Periodic Paralysis: Pursuing a Protocol

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PERIODIC PARALYSIS: PURSUING A PROTOCOL

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

KIRSTEN DENMAN
B.S., Wright State University, 2020

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ABSTRACT

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Periodic Paralysis: Pursuing a Protocol

Hypokalemic Periodic Paralysis is an autosomal dominant disease of skeletal muscle in which patients experience episodes of weakness. There is currently no highly effective therapy. A mouse model has been created to study the disease to better understand the physiological changes leading to weakness and to develop novel treatments. However, the work done with the mouse model has used ever-changing protocols and the data produced have been insufficient to answer key questions and bring treatments closer to clinical trials. I evaluated factors of temperature, insulin, potassium concentrations, and length of protocol or exercise, to develop a protocol that reproducibly triggers weakness. I was able to successfully create a protocol that consistently produces paralysis (35°C, insulin, 4.75 to 1 mM K+, long protocol or exercise) to study the underlying pathophysiology and to evaluate novel therapy.
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Introduction

Clinical aspects of hypokalemic periodic paralysis:

HypoKalemic Periodic Paralysis (HPP) is an autosomal dominantly inherited disease, in which patients experience attacks of weakness lasting hours to days. Symptoms also include muscle stiffness and myopathy later in life. The timing of attacks is highly variable; the most common environmental triggers are high-carbohydrate meals, rest after exercise, and temperature changes. Historically, clinical diagnosis was made by infusion of a glucose and insulin challenge, which lowered serum K. If the patient developed weakness they were diagnosed with HPP, now diagnosis is generally made through genetic testing and family history (Wu et al, 2011).

The genetic cause of the mutation has been known for decades. HPP is caused by point mutations, usually Argentine to Histadine, in either Nav1.4 or Cav1.1 channels’ voltage sensing region - creating a gating pore, through which monovalent cations pass, depolarizing the muscle membrane. The gating pore is independent from the normal voltage-gated pore. The mutations occur in the voltage sensing region of the Nav1.4 channel and are almost always at an Arginine residue (Wu et al, 2011).

Currently, the field has not successfully conducted experiments to prove hypotheses of the underlying physiological cause of disease or rigorously tested drugs for the disease. To develop more effective therapy it is necessary to achieve a deeper understanding of the mechanisms responsible for attacks of weakness. To achieve a deeper understanding of the disease and more effective therapies a mouse model needed to be created. The focus of the thesis is creating a
successful protocol so experiments testing hypotheses and developing treatments can be conducted.

**Creation of a Mouse Model:**

The reasoning behind creating an animal model is two-fold; to understand the underlying mechanisms and create novel treatments. Creating a mouse model in 2011 was a crucial step to being able to conduct experiments to study the disease. I used the mouse containing the Nav1.4-R669H mutation responsible for HPP in patients. This mouse was generated in 2011 by “introducing in the mouse ortholog” (Wu et al, 2011, p. 4083) an arginine-to-histamine mutation in the S4 voltage-sensing domain. The Nav1.4-R669H mutants had no visible changes or viability to maturity changes compared to the WT mice. Grip strength was only measured in heterozygous (m/) and wild type (+/) mice where no statistical differences were found. Soleus muscle in vitro force at normal 4.75 mM K+ was significantly lower (P<0.0001) in the homozygous (m/m) than +/+ mice, there was no significant difference between m/+ mice and +/+ mice (Wu et al, 2011). The creation of a genetic mouse model was an exciting step forward for the field. Now that a genetic model existed a protocol to induce the symptoms of weakness was needed.

**Attempts to trigger weakness in mouse models of HPP:**

Once it was determined the mouse model was viable, a protocol to consistently induce attacks of weakness was needed to study the disease. In the initial paper published with the mouse model, ex-vivo soleus muscles were studied where the muscle was anchored to a dish on one tendon and...
a force transducer on the other tendon measuring force generation due to muscle stimulation.

Potassium in the perfusion solution was started at 4.75 mM K then dropped to 2 mM K, which induced sustained loss of force; the wild type, +/+, mice by about 10%, heterozygote, m/+, mice by about 40%, and homozygote, m/m, mice by about 90% (Wu et al, 2011). The severe loss of force in m/m mice in response to lowered K showed the mouse model successfully mimics the human heterozygote experience of paralysis in low serum K. Normal plasma K in humans ranges from 3.0 mM to 5.0 mM (Walker et al., 1990). The same protocol was also used switching the 2 mM K to 3 mM K. This produced oscillations where force would decrease and increase in a sinusoidal-like pattern in both +/m and m/m, which were consistent between the two muscles within the same mouse in separate baths, but variable between mice (Wu et al, 2011). The less robust phenotype of weakness at higher low K values supports using low K values under 2 mM K to induce severe weakness.

