Filatov, Pinter, and Rich 2005). Thus, unless the holding potential is kept at the value of the resting potential, gating parameters for Na\(^+\) channels are likely to be altered. Holding potentials during voltage clamp studies are almost always more negative than the resting potential. This is done to relieve Na\(^+\) channel inactivation.

To mitigate concerns about the effects of voltage clamp on H-H parameters, I have fit whole APs recorded in current clamp mode from unperturbed mammalian muscle...
fibers at their normal resting potential. High resistance electrodes filled with 3 M KCl are used for these recordings and single action potentials are recorded soon after impalement (M. M. Rich et al. 1998; M. M. Rich and Pinter 2001; Mark M. Rich and Pinter 2003). The combination of use of high resistance electrodes (~20 MΩ when filled with 3 M KCl), the recording of action potentials within 1-2 minutes of impalement, and the normal resting potential avoid the issues that alter gating of Na\(^+\) channels in previous studies used to derive H-H parameters.

The goal of this Aim was to determine whether H-H parameters are sufficient to accurately model action potentials from skeletal muscle. Specifically, I set out to find a set of parameters for the H-H equations that will qualitatively reproduce the shape of skeletal muscle APs, compare the resulting parameter ranges with previously published parameter sets, and identify connections between parameters and AP features. While fitting whole APs is a more direct and efficient way of finding a set of parameters, the reason it has not been done previously is that it is significantly more difficult to perform. There are many parameters that have to be adjusted and their sensitivity to changes is such that slight errors leads to complete failure of the simulation to produce an action potential. I have overcome these difficulties by breaking the problem down into discrete steps and have been able to derive parameters that more accurately simulate action potentials than parameters used previously. To my knowledge this is the first time anyone has used this approach. By obtaining the entire parameter set from a single prep in a single species, I hoped to gain a more accurate representation of the electrical behavior of the cells we study in our laboratory. I was able to reproduce action potential rate of rise, peak, rate of fall and half width with H-H parameters derived from fitting of
action potentials. My ability to accurately model action potentials both shows that the H-H model is sufficient and that we can use the H-H model in the future to explore failure of action potential generation in skeletal muscle in various disease states.

**Methods**

*Model Structure*

As in Aim 1, I am modeling a short segment of muscle fiber as an intracellular compartment with a t-tubule compartment connected to the extracellular space via an access resistance ($R_a$). The t-tubule compartment is a radial cable consisting of a series of concentric shells. Each adjacent shell is connected by a luminal conductance ($G_L$). Each shell is also individually connected to the intracellular space by a capacitance ($C_m$) in parallel with a leak conductance ($g_{leak}$). For this aim, I have added the voltage-gated conductances $g_{Na}$ and $g_{Kdr}$ to the intracellular compartment and each t-tubule shell according to the H-H formulation. (see Figure 3) The parameters I am optimizing are the three max conductances: $\bar{g}_{Na}$, $\bar{g}_K$, $g_{leak}$, and the fifteen parameters of the rate coefficients: $\bar{V}_m$, $k_\alpha m$, $k_\beta m$, $\bar{\alpha}_m$, $\bar{\beta}_m$, $\bar{V}_h$, $k_\alpha h$, $k_\beta h$, $\bar{\alpha}_h$, $\bar{\beta}_h$, $\bar{V}_n$, $k_\alpha n$, $k_\beta n$, $\bar{\alpha}_n$, $\bar{\beta}_n$. 
Figure 3: Model schematic with active conductances in the intracellular and t-tubular compartments.
Experimental Data

To obtain values for each of these parameters, I am fitting recordings of current clamp experiments from the Rich lab in intact fibers in the EDL muscle of wild-type mice. Each experiment includes recordings of single APs and the passive voltage response to a hyperpolarizing current pulse. Recordings were chosen from datasets from two different experimenters. Criteria for inclusion was a resting membrane potential equal to or more hyperpolarized than -80 mV.

Eight action potential traces were chosen for fitting of action potentials with Hodgkin-Huxley model parameters. The intracellular recordings were made by two different experimenters using different recording rigs and recording software. Four fibers were used from recordings by experimenter one from two mice on different recording days. Four fibers were used from recordings by experimenter two from four mice on four different recording days. Both experimenters used the same preparation: non-dissociated fibers in intact wild-type EDL muscle dissected tendon to tendon.

Evaluation of Existing Literature Parameters

I began by examining the quality of fit using previously published Hodgkin-Huxley model parameters (S. C. Cannon, Brown, and Corey 1993; Filatov, Pinter, and Rich 2005). The model by Cannon et al is a simplified model of skeletal muscle that uses a single, lumped t-tubule compartment connected to an intracellular compartment. The parameters were assembled from several previously published analyses of voltage-clamp data. Na⁺ channel rate constants ($\bar{\alpha}_m$, $\bar{\beta}_m$, $\bar{\alpha}_h$, $\bar{\beta}_h$), steepness factor $k_{\alpha} h$, and maximum conductance ($\bar{g}_{Na}$) were taken from a study using rat EDL and sternomastoid muscle
(Pappone 1980). Na\(^+\) channel voltage dependence and the other three steepness factors \((\bar{V}_m, \bar{V}_h, k_\alpha m, k_\beta m, k_\beta h)\) were taken from data on frog sartorius muscle (Adrian and Peachey 1973) and adjusted to account for differences in voltage dependence in mammalian muscle (Adrian and Marshall 1977; Almers, Roberts, and Ruff 1984). All K\(^+\) channel parameters \((\bar{g}_K, \bar{V}_n, k_\alpha n, k_\beta n, \bar{\alpha}_n, \bar{\beta}_n)\) came from a study by Beam and Donaldson in rat omohyoid muscle (Beam and Donaldson 1983). A study of Cl\(^-\) conductance in rat diaphragm was used to determine the value of \(g_{\text{leak}}\) near resting membrane potential (Palade and Barchi 1977). Most other models published since have borrowed freely from this model’s parameters, generally leaving them unchanged from the values used by Cannon et al. (Wallinga et al. 1999; Fortune and Lowery 2009). The model by Filatov et al. used the Cannon values as a starting point, but made significant adjustments to several parameters to get the model simulations to match behavior they had observed in their own experiments (Filatov, Pinter, and Rich 2005). The specific parameters that differed were \(\bar{g}_{Na}, \bar{g}_K, g_{\text{leak}}, \bar{V}_m, \bar{V}_h, \bar{V}_n, \bar{\alpha}_m, \bar{\beta}_m, \bar{\beta}_h, \bar{\alpha}_n\). Their model structure consisted of an intracellular compartment and a longitudinal t-tubule cable.

Figure 4 shows simulated action potentials from the two parameter sets plotted overlying one of the eight action potentials recorded in the Rich lab. Membrane reversal potential was matched to the recorded trace by adjusting \(E_L\), the model parameter that controls resting potential. Input current for simulations was set to a value that produced APs with a rate of rise as close as possible to that of the experimental traces. Despite these adjustments, neither set of previously used parameters resulted in simulations that closely approximated the recorded action potential. In both cases the simulated action potential is substantially narrower than the recorded action potential. This is due to both
a more rounded action potential peak as well as a significantly slower falling phase of the recorded action potential.
Figure 4.
Figure 4: Simulated action potentials using Hodgkin-Huxley equations with parameters from previous studies (S. C. Cannon, Brown, and Corey 1993; Filatov, Pinter, and Rich 2005). Shown in both traces in blue is an action potential recorded intracellularly using sharp electrodes. In the example shown the resting potential was -80 mV and the action potential peaked near +35 mV. Shown superimposed on the recorded action potential in each trace in orange is the simulated action potential using parameters from the citation referenced in the figure.
General Fitting Approach

While previous efforts at parameter derivation for the Hodgkin-Huxley model have focused on fitting the individual ionic components of the membrane currents involved (Hodgkin and Huxley 1952d; Adrian, Chandler, and Hodgkin 1970; Ildefonse and Rougier 1972; Campbell and Hille 1976; Pappone 1980; Beam and Donaldson 1983), none have attempted to fit the AP trace itself. Of the models that generated APs from the derived parameters, no direct comparison was made to real traces (Adrian and Peachey 1973; S. C. Cannon, Brown, and Corey 1993; Filatov, Pinter, and Rich 2005; Wallinga et al. 1999; Fraser, Huang, and Pedersen 2011). A likely reason for this is that the AP shape is complex and requires a multi-layered approach for fitting successfully. Generation of simulated APs using the Hodgkin-Huxley model requires simultaneous solution of a minimum of ten equations, each with parameters that must be adjusted during fitting. This requires curve-fitting to be done on top of model simulation runs.

Despite the difficulty in directly deriving Hodgkin-Huxley parameters from action potentials, I decided to take this approach to modeling of action potentials. One of the reasons for taking this approach is concern that methods used in voltage clamp recording may alter Na+ channel behaviors (see introduction for details). Furthermore, as my goal is to optimize simulation of real action potentials, simulation of each current separately is not a current goal. Finally, as computing power has increased, running simulations is faster than in the past.

