Altered Skeletal Muscle Excitation-Contraction Coupling in the R6/2 Transgenic Mouse Model for Huntington's Disease

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ALTERED SKELETAL MUSCLE EXCITATION-CONTRACTION COUPLING IN THE R6/2 TRANSGENIC MOUSE MODEL FOR HUNTINGTON’S DISEASE

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

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ABSTRACT


Huntington’s disease (HD) has classically been categorized as a neurodegenerative disorder. However, the expression of the disease-causing mutated huntingtin gene in skeletal muscle may contribute to the symptoms of HD, namely those that involve involuntary muscle contraction. In the R6/2 transgenic mouse model of HD, we previously observed ion channel defects that could contribute to involuntary muscle contraction. Here, in R6/2 muscle we investigated the consequence of these ion channel defects on action potentials (APs), the first step in excitation-contraction (EC) coupling. We found that the ion channel defects were associated with depolarizing the baseline membrane potential during AP trains. We also observed changes in the AP waveform in R6/2 muscle, including a prolonged falling phase, which was associated with reduced K\(^+\) channel expression (another ion channel defect). Next, we investigated the consequence of prolonged APs on intracellular Ca\(^{2+}\) release flux, the second step in EC coupling. We observed an increase in Ca\(^{2+}\) release flux duration, which compensated for a reduction in peak Ca\(^{2+}\) release flux, resulting in normal levels of Ca\(^{2+}\) available for contraction in R6/2 muscle. Finally, we investigated the consequence of prolonged APs and normal levels of Ca\(^{2+}\) available for contraction on muscle force generation, the final step in EC coupling. We found that, when accounting for muscle atrophy, the force generated by one AP (twitch) was normal in R6/2 mice. This could be explained by the reduced parvalbumin...
and normal levels of Ca\textsuperscript{2+} available for contraction we observed in R6/2 muscle. We conclude that downregulation of K\textsuperscript{+} channels to prolong APs is a compensatory mechanism for muscle weakness that leads to increased Ca\textsuperscript{2+} release duration and force production in R6/2 muscle. This is the first study to examine the entire EC coupling sequence in HD muscle, revealing the importance of the AP waveform in contributing to muscle force generation.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I: INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II: BACKGROUND</td>
<td>5</td>
</tr>
<tr>
<td>III: SPECIFIC AIM 1</td>
<td>26</td>
</tr>
<tr>
<td>IV: SPECIFIC AIM 2</td>
<td>69</td>
</tr>
<tr>
<td>V: SPECIFIC AIM 3</td>
<td>92</td>
</tr>
<tr>
<td>VI: DISCUSSION</td>
<td>120</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>131</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Statistical methodology for between groups comparison</td>
<td>35</td>
</tr>
<tr>
<td>2. Action potential (AP) parameters and single AP analysis</td>
<td>39</td>
</tr>
<tr>
<td>3. Action potential peak amplitude and max repolarization</td>
<td>45</td>
</tr>
<tr>
<td>4. Action potential max rate-of-rise</td>
<td>48</td>
</tr>
<tr>
<td>5. 40% decay (D40) and 80% decay (D80)</td>
<td>50</td>
</tr>
<tr>
<td>6. Relationship between AP peak amplitude and max rate-of-decay</td>
<td>53</td>
</tr>
<tr>
<td>7. K_v mRNA expression</td>
<td>55</td>
</tr>
<tr>
<td>8. Recovery APs</td>
<td>59</td>
</tr>
<tr>
<td>9. Microscope setup, representative raw traces, and conversion of fluorescence to Ca^{2+} release flux</td>
<td>81</td>
</tr>
<tr>
<td>10. R6/2 action potential-evoked Ca^{2+} release flux</td>
<td>83</td>
</tr>
<tr>
<td>11. TEA action potential-evoked Ca^{2+} release flux</td>
<td>85</td>
</tr>
<tr>
<td>12. TEA action potential-evoked Ca^{2+} release flux for each fiber</td>
<td>87</td>
</tr>
<tr>
<td>13. Twitch force</td>
<td>102</td>
</tr>
<tr>
<td>14. Force-frequency relationship</td>
<td>104</td>
</tr>
<tr>
<td>15. Myosin heavy chain</td>
<td>106</td>
</tr>
<tr>
<td>16. Myosin light chain</td>
<td>107</td>
</tr>
<tr>
<td>17. SERCA and parvalbumin</td>
<td>108</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Within group AP comparison</td>
<td>41</td>
</tr>
<tr>
<td>2. Curve fit values for AP recovery</td>
<td>60</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

HD is the most prevalent of the CAG repeat expansion disorders, affecting about 1 in 10,000 people (Budworth & McMurray, 2013). The incidence of HD varies from region to region with the highest prevalence in North America (Rawlins et al., 2016). In the United States, it is estimated that as many as 40,000 persons have manifest HD, of which 21,100 have been diagnosed (Yohrling et al., 2020). HD is an adult-onset disease that causes involuntary movement, cognitive decline, and psychiatric abnormalities that progress over about 15 to 20 years (Bates, Tabrizi, & Jones, 2014). At the end-stage of the disease, patients are often unable to walk, speak, or swallow due to loss of voluntary muscle control (Nance & Sanders, 1996). Premature death is usually caused by aspiration induced pneumonia, cardiovascular disease, or suicide (Sørensen & Fenger, 1992). HD is caused by a mutation of the huntingtin (HTT) gene, whereby an abnormal number of CAG repeats (> 35) leads to disease symptoms. The precise mechanism by which the CAG repeat expansion of huntingtin leads to the symptoms of HD is not fully understood, making it difficult to establish an effective treatment.

The difficulty in establishing an effective treatment for HD arises from the many functions of the protein product for huntingtin. The wild-type huntingtin protein interacts with over 400 other proteins, including those associated with transcription, vesicle transport, cell division, ciliogenesis, endocytosis, and autophagy (Saudou & Humbert, 2016; Vitet, Brandt, & Saudou, 2020). Additionally, the expanded polyglutamine repeats (coded by the expanded CAG repeats) of the mutated huntingtin protein causes protein
aggregation and the formation of cellular inclusions, which aberrantly bind to other proteins, considered a toxic gain-of-function (S. Li & Li, 2006). There is also a growing body of evidence, including from our lab showing that the CAG repeats of huntingtin RNA aberrantly bind to proteins (Heinz, Nabariya, & Krauss, 2021; D. R. Miranda et al., 2017). As can be imagined, the loss of normal huntingtin function, and the toxic gain-of-function caused by mutated huntingtin RNA and protein product lead to many aberrant cellular processes that lead to disease symptoms (Francelle, Galvan, & Brouillet, 2014; Leavitt, Kordasiewicz, & Schobel, 2020; Saudou & Humbert, 2016; Vitet et al., 2020).

Currently, treatments, such as antisense oligonucleotides that target the degradation of huntingtin and/or mutant huntingtin RNA are in development. The general route of administration for these treatments in clinical trials has been to target the brain (Dash & Mestre, 2020; Leavitt et al., 2020; Marxreiter, Stemick, & Kohl, 2020). The brain is targeted because the striatum of the brain is selectivity vulnerable to the effect of mutant huntingtin and is among one of the first brain regions to degenerate (Blumenstock & Dudanova, 2020; Francelle et al., 2014; Ponzi et al., 2020). Synaptic dysfunction (e.g. cortical-striatal synapse dysfunction) is thought to be the primary cause of striatal degeneration (Blumenstock & Dudanova, 2020; Ponzi et al., 2020; Tyebji & Hannan, 2017; Vitet et al., 2020) and it is likely that cortical-striatal synapse dysfunction leads to the cognitive, psychiatric, and choreic symptoms of HD (Rüb et al., 2016). This is because the striatum and cortex are involved in the action of voluntary movement, cognition, and behavior (Middleton & Strick, 2000; Ponzi et al., 2020; Rüb et al., 2016; Vitet et al., 2020). Therefore, degeneration of the striatum and cortex are likely to lead to
disinhibition of voluntary movement (chorea) and affect (psychiatric disorders), as well cognitive deficits.

Targeting brain pathology in HD seems promising, but it may be necessary to also target the peripheral tissues to alleviate or prevent the full spectrum of symptoms, especially the muscle-related symptoms. Muscle-related symptoms include the symptoms that involve involuntary muscle contraction, such as dystonia (abnormal posturing), muscle rigidity (sustained muscle contraction), and motor impersistence (inability to maintain contraction) (Gordon, Quinn, Reilmann, & Marder, 2000; Roth, 2019; Shannon, 2011), as well as reduced strength (Busse, Hughes, Wiles, & Rosser, 2008), which may contribute to impairments in balance and mobility (Cruickshank, Reyes, Peñailillo, Thompson, & Ziman, 2014). The contribution of muscle intrinsic defects to the muscle-related symptoms HD is unclear, as most HD research has focused on neurodegeneration. Thus, it is important to characterize HD muscle defects to better define targets for treatment(s).

The purpose of the following studies was to investigate muscle function in HD skeletal muscle to determine the physiological defects leading to aberrant contraction. The primary function of muscle is to contract, and this occurs by a process called excitation-contraction coupling. Action potentials (APs) are the first step in excitation-contraction coupling (CHAPTER III). APs lead to the second step of EC coupling, intracellular Ca²⁺ release (CHAPTER IV). When intracellular Ca²⁺ is released into the myoplasm (cytosol of muscle cells), it activates the contractile filaments of the muscle fiber (cell), leading to the final step of excitation-contraction coupling: muscle
contraction (CHAPTER V). Skeletal muscle contraction generates force. Thus, Ca$^{2+}$ “couples” APs (excitation) with muscle force generation (contraction).

Here, in the natural order of EC coupling events, it is shown that, in the muscle of the R6/2 transgenic model for HD, changes in the AP waveform (CHAPTER III) lead to changes in intracellular Ca$^{2+}$ release that could compensate for muscle weakness (CHAPTER IV), resulting in normal force generation after accounting for muscle atrophy (CHAPTER V). Since muscle function defects in HD have not yet been fully characterized, we took an integrative approach, investigating the overall function of R6/2 muscle. Our results point to a compensation mechanism to counter muscle atrophy in HD muscle, leading the way for future studies to determine the role of mutant huntingtin expression in muscle, as well as to elucidate the molecular events leading to muscle atrophy, which will progress the development of muscle-specific targets for the treatment of HD.

In the next chapter (CHAPTER II), background information describing the resting membrane potential, the AP waveform, EC coupling, skeletal muscle function, and K$^+$ build-up will be presented. This will be followed by a brief review of skeletal muscle defects in HD and how they relate the studies in CHAPTERS III-IV. Finally, the Specific Aims of these studies in will be described.
II: BACKGROUND

RESTING MEMBRANE POTENTIAL

This electrical potential difference (voltage) across a cell membrane is termed membrane potential (Raghavan, Fee, & Barkhaus, 2019). Excitation refers to a change in the polarity of the membrane potential, which requires the cell to first be polarized at rest. Therefore, understanding the basis of the resting membrane potential of excitable cells helps to understand excitation, the first step in excitation-contraction (EC) coupling.

The resting membrane potential is determined by the distribution of ions across the cell membrane, the most abundant of which are sodium (Na\(^+\)), potassium (K\(^+\)), chloride (Cl\(^-\)), and calcium (Ca\(^{2+}\)). The distribution of these ions is largely established by Na\(^+\)/K\(^+\) ATPase, which transports 3 Na\(^+\) ions out the cell and 2 K\(^+\) ions into the cell, creating an uneven distribution of positive charge across the membrane (Brodie & Sampson, 1985). It is due to the net removal of positive charge from the cell that creates a negative resting membrane potential inside the cell. The resting membrane potential varies between cell types; the average resting potential for a neuron is about −70 mV and the average resting potential for muscle fibers is about −90 mV (Cunningham, Carter, Rector, & Seldin, 1971; Hopkins, 2006).

The membrane is highly resistant to the flow of charge and acts as an insulator, so ions can only cross, in large amounts through species-specific channels, called ion channels. In resting muscle, Cl\(^-\) is the most permeable ion because
Cl\textsuperscript{−} channels (CIC-1) are open at rest. Likewise, K\textsuperscript{+} is permeable because inwardly rectifying K\textsuperscript{+} (Kir) channels, specifically Kir2.1 and Kir2.2 are open at rest in muscle (DiFranco, Yu, Quiñonez, & Vergara, 2015; Kubo, Baldwin, Jan, & Jan, 1993).

The resting membrane potential, and the membrane potential in general are determined by the most permeable ion because that ion is able to move closer its equilibrium potential, which is the potential at which that ion is electrically and chemically balanced across the membrane. The equilibrium potential can be determined by the Nernst equation:

\[ V_m = \frac{R T}{z F} \ln \left( \frac{[X]_o}{[X]_i} \right) \]

where \( V_m \) is the membrane voltage, \( R \) is the gas constant, \( T \) is the absolute temperature, \( z \) is the charge of the ion, \( F \) is Faraday’s constant, \([X]_o\) is the concentration of the ion outside of the cell, and \([X]_i\) is the concentration of ion inside of the cell. For example, the equilibrium potential for Na\textsuperscript{+} under normal conditions would be as follows:

\[ E_{Na} = V_m = \frac{R T}{F} \ln \frac{145}{12} \]

In this case, the value of \( z \) is positive, \( R, T, \) and \( F \) are constants and there is a 145 mM concentration of Na\textsuperscript{+} outside of the cell and a 12 mM concentration of Na\textsuperscript{+} inside of the cell. Assuming a temperature of 37°C, the equilibrium potential for Na\textsuperscript{+} is 67 mV.

The values used for the concentration of Na\textsuperscript{+} inside and outside of the cell in the above equation are physiological values. The difference in concentration creates a large
electrochemical gradient across the membrane for Na\(^+\). There is also a large
electrochemical gradient for K\(^+\). The equilibrium potential for K\(^+\) is about -98 mV, almost
the polar opposite of the equilibrium potential for Na\(^+\). The equilibrium potential for Na\(^+\)
and K\(^+\) are important in shaping action potentials (APs), which will be described in the
next section.

It should be mentioned that all permeable ions contribute the resting potential of
cells. To determine the contribution of all the major ions to the resting membrane
potential, the Goldman-Hodgkin-Katz (GHK) equation may be used:

\[
E_m = \frac{RT}{F} \ln \left( \frac{P_{Na}[Na^+][o]+P_{K}[K^+][o]+P_{Cl}[Cl^-][i]}{P_{Na}[Na^+][i]+P_{K}[K^+][o]+P_{Cl}[Cl^-][o]} \right)
\]

The GHK equation presents an accurate estimation of the actual membrane potential of
excitable cells. Not only does the GHK equation consider the concentrations of all the
major ions, it also takes into account the relative permeability of each ion.

Overall, the resting membrane potential of excitable cells is determined by the
distribution of ions across the membrane, which creates the electrochemical gradient
necessary for AP propagation. In skeletal muscle, CIC-1 and Kir channels, along with
Na\(^+\)/K\(^+\) ATPase maintain the resting membrane potential at ~-90 mV. Excitation occurs
when the polarity of the cell changes to more positive values, which is called
depolarization (Cowan, 1937). Depolarization changes the relative ionic permeabilities of
the membrane by activating voltage-gated ion channels. As will be explained in the next
section, opening of these voltage-gated ion channels leads to changes in the membrane
potential called APs.
ACTION POTENTIALS

The excitation step of excitation-contraction (EC) coupling involves the movement of ions across the cell membrane, which produces changes in membrane potential measured as APs. The rising phase and peak of APs are shaped by Na\(^+\) ions, and the falling phase is shaped by K\(^+\) ions. Much of the pioneering work and ideas describing the nature of APs were published in the mid-20\(^{th}\) century by Hodgkin, Huxley, and colleagues, who first experimented with squid giant axons (Hodgkin & Huxley, 1939, 1945, 1952; Nastuk & Hodgkin, 1950). Now, it is known that APs occur in all excitable cells, including neurons, muscle fibers, and cardiac cells. In general, an AP can be described as an electrical signal that transmits information rapidly, such as from the brain to muscle.

The initial trigger for skeletal muscle APs begins in the neuron. For example, if we choose to wiggle our toes, APs will fire in the brain and propagate to the motor neurons of the spinal cord. Motor neurons form junctions with muscle fibers called neuromuscular junctions (NMJs). When excited by an AP, the motor neuron will release the chemical transmitter, acetylcholine (ACh). ACh binds to ACh receptors (AChRs) on the muscle fiber. AChRs are ion channels, and when bound to ACh, cause an influx (movement of ions from outside to inside the cell) of Na\(^+\) ions, which depolarizes the surface of the muscle fiber membrane (sarcolemma).

If the depolarization of the sarcolemma is large enough, voltage-gated Na\(^+\) (Nav1.4) channels will open (Bezanilla, 2007; Goldin et al., 2000), making the fiber more permeable to Na\(^+\) ions, which brings the membrane potential closer to the equilibrium potential for Na\(^+\). As stated in the previous section (RESTING MEMBRANE
POTENTIAL), the equilibrium potential of Na$^+$ is about 67 mV. Due to the electrochemical gradient of Na$^+$ across the sarcolemma, there is a large difference between the resting membrane potential (-90 mV) and the Na$^+$ equilibrium potential (+67 mV). Therefore, when $\text{Na}_V 1.4$ channels open, there is a strong electromotive force, called driving force that causes Na$^+$ ions to move into the cell. It is this influx of Na$^+$ ions moving toward their equilibrium potential when $\text{Na}_V 1.4$ channels are open that shape the rising of phase of an AP.

In addition to $\text{Na}_V 1.4$ channels, sufficient depolarization of the sarcolemma opens voltage-gated K$^+$ ($\text{K}_V$) channels, which are called delayed rectifiers because they open slower than $\text{Na}_V 1.4$ channels and rectify the membrane potential (Bezanilla, 2007; DiFranco, Quinonez, & Vergara, 2012). Rectification and repolarization have similar meaning, in that they both describe bringing the membrane potential back to rest after being depolarized. Opening of $\text{K}_V$ channels causes the efflux (movement of ions from inside to outside the cell) of K$^+$ ions, due to the driving force created by the electrochemical gradient for K$^+$. Since the K$^+$ equilibrium potential (-98 mV) is more negative than the resting membrane potential (-90 mV), K$^+$ efflux repolarizes the membrane potential and shapes the falling phase of the AP.

To summarize, the AP waveform is shaped by Na$^+$ and K$^+$ ions rapidly moving across the cell membrane (in opposite directions) through open $\text{Na}_V 1.4$ and $\text{K}_V$ channels, respectively. This rapid movement of charged particle (ions) creates an electrical current. Hence, $\text{Na}_V 1.4$ and $\text{K}_V$ channel currents shape the AP waveform. APs are the first step in EC coupling and, as will be described further in the next section, indirectly control intracellular Ca$^{2+}$ release.
EXCITATION-CONTRACTION COUPLING

Excitation-contraction (EC) coupling is the process by which APs lead to intracellular Ca\(^{2+}\) release and muscle contraction. The key components of EC coupling are the voltage-gated Ca\(^{2+}\) channel (Cav1.1) and the ryanodine receptor (RyR1), which are both Ca\(^{2+}\) ion channels. However, Cav1.1 currents are not required for muscle contraction and Cav1.1 acts more as a voltage-sensor (Dayal et al., 2017; Tuluc et al., 2009) (Agrawal, Suryakumar, & Rathor, 2018; Hernández-Ochoa & Schneider, 2018).

Cav1.1 is embedded within the cell membrane of muscle fibers, specifically the transverse tubule membrane. RyR1 is embedded within the sarcoendoplasmic reticulum (SR), which stores Ca\(^{2+}\) within muscle fibers. RyR1 is the intracellular Ca\(^{2+}\) release channel of skeletal muscle. In neurons and cardiac cells, RyRs open in response to binding Ca\(^{2+}\), which comes into the cell from the extracellular space through Cav channels. Since Ca\(^{2+}\) influx is not required for skeletal muscle contraction, what separates skeletal muscle from other excitable cells is that Cav1.1 and RyR1 physically interact to cause intracellular Ca\(^{2+}\) release. The cytoplasmic linker region between repeats II and III of Cav1.1, and the auxiliary protein, STAC3 are both required for this interaction (Agrawal et al., 2018; Hernández-Ochoa & Schneider, 2018; Tanabe, Beam, Adams, Niidome, & Numa, 1990). As will be described next, the interaction between Cav1.1 and RyR1 is what couples excitation to contraction in muscle.

It is the unique architecture and highly organized structure of skeletal muscle fibers that places Cav1.1 in close proximity to RyR1. The SR and transverse tubule membrane form what are called triad junctions, named so due to their appearance in cross-sectional tissue slices, whereby a tubule is surrounded by SR membrane on either
side (Romer et al., 2021). At the triad junctions, the transverse tubule and SR membranes are only nanometers apart, allowing Cav1.1 in the transverse tubules to physically interact with RyR1 in the SR. Essentially, the physical interaction between Cav1.1 and RyR1 unifies the opening of both channels, so when Cav1.1 begins to open, so does RyR1. This is how APs control intracellular Ca\(^{2+}\) release.

The depolarization of the transverse tubule membrane caused by APs pulls on the positively charged domains of Cav1.1 causing conformational changes in the channel (Allard, 2018; Hernández-Ochoa & Schneider, 2018). The currents created by the movement of charge within Cav1.1 when it changes conformation can be measured. These currents, termed charge movement precede but coincide with the intracellular release of Ca\(^{2+}\) and muscle contraction, demonstrating the direct link between Cav1.1 and intracellular Ca\(^{2+}\) release (Endo, 1977; Imagawa, Smith, Coronado, & Campbell, 1987; Martonosi, 1984; Tanabe et al., 1990) (Geeves, Fedorov, & Manstein, 2005; Godt 1974). Once Ca\(^{2+}\) is released into the myoplasm from the SR through RyR1, it is free to activate muscle contraction. Thus, intracellular Ca\(^{2+}\) provides the link between excitation and contraction.

The EC coupling machinery of muscle is highly regulated and is modulated by the metabolic state of the cell. The is important for HD because metabolic dysfunction is a hallmark of HD cellular pathology. Modifications of Cav1.1 and/or RyR1 can lead to muscle dysfunction (Agrawal et al., 2018). Of the primary components of EC coupling, RyR1 is of the most highly regulated and susceptible to changes in the cellular environment. A major regulator of RyR1 is FK506-binding protein (FKBP12), which increases the open probability of RyR1 by dissociating from the channel (Brillantes et al.,
1994). Phosphorylation of RyR1 by cAMP-dependent protein kinase (PKA), a key mediator of energy metabolism can cause FKBP12 to dissociate from RyR1 (Reiken et al., 2003). In addition, RyR1 has phosphorylation sites for calmodulin kinase II (CaMKII), which also increases the channels open probability (Gehlert, Bloch, & Suhr, 2015).

In addition to phosphorylation, RyR1 can be modified by nitrosylation, carbonylation, and glutathionylation, which can occur in HD mouse model brain, heart, and skeletal muscle (Dridi et al., 2020). Under various stress conditions like these, RyR1 can become unstable and let Ca$^{2+}$ out the SR unregulated. This is called Ca$^{2+}$ leak, which leads to reduced Ca$^{2+}$ in the SR and lower Ca$^{2+}$ release, which may be what occurs in HD mouse model muscle (Braubach et al., 2014; Dridi et al., 2020). Oxidative stress, which coincides with metabolic dysfunction, and is another hallmark of HD cellular pathology also leads to RyR1 leak (Andersson et al., 2011; Stamler & Meissner, 2001). Thus, under cellular stress conditions, RyR1 is sensitive to dysregulation and Ca$^{2+}$ leak.

Dysregulation of RyR1 can also lead to disease and cause defects in muscle function. Calmodulin (CaM), which is activated by intracellular Ca$^{2+}$ release plays a protective role by interacting with RyR1 and blocking RyR1 nitrosylation and oxidation sites. However, cellular stress conditions can disrupt the interaction between CaM and RyR1, leading to alterations in muscle function (Moore et al., 1999). In addition to the RyR1 nitrosylation observed in HD, nitrosylation of RyR1 has been observed in sarcopenia (age-related muscle atrophy), and malignant hyperthermia, a disease caused by a mutation in RyR1 or Cav1.1 (Agrawal et al., 2018; Dridi et al., 2020). Additionally, mutations in Cav1.1 have been associated with muscle dysfunction and congenital...
myopathy (Agrawal et al., 2018). Overall, there are many potential points of dysfunction in the EC coupling machinery, many of which are related to metabolic defects and oxidative stress, both hallmarks of HD cellular pathology.

