1-1-2011

Altered Calcium handling in cerebellar Purkinje neurons with the malignant hyperthermia mutation, RyR1-Y522S/+ 

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ALTERED CALCIUM HANDLING IN CEREBELLAR PURKINJE NEURONS WITH THE MALIGNANT HYPERTERMIA MUTATION, RYR1^{Y522S/+}

A Thesis
Presented to
The Faculty of Natural Sciences and Mathematics
University of Denver

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
George C. Talbott
June 2011
Advisor: Nancy M. Lorenzon, PhD
Abstract

To investigate the etiology of malignant hyperthermia and central core disease, mouse models have recently been generated and characterized (Chelu et al., 2006). These RyR$^{Y522S/+}$ knock-in mutant mice provide an excellent tool to investigate calcium dysregulation, its pathological consequences, and potential therapeutic approaches. Skeletal muscle harboring this mutation exhibits calcium leak from internal stores and an increased sensitivity to activation by caffeine, voltage, and temperature (Durham et al., 2008). Although alterations in RyR1 channel function and resultant changes in cellular function have been characterized in skeletal muscle, the effects of MH mutations in RyR1 on central nervous system function have not been investigated. Since RyR1 is highly expressed in cerebellar Purkinje neurons, our goal is to investigate whether the RyR$^{Y522S/+}$ mutation causes altered calcium handling and whether potential calcium dysregulation alters cellular function.
Acknowledgements

I would like to thank Nancy Lorenzon, PhD for being a wonderful advisor and mentor. I definitely could not have completed this project without her invaluable support and patience. I would also like to thank my committee for their assistance, guidance and input. My project would not be the same without their help. I would also like to thank Nathan Duval for being a springboard for my ideas and a great friend. I want to thank all of the graduate students in Biology for making DU a fun and exciting research environment. Finally, I would like to thank Meagan Thompson for putting up with me for all these years and miles traveled.
# Table of Contents

Chapter One: Introduction .................................................................................................. 1  
Ryanodine Receptor 1 ............................................................................................. 2  
Figure 1: Schematic of RyR1 regulation .................................................... 4  
Malignant Hyperthermia ......................................................................................... 7  
Figure 2: RyR1 is expressed in the cerebellum .......................................... 9  
Purkinje neurons ..................................................................................................... 9  
Figure 3: Calcium signaling in cerebellar Purkinje neurons ..................... 11  

Chapter Two: Methods ..................................................................................................... 14  
Genotyping of Y522S-RyR1 mice ........................................................................ 14  
Dissociation of cerebellar cells ............................................................................. 14  
Identification of dissociated Purkinje neurons ...................................................... 15  
Figure 4: Visual identification of dissociated Purkinje neurons ............... 16  
Immunohistochemistry ......................................................................................... 16  
Calcium Imaging Experiments ............................................................................. 17  
Dose response to caffeine ..................................................................................... 18  
Depolarization-induced calcium release ............................................................... 18  
Store content and SOCE ....................................................................................... 18  
Statistical Analysis ................................................................................................ 18  

Chapter Three: Results ...................................................................................................... 20  
Cerebellar structure and Purkinje cell morphology is not grossly altered in Y522S-RyR1/+ mice .......................................................... 20  
Figure 5: Morphology of RyR1-Y522S/+ Purkinje neurons .................... 21  
Decreased threshold for activation in RyR1-Y522S/+ channels .................. 21  
Figure 6: Individual versus sequential caffeine dose response ............... 23  
Figure 7: Decreased threshold for activation in RyR1 \( ^{Y522S/+} \) channels ..... 26  
Increased intracellular calcium concentration in RyR1-Y522S/+ Purkinje neurons ............................................................................................................................... 27  
Figure 8: Increased intracellular calcium concentration in RyR-Y522S/+ Purkinje neurons .......................................................... 28  
Intracellular calcium stores are unaffected by the RyR1-Y522S/+ mutation .... 29  
Figure 9: ER calcium store content is unaffected by the RyR1 \( ^{Y522S/+} \) mutation as assessed by maximal doses of caffeine ............................. 31  
Figure 10: Store content assessed by thapsigargin ................................. 33  
Store-operated calcium entry - a compensatory mechanism? ................. 34  
Figure 11: SOCE-a compensatory mechanism? ................................. 36  
Effects of altered RyR1 function on cellular function ................................. 37  
Figure 12: Effects of the Y522S mutation on calcium dynamics .......... 40  

Chapter Four: Discussion .................................................................................................. 42  

Chapter Five: Summary .................................................................................................... 50
References