Fitting Passive Parameters
There are too many parameters used in Hodgkin-Huxley modelling of action potentials to attempt to derive them all simultaneously. I thus began by deriving parameters that control the passive properties of fibers. The reversal potential of leak conductance ($E_{\text{leak}}$) and the amount of passive leak conductance ($g_{\text{leak}}$) for each fiber was determined by fitting the fiber response to injection of hyperpolarizing current pulses (Fig 5). ‘$g_{\text{leak}}$’ in the Hodgkin-Huxley model represents conductance of multiple ionic species, including Cl$^-$, K$^+$, and a small amount of Na$^+$, that is not voltage-activated. This conductance is very small compared with the voltage-activated Na$^+$ and K$^+$ conductances, but is the dominant conductance at rest. Cell membrane capacitance ($C_m$) was chosen as 0.9 uF/cm$^2$ based on our own previous work and others (Romer et al. 2021; Hodgkin and Nakajima 1972). $E_{\text{leak}}$ was set to match fiber resting potential just prior to the hyperpolarizing pulse. The fitting algorithm was run to determine the value of $g_{\text{leak}}$ that produced the best fit to the time-course and magnitude of the hyperpolarizing pulse. ‘Best fit’ was determined visually, with emphasis placed on fitting the curve during the initial hyperpolarization. The late portion of the response to a 200 ms injection of current was de-emphasized because it may be affected by slowly gating channels not in the model or by K$^+$ and Cl$^-$ shifts following a change in steady-state membrane potential.
Figure 5.
Figure 5: **Illustration of the steps used for deriving the H-H parameters responsible for passive properties of muscle fibers.** Shown in blue in each trace is a recorded response from a muscle fiber to a 200 ms injection of a small amount of hyperpolarizing current. In the upper left trace $E_{\text{leak}}$ was set at its default value of -85 mV. In the upper right trace $E_{\text{leak}}$ has been adjusted such that the resting potential is now correct. However, the membrane resistance is incorrect such that the response to current injection is too small. The lower left trace is after allowing the fitting algorithm to run with $g_{\text{leak}}$ selected as the parameter to fit. The input resistance has been increased so that the response to current injection is correct. In the lower right is the final fit. The initial portion of the response to current injection is shown on an expanded time scale showing that the time constant was well fit. I did not attempt to fit the slow hyperpolarization as this may be due to activation of other channel types not included in this model.
Fitting AP Parameters

After $g_{\text{leak}}$ was determined, parameters for the H-H voltage gated Na$^+$ conductance ($g_{Na}$) and voltage gated K$^+$ (delayed rectifier) conductance ($g_{Kdr}$) were determined by fitting the AP. For each AP, time points from experimental traces were divided into three phases: the rising phase, the peak, and the falling phase. The peak includes time points from both the late rising phase and the early falling phase. Fits of whole APs included time points for all three phases from the beginning of the rising phase to the end of the falling phase. The stimulus artifact and early subthreshold portions of the AP were excluded from fitting.

Each phase of simulated APs is controlled by different groups of model parameters. There are five parameters each for the activation of $g_{Na}$ and $g_{Kdr}$ and five for inactivation of $g_{Na}$ as well as the maximum conductances, $\tilde{g}_{Na}$ and $\tilde{g}_{K}$, for a total of seventeen parameters remaining to be fit for each AP trace. The rising phase and peak timing is mainly controlled by $g_{Na}$ activation (the ‘$m$’ parameters: $\bar{V}_m$, $k_\alpha m$, $k_\beta m$, $\bar{\alpha}_m$, $\bar{\beta}_m$). Peak height and early repolarization is mostly controlled by $g_{Na}$ inactivation (the ‘$h$’ parameters: $\tilde{V}_h$, $k_\alpha h$, $k_\beta h$, $\bar{\alpha}_h$, $\tilde{\beta}_h$). Late repolarization is driven by a combination of $g_{Na}$ inactivation and $g_{Kdr}$ activation ($g_{Kdr}$ activation, ‘$n$’ parameters: $\bar{V}_n$, $k_\alpha n$, $k_\beta n$, $\bar{\alpha}_n$, $\tilde{\beta}_n$). $E_{\text{leak}}$ was adjusted to match the resting potential of the AP being fitted. Simulated input current duration was matched to the experimental record. For each trace, input current magnitude was adjusted so that the initial passive membrane response preceding AP threshold had a similar rate of rise as the experimental trace.

The steps taken for fitting APs is outlined below:
1. Select the AP trace to be fit. To be selected, the traces had to have a resting membrane potential < -80 mV. Each AP trace recorded for each fiber also has a hyperpolarizing pulse for determination of passive properties.

2. Select the specific time points of the AP phase(s) to be fit.

3. Select which parameters to fit, non-selected parameters were held constant.

4. Set the upper and lower bounds (determined empirically) for the parameters to be fit.

5. Run a simulation using initial input values for all parameters. Initial H-H parameter values were taken from Cannon et al. (S. C. Cannon, Brown, and Corey 1993).

6. Take the output of this simulation and use it as the input to Matlab’s curve-fitting function (lsqcurvefit). The lsqcurvefit function adjusts the parameters being fit (within the bounds set previously) to try to minimize the difference between the simulated trace (voltage versus time) and the experimental trace.

7. Steps 5 and 6 were automatically repeated by the fitting algorithm many times using the result of step 6 as the new initial values until the fitting function exceeded a predetermined number of maximum evaluations or the fitting algorithm reached a local minimum.

8. A final simulation was run with the final parameter set for that fitting run and the result of the fitting were displayed as a graph of voltage vs time.

9. Using this graph, the fit quality was assessed by comparing the experimental trace to the simulated trace to see whether the fit had improved. If the fit needed improvement, several choices had to be made: the initial values could be adjusted
and the same parameters re-fit, a different subset of parameters could be chosen for the next fitting run, or different time points corresponding to different phases of the AP could be chosen for further refinement of fit to a specific portion of the trace.

10. Steps 2 through 9 were repeated, focusing on different phases of the AP trace, until all parameters had been fit and the fit stopped improving.

A few selected steps in the process for fitting action potentials are shown in Figure 6. An initial fit was performed on the whole AP trace allowing all seventeen parameters to vary. If the fitting run produced an acceptable rough fit, the results were used as the initial values for the next fitting run. If the initial fit was poor, the initial values of the parameters were adjusted by hand or the parameter set to be fit was reduced to a smaller number of parameters and the fitting algorithm was run again until an acceptable rough fit was achieved. The parameters that were allowed to vary were adjusted over many iterations to achieve an acceptable rough fit. During the early fitting process, I focused on getting the timing of the simulated AP peak close to the timing of the experimental peak. To do this, I selectively fit the parameters that controlled the voltage dependence of the rest of the model and were most likely to have a direct effect on peak height and peak timing: $\bar{V}_m$, $\bar{V}_h$, $\bar{V}_n$ and $\bar{g}_{Na}$ and $\bar{g}_K$. While the range of values tested for $\bar{g}_K$ did not have a direct effect on peak height or timing, $\bar{g}_K$ had to be allowed to vary while $\bar{g}_{Na}$ was being fitted or the resulting fit was poor.
Once the simulated peak timing was near experimental peak timing and the rough fits of the whole AP started to improve, I started refining the fits for the three AP phases. Because the early part of the fitting process was mainly concerned with peak timing, the parameters for $g_{Na}$ activation (‘$m$’ parameters) had been partially fit by this point. I next focused on fitting the AP peak magnitude and the repolarization phase which are controlled by $g_{Na}$ inactivation (the five ‘$h$’ parameters) and $g_{Kdr}$ activation (the five ‘$n$’ parameters). During this part of the fitting process I allowed only those parameters to vary, holding the rest constant. I again used an iterative process to fit both the AP peak and the AP repolarization phases. If the fits were improving, I used the results as the initial values for the following runs. If the fits did not improve, I adjusted the values by hand, guided by values from previous fitting results before running the fitting algorithm again. Finally, the whole AP fit was refined allowing the fifteen parameters controlling $g_{Na}$ and $g_{Kdr}$ (‘$m$’, ‘$h$’, and ‘$n$’ parameters) to vary. Each iteration used the previous results as initial values if the fits were improving. After multiple iterations, a ‘best fit’ was determined when the parameters were not changing during a fitting run and the whole AP was fit well overall as judged by eye.
Figure 6.
Figure 6: **Stepwise fitting of action potentials.** Shown in each panel is the same recorded action potential in blue. In the top row the amount of current injected and $E_{\text{leak}}$ were adjusted as indicated. In the second row are shown intermediate steps in which various combinations of parameters $\bar{g}_{Na}, \bar{g}_K, \bar{V}_m, \bar{V}_h,$ and $\bar{V}_n$ led to hypoexcitability with no AP (left), hyperexcitability (middle) or steady depolarization (right) due to too much resting $g_{Na}$. In the bottom row are shown the final steps in optimizing the fit. In the lower left, a set of parameters was found that led to reasonable simulation of the rising phase, falling phase and an approximation of half width. Adjusting this parameter set allowed for accurate simulation of AP peak and half width as shown in the lower right panel.
Analysis

A key decision was how to evaluate the quality of fit of the modeled APs. The approach I took was to perform qualitative fits by eye rather than attempting to quantitate the quality of the fit. One reason for this is that it is difficult to come up with a quantitative approach that is not greatly affected by timing of the action potential peak. Slight differences in timing cause large measures of difference in the fit. Fitting qualitatively was also fast as it did not require additional computing power and writing of software to judge the quality of the fit. My approach does not eliminate the possibility of performing quantitative analysis of the fit at a later date.