Ex-vivo force experiments were once again studied in the next paper by the same group. However, some of the experimental protocols were altered. This time the perfusion solution included insulin. It was stated that the reason for this inclusion was the possibility of increased muscle viability and modulating many intracellular functions ranging from activation of multiple G-protein cascades to rates of ion transporters. During the 30 minute low K phase, there were some oscillations in force. All three genotypes experienced a loss of force during low K; +/+ was down 30% (n=8), +/- was down 60% (n=7), and m/m was down 90% (n=8), no comparative statistics were performed (Wu et al, 2013).
In a third paper, by the same group, ex-vivo force experiments were performed again - with some variation in the experimental protocols. In some of the experiments “insulin [was used] to prolong the viability of the ex vivo preparation. Subsequently, we found there was no improvement, and so insulin was omitted for the majority of the data in this paper” (Mi et al, 2019, p. 556). However, there was no comparison of the drop in force during perfusion of solution containing low K with or without insulin. For the first set of experiments using insulin, there was only an n=1 for m/+, both soleus muscles were used. The second set had a mix of insulin and no insulin, which ones had insulin or how many of each was not discussed. In additional experiments, periods of acidosis (pH = 6.75) were induced by 25% CO2 bubbling the perfusion solution. Recovery back to a pH of 7.4 induced a loss of force in m/m by about 70% at the minimum of a single oscillation (n=6), WT had no loss of force (n=7), and there were no +/-m data. Loss of force was due to acidosis, not low O2, as N2 was tried as a substitute and loss of force did not occur (no data or statistics were shown for the N2 substitution) (Mi et al, 2019).

In the most recent paper by the same group, ex-vivo soleus force experiments were once again performed with variations in experimental protocols. This time all force experiments were done with no insulin, no explanation was provided on why insulin was not included. The group has not been consistent in the duration of low K for inducing weakness, with times ranging from 30 minutes to 70 minutes. In their experiments, weakness was never maintained. Instead, the severity of weakness oscillated and steady state was never achieved (Quiñonez et al., 2023). This makes studying the underlying mechanisms difficult as the severity of weakness is always changing unpredictably.
The changes in protocol between papers and within papers bring into question the repeatable and verifiable quality of these experiments when a key ingredient such as insulin is included or excluded without sufficient understanding. To repeat experiments with confidence, test treatments, and explore underlying mechanisms, a consistent protocol is needed.

**Measuring CMAPS:**

The initial paper also measured CMAPs (compound muscle action potentials), the summation of action potentials from the individual muscle fibers when the muscle was stimulated by the sciatic nerve, in-vivo. Serum K+ was lowered by an insulin and glucose challenge in mice pretreated with sodium polystyrene sulfonate (48 hrs). Manipulating plasma insulin and glucose is a highly challenging experiment. The initial lowering of serum K+ (WT 2.79 ± 0.21 mM, n = 16; +/-m 2.65 ± 0.07 mM, n=8; m/m 2.24 ± 0.08 mM, n=7) did not cause observable weakness or basal twitch force between genotypes, only m/m had lower CMAP amplitude (P<0.05). After 2 hours of glucose and insulin infusion serum K+ decreases further (WT 2.25 ± 0.13 mM, n = 16; +/-m 1.97 ± 0.34 mM, n=8; m/m 1.51 ± 0.14 mM, n=7). The sodium polystyrene sulfonate (a drug used for reducing serum K+ by binding K+ in the intestines preventing absorption) was added because the force reduction was variable, sometimes only decreasing by 50%. The muscle force with sodium polystyrene sulfonate, insulin, and glucose did not drop as much or steeply as the CMAP did (Wu et al, 2011). These CMAP experiments further demonstrate the need for insulin to be considered as a variable in a successful protocol.
Understanding the Physiology Underlying Weakness:

Resting Membrane Potential

Depolarized resting membrane potential due to low K+ is one of the defining and mystifying, characteristics of hypokalemic periodic paralysis. Resting membrane potential is also a key measurement in understanding the underlying physiological changes occurring when patients experience symptoms. Current clamp experiments to measure resting membrane potentials in the soleus muscles of the mouse model was a logical step; at 4.75 mM K+; +/+ fibers were $-72.6 \pm 0.66$ mV and +/m fibers were $-73.6 \pm 0.41$ mV (P=0.21). The resting membranes at 2 mM K+ for +/+ fibers were $-81.7 \pm 0.82$ mV and +/m fibers were $-70.6 \pm 0.40$ mV (P<0.001) at 37˚C. Thus +/+ hyperpolarized and +/m depolarized, not as much as expected for weakness to occur or correlate with what is believed to occur clinically. No data were shown for m/m mice (Wu et al, 2011). Resting membrane potentials using current clamp experiments were checked again in a later paper in EDL muscles at 37˚C. One +/+ fiber hyperpolarized to about -90 mV at 2 mM and 1 mM K+, and depolarized to -50 mV at 0.5 mM K+ (n=1). One m/m fiber depolarized to about -65 mV at 1 mM K+ (n=1) and another m/m fiber depolarized to about -50 mV (n=1) at 2 mM K (Quiñonez et al., 2023). These experiments with their low n values suggest lowered extracellular K+ does cause depolarization of resting membrane potential in m/m mice, and hyperpolarization in +/+ mice (Wu et al., 2011).