In summary, EC coupling is mediated by the interaction between Cav1.1 and RyR1. Because of the physical coupling between Cav1.1 and RyR1, activation of Cav1.1 by APs almost simultaneously activates RyR1, which causes intracellular Ca\(^{2+}\) release. Cav1.1 and RyR1 are highly regulated, particularly RyR1 under conditions of metabolic dysfunction and oxidative stress, which are characteristic of HD pathology. Dysfunction of Ca\(^{2+}\) release in HD muscle is assessed in CHAPTER IV. Based on the results of CHAPTER IV, we propose that prolonged APs activate more Cav1.1/RyR1 channels and/or keep the RyR1 channels open longer, thereby increasing the duration of Ca\(^{2+}\) release. It is shown that the increase in Ca\(^{2+}\) release duration compensates for RyR1 dysfunction (decreased peak Ca\(^{2+}\) release flux) in R6/2 muscle, which seems to increase the strength of contraction (CHAPTER V). The next section will describe skeletal muscle function, which is to contract, the last step of EC coupling.

SKELETAL MUSCLE FUNCTION

Contraction is the primary function of skeletal muscle and is the last step of EC coupling. Skeletal muscle fibers are composed of repeated contractile units called sarcomeres. Under an electron microscope, each sarcomere appears as several distinct bands of varying density due to the organization of the contractile filaments within each fiber. These contractile filaments are proteins called myofibrils and their organization makes muscle fibers appear striated. The two types of contractile filaments are the thick
(myosin) filaments and the thin (actin) filaments. According to sliding filament theory, during muscle fiber contraction, each sarcomere shortens due to the actin filaments sliding past the myosin filaments towards the center of the sarcomere (Sweeney & Hammers, 2018).

The interaction between the myosin and actin filaments occur only in the presence of Ca\(^{2+}\) and ATP. In the absence of Ca\(^{2+}\), tropomyosin and troponin C block the actin binding site for myosin. When Ca\(^{2+}\) is present, Ca\(^{2+}\) binds to troponin C and causes a conformational change in tropomyosin that unblocks the actin binding site for myosin and allows the proteins to interact. The catalytic “myosin head” of myosin binds to the actin binding site and acts as a molecular motor that slides actin filaments towards the center of the sarcomere. Myosin and its various isoforms determine the sarcomere shortening velocity through their rate of ATPase activity. When ATP binds to a myosin head, it is hydrolyzed to inorganic phosphate (P\(_i\)) and ADP. The hydrolysis of ATP causes a power-stroke, whereby the myosin head binds to actin and, upon the release of P\(_i\) and ADP, moves, like a lever, causing the actin filament to slide toward the center of the sarcomere. When another ATP molecule binds to the myosin head, the myosin head detaches from the actin binding site. The hydrolysis of that ATP molecule causes the myosin head to bind to a new actin binding site and repeat the power-stroke process on an adjacent portion of the actin filament. The Ca\(^{2+}\) and ATP dependent process of myosin binding to actin to cause sarcomere shortening is called cross-bridge cycling (Sweeney & Hammers, 2018). Cross-bridge cycling is the basis for sarcomere shortening and muscle function, which is to produce tension and generate force.
As stated above, myosin has multiple isoforms, which determine the strength of muscle contraction. The thick filament is composed of a pair of myosin heavy chains (MyHC), a pair of essential myosin light chains (MyLC1/3), and a pair of regulatory myosin light chains (MyLC2) (Ojima, 2019; S. Schiaffino & Reggiani, 1996). MyHC determines contraction velocity, with a faster velocity generating more force, and also determines fiber type. From slowest to fastest contraction velocity, the adult isoforms of MyHC are MyHC1 (coded by Myh7), MyHC2a (coded by Myh2), MyHC2x (coded by Myh1), and MyHC2b (coded by Myh4). During development, and in R6/2 muscle (D. R. Miranda et al., 2017)(this study), embryonic MyHC (coded by Myh3) and neonatal MyHC (coded by Myh3 and Myh8) are also expressed in skeletal muscle (Condon, Silberstein, Blau, & Thompson, 1990; Sotelo, 2021). Essential MyLCs are required for force production and include MyLC1f, MyLC1s, and MyLC3s (splice variants of Myl1) (Strehler, Periasamy, Strehler-Page, & Nadal-Ginard, 1985; Weeds & Lowey, 1971). The “f” stands for fast-type fibers (MyHCII_), and the “s” stands for slow-type fibers (MyHC1) to demarcate the MyHC isoform that the MyLC isoform is associated with. Regulatory MyLCs can be phosphorylated by myosin light chain kinase to increase the velocity of MyHC and potentiate contraction strength (Stull, Kamm, & Vandenboom, 2011). The two isoforms of regulatory MyLC are MyLC2f (coded by Myph) and MyLC2s (coded by Myl2) (Gannon, Doran, Kirwan, & Ohlendieck, 2009; Stefano Schiaffino & Reggiani, 2011). Changes in MyLC are more sensitive to fiber-type transition (Leeuw & Pette, 1996), which occurs in HD muscle (Hering, Braubach, Landwehrmeyer, Lindenberg, & Melzer, 2016; Mielcarek et al., 2015; Strand et al., 2005). Overall, the isoforms of myosin that are expressed in a muscle determine the
strength of individual cross-bridges, which form the basis for the muscle’s maximum strength capacity. Maximum strength is achieved when all the cross-bridges in the fiber are activated by Ca²⁺.

The concentration of Ca²⁺ released from the RyR1 determines the number of cross-bridges that are activated (Stein, Bobet, Oğuztöreli, & Fryer, 1988), thus determining the strength of contraction in a Ca²⁺-dependent manner. The majority of Ca²⁺ released from RyR1 binds troponin C to activate contraction, but it can also bind to calmodulin (CaM), which then activates effectors like, calmodulin kinase II (CaMKII), calmodulin kinase IV (CaMIV), and calcineurin (Gehlert et al., 2015). CaM can activate myosin light chain kinase, which phosphorylates regulatory MyLC and potentiates cross-bridge cycling, thereby making contraction velocity more sensitive to Ca²⁺ (Levine, Kensler, Yang, Stull, & Sweeney, 1996; Tubman, MacIntosh, & Maki, 1996). Calcineurin activates a signaling cascade that leads to the expression of genes associated with changes in muscle fiber-type (Tavi & Westerblad, 2011). In addition to the many downstream effects of Ca²⁺, Ca²⁺ influx into mitochondria has been shown to increase electron transport chain activity and ATP production (Glancy, Willis, Chess, & Balaban, 2013; Korzeniewski, 2007; McMillin & Madden, 1989), which is thought to sustain the ATP-dependent processes of muscle contraction, including cross-bridge cycling. There are also Ca²⁺ binding proteins in the myoplasm that can affect the amount of Ca²⁺ available to bind troponin C, including parvalbumin. Parvalbumin is primarily expressed in fast-type fibers and reductions in parvalbumin can indicate fiber-type switching (Berchtold, Brinkmeier, & Müntener, 2000; Green et al., 1984). To end muscle
contraction by removing Ca\textsuperscript{2+} from the myoplasm, the sarcoendoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) transports Ca\textsuperscript{2+} back into the SR.

SERCA is located within the SR membrane and uses ATP to pump Ca\textsuperscript{2+} back into the SR. There are four known isoforms of SERCA: SERCA1 and SERCA 1b (splice variants of \textit{ATP2A1}), and SERCA2 and SERCA2b (splice variants of \textit{ATP2Ab}) (Talmadge & Paalani, 2007; Wuytack et al., 1992). SERCA1b is expressed only during muscle development and SERCA2b is expressed only at very low levels in skeletal muscle. SERCA1 is associated with fast-type fibers and SERCA2 is associated with slow-type fibers, and changes in SERCA isoforms can be an early indicator of fiber-type transition, changes in MyLC and parvalbumin (Green et al., 1984; Talmadge & Paalani, 2007; Wuytack et al., 1992). SERCA1/2 contributes to the relaxation time of muscle force generation by determining the rate at which Ca\textsuperscript{2+} is removed from the myoplasm (Sotelo, 2021). Once Ca\textsuperscript{2+} is returned to the SR by SERCA1/2, the muscle no longer contracts, and the EC-coupling event is over.

An important structural feature of muscle fibers that effects EC coupling, and muscle function is the transverse tubules. Muscle fibers are relatively large, ranging from 50 to 100 \(\mu\)m in diameter (A. Dulhunty, 2006). By diffusion, Ca\textsuperscript{2+} would take seconds to travel from the fibers plasma membrane (sarcolemma) to the tightly packed contractile filaments within the interior. To compensate for the size of the cell, it is necessary for the sarcolemma to intrude into the interior of the cell for AP propagation. These intrusions or invaginations of the sarcolemma form pores at the surface of the cell which then take the form of tubules. The tubules form a system that transverse the length of the muscle fiber called the transverse tubule system (t-tubule system). The t-tubules form junctions with
the terminal cisternae of SR. Thus, APs can propagate into the interior of the muscle fiber in close proximity to the SR and myofibrils allowing for muscle contraction to occur within milliseconds.

**T-TUBULE K\(^+\) BUILDUP**

Transverse tubule K\(^+\) buildup is one of the most accepted theories for explaining the progressive decrease in AP amplitude during AP trains. This phenomenon is important for understanding possible mechanisms for muscle weakness in HD. The theory states that not all the K\(^+\) leaving the cell during AP trains directly enters the extracellular space where it would diffuse and/or be transported back into the cell, making its contribution to the concentration of extracellular K\(^+\) negligible. Instead, K\(^+\) enters the t-tubules where it accumulates (Beam & Donaldson, 1983; DiFranco et al., 2012). This K\(^+\) build-up changes the relative concentrations of K\(^+\) across the t-tubule membrane and decreases the driving force for K\(^+\). The lack of membrane repolarization due to the decrease in K\(^+\) driving force causes a gradual depolarization of the membrane potential during AP trains.

As the membrane potential depolarizes, the amplitude of APs begins to decrease due to the gating properties of Nav1.4 channels. The membrane potential must be adequately repolarized between APs to relieve Nav1.4 channels from inactivation (Ruff, 1996). The Nav1.4 channel has three different states: the closed state, the open state, and the inactive state (Bagal, Marron, Owen, Storer, & Swain, 2015). At rest, Nav1.4 channels are in the closed state and will open when the membrane potential reaches a threshold of about -55 mV. When the membrane potential reaches threshold, Nav1.4
channels open (open state) and trigger an AP. After a few milliseconds, Nav1.4 channels inactivate (inactive state) and cannot be opened. Only when the membrane potential is sufficiently repolarized will Nav1.4 channels return to their closed state, enabling them to open again.

When the membrane depolarizes during AP trains (in theory due to K⁺ buildup) many Nav1.4 channels are inactivated. Since inactive Nav1.4 channels cannot be opened, there is a decrease in Na⁺ influx, which causes a decrease in the peak amplitude of APs. To support this, in a study where the membrane potential was depolarized by extracellular K⁺, which mimics t-tubule K⁺ buildup, the peak amplitude of APs was reduced (Rich & Pinter, 2003). Interestingly, Kir channels may play an important role in counterbalancing t-tubule K⁺ build up (DiFranco et al., 2015; Hibino et al., 2010; Kubo et al., 1993) by allowing the influx of K⁺ during AP trains. As will be seen in Specific Aim 1 (CHAPTER III), the lack of ClC-1 and Kir currents in R6/2 muscle are associated with increased membrane depolarization during AP trains, possibly resulting in muscle weakness (CHAPTER V).

HUNTINGTON’S DISEASE SKELETAL MUSCLE DEFECTS

It is still unclear how mutated huntingtin expression leads to the many cellular defects in HD skeletal muscle. The extension of polyglutamine repeats in the mutated huntingtin protein (mHTT) leads to the activation of several cellular stress responses, but these responses seem to be altered. The disease-causing mutated huntingtin protein (mHTT) is expressed in the skeletal muscle of HD patients and HD mouse models, where it tends to misfold, aggregate, and form inclusions (Hoogeveen et al., 1993; S. Li,
Schilling, Young, Li, & Ross, 1993) (Turner, Cooper, & Schapira, 2007). (Bondulich et al., 2017; Cheng et al., 2013; Mielcarek et al., 2015; Moffitt, McPhail, Woodman, Hobbs, & Bates, 2009; Orth, Cooper, Bates, & Schapira, 2003; Sathasivam et al., 1999; B. E. Wade et al., 2014; Weiss et al., 2009). In addition, in R6/2 muscle fibers, we observed nuclear aggregates of huntingtin RNA (D. R. Miranda et al., 2017). In HD models, including the R6/2 model, mHTT is associated with enhancement of cellular stress pathways, including the activation of heat shock proteins, which would normally prevent protein aggregation (Labbadia et al., 2011; Neueder et al., 2017). However, the heat shock response is altered in R6/2 muscle and unable to prevent mHTT misfolding, aggregation, and inclusion formation, which seems to contribute to muscle atrophy (Fujimoto et al., 2005). The autophagy response, which normally clears misfolded proteins is also elevated in R6/2 muscle (She et al., 2011), but this pathway is also altered. The altered autophagy pathway is possibly due to a direct interaction between mHTT and autophagy-related proteins, which is associated with dysregulation of mammalian target of rapamycin (mTOR), an activator of autophagy (Fox et al., 2010; Rui et al., 2015). Dysregulated autophagy has been linked to oxidative stress and mitochondrial defects, as oxidative stress and autophagy are both reduced in response to the mitochondria-specific antioxidant, MitoQ in R6/2 muscle (Pinho et al., 2020), and autophagy-related mitochondrial defects have been observed in HD myoblast (Squitieri et al., 2010). Overall, the misfolding of mHTT is associated with an increased, but dysregulated heat shock and autophagic response. The dysregulated autophagic response has been linked to oxidative stress and mitochondrial dysfunction, which are also characteristic of HD cellular pathology.
Deficits in energy metabolism and mitochondrial dysfunction (which leads to oxidative stress) are prominent features of HD skeletal muscle. It has been demonstrated that HD patients and R6/2 mice are in state of negative energy balance despite normal movement, indicating metabolic dysfunction (Goodman et al., 2008). Studies of HD patients and animal models have shown deficits in muscle energy metabolism, such as increased ATP consumption (Koroshetz, Jenkins, Rosen, & Beal, 1997; Lodi et al., 2000; Tsang et al., 2006), decreased mitochondrial respiration (Arenas et al., 1998; Gehrig et al., 2017; Kojer et al., 2019; Kosinski et al., 2007; Rodinova et al., 2019), altered Krebs cycle activity (Kojer et al., 2019; Rodinova et al., 2019; Tabrizi et al., 2000), and abnormalities in oxygen consumption (Andrea Ciammola et al., 2011). Some of these deficits, which include changes in gene expression can be reduced by exercise (Frese et al., 2017; Mueller et al., 2017). (Mueller et al., 2019; Paré & Jasmin, 2017), upregulating PGC-1α (key protein involved in mitochondrial regulation) (Chaturvedi et al., 2009; Johri et al., 2011), reducing glucocorticoids (stress hormone) levels (Dufour & McBride, 2019), and promoting appetite (Sjögren et al., 2019), (Sjögren et al., 2017). Overall, nutrient and energy metabolism, including deficits in mitochondrial utilization of oxygen and metabolites is highly dysregulated in HD muscle. How mutant huntingtin expression leads to metabolic dysfunction in HD muscle is still unclear.

Many of cellular defects in HD muscle described above seem to lead to muscle atrophy, and this is likely due to apoptosis. Markers for apoptosis are elevated in HD patient derived muscle cultures and HD mouse models (A. Ciammola et al., 2006; Ehrnhoefer et al., 2013; McConoughey et al., 2010; She et al., 2011).
Signs of apoptosis include a dissipated mitochondrial membrane potential which can be induced by high Ca$^{2+}$ levels (A. Ciammola et al., 2006; Gizatullina et al., 2006; J. Sassone et al., 2010). Ca$^{2+}$ homeostasis is dysregulated in HD mouse model muscle (Braubach et al., 2014; Dridi et al., 2020), so it is possible that mitochondrial dysfunction and high Ca$^{2+}$ lead to apoptosis and muscle atrophy in HD muscle.

Muscle atrophy in HD is not due to denervation, however there are defects in HD motor neurons. We have confirmed, using morphology, molecular biology, and electrophysiology that R6/2 muscle is not denervated (Khedraki et al., 2017), which was alluded to previously (Mielcarek et al., 2015; Ribchester et al., 2004). However, abnormalities and alterations in neuromuscular transmission have been observed in a fly and several mouse models of HD (de Aragão et al., 2016; Khedraki et al., 2017; Romero et al., 2008; Rozas, Gómez-Sánchez, Tomás-Zapico, Lucas, & Fernández-Chacón, 2011; Priscila A. C. Valadão et al., 2018). Neuromuscular transmission refers the process at the NMJ, whereby APs from the motor neuron are converted to a chemical signal (ACh) and then back to an electrical (APs) in the corresponding muscle fiber. In addition to abnormal neuromuscular transmission, motor neuron atrophy has been observed in the R6/2 and other mouse models for HD (Priscila Aparecida Costa Valadão et al., 2019; Priscila Aparecida Costa Valadão et al., 2017; A. Wade, Jacobs, & Morton, 2008). Also, R6/2 motor units have been shown to be reduced (Bondulich et al., 2017; Mielcarek et al., 2015). Thus, it is possible that motor neuron defects and abnormal neuromuscular transmission contribute to muscle atrophy in HD.

The contribution of muscle atrophy to muscle weakness in HD is not fully understood. Reduced muscle force has been reported in HD mouse models, and has been
attributed to atrophy (Mielcarek et al., 2015), reduced MyLC expression (Hering et al., 2016), and RyR1 Ca\(^{2+}\) leak (Dridi et al., 2020). In the following studies, we provide evidence to suggest that alterations in the AP waveform and reduced parvalbumin help to compensate for the muscle weakness caused by atrophy in R6/2 muscle, however reduced ClC-1 and Kir currents and saturation of parvalbumin are unable to compensate during stronger (high-frequency) contractions.

SPECIFIC AIMS

We previously observed a reduction in ClC-1 and Kir currents, as well as prolonged APs in R6/2 muscle (Waters, Varuzhanyan, Talmadge, & Voss, 2013). The increased duration of R6/2 APs suggests that Kv currents are reduced in R6/2 muscle. To sustain muscle contraction and generate force, the baseline membrane potential during AP trains must be sustained at levels that allow for the regeneration of Na\(_V\)1.4 channel currents. Since ClC-1 and Kir currents maintain the baseline membrane potential, and Kv currents repolarize the membrane potential, we hypothesized that, during AP trains the baseline membrane potential would depolarize to the point of inactivating Na\(_V\)1.4 channels. Consequently, the reduction in active Na\(_V\)1.4 channels and decrease in Na\(^+\) currents would diminish the peak amplitude of APs. The reduction in AP amplitude would then lead to decreased intracellular Ca\(^{2+}\) release and force generation, explaining muscle weakness in HD. We tested this hypothesis with three Specific Aims.

Specific Aim 1 (CHAPTER III) was to establish if reduced ClC-1 and Kir currents in R6/2 muscle depolarize the baseline membrane potential during AP trains, and if this decreases the amplitude of APs. While the long-term goal of our lab is to
understand EC coupling during long AP trains (like in Aim 1), we analyzed intracellular Ca\(^{2+}\) release in response to only single APs for the following reasons: 1) We need to establish methods for analyzing the fluorescence signal during AP trains. The peak of the fluorescence signal diminishes after the beginning of high-frequency AP trains, making it difficult to detect the fluorescence signal towards the end of the trains using our analysis software (pClamp). Also, during trains, the fluorescence signal doesn’t return to baseline after each AP. Thus, calculating the Ca\(^{2+}\) release flux from the fluorescence signal during trains is not as straightforward as analyzing the fluorescence signal from one AP, which has clear boundaries. 2) Because Ca\(^{2+}\) handling during trains is complex and incompletely understood, interpreting the results may require further experimentation to determine the role of aberrant processes that may be occurring during trains in R6/2 muscle, including Na\(^+\)/K\(^+\) ATPase activity, extracellular Ca\(^{2+}\) influx, Ca\(^{2+}\) activated K\(^+\) channels, and other potential ion channels, which is beyond the scope of these studies. Therefore, the focus of Aims 2 and 3 were on single APs and force-frequency curves.

Specific Aim 2 (CHAPTER IV) was to determine if the increased duration of APs in R6/2 muscle corresponded to an increase in the duration of Ca\(^{2+}\) release. Specific Aim 3 (CHAPTER V) was to ascertain the contribution of muscle atrophy, prolonged APs (evokes changes in intracellular Ca\(^{2+}\)), and reduced ClC-1 currents to R6/2 muscle function. Each step of EC coupling was examined in each aim: The first step (excitation) in Aim 1, the second step (intracellular Ca\(^{2+}\) release) in Aim 2, and the third step (contraction) in Aim 3.
In the following chapter (CHAPTER III), the results of Aim 1 will be described. This has been published previously (D. R. Miranda et al., 2020). The results of Aim 2 (CHAPTER IV) and Aim 3 (CHAPTER V) have not yet been published.
III: SPECIFIC AIM 1

Mechanisms of altered action potentials in Huntington’s disease skeletal muscle
Introduction

Huntington’s disease is a monogenic disorder caused by a CAG repeat expansion mutation of the huntingtin (HTT) gene. Mutant HTT is expressed ubiquitously and leads to central and peripheral pathology (Mielcarek, 2015; Jenny Sassone, Colciago, Cislaghi, Silani, & Ciammola, 2009; van der Burg, Björkqvist, & Brundin, 2009). The motor symptoms of Huntington’s disease include involuntary muscle contractions that can lead to chorea (irregular jerky movements), bradykinesia (slow movement), dystonia (abnormal positioning), and muscle rigidity. HD patients also suffer from reduced lower limb muscle strength (Busse et al., 2008), which may contribute to impairments in balance and mobility (Cruickshank et al., 2014).

We previously discovered a decrease in skeletal muscle Cl⁻ channel (ClC-1) and inwardly rectifying K⁺ channel (Kir) currents, as well as aberrant splicing of the gene encoding ClC-1, Clcn1 in the R6/2 transgenic mouse model for Huntington’s disease (Waters et al., 2013). We found that the reduction in ClC-1 currents, and Clcn1 aberrant splicing progressed over the same time course as the disease, suggesting that ClC-1 defects contribute to the overall pathology of Huntington’s disease (D. R. Miranda et al., 2017). A likely mechanism by which reduced ClC-1 and Kir currents affect Huntington’s disease pathology is by prolonging muscle action potentials (APs) during repetitive stimulation and altering muscle contraction.