I chose to compare four features to judge quality of fit of the modeled APs to the experimental recordings: AP magnitude (measured from resting potential to peak), AP half-width, maximum rate of rise, and maximum rate of repolarization (Figure 7, Table 4). A paired student’s t-test was used to compare the four AP features from recorded APs to those from simulated APs (Figure 9). Bonferroni correction was used to account for the multiple comparisons. Following Bonferroni correction, none of the differences were statistically significant at p <0.05. Statistical analysis was done in OriginPro (OriginLab Corporation 2019b).
Figure 7.
Figure 7: **Illustration of the four features used in analysis of the quality of fit of action potentials.** Action potential amplitude and half width are represented with the vertical and horizontal lines terminating in arrowheads and were the primary focus. Rate of rise and decay are represented by the sloped rising and falling lines without arrowheads and were the secondary focus.
Results

Shown in Fig 8 are examples of the final fits arrived at for 3 different recorded action potentials. Each simulated AP is produced from a unique set of parameters obtained by the fitting procedure (Table 5). Compared with experimental traces, simulated mean AP amplitudes, half width, rate of rise and rate of decay were all close enough to the values from recorded action potentials that none of the differences were statistically significant (Table 4, \( p = 0.10 \) for rate of rise, and \( p = 0.14 \) for rate of decay (t-test without Bonferroni correction)).
Figure 8.

Experimentor 1
fiber 4

Experimentor 2
fiber 2

Experimentor 2
fiber 4
Figure 8: **The final fits for three recorded action potentials.** In all three cases I was able to closely simulate the amplitude, half width, rising phase and falling phase. In the example at the top there is deviation of the initial and late parts of the simulated response from the recorded AP. In the middle example all aspects of the response were optimized while the response on the bottom is nicely fit for all aspects except the initial current injection.
We compared the experimental APs with simulated APs using parameters we derived from our fitting procedure to values from the literature used in our model. The same four AP features were used as a means of comparison (Table 4). APs from simulations using parameters from Cannon et al. had the same amplitude as the average of our experimental traces (120.5 mV). The rate of rise (358.5 mV/ms) and rate of repolarization (-152.6 mV/ms) were also well within range of our experimental values (399.5 ± 114 mV/ms and -113.7 ± 91.7 mV/ms, respectively). The one AP feature from this parameter set that fell outside of range was half-width, which was smaller than our experimental values (0.83 ms, Cannon et al.; 1.06 ± 0.12 ms, experimental). APs from simulations using parameters from Filatov et al. had all four features outside of our experimental ranges: AP magnitude was greater (138.1 mV, Filatov et al.; 120.5 ± 9.3 mV, experimental), AP half-width was much smaller (0.65 ms, Filatov et al.; 1.06 ± 0.12 ms, experimental), rate of rise was much steeper (867 mV/ms, Filatov et al.; 399.5 ± 114 mV/ms, experimental), and rate of repolarization was also steeper (-244.6 mV/ms, Filatov et al.; -113.7 ± 91.7 mV/ms, experimental). Overall, parameters derived by fitting whole AP traces yielded simulated APs that more closely match real data than the parameters available from the literature.
Table 4.

<table>
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<tr>
<th></th>
<th>Experimental APs (n = 8, mean ± SD)</th>
<th>APs using fitted parameters (n = 8, mean ± SD)</th>
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<th>APs using Filatov, et al. parameters</th>
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<td>AP amplitude (mV)</td>
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<td>Max rate of rise (mV/ms)</td>
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<td>Max rate of decay (mV/ms)</td>
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<td>-152.6</td>
<td>-244.6</td>
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</table>
Table 4: The values for the four AP features used to judge quality of the fit. Our derived parameters were best for half width and decay rate. Both our parameters and those from Cannon et. al. fit amplitude and rate of rise well. The parameters from Filatov et. al did worst in simulating the four parameters.
Figure 9.
Figure 9: **Evaluation of quality of fit.** Shown are plots of the four AP characteristics measured from the eight recorded APs and the corresponding value for each modeled action potential. To compare the four AP features from recorded APs to those from simulated APs, the paired student’s t-test was used. Bonferroni correction was used to account for the multiple comparisons. Following Bonferroni correction, none of the differences were statistically significant at p <0.05.
Shown in Table 5 are the derived Hodgkin-Huxley parameters which led to the best fit of the recorded action potentials. Included for comparison are the parameters from two previous modeling studies. While many of the parameters we derived agreed well with previously used parameters, there were a number of differences. Compared with the parameters from Cannon et al., our parameters for voltage dependence ($\overline{V}_m$, $\overline{V}_h$, $\overline{V}_n$) and the maximum Na$^+$ conductance ($g_{Na}$) were quite similar. The main differences were seen in the steepness factors ('$k$' values) and the rate constants ('$\alpha$' and '脒' values) with the exception of $k_\alpha m$, $k_\alpha h$, and $\overline{\beta}_m$. Our values for maximum conductances $g_{Kdr}$ and $g_{leak}$ also differed from this parameter set. In contrast, our parameters for voltage dependence of Na$^+$ and K$^+$ activation ($\overline{V}_m$, $\overline{V}_n$) and the maximum Na$^+$ conductance ($g_{Na}$) were quite different from Filatov et al. There were also several differences seen in the steepness factors and rate constants. Notable exceptions in these groups were $k_\alpha m$, $k_\alpha h$, $\overline{\beta}_h$, and $\overline{\beta}_n$. Figure 10 shows how the ranges of the derived parameters (orange bars) compare with the ranges of the two sets of parameters from the literature (blue open boxes).

In order to understand the importance of the differences between our derived parameters and those used previously in modeling studies, we undertook sensitivity analysis. Our goal was to identify which parameters play the most important role in determining action potential shape. If differences in our parameter values from those in the literature are in parameters that have little effect on AP shape, those differences can likely be ignored moving forward. However, if a parameter has both a significant impact on action potential shape and is one for which we derived a different value from that used in previous studies, the difference needs to be paid attention to in future modeling studies.
Figure 10.
Figure 10: **Fitted parameter ranges versus literature parameter ranges.** Shown are plots of the ranges of fitted parameters (orange bars) overlaid on the ranges of literature parameters (light blue boxes). Parameters are grouped according to type and units.

Upper left, maximum conductances. Upper right, voltage dependence of gating parameters. Lower left, steepness factors. Lower right, rate constants.
<table>
<thead>
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<th>Filatov et al.</th>
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<td>10</td>
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<tr>
<td>$k_{\beta} m$</td>
<td>mV</td>
<td>23.5, 35.3</td>
<td>31.2</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>$\bar{a}_m$</td>
<td>1/(ms*mV)</td>
<td>0.091, 0.223</td>
<td>0.179</td>
<td>0.288</td>
<td>1</td>
</tr>
<tr>
<td>$\bar{\beta}_m$</td>
<td>1/ms</td>
<td>0.65, 0.99</td>
<td>0.81</td>
<td>1.38</td>
<td>2</td>
</tr>
<tr>
<td>$\bar{V}_h$</td>
<td>mV</td>
<td>-44.8, -34.9</td>
<td>-39.4</td>
<td>-45</td>
<td>-35</td>
</tr>
<tr>
<td>$k_{\alpha} h$</td>
<td>mV</td>
<td>14.2, 33.9</td>
<td>22.9</td>
<td>14.7</td>
<td>14.7</td>
</tr>
<tr>
<td>$k_{\beta} h$</td>
<td>mV</td>
<td>3.0, 5.9</td>
<td>4.2</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>$\bar{a}_n$</td>
<td>1/ms</td>
<td>0.0034, 0.0052</td>
<td>0.0043</td>
<td>0.0081</td>
<td>0.0081</td>
</tr>
<tr>
<td>$\bar{\beta}_n$</td>
<td>1/ms</td>
<td>6.5, 11.5</td>
<td>8.8</td>
<td>4.4</td>
<td>8</td>
</tr>
<tr>
<td>$\bar{V}_n$</td>
<td>mV</td>
<td>-43.8, -36.6</td>
<td>-40.1</td>
<td>-40</td>
<td>-32</td>
</tr>
<tr>
<td>$k_{\alpha} n$</td>
<td>mV</td>
<td>3.8, 6.3</td>
<td>5.2</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>$k_{\beta} n$</td>
<td>mV</td>
<td>16.4, 22.3</td>
<td>19.0</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>$\bar{a}_n$</td>
<td>1/(ms*mV)</td>
<td>0.0136, 0.0167</td>
<td>0.0148</td>
<td>0.0131</td>
<td>0.020</td>
</tr>
<tr>
<td>$\bar{\beta}_n$</td>
<td>1/ms</td>
<td>0.056, 0.120</td>
<td>0.084</td>
<td>0.067</td>
<td>0.067</td>
</tr>
<tr>
<td>$\bar{g}_{Na}$</td>
<td>mS/cm²</td>
<td>115, 167</td>
<td>136</td>
<td>150</td>
<td>500</td>
</tr>
<tr>
<td>$\bar{g}_K$</td>
<td>mS/cm²</td>
<td>5.3, 18.5</td>
<td>12.0</td>
<td>21.6</td>
<td>30</td>
</tr>
<tr>
<td>$g_{\text{leak}}$</td>
<td>mS/cm²</td>
<td>0.21, 0.38</td>
<td>0.30</td>
<td>0.75</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Table 5: The derived H-H parameters. Shown are the units of the parameters, the range and mean for each derived value for my fits of the eight recorded action potentials as well as the literature values for the parameters used in two previous modeling studies.
Sensitivity analysis

A representative parameter set from our fits of the eight recorded action potentials was chosen for sensitivity analysis. The one-parameter-at-a-time (OAT) approach was used, by which each parameter is varied individually by a small amount and the output (in this case, a simulated AP) is assessed for any differences from the original simulation trace. I chose to test each parameter by an increase of 5% and by a decrease of 5% from the original value. 5% was chosen as a 10% change often changed action potentials so dramatically that there was complete failure. A 1% change often led to changes that were very small and thus difficult to quantitate. The effect of these parameter changes was quantified by how they altered four AP features of interest: AP magnitude, AP half-width, rate of rise, and rate of repolarization. These effects were reported as a percent change for each AP feature for each parameter tested.