Action Potentials

Current clamp recordings showed changes in +/m compared to WT including reduced action potential amplitude, reduced maximal rate of rise, and an increased width (Wu et al., 2011).
These alterations are in agreement with fibers biopsied from patients (Jurkat-Rott et al, 2000).

Intracellular recordings measuring resting membrane potential, input resistance need to be repeated to have statistically significant numbers and done in repeatable experimental conditions where weakness has been shown to be consistently induced in force experiments, in order to move the field forward.

**Inactivation of Nav1.4 Channels**

Whether or not Nav1.4 channel function was impacted by the point mutation causing HPP was explored through voltage clamp recordings, which demonstrated enhancement of both slow inactivation and fast inactivation with a -10mV shift in m/m compared to +/+ (Wu et al., 2011). This suggested Nav1.4 channel gating is altered and likely plays a role in the development of weakness - an additional area to be further investigated in the future.

**Intracellular Ca2+**

Another step in excitation contraction coupling is elevation of intracellular Ca. Ca release was studied under voltage clamp conditions using low-affinity Ca2+ indicator OGN-5. The Ca peak for homozygous NaV1.4-R669H fibers ($\Delta F/F_0$ was 0.72 ± 0.074, n = 8 fibers) was identical to WT at 4.75 mM K+. Of note, the m/m muscle fibers had smaller $\Delta V$ for action potentials (102 ± 2.3 mV n = 6) than +/+ fibers 108 ± 1.2 mV n = 13; p < 0.05). The width of the induced action potential was not discussed or shown. These experiments were done in dissociated fibers (fibers are highly stressed both chemically and physically) and resting potential was supplied by the experimenter, not by the physiology of the muscle fibers (DiFranco & Cannon, 2022). It is
worth considering if the use of voltage clamp measurements to study a disease where changes in membrane potential are at the core of the disease, provide useful information about the underlying mechanism.

There is a possible correlation between the development of myopathy and permanent weakness in CaV1.1-R528H patients and the reduced intracellular Ca release (Jurkat-Rott et al, 2009), as WT mice show an age-dependent decrease in Ca release - it is known weakness and myopathy is also associated with aging (Jimenez-Moreno et al., 2008). Whether intracellular Ca2+ release is altered during force generation is a topic for further investigation.

**Na/K/ATP Pump**

Ouabain was used to understand the role the Na/K/ATP pump plays in HPP. The Na/K/ATP pump inhibitor Ouabain (1 uM) when applied initiated a fall in force for m/m mice at 4.75 mM K+, when K+ was then lowered to 3 mM force fell sharply, recovery either did not occur or appeared as an oscillation upon returning to 4.75 mM K+. Recovery appeared to occur once Ouabain was washed out (1 mouse, 2 soleus muscles). In +/+ mice Ouabain did not appear to have any effect on its own, and when paired with 3 mM K+ there was only a force reduction of 10% - insignificant (1 mouse, 2 soleus muscles) (Wu et al., 2011). Suggesting Na/K/ATP pump function plays an increased role due to changes caused by the mutation and is crucial in bringing the muscle back to a functioning level.
Treatment of HPP in mouse models of HPP:

The primary treatment for HPP is carbonic anhydrase inhibitors, which are believed to work by changes to pH. However, they are usually not effective in patients with a Na+ channel mutation. More effective treatments are needed for HPP Patients.

Diuretic: Acetazolamide

Acetazolamide is a carbonic anhydrase and diuretic, which has been shown to be effective in reducing weakness in the Cav1.1 HPP mouse model (Wu et al., 2013). When used as a 200 uM pretreatment on +/-m mouse EDL and soleus muscles and then exposed to 2 mM K+ it was not successful in preventing weakness 8 out of 9 times, both the soleus (n=5) and EDL (n=4). Acetazolamide was also tried as a protective pretreatment for weakness induced by post-acidosis at 100 uM. In the Nav1.4 model, the pretreatment caused a more severe post-acidosis episode - making the symptoms worse (P<0.05, n=3) (Wu et al., 2011). However, in the Cav1.1 model pretreatment provided some protection against post-acidosis loss of force (P<0.01, n=3) (Wu et al., 2013). This is consistent with what has been shown clinically, acetazolamide is helpful in the Cav1.1 mutation but not the Nav1.4 mutation (Matthews et al., 2011). While acetazolamide can be beneficial in hypokalemic periodic paralysis with the Cav1.1 mutation in the hypokalemic periodic paralysis NaV1.4-M1592V mutation it is either not helpful or makes attacks of weakness worse (Wu et al., 2013). These data demonstrate the need for a new treatment for people with HPP due to Nav1.4 channel mutation.
**NKCC Inhibitor: Bumetanide**