The objective of this study was to determine the effect of the reduced ClC-1 and Kir currents on APs as well as to identify any additional ion channel defects that affect single or repetitive APs. We measured single APs and trains of APs (20-60 Hz) from the skeletal muscle fibers of R6/2 mice and their age-matched wild type (WT) littermates.
Each AP was analyzed for the maximum (max) rate-of-rise, peak amplitude, 40% decay, 80% decay, and max repolarization. To determine which changes in R6/2 APs were caused by the reduced ClC-1/Kir currents, we pharmacologically blocked ClC-1 and Kir currents in WT fibers to the same extent they are reduced in R6/2 skeletal muscle. The reduced R6/2 ClC-1 and Kir currents cause a significant depolarization in the baseline membrane potential between APs during a train (measured by the max repolarization), which results in inhibition of the fast voltage-gated sodium channels, Nav1.4. In addition, we found a significant prolonging of single and repetitive APs in R6/2 muscle that is explained by reduced expression of voltage-gated K⁺ channels (Kᵥ). Beyond revealing the effect of reduced ion channel activity on R6/2 muscle APs, this study provides one of the most complete examinations of the dynamic properties of WT APs during repetitive stimulation. Our work demonstrates an important role of Cl⁻ movement during normal repetitive activity in WT muscle as well as a striking capacity of WT and disease muscle to maintain AP firing throughout periods of considerable Naᵥ1.4 inhibition.
Materials and Methods

Ethical Approval

All animal procedures were performed in accordance with the policies of the Animal Care and Use Committee of Wright State University. We established a breeding colony in the Wright State University Laboratory Animal Resources Facility that consisted of one wild-type B6CBA female with an ovarian transplant (hemizygous for Tg(HDexon1)62Gpb) and one wild-type male (B6CBAF1/J) purchased from The Jackson Laboratory, stock #002810 (RRID: IMSR_JAX:002810). A total of 4 male and 4 female R6/2, as well as 5 male and 5 female WT mice produced from 6 breeding pairs were used for this study. Tail samples cut between 7 and 14 d of age were genotyped at Laragen Inc. (Culver City, CA). Mice were housed with wild-type littermates (control) in sex-matched cages after weaning at ∼14 d of age. Environmental conditions were maintained with a 12-h light/dark cycle and constant temperature (21–23°C) and humidity (55 ± 10%). The cages contained corncob bedding (Harlan Teklad 7902) and environmental enrichment (mouse house and cotton nestlet). Mice were supplied with dry chow (irradiated rodent diet; Harlan Teklad 2981) and water ad libitum. Beginning at 10 weeks of age, all cages housing R6/2 mice were supplied with a Petri dish containing moist chow (dry chow soaked in water) to ensure adequate nutritional intake in the symptomatic mice. For the 9-AC/Ba^{2+} group, wild type, noncarrier (B6CBA-Tg(HDexon1)62Gpb/1J wild type mice) mice from the Jackson Laboratory, stock #100011 (RRID: IMSR_JAX:100011) were used and housed under the same conditions as the R6/2 mice. A total of 3 female WT mice produced from one breeding pair were used for the 9-AC/Ba^{2+} group.
Clinical and behavioral assessment

The health and phenotype severity assessment for HD mice, as described previously (Waters et al., 2013; Miranda et al., 2017), were performed weekly for mice 8–10 weeks of age, every other day for mice 10 weeks of age, and daily for mice ≥11 weeks of age. Assessment categories included physical condition, activity level, and weight loss. Of the behavioral measures, mouse weight has been a reliable and easy to obtain measurement to assess disease progression. We previously showed that R6/2 weight stopped increasing from 6 to 10 weeks of age and decreased after 10 weeks of age (Miranda et al., 2017).

Electrophysiology

Mice were euthanized by inhalation of a saturating dose of isoflurane (~2 g/L), followed by cervical dislocation. All R6/2 and control mice were between 11 and 13 weeks of age. Hind limb flexor digitorum brevis and interosseous muscles were dissected and enzymatically dissociated at 35-36 °C under mild agitation for ~1 h using 1,000 U/mL of collagenase type IV (Worthington Biochemical). Collagenase was dissolved in an extracellular solution (below). Dissociation was completed using mild trituration in extracellular solution with no collagenase. The fibers were allowed to recover at 21–23 °C for 1 hour before electrical measurements were recorded.

Fibers were visualized in an Olympus BX51WI or IX71 microscope. The BX51WI microscope was equipped with 10x (UMPLFLN10XW) and 40x (LUMPLFLN40XW) objectives, and the IX71 microscope was equipped with 10x (UPlanFLN) and 20x (UPlanFLN) objectives. Electrical properties were measured under
standard current-clamp conditions at 21–23°C using two borosilicate intracellular microelectrodes with a 1.5 mm outside diameter and 0.86 mm inside diameter (Sutter Instruments), an Axoclamp 900A amplifier, a Digidata 1550 digitizer, and pCLAMP 10 or 11 data acquisition and analysis software (Molecular Devices). The Axoclamp 900A was equipped with a virtual-ground headstage (VG-9Ax100). Reference electrodes were placed in separate cups containing 3 M KCl and connected to the extracellular fluid via agar bridges. Electrodes were impaled ~10 µm apart from each other. The voltage-sensing electrode was connected with an Axoclamp HSx1 headstage and the current-passing electrode was connected with an Axoclamp HSx10 headstage that was modified to have a 2 MΩ output resistor (HSx5). Both the current-passing and voltage-sensing electrodes were filled with the same internal solution (below). Electrode resistances for each electrode were between 10 and 15 MΩ. Data were acquired at 100 kHz. Current and voltage records were low-pass filtered with the internal Axoclamp 900A filters at 6 kHz.

Upon impalement of each fiber, the intracellular solution was allowed to equilibrate with the sarcoplasm for 20 minutes and the baseline membrane potential was maintained at ~−85 mV by injecting a constant negative current. Fibers that exceeded −20 nA of holding current, which indicated membrane damage, were excluded from the study. Action potentials (APs) were elicited by 0.2 ms current pulses with an amplitude equal to 1.1x the threshold for firing an AP. A single AP was triggered 75 ms before each train to use as a reference. Trains of APs, 5 s in duration and of increasing frequency (from 20 to 40 to 60 Hz) were measured from each fiber with at least 1 min between each train. If a fiber did not fully repolarize within 1 min after each train, the holding current was adjusted to bring the membrane potential back to −85 mV. To examine recovery, 7
APs at 0.5 Hz were recorded after each train. To prevent contractions, 70 mM EGTA was added to the intracellular solution.

*Intracellular and extracellular buffers for electrophysiology:*

**Extracellular solution (in mM):** 139 NaCl, 4 KCl, 5 CaCl$_2$, 2 MgCl$_2$, 5 glucose, 1 NaH$_2$PO$_4$, 10 MOPS, and pH 7.4 (NaOH). 0.03 9-anthracene carboxylic acid (9-AC) and 0.00073 BaCl$_2$ added when partially blocking CIC-1 and Kir respectively.

**Intracellular solution (in mM):** 5 MgCl$_2$, 5 ATP disodium, 5 phosphocreatine disodium, 5 glutathione, 40 MOPS, 70 EGTA, and pH 7.2 (KOH).

*Chemicals*

Chemicals were purchased from Fisher Scientific; exceptions include CaCl$_2$ and MgCl$_2$ stock solutions (TekNova), ATP di-Na (Sigma-Aldrich), BaCl$_2$ (Matheson, Colman and Bell), and anthracene-9-carboxylic acid (Tocris).

*RNA extraction and real-time PCR*

Total RNA was isolated from the interosseous of 11 to 12 week old R6/2 mice and their age-matched WT littermates using TRIzol reagent (#15596018; Thermo Fisher Scientific) according to the manufacturer's instructions. One µg of total RNA was used for reverse transcription with the High-Capacity cDNA Reverse Transcription Kit (#4368814, Thermo Fisher Scientific). The real-time PCR reaction was carried out in a QuantStudio Real-Time PCR System (Thermo Fisher Scientific) using SYBR Green Real-time PCR Master Mix (#A25742; Thermo Fisher Scientific). Expression data were
normalized to the housekeeping gene β-actin and the relative mRNA expression was determined by the comparative Ct (2−ΔΔCt). PCR was performed using primers for Kv3.4 (F: 5′-TGG GCT GTG GTC ACC ATG AC-3′; R: 5′-CTC TCG ACC ACA CCC TCT TCC-3′), Kv1.5 (F: 5′-TCCGACGGCTGGACTCAATAA-3′; R: 5′-CAGATGGCCTTCTAGGCTGTG-3′) and Kv1.4 (F1: TGC TGG GAA TGG TGA AGT G; R1: GGA AGG TAG AGA AGG TGG TAG A; F2: GAA AGC AGG AAA TGA AGA GCA TC; R2: GTT GCA GCG TGG AAA AGG; F3: GCT CAC TCC AGG GCA GCT GCA GCT GCT GCT; R3: TCA CGC ATG CTG GCT CTT AGG GTG TGG CCC) and β-actin (F: 5′-ACTGTGAGTGTCGCGTCCA-3′; R: 5′-GTCATCCATGCGAACTGGT-3′).

Statistics

Between Groups Comparisons: A repeated measures ANOVA was run at each frequency of stimulation for each AP parameter of interest (max rate-of-rise, peak amplitude, 40% decay, 80% decay, and max repolarization). The independent variable was group, which included wild type (WT) fibers, transgenic R6/2 Huntington’s disease (R6/2) fibers, and WT fibers exposed to 9-AC and Ba^{2+} to inhibit ClC-1 and Kir (9-AC/Ba^{2+}). The repeated measure was AP number. The repeated measure accounts for potentially correlated measurements from the same muscle fiber across APs. A random effect for mouse was also included to account for potentially correlated measurements between fibers from the same mouse at each AP. An autoregressive covariance structure, which allows observations further apart in time to be less highly correlated than observations closer together in time, was found to be the best fit by Akaike’s Information
Criterion (AIC). Denominator degrees of freedom were calculated via the Kenward-Roger degrees of freedom approximation (Kenward & Roger, 1997). A flow chart of the statistical procedure for assessing between group effects is shown in Figure 1.
Figure 1. Statistical methodology for between groups comparison.
The presence of a two-way interaction between group and AP number was tested first. A significant interaction indicates that the mean difference between groups was not constant across all APs. When a significant interaction was not present, the interaction term was removed and the means were directly compared using Tukey’s post hoc multiple comparison procedure. In the presence of a significant interaction, a series of repeated measures ANOVAs were run for each pairwise combination of groups. When there was no significant interaction for a pairwise combination of groups, the groups were compared directly using an ANOVA. When there was a significant interaction between pairs of groups, post hoc multiple comparisons were carried out for each AP number via the Bonferonni sequentially rejective multiple comparison procedure (Holm, 1979). The procedure ensures that the type I error rate for each ANOVA is at most $\alpha = 0.05$.

**Within Groups Comparisons:** A repeated measures ANOVA was run for each AP parameter of interest to determine if the parameter changed over the time course of the AP train. An independent variable for group (WT, R6/2, 9-AC/Ba$^{2+}$) was not included, since a separate ANOVA was run within each group at each frequency. Therefore, there was only the repeated measure for AP and the random effect for mouse. An autoregressive covariance structure was once again found to be the best fit by AIC. Denominator degrees of freedom were calculated via the Kenward-Roger degrees of freedom approximation (Kenward & Roger, 1997). All APs in the train were compared to the first single AP recorded before the train with Bonferonni’s sequentially rejective multiple comparison procedure being used to control the type I error rate for the pair-wise post hoc comparisons.
Results

Action potentials (APs) were recorded from single muscle fibers using two intracellular microelectrodes (a voltage-sensing and a current-passing electrode) from R6/2 mice and their WT littermates. Because ClC-1 and Kir currents have been shown to be reduced in R6/2 skeletal muscle by about 70% and 30% respectively (D. R. Miranda et al., 2017; Waters et al., 2013), APs were measured from WT fibers in the presence of 9-AC (30 µM) and Ba²⁺ (750 nM). The inhibition of ClC-1 and Kir currents by 30 µM 9-AC and 750nM Ba²⁺ was based on dose response curves obtained from rodent muscle (Palade & Barchi, 1977; Quayle, McCarron, Brayden, & Nelson, 1993) and was used to mimic the conditions observed in R6/2 fibers. AP train comparisons between WT, R6/2, and WT fibers in the presence of 9-AC and Ba²⁺ (9-AC/Ba²⁺) were analyzed quantitatively by using the statistical method diagramed in Figure 1. Each AP was analyzed for the max rate-of-rise, peak amplitude, 40% decay, 80% decay, and max repolarization (Figure 2A (top)). The max rate-of-rise was defined by the maximum slope of the AP rising phase, as determined by the first derivative of each AP (Figure 2A (middle)). The peak amplitude was the peak voltage above 0 mV. The 40% and 80% decay was the duration between the peak amplitude and the voltage at which the falling phase of the AP decays to 40% or 80% of the total voltage; the total voltage being the difference between the peak amplitude of each AP and the baseline membrane potential (~–85 mV). The max repolarization is the most negative voltage after each AP. Each AP was evoked by a 0.2 ms current stimulus (Figure 2A (bottom)).
Single action potential analysis

To assess single APs, we examined an isolated AP that was recorded before the first 20 Hz AP train from each fiber. This AP was not affected by any physiological changes that may have occurred during AP train recordings. Figure 2B shows the average single AP for WT, R6/2, and 9-AC/Ba\(^{2+}\) fibers and Figure 2C-2F shows boxplots of the average max rate-of-rise, peak amplitude, 40% decay, and 80% decay. The max repolarization was not analyzed because the voltage potential was allowed to return to rest (~–85 mV) after each single AP.

The results show that the peak amplitude of R6/2 APs was significantly lower (23.68 ± 3.50 mV) than both WT (37.06 ± 2.89 mV) and 9-AC/Ba\(^{2+}\) (43.99 ± 3.58 mV) (Figure 2C). This was consistent with the max rate-of-rise, which although not reaching statistical significance trended lower in R6/2 fibers (244.4 ± 33.23 mV/ms) compared to both WT (320.56 ± 27.40 mV/ms) and 9-AC/Ba\(^{2+}\) (309.43 ± 34.82 mV/ms) (Figure 2D). The difference in the R6/2 AP falling phase relative to WT and 9-AC/Ba\(^{2+}\) was more apparent. The 40% decay time was significantly higher in R6/2 fibers (2.72 ± 0.17 ms) compared to both WT (1.48 ± 0.14 ms) and 9-AC/Ba\(^{2+}\) (1.15 ± 0.16 ms) (Figure 2E). Similarly, the 80% decay time was significantly higher in R6/2 fibers (11.16 ± 0.61 ms) compared to WT (4.72 ± 0.53 ms) and 9-AC/Ba\(^{2+}\) (3.50 ± 0.55 ms) (Figure 2F). The decrease in the R6/2 AP peak amplitude suggests a Na\(^{+}\) channel defect and the increase in decay time suggests a K\(^{+}\) channel defect. Since the peak amplitude and decay time of 9-AC/Ba\(^{2+}\) APs were not significantly different than WT, reduced ClC-1 and Kir currents do not likely explain the differences between R6/2 and WT APs for these parameters.
**Figure 2. Action potential (AP) parameters and single AP analysis.** (A) Representative AP with peak amplitude, 40% decay, 80% decay, and max repolarization labeled (top). The max rate-of-rise (dV/dt) was determined by taking the first derivative of the AP (middle). Also shown is the 0.2 ms current pulse used to stimulate each AP (bottom). (B) Average single AP for WT (n = 6 mice, 17 fibers), R6/2 (n = 4 mice, 13 fibers), and 9-AC/Ba²⁺ (n = 3 mice, 16 fibers). Box plots for; (C) peak amplitude (WT vs R6/2 p = 0.0358, WT vs 9-AC/Ba²⁺ p = 0.34, and R6/2 vs 9-AC/Ba²⁺ p = 0.0087); (D) max rate-of-rise (p = 0.25); (E) 40% decay (WT vs R6/2 p = 0.0008, WT vs 9-AC/Ba²⁺ p = 0.33, and R6/2 vs 9-AC/Ba²⁺ p = 0.0007); and (F) 80% decay (WT vs R6/2 p < 0.0001; WT vs 9-AC/Ba²⁺ p = 0.26, and R6/2 vs 9-AC/Ba²⁺ p < 0.0001). Data compared using a one-way ANOVA, boxplots show the 25th and 75th percentile (box), mean (dot), median (line), and 1.5 interquartile range (error bars). The horizontal lines with an asterisk indicate a significant difference (α = 0.5).
Peak amplitude and max repolarization during trains

Trains of APs were recorded with increasing stimulation frequency (20 to 40 to 60 Hz). Representative WT (left) and R6/2 (right) 60 Hz train recordings are shown in Figure 3A; insets show the first AP in the train with the peak amplitude and max repolarization labeled. Over the duration of the trains, there were significant changes in peak amplitude within the WT, R6/2, and 9-AC/Ba²⁺ groups at 20, 40, and 60 Hz (Table 1.) This was determined by comparing each AP in the train to the single AP recorded before each train (Figure 3A). To compare WT, R6/2, and 9-AC/Ba²⁺ AP trains, we first tested for an interaction (Figure 1). Because there was no significant interaction between the three groups for peak amplitude at 20 Hz (Figure 3B, \( p = 0.56 \) for 20 Hz 3 group interaction), all three groups were compared using Tukey’s multiple comparisons procedure (†), which treats each train as a whole. As shown in Figure 3B, the peak amplitude of R6/2 APs at 20 Hz was significantly lower than both WT and 9-AC/Ba²⁺, whereas WT and 9-AC/Ba²⁺ APs were not different from each other. At 40 Hz, the peak amplitude remained significantly lower in R6/2 fibers compared to WT and 9-AC/Ba²⁺, and there was no significant difference between WT and 9-AC/Ba²⁺ fibers (Figure 3B, \( p = 0.95 \) for 3 group interaction).
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Table 1. Within group AP comparison. All APs in the train were compared to the first single AP recorded before the train with Bonferonni’s sequentially rejective multiple comparison procedure.
For the peak amplitude at 60 Hz, there was a significant interaction between all three groups (Figure 3B, \( p < 0.0001 \) for 3 group interaction), which implies that the mean difference between groups was not constant across all APs. Therefore, we determined if there was an interaction between pairs of groups (Figure 1). There was no significant interaction between WT vs 9-AC/Ba\(^{2+}\), so these groups were compared directly, yielding no significant difference (Figure 3B, \( p = 0.32 \) for pairwise interaction). A significant pairwise interaction between WT vs R6/2 (\( p < 0.0001 \)) and R6/2 vs 9-AC/Ba\(^{2+}\) (\( p < 0.0001 \)) lead to the more strict Bonferroni multiple comparisons procedure (\( \beta \)), which also yielded no significant differences (Figure 2B). The primary interaction between all three groups was driven by the sudden drop in peak amplitude from the first AP in the train to the second AP in the train in R6/2 fibers (Figure 3B). This abrupt decrease likely occurred because of the corresponding depolarization in membrane potential (shift in max repolarization), which would cause fast inactivation of the skeletal muscle voltage-gated Na\(^+\) channel, Nav1.4 (Catterall, 2012; Featherstone, Richmond, & Ruben, 1996). When the data set was analyzed from the second AP onward (AP2–300), the interaction was no longer significant (\( p = 0.22 \) for 60 Hz 3 group interaction). Tukey’s multiple comparison procedure was then employed for AP2–300 and, consistent with the 20 and 40 Hz trains, the peak amplitude of R6/2 APs at 60 Hz was significantly lower than both WT (\( p = 0.008 \)) and 9-AC/Ba\(^{2+}\) (\( p = 0.029 \)). In addition, WT was not significantly different than 9-AC/Ba\(^{2+}\) (\( p = 0.34 \)). Overall, the peak amplitude of R6/2 APs was consistently reduced compared to WT and 9-AC/Ba\(^{2+}\). Because of the similarity between WT and 9-AC/Ba\(^{2+}\), the decrease in CIC-1 and Kir currents do not likely explain the reduction in R6/2 peak amplitude.
The max repolarization was the most negative potential at the end of each AP and describes the extent of repolarization before the next AP in the train (Figure 3C). During each train, there were significant changes in the max repolarization within the WT, R6/2, and 9-AC/Ba\(^{2+}\) groups at 20, 40, and 60 Hz (Table 1.) Between groups, there was no significant difference in the max repolarization at 20 Hz (Figure 3C, \(p < 0.0001\) for 3 group interaction). At 40 Hz, the max repolarization of R6/2 APs jumped from \(-85.29 \pm 0.16\) mV (initial single AP) to \(-78.83 \pm 0.65\) mV (AP1 of the train) and continued to depolarize to \(-72.50 \pm 0.74\) mV by the end of the train. In contrast, the WT max repolarization gradually depolarized from \(-86.02 \pm 0.27\) mV to \(-75.71 \pm 1.30\) mV over the duration of the train. Thus, the max repolarization was significantly more depolarized in R6/2 fibers compared to WT at 40 Hz (Figure 3C, \(p < 0.0001\) for 3 group interaction). In the presence of 9-AC/Ba\(^{2+}\), the max repolarization was significantly more depolarized than WT from AP8 to AP110 but not significantly different from R6/2 fibers at any AP in the 40 Hz train (Figure 3C). Overall, the R6/2 max repolarization was more similar to 9-AC/Ba\(^{2+}\) than WT at 40 Hz.

A striking feature of the max repolarization at 60 Hz was the large step from \(-85.50 \pm 0.26\) mV after the single AP to \(-69.79 \pm 0.86\) mV for AP1 in R6/2 fibers (Figure 3C). A similar but less dramatic step was observed in the presence of 9-AC/Ba\(^{2+}\). By the end of the 60 Hz train, the max repolarization of R6/2 and 9-AC/Ba\(^{2+}\) fibers were \(-63.64 \pm 1.65\) and \(-63.15 \pm 1.07\) mV, respectively. In contrast, the max repolarization of WT fibers at 60 Hz underwent a more gradual change from \(-85.53 \pm 0.25\) mV at baseline to \(-70.05 \pm 1.21\) mV by the end of the train making it significantly different than R6/2 and 9-AC/Ba\(^{2+}\) fibers throughout the 60 Hz train (Figure 3C, \(p < 0.0001\) for 3 group interaction).
interaction). Highlighting their similarities, the R6/2 and 9-AC/Ba\textsuperscript{2+} fibers were only statistically different at AP1 and AP2 (Figure 3C). Overall, exposure of WT fibers to 9-AC/Ba\textsuperscript{2+} largely replicated the AP max repolarization in R6/2 fibers, suggesting that reduced ClC-1 and Kir currents alone can largely explain the shift in the max repolarization of R6/2 APs.
Figure 3. Action potential peak amplitude and max repolarization. (A) Representative WT (left) and R6/2 (right) 60 Hz AP trains with peak amplitude and max repolarization labeled (insets). The x-axis breaks separate the 1st single AP and recovery APs from the train. (B) Graph of average peak amplitude and (C) max repolarization at 20 Hz (left panels), 40 Hz (middle panels), and 60 Hz (right panels) for WT [n = 17 fibers (20 and 40 Hz) and 16 fibers (60 Hz) from 6 mice], R6/2 [n = 13 fibers (20 Hz), 11 fibers (40 Hz) and 12 fibers (60 Hz) from 4 mice], and 9-AC/Ba²⁺ [n = 16 fibers (20 Hz and 40 Hz) and 15 fibers (60 Hz) from 3 mice]. Each AP is shown as an average ± SEM. Black font signifies a significant difference and gray font indicates no significant difference between groups. The black lines indicate which APs were significantly different. † = Tukey’s comparison procedure. ß = stepdown Bonferonni comparison procedure. No symbol = direct comparison.
**Max rate-of-rise**

The max rate-of-rise was the maximum slope of the AP rising phase, which was calculated by taking the first derivative of each AP. Figure 4A shows the derivative of the 1st and last 3 APs of representative WT (left) and R6/2 (right) 60 Hz trains. Within the 20, 40, and 60 Hz AP trains, the max rate-of-rise significantly decreased for WT, R6/2 and 9-AC/Ba²⁺ (Table 1). The least dramatic changes occurred in WT fibers at 20 Hz, in which the max rate-of-rise significantly decreased only in some APs (Table 1). In comparing groups, it appeared that the R6/2 max rate-of-rise was lower than WT and 9-AC/Ba²⁺ (Figure 4). In response to repetitive stimulation, the R6/2 max rate-of-rise decreased by 11% at 20 Hz, 33% at 40 Hz, and 60% at 60 Hz. The change in R6/2 max rate-of-rise was about 2-fold more than WT, which decreased by 7% at 20 Hz, 15% at 40 Hz, and 27% at 60 Hz. The 9-AC/Ba²⁺ max rate-of-rise began at levels close to WT and decreased by 9% at 20 Hz, 23% at 40 Hz, and 50% at 60 Hz. However, the max rate-of-rise was not significantly lower in R6/2 fibers compared to WT or 9-AC/Ba²⁺ at any frequency, based on the direct ANOVA comparison and the stepdown Bonferroni procedure (Figure 4B). To assess if this was due to high variability or the strict correction made to the p-values by the multiple comparison procedure in the presence of between groups interactions, we compared specific APs using a standard one-way ANOVA. The max rate-of-rise for AP1 was not significantly different between WT, R6/2, and 9-AC/Ba²⁺ fibers at 20 Hz (p = 0.24), 40 Hz (p = 0.30), or 60 Hz (p = 0.36). Similarly, the max rate-of-rise of the last AP in the train was not significantly different between R6/2, WT, and 9-AC/Ba²⁺ at 20 Hz (AP100, p = 0.23) or 40 Hz (AP200, p = 0.11). At 60 Hz, there was a substantial drop in max rate-of-rise at AP2 in R6/2 fibers, which prompted us
to test for differences at AP2 and AP300 (Figure 4B). Indeed, the R6/2 max rate-of-rise was significantly lower than WT at AP2 (R6/2: 160.88 ± 32.66 mV/ms, WT: 285.47 ± 32.65 mV/ms, p = 0.0406) and at AP300 (R6/2: 89.64 ± 16.90 mV/ms, WT: 210.64 ± 33.13 mV/ms, p = 0.02807). The R6/2 max rate-of-rise was not significantly different than 9-AC/Ba²⁺ at AP2 (269.44 ± 9.03 mV/ms, p = 0.14) or AP300 (141.54 ± 7.84 mV/ms, p = 0.52). Thus, whereas high variability drove the lack of statistical significance in AP max rate-of-rise between groups at 20 and 40 Hz, there was evidence that the AP max rate-of-rise was lower in R6/2 fibers compared to WT at 60 Hz. Exposure of WT fibers to 9-AC/Ba²⁺ had little effect on the AP max rate-of-rise, suggesting that reduced ClC-1 and Kir currents in R6/2 skeletal muscle does not explain changes in the AP rising phase during repetitive firing.
Figure 4. Action potential max rate-of-rise. (A) First and last 3 APs from representative WT (left) and R6/2 (right) 60 Hz AP trains with max rate-of-rise labeled. The x-axis breaks separate the 1st single AP and recovery APs from the train. (B) Graph of max rate-of-rise at 20 Hz (left), 40 Hz (middle), and 60 Hz (right) for WT [n = 17 fibers (20 Hz and 40 Hz), and 16 fibers (60 Hz) from 6 mice], R6/2 [n = 13 fibers (20 Hz), 11 fibers (40 Hz), and 12 fibers (60 Hz) from 4 mice], and 9-AC/Ba²⁺ [n = 16 fibers (20 Hz and 40 Hz) and 15 fibers (60 Hz) from 3 mice]. Data shown as average ± SEM. Black font signifies a significant difference between groups. Gray font signifies no significant difference. Black lines signify which APs are significantly different between groups. † = Tukey’s comparison procedure. ß = stepdown Bonferronni comparison procedure. No symbol = direct comparison.
**Falling phase**

The falling phase of each AP consisted of a fast and slow portion. The fast portion was analyzed using the 40% decay time and the slow portion analyzed using the 80% decay time, both from the peak. APs from the beginning and end of a representative WT (*left*) and R6/2 (*right*) 60 Hz train are shown in Figure 5A with the 40% (D40) and 80% (D80) decay points labeled. Within all groups, the 40% decay did not significantly change during 20 Hz trains and remained constant in WT fibers at all frequencies (Table 1). Within the R6/2 and 9-AC/Ba²⁺ groups, the 40% decay significantly prolonged over the duration of the 40 Hz and 60 Hz trains (Table 1). Between groups, the 40% decay of R6/2 APs was significantly prolonged compared to WT and 9-AC/Ba²⁺ at 20, 40, and 60 Hz stimulation, whereas the 40% decay in WT fibers was not significantly different than 9-AC/Ba²⁺ fibers (Figure 5B, $p = 0.84$ for 3 group interaction).