We found that AP shape is much more sensitive to changes in Na\(^+\) conductance parameters than K\(^+\) conductance parameters (Table 6). Additionally, there was more sensitivity to the Na\(^+\) activation (‘\(m\)’) parameters as a group than to the Na\(^+\) inactivation (‘\(h\)’) parameters. There was greatest sensitivity to changes in \(\bar{V}_m\) and \(\bar{V}_h\), the two parameters that set the voltage dependence of the Na\(^+\) activation and inactivation rate coefficients, respectively. A ±5% change to these parameters changed all four AP features: the AP rate of rise and rate of repolarization by around ±30% and AP magnitude and half-width by roughly ±15%. The next most important parameters were \(k_{\alpha m}\), \(\bar{\beta}_h\), and \(\bar{\alpha}_m\). Changes to these parameters also altered all four AP features of interest, but to a lesser degree with a ±5% change resulting in ±10-15% change in rates of rise and repolarization and ±5-10% change in AP magnitude and half-width. The model was
moderately sensitive to changes in $k_{\beta h}$: AP rate of rise and rate of repolarization changed by slightly more than $\pm 5\%$, AP magnitude changed less than $5\%$, but half-width was not affected by a $\pm 5\%$ change. The model was much less sensitive to changes in $\bar{\beta}_m$, $k_{\beta m}$, $k_{\alpha h}$ and $\bar{\alpha}_h$. A $\pm 5\%$ change to any one of these parameters had virtually no effect on half-width and resulted in less than $\pm 5\%$ change to any of the other three AP features.

The Na$^+$ channel gating parameters ranked in order of model sensitivity are, from highest to lowest: $\bar{V}_m$, $\bar{V}_h$, $k_{\alpha m}$, $\bar{\beta}_h$, $\bar{\alpha}_m$, $k_{\beta h}$, $\bar{\beta}_m$, $k_{\beta m}$, $k_{\alpha h}$, $\bar{\alpha}_h$.

The model was relatively insensitive to changes in any of the K$^+$ channel gating parameters. Changes to these parameters had the most effect on the rate of repolarization. They had a very minor effect, if any, on AP magnitude, and had virtually no effect on AP half-width or rate of rise. This was surprising to us as K$^+$ conductance is generally thought to play a central role in the rate of AP repolarization. Two parameters, $\bar{V}_n$ and $\bar{\alpha}_n$, which set the voltage dependence of the K$^+$ activation rate coefficients and scale the rate of K$^+$ activation, did change the rate of AP repolarization, but by less than $\pm 5\%$. The K$^+$ channel gating parameters ranked in order of model sensitivity are: $\bar{\alpha}_n$, $\bar{V}_n$, $k_{\alpha n}$, $k_{\beta n}$, $\bar{\beta}_n$.

The model is also less sensitive than might be expected to changes in the maximal Na$^+$ conductance ($\bar{g}_{Na}$), given the relative sensitivity to the Na$^+$ gating parameters. A $\pm 5\%$ change caused less than $10\%$ change in rate of rise or repolarization, and less than $5\%$ change in peak height. Sensitivity to changes in either the maximal K$^+$ conductance ($\bar{g}_K$) or the constant leak conductance ($g_{\text{leak}}$) is low. None of these conductance parameters has any appreciable effect on AP half-width.
Listed in Table 7 is the relationship between the direction of change for each parameter and the gross effects on specific AP features. For example, increasing the absolute magnitude of $\bar{V}_m$ from -41 mV to -45 mV markedly increases both the rate of rise and rate of repolarization, increases peak height, and decreases half-width. Decreasing $\bar{V}_m$ from -41 to -37 mV has the opposite effect: decreased rates of rise and repolarization, decreased peak height, and increased half-width. A similar symmetric relationship holds for all parameters listed. If an increase in the magnitude of a parameter has a specific effect on an AP feature, a decrease of that parameter has the opposite effect on that same AP feature.
Table 6: Sensitivity analysis of H-H parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AP magnitude % change (mean)</th>
<th>AP half-width % change (mean)</th>
<th>Rate of rise % change (mean)</th>
<th>Rate of repolarization % change (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\bar{V}_m$</td>
<td>12</td>
<td>19</td>
<td>35</td>
<td>28</td>
</tr>
<tr>
<td>$k_\alpha m$</td>
<td>5.5</td>
<td>9.1</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>$k_\beta m$</td>
<td>0.5</td>
<td>0</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>$\bar{a}_m$</td>
<td>4.2</td>
<td>4.5</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>$\bar{B}_m$</td>
<td>1.2</td>
<td>0</td>
<td>3.4</td>
<td>2.8</td>
</tr>
<tr>
<td>$\bar{V}_n$</td>
<td>12</td>
<td>15</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>$k_\alpha h$</td>
<td>0.1</td>
<td>0</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>$k_\beta h$</td>
<td>2.8</td>
<td>0</td>
<td>7.6</td>
<td>6.8</td>
</tr>
<tr>
<td>$\bar{a}_h$</td>
<td>0.04</td>
<td>0</td>
<td>0.1</td>
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<tr>
<td>$\bar{B}_h$</td>
<td>5.2</td>
<td>9.1</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>$\bar{V}_n$</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>4.1</td>
</tr>
<tr>
<td>$k_\alpha n$</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>$k_\beta n$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>$\bar{a}_n$</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>4.4</td>
</tr>
<tr>
<td>$\bar{B}_n$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
</tr>
<tr>
<td>$\bar{g}_{Na}$</td>
<td>2.6</td>
<td>0</td>
<td>7.3</td>
<td>6.5</td>
</tr>
<tr>
<td>$\bar{g}_{K}$</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
<td>1.8</td>
</tr>
<tr>
<td>$g_{leak}$</td>
<td>0.5</td>
<td>0</td>
<td>0.9</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Table 6: Sensitivity of model output to ± 5% change of each parameter. Parameter name is labeled with the color representing the largest mean change to an AP feature caused by a 5% change to that parameter.

<table>
<thead>
<tr>
<th>&gt;20% change</th>
<th>10-20% change</th>
<th>5-10% change</th>
<th>1-5% change</th>
<th>&lt;1% change</th>
</tr>
</thead>
</table>


Table 7: Effects of H-H parameters on AP characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Peak height</th>
<th>$\frac{1}{2}$ width</th>
<th>Rate of rise</th>
<th>Rate of repolarization</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\bar{V}_m$</td>
<td>++</td>
<td>- -</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>$k_\alpha m$</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$k_\beta m$</td>
<td>~+</td>
<td>0</td>
<td>~+</td>
<td>~+</td>
</tr>
<tr>
<td>$\bar{\alpha}_m$</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$\bar{\beta}_m$</td>
<td>~-</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\bar{V}_h$</td>
<td>~-</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$k_\alpha h$</td>
<td>~-</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$k_\beta h$</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\bar{\alpha}_h$</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>$\bar{\beta}_h$</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\bar{V}_n$</td>
<td>~-</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>$k_\alpha n$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$k_\beta n$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+ (late)</td>
</tr>
<tr>
<td>$\bar{\alpha}_n$</td>
<td>~-</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>$\bar{\beta}_n$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>- (late)</td>
</tr>
<tr>
<td>$g_{Na}$</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$g_{K}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>~+</td>
</tr>
<tr>
<td>$g_{\text{leak}}$</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>~-</td>
</tr>
</tbody>
</table>
Table 7: Gross effects of *increasing the absolute magnitude of each parameter on specific AP features. Below is the key to Table 7. (*Note: A decrease in absolute magnitude of a given parameter produced a similar change in the opposite direction.)

<table>
<thead>
<tr>
<th>Strong increase</th>
<th>Increase</th>
<th>Weak increase</th>
<th>Strong decrease</th>
<th>Decrease</th>
<th>Weak decrease</th>
<th>No change</th>
</tr>
</thead>
<tbody>
<tr>
<td>++</td>
<td>+</td>
<td>~+</td>
<td>- -</td>
<td>-</td>
<td>~-</td>
<td>0</td>
</tr>
</tbody>
</table>
Discussion

The ability to derive Hodgkin-Huxley parameters from recording of single action potentials

All previous studies I am aware of derived H-H parameters for simulation of action potentials from voltage clamp recordings of individual currents. I used a method, never used previously, to derive H-H parameters for action potential simulation directly from the action potentials to be simulated. An advantage of this approach is that it does not require voltage clamp recording of Na\(^+\) and K\(^+\) currents in isolation to derive the parameters. The ability to directly derive H-H parameters without performing voltage clamp recordings has several significant advantages. One of the primary advantages is that obtaining well clamped Na\(^+\) currents from intact muscle fibers is extremely challenging as the Na\(^+\) current is large and very fast; both properties make it challenging to maintain control over voltage. As outlined in the introduction to this chapter, another advantage is that avoiding voltage clamp allows one to derive H-H parameters from ion channels that are relatively unperturbed. The formation of tight seals during patch clamp recordings, disruption of intracellular milieu and the application of holding potentials to the voltage clamped membrane all appear to alter Na\(^+\) channel gating. Since our goal is to derive parameters to accurately simulate action potentials, it makes sense to derive the parameters from the cell of interest under the conditions of interest. To this end, my parameters were derived directly from current clamp recordings in the same preparation we use to study muscle electrophysiology. By taking this approach, I have avoided the issues that can be introduced with voltage clamping. Furthermore, all parameters were
derived from the same tissue in the same species under the same experimental conditions. This is a distinct improvement over other available models of skeletal muscle which relied on parameters combined from unrelated voltage clamp studies of multiple muscle types and multiple species.