Chloride accumulation inside muscle fibers is thought to play a role in attacks of weakness in HPP. Bumetanide, an NKCC inhibitor and diuretic, was tested in multiple scenarios to determine whether lessening Cl movement into muscle could lessen weakness. Post-acidosis loss of force was inhibited by a 0.5 uM pretreatment (n=3). Bumetanide at 75 uM was also used as a treatment for weakness due to 2 mM K exposure. When bumetanide and 2 mM K+ were used together and then bumetanide was washed out, and 2 mM K was retained, force was maintained for the duration of the low K and then had supramaximal recovery for both +/+ and +/-m. m/m only had a small loss of force during bumetanide compared to 80% loss of force after bumetanide in low K. Bumetanide was used as a rescue during the 2 mM K; it rescued force for all three genotypes, +/+ did not have a full recovery till return to 4 mM K, m/m had a 110% recovery but started dropping at the end of the protocol prior to returning to 4.75 mM K, then had a 120% recovery in return to normal K (Wu et al., 2013). Bumetanide was only effective when extracellular Cl- was present, thus suggesting bumetanide’s inhibitions of the NKCC transporter are Cl dependent and present a possible treatment for hypokalemic periodic paralysis. However, bumetanide was only tested in post-acidosis experiments with low n-values, so I would like to see this repeated with more rigorous testing.

**Kv7 Opener: Retigabine**

Retigabine, a K+ channel opener, was applied at 10 uM prior to lowering K+ to 2 mM. It made force in mutant muscle comparable to WT (n=2). Retigabine was also shown to be an effective
rescue drug (n=2); improving force by $83.2\% \pm 0.021$ (n=3), when compared to the opposite side EDL muscle of the same mouse without retigabine, after loss of force has already begun due to 2 mM K. Retigabine was also shown in intracellular recordings to prevent depolarization and rescue depolarization induced by low K+ (n=1 1mM K+, n=1 2mM K) (Quiñonez et al., 2023). K+ channel openers such as retigabine may be a promising therapeutic avenue. The creation of a consistent protocol would allow us to determine whether we can reproduce the results, which if successful would help move closer to clinical trials and treatments for people.

There are many exciting findings in the field and promising areas to explore, but when multiple protocols are used and the protocols yield inconsistent results, it is difficult to know if drug treatments are effective or if the result is simply due to chance. To take the next steps in verifying the findings, understanding the underlying mechanisms, bringing novel treatments to people, and continuing the progress, a consistent experimental protocol is needed to understand the underlying mechanisms and bring novel treatments to people.
Methods

Animals

All animal procedures were performed in accordance with the policies of the Animal Care and Use Committee of Wright State University and were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

NaV1.4 sodium channel Scn4a p.R663H missense mutation mouse model on 129/Sv background was used (Wu et al., 2011). Mice were genotyped and the homozygous and wild type littermate mice were used for experiments. In addition, mice expressing GCAMP6f (Chen et al., 2013) in skeletal muscle were generated by crossing floxed GCAMP6f (GCAMP) mice (Jackson Labs, B6J.Cg-Gt(ROSA)26Sortm95.1(CAG-GCaMP6f)Hze/MwarJ, cat #028865) with mice expressing parvalbumin promoter driven Cre (Jackson Labs, B6.129P2-Pvalbtm1(cre)Arbr/J, cat# 030218). The GCAMP mice were crossed with the hypokalemic mice for two generations to obtain mice expressing GCAMP, which were also either homozygous for knockout of Scn4a p.R663H or wild type.

Mice were sacrificed using CO2 inhalation followed by cervical dislocation. In a few specified cases isofluorane inhalation followed by cervical dislocation or guillotine was used for sacrifice to ensure there were no differences due to euthanasia.

Ex Vivo Force Recordings:
All experiments were performed on *extensor digitorum longus* (EDL) muscles at either 21–23 °C or 35°C as shown in Fig 1. The EDL muscle was dissected, placed in a custom recording chamber - the chamber was 3D printed with a rectangular chamber to allow for easy setting of the muscle but minimize extra volume contributing to lowering of temperature. The muscle was continuously perfused with Ringer solution containing (in mM): 118 NaCl, 3.5 KCl, 1.5 CaCl2, 0.7 MgSO4, 26.2 NaHCO3, 1.7 NaH2PO4, with 5.5 glucose and maintained at pH 7.3–7.4 by aeration with 95% O2 and 5% CO2. The KCl concentrations were altered to either 4.75mM or 1mM - osmolarity was adjusted by the addition of NaCl, as outlined in the results. The proximal tendon was tied with a 6–0 caliber silk suture to a bar and the distal tendon was tied to a hook and attached to a force transducer (Aurora Scientific). The EDL was stimulated with two platinum electrodes placed parallel to the muscle in the bath stimulating the muscle with 2 ms pulses once every minute. When exercise protocols were used, 12, 100hz trains of 45 pulses were performed over two minutes. For a few experiments, nerve stimulation was used by a suction electrode. The optimal length for maximal force recording was determined by adjusting the tension of the muscle until maximal twitch force was achieved. Force was recorded using a CED 1401 A to D board using Spike2 software (Cambridge Electronic Design Limited). No filtering was applied to the signal. The Data was exported into Matlab 2019b and analyzed by code written by Jessica Myers utilizing the find peaks function. Statistical analysis of unpaired t-tests and charts were made using Origin 2019b.
Results