The 80% decay time significantly increased within all groups at all frequencies (Table 1). The between groups comparison for the 80% decay was similar to that of the 40% decay. R6/2 APs had a significantly higher decay time than both WT and 9-AC/Ba²⁺ at all frequencies (Figure 5C). Because of the shift in the max repolarization (Figure 3C), many of the APs from R6/2 and 9-AC/Ba²⁺ fibers at 60 Hz did not decay to 80% before the next AP fired, especially by the end of the train. Therefore, 7 of the R6/2 and 10 of the 9-AC/Ba²⁺ fibers could not be used for the 80% decay results. Overall, this data shows that there was an increase in both the 40% and 80% AP decay time in R6/2 muscle fibers compared to WT, which was not explained by the partial block of CIC-1 and Kir currents, suggesting a K⁺ current defect.
Figure 5. **40% decay (D40)** and **80% decay (D80)**. (A) First and last 3 APs from representative WT (left) and R6/2 (right) 60 Hz AP trains with points of analysis labeled. The x-axis breaks separate the 1st single AP and recovery APs from the train. (B) Graph of 40% decay for WT \[n = 17\] fibers (20 Hz and 40 Hz) and 16 fibers (60 Hz) from 6 mice, R6/2 \[n = 13\] fibers (20 Hz), 11 fibers (40 Hz), and 12 fibers (60 Hz) from 4 mice, and 9-AC/Ba^{2+} \[n = 16\] fibers (20 Hz and 40 Hz) and 14 fibers (60 Hz) from 3 mice. (C) Graph of 80% decay for WT \[n = 17\] fibers (20 Hz and 40 Hz) and 15 fibers (60 Hz) from 6 mice, R6/2 \[n = 13\] fibers (20 Hz), 9 fibers (40 Hz), and 5 fibers (60 Hz) from 4 mice, and 9-AC/Ba^{2+} \[n = 16\] fibers (20 Hz and 40 Hz) and 4 fibers (60 Hz) from 3 mice. For B and C, decay values shown for 20 Hz (left panels), 40 Hz (middle panels), and 60 Hz (right panels) stimulation. Data shown as average ± SEM. Black font signifies a significant difference between groups. Gray font signifies no significant difference. Black lines
signify which APs are significantly different between groups. † = Tukey’s comparison procedure. β = stepdown Bonferonni comparison procedure. No symbol = direct comparison.
Increasing R6/2 peak amplitude to WT levels

Previous studies suggest that a decrease in AP peak amplitude can increase the duration of the falling phase (Beam & Donaldson, 1983; Hodgkin & Katz, 1949). To test this, we experimentally increased the peak amplitude of R6/2 APs (High Stim) to ~50 mV, which was slightly above WT levels, by increasing the amplitude of the stimulus pulse in 25 nA intervals (Figure 6A). Because the High Stim APs had different peak characteristics than normal APs (Figure 6A), the 40% and 80% decay for the High Stim APs were not comparable to those of normal APs. Thus, to examine the falling phase, we determined the max rate-of-decay using the first derivative of each High Stim AP (Figure 6A&B). We focused on the decay rates after the rapid voltage changes caused by the high stimulus current had subsided, shown in Figure 6A&B as the region bracketed by dashed vertical lines. This region of the High Stim APs was most comparable to regular APs from WT and R6/2 fibers. As can be seen in Figure 6B, increasing the AP peak had little effect on the rate of repolarization. To better examine the relationship between peak amplitude and falling phase, we plotted the max rate-of-decay as a function of peak amplitude for regular WT and R6/2 APs as well as High Stim R6/2 APs; each group was fit with a line (Figure 6C). The WT slope (1.72 ± 0.16 ms⁻¹) was greater than the R6/2 slope (0.79 ± 0.07 ms⁻¹), suggesting that the WT max rate-of-decay was more steeply dependent on peak amplitude than the R6/2 max rate-of-decay. In addition, the High Stim R6/2 slope (0.50 ± 0.14 ms⁻¹) was lower than the R6/2 slope, suggesting that the prolonged falling phase of R6/2 APs was not caused by a decrease in peak amplitude. Most likely, the prolonged repolarization of R6/2 muscle was caused by a reduction in voltage-dependent K⁺ current.
Figure 6. Relationship between AP peak amplitude and max rate-of-decay. (A) Representative recording of R6/2 APs with increasing peak amplitudes. (B) First derivative of the APs from (A) to determine max rate-of-decay. Traces in A and B are color-coded and the dotted lines bracket the region used for analysis. (C) Plot of AP max rate-of-decay vs. AP peak amplitude for WT (n = 6 mice, 17 fibers, 57 APs), R6/2 (n = 4 mice, 13 fibers, 46 APs), and R6/2 High Stim. (n = 2 mice, 11 fibers, 59 APs) with linear regression lines.
Decreased voltage-gated $K^+$ channel mRNA in HD skeletal muscle

To determine if the prolonged falling phase of R6/2 APs was due to reduced voltage-gated $K^+$ channel (Kv) current, we measured the mRNA expression levels of three Kv genes reported to be expressed in mouse skeletal muscle (DiFranco et al., 2015; Lesage, Attali, Lazdunski, & Barhanin, 1992; D. Vullhorst, Klocke, Bartsch, & Jockusch, 1998). We found that, compared to WT, Kv1.5 was reduced by 43% and Kv3.4 was reduced by 81% in R6/2 skeletal muscle (Figure 7). We also tested three different primers sets for Kv1.4. The very high Ct levels suggested that Kv1.4 was expressed at very low levels in both WT and R6/2 skeletal muscle (not shown). Overall, the reduction in Kv1.5 and Kv3.4 mRNA help explain the prolonged decay time of R6/2 APs.
Figure 7. Kv mRNA expression. The expression of Kv1.5 and Kv3.4 relative to β-actin was significantly reduced in R6/2 skeletal muscle (n = 4) compared to WT (n = 4). Data compared using a two-sample T-test. Columns shown as mean ± SEM. * p = 0.010, ** p = 1.592 x 10^{-9}. 
Action Potential Recovery

Following each AP train, 7 APs were recorded at 0.5 Hz to assess recovery from the train. The first recovery AP was recorded 125 ms after the last AP in the train. Thus, recovery occurred over two phases, a fast phase within the first 125 ms after the train and a more gradual phase during the 0.5 Hz recovery period, which followed an exponential time course (Figure 8). Full recovery of each AP parameter did not often occur within the ~15 s recovery period. However, as can be seen by comparing the first data points of each frequency panel in Figures 2-5, full recovery was achieved during the 1-3 min rest period between the 20, 40, and 60 Hz trains.

For analysis of the peak amplitude and max rate-of-rise recovery, the last AP in the train was set to 0 and the recovery values were normalized to the first single AP. The recovery values were fitted with the equation,

\[ y = y_0 + A \left(1 - e^{-\frac{x}{\tau}}\right) \]

where \( y_0 = y\)-intercept, \( A = \text{amplitude} \), and \( \tau = \text{time constant} \). For the analysis of the max repolarization, 40% decay, and 80% decay recovery, the first single AP was set to 0 and recovery values were normalized to the last AP in the train. The recovery values were fitted with the equation,

\[ y = y_0 + A \left(e^{-\frac{x}{\tau}}\right) \]
where $y_0 = y$-intercept, $A = \text{amplitude}$, and $\tau = \text{time constant}$. The $\tau$ and $R^2$ values for all fitted curves are shown in Table 2. Note, recovery was not analyzed for AP parameters that did not significantly change during a train in the within groups comparison (Table 1), such as the peak amplitude of APs in R6/2 fibers during 20 Hz stimulation.

As shown in Figure 8A, the peak amplitude in R6/2 fibers recovered to a greater extent than in WT and 9-AC/Ba$^{2+}$ fibers at 40 and 60 Hz. The rapid jump in peak amplitude from the last AP in the train (open blue circle) to the first recovery AP (initial 125 ms recovery period) was more pronounced in R6/2 fibers compared to WT and 9-AC/Ba$^{2+}$ and was larger with increasing frequency. In addition to the initial jump in recovery, the time constant values (Table 2) describe a faster recovery in R6/2 fibers compared to WT and 9-AC/Ba$^{2+}$. The rapid 125 ms recovery of the peak amplitude in R6/2 fibers could be partially explained by relief of Na$\text{V}_{1.4}$ fast-inactivation, as would result from a quick recovery of the max repolarization.

The recovery of max repolarization (Figure 8B) was most similar between R6/2 and 9-AC/Ba$^{2+}$ fibers, which both differed from WT fibers, especially at 60 Hz. At 40 Hz and 60 Hz, the R6/2 and 9-AC/Ba$^{2+}$ max repolarization recovered more than WT during the initial 125 ms and had a faster time constant (Table 2). Also at 60 Hz, the R6/2 and 9-AC/Ba$^{2+}$ fibers had a more complete overall recovery during the 15 s recovery phase compared to WT. The 9-AC/Ba$^{2+}$ recovery became closer to R6/2 with increasing frequency where, at 60 Hz, they became nearly identical (Figure 8B).

The recovery of the max rate-of-rise was similar between groups at all frequencies in terms of time constant (Table 2) and extent of recovery, but differed in rapid initial recovery (Figure 8C). The fast recovery at 60 Hz in R6/2 and 9-AC/Ba$^{2+}$ fibers was
nearly double that of WT fibers. This is somewhat similar to the trend observed in the peak amplitude recovery and is likely heavily influenced by the max repolarization.

Recovery of the 40% decay was only analyzed for the R6/2 and 9-AC/Ba\(^{2+}\) fibers at 40 and 60 Hz. This is because there was no statistical change in the 40% decay in WT fibers at any frequency, or in R6/2 and 9-AC/Ba\(^{2+}\) fibers at 20 Hz (Table 1). Despite significant and apparent differences in the 40% decay between R6/2 and 9-AC/Ba\(^{2+}\) fibers at 40 and 60 Hz, there were no clear differences in the initial rapid recovery (Figure 8D) or in the rate of exponential recovery (Table 2).

Recovery of the 80% decay (Figure 8E) differed from the trend observed in the 40% decay. During the first 125 ms after the trains, there was little to no recovery in the 80% decay of R6/2 or 9-AC/Ba\(^{2+}\) fibers at any frequency; WT fibers continued to prolong (worsen) during the initial 125 ms recovery period at all frequencies. Unexpectedly, after 20 Hz trains, the 80% decay in R6/2 fibers continued to prolong instead of shorten during the 0.5 Hz recovery. This was primarily due to two R6/2 fibers in which the 80% decay did recover. Notably, the peak amplitude, max rate-of-rise, 40% decay, and max repolarization did recover in these two fibers, suggesting the 80% decay of APs is controlled separately from the other parameters. Otherwise, the 80% decay of WT APs recovered at the slowest rate during the 0.5 Hz recovery (Table 2). In R6/2 and 9-AC/Ba\(^{2+}\) fibers, the AP 80% decay recovered at a similar rate during the 0.5 Hz train, although the extent of recovery was less in R6/2 fibers. Overall, the 80% decay recovery was greater in the 9-AC/Ba\(^{2+}\) fibers than in WT and R6/2.
Figure 8. Recovery APs. 1st single AP (open green circle), last AP in train (open blue circle), and recovery APs (filled black circles) for (A) peak amplitude, (B) max repolarization, (C) max rate-of-rise, (D) 40% decay, and (E) 80% decay at 20 Hz (left panels), 40 Hz (middle panels), and 60 Hz (right panels) with recovery APs fitted with an exponential equation. Recovery was not analyzed for AP parameters that did not significantly change during a train in the within groups comparison.
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th></th>
<th>R6/2</th>
<th></th>
<th>9-AC/Ba²⁺</th>
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<tbody>
<tr>
<td></td>
<td>τ ± SE (s)</td>
<td>R²</td>
<td>τ ± SE (s)</td>
<td>R²</td>
<td>τ ± SE (s)</td>
<td>R²</td>
</tr>
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<td>20 Hz Peak Amplitude</td>
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<td>N/A</td>
<td>0.94 ± 0.20</td>
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<tr>
<td>40 Hz Peak Amplitude</td>
<td>1.15 ± 0.11</td>
<td>0.997</td>
<td>0.83 ± 0.18</td>
<td>0.991</td>
<td>1.20 ± 0.24</td>
<td>0.983</td>
</tr>
<tr>
<td>60 Hz Peak Amplitude</td>
<td>1.13 ± 0.09</td>
<td>0.997</td>
<td>0.84 ± 0.13</td>
<td>0.995</td>
<td>1.21 ± 0.13</td>
<td>0.995</td>
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<tr>
<td>20 Hz Max Repolarization</td>
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<td>N/A</td>
<td>1.19 ± 0.09</td>
<td>0.997</td>
<td>0.85 ± 0.11</td>
<td>0.997</td>
</tr>
<tr>
<td>40 Hz Max Repolarization</td>
<td>2.29 ± 0.33</td>
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<td>1.23 ± 0.12</td>
<td>0.996</td>
<td>1.10 ± 0.16</td>
<td>0.993</td>
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<tr>
<td>60 Hz Max Repolarization</td>
<td>2.57 ± 0.52</td>
<td>0.976</td>
<td>1.25 ± 0.11</td>
<td>0.996</td>
<td>1.06 ± 0.17</td>
<td>0.991</td>
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<tr>
<td>20 Hz Max Rate-of-Rise</td>
<td>2.42 ± 0.71</td>
<td>0.950</td>
<td>1.46 ± 0.16</td>
<td>0.993</td>
<td>2.31 ± 0.50</td>
<td>0.972</td>
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<tr>
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<td>1.17 ± 0.09</td>
<td>0.997</td>
<td>1.70 ± 0.02</td>
<td>0.999</td>
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<tr>
<td>60 Hz Max Rate-of-Rise</td>
<td>1.56 ± 0.19</td>
<td>0.992</td>
<td>1.32 ± 0.11</td>
<td>0.997</td>
<td>1.34 ± 0.10</td>
<td>0.997</td>
</tr>
<tr>
<td>20 Hz 40% Decay</td>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>40 Hz 40% Decay</td>
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<td>N/A</td>
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<td>1.14 ± 0.10</td>
<td>0.997</td>
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<tr>
<td>60 Hz 40% Decay</td>
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<td>N/A</td>
<td>1.32 ± 0.16</td>
<td>0.993</td>
<td>0.84 ± 0.09</td>
<td>0.997</td>
</tr>
<tr>
<td>20 Hz 80% Decay</td>
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<td>1.08 ± 0.12</td>
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<td>2.05 ± 0.31</td>
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<tr>
<td>40 Hz 80% Decay</td>
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<td>0.984</td>
<td>1.28 ± 0.14</td>
<td>0.994</td>
</tr>
<tr>
<td>60 Hz 80% Decay</td>
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<td>1.03 ± 0.05</td>
<td>0.999</td>
<td>1.06 ± 0.12</td>
<td>0.995</td>
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</table>

Table 2. Curve fit values for AP recovery. The τ ± standard error and $R^2$ values for curves fitted to recovery APs. N/A is listed where recovery was not analyzed because the respective AP parameters did not significantly change during a train in the within groups comparison.
Discussion

Our previous work revealed that skeletal muscle from transgenic R6/2 Huntington’s disease mice is hyperexcitable owing to decreased ClC-1 and Kir currents (D. R. Miranda et al., 2017; Waters et al., 2013). In this study, we examined action potential (AP) repetitive firing in R6/2 skeletal muscle and, by pharmacologically blocking ClC-1 and Kir currents in WT muscle to R6/2 levels, we show which changes in R6/2 APs were caused by decreased ClC-1 and Kir currents. This led us to discover a decrease in voltage-gated K\textsuperscript{+} channel (K\textsubscript{V}) mRNA expression in R6/2 skeletal muscle. Our analyses also provide insights into the dynamic changes that occur during repetitive firing in WT muscle.

Action potentials in Huntington’s disease skeletal muscle

In isolation and during repetitive firing, R6/2 skeletal muscle APs had significantly lower peaks, significantly prolonged decay times, and tended to have a slower rates-of-rise compared to WT APs. The partial block of ClC-1 and Kir currents in WT muscle (9-AC/Ba\textsuperscript{2+}) did not replicate these changes, which is consistent with the established roles of Cl\textsuperscript{-} and K\textsuperscript{+} currents in maintaining the resting membrane potential of skeletal muscle fibers (Hodgkin & Horowicz, 1959). Indeed, the extent to which the membrane potential returned to rest after each AP during repetitive firing (max repolarization) was significantly attenuated in both R6/2 and 9-AC/Ba\textsuperscript{2+} fibers compared to WT, indicating that reduced ClC-1 and Kir currents in R6/2 muscle cause a shift in max repolarization to more depolarized levels. Furthermore, this indicates that the max repolarization during AP trains is regulated by ion channels (and perhaps transporters)
that are independent from the ion channels that determine the rate of AP repolarization (measured by the 40% and 80% decay in this study).

The most widely accepted explanation for the depolarized shift in max repolarization during repetitive stimulation is $K^+$ accumulation in the transverse tubules (t-tubules), which shifts the Nernst potential for $K^+$ to cause depolarization of the resting membrane potential (Adrian & Bryant, 1974; Wallinga et al., 1999). 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 & Bryant, 1974; Palade & Barchi, 1977). However, when ClC-1 currents are decreased (as in HD muscle) or partially blocked (as in 9-AC/Ba$^{2+}$ muscle), the effect of $K^+$ build-up is exaggerated. Whether $K^+$ build-up is the sole cause of the steady depolarization remains unknown.

$K^+$ buildup in the t-tubules is expected to occur gradually during repetitive firing such that there would be a gradual shift in max repolarization if it is the primary driver of the depolarization. Though a gradual shift in max repolarization was observed in WT fibers, the depolarized shift was abrupt in R6/2 and 9-AC/Ba$^{2+}$ fibers, particularly at high frequencies of stimulation. We propose that the activation of a subthreshold current may be responsible for this rapid depolarization. For example, persistent inward Na$^+$ current is a voltage activated current that is an important trigger of myotonic APs in muscle lacking ClC-1 channels (Hawash, Voss, & Rich, 2017; Metzger, Dupont, Voss, & Rich, 2020). Activation of persistent inward Na$^+$ current is likely to be increased in R6/2 and 9-AC/Ba$^{2+}$ fibers by the lack of full repolarization following APs. Furthermore, the depolarization caused by persistent inward Na$^+$ current will be amplified in R6/2 and 9-
AC/Ba$^{2+}$ fibers compared to WT fibers because of the increased membrane resistance (Waters et al., 2013). The combination of increased activation of a subthreshold current and its amplified effect on membrane potential may lead to the sudden lack of repolarization following APs.

The low peak amplitude and tendency towards a slower rate-of-rise observed in R6/2 APs was not likely due to reduced CIC-1 and Kir currents because of the normal rising phase in 9-AC/Ba$^{2+}$ fibers, suggesting the presence of other ion channel defects. The rising phase of an AP, which includes the rate-of-rise and peak amplitude, has classically been attributed to Na$^+$ influx (Hodgkin & Huxley, 1952), which in skeletal muscle is mediated by the voltage-gated Na$^+$ channel, Nav1.4 (Goldin et al., 2000). Therefore, it is likely that there is a disruption in Nav1.4 activity in R6/2 skeletal muscle. The disruption is not likely caused by a reduction in the Na$^+$ equilibrium potential because we controlled the intracellular and extracellular Na$^+$ concentrations. More likely, there is a decrease in Nav1.4 current or a shift in the voltage-dependence of Nav1.4 activation and/or inactivation. There is evidence of altered Nav1.4 activity in other HD cell types. In striatal neurons, the β4 subunit, which is involved in the voltage-dependence of Na$^+$ channel activation, was shown to be down-regulated in HD mouse models and human patients (Oyama et al., 2006). Further studies will be needed to determine if Nav1.4 influences R6/2 APs in skeletal muscle.

The most dramatic difference between R6/2 and WT APs was the decay time. Compared to WT, the falling phase of R6/2 APs was markedly prolonged and was not due to reduced CIC-1 and Kir currents. Prolonged decay has been associated with low peak amplitude (Beam & Donaldson, 1983; Hodgkin & Katz, 1949). However, the low
peak of R6/2 APs did not likely cause the prolonged decay time because injecting current to increase the peak of R6/2 APs did not increase the rate of decay time. Therefore, since Kv currents are responsible for the falling phase of APs (Beam & Donaldson, 1983; DiFranco et al., 2015), we measured the expression level of Kv1.5 and Kv3.4 in R6/2 skeletal muscle.