One possible outcome of my efforts was that I would be unable to accurately simulate single action potentials. In this case there would have been two possible explanations for failure: 1) The problem is too difficult, one must use voltage clamp recordings of currents to break the problem down into manageable chunks, 2) The Hodgkin-Huxley model lacks too many features of real ion channels and thus is insufficient to simulate muscle action potentials. Because I succeeded in accurately simulating action potentials, I can reject both of these conclusions. I conclude both that it is possible to directly derive H-H parameters from the action potentials to be modelled and that the H-H model is sufficient to accurately simulate single action potentials in skeletal muscle.

Features that can be added to the model I created to more accurately simulate action potentials.

While the focus of this Aim was on replication of single APs, the basic H-H model may not be able to replicate behaviors over longer times scales. A question that I have not addressed in my study is whether the H-H model is sufficient to model action potentials in other situations such as during depolarization of muscle or during repetitive firing of action potentials. In both cases, the lack of inclusion of slow and ultra-slow
inactivation of Na\(^+\) channels in the H-H model may prove a limitation. The H-H model includes only fast inactivation of Na\(^+\) channels.

Another limitation of the H-H model is that it has only one voltage gated K\(^+\) channel. Skeletal muscle expresses several isoforms of Kv channels (Jurkat-Rott and Lehmann-Horn 2004; DiFranco, Quinonez, and Vergara 2012) and it is likely that more than one contributes to AP repolarization. Some of these Kv channels may inactivate (Adrian, Chandler, and Hodgkin 1970; DiFranco, Quinonez, and Vergara 2012). The H-H model does not include inactivation for K\(^+\) channels and combines Kv conductances into a single delayed rectifier K\(^+\) conductance. Both of these limitations may be important during both depolarization and repetitive firing.

Finally, the H-H model has only one leak conductance, representing both the K\(^+\) and Cl\(^-\) channels open at the resting potential. In the H-H model, \(g_{\text{leak}}\) is a passive, linear conductance. This is likely not the case in real muscle. The muscle Cl\(^-\) channel (ClCn1) is voltage dependent (Waters et al. 2013). In addition, the resting K\(^+\) conductance demonstrates rectification with depolarization such that the leak K\(^+\) conductance decreases with depolarization. While it is possible that these two effects cancel each other out, it seems likely that they will impact modeling of depolarization-induced changes in excitability.

These limitations of the current model can be addressed moving forward. The design of the model makes it possible to modify each conductance independent of other conductances. The hope is that as more conductances are added to the model, it will be able to accurately simulate more and more complex behaviors of muscle. A question that
can be asked at each step is what specific behavior of muscle requires the addition of each new feature incorporated into the model.

Another improvement that can be added to the model in the future, is the cable shape of skeletal muscle. I used a single intracellular compartment in my model. The use of a single intracellular compartment does not allow for study of action potential propagation down the length of a muscle fiber. To generate a cable model, one simply needs to replicate the t-tubule and intracellular compartments I used to model a short segment of muscle. The addition of the cable spatial arrangement is likely to impact passive properties of the model by increasing total capacitance and thus could impact single action potentials. However, the addition of a longitudinal cable model will also slow down simulation time considerably. This is likely to become an issue since the optimization process requires the simulation to be executed many times.

*The relative importance of different Hodgkin-Huxley parameters in accurate modeling of APs*

The H-H model includes 18 parameters. While many of the values for Hodgkin-Huxley parameters I derived agreed well with the values used previously, others did not. In order to interpret the importance of these differences I performed sensitivity analysis on all 18 H-H parameters. Not too unexpectedly, the parameters most important in determining the rate of rise and amplitude of action potentials were primarily involved in the voltage dependent behavior of Na⁺ channels. What was surprising was that Na⁺ channel gating parameters were also most important in determining the rate of AP decay. In no case did a 5% change in a parameter controlling voltage gated K⁺ channels cause
more than a 5% change in any of the four action potential properties I analyzed in my sensitivity analysis. A benefit of having done this analysis is that if others want to derive H-H parameters for action potentials in other tissues, my study provides guidance as to which parameters must be altered in very small steps during the fitting process and which ones can be varied in larger steps.

I found parameters controlling the rising phase and peak of the AP require greater care when optimizing than those involved in the falling phase. Another conclusion is that parameters representing relief of fast inactivation are much more forgiving \((k_\alpha h, \bar{\alpha}_h)\) than parameters involved in entry into inactivation \((k_\beta h, \bar{\beta}_h)\). Another lesson I learned from doing this analysis is that fitting parameters for each gating variable \((m, h, n)\) as a group works better than fitting them individually. My interpretation is that there are interdependencies both among the parameters for each gating variable and between the parameters affecting activation or inactivation of Na\(^+\) or K\(^+\) channels. For example, when I allowed simultaneous fitting of the forward \((\alpha)\) and reverse \((\beta)\) parameters for a given gating variable, the fitting algorithm reach a better fit with fewer fitting runs. In addition, certain phases of the AP are controlled by more than one gating variable (for example, AP peak, which is controlled by both ‘\(m\)’ and ‘\(h\)’ parameters). Ignoring these interdependencies led to wasted time and effort due to manipulations of parameters that yielded only minor improvements in fit.

A number of the H-H parameters I derived differed substantially from values used for previous modeling studies. Sensitivity analysis allowed me to determine which of these differences were responsible for my ability to more accurately simulate action potentials and which differences were unimportant. For example, my values for \(k_\alpha n\) and
$k_\beta n$ representing the steepness of voltage dependence of the forward and reverse rate constants for $K^+$ channel activation differed by 30 and 100% from previously used values. Sensitivity analysis suggests these differences had little effect on the earlier AP phases, but could have affected late repolarization which we were not comparing at this time.

Both Cannon and Filatov parameters underestimated the half-width. Based on my sensitivity analysis, this is most likely to be caused by $\bar{V}_m, \bar{V}_h, k_\alpha m, \bar{\alpha}_m$, or $\bar{\beta}_h$. These parameters set the voltage dependence of $Na^+$ activation ($\bar{V}_m$) and inactivation ($\bar{V}_h$), the steepness of voltage dependence of $Na^+$ activation ($k_\alpha m$), and scale the forward rates of activation ($\bar{\alpha}_m$) and inactivation ($\bar{\beta}_h$). Comparing my fit values for these parameters with the literature values, it appears that Cannon differed primarily in $\bar{\beta}_h$, while Filatov differed primarily in $\bar{V}_m$. However, when the results of the sensitivity analysis are combined with the parameter values from Table 5 and the effects of changing a given parameter from Table 7, it can be seen that for the Cannon model, two additional parameters contributed to a narrower half-width: $\bar{V}_m$ and $\bar{\alpha}_m$. Conversely, the most important determinant of half-width for Filatov was $\bar{\alpha}_m$ rather than $\bar{V}_m$. This is because Filatov’s more depolarized $\bar{V}_m$ on its own would have the effect of increasing half-width, but the five-fold increase in $\bar{\alpha}_m$ overpowers this effect and decreases AP half-width. This demonstrates the utility of the analysis provided here to improve AP simulation, enabling a future modeler to fine tune their parameters.

Finally, a parameter with significant effects on both the rising and falling phase of the action potential was $\tilde{g}_{Na}$. My mean derived value for $\tilde{g}_{Na}$ of 136 was close to the value of 150 used by Cannon et. al, but appears to differ by more than 3-fold from the
value used by Filatov et. al. However, this difference is misleading. The study by Filatov et. al. included slow inactivation of Na\(^+\) channels, which is significant at a resting potential of -85 mV, such that \(\bar{g}_{Na}\) was close to 150.

In conclusion, I set out determine whether it was possible to derive a set of H-H parameters by fitting action potentials. My approach was successful and it thus appears that H-H parameters can be derived directly. This bypasses the need for voltage clamp studies in tissues where voltage clamp is difficult due to space clamp issues. My ability to accurately simulate single action potentials makes possible a number of modeling studies of skeletal muscle excitability moving forward. The initial results of one of those studies is presented in my next chapter. Other studies to be performed in the future are discussed in my summary chapter.
Chapter VI: Modeling of action potential-induced depolarization of t-tubules

Introduction

Excitation contraction coupling (ECC) was first defined by Alexander Sandow as the series of events spanning action potential initiation in the surface membrane of skeletal muscle to the beginning of force generation by fibers (Kahn and Sandow 1950; Sandow 1952). In the close to 70 years since Sandow’s initial work, a great deal of progress has been made in understanding the sequence of events involved in ECC.

ECC causes translation of action potentials in the surface membrane into $\text{Ca}^{2+}$ release from the sarcoplasmic reticulum (SR) in several steps, each involving a different set of ion channels (Calderón, Bolaños, and Caputo 2014; Allard 2018; Hernández-Ochoa and Schneider 2018). First, muscle action potentials initiated by opening of $\text{Na}^{+}$ channels in the sarcolemma cause depolarization of a network of membrane invaginations in muscle known as the transverse tubules (t-tubules) (Adrian, Costantin, and Peachey 1969). Depolarization in the t-tubules triggers outward movement of the positively charged S4 alpha helices of Cav1.1; termed gating charge movement (Bannister and Beam 2013). Finally, the movement of gating charges of Cav1.1 channels triggers opening of ryanodine receptors (RyR) on the SR allowing for $\text{Ca}^{2+}$ to exit into the cytoplasm (Melzer, Herrmann-Frank, and Lüttgau 1995; Dulhunty 2006; Hernández-Ochoa and Schneider 2018). $\text{Ca}^{2+}$ release from the SR increases over a relatively wide range of voltages: it begins to be triggered when there is transient depolarization to -30 to -20 mV and becomes maximal when the transient depolarization is above +10 mV (Z. M.
Thus, to get maximal Ca\textsuperscript{2+} release, the interior of the fiber must depolarize to above +10 mV.