EDL muscles from the Nav1.4-R669H HypoKalemic Periodic Paralysis mouse model were studied. Ex-vivo muscle force measurements were performed by stimulating the muscle with parallel electrodes. Force was compared in normal K+ conditions to low K+ conditions to see if weakness could be consistently induced and maintained. Muscle force was measured from the baseline to the peak of the single twitch in response to stimuli. One concern is a paper that showed force could be evoked from muscle stimulation when TTX was present blocking the Nav1.4 channels, preventing action potentials from occurring (Cairns et al., 2007). The interpretation was the depolarizing stimuli were of sufficient amplitude to directly trigger Ca elevation in muscle fibers such that the generation of action potentials was not necessary for contraction (Cairns et al., 2007). However, when I used 1 uM TTX during muscle stimulation to prevent action potential generation, no twitch force was produced, n=2, suggesting my stimulation protocol requires action potential generation for force production.

Previous force recordings done in the lab showed a robust weakness phenotype in m/m HPP EDL muscles, n=4, and sustained normal force in +/+ EDL muscles, n=4, at room temperature with 20 units/L insulin in 1 mM K+ compared to 3.5 mM K+ at room temperature with insulin. When attempts to replicate the results were made neither I nor the original experimenter, Murad Nawaz, were able to reproduce the findings of weakness (Fig 2). Insulin was used, as it has historically been used to clinically diagnose patients by inducing attacks of weakness with insulin and glucose injections (Grob et al., 1957). Insulin has been reported to aid in
hypokalemic paralytic phenotype induction (Wu et al., 2011). However, it was later stated “insulin [was used] to prolong the viability of the ex vivo preparation. Subsequently, we found there was no improvement, and so insulin was omitted for the majority of the data in this paper” (Mi et al, 2019, p. 556). Therefore insulin is a variable considered throughout the experiments conducted.

In the process of understanding why Murad Nawaz and I could not reproduce his original results, the method of euthanasia was investigated. In previous publications, isoflurane had been used as the method of euthanasia (Wu et al., 2011; Wu et al., 2013; Mi et al, 2019; DiFranco & Cannon, 2022; Quiñonez et al., 2023). Murad Nawaz used isoflurane as well, whereas I had used CO2 inhalation. We began by switching our respective methods of euthanasia and neither of us achieved weakness. Then we used muscles from the same mouse. We did two mice euthanized by isoflurane and two mice euthanized by CO2 euthanizing a mouse and each studying force in an opposite leg EDL muscle simultaneously. In addition, we tried one person performing the dissection and the other person running the experiment and switched roles. Finally, we had 2 mice sacrificed by guillotine where we each did an EDL from the mouse to eliminate any potential side effects caused by the 2 inhaled methods of euthanasia. Regardless of the method of euthanasia, we were unable to reproduce the initial findings.

I considered whether 3.5 mM K+ for normal K+ was already too low. Normal K+ is usually between 3 and 5 mM in plasma, which suggests I was on the low end of normal (Walker et al., 1990). Previous papers have shown oscillations in the degree of weakness in HypoKalemic
periodic paralysis muscle at 3 mM K+ (Wu et al., 2011). I made solution increasing normal K+ from 3.5 mM to 4.75 mM, as 4.75 mM was used in previous publications (Wu et al., 2011; Wu et al., 2013; Mi et al, 2019; DiFranco & Cannon, 2022; Quiñonez et al., 2023). In addition, HypoKalemic Periodic Paralysis is highly variable in onset and duration, so I decided to create a long protocol to try to eliminate as much variability as possible. The long protocol begins with the muscle in normal K+ for 60 minutes, followed by low K+ for 135 minutes, with a return to normal K+ for 25 minutes. When muscle underwent the long protocol with normal K+ increased to 4.75 mM and insulin at room temperature, weakness did not occur, n = 3 (Fig 3).

I raised the temperature from room temperature (22˚C) to a physiologically relevant temperature (35˚C) in an attempt to induce the phenotype of weakness (Flouris et al., 2015). When the long protocol was done at 35˚C without insulin weakness in response to low K+, 1 mM, did not occur in m/m muscles (n=5, Fig 4). This produced baseline force changes in both the +/+ and m/m mice independent from stimulation. In both genotypes, the baseline would slowly and smoothly increase then decrease over minutes.

I considered how to measure force with the variable baseline. If the muscle is generating maximal force and the baseline increases, the force measurement would decrease as measured from the baseline, but the peak would not have changed (Fig 5). If force is only measured using sweeps rather than the continuous recording that we employ, changes in baseline force might be missed, which would impact measurements. At each measurement, the baseline would be only relative to itself during the brief time the twitch occurs - appearing to be flat. Our impression is
that other labs in the field measure force only during twitch and may not notice the baseline changes. If force is measured only by the peak and changes in the baseline are not considered the measurement of force could increase even though the same functional force (change from baseline to peak) is produced (see Figure 5B top and bottom plot).