Voltage-gated K⁺ channel expression in HD skeletal muscle

We found that mRNA levels of Kv1.5 and Kv3.4 were lower in R6/2 skeletal muscle compared to WT, which helps explain the prolonged decay time we observed in R6/2 APs. Similarly, APs in R6/2 medium spiny neurons have a prolonged decay time and have decreased Kv2.1 expression (Ariano, Cepeda, et al., 2005; Klapstein et al., 2001). There are several factors that may lead to decreased Kv3.4 expression in R6/2 skeletal muscle. Skeletal muscle Kv3.4 expression decreases in denervated muscle, is higher in fast-twitch muscle fibers, and increases with age until adulthood in mice (D. Vullhorst et al., 1998). We can exclude a relation to denervation since morphological, physiological, and molecular experiments have shown that R6/2 skeletal muscle is not denervated (Khedraki et al., 2017). Fiber type switching may help explain the reduced Kv3.4 levels since a shift to slow and neonatal myosin heavy chain occurs in R6/2 muscle (Mielcarek et al., 2015; D. R. Miranda et al., 2017; Ribchester et al., 2004; Strand et al., 2005). Additionally, we have shown that R6/2 skeletal muscle is not fully matured (D. R. Miranda et al., 2017). Thus, it may be that a transition to slow fiber types and disruption in muscle maturation explain the reduced Kv3.4 levels. Additionally, transcription factor activity for Kv3.4 may be altered in R6/2 skeletal muscle. Kcna4, the gene that codes for
Kv3.4 contains binding sites for Sp-1 and RORα1 (Euler et al., 2012; Detlef Vullhorst, Jockusch, & Bartsch, 2001), transcription factors that have been shown to bind, or are thought to bind mutant huntingtin protein (Bradford et al., 2009; Jiang, Diaz-Castro, Looger, & Khakh, 2016). This pathway has been linked to the downregulation of proteins such as Kir4.1 and to HD pathology such as dysregulated intracellular Ca\textsuperscript{2+} signaling (Bradford et al., 2009; Jiang et al., 2016).

Kv1.5 expression changes throughout skeletal muscle development and plays an important role in myocyte proliferation (Lesage et al., 1992; Villalonga et al., 2008). Nerve growth factor has been shown to upregulate Kv1.5 and cause a decrease in AP decay time in myocytes cultured from 1-2 day old rat skeletal muscle (Vigdor-Alboim et al., 1999). The lack of nerve growth factor in R6/2 skeletal muscle may lead to decreased Kv1.5 expression and muscle atrophy (Alberch, Pérez-Navarro, & Canals, 2004; Ruberti et al., 2000). Also, Kv1.5 expression has been shown to be promoted by Sp-1, which in turn was shown to have increased activity in response to oxidative stress in myocytes (Fountain et al., 2007). It is well established that mitochondrial dysfunction and oxidative stress play a pivotal role in HD pathology in both skeletal muscle and the CNS (Tobore, 2019; Zielonka, Piotrowska, Marcinkowski, & Mielcarek, 2014). Since the expression of Kv1.5 and Kv3.4 is decreased in R6/2 skeletal muscle, it may be that Sp-1 can no longer respond to oxidative stress because it is bound to mutant huntingtin in HD.

*Action potential recovery*

We measured AP recovery for ~15 s following each AP train. The recovery followed two phases, a fast recovery occurring 125 ms after each AP train, and a slow
recovery occurring over the remaining time period. During the fast phase, the max repolarization recovered by approximately 30-50% in R6/2 and 9-AC/Ba2+ fibers but only 10% in WT fibers after the 40 Hz and 60 Hz trains. The rapid recovery of the max repolarization would increase the degree of Nav1.4 relief from inactivation, which would explain the more rapid recovery in peak amplitude and max rate-of-rise we observed in R6/2 and 9-AC/Ba2+ fibers compared to WT.

The slow recovery phase of the AP max repolarization may be explained by the flux of K+ and Cl− from the t-tubules after sustained stimulation (Adrian & Bryant, 1974; Jack, Noble, & Tsien, 1983). Our interpretation is that the reduction in ClC-1 and Kir currents limit the movement of K+ into the t-tubules during trains in R6/2 and 9-AC/Ba2+ fibers. Thus, buildup of K+ in t-tubules contributes more to the max repolarization of WT fibers than it does to R6/2 and 9-AC/Ba2+ fibers. This likely occurs because the mass movement of K+ into the t-tubules must be accompanied by counter ions in order to maintain bulk electroneutrality, which is achieved with Cl− movement through ClC-1 in WT muscle (Bretag, 1987; Cairns, Hing, Slack, Mills, & Loiselle, 1997; Coonan & Lamb, 1998; A. F. Dulhunty, 1979; Jentsch, Steinmeyer, & Schwarz, 1990; Koch et al., 1992; Miller, 2006). Because ClC-1 currents are severely restricted in R6/2 and 9-AC/Ba2+ fibers, K+ fluxes may be decreased. Overall, this suggests that ClC-1 and Kir dampen rapid voltage changes in WT fibers caused by subthreshold currents but facilitate the bulk movement of K+ and Cl−. This highlights a key role of Cl− movement in WT muscle, which is consistent with work examining the role of ClC-1 in regulating muscle during normal activity (de Paoli, Ørtenblad, Pedersen, Jørgensen, & Nielsen, 2010; Thomas Holm Pedersen, de Paoli, Flatman, & Nielsen, 2009; T. H. Pedersen, Macdonald,
de Paoli, Gurung, & Nielsen, 2009; T. H. Pedersen, Riisager, de Paoli, Chen, & Nielsen, 2016) and a recent study has described Cl\textsuperscript{-} movement with a four-electrode technique (Heiny & Cannon, 2019). In contrast to WT, R6/2 and 9-AC/Ba\textsuperscript{2+} fibers are more prone to rapid voltage changes caused by subthreshold currents but less susceptible to slow voltage changes caused by altered ion gradients.

Unlike the other AP parameters, there were no changes in the AP decay time during the fast recovery period in WT, R6/2, or 9-AC/Ba\textsuperscript{2+} fibers. Moreover, the 80\% decay time of WT APs continued to prolong during the fast recovery period. The recovery of the 40\% and 80\% decay in R6/2 and 9-AC/Ba\textsuperscript{2+} fibers occurred with a time constant of 1-2 s, which is consistent with the reported recovery from inactivation for Kv, including Kv3.4 (ex., 1.8 s time constant in dorsal root ganglion neurons) (Ritter, Ho, O’Leary, & Covarrubias, 2012). Thus, it is likely that recovery from inactivation of the remaining Kv1.5 and Kv3.4 explains the exponential recovery phase recorded for R6/2 and 9-AC/Ba\textsuperscript{2+} fibers. Moreover, the prolonged recovery of the AP max repolarization in WT fibers may further delay the recovery from inactivation of Kv1.5 and Kv3.4.

**Conclusion**

Broadly, this study shows that APs in WT and disease muscle undergo significant and progressive changes in the rising and falling phases, peak, and baseline (max repolarization) during trains of activity. The dynamic nature of APs has implications for basic assessments of muscle function. For example, changes in electromyography (EMG) records are sometimes assumed to reflect changes in muscle function under the assumption that muscle APs are unchanging because of their all-or-none characteristics.
However, it is likely that changes in EMG waveforms simply reflect changes in the APs of individual muscle fibers, such as the widening of the AP during trains as shown in this study.

During trains, we found a substantial decrease in the AP max rate-of-rise, particularly in R6/2 fibers at high frequencies of stimulation. This was likely because the depolarizing shift in the max repolarization inactivated many Na\textsubscript{v}1.4 channels. Despite the inactivation of Na\textsubscript{v}1.4, the AP peak only decreased by only a few mV. Our interpretation is that a corresponding inactivation of Kv channels during trains, as indicated by the widening of the APs, served to maintain the AP peak. In short, because of the reduced K\textsuperscript{+} currents, less Na\textsuperscript{+} current was needed to achieve a near normal AP peak. Eventually, the declining AP peak will cause muscle weakness once Cav1.1 is insufficiently activated. Weakness or fatigue is expected to occur sooner in R6/2 muscle than WT because of the consistently reduced AP peak in R6/2 fibers. This work provides the foundation to determine the role of AP defects in HD muscle dysfunction and to determine the ion channel regulatory events that modulate APs in WT and disease muscle.
IV: SPECIFIC AIM 2

Prolonged action potentials and increased Ca$^{2+}$ release duration in Huntington’s disease skeletal muscle
Introduction

The huntingtin mutation in HD causes a host of cellular defects in multiple organ systems, manifesting in neurodegeneration, cachexia, and systemic metabolic dysfunction (Chuang & Demontis, 2021). The symptoms of HD include impairment of voluntary movement, diminished muscle strength, and mobility problems (Bates et al., 2014; Busse et al., 2008; Cruickshank et al., 2014). Because many HD symptoms are related to skeletal muscle function, skeletal muscle seemingly plays an important role in the muscle-related symptoms of HD.

In Specific Aim 1 (CHAPTER III), we observed changes in the R6/2 muscle AP waveform, including reduced amplitude and prolonged duration (D. R. Miranda et al., 2020), the latter of which was associated with reduced Kv 1.5 and Kv3.4 mRNA (D. R. Miranda et al., 2020). Our observations of these AP defects led us to explore the link between the AP waveform and intracellular Ca\(^{2+}\) release in R6/2 muscle. Although our long-term goal is to understand intracellular Ca\(^{2+}\) signaling during AP trains, like the trains measured in Specific Aim 1 (CHAPTER III), because Ca\(^{2+}\) handling during trains is complex and incompletely understood, the focus of this study was on single APs.

In skeletal muscle, intracellular Ca\(^{2+}\) is the key signaling molecule mediating the communication between APs and muscle contraction. In this study, we simultaneously measured single APs and intracellular Ca\(^{2+}\) from R6/2 muscle fibers. We hypothesized that prolonged APs correspond to an increase in Ca\(^{2+}\) release duration, which was previously proposed by Sandow and colleagues (Sandow, Taylor, & Preiser, 1965). They proposed that AP duration determines the duration and intensity of muscle contraction by controlling the amount of Ca\(^{2+}\) accumulated in the myoplasm. They hypothesized that
when an AP reaches a specific voltage, called the mechanically effective period (MEP), intracellular Ca\(^{2+}\) release is activated; prolonged APs increase the duration of the MEP and, therefore the duration of intracellular Ca\(^{2+}\) release (Sandow et al., 1965).

Since 1965, the idea that AP duration controls Ca\(^{2+}\) release duration has been underexplored, it is only recently that this idea has again emerged as an important aspect of EC coupling (Banks et al., 2021; Wang et al., 2021). The duration of APs in cardiac muscle is expected to regulate intracellular Ca\(^{2+}\) release (Bouchard, Clark, & Giles, 1995). However, in skeletal muscle, it is often thought that the AP serves more as a switch for Ca\(^{2+}\) release, whereby the peak amplitude of the AP (Cairns, Buller, Loiselle, & Renaud, 2003; Gong, Legault, Miki, Seino, & Renaud, 2003; Yensen, Matar, & Renaud, 2002) is sufficient to determine the amount of intracellular Ca\(^{2+}\) that is released. We find that prolonged APs are indeed associated with increased Ca\(^{2+}\) release duration in R6/2 muscle, and that the increase in duration compensates for the reduction in peak Ca\(^{2+}\) release flux.
Methods

Animals (similar procedure described in CHAPTER III methods)

All animal procedures were performed in accordance with the policies of the Animal Care and Use Committee of Wright State University. To study HD skeletal muscle, we used the R6/2 mouse model, which is transgenic for exon 1 of the human huntingtin gene (Mangiarini et al., 1996). An R6/2 mouse breeding colony was established at the Wright State University Laboratory Animal Resources Facility. Each R6/2 breeding pair consisted of a wild-type B6CBA female with an ovarian transplant (hemizygous for Tg(HD exon1)62Gpb) and a wild-type male (B6CBAF1/J) purchased from The Jackson Laboratory, stock #002810 (RRID: IMSR_JAX:002810). A total of 2 male and 2 female diseased R6/2 mice from 3 breeding pairs were used in this study. For genotyping, tail samples were obtained between 7 and 14 days of age and sent to Laragen Inc. (Culver City, CA). Mice were housed with wild-type (WT) littermates in sex-matched cages after weaning at ~14 days of age.

Environmental conditions were maintained with a 12-h light/dark cycle and constant temperature (21–23°C) and humidity (55 ± 10%). The cages contained corncob bedding (Harlan Teklad 7902) and environmental enrichment (mouse house and cotton nestlet). Mice were supplied with dry chow (irradiated rodent diet; Harlan Teklad 2981) and water ad libitum. Beginning at 10 weeks of age, all cages housing R6/2 mice were supplied with a Petri dish containing moist chow (dry chow soaked in water) to ensure adequate nutritional intake in the symptomatic mice. The health and phenotype severity assessment, as described previously (D. R. Miranda et al., 2020; Waters et al., 2013) were performed weekly for mice 8–10 weeks of age, and daily for mice ≥11 weeks of age.
Assessment categories included physical condition, activity level, and weight loss. Of the behavioral measures, mouse weight has been a reliable and easy to obtain measurement to assess disease progression. We previously showed that the body weight of R6/2 mice does not change from 6 to 10 weeks of age and decreases after 10 weeks of age (Miranda et al., 2017).

For the control mice, a B6CBA (background strain for R6/2 mice) mouse breeding colony was established, and consisted of noncarrier (B6CBA-Tg(HDexon1)62Gpb/1J) mice from the Jackson Laboratory, stock #100011 (RRID:IMSR_JAX:100011). The control mice were housed under the same conditions as the R6/2 mice. A total of 4 male and 5 female WT mice produced from 5 breeding pairs were used as controls for the diseased R6/2 mice, and for the TEA experiments. All mice were age-matched and between 83 and 111 days of age. Before harvesting muscles for experimentation, all mice were euthanized by inhalation of a saturating dose of isoflurane (~2 g/L), followed by cervical dislocation.

Action Potential and Ca\(^{2+}\) Measurements

Hind limb flexor digitorum brevis (FDB) and interosseous (IO) muscles were dissected and enzymatically dissociated at 35 °C under mild agitation for ~1 h using 1,000 U/mL of collagenase type II (Worthington Biochemical). Collagenase was dissolved in an extracellular solution (below). For the TEA experiments, the collagenase was dissolved in Leibovitz’s L-15 (Company) containing 10% fetal bovine serum (FBS) (Company). L-15 and FBS were used for the TEA and not R6/2 experiments because we transitioned to using L-15 and FBS (for less leaky fibers) after the R6/2 experiments were
completed. Dissociation was completed using mild trituration in the absence of collagenase. The fibers were allowed to recover at 21–23 °C for 1 hour before electrical measurements were recorded.

Fibers were visualized on an Olympus IX71 microscope equipped with 10x (UPlanFLN), 20x (UPlanFLN), and 40x (UPlanFLN) objectives. Electrical properties were measured under standard current-clamp conditions at 21–23°C using two borosilicate intracellular microelectrodes (Sutter Instruments), an Axoclamp 900A amplifier, a Digidata 1550 digitizer, and pCLAMP 11 data acquisition and analysis software (Molecular Devices). A reference electrode, grounded to the HSx1 headstage was placed into a cup containing 3 M KCl and connected to the extracellular fluid via agar bridges. Electrodes were impaled ~10 µm apart from each other. The voltage-sensing electrode was connected to an Axoclamp HSx1 headstage and the current-passing electrode was connected to an Axoclamp HSx10. Both the current-passing and voltage-sensing electrodes were filled with the same internal solution containing the fluorescent Ca\(^{2+}\) indicator dye, fluo-4 to measure changes in intracellular Ca\(^{2+}\) (see below). Data were acquired at 100 kHz. Current and voltage records were low-pass filtered with the internal Axoclamp 900A filters at 6 kHz.

Upon impalement of each fiber, the intracellular solution was allowed to equilibrate with the sarcoplasm for 20 minutes while the baseline membrane potential was maintained between –85 and –80 mV by injecting a constant negative current. Fibers that exceeded –20 nA (–25 nA for TEA experiments) of holding current were excluded from the study. A holding current of -25 nA was used for the TEA experiments because the holding current tended to increase when increasing the concentration of TEA.
in the same fiber. Action potentials (APs) were elicited by a 0.2 ms current pulse with an amplitude equal to 1.1x the threshold for firing an AP. APs and intracellular Ca\(^{2+}\) were measured simultaneously. Figure 9A shows the microscope setup for fluorescent measurements. Fluorescence data was detected through a 40x oil-immersion objective (UPlanFLN). A 470 nm wavelength light-emitting diode (Thorlabs, M470L3) was used for excitation, and emission was captured by a photo multiplier tube (PMT) after passing through a 525 nm wavelength filter (Thorlabs). The fluorescence signal from the PMT was digitalized and filtered at 6 kHz. The sensitivity of the PMT was adjusted according to each fiber. For each fiber, the background fluorescence of the dish was recorded so that it could be subtracted from the fluorescent signal for analysis. In the TEA experiments, simultaneous measurements of APs and intracellular Ca\(^{2+}\) were measured from the same fiber with three different concentrations of TEA (see below).

**Extracellular and Intracellular Buffers**

**Extracellular solution (in mM):** 144 NaCl, 4 KCl, 1.2 CaCl\(_2\), 0.6 MgCl\(_2\), 5 glucose, 1 NaH\(_2\)PO\(_4\), 10 MOPS, and pH 7.4 (NaOH).

**Extracellular solution for 0 mM TEA (in mM):** 144 NaCl, 4 KCl, 1.2 CaCl\(_2\), 0.6 MgCl\(_2\), 5 glucose, 1 NaH\(_2\)PO\(_4\), 10 MOPS, and pH 7.4 (NaOH) + 0.2% FBS and 50 µM BTS.

**Extracellular solution for 1 mM TEA (in mM):** 140 NaCl, 4 KCl, 1.2 CaCl\(_2\), 0.6 MgCl\(_2\), 5 glucose, 1 NaH\(_2\)PO\(_4\), 10 MOPS, 1 mM TEA-OH, 12 mM D-mannitol, and pH 7.4 (NaOH).

**Extracellular solution for 10 mM TEA (in mM):** 144 NaCl, 4 KCl, 1.2 CaCl\(_2\), 0.6 MgCl\(_2\), 5 glucose, 1 NaH\(_2\)PO\(_4\), 10 MOPS, 10 mM TEA-OH, and pH 7.4 (NaOH).
Intracellular solution (in mM): 90 K-methanesulfonate, 2 Ca(OH)$_2$, 3 or 5 MgCl$_2$, 5 ATP disodium, 5 phosphocreatine disodium, 5 glutathione, 20 MOPS, 20 EGTA, and pH 7.2 (KOH). 0.02 fluo-4 added for intracellular Ca$^{2+}$ measurements.

Chemicals

Chemicals were purchased from Fisher Scientific; exceptions include CaCl$_2$ and MgCl$_2$ stock solutions (TekNova), ATP di-Na (Sigma-Aldrich).

Ca$^{2+}$ Analysis

The time course of free [Ca$^{2+}$] was estimated from the subtracted fluorescence signal as described previously (Prosser, Hernández-Ochoa, Zimmer, & Schneider, 2009). First, the fluorescence signal (F) was divided by the resting fluorescence (F$_0$) to obtain F/F$_0$. F/F$_0$ was converted to the time course of free [Ca$^{2+}$] with the following equation:

\[
[Ca^{2+}](t) = K_{D,flu4} \ast \left( \frac{F}{F_0} + \frac{d(F/F_0)}{dt} \ast \frac{1}{k_{off,flu4}} \right) \ast [Ca^{2+}]_{rest}
\]  

(Eq. 1)

where $K_{D,flu4}$ is the dissociation constant of fluo-4 (1 µM), $k_{off,flu4}$ is the off-rate constant of fluo-4 (90 s$^{-1}$), and [Ca$^{2+}$]$_{rest}$ is the resting myoplasmic Ca$^{2+}$ concentration. [Ca$^{2+}$]$_{rest}$ was determined using the MAXCHEALTOR program and the concentrations of Ca$^{2+}$, Mg$^{2+}$, ATP, and EGTA used in our intracellular solution (see above). It was assumed that 70% of the intracellular solution equilibrated with the sarcoplasm of the muscle fiber and the concentration of Ca$^{2+}$ already in the sarcoplasm was 70 nM, as reported previously in R6/2 skeletal muscle (Braubach et al., 2014). Thus, 70% of the [Ca$^{2+}$] calculated using MAXCHEALATOR was added to 30% of the [Ca$^{2+}$] reported.
previously by Braubach et al., to obtain $[\text{Ca}^{2+}]_{\text{rest}} = 36.4$ nM in the presence of 5 mM Mg$^{2+}$ and $[\text{Ca}^{2+}]_{\text{rest}} = 36.1$ nM in the presence of 3 mM Mg$^{2+}$. The time course of free $[\text{Ca}^{2+}]$ estimates the Ca$^{2+}$ released into the myoplasm and accounts for the Ca$^{2+}$ bound to fluo-4. However, the time course of free $[\text{Ca}^{2+}]$ does not account for the Ca$^{2+}$ that binds to EGTA.

Under conditions of 20 mM EGTA, we assume that EGTA is the primary binding site for Ca$^{2+}$ in the myoplasm. With EGTA in the intracellular solution, the following equation provides a good approximation of Ca$^{2+}$ release flux:

$$
\frac{d[\text{Ca}:\text{EGTA}](t)}{dt} = k_{\text{on},\text{EGTA}} \cdot [\text{Ca}](t) \cdot ([\text{EGTA}]_{\text{total}} - [\text{Ca}:\text{EGTA}]) - k_{\text{off},\text{EGTA}} \cdot [\text{Ca}:\text{EGTA}](t)
$$

(Eq. 2)

where $[\text{Ca}:\text{EGTA}]$ is the concentration of Ca$^{2+}$ bound to EGTA, $k_{\text{on},\text{EGTA}}$ is the on-rate constant of EGTA (15 µM$^{-1}$s$^{-1}$), $k_{\text{off},\text{EGTA}}$ is the off-rate constant of EGTA (1.2 s$^{-1}$), and $[\text{EGTA}]_{\text{total}}$ is the concentration of EGTA in the intracellular solution (20 mM). The initial concentration for Ca$^{2+}$ bound to EGTA ([Ca:EGTA] = 1399.93 µM) was determined by using MAXCHEALTOR. Based on Equation 1 and Equation 2, it is assumed that Ca$^{2+}$ binds primarily to the dye (Eq. 1) and EGTA (Eq.2), bypassing endogenous Ca$^{2+}$ buffers, including parvalbumin, ATP, troponin C (EGTA also blocks contraction), and SERCA (Hollingworth & Baylor, 2013; Prosser et al., 2009). However, it cannot be ruled out that the endogenous Ca$^{2+}$ buffers, especially those with relatively high affinities for Ca$^{2+}$, like parvalbumin also bind to intracellular Ca$^{2+}$. Since the
dissociation constant for Ca\textsuperscript{2+} binding to EGTA in our system is 0.08 µM, and the
dissociation constant for Ca\textsuperscript{2+} binding to parvalbumin has been calculated as 0.012 µM in
mouse EDL (Hollingworth & Baylor, 2013), the affinity of Ca\textsuperscript{2+} to parvalbumin is higher
than it is for EGTA. In future studies, we plan to measure APs and intracellular Ca\textsuperscript{2+}
without EGTA \textit{via} field stimulation, which does not directly alter the intracellular
environment. For these studies, we will model the contribution of parvalbumin and other
Ca\textsuperscript{2+} buffers to the Ca\textsuperscript{2+} release flux.

With the gadget integration tool in OriginPro 2021 (OriginLab Corporation), the
integral of the Ca\textsuperscript{2+} release flux was used to assess the peak Ca\textsuperscript{2+} release flux, full-width
half-maximum (FWHM), and the Ca\textsuperscript{2+} available for contraction. The baseline for the
integral/area under the curve (AUC) was set to “y = 0.” The peak Ca\textsuperscript{2+} release flux was
the maximum height of the AUC, the FWHM was the duration of the AUC 50% from the
maximum height, and the Ca\textsuperscript{2+} available for contraction was the AUC value.

\textit{Statistics}

\textbf{R6/2 AP and Ca\textsuperscript{2+} release flux:} To determine whether a significant mean
difference exists between control and R6/2 mice, a series of mixed effects ANOVAs were
run with the AP and Ca\textsuperscript{2+} release flux parameters as dependent variables and genotype as
the independent variable. Multiple measurements were taken from each mouse, so a
random effect for the mouse was included to account for potentially correlated
measurements from the same mouse.