A necessary step in the process of successful ECC is the spread of depolarization from the surface membrane into the center of the fiber. If depolarization fails to spread to the interior of the fiber, Ca\textsuperscript{2+} release would only be maximal from SR located near the surface membrane and force generation would be impaired. Muscle fibers are large, multinucleated cells, such that depolarization must travel up to 50 um from the surface to reach the center of a fiber (Adrian, Costantin, and Peachey 1969). It is widely accepted that the way depolarization travels from the surface of the fiber to the center is via t-tubules, by active AP propagation along the t-tubule membrane (Calderón, Bolaños, and Caputo 2014; Allard 2018; Hernández-Ochoa and Schneider 2018).

Several lines of evidence have led to the conclusion that action potentials travel to the center of fibers via the T-tubule system (TTS). One of the foundational studies was performed by Adrian et. al. (Adrian, Costantin, and Peachey 1969). In that study isolated frog muscle fibers were treated with tetrodotoxin to block action potentials. Muscle fibers were voltage clamped with two electrodes and the degree of depolarization was varied while fibers were imaged. Via imaging they were able to determine whether contraction occurred in only superficial myofibrils or whether it involved the entire fiber. When an action potential waveform was used as the command voltage, they found that this was just sufficient to cause contraction of the entire fiber. They concluded, “it seems that an action potential at the surface of a frog striated muscle fibre could just, but only just, activate the axial myofibrils by electrotonic spread along the T-system.”
Another line of work suggesting action potential propagation in t-tubules is necessary comes from experiments using glycerol-induced detubulation. In those experiments cells were rapidly swelled and shrunk due to rapid changes in extracellular osmolarity (see (B. Eisenberg and Eisenberg 1968) for details of procedure). The rapid swelling and shrinking rips the t-tubule membrane away from the surface. It was found that after detubulation, contraction was greatly reduced. The interpretation has been that detubulation prevents muscle contraction because APs cannot reach the voltage-sensor/calcium release structures to initiate the calcium release. Following detubulation APs can still propagate along the surface of these fibers. They are, however, slightly altered in shape and lack the early afterpotential usually seen in skeletal muscle after repetitive firing (Gage and Eisenberg 1969; Lännergren and Westerblad 1987). This early afterpotential has been attributed to charging the t-tubule membrane since the time-course of the later phase is similar to the membrane time constant.

In amphibian muscle it has been shown that under passive conditions, the t-tubule response is slower than the surface membrane response (J. A. Heiny and Vergara 1982; Ashcroft, Heiny, and Vergara 1985). This is thought to be due to both a high t-tubule access resistance as well as a high luminal resistance (Adrian, Costantin, and Peachey 1969; Adrian and Peachey 1973; Ashcroft, Heiny, and Vergara 1985). In mouse muscle, however, depolarization of the t-tubule membrane occurs almost simultaneously with the surface membrane. Using a voltage-dependent dye Woods et. al found that 95% of the steady state value for the TTS fluorescence was achieved within 0.1 ms (Woods et al. 2005). The finding of nearly simultaneous depolarization of t-tubules and surface
membrane calls into question the requirement of sequential Na\(^+\) channel opening radially along the t-tubule membrane.

Based on modelling of the t-tubule response to current injection performed in Aim 1 of my thesis, I began to question whether depolarization spreads to the interior of muscle fibers via AP propagation along t-tubule membrane. When an AP is triggered at the membrane surface, an influx of Na\(^+\) ions causes current to flow through the intracellular space. This current flow would allow electrotonic spread of depolarization over short distances. Given that skeletal muscle fibers have a relatively large space constant relative to their diameter (Luff and Atwood 1972), sufficient transverse depolarization may occur to trigger Ca\(^{2+}\) release from the SR. In the simulation, the interior of the fiber is modeled as a single compartment containing high levels of ions, such as K\(^+\), such that the intracellular solution has low resistance and the compartment is close to isopotential. While this is certainly an oversimplification, it caused me to consider the possibility that current flow through the intracellular compartment may be an easier path for spread of depolarization to the interior of the fiber than AP propagation along the t-tubule membrane. If this hypothesis is correct, it would change our understanding of the process of excitation in skeletal muscle.

The goal of this Aim was to explore the possibility that depolarization spreads to the center of muscle fibers via currents in the intracellular space rather than requiring activation of Na\(^+\) channels in the t-tubules. Initial simulations suggest the resistance to current flow in t-tubules plays a critical role in the transmembrane potential of t-tubules in the center of the fiber. Using the value of resistance of extracellular solution, the
estimated t-tubule resistance would lead to an adequately depolarized transmembrane t-tubule potential to cause muscle contraction.

Methods

One parameter set was chosen from Aim 2 (Experimenter 2, fiber 2). The same model structure as in Aim 2 was used for this study. Two sets of simulations of single APs were compared, each set using two conditions for Na\(^+\) channel density in the t-tubule compartment: 1) t-tubule Na\(^+\) channel density set equal to the Na\(^+\) channel density of the surface membrane (intracellular compartment) (t-tubule gNa ‘on’), and 2) t-tubule Na\(^+\) channel density set to zero (t-tubule gNa ‘off’), but with surface membrane Na\(^+\) density unchanged. For the first set, t-tubule luminal conductance (\(G_L\)), representing the conductivity of fluid in the t-tubules, was 8 mS/cm. This is close to the conductivity of extracellular solutions typically used in electrophysiology, such as Ringer’s or Tyrode’s solutions. For the second set, \(G_L\) was reduced to 3.7 mS/cm, the value used in the model by Wallinga et al. (Wallinga et al. 1999). They had chosen this value empirically based on their model simulations of AP conduction velocity in the t-tubules. For both sets of simulations, peak voltages taken at the surface and at each of the t-tubule shells were compared for the t-tubule \(g_{Na} \) ‘on’ and ‘off’ conditions.

Preliminary Results
The first set of simulations was performed with t-tubule luminal conductance at 8 mS/cm. This is close to the conductivity of Ringer’s solution. Most published models have used slightly higher values for $G_L$, around 10 mS/cm (Adrian and Peachey 1973; DiFranco and Vergara 2011; DiFranco et al. 2013; Vergara et al. 2014). When Na\(^+\) channel density of the t-tubules was equal to the surface, peak transmembrane voltages in the t-tubules were slightly greater than at the surface. With t-tubule Na\(^+\) channel density at zero, significant passive depolarization in the t-tubule compartment still occurred when an AP was triggered at the surface (Figure 11). Compared with the surface AP, the peaks were lower. However, peak voltages were still greater than +20 mV. This suggests that spread of depolarization through the t-tubule compartment may still occur rapidly regardless of availability of Na\(^+\) channels in the t-tubules.
Figure 11.
Figure 11: **Depolarization within the t-tubules in the presence and the absence of a t-tubular Na\(^+\) conductance: dependence on t-tubule luminal conductance.** APs from the surface/intracellular compartment (light blue) overlaid on peak voltages from selected shells at specified depths within the t-tubule system. Left: When t-tubule Nav channel density is equal to surface Nav channel density, voltages within the t-tubules peak at higher voltages than the surface AP. Middle: Nav channel density is zero in t-tubules, yet depolarizations still occur. These depolarizations have similar shape as surface APs, but are wider and have lower amplitude. Right: t-tubule peak voltages plotted versus radial depth from the surface. Black dots are peak voltages with t-tubule $g_{Na}$ present and equal to surface $g_{Na}$. Red dots are peak voltages with zero $g_{Na}$ in t-tubules. Top row: T-tubule luminal conductance ($G_L$) equal to 8 mS/cm. Bottom row: T-tubule luminal conductance equal to 3.7 mS/cm (Wallinga et al. 1999).
In Aim 1, we had observed that a finite t-tubule luminal conductance caused a voltage gradient in the t-tubules at subthreshold step potentials during voltage clamp. This luminal conductance would also determine the gradient of peak voltages in the t-tubules during an AP. If $G_L$ in mammalian muscle is considerably lower than we had supposed, this would affect the peak voltage reached by the deeper regions of the muscle fiber. One model had chosen a $G_L$ of 3.7 based on their simulations of AP propagation in the t-tubule compartment (Wallinga et al. 1999). They had chosen this value to get an AP conduction velocity of 2.5 cm/s in the t-tubules. This value for $G_L$ was subsequently adopted by two other modeling studies on surface AP conduction velocity (Fraser, Huang, and Pedersen 2011; Fortune and Lowery 2012).