Nerve stimulation experiments were conducted as nerve stimulation is more physiologically relevant and requires propagation of action potentials away from the neuromuscular junction. Nerve stimulation with a long protocol at 35°C with insulin using 4.75 mM normal K+ produced weakness in low (1mM) K+. However, there was more variability in eliciting and maintaining weakness than I had hoped (Fig 6). In addition, a larger change in the baseline was seen.

I investigated if the large baseline changes were part of the disease model or separate from the genetic mutation. The integral under the baseline was taken to evaluate if there was a difference in severity between +/- (n=10) and m/m (n=7). There was not a statistically significance difference (p=0.8) showing the baseline shifts are due to experimental parameters not the mouse genotype (Fig 7). In addition, baseline increases still occurred when TTX was present and muscle generated no force in response to stimulation. These data prove that the contraction is occurring independent of action potentials, suggesting a novel process is responsible.

To see if nerve stimulation was necessary for inducting the phenotype of weakness the same protocol from the nerve stimulation experiments was done with muscle stimulation. The long protocol at 35°C with insulin using 4.75 mM normal K+ using muscle stimulation produced
sustained weakness. The onset of weakness was variable from 65 minutes to 115 minutes in 1 mM K+, but once weakness occurred it was maintained for the remainder of the 135 minutes (n=5) in 1 mM K+. Recovery began when K+ was returned to normal. Weakness did not occur in any of the +/+ mice (n=5) (Fig 8). This was a highly successful protocol for inducing severe weakness comparable to patient reports of paralysis.

After creating a successful protocol for inducing muscle weakness, I wanted to determine whether it could be done more quickly, as the protocol is long. Many patients experience weakness post-exercise (Elbaz A, et al., 1995); I tested if adding an exercise protocol could shorten the time necessary to induce weakness. To mimic this trigger in EDL muscles ex-vivo in solution containing insulin, a protocol of 12, 100 Hz trains consisting of 45 pulses was delivered every 30 seconds for 6 minutes in 3.5 mM K+. This was followed by a single 2 ms pulse every minute for 10 pulses, prior to switching the solution to 1 mM K+. Force was then followed for 1 hour at room temperature. The exercise protocol triggered weakness in normal K+ in both m/m and +/+ mice; with a clear and consistent drop in force in low K+ after 1 hour in m/m mice (n=4), but not in +/+ mice (n=3), p ≤ 0.05 (Fig 9). This presents an alternative protocol for studying HypoKalemic Periodic Paralysis.

In order to better understand the mechanism underlying weakness, I wished to perform Ca2+ imaging. HypoKalemic mice were crossed with GCAMP mice, expressing a fluorescent Ca2+ binding dye. This would allow for further study of where the excitation-contraction coupling process breaks down as elevation of intracellular Ca2+ could be measured in relationship to
action potential amplitude. First I wanted to make sure the phenotype was not affected by the
cross of the two mutants. When m/m mice force was measured using a long protocol at 35°C
with insulin using 4.75 mM normal K+, loss of force in +/+ mice (n=5) was similar to loss of
force in m/m mice (Fig 10, n = 6, p =0.072). These data suggest the crossing of lines had some
unexpected effects causing decreased weakness in m/m mice and increased weakness in +/+ mice, possibly through changes in genetic background or Ca2+ buffering due to the GCAMP
Ca2+ binding, which could be investigated in future studies.
Discussion

The mutations responsible for HPP have been known for close to 30 years and the first mouse model was created over a decade ago. However, the ability to study the disease has been hampered by a lack of a standard protocol to induce weakness.

I took the approach of using known triggers in patients to trigger weakness in EDL muscles from mice having a Nav1.4 mutation known to cause HPP. The combination of insulin, physiological temperature, and a long protocol provided a method for a standard protocol to induce weakness. Incorporating exercise shortened the protocol and proved its potential as a method for future experiments.

The goal of my work was to develop an improved understanding of the mechanisms underlying episodes of weakness with the long-term goal of improving therapy. Prior to pursuing those goals I needed to establish a way to consistently induce weakness. An unexpected finding was that there is likely a novel mechanism that causes muscle force generation independent of action potentials.

Development of Protocols to Consistently Induce Weakness:

The mechanism underlying post exercise weakness is not well understood. One possible explanation is that exercise effectively creates a recovery from acidosis protocol. During exercise muscle pH becomes more acidic and then recovers back to normal with rest (Juel, 2007). A previous paper demonstrated post-acidosis as a robust trigger of weakness in HPP (Mi et al,
Modulating pH in our experimental setting is challenging as we use a bicarbonate buffer bubbled with 95% O2 and 5% CO2. pH is set by the percentage of CO2 in the gas, which is expensive to change. The exercise protocol performed in this paper could be an effective alternative.

Membrane depolarization-induced inactivation of Nav1.4 channels due to the closing of Kir channels is thought to explain weakness, but this has not been clearly demonstrated (Struyk and Cannon, 2008). Experiments proving these changes have not been shown with a sufficient n, where the membrane resting potential was not artificially altered. The development of a protocol that induces sustained weakness will allow for intracellular recordings to be done quantitating changes in resting potential and action potential amplitude between normal conditions and conditions in which muscle is weak.