\textbf{TEA AP and Ca\textsuperscript{2+} release flux:} The AP and Ca\textsuperscript{2+} release flux parameters were
compared between 0 mM, 1 mM, and 10 mM TEA with a repeated measures ANOVA.
The repeated measure accounts for potentially correlated measurements from the same muscle fiber over time. A random effect for mouse was included to account for the potentially correlated measurements from muscle fibers from the same mouse. A first-order ante-dependent covariance structure was fit to the model to account for variance within and across concentrations. Post-hoc multiple comparisons were carried out via the Bonferonni sequentially rejective multiple comparison procedure (Holm, 1979). The type I error was set \( \alpha = 0.05 \) and SAS version 9.4 (SAS Institute, Inc., Cary, NC) was through the analysis.
Results

To determine whether prolonged APs in R6/2 muscle correspond to an increase in Ca\(^{2+}\) release duration, single APs and intracellular Ca\(^{2+}\) were measured simultaneously from control and R6/2 FDB and IO muscle fibers. Intracellular Ca\(^{2+}\) was measured with the fluo-4 Ca\(^{2+}\) indicator dye, which was added to the intracellular solution of the voltage-sensing and current-passing microelectrodes (Figure 9A). Representative control and R6/2 traces showing an AP, intracellular fluorescence signal, and stimulus are shown in Figure 9B. To further investigate the association between prolonged APs and Ca\(^{2+}\) release duration, we used TEA to block \(K_V\) channels and prolong APs in WT muscle. Finally, as to study all the major components of EC coupling, we examined the twitch force kinetics of R6/2 and control EDL muscle to link APs, Ca\(^{2+}\) release, and force generation.

*Increased AP-Evoked Ca\(^{2+}\) Release Duration in R6/2 Muscle*

The averaged control and R6/2 AP and corresponding Ca\(^{2+}\) release flux are shown in Figure 10A. As we have reported previously (D. R. Miranda et al., 2020), the R6/2 APs were significantly prolonged, as shown by the increase in 40% (D40; data shown, values presented as mean ± standard error; control: 0.66 ± 0.12 ms, R6/2: 1.62 ± 0.12 ms, \(p < 0.0001\)) and 80% (D80; data now shown, control: 1.94 ± 0.85 ms, R6/2: 8.05 ± 0.81 ms, \(p < 0.0001\)) decay time.
Figure 9. Microscope setup, representative raw traces, and conversion of fluorescence to Ca^{2+} release flux. (A) Microscope setup for intracellular fluorescence detection. Fluo-4 Ca^{2+} indicator dye contained within intracellular microelectrode, excited with 470 nm light emitting diode (LED), and 525 nm emission captured by photomultiplier tube (PMT). (B) Representative control and R6/2 action potential, fluo-4 signal, and current stimulus. (C) Representative control fluorescence signal (F) showing baseline fluorescence (F_0) converted to F/F_0 then Ca^{2+} release flux.
The AP peak amplitude was also significantly reduced (data not shown, control: 41.77 ± 2.99 mV, R6/2: 29.21 ± 3.02 mV, p = 0.026) and the maximum rate-of-rise significantly slower (data not shown, control: 634.49 ± 39.25 mV/ms, R6/2: 415.36 ± 39.49 mV/ms, p = 0.012) in R6/2 muscle. These results confirm that APs in R6/2 muscle are slower and have reduced amplitudes (CHAPTER III)(D. R. Miranda et al., 2020; Waters et al., 2013) and that fluo-4 does not affect APs.

The Ca\(^{2+}\) release flux was calculated from the AP-evoked intracellular fluorescence signal (Figure 9C, left) as described previously (Prosser et al., 2009). Briefly, the fluorescence signal was normalized to the baseline fluorescence (Figure 9C, middle) then kinetically corrected to obtain the Ca\(^{2+}\) release flux signal (Figure 9C, right, Eq. 1 and 2 in methods). From the Ca\(^{2+}\) release flux signal, the peak Ca\(^{2+}\) release flux, full-width half-maximum (FWHM), and Ca\(^{2+}\) available for contraction were determined, as described in the methods section and illustrated in Figure 10B. The peak Ca\(^{2+}\) release flux was significantly higher in control muscle compared to R6/2 muscle (Figure 10C), which may be due to a dysfunctional ryanodine receptor (RyR1) in HD muscle (Dridi et al., 2020). The FWHM, which is the duration of the Ca\(^{2+}\) release flux was significantly longer in R6/2 muscle compared to controls (Figure 10D). The increase in Ca\(^{2+}\) release duration helps compensate for the reduced Ca\(^{2+}\) release flux in R6/2 muscle because there was no significant difference in the Ca\(^{2+}\) available for contraction between R6/2 and control muscles (Figure 10E), although the compensation may not be complete because there was a trend towards lower level of Ca\(^{2+}\) available for contraction in R6/2 muscle (p = 0.071).
Figure 10. R6/2 action potential-evoked Ca\textsuperscript{2+} release flux. (A) Average control (n = 4 mice, 11 fibers) and R6/2 (n = 4 mice, 12 fibers) action potential and corresponding Ca\textsuperscript{2+} release flux. (B) Representative control Ca\textsuperscript{2+} release flux signal with peak Ca\textsuperscript{2+} release flux, full-width half-maximum (FWHM), and Ca\textsuperscript{2+} available for contraction (area under the curve [colored in gray]) labeled. Box and whisker plots for (C) peak Ca\textsuperscript{2+} release flux, (D) FWHM, and (E) estimation of Ca\textsuperscript{2+} available for contraction. Box and whisker plots show the 25\textsuperscript{th} and 75\textsuperscript{th} percentile (box), mean (white box), median (line), and 1.5 interquartile range (error bars). Significant values (p < 0.05) are shown in figure.
Overall, since R6/2 APs are prolonged, and Ca\(^{2+}\) release duration is increased, it is possible that the prolonged AP contributes to the increase in Ca\(^{2+}\) released. However, it cannot be ruled out that the disease-state leads to increased Ca\(^{2+}\) release duration, independent of AP duration. To test this, we used TEA to block Kv channels and prolong APs in WT muscle.

**Blocking Kv Channels Leads to Increased Ca\(^{2+}\) Release Duration**

To help confirm the relationship between prolonged APs and Ca\(^{2+}\) release duration, APs and Ca\(^{2+}\) release flux were simultaneously recorded from WT muscle in the presence of three concentrations of tetraethylammonium (TEA): 0 mM, 1 mM, and 10 mM in the same fiber. TEA prolongs APs by decreasing Kv channel conductance in muscle (Hille, 1967; Stanfield, 1970). Figure 11A shows the average APs and corresponding Ca\(^{2+}\) release flux for all three concentrations of TEA, with the peak amplitude, 40% decay time (D40), and 80% decay time (D80) of the AP labeled. As expected, the peak amplitude of the APs did not significantly change in response to blocking Kv channels with TEA (Figure 11B). The duration of the early falling phase of the AP (D40) significantly increased with increasing TEA concentration (Figure 11C). Similarly, the duration of the late falling phase of the AP (D80) significantly increased from 1 to 10 mM TEA and from 0 to 10 mM TEA (Figure 11D). Overall, these results confirm that TEA prolongs APs.
Figure 11. TEA action potential-evoked Ca\(^{2+}\) release flux. (A) Average action potential and corresponding Ca\(^{2+}\) release flux in 0, 1, and 10 mM TEA (n = 5 mice, 12 fibers). Box and whisker plots for action potential (B) peak, (C) D40, and (D) D80; and (E) peak Ca\(^{2+}\) release flux, (F) FWHM, and (G) Ca\(^{2+}\) available for contraction in 0, 1, and 10 mM TEA. Box and whisker plots show the 25\(^{th}\) and 75\(^{th}\) percentile (box), mean (white box), median (line), and 1.5 interquartile range (error bars). Significant values (p < 0.05) are shown in figure.
We observed no significant changes in the peak Ca\textsuperscript{2+} release flux in response to TEA (Figure 11E), but the FWHM significantly increased from 0 to 1 mM TEA and from 0 to 10 mM TEA (Figure 11F). The Ca\textsuperscript{2+} available for contraction increased only from 0 to 10 mM TEA (Figure 11G). Overall, the Ca\textsuperscript{2+} release duration was increased in response to 1 mM TEA, and further increased by 10 mM TEA in parallel to increases in AP duration (D40 and D80), suggesting that prolonged APs increase Ca\textsuperscript{2+} release duration.

Because the box and whisker plots in Figure 11 do not capture the progression of each AP and Ca\textsuperscript{2+} release flux parameter with increasing TEA in individual fibers, scatter plots with lines connecting the same fiber for each TEA concentration are shown in Figure 12. Figure 12 also includes the max rate-of-rise which significantly decreased from 0 to 1 mM TEA and 0 to 10 mM TEA (Figure 12A). Figure 12 helps to illustrate the changes in each parameter with increasing TEA concentration. It can be seen that the changes in D40 and D80 do not vary as much as the Ca\textsuperscript{2+} release flux parameters.

In summary, since the D40, D80, FWHM, and Ca\textsuperscript{2+} available for contraction increased when the TEA concentration was increased, it seems that prolonged APs are associated with increased Ca\textsuperscript{2+} release duration and Ca\textsuperscript{2+} available for contraction. The decrease in max rate-of-rise likely does not significantly increase Ca\textsuperscript{2+} release duration because the rising phase of the AP is much faster than the falling phase. A slower max rate-of-rise indicates a slight increase in the duration of the AP rising phase (Figure 11A), which is likely too small and too early to significantly influence the Ca\textsuperscript{2+} release duration.
Figure 12. TEA action potential-evoked Ca²⁺ release flux for each fiber. (A) Action potential max rate-of-rise, (B) D40, and (C) D80; and Ca²⁺ release flux (D) peak, (E) FWHM, and (F) Ca²⁺ available for contraction in 0, 1, and 10 mM TEA (n = 5 mice, 12 fibers). Each line connects the same fiber across TEA concentrations, and each color represents the same fiber across all plots. Significant values (p < 0.05) are shown in figure.
Discussion

We have shown that AP duration in R6/2 muscle is associated with an increase in Ca\(^{2+}\) release duration. We also showed that TEA prolongs APs and increases Ca\(^{2+}\) release duration. These results support the idea that AP duration controls the duration of Ca\(^{2+}\) release, which may be a compensatory mechanism to counter muscle weakness in HD skeletal muscle.

*Prolonged APs and Ca\(^{2+}\) Release Duration*

We have previously shown that APs are prolonged in R6/2 skeletal muscle (D. R. Miranda et al., 2020; Waters et al., 2013). Here we show that Ca\(^{2+}\) release duration in R6/2 muscle is also prolonged, likely as a result of prolonged APs. To support this, we blocked Kv channels with TEA to prolong APs in WT muscle and found that TEA also increases the Ca\(^{2+}\) release duration. So it seems that AP duration regulates Ca\(^{2+}\) release duration, as Sandow and colleagues proposed in 1965 (Sandow et al., 1965), before intracellular Ca\(^{2+}\) could reliably be measured (Takahashi, Camacho, Lechleiter, & Herman, 1999). Since 1965, several studies have utilized fluorescent Ca\(^{2+}\) binding techniques to provide evidence for Sandow and colleagues hypothesis. Low concentrations of the drug 4-DAP, which blocks K\(^+\) conductance was reported to prolong APs and increase the duration of the intracellular Ca\(^{2+}\) signal in frog muscle (Miledi, Parker, & Zhu, 1984). Also, Ni\(^{2+}\) was shown to cause an increase in the duration of AP repolarization and a slow intracellular Ca\(^{2+}\) falling phase in frog muscle (Delay, Ribalet, & Vergara, 1986). Recently, Wang and colleagues (2021) investigated the entire EC coupling sequence in mouse muscle by measuring APs and intracellular Ca\(^{2+}\)
simultaneously, as well as twitch force. They found that the AP area was closely associated with Ca\(^{2+}\) release upon membrane depolarization (pre-print) (Wang et al., 2021). These studies, along with our own results support the idea that APs regulate intracellular Ca\(^{2+}\) release in skeletal muscle.

**Voltage Control of Ca\(^{2+}\) Release**

Intracellular Ca\(^{2+}\) release in skeletal muscle is dependent on changes in membrane potential. Physiologically, the movement of ions across the membrane during muscle contraction creates changes in voltage that are measured as APs. APs depolarize the transverse tubule membrane, which in the current model of EC coupling causes conformational changes in the voltage-gated Ca\(^{2+}\) channel (Cav1.1) by pulling on positively charged residues (Bannister & Beam, 2013; A. Dulhunty, 2006; Hernández-Ochoa & Schneider, 2018; Melzer, Herrmann-Frank, & Lüttgau, 1995). Sufficient depolarization causes conformational changes in Cav1.1 that open the RyR1, which allows for the release of Ca\(^{2+}\) from the SR into the sarcoplasm (García, Tanabe, & Beam, 1994; Kovács, Ríos, & Schneider, 1979; Rios & Brum, 1987). Conformational changes in Cav1.1, as measured by charge movement are both voltage and time dependent (Schneider & Chandler, 1973), which could mean that the duration of an AP can regulate the duration of time that the RyR1 is open. That sustained depolarization within physiological (AP) range leads to increased charge displacement (Ferreira Gregorio, Pequera, Manno, Ríos, & Brum, 2017) supports the idea that increased AP duration can lead to additional charge movement, keeping the RyR1 open longer, and allowing Ca\(^{2+}\) to continue leaving the SR. Recently, Banks and colleagues (2021) found that a normal AP
moves only 65% of the muscle fibers maximum available charge (Banks et al., 2021),
again suggesting that additional charge movement could occur during a longer AP.
Therefore, it is likely that the sustained depolarization of a prolonged AP, such as the
APs in R6/2 muscle can induce sustained Ca\(^{2+}\) release.

*Prolonged APs as a Possible Compensation Mechanism in HD Muscle*

We have shown that the peak Ca\(^{2+}\) release flux is significantly decreased in R6/2
muscle, in agreement with a previous study (Braubach et al., 2014). The decrease in peak
Ca\(^{2+}\) release flux may be due to a leaky RyR1, which is excessively phosphorylated,
oxidated, and nitrosylated in the diaphragm of the Q175 HD mouse model (Dridi et al.,
2020). Leak of Ca\(^{2+}\) from the RyR1 may lead to a decrease in SR Ca\(^{2+}\) content, which has
been reported in R6/2 muscle (Braubach et al., 2014) and could reduce Ca\(^{2+}\) release by
diminishing the driving force of Ca\(^{2+}\) from the SR to the sarcoplasm. Interestingly,
despite the decrease in peak Ca\(^{2+}\) release flux, the level of Ca\(^{2+}\) available for contraction
was normal in R6/2 muscle, which is likely due to the increase Ca\(^{2+}\) release duration.
Therefore, it seems that the increase in Ca\(^{2+}\) release duration made up for the reduction in
peak Ca\(^{2+}\) release flux, suggesting that downregulation of Kv channels and AP widening
(D. R. Miranda et al., 2020) compensate for RyR1 defects.

*Conclusion*

Few studies have measured the effects of the AP waveform on intracellular Ca\(^{2+}\) release
in skeletal muscle (Delay et al., 1986; Miledi et al., 1984; Wang et al., 2021). More
studies have focused on the effect of AP duration on muscle force generation, but do not
measure intracellular Ca\textsuperscript{2+}. For example, in rat diaphragm, it was shown that both TEA and 4-DAP increase AP duration and force generation (Delbono & Kotsias, 1987). Another study showed that TEA and 4-AP increased twitch force in the diaphragm, EDL, and soleus (van Lunteren, Moyer, & Pollarine, 2007). Interestingly, AP duration does not seem to increase twitch force when the increase in duration is induced by raising the concentration of extracellular K\textsuperscript{+} (Yensen et al., 2002). More studies measuring APs and Ca\textsuperscript{2+} release simultaneously in skeletal muscle may be necessary to fully understand the role of AP duration on intracellular Ca\textsuperscript{2+} release duration, especially during tetanic force contraction.
V: SPECIFIC AIM 3

Huntington’s disease skeletal muscle twitch force is normal when accounting for muscle atrophy.
Introduction

HD is a monogenic disorder caused by a CAG repeat expansion mutation of the **huntingtin** gene that leads to central and peripheral pathology (Mielcarek, 2015; Jenny Sassone et al., 2009; van der Burg et al., 2009). HD patients have reduced lower limb muscle strength (Busse et al., 2008), which may contribute to impairments in their balance and mobility (Cruickshank et al., 2014). To determine the extent to which intrinsic muscle defects contribute to aberrant muscle function in HD, we investigated muscle force generation in R6/2 muscle.

We have previously observed muscle defects in the R6/2 mouse that may contribute to impairments in muscle function (Khederaki et al., 2017; D. R. Miranda et al., 2020; D. R. Miranda et al., 2017; Romer et al., 2021; Waters et al., 2013). This includes changes in the baseline membrane potential during AP trains, which we showed was associated with a reduction in ClC-1 and Kir currents (CHAPTER III)(D. R. Miranda et al., 2020). We also observed changes in the R6/2 muscle AP waveform, namely a low peak amplitude and prolonged falling phase, which worsened during AP trains (CHAPTER III)(D. R. Miranda et al., 2020) Because APs are involved in the first step in excitation-contraction (EC) coupling, the sequence of molecular events that lead to muscle contraction, we hypothesized that the altered R6/2 AP waveform and/or reduced ClC-1 and Kir currents could lead to muscle weakness. We find that reduced parvalbumin, and possibly AP duration lead to normal twitch force after accounting for muscle atrophy.
Methods

Animals (similar to CHAPTER III and IV methods)

All animal procedures were performed in accordance with the policies of the Animal Care and Use Committee of Wright State University. To study HD skeletal muscle, we used the R6/2 mouse model, which is transgenic for exon 1 of the human huntingtin gene (Mangiarini et al., 1996). An R6/2 mouse breeding colony was established at the Wright State University Laboratory Animal Resources Facility. Each R6/2 breeding pair consisted of a wild-type B6CBA female with an ovarian transplant (hemizygous for Tg(HDexon1)62Gpb) and a wild-type male (B6CBAF1/J) purchased from The Jackson Laboratory, stock #002810 (RRID: IMSR_JAX:002810). A total of 3 male and 3 female R6/2 mice from 2 breeding pairs were used in this study. For genotyping, tail samples were obtained between 7 and 14 days of age and sent to Laragen Inc. (Culver City, CA). Mice were housed with wild-type (WT) littermates in sex-matched cages after weaning at ∼14 days of age.

Environmental conditions were maintained with a 12-h light/dark cycle and constant temperature (21–23°C) and humidity (55 ± 10%). The cages contained corncob bedding (Harlan Teklad 7902) and environmental enrichment (mouse house and cotton nestlet). Mice were supplied with dry chow (irradiated rodent diet; Harlan Teklad 2981) and water ad libitum. Beginning at 10 weeks of age, all cages housing R6/2 mice were supplied with a Petri dish containing moist chow (dry chow soaked in water) to ensure adequate nutritional intake in the symptomatic mice. The health and phenotype severity assessment, as described previously (D. R. Miranda et al., 2020; D. R. Miranda et al., 2017; Waters et al., 2013) were performed weekly for mice 8–10 weeks of age, and daily
for mice ≥11 weeks of age. Assessment categories included physical condition, activity level, and weight loss. Of the behavioral measures, mouse weight has been a reliable and easy to obtain measurement to assess disease progression. We previously showed that the body weight of R6/2 mice does not change from 6 to 10 weeks of age and decreases after 10 weeks of age (D. R. Miranda et al., 2017).

For the control mice, a B6CBA (background strain for R6/2 mice) mouse breeding colony was established, and consisted of noncarrier (B6CBA-Tg(HDexon1)62Gpb/1J) mice from the Jackson Laboratory, stock #100011 (RRID:IMSR_JAX:100011). The control mice were housed under the same conditions as the R6/2 mice. A total of 2 male and 1 female WT mouse produced from 1 breeding pair was used as controls for the diseased R6/2 mice. All mice were age-matched and between 85 and 92 days of age. Before harvesting muscles for experimentation, all mice were euthanized by inhalation of a saturating dose of isoflurane (~2 g/L), followed by cervical dislocation.

Isometric Force Measurements Setup

After the extensor digitorum longus (EDL) muscle was removed, it was incubated in 300 nM α-bungarotoxin for 1 hour at 21–23 °C to eliminate changes in neuromuscular transmission (Khedraki et al) contributing to the force measurements. After the incubation, the EDL was transferred to a custom 3D-printed recording chamber. The proximal tendon was attached to the chamber with a 6-0 caliber silk suture. The suture was also used to tie the distal tendon to a hook, which was then attached to a 300D-300C-LR force transducer (Aurora Scientific). Isometric force measurements were recorded at
21–23°C, consistent with the AP and intracellular Ca\textsuperscript{2+} recordings. The extracellular buffer (see below) was continuously gassed with 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The EDL was stimulated with two silver electrodes placed in the bath. An S-900 pulse generator and S-910 Stimulus Isolation Unit (Dagan) were used to elicit 2.0 ms voltage pulses with an amplitude of 100 V, which were triggered using pCLAMP 10 data acquisition and analysis software. The force transducer was controlled by a 305C two-channel controller (Aurora Scientific), and the mechanical force was digitized by a Digidata 1550B digitizer (Molecular Devices). The optimal length of the muscle was determined by adjusting the tension of the muscle until the maximal twitch force was achieved. To determine the effect of ClC-1 and Kir currents, the same concentrations of 9-AC and Ba\textsuperscript{2+} used for AP train recordings (D. R. Miranda et al., 2020) were used for force recordings (30 µM 9-AC and 750 nM Ba\textsuperscript{2+}).

**Extracellular solution for twitch force measurements (in mM):** 118 NaCl, 3.5 KCl, 1.5 CaCl\textsubscript{2}, 0.7 MgSO\textsubscript{4}, 1.7 NaH\textsubscript{2}PO\textsubscript{4}, 26.2 NaHCO\textsubscript{3}, 5.5 glucose (pH 7.4). 0.03 9-anthracene carboxylic acid (9-AC) and 0.00073 BaCl\textsubscript{2} added when partially blocking ClC-1 and Kir respectively.

**Force-Frequency/Twitch Protocol and Analysis**

To determine the force-frequency relationship, the EDL was stimulated with 15 pulse trains of increasing frequency (0.3 – 160 Hz) to obtain tetani. The muscle was allowed to rest 1 minute between each stimulation train. The single twitch analysis was derived from the first twitch of the 0.3 Hz train.
For the twitch analysis, the absolute (raw) peak amplitude, time-to-peak (10-90%), half-width, and time to half-maximum relaxation were analyzed using pClamp 11 software. The absolute peak twitch force was normalized to the weight of the corresponding EDL muscle to obtain the specific peak twitch force. Absolute peak tetanic force was also analyzed using pClamp 11 software and was normalized to the weight of the corresponding EDL muscle to obtain the specific peak tetanic force. For each muscle, force-frequency curves were constructed from the peak tetanic force values and fitted with a Boltzmann curve in Origin Pro. The Boltzmann curves were averaged to obtain minimum and maximum force values, as well as the frequency of half-maximum force.

**Protein and mRNA Measurements**

The *gastrocnemius* muscle was removed from mid-stage (6-9 weeks) R6/2 and WT mice, snap frozen in liquid nitrogen, and stored at -80°C. Muscles were shipped to the lab of Dr. Robert J. Talmadge of California State Polytechnic University, Pomona (Sotelo, 2021).

**Western Blots:** Muscle samples were homogenized in RIPA buffer, with protease and phosphatase inhibitors added. After centrifugation, the supernatants (cytosolic and membrane fraction) were analyzed for SERCA isoforms and parvalbumin, and the pellets (myofibril fraction) were analyzed for MyHC and MyLC isoforms. Protein was quantified via the Bradford method (Bio-Rad). Protein samples were run through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose (NC) membrane (Trans-Blot® Turbo™ Ready-to-Assemble Transfer Kit).
Ponceau Red was used to assess uniform protein loading. To assess SERCA1 content, membranes were probed with IIH11 monoclonal mouse primary antibodies (Affinity 11). To assess SERCA2, blots were probed with 2A7 mouse monoclonal primary antibodies (Invitrogen). For parvalbumin, blots were probed with PA1-933 polyclonal rabbit primary antibodies (Affinity BioReagents). For secondary antibodies, anti-mouse (for SERCA) or anti-rabbit (for parvalbumin) IgG conjugated with horseradish peroxidase (HRP) from Cell Signaling Technology was used. Proteins were visualized with chemiluminescence using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) and the FluorChem (Alpha Innotech) Imaging System. Molecular weight markers (PageRuler Plus pre-stained protein ladder) were rendered fluorescent with a Li-Cor® WesternSure® Pen prior. Band intensities were acquired by scanning line densitometry of all lanes simultaneously. Band intensities were normalized to Ponceau Red staining (which was not different across groups).