To test the effect of a reduced t-tubule luminal conductance on the spread of both active and passive depolarization in the t-tubules, the simulations were repeated with $G_L$ reduced to 3.7 mS/cm. When Na$^+$ channel density in the t-tubules was equal to Na$^+$ channel density at the surface, the peak voltages at the center of the fiber were about 10 mV greater than at the surface (Figure 11, bottom left). When there was a Na$^+$ channel density of zero in the t-tubules, a much steeper drop in peak voltages occurred compared with the simulations with a higher $G_L$ (Figure 11, middle, lower panel). Peak voltages at the center of the fiber were +2 mV, roughly 30 mV lower than the peak voltage at the surface. While ECC may still occur, this would leave skeletal muscle with a very low safety factor. A small depolarization of membrane reversal potential could lead to failure of Ca$^{2+}$ release in this situation.
Discussion

*The effect of t-tubules on depolarization of muscle fibers*

80% of the surface membrane of a skeletal muscle fiber is in the t-tubules. Since capacitance is directly proportional to membrane area this means that 80% of the capacitance of a skeletal muscle fiber is due to membrane in the t-tubule. The lower the capacitance, the less Na\(^+\) current is needed to generate an action potential. While the t-tubules are necessary for ECC, the additional capacitance they confer may pose a significant impediment to generation of APs when Na\(^+\) channels are inactivated. The goal of this aim was to explore how the muscle overcomes this impediment to achieve rapid depolarization of t-tubules.

When I eliminated Na\(^+\) conductance in t-tubules, the degree of depolarization of t-tubules in the center of the fiber depended critically on the resistance to radial current flow through t-tubules. With relatively low radial resistance, depolarization spread readily to the center of the fiber, but with only a 2-fold increase there was a significant radial drop in peak voltages. Unfortunately, one cannot measure t-tubule luminal conductance directly. Luminal conductance values have either been estimated from the conductivity of the external solution used for experiments or derived from analysis of impedance measurements from skeletal muscle. Impedance measurements of skeletal muscle preparations from several non-mammalian species have been interpreted using various model structures and have led to differing values. It was postulated these differences may be due to differences in t-tubule geometry among the species studied (R. S. Eisenberg 2010). An analysis of rat skeletal muscle impedance concluded that t-tubule
lumen resistivity was 154 ± 27 Ohm cm which corresponds to a $G_L$ of 5.5 - 7.8 mS/cm (Thomas H. Pedersen, L-H Huang, and Fraser 2011).

While the overall resistance of the TTS appears to be critically dependent on $G_L$, it is also a direct consequence of the geometry of the T-system itself, as seen in Aim 1. Both fiber size and t-tubule diameter impacted the radial voltage gradient. This voltage gradient becomes larger as radial resistance in the TTS increases. My preliminary results suggest that although the presence of a Na$^+$ conductance in the t-tubule membrane may not be strictly necessary for adequate depolarization of the interior of the fiber, it may help offset the resistance of the TTS. To more fully explore the functional significance of Na$^+$ channels in the t-tubules, future modeling studies will be needed to compare the timing of radial depolarization with the speed of AP propagation along the t-tubule membrane. If depolarization occurs more quickly than APs can travel along the membrane, this would strengthen the hypothesis that intracellular spread of depolarization within muscle fibers is primarily electrotonic and does not depend on activation of Na$^+$ channels in the t-tubules.

The function of t-tubules

Despite my simulation being inconclusive, I hope to continue to explore the following hypothesis regarding t-tubule function. For ECC to occur, Cav1.1 channels coupled to RYRs on the SR in the center of the fiber must be able to sense that there has been a change in the transmembrane potential. The reason for this need is that the change in transmembrane potential triggers the movement of gating charges in Cav1.1 channels, which in turn triggers opening of RYRs and release of Ca$^{2+}$ from the SR. If muscle fibers
did not have t-tubules, the interior of the fiber would depolarize just fine during an action potential, but there would be no way for Cav1.1 channels to know that the potential had changed. To sense a change in voltage there must be a reference voltage for comparison. The reference voltage (ground) is present in the extracellular space. The problem for muscle becomes, how can Cav1.1 channels in the interior of the fiber compare the intracellular voltage to ground? The only way to do this is to bring a narrow channel of extracellular fluid, separated by the cell membrane, into the center of the fiber. This extracellular fluid is connected by narrow channel of saline to the ground potential. I propose this is the true function of t-tubules: not to allow for propagation of action potentials, but to allow for comparison of the intracellular potential to ground.

My hypothesis may seem like a semantic difference that has little to no functional consequence. However, if action potential conduction into t-tubules is not part of the ECC cascade of events, failure of ECC during depolarization cannot be due to failure of AP propagation into t-tubules. This possibility has changed the Rich lab’s approach to studies of depolarization-induced failure of ECC. If AP invasion into t-tubules is not a critical step in ECC, it becomes less urgent to perform studies requiring rapid imaging of voltage sensitive dyes to follow the spread of depolarization into the center of the fiber. The lab will be guided by the results of continued modeling efforts to determine whether AP invasion into t-tubules appears essential for successful ECC.
Accurate modeling of action potentials will help us to explore excitation of muscle as well as the process of excitation contraction coupling in various muscle diseases. One frustration in the field has been that identification of the ion channels involved in various diseases has led to only limited advances in treatment of the diseases. By better understanding of the roles of various ion channels (through selective manipulation of individual currents using modeling) it may be possible to identify new therapeutic targets. Having a spatially correct model that can accurately replicate electrophysiologic behaviors will, with the addition of the channels of interest, enable precise testing of channel functions to target specific muscle fiber behavior.

Questions that can be explored using the model I have generated:

The role of NaPIC in generation of myotonia in the muscle disease myotonia congenita

The identification of loss of function mutations of the ClCn1 gene as the cause of myotonia congenita (Steinmeyer et al. 1991) has not led to any advances in therapy. One reason for this is an incomplete understanding of the mechanisms responsible for pathologic depolarization. In myotonia congenita there are both a steady and a transient depolarization that combine to trigger involuntary firing of muscle action potentials in myotonia (Metzger et al. 2019). (see Figure 12) It is the Rich lab’s current hypothesis...
that a Na\(^+\) persistent inward current (NaPIC) is responsible for the transient depolarization, which depolarizes the fiber to action potential threshold during myotonia.

Figure 12.
Figure 12: **Two contributors to the depolarization that triggers myotonia.** On top is an intracellular recording of action potentials from a normal mouse skeletal muscle fiber. In normal muscle, as soon as voluntary firing of muscle action potentials stops, muscle hyperpolarizes and relaxes. On the bottom are action potentials from a myotonic mouse muscle. Unlike normal muscle, there is continued firing of action potentials following cessation of voluntary firing. The cause of involuntary firing is a combination of a steadily increasing depolarization (green), such that the membrane potential does not return to the resting membrane potential (RMP), indicated by a thin black line, between action potentials, and a transient depolarization (red), which occurs prior to each myotonic action potential.
The Rich lab recently determined that NaPIC is an important contributor to the repetitive firing occurring during myotonia (Hawash, Voss, and Rich 2017). NaPIC is present in normal skeletal muscle and lacks the fast inactivation which is the hallmark of the Na\(^{+}\) channels that are responsible for triggering action potentials (Gage, Lamb, and Wakefield 1989). In muscle, NaPIC is sensitive to low doses of tetrodotoxin, suggesting it is carried by Nav1.4 channels (Hawash, Voss, and Rich 2017). It is likely that muscle NaPIC derives from a small subset of Nav1.4 channels that are in a different conformation from fast-inactivating Nav1.4 channels. This understanding is based on recordings from frog skeletal muscle, in which single Na\(^{+}\) channels shifted modes between a normal, fast-inactivating mode and a mode lacking fast inactivation (J. B. Patlak and Ortiz 1986). For clarity, I will term the Na\(^{+}\) channels responsible for action potentials “fast-inactivating Na\(^{+}\) channels” as they stay open for no more than a few milliseconds before inactivating. This is in contrast to channels in the NaPIC mode, which can stay open for seconds without inactivating. Because the same channel type is responsible for both NaPIC and fast inactivating Na\(^{+}\) channels, there is no way to distinguish the channels pharmacologically or genetically.

The role of channels in the NaPIC mode in regulating excitability has primarily been studied in neurons. It is well established that channels in the NaPIC mode help maintain repetitive firing of neurons in response to sustained depolarization (Bean 2007; Heckman and Enoka 2012). Neurons have many weak synapses that are activated asynchronously. Thus, neurons experience steady depolarization from asynchronous activation of their many inputs, which is converted to repetitive firing. The greater the depolarization of the neuron, the greater the firing rate. This is known as the F-I
relationship – F represents firing frequency and I represents injected current. In lower motor neurons, this relationship is one of the primary means of muscle force regulation (Heckman and Enoka 2012).

The F-I relationship in motor neurons is governed by currents that are active at membrane potentials more negative than action potential threshold (subthreshold currents). These currents consist of both non-voltage gated (leak) currents and voltage-gated currents which activate at potentials negative to action potential threshold (Iglesias et al. 2011; Nardelli et al. 2017). In neurons, subthreshold currents include depolarizing currents carried by channels in the NaPIC mode and Ca$^{2+}$ channels, as well as hyperpolarizing currents carried by K$^+$ channels (Bean 2007; Heckman and Enoka 2012). The ratio of depolarizing to hyperpolarizing current determines whether the neuron will reach threshold and fire. When the ratio of depolarizing to hyperpolarizing subthreshold current is high, there is rapid depolarization toward action potential threshold and a high F-I gain (a high firing rate for a given current injection). When the depolarizing to hyperpolarizing subthreshold current ratio is low, the F-I gain is low and, in the extreme case, neurons can fire a single action potential, but cannot fire repetitively (Nardelli et al. 2017). Manipulation of NaPIC in neurons via dynamic clamp has a dramatic effect on the F-I relationship and ability of neurons to fire repetitively (Nardelli et al. 2017).