We will begin by measuring membrane potential changes due to lowering K+ with insulin at 35°C to see how much depolarization is triggered. We can infer the closure of Kir channels by measuring input resistance and membrane time constant. A higher input resistance would signal the closing of Kir channels.

We can then study the effect of depolarization on excitability. Is the depolarization sufficient to cause loss of excitability? This will be measured by measuring the response of fibers to injection of depolarizing current. In conditions where the membrane potential was controlled by current injection, action potentials were shown to have lower peaks in HPP mutants than wild type
(DiFranco & Cannon, 2022) and more hyperpolarized voltage dependence of inactivation (Wu et al, 2011). Questions to be asked include: Can action potentials be initiated? At what voltage do action potentials peak? Is the depolarization during action potentials sufficient to trigger normal Ca transients?

This knowledge combined with data showing action potential peaks fail to cause increase in intracellular Ca2+ before the action potentials themselves fail (Wang et al., 2022) brings up the question of where does the initial breakdown in the excitation contraction coupling cascade occur? This question could also be addressed using Ca2+ imaging techniques to correspond action potential peaks with changes in intracellular Ca2+. However, GCAMP mice would not be useful for these studies as they do not have a phenotype of weakness in mice carrying the HPP mutation. However, the GCAMP HPP mutant mice not having a phenotype of weakness does open up the possibility of Ca2+ buffering being a potential avenue for therapeutic treatment.

A consistent phenotype of weakness will also provide the ability to test pharmacological treatments. While retigabine has been shown to be a promising therapeutic agent, the n for the experiments was extremely low (Quiñonez et al., 2023). Prior to retigabine being investigated in clinical trials, further studies using a protocol with reproducible weakness yielding an increased n are needed. My work makes this possible.
Spontaneous Generation of Force:

It is currently thought that muscle stiffness in ion channelopathies is due to the unwanted firing of action potentials. HPP is known not to have unwanted firing of action potentials (known as myotonic discharges), but patients do experience muscle tightness during attacks of weakness. A patient with HPP who spoke to the Rich lab stated that she has painful contractions of muscle during attacks of paralysis. Given the lack of evidence for the spontaneous firing of action potentials in patients, the mechanism underlying the muscle contraction responsible for unstimulated force generation (the shift in baseline) is unlikely to be due to the firing of action potentials. Preliminary data by Chris Dupont showed baseline force generation in Hyperkalemic Periodic Paralysis occurred when the muscle was electrically silent.

To explore the mechanism underlying baseline force generation I performed experiments in which I applied 1 uM TTX, a Nav1.4 blocker to m/m HPP muscle that was exposed to insulin at 35°C. In both experiments, TTX did not prevent baseline force generation. These data prove spontaneous firing of action potentials is not the mechanism underlying baseline force generation and indicate a novel mechanism is responsible.

One possibility is swelling of the muscle due to changes in K+ buffering, which increases osmolarity inside muscle fibers compared to the extracellular compartment. In the future, this could be studied by imaging muscle fibers during tightness to measure to see if swelling does occur. Action potentials occur at the beginning of the excitation contraction coupling cascade leaving the possibility later steps are activated during electrically silent tightness. Whether or not
there is elevation of intracellular Ca2+ could be studied as baseline force generation does occur in mice expressing GCAMP.

Conclusion:

My work opens up the possibility for detailed studies of mechanisms underlying both weakness and muscle stiffness in HPP. The hope is that this leads to the discovery of novel therapy for the affected population.
Figure 1

Measuring EDL Force

A

B

Force

1 Twitch/minute

5mN

60s
Fig 1: The setup used for force experiments.

A) Force recordings were done ex-vivo using mouse EDL muscles dissected tendon to tendon and anchored to the dish and a fixed force transducer. Bicarbonate buffered saline was continuously perfused. Contractions were triggered by a pulse generator controlled digitally. Muscle was directly stimulated with two parallel platinum electrodes. Force was then recorded in Spike and analyzed in Matlab.

B) An example +/+ trace. Each line is a single twitch force in response to a 2ms pulse. The force is quantitated as the difference between the peak and the baseline.
Figure 2

A Nawa: Room Temperature & Insulin

B Denman: Room Temperature & Insulin
Fig 2: Irreproducible weakness in response to stimulation measured in 3.5 mM K+ and 1 mM K+ (gray) at room temperature.

A) The graph shows averaged values with the standard deviations recorded by Nawaz. Each muscle began in normal, 3.5 mM K+ and then K+ was lowered to 1 mM where the m/m mice achieved and maintained weakness (n=4), followed by force recovery in a return to normal K+. The +/+ mice (n=4) maintained force throughout the experiment. 20 units/L of insulin were present throughout the experiment.

B) Attempts to replicate the data in A under the same experimental conditions were unsuccessful. m/m mice did not become weak.
Figure 3

Long Protocol with Insulin at Room Temperature
Fig 3: Long Protocol measuring EDL twitch force in response to 2 ms muscle stimulation measured in 4.75 mM K+ and 1 mM K+ (gray) at room temperature with insulin.