Myosin Analyses: To assess the proportional content of MyHC isoforms, myofibrillar protein samples (i.e., the resuspended pellets) were subjected to a high resolution SDS-PAGE procedure (Talmadge & Roy, 1993). To assess the protein content of MyLC isoforms, protein samples were run through an SDS-PAGE protocol adapted from Laemmli (1970)(Laemmli, 1970).

RT-qPCR: Muscle samples were homogenized in TRIzol™ Reagent and RNA was isolated. The RNA was quantified via spectrophotometry at A260. The purity of the RNA was determined using the A260:A280 ratio. All samples had A260:A280 ratios above 1.95, well above the minimal purity (A260:A280 of 1.8). For mRNA quantification, Real-Time (Quantitative) Polymerase Chain Reaction was performed with
primer probes for each gene of interest using the TaqMan® Assay in an MJ Research DNA engine Opticon 2 thermocycler. Samples underwent 40 cycles of 95 °C for 20 seconds, and 60 °C for 1 min, followed by fluorescence detection. Cycle threshold (CT) values were determined for each sample (mid exponential phase), allowing the use of the delta delta CT method to calculate relative expression against actin (Act1).

Statistics

**Twitch Force:** To determine whether a significant mean difference exists between control, 9-AC/Ba²⁺, and R6/2 mice, a series of mixed effects ANOVAs were run with the twitch force parameters and EDL weights as dependent variables and group as the independent variable. Multiple measurements were taken from each mouse, so a random effect for the mouse was included to account for potentially correlated measurements from the same mouse. Post-hoc multiple comparisons for statistically significant variables were carried out via Tukey’s multiple comparison procedure.

**Force-frequency relationship:** To determine whether any significant mean difference exists between control, 9-AC/Ba²⁺, and R6/2 mice across various frequencies, a series of repeated measures ANOVAs were run with peak tetanic force as dependent variables. The repeated measure was the stimulation frequency since the same mice were measured across the range of frequencies. Multiple measurements were taken from some mice, so a random effect for the mouse was included to account for potentially correlated measurements from the same mouse at the same frequency. Post-hoc comparisons between the three groups at similar frequencies were carried out via the Bonferroni sequentially rejective multiple comparison procedure.
Results

Isometric force was measured in ex vivo experiments from the extensor digitorum longus (EDL) muscle. The EDL was placed into a custom recording chamber filled with extracellular solution. The ex vivo experiments allowed for the addition of 9-AC and Ba$^{2+}$ to the extracellular solution to partially block ClC-1 and Kir currents, and mimic the phenotype of R6/2 muscle (D. R. Miranda et al., 2017; Waters et al., 2013), as was done in Specific Aim 1 (CHAPTER III). It should be pointed out that the AP train (CHAPTER III) and AP/Ca$^{2+}$ (CHAPTER IV) measurements were recorded from single muscle fibers (FDB and IO muscle) in the presence of high intracellular EGTA, while the present ex vivo force measurements were recorded from whole muscle (EDL) without any direct changes made to the intracellular environment. Therefore, during force measurements, Ca$^{2+}$ was able to bind parvalbumin, ATP, troponin C, and SERCA instead of EGTA, more accurately recapitulating the dynamics intracellular Ca$^{2+}$ that occur in vivo. Thus, one caveat to directly comparing the data between APs, Ca$^{2+}$, and force generation is that APs/Ca$^{2+}$ experiments were carried out within a different system than the force measurements. However, this caveat is also an advantage, in that the following ex vivo force experiments are more physiologic than the AP/Ca$^{2+}$ measurements.

Normal R6/2 Specific Twitch Force

The average absolute (raw) twitch force for control, R6/2, and 9-AC/Ba$^{2+}$ muscle are shown in Figure 13A. Without normalizing the force data to muscle size, the peak absolute twitch force was not significantly reduced in R6/2 muscle compared to the controls and 9-AC/Ba$^{2+}$ (Figure 13B), but trended lower ($p = 0.084$). A decrease in R6/2
absolute twitch force is expected, not only because reduced absolute peak twitch force has been reported in R6/2 EDL previously (Hering et al., 2016; Mielcarek et al., 2015), but also because of severe muscle atrophy, which is a hallmark characteristic of R6/2 muscle (She et al., 2011). In this study, the EDL weights of the R6/2 mice were significantly reduced and about half the size of control and 9AC/Ba²⁺ EDLs (Figure 13C). To account for this extreme atrophy, we normalized the absolute force to the EDL weight to obtain the specific force. Once the atrophy was accounted for, the peak specific force was not significantly different between R6/2, control, and 9-AC/Ba²⁺ muscle (Figure 13D) and did not trend lower in R6/2 muscle. The average control, 9-AC/Ba²⁺, and R6/2 specific twitch force are shown in Figure 13E. As can be seen, there were no differences in the time-to-peak (from 10 to 90% of the peak) (Figure 13F), half-width (Figure 13G), or half-maximum relaxation time (Figure 13H). These results show that, when accounting for muscle atrophy, twitch force is normal in R6/2 muscle.

**Force-Frequency Relationship**

Peak force generation was measured in response to 15 pulse stimulation trains of increasing frequency ranging from 0.3 Hz to 160 Hz. There was not a significant difference in the absolute force (Figure 14A) between control, R6/2, and 9-AC/Ba²⁺ between 0.3 Hz and 30 Hz, although R6/2 force trended lower than control at 20 Hz (p = 0.093), and trended lower than both control (p = 0.0501) and 9-AC/Ba²⁺ (p = 0.084) at 30 Hz. Between 40 Hz and 160 Hz, R6/2 tetanic force was significantly lower than control and 9-AC/Ba²⁺ muscle. The absolute force-frequency relationship would suggest that reduced ClC-1 and Kir currents do not contribute to muscle weakness in R6/2 muscle.
Figure 13. Twitch force. (A) Average absolute and specific (E) twitch force for control (n = 4 mice, 7 muscles), R6/2 (n = 5 mice, 9 muscles), and 9-AC/Ba²⁺ (n = 4 mice, 7 muscles). Box and whisker plots for (B) absolute peak (C) EDL weight, (D) specific peak, (F) time-to-peak, (G) half-width, and (H) half-max relaxation time. Box and whisker plots show the 25th and 75th percentile (box), mean (white box), median (line), and 1.5 interquartile range (error bars). Significant values (p < 0.05) shown in figure.
because the 9-AC/Ba\(^{2+}\) group was not significantly different from controls. However, R6/2 muscle is known to have defects in addition to reduced ClC-1 and Kir currents, including membrane hyperexcitability (Waters et al., 2013) and atrophy (Figure 13C). Therefore, reduced ClC-1 and Kir currents in WT muscle (9-AC/Ba\(^{2+}\) group) do not fully replicate the HD phenotype and the interaction between reduced ClC-1/Kir currents and other cellular defects. Thus, reduced ClC-1/Kir currents in R6/2 muscle still may contribute to muscle weakness.

After accounting for muscle atrophy by normalizing the force to muscle size and obtaining the specific force (Figure 14B), there was no significant difference between control, R6/2, and 9-AC/Ba\(^{2+}\) force at any frequency. Still, R6/2 force trended lower than control force throughout the entirety of the plateau phase of the curve at 80 Hz (p = 0.082), 100 Hz (p = 0.059), 120 Hz (p = 0.059), 140 Hz (p = 0.066), and 160 Hz (p = 0.08). These data suggest that muscle atrophy only partially contributes to muscle weakness during tetanic force trains. The additional weakness may be due to reduced ClC-1 and Kir currents.

*Altered R6/2 Myosin, SERCA, and Parvalbumin*

The normal specific twitch force in R6/2 muscle could be explained by atrophy and normal Ca\(^{2+}\) available for contraction (CHAPTER IV), but since the Ca\(^{2+}\) available for contraction trended lower in R6/2 muscle (CHAPTER IV), it is possible that other factors contribute to normal specific twitch force. Therefore, we investigated several other potential mechanisms for normal twitch force. One of the major proteins responsible for determining the strength of mechanical contraction in muscle fibers is
Figure 14. Force-frequency relationship. (A) Average absolute and (B) specific peak tetanic force in response to increased stimulation frequency between control (n = 4 mice, 7 muscles), R6/2 (n = 5 mice, 9 muscles), and 9-AC/Ba²⁺ (n = 4 mice, 7 muscles). * = significant difference between control and R6/2 (p ≤ 0.0035).
myosin. We have previously shown that myosin heavy chain (MyHC) isoforms are altered in end-stage R6/2 muscle (Miranda et al., 2017). Here we measured MyHC isoforms from mid-stage R6/2 gastrocnemius muscle at the mRNA and protein level. Both MyHC1 and neonatal MyHC mRNA were significantly increased in R6/2 muscle compared to the muscle of WT littermates (Figure 15A), suggesting a disruption of muscle maturation early in disease progression (Miranda et al., 2017). However, at the protein level, there were no differences in MyHC expression (Figure 15B), indicating that myosin may not be contributing to normal force production. Myosin light chain (MyLC) is involved in regulating the binding of MyHC and therefore may directly be involved in twitch potentiation. To explore this, we measured MyLC isoforms at the mRNA and protein level. While MyLC1f and MyLC2f mRNA were significantly reduced in R6/2 muscle (Figure 16C), there were no differences at the protein level (Figure 16D).

Sarcoendoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) acts as the primary removal mechanism of Ca\(^{2+}\) from the myoplasm. A decrease in SERCA activity or protein expression would suggest that Ca\(^{2+}\) remains in the sarcoplasm where it can bind to troponin C and activate contraction. In mid-stage R6/2 muscle, we measured two of the primary isoforms of SERCA (SERCA1 and SERCA2) at the mRNA and protein level. We found that SERCA1 was reduced and SERCA2 increased at the mRNA level in R6/2 muscle, but there were no differences at the protein level (Figure 17A-B).
Figure 15. Myosin heavy chain. Differences in (A) mRNA and (B) protein expression of myosin heavy chain (MyHC) isoforms in WT (n = 5) and R6/2 (n= 5) muscle. * = significant difference (p < 0.05).
Figure 16. Myosin light chain. Differences in (A) mRNA and (B) protein expression of myosin light chain (MyLC) isoforms between WT (n = 5) and R6/2 (n= 5) mice. * = significant difference (p < 0.05).
Figure 17. **SERCA and parvalbumin.** Differences in \((A)\) mRNA and \((B)\) protein expression of SERCA isoforms and parvalbumin between WT \((n = 5)\) and R6/2 \((n = 5)\) mice. \(* = \) significant difference \((p < 0.05)\).
Parvalbumin is a Ca\textsuperscript{2+} binding protein that resides in the myoplasm of fast-twitch muscle fibers (Berchtold et al., 2000). We measured the expression of parvalbumin at the mRNA and protein level. In mid-stage R6/2 muscle, both parvalbumin mRNA and parvalbumin protein were reduced (Figure 17A-B). These findings suggest that the level Ca\textsuperscript{2+} binding potential is reduced in R6/2 muscle, which may implicate parvalbumin pathology as a contributor to normal specific twitch force.
Discussion

To determine whether APs and/or reduced ClC-1 and Kir currents contribute to changes in muscle force generation in R6/2 muscle, we measured twitch and tetanic force from R6/2 muscle and from WT muscle with ClC-1/Kir channels partially blocked with 9-AC/Ba^{2+}. We showed that specific twitch force was normal in R6/2 muscle, after accounting for atrophy. We also showed reductions in tetanic force in R6/2 muscle, which is possibly due to reduced ClC-1/Kir currents. In addition, we found a significant reduction in parvalbumin from mid-stage R6/2 muscle, which may contribute to the normal twitch force we observed.

Theoretical Basis of Reduced Force from Reduced ClC-1/Kir Currents in R6/2 Muscle

We previously showed that ClC-1 currents are reduced by 70% and Kir currents reduced by 30% in R6/2 muscle (Miranda et. al, 2017). We therefore used concentrations of 9-AC and Ba^{2+} that would partially block ClC-1 and Kir channels by 70% and 30%, respectively. Previously, we used this strategy to measure the effect of reduced of ClC-1/Kir currents on AP trains. We found that reduced ClC-1 and Kir currents were associated with the depolarization of the baseline membrane potential during AP trains (D. R. Miranda et al., 2020)(CHAPTER III), which could lead to decreased muscle force generation.

The movement and balance of Cl\(^{-}\), K\(^{+}\), and Na\(^{+}\) ions across the cell membrane of muscle fibers is important for sustained membrane excitability and muscle contraction. ClC-1 and Kir channels are the primary channels responsible for keeping the resting membrane potential of skeletal muscle fibers at about -90 mV (Goldin et al., 2000).
Depolarizations of the membrane potential (values more positive than -90 mV) are ultimately restored by ClC-1 and Kir currents. During muscle contraction, voltage-activated Na\(^+\) and K\(^+\) currents form APs in the membrane potential waveform while ClC-1 and Kir currents influence the baseline membrane potential (the membrane potential between each AP during an AP train) by keeping it closer to -90 mV (D. R. Miranda et al., 2020)(CHAPTER III).

ClC-1 and Kir currents help to maintain the excitability of muscle fibers. ClC-1 and Kir channels, primarily ClC-1 channels (Tekle, Astumian, & Chock, 1990; Wright, 2004), are important for sustained muscle contraction/AP trains because in cases where the membrane potential becomes too depolarized (more positive than about -50 mV), the voltage-gated Na\(^+\) (Nav1.4) channels can no longer conduct current, and APs can no longer fire. This occurs in myotonia (Wheeler, Lueck, Swanson, Dirksen, & Thornton, 2007), where a lack of membrane potential stabilization from decreased ClC-1 function renders muscle fibers inexcitable (Myers et al., 2021). The AP amplitude also decreases as the membrane depolarizes during AP trains (CHAPTER III). The amplitude of an AP is an indicator of the level of membrane depolarization, which is necessary to evoke voltage-activated intracellular Ca\(^{2+}\) release and muscle contraction. Thus, a decrease in AP amplitude signifies insufficient depolarization of the membrane, which leads to less intracellular Ca\(^{2+}\) release and weaker muscle contraction.

**Normal R6/2 Twitch Force**

Muscle twitch force is the force produced by a single AP (Watanabe & Wada, 2020), therefore it is difficult to explain the effect of reduced ClC-1 and Kir currents on
twitch force, since there is no changes in baseline membrane potential during a single AP. Our data suggest that reduced ClC-1/Kir currents do not affect twitch force, at least when acutely blocked in WT muscle. However, we previously showed that R6/2 muscle APs are significantly prolonged (CHAPTER III) (Waters et al., 2013), which could explain the normal specific twitch force. When not accounting for muscle atrophy, we showed that the peak twitch force in R6/2 muscle trended low when compared to controls and 9-AC/Ba2+. This is in line with previous studies that reported reduced non-normalized peak twitch force in R6/2 EDL (Hering et al., 2016; Mielcarek et al., 2015). However, non-normalized force data does not account for the atrophy that is characteristic of R6/2 muscle (She et al., 2011).

To account for the contribution of muscle atrophy in R6/2 mice, we normalized our force data to muscle size (specific force). Surprisingly, the specific peak twitch force was normal in R6/2 mice. In contrast, another study has reported that specific peak twitch force is reduced in R6/2 EDL (Hering et al., 2016), which may be due to differences in experimental conditions. For example, Hering and colleagues used a vertically mounted test chamber, whereas we used a horizontally mounted test chamber. It is unclear how a vertical versus horizontal test chamber would cause differences in muscle force, but there were also some stark differences in peak absolute force. In Hering and colleagues’ study (in the same mouse model and muscle as our study), the average WT absolute peak twitch force was ~0.02 N and the R6/2 peak twitch was ~0.004 N (Hering et al., 2016), which are approximately 3.5-fold and 10-fold lower than our average values for control (~0.07 N) and R6/2 (~0.04 N) absolute peak twitch force, respectively (Figure 13B). Our results agree with data obtained from Q175 mice by Dridi and colleagues who found no
differences in EDL specific force, although specific force was reduced in the diaphragm and restored by drugs that fix RyR1 leak (Dridi et al., 2020). Also, in the BACHD mouse model, there was no reported differences in diaphragm twitch force (Priscila A. C. Valadão et al., 2018), suggesting differences in HD mouse models.

In addition to observing no differences in peak specific twitch force, we also did not observe changes in time-to-peak, half-maximum relaxation time, or half-width in R6/2 EDL. This is in contrast to previous studies showing that both rise time and $\frac{1}{2}$ relaxation time are increased in R6/2 EDL (Bondulich et al., 2017; Hering et al., 2016; Mielcarek et al., 2015). Again, this discrepancy may be to differences in experimental setup and testing chamber, or possibly due to differences in protocols. We have observed that the time-to-peak, half-maximum relaxation time, and half-width of the R6/2 twitch increases more than controls with continued stimulation (unpublished data). For our single twitch data, we analyzed only the first twitch to be recorded from the muscle.

Overall, a normal specific twitch force in R6/2 muscle would suggest that muscle weakness in HD is due to atrophy, which corresponds to the normal levels of $\text{Ca}^{2+}$ available for contraction observed in R6/2 muscle (CHAPTER IV). Because the normal $\text{Ca}^{2+}$ available for contraction was a result of increased $\text{Ca}^{2+}$ release duration that compensated for reduced peak $\text{Ca}^{2+}$ release flux, and the increased $\text{Ca}^{2+}$ release duration was associated with prolonged APs in R62 muscle (CHAPTER IV), it seems that the prolonged APs compensated for muscle weakness. This could be explained by $\text{Ca}^{2+}$ increasing the number of cross-bridges cycles, which are activated when $\text{Ca}^{2+}$ binds to troponin C. If it is assumed that the concentration of troponin C binding sites for $\text{Ca}^{2+}$ is 240 $\mu$M (Baylor & Hollingworth, 2007), then increasing the $\text{Ca}^{2+}$ available for
contraction in R6/2 muscle (~105 µM) should activate more cross-bridges and increase force production. These results emphasize the importance of the integrative and broad approach we took in studying HD muscle function. For example, we, as well as Braubach and colleagues both found that the peak Ca²⁺ release flux was reduced, and that APs were prolonged in R6/2 muscle (CHAPTER IV) (Braubach et al., 2014), yet Braubach and colleagues did not consider whether the APs affected the Ca²⁺ release flux, which may be why they did not measure the duration of Ca²⁺ release. Also, Braubach and colleagues (2014) did not measure muscle force, while in other studies, force, but not APs and Ca²⁺ release flux were measured (Bondulich et al., 2017; Hering et al., 2016; Mielcarek et al., 2015). Thus, by measuring the entire sequence of events in EC coupling, our interpretation may be more complete.

**Contribution of Muscle Atrophy and Ion Channel Defects to Weakness in HD**

We showed that the absolute tetanic force in R6/2 muscle was lower than controls, in line with previous studies (Bondulich et al., 2017; Hering et al., 2016; Mielcarek et al., 2015). The specific tetanic force also trended lower in R6/2 muscle, in line with a previous study (Hering et al., 2016). Since the 9-AC/Ba²⁺ muscle was not significantly different than controls, it would seem that reduced ClC-1 and Kir currents do not lead to muscle weakness. However, this may not be the case. In Specific Aim 1, we showed that reduced ClC-1 and Kir currents depolarize the baseline membrane potential during 40 Hz and 60 Hz AP trains, but not 20 Hz AP trains (CHAPTER III) and it was at 40 Hz that the R6/2 force significantly diverged from controls in the force-frequency (FF) curve. Thus, it may be possible that, since the resting membrane potential
of R6/2 muscle is already depolarized (Ribchester et al., 2004), the additional
depolarization during high frequency stimulation (40 Hz and above) in the FF curve
could lead to a decline in AP amplitude and a decrease in force generation. This decline
in AP amplitude may not have shown up in the AP train recordings because the
membrane potential was held at -85 mV (CHAPTER III), artificially hyperpolarizing the
R6/2 fibers. Furthermore, within the first 15 APs of the AP trains (corresponding to the
force-frequency curve), the R6/2 membrane potential depolarized immediately, while the
9-AC/Ba^{2+} membrane potential depolarized gradually (CHATPTER III), which could
also lead to a decrease in AP amplitude in R6/2 muscle but not 9-AC/Ba^{2+} muscle within
the short (15 pulse) trains of the force-frequency curve. Additionally, since the AP
amplitude was already reduced in R6/2 muscle (CHAPTER III and IV), it could decline
to point of not generating force faster than in 9-AC/Ba^{2+} muscle, where the AP amplitude
is normal. Overall, based on our integrative approach, we conclude that it is still possible
the reduced ClC-1 and Kir currents, in combination with reduced AP amplitude in R6/2
muscle leads to reduced tetanic force. Aside from reduced ClC-1 and Kir currents,
muscle weakness in R6/2 muscle (after accounting for atrophy) could also be due to
muscle remodeling, including an increase in type I from type II fibers, and alterations in
gene expression (Hering et al., 2016; Mielcarek et al., 2015). However, these factors may
not explain normal specific twitch force and other factors may also be involved.

Other Possible Factors Contributing to Normal R6/2 Twitch Force

There are several possible mechanisms by which low Ca^{2+} release can produce
normal force, some of which we investigated. One possible mechanism is that Ca^{2+}
sensitivity is high in R6/2 muscle. \( \text{Ca}^{2+} \) sensitivity is controlled by regulatory MyLC which, when phosphorylated can increase the binding capacity of MyHC and increase force production (Ryder, Lau, Kamm, & Stull, 2007). We observed a reduction in regulatory MyLC2f mRNA in mid-stage R6/2 muscle. Similarly, reduced phosphorylatable fast myosin light chain mRNA was reduced (Strand et al., 2005) and regulatory MyLC protein unchanged in end-stage R6/2 muscle (Hering et al., 2016). There is still the possibility that the phosphorylation state of regulatory MyLC is increased in R6/2 muscle. To support this, increased regulatory MyLC phosphorylation has been observed in Q175 mouse cardiomyocytes along with increased AP duration and normal peak force (Joviano-Santos et al., 2019). Thus, the possibility that \( \text{Ca}^{2+} \) sensitivity is high in R6/2 muscle remains open for future studies.

Another potential mechanism by which low \( \text{Ca}^{2+} \) could lead to normal force production involves the dynamics of \( \text{Ca}^{2+} \) after it has been released from the SR. For example, the reuptake of \( \text{Ca}^{2+} \) back into the SR by SERCA could be decreased, which would increase the amount of \( \text{Ca}^{2+} \) available in the sarcoplasm to activate the contractile filaments. However, it does not seem that SERCA is involved in the normal specific twitch force of R6/2 muscle because we observed no changes in SERCA protein. Still, it is possible that the function of SERCA is altered in R6/2 muscle. This is supported by reduced SERCA1 mRNA and increased SERCA2 mRNA, suggesting both fiber-type switching and dysfunctional SERCA in mid-stage R6/2 muscle. Indeed, in their analysis of \( \text{Ca}^{2+} \) homeostasis in R6/2 muscle, Braubach and colleagues showed that \( \text{Ca}^{2+} \) removal was decreased, probably due to slowed SERCA function (Braubach et al., 2014). Future
studies of the direct contribution of SERCA to Ca\textsuperscript{2+} reuptake in HD skeletal muscle may confirm this.