If NaPIC has a similar function in triggering repetitive firing in muscle, it could play a central role in triggering myotonia. How would it do this? Part of the answer is that the “persistent” part of NaPIC’s name can be misleading. Channels in the NaPIC mode have a more negative voltage dependence of activation relative to fast-inactivating Na$^+$ channels (Gage, Lamb, and Wakefield 1989). Thus, they open at voltages negative
to action potential threshold and bring the fiber to threshold such that an action potential
is triggered. The channels then close during repolarization following the action potential.
Therefore, during myotonia, NaPIC may not be a persistent current, but may instead be a
transient current that contributes to the transient depolarization that triggers each action
potential.

Simulation of repetitive firing of action potentials in muscle is possible using the
model I have generated. Using another module developed for this model (not used
during fitting) the percentage of Na\(^+\) channels in the NaPIC mode can be easily
manipulated to determine the parameters such as current density and voltage dependence,
which are required to generate myotonia. Of particular interest is the kinetics of NaPIC
required for generation of myotonia as the current must activate during an action
potential and deactivate during repolarization. This suggests that the current must have
rapid kinetics. I am unaware of any studies of the kinetics of NaPIC activation and
deactivation required to generate myotonia.

**Theoretical exploration of the role of subthreshold currents in generation of myotonia**

Subthreshold currents are active at voltages more negative than action potential
threshold. The balance of inward and outward subthreshold currents involves both a) ion
channels that are open at rest and contribute to regulation of resting potential and b) ion
channels not open at rest, which activate in the voltage range between resting potential
and action potential threshold. This second type of ion channel can have profound effects
on repetitive firing without having any effect on resting potential or properties of single
action potentials (Hawash, Voss, and Rich 2017; Dupont et al. 2019). While
subthreshold currents may be small, they can determine whether an action potential fires, and thus play a major role in regulation of muscle excitability.

I propose that disorders of muscle excitability are caused by an elevation of the depolarizing to hyperpolarizing subthreshold current ratio. This framework could explain why mutations in both Na⁺ channels and Cl⁻ channels can trigger myotonia. Either increasing subthreshold depolarizing current (due to increases in NaPIC caused by mutation of Na⁺ channels (S. C. Cannon, Brown, and Corey 1991; El-Bizri et al. 2011)) or decreasing subthreshold hyperpolarizing current (due to loss-of-function mutations of ClC-1 Cl⁻ channels (Steinmeyer et al. 1991)) will increase the depolarizing-to-hyperpolarizing subthreshold current ratio.

Hyperpolarizing subthreshold currents are carried by Cl⁻ and K⁺ channels. Understanding the role of ClC-1 chloride channels in regulation of muscle excitability is complicated by the fact that the Cl⁻ reversal potential can be either more depolarized or more hyperpolarized than the resting membrane potential. Normally, the Cl⁻ reversal potential is slightly more depolarized than the resting potential (Aickin, Betz, and Harris 1989; Judith A. Heiny, Cannon, and DiFranco 2019). This might make one think that ClC-1 channels function to increase excitability. However, during periods of depolarization, such as at the end of action potentials and after K⁺ build-up in t-tubules (see below), ClC-1 chloride channels function to hyperpolarize muscle as the Cl⁻ reversal potential is more negative than the depolarized membrane potential (Stephen C. Cannon 2015; Bækgaard Nielsen et al. 2017). In this situation, ClC-1 channels are the biggest contributor to hyperpolarizing subthreshold currents because they are responsible for 70-
80% of resting membrane conductance (Palade and Barchi 1977; Thomas Holm Pedersen et al. 2016).

Using the model I have generated it will be possible to explore whether there is a set ratio of depolarizing to hyperpolarizing subthreshold currents at which myotonia is generated. In addition, it is possible to manipulate Cl\(^-\) and K\(^+\) conductances separately to determine the unique effects of each of these conductances. The hope is that modeling will allow us to refine and generate hypotheses regarding the role of each ion channel type in generation of myotonia.

**The effect of K- Build-up in t-tubules on steady depolarization of the membrane potential during repetitive firing**

As mentioned above, there are two depolarizations that combine to trigger myotonia. I hypothesize that NaPIC is responsible for the transient depolarization. One likely contributor to the steady depolarization is build-up of K\(^+\) in the t-tubules, which shifts the Nernst potential for K\(^+\) to cause depolarization of the resting membrane potential (Adrian and Bryant 1974; Adrian and Marshall 1976; Wallinga et al. 1999; Fraser, Huang, and Pedersen 2011). Normally, ClC-1-mediated Cl\(^-\) current, which accounts for 70%-80% of resting muscle membrane conductance, offsets the depolarizing influence of K\(^+\) accumulation and thus lessens the steady depolarization (Adrian and Bryant 1974; Palade and Barchi 1977; Steinmeyer et al. 1991; Steinmeyer, Ortland, and Jentsch 1991; Allen, Lamb, and Westerblad 2008). However, in myotonia congenita, Cl\(^-\) conductance is decreased/absent such that K\(^+\) build-up is thought to cause significant depolarization.
It is well known that repeated activation of muscle causes a net efflux of $K^+$ and net influx of $Na^+$, reducing $Na^+$ and $K^+$ gradients across the cell membrane (Sjogaard, Adams, and Saltin 1985; Hodgkin and Horowicz 1959; Torben Clausen 2003). Previous studies have attempted to model $K^+$ build-up. Adrian and Marshall used modeling to study the contribution of a reduced chloride leak conductance versus t-tubule $K^+$ accumulation as potential triggers of myotonia in myotonia congenita (Adrian and Marshall 1976). Cannon and colleagues’ model (S. C. Cannon, Brown, and Corey 1993) focused on the mechanisms triggering myotonia. They did phase plane analysis of a reduced version of the model to study the effects of increased extracellular $K^+$ combined with some fraction of $Na^+$ channels that were non-inactivating (NaPIC). Wallinga et al. (Wallinga et al. 1999) studied the effect of $K^+$ accumulation in the t-tubules on AP shape, and focused on the mechanisms of $K^+$ clearance ($Kir$ and $Na^+-K^+$ pumps) for maintaining excitability. Fortune and Lowery (Fortune and Lowery 2009) studied fatigue and the effects of extracellular $K^+$ on changes in AP shape and conduction velocity.

The model I have generated allows for more accurate modeling of $K^+$ diffusion out of the t-tubules than previously possible as we have more faithfully recreated the spatial arrangement of t-tubules than in previous models (using optical data from the Voss lab). While this may seem like a detail, it is the essence of modeling $K^+$ build-up in t-tubules. Another module being developed for this model that was not used during fitting will allow future studies that combine $K^+$ build-up with NaPIC to see whether we can recreate the combination of steady and transient depolarizations that cause myotonia. The key outcome will be determining whether the contributors identified are sufficient to fully recreate myotonia.
Exploration of the depolarization-induced failure of EC coupling

Finally, the Rich lab has recently been performing studies of depolarization-induced failure of excitation contraction coupling triggered by elevation of extracellular K\(^+\) in mouse muscle. These studies are relevant to understanding hyperkalemic periodic paralysis and other disorders in which depolarization contributes to failure of muscle excitation. Surprisingly, failure of action potential generation with depolarization appears to be graded such that in individual fibers there was no single resting potential that could be identified at which excitation failed. In contrast, failure of the Ca\(^{2+}\) transient with depolarization of individual fibers is sudden, occurring over 1 to 2 mV of resting potential. The cause of the sudden failure of the Ca\(^{2+}\) transient appears to be a combination of the depolarization induced reduction in action potential peak and the voltage dependence of the Ca\(^{2+}\) transient on action potential peak. This work overturns the widely accepted view that action potentials are always all-or-none and provides a quantitative framework for use in studies of depolarization-induced failure of excitation contraction coupling.

Using the model I have generated it will be possible to explore whether the H-H parameters for Na\(^+\) channels derived from my fitting of action potentials lead to graded failure of excitation as found by the Rich lab or whether they lead to all or none failure. If the derived parameters generate graded failure it will suggest we understand the process, but have ignored the implications of the model for depolarization induced failure of excitability. If not, we may have to alter our understanding of Na\(^+\) channel behavior.
Addition of Cav1.1 channels to the t-tubules in my model will allow us to explore whether all or none failure of APs is responsible for the near all or none failure of the Ca^{2+} transient can be recreated. If not, it will suggest there are aspects of excitation contraction coupling that are not yet understood. This would be significant as the field currently views the action potential induced activation of Cav1.1 as well understood. My finding that action potential invasion into t-tubules may not be required for depolarization of t-tubules, together with these modeling studies has the potential to significantly alter our understanding of the process of excitation contraction coupling.

Summary:

I have established a model of skeletal muscle excitation that is more accurate in both its structural and electrophysiologic features than any model previously generated. Combining this model with data acquired from intracellular recordings obtained in the Rich lab will allow for generation and testing of hypotheses relating to muscle diseases such as myotonia congenita, periodic paralysis and ICU acquired weakness.
Chapter VIII

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Appendix A

Abbreviations:

AP- Action Potential
ECC- Excitation Contraction Coupling
F-I- Frequency-Current (I) relationship
HD- Huntington’s Disease
H-H- Hodgkin Huxley
HPP- Hyperkalemic Periodic Paralysis
NaPIC- Na⁺ Persistent Inward Current
RYR- Ryanodine Receptor
R6/2- A mouse model of Huntington’s Disease
SA- Surface Area
SR- Sarcoplasmic Reticulum
SERCA- Sarco/Endoplasmic Reticulum Ca²⁺ ATPase
TTS- T-tubule System