An example m/m trace; 60 minutes in normal K+, 135 minutes in low K+, 25 minutes in normal K+. No clear weakness developed.
Figure 4

Long Protocol at 35C

A

B
Fig 4. Long Protocol measuring EDL twitch force in response to 2 ms muscle stimulation measured in 3.5 mM K+ and 1 mM K+ (gray) at 35°C, without insulin.

A) An example m/m trace 60 minutes in normal K+, 135 minutes in low K+, 25 minutes in normal K+. Some weakness developed, but weakness was incomplete.

B) Mean data with standard deviations for n=3 m/m muscles.
Figure 5

A Measuring Force 2 Ways  
B Plots of Measured Force

Peak Force From Initial

Peak Force From Baseline

Minutes

Minutes
Fig 5: The issue of changing baseline force on measurement of evoked force.

A) Initial peak force is measured, 1, and subsequent measurements are normalized to the initial peak force. Peak force measured in normal K+ at 35°C with insulin is either 1.59 or 0.52 depending on what baseline is used. If the initial baseline force is used force is 1.59. If the current baseline is used it is 0.52.

B) Twitch force values from the EDL are plotted for 60 minutes in normal K+ with insulin measuring force relative to the initial baseline (upper trace) or the current baseline (lower trace).
Figure 6

Nerve Stimulation

A

Mutant Example

B
Fig 6. Nerve stimulated EDL twitch force using the long protocol in 4.75 mM K+ and 1 mM K+ (gray) at 35°C with insulin.

A) An example m/m trace; 60 minutes in normal K+, 135 minutes in low K+, 25 minutes in normal K+. Weakness developed, but loss of force was not complete or sustained at the smallest force and there was partial recovery in low K+.

B) Average normalized EDL twitch force m/m traces with standard deviations, n = 5.
**Figure 7**

A. Example Trace with Stimulation

B. Example Trace without Stimulation

C. Box Plot of Baseline Integrals over 1hr
Fig 7: Contraction without stimulation at 35°C in solution containing 3.5 mM K+ and insulin

A) Example trace of twitch force was recorded in response to 2 ms muscle stimulations in normal K+ conditions (3.5 mM K+) and low K+ conditions (1 mM K+) to try to induce weakness in HypoKalemic Mutants. The gray box denotes when low K+ conditions were present. Weakness was not induced. However, changes in baseline force did occur.

B) Baseline force was recorded for 1 hr without stimulation, m/m muscle was still excitable (not shown). The shaded area indicates where the integral under the baseline was calculated to measure both the intensity and duration of the baseline shift.

C) Box plot showing the integrals under the baseline for the first hour were calculated for +/- (n=7) and m/m (n=10), p=0.8.
Figure 8

A Protocol for Weakness

A

1 mM K+

B

Normalized Twitch Force

Mutant WT

5mN

A+/+ (n=5)
m/m (n=5)

0 50 100 150 200 250

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6
Fig 8. Long Protocol measuring EDL twitch force in response to 2 ms muscle stimulation measured in 4.75 mM K+ and 1 mM K+ (gray) at 35°C with insulin.

A) Example traces of +/+ and m/m 60 minutes in normal K+, 135 minutes in low K+, 25 minutes in normal K+. Complete loss of force occurred in the m/m muscle while force declined by 40% in the +/+ muscle.

B) Average normalized EDL twitch force m/m traces with standard deviations. All m/m had complete loss of force generation during low K+ sustained until return to normal K+. N = 5 for both groups
Figure 9

A  Example Traces of Exercise Protocol

B  Box Plots of Force
Fig 9: Exercise Induced Weakness.

A) Example traces of +/+ and m/m force at 35°C with insulin. Muscles were stimulated with 10, 2 ms pulses in normal K+ (3.5 mM) followed by 12 100 Hz trains of 45 pulses every 30 seconds with 10 additional 2ms pulses. The solution was then switched to low K+ (1mM) for 60 minutes, a 2ms pulse was applied every minute (gray in the figure). The +/+ EDL muscles maintained force in low K+ whereas the -/- EDL muscles had weakness. Upon return to normal K+ for 10 minutes, +/+ continued maintaining force and -/- recovered force.

B) Box plots of +/+ (n=2) and -/- (n=4) EDL muscles showing the last twitch force in normal K+ (normalized to 1), compared to the last twitch force in low K+, and the last twitch force of recovery in normal K+. The box's edges are the lower quartile and upper quartile with the center line being the median, the whiskers are the minimum and maximum.
Figure 10

Gcamp x HypoKalemic Periodic Paralysis

A

WT

Mutant

B

WT

Mut

1 mM K+
Fig 10. HypoKalemic Periodic mice expressing GCAMP have loss of force in 1 mM K+ that is similar to the loss of force in +/+ mice expressing GCAMP.

A) Example traces of +/+ and m/m with 60 minutes in normal K+, 135 minutes in low K+, 25 minutes in normal K+.

B) Average normalized EDL twitch force m/m traces with standard deviation. A trend toward more weakness was seen in the +/+ mice than in the m/m mice, neither had a complete loss of force.
Literature Cited