\textit{Reduced Parvalbumin Enhances Twitch but not Tetanic Force}

Based on our results, the most probable contributing factor to normal specific twitch force in R6/2 muscle, in addition to the normal levels of Ca\textsuperscript{2+} available for contraction (CHAPTER IV) is a reduction in parvalbumin. Parvalbumin is a major sarcoplasmic Ca\textsuperscript{2+} binding protein and, in its absence or depletion Ca\textsuperscript{2+} is free to activate the contractile filaments and generate force (Murphy et al., 2012; Röhrle, Neumann, & Heidlauf, 2016). Thus, the reduction in parvalbumin we observed in mid-stage R6/2 muscle could lead to a higher level of intracellular Ca\textsuperscript{2+} in R6/2 muscle. Further research on late-stage R6/2 mice will be necessary, but we assume that the reduction in parvalbumin protein will be sustained, or even decreased further with disease progression, as do many of the muscle defects we have previously observed in R6/2 muscle (D. R. Miranda et al., 2017). To support this, reduced parvalbumin mRNA has been observed in end-stage R6/2 mouse muscle (Strand et al., 2005).

While we observed normal specific twitch force in R6/2 muscle, tetanic force was reduced. If reduced parvalbumin was responsible for higher Ca\textsuperscript{2+} availability in R6/2 muscle at low frequency stimulation, then it is possible that at higher frequency stimulation parvalbumin becomes saturated and the level of Ca\textsuperscript{2+} available for contraction becomes similar between R6/2 and control. In cases of saturated parvalbumin, the R6/2 force is no longer potentiated and the reduction in in peak Ca\textsuperscript{2+} release flux leads to muscle weakness. Future studies utilizing genetic manipulation of the parvalbumin gene
will be needed to confirm the role of parvalbumin in regulating muscle force in R6/2 muscle.

**Conclusion**

In this study, we blocked neuromuscular transmission and directly stimulated muscle to generate force, so it is unlikely that the changes in muscle force we observed in R6/2 muscle were due to neuronal defects. We observed a trend towards a lower peak twitch force in R6/2 muscle, as would be expected for muscle weakness. Surprisingly, the peak twitch force was normalized after accounting for muscle atrophy. Based on this, it would seem that muscle twitch force is reduced in R6/2 muscle solely due to atrophy. However, in Specific Aim 2 (CHAPTER IV) we observed a decrease in peak Ca\(^{2+}\) release flux in R6/2 muscle. In CHAPTER IV, the prolonged duration of R6/2 APs was associated with an increase in Ca\(^{2+}\) release duration, which compensated for the reduction in peak Ca\(^{2+}\) release flux, leading to a trend towards reduced Ca\(^{2+}\) available for contraction. The reduction in Ca\(^{2+}\) available for contraction would decrease the peak twitch force. Since we observed a reduction in parvalbumin in R6/2 muscle, it seems that parvalbumin compensated for this reduction in Ca\(^{2+}\) available for contraction, allowing for normal peak twitch force after accounting for atrophy. Overall, in the case of twitch force, we hypothesize that the prolongation of APs in R6/2 muscle (CHAPTER III & IV) (Waters et al., 2013), in combination with reduced parvalbumin leads to higher levels of myoplasmic Ca\(^{2+}\), which increases atrophy-corrected force production to normal levels (Borja-Oliveira, Pertinhez, Rodrigues-Simioni, & Spisni, 2009; Williams & Barnes, 1989). The prolongation of APs in R6/2 muscle may be due to a positive inotropic effect,
where force generation is increased by compensatory mechanisms such as
downregulation of Kv channels (CHAPTER III), as is thought to occur in cardiac muscle
in response to heart failure (Wickenden et al., 1998).

The remarkable compensatory effect of prolonged APs, in addition to reduced
parvalbumin in R6/2 muscle seems to breakdown during sustained contraction at higher
frequencies. We observed that tetanic force was reduced in R6/2 muscle and trended
reduced after accounting for atrophy. This reduction could be due to saturation of
parvalbumin, as well as the degradation of APs and/or Ca\(^{2+}\) availability. For example, the
peak amplitude of APs could decrease (due to reduced CIC-1/Kir-evoked membrane
depolarization) to the point where the duration of the APs no longer compensates for
reductions in the peak Ca\(^{2+}\) release flux by increasing the duration of Ca\(^{2+}\) release.
Overall, our results support other studies showing that muscle weakness in HD is not
only due to atrophy, but other muscle specific defects (Bondulich et al., 2017; Dridi et al.,
2020; Hering et al., 2016; Mielcarek et al., 2015). Therefore, treatments targeting
prevention of muscle atrophy and other muscle defects may be beneficial for HD patients.
VI: DISCUSSION

Skeletal muscle EC coupling is the sequence of cellular events that lead to muscle contraction, the primary function of skeletal muscle. HD patients suffer from muscle rigidity, dystonia, and motor impersistence, which leads to abnormal movement (Bates et al., 2014), yet EC coupling in HD skeletal muscle has not yet been fully characterized. Therefore, we investigated the full sequence of EC coupling events in the R6/2 transgenic mouse model for HD.

The first step of EC coupling is the excitation step, which refers to the electrical activation of muscle via changes in the membrane potential, measured as APs. The AP waveform depends on ionic currents, primarily Nav1.4 and Kv currents, however, ClC-1 and Kir currents may also influence the AP waveform, especially during AP trains (T. H. Pedersen et al., 2016; Wallinga et al., 1999). In Specific Aim 1, we examined AP trains in R6/2 muscle, previously observed to have reduced ClC-1 and Kir currents (Waters et al., 2013). The reduction in ClC-1 and Kir currents was associated with a more depolarized baseline membrane potential during 40 Hz and 60 Hz AP trains in R6/2 muscle, yet, although reduced the peak amplitude of the APs persisted at a level that would not be expected to lead to muscle weakness (Cairns et al., 2003; Gong et al., 2003; Yensen et al., 2002). However, we held the membrane potential at -85 mV, which is more negative than what has been reported for the membrane potential in R6/2 muscle (Ribchester et al., 2004). Therefore, depolarization caused by ClC-1/Kir currents in combination with a reduction in AP peak amplitude we could lead to muscle weakness. We also showed that the falling phase of the R6/2 APs were significantly prolonged, regardless of single or repeated stimulation. The AP falling phase is shaped by Kv channels (DiFranco et al.,
2012), so we investigated the mRNA levels of two major Kv channel isoforms, Kv1.5 and Kv3.4 and found that both were significantly reduced in R6/2 muscle. For Specific Aim 1, we conclude that the peak amplitude of R6/2 APs and reduced ClC-1/Kir currents may contribute to muscle weakness and that prolonged APs are due to a reduction in Kv channels.

The second step of EC coupling involves the activation of intracellular Ca\(^{2+}\) release by APs. In Specific 2, we simultaneously measured APs and intracellular Ca\(^{2+}\) release in R6/2 muscle. We had previously shown that single APs are prolonged in R6/2 muscle (Waters et al., 2013), which led us to analyze several aspects of the Ca\(^{2+}\) release flux, including the Ca\(^{2+}\) release duration. There was a significant reduction in the peak Ca\(^{2+}\) release flux, as had been previously reported (Braubach et al., 2014), but there was also a significant increase in the Ca\(^{2+}\) release duration. Also, the Ca\(^{2+}\) available for contraction was normal in R6/2 muscle, demonstrating that the increase in Ca\(^{2+}\) release duration compensates for the reduction in peak Ca\(^{2+}\) release flux. Recently, the increase in Ca\(^{2+}\) release duration compensating for reduced peak Ca\(^{2+}\) release flux was also shown R6/2 pyramidal neurons (Oikonomou, Donzis, Bui, Cepeda, & Levine, 2021). For Specific Aim 2, we conclude that an increase in AP duration increases the amount of Ca\(^{2+}\) released per AP in R6/2 muscle.

In the third step of EC coupling, Ca\(^{2+}\) activates the contractile filaments, which causes muscle contraction and force generation. In Specific Aim 3, we examined force generation in R6/2 muscle. To do this, we measured isometric twitch force, which is the force generated by a single AP, as well as isometric tetanic force, the force generated by trains of APs. When not accounting for muscle atrophy, the twitch force in R6/2 muscle
was reduced. However, atrophy is a prominent feature of R6/2 muscle, so we normalized the twitch force to muscle size, to account for atrophy. When accounting for atrophy, the twitch force in R6/2 muscle was normal. In contrast, the tetanic force was reduced even after accounting for atrophy, suggesting that muscle weakness in HD is due to atrophy and other muscle defects, including reduced ClC-1/Kir currents and parvalbumin.

Together, the results of Specific Aims 1 through 3 would suggest that prolonged APs in R6/2 muscle serve to increase the Ca\(^{2+}\) release duration. The increase in Ca\(^{2+}\) release duration compensates for the decrease in peak Ca\(^{2+}\) release flux, which leads to normal levels of Ca\(^{2+}\) available for contraction. The normal levels of Ca\(^{2+}\) available for contraction then contribute to normal twitch force per muscle size. In addition, reduced parvalbumin in R6/2 muscle may also contribute to normal specific twitch force. During tetanic muscle force, it is hypothesized that in addition to atrophy, decreased ClC-1 and Kir currents, and saturated parvalbumin contribute to weakness in R6/2 muscle. Overall, it seems that atrophy, along with other muscle defects lead to muscle weakness, and that the downregulation of Kv channels is a compensatory mechanism to counter atrophy and weakness by increasing intracellular Ca\(^{2+}\) release and increasing force production.

\textbf{NOTE:} In searching the literature for possible mechanisms by which Kv channels could be downregulated in R6/2 muscle, other than those mentioned in Specific Aim 1 (CHAPTER III), there was not enough evidence to support any one possibility. For this reason, and because HD cellular pathology is incompletely understood, portions of this discussion consist of speculation and conjecture.
Expression of Kv11.1 in Atrophied Muscle

A Kv channel expressed in atrophied skeletal muscle may be present in HD muscle and contribute to prolonged APs. The erg K+ channel (Kv11) is primarily expressed in fully developed cardiac muscle and neurons, but is also expressed at very low levels in mature muscle fibers, as well muscle fibers undergoing embryonic development (Simone et al., 2003; Xun Wang 2006). In addition, Kv11 is expressed in atrophied mouse skeletal muscle and has been shown to induce atrophy by activating the ubiquitin proteasome pathway (Xun Wang 2006). Recently, it was shown that Kv11.1 activates several genes related to muscle atrophy, including SMAD and interferon (Pond, Whitmore, Thimmapuram, & Hockerman, 2021) and is expressed in the human tissue of cancer patients and elderly with atrophied muscle (Zampieri et al., 2021), indicating that it may be expressed in the atrophied muscle of HD mouse models and HD patients. When C2C12 myotubes were transduced with human Kv11.1, basal intracellular Ca2+ was elevated and calpains were activated (Whitmore et al., 2020), which occurs in HD neurons and may occur in HD muscle. In cardiac muscle, different combinations of Kv11 isoforms results in changes in AP duration (Larsen & Olesen, 2010). Although these APs are 300 – 400 ms in duration in cardiac muscle, and are activated at -30 mV, it is possible that they may be differentially regulated in skeletal muscle and expressed in combination with other Kv channels, like Kv3.4, Kv1.5, to produce prolonged APs. By elucidating the role Kv channel expression and regulation in HD muscle, it is possible that they may become a target for treatment of HD muscle symptoms, as well as symptoms resulting from neuronal dysfunction, since differences in AP duration have also been observed in HD neurons.
AP and K⁺ channel Defects in HD Striatal Neurons

Though we are the first to characterize APs in HD skeletal muscle, changes in the AP waveform in the striatal neurons of several HD mouse models have been observed previously. The striatum of the brain and the neurons it contains are selectively vulnerable to the huntingtin mutation and degeneration of this region is thought to be the epicenter of HD symptoms (Bates et al., 2014). In the striatal spiny neurons of R6/2 mice, APs were shown to be prolonged, but were otherwise normal (Klapstein et al., 2001). The amplitude of APs were reduced in the medium spiny neurons of Q175 mice, but the duration of the AP was not reported (Heikkinen et al., 2012). In Q175 mice, it was found that dopaminergic neurons from the direct pathway (D1) differ from the indirect pathway (D2), for example the AP amplitude was reduced and rise time increased in D1 but not D2 neurons (J. W. Goodliffe et al., 2018). Interestingly, indirect pathway striatal neurons were shown to have a low AP amplitude in 3-month-old Q175, but not 12-month-old Q175 mice (Sebastianutto, Cenci, & Fieblinger, 2017). In the medium spiny neurons of a modified BACHD HD mouse model that expresses the huntingtin mutation only in the nucleus (BACHDΔN17), the rise and falling phase of APs was increased (J. Goodliffe, Rubakovic, Chang, Pathak, & Luebke, 2020). Though there are differences between mouse models and cell types, overall, there seems to be a pattern of increased AP duration and reduced amplitude in striatal neurons. Like in skeletal muscle, the prolonged striatal APs seem to be due to a reduction in K⁺ currents.

Reduced K⁺ currents have been observed in the striatal neurons of several mouse models of HD. Inward and outward K⁺ currents were shown to be reduced and associated with reduced Kir2.1, Kir2.3, and Kv2.1 protein in R6/2 and R6/1 medium
spiny neurons (Ariano, Cepeda, et al., 2005; Ariano, Wagle, & Grissell, 2005). In striatal neurons of the indirect pathway, inward and voltage-gated K⁺ currents were reduced in R6/2 and Q175 mice (Sebastianutto et al., 2017). Using proteomic analysis of Q175 brain tissue, Kir2.3 expression was found to be reduced (Langfelder et al., 2016). In the BACHDΔN17 model, several K⁺ channels were shown to be reduced, including Kv11.1 (Gu et al., 2015). Since we have previously shown a reduction in Kir currents and Kir mRNA expression (D. R. Miranda et al., 2017; Waters et al., 2013), as well as reduced Kv channel mRNA in R6/2 muscle (D. R. Miranda et al., 2020), there seems to be a shared mechanism of pathology between the striatum and muscle in HD. While not yet elucidated, it would seem that this mechanism could involve the cell-autonomous expression of mutated huntingtin in each tissue separately, rather than striatal defects leading to muscle defects. This also seems to be the case for the dysregulation of Ca²⁺ in HD.

**Dysregulation of Ca²⁺ Homeostasis in HD**

In muscle and in the nervous system, Ca²⁺ is arguably the most important signaling molecule, due to the diversity of its effectors, and the important role it plays in the primary function of neurons (transmitting and storing information) and muscle cells (contraction). For these reasons, Ca²⁺, as a secondary messenger must be tightly regulated and kept out of the cytosol, unable to freely bind to its effectors. In normal functioning muscle cells and neurons, Ca²⁺ is stored in the endoplasmic reticulum (ER), called the sarcoplasmic reticulum (SR) in muscle. Ca²⁺ is released from the ER/SR primarily through the ryanodine receptor (RyR) channel during contraction, but also from the
inositol 3-phosphate receptor (IP₃R) channel through various signaling pathways. Under pathological conditions, these Ca²⁺ release channels can leak Ca²⁺ into the cytosol unregulated. Ca²⁺ leak from the ER/SR can cause basal cytosolic Ca²⁺ levels to rise, which alters Ca²⁺ homeostasis and can lead to cell death.

Dysregulation of Ca²⁺ homeostasis occurs in mouse and cell models of HD, including muscle cells and neurons. In striatal medium spiny neurons (MSNs), mutant huntingtin has been shown to bind to the IP₃R, leading to Ca²⁺ leak from the ER and increased basal cytoplasmic Ca²⁺ (Tang et al., 2004). One of the defects caused by Ca²⁺ leak is depletion of Ca²⁺ from the ER/SR. Depletion of Ca²⁺ can cause ER/SR stress and activate of store-operated Ca²⁺ entry (SOCE), a process that refills the ER/SR directly from the extracellular space by activating Ca²⁺ influx, through channels such as Orai1 (Michelucci, García-Castañeda, Boncompagni, & Dirksen, 2018). Activation of SOCE is thought to cause ER stress and cytosolic Ca²⁺ accumulation, which ultimately leads to cell death in HD neurons (Kraskovskaya & Bezprozvanny, 2021). Whether this occurs in HD muscle is unknown, but SOCE, which can also be activated by opening the IP₃R in skeletal muscle, has been shown to be associated with muscle atrophy, oxidative stress, and mitochondrial dysfunction (Conte et al., 2021; Lilliu, Koenig, Koenig, & Frieden, 2021; Michelucci, Liang, Protasi, & Dirksen, 2021), all of which are pathologies of HD muscle. Additionally, RyR1 Ca²⁺ leak has been shown in the muscle of the Q175 HD mouse model (Dridi et al., 2020) and reduced SR Ca²⁺ store content has been reported in R6/2 muscle (Braubach et al., 2014), pointing to activation of SOCE and increased cytosolic Ca²⁺ in HD muscle. Based on recent findings, it seems unlikely that SOCE contributes to AP-evoked intracellular Ca²⁺ because blocking SOCE does not affect AP-
evoked intracellular Ca\(^{2+}\) in R6/2 pyramidal neurons (Oikonomou et al., 2021), however, SOCE may still play role in the dysregulation of Ca\(^{2+}\) homeostasis in HD muscle. Other Ca\(^{2+}\) channels, such as the L-type Ca\(^{2+}\) channels are more likely to contribute AP-evoked intracellular Ca\(^{2+}\) in HD muscle.

L-type Ca\(^{2+}\) channels are a class of voltage-gated Ca\(^{2+}\) channels (C\(_V\)) that are expressed in excitable tissues (Lipscombe, Helton, & Xu, 2004). In neurons, L-type Ca\(^{2+}\) channels are activated in response to APs and cause an influx of Ca\(^{2+}\) into the cell where it binds to the RyR and causes Ca\(^{2+}\) release, in a process known as calcium induced calcium release (Verkhratsky & Shmigol, 1996). In skeletal muscle cells, the primary L-type Ca\(^{2+}\) channel is Cav1.1, also called the dihydropyridine receptor (DHPR). In contrast to the L-type Ca\(^{2+}\) channels in neurons, the influx of Ca\(^{2+}\) through Cav1.1 is not required for RyR Ca\(^{2+}\) release, at least in normal functioning cells (Dayal et al., 2017; Tuluc et al., 2009), rather Cav1.1 physically interacts with RyR to cause Ca\(^{2+}\) release (Agrawal et al., 2018; Hernández-Ochoa & Schneider, 2018; Tanabe et al., 1990). However, since Cav1.1 conducts Ca\(^{2+}\) influx in skeletal muscle, it may contribute to AP-evoked intracellular Ca\(^{2+}\) under pathological conditions, such as in HD. In support of this, in HD mouse model cortical neurons, Cav1.2 expression and L-type Ca\(^{2+}\) currents were increased (A. S. Miranda et al., 2019). Recently, it was shown that nifedipine, an L-type Ca\(^{2+}\) channel blocker decreased AP-evoked intracellular Ca\(^{2+}\) in R6/2 pyramidal neurons (Oikonomou et al., 2021). These studies suggests that increased Ca\(^{2+}\) influx thorough L-type Ca\(^{2+}\) channels contribute to increased AP-evoked intracellular Ca\(^{2+}\) in HD neurons, and possibly muscle. L-type Ca\(^{2+}\) channel expression has not been measured in HD muscle, but, in R6/2 muscle, L-type Ca\(^{2+}\) current was predicted to be increased at membrane.
potentials less than 0 mV (Braubach et al., 2014). Also, increased Cav1.2 expression has been observed in response to exercise and fiber type switching (Jeftnija et al., 2007). Since fiber type switching occurs in HD muscle (Hering et al., 2016), it is possible that Cav1.2 expression is increased, which could cause increased L-type Ca\(^{2+}\) currents and contribute to AP-evoked intracellular Ca\(^{2+}\). Still, further studies exploring the nature of Ca\(^{2+}\) influx in HD muscle are necessary to elucidate the role of L-type Ca\(^{2+}\) channels in intracellular Ca\(^{2+}\) signaling and Ca\(^{2+}\) homeostasis.

Mitochondria are critical for Ca\(^{2+}\) homeostasis in muscle fibers and neurons because they can uptake, extrude, and store Ca\(^{2+}\) (A. Li, Yi, Li, & Zhou, 2020). When mitochondrial Ca\(^{2+}\) uptake is too high, the mitochondria become overloaded with Ca\(^{2+}\) and the mitochondrial membrane potential dissipates, leading to the opening of the mitochondrial permeability transition pore (mPTP), which releases activators of cell death, such as cytochrome C (A. Li et al., 2020) (Zhang, Zhou, Crowley-McHattan, Wang, & Li, 2021). Mitochondrial Ca\(^{2+}\) overload can be caused by increased cytosolic Ca\(^{2+}\), which has been shown in HD neurons (Kraskovskaya & Bezprozvanny, 2021), and implied in HD muscle (Braubach et al., 2014; Dridi et al., 2020). In HD neuron cell models, mutated huntingtin was shown to localize to mitochondria and increase the susceptibility mPTP opening by Ca\(^{2+}\) overload (Choo, Johnson, MacDonald, Detloff, & Lesort, 2004). Similarly, mitochondria from HD patient lymphoblast and HD mouse model brains were more susceptible to depolarization by Ca\(^{2+}\) overload (Panov et al., 2002). In R6/2 muscle, mitochondria are also more susceptible to Ca\(^{2+}\) induced mPTP opening (Gizatullina et al., 2006) and in HD patient cultured muscle cells, the mitochondrial membrane potential is altered (J. Sassone et al., 2010). In addition, muscle
cells cultured from HD patients showed signs of apoptosis with a dissipated mitochondrial membrane potential and increased cytochrome C release (A. Ciammola et al., 2006). These studies suggest that in HD skeletal muscle, mitochondrial Ca\textsuperscript{2+} overload could lead to cell death, and therefore muscle atrophy.

Overall, it appears that Ca\textsuperscript{2+} handling is impaired similarly in both neurons and muscle. Based on the above evidence, cell-autonomous expression of mutant huntingtin could be leading to dysregulation of Ca\textsuperscript{2+} homeostasis in each tissue separately. To confirm this, further studies are needed to determine if mutant huntingtin interacts with the IP\textsubscript{3}R and mitochondrial membrane in muscle cells. If so, leak of Ca\textsuperscript{2+} from the IP\textsubscript{3}R could lead to cytosolic buildup of Ca\textsuperscript{2+}, which would then cause mitochondrial Ca\textsuperscript{2+} overload and opening of the mPTP, which would lead to apoptosis in the brain (neurodegeneration) and muscle (atrophy) separately. Thus, prevention of cytosolic Ca\textsuperscript{2+} buildup would be beneficial for treating both brain and muscle pathology in HD.

_Treating the Whole Body_

The molecular underpinning of how the huntingtin gene mutation leads to cellular pathology is unclear. The expanded polyglutamine repeats of the mutated huntingtin protein, as well as the loss of normal huntingtin function both lead to cellular defects (Bates et al., 2014). Additionally, the CAG repeat expansion of huntingtin mRNA may also lead cellular defects (D. R. Miranda et al., 2017). Furthermore, there is likely an interaction between the loss of huntingtin function, the gain of mutant huntingtin function, and the cellular defense mechanisms involved to compensate for both. Ultimately, the cell is unable to defend against the huntingtin mutation, leading to
dysfunction and cell death in both neurons and muscle fibers. It is possible that since the brain and skeletal muscle are the two most metabolically active tissues in the body, that these tissues are more susceptible, or at least succumb faster to the negative effects of mutant huntingtin. Now that many of the general cellular defects associated with neurons have been discovered, it may be advantageous to continue determining whether similar defects are present in skeletal muscle, and other excitable tissues, such as the heart, or even non-excitable tissues. In this way, it may be possible to develop a treatment that has the same target in multiple tissues (e.g. L-type Ca^{2+} channels). Other treatments targeting both the brain, skeletal muscle, and other peripheral tissues, such as a mutant huntingtin reducing drug that can be carried into cells from the bloodstream and cross the blood-brain barrier would be ideal.


Dayal, A., Schrötter, K., Pan, Y., Föhr, K., Melzer, W., & Grabner, M. (2017). The Ca2+ influx through the mammalian skeletal muscle dihydropyridine receptor is irrelevant for muscle performance. *Nature Communications, 8*(1), 475. doi: 10.1038/s41467-017-00629-x


García, J., Tanabe, T., & Beam, K. G. (1994). Relationship of calcium transients to calcium currents and charge movements in myotubes expressing skeletal and
cardiac dihydropyridine receptors. *Journal of General Physiology, 103*(1), 125-147. doi: 10.1085/jgp.103.1.125


knock-in mouse model of Huntington's disease. *PLOS ONE, 4*(11), e8025. doi: 10.1371/journal.pone.0008025


143


148