51
Chapter One: Introduction

Calcium plays an important role in cellular signaling and function, yet calcium can also trigger pathology and cell death. Cytosolic calcium originates from two main sources: influx of extracellular Ca\(^{2+}\) through voltage- or ligand-gated ion channels in the plasma membrane and release from intracellular Ca\(^{2+}\) stores through ryanodine receptor (RyR) or inositol 1,4,5-trisphosphate receptor (IP\(_3\)R) channels in the sarcoplasmic (SR) or endoplasmic (ER) reticular membrane. Cytosolic calcium can act as a second messenger through downstream activation of a myriad of effectors; among these targets are calpains and other apoptotic machinery, so free calcium must be tightly regulated. Extensive measures are employed by a cell to maintain calcium homeostasis, but even slight alterations in these tightly controlled processes can disrupt the fine balance of control. Furthermore, calcium dysregulation is the underlying mechanism for a number of different human disorders. Malignant hyperthermia, for example, is a skeletal muscle disease that is associated with disrupted function of the RyR1 in the presence of certain triggering agents, including volatile anesthetics, depolarizing muscle relaxants, and environmental or exertional heat stress (Durham et al., 2008). The RyR1 is the “skeletal muscle” isoform of the RyR and is highly expressed in this tissue, but also exhibits limited expressions by certain cell types within the brain (Giannini et al., 1995). Cerebellar Purkinje neurons (PN) are one of these cell types, and calcium dysregulation within PNs could have profound effects on not just cellular function, but organismal
function as well. Purkinje neurons are the integrator and sole output of the cerebellar cortex (Ito et al., 1984), therefore serving as an essential step in the cerebellar signaling pathway. Disruptions in calcium signaling in these cells can result in loss of motor coordination and ataxia, but the specific effects of altered RyR1-mediated calcium release on PN and/or cerebellar function have not been determined. Furthermore, the normal functional role of the RyR1 in PNs is not fully understood. Recently, a model of malignant hyperthermia (MH) has been created in a knock-in mouse by substitution of a tyrosine for a serine at residue 522 (Y522S) in RyR1 (Chelu et al., 2006). The function of the mutant form of this channel has been characterized in skeletal muscle, and the heterozygous form of this mutation has been shown to exhibit a decreased threshold for activation by agonists and an increased intracellular calcium concentration at physiological temperatures, likely due to enhanced basal channel activity of the RyR1 (Chelu et al., 2006; Durham et al., 2008). This “hyperactive” RyR1 could serve as a unique tool to assess the function of RyR1 in PNs, the effects of altered RyR1 release in PNs, and the neurological consequences of MH. To understand altered function of the RyR1 in PNs, however, it is first important to understand what is known about the normal functioning of the channel.

**Ryanodine Receptor 1**

The RyR1 is a calcium release channel on the SR membrane that acts as a source of calcium for excitation-contraction coupling in skeletal muscle (Otsu et al., 1990). In PNs, however, the function of this channel in the ER membrane is less clear. Three isoforms of the RyR are expressed in mammals and each of these three isoforms functions as a calcium release channel. There are two known modes of activation
associated with the RyR. The first is calcium-induced calcium release (CICR), whereby the calcium fluxed through voltage-gated calcium channels serves as the agonist for the RyR (Endo, 1977). The second is mechanical coupling, whereby membrane depolarization induces a conformational change in L-type voltage-gated calcium channels (DHPR) that triggers release of Ca$^{2+}$ through the RyR1 by a direct physical interaction (Tanabe et al., 1990). The RyR1 is unique in that it is the only isoform of the three known to be activated by mechanical coupling (Tanabe et al., 1990).

The RyR1 was originally isolated in skeletal muscle, where it is highly expressed and localized to the junctional SR, defined by the close proximity of T-tubule invaginations to terminal cisternae of the SR. Freeze-fracture analysis of junctions in skeletal muscle (Protasi et al., 1997) revealed tightly compacted, two-dimensional arrays of RyR1s on the SR membrane and clustering of four DHPRs, in opposition to every other RyR, in the sarcolemmal membrane. Each DHPR interacts with a different subunit of the RyR1, which is a homotetramer with a molecular mass of over 2 MDa. Each RyR1 subunit is approximately 565 KDa (Inui et al., 1987; Lai et al., 1988), and this large size makes the RyR the largest known ion channel. The majority of the RyR1 mass, about 80%, resides within the cytoplasm, while only 20% of the protein is located in the luminal and transmembrane space (Hamilton, 2005). The N-terminal domain comprises the large cytoplasmic portion of the RyR, while the C-terminal domain forms the pore and transmembrane region of the channel (Fill et al., 2002).

Regulation of this enormous channel is quite complex (Fig 1); there are three main regulatory domains controlled by calcium, ATP and magnesium binding, two of which are cytoplasmic. The first is a high affinity, specific cytoplasmic calcium activation site
known as the A-site, which also allows competitive binding of magnesium and monovalent cations (reviewed by Laver, 2005). The second regulatory domain is a non-specific, low affinity, inhibitory cytoplasmic region known as an I-site. Magnesium potently inhibits the channel when bound to either or both sites, while calcium can act as both an activator and an inhibitor depending upon cytoplasmic calcium concentration (reviewed by Laver, 2005). Isolated RyR1s are activated at approximately 1 µM [Ca^{2+}]_i and inhibited at approximately 1 mM [Ca^{2+}]_i (Meissner 1994). There is also evidence of a luminal regulatory domain sensitive to calcium levels, but it is unknown exactly what region of the RyR1 serves as this luminal site or whether luminal regulation is controlled by an associated protein.

Figure 1: Schematic of RyR1 regulation

Adapted from Lanner et al., 2005
There are two theories regarding luminal calcium regulation of the channel. The first theory, direct luminal activation, posits that calcium binding to a regulatory domain within the lumen allosterically modifies A-site affinity to Mg\(^{2+}\) (reviewed by Laver, 2007). Once Mg\(^{2+}\) inhibition of the A-site has been relieved, calcium and ATP are free to bind and activate the channel. The second theory, calcium feed-through, involves the release of luminal calcium through the channel when the concentration of calcium within the ER store is high and relieving Mg\(^{2+}\) inhibition by competitively binding at the A-site (reviewed by Laver, 2007). These theories are not mutually exclusive, and it is more than likely that both play a role in RyR activation.

One potential candidate for a luminal regulatory domain is calsequestrin. Calsequestrin interacts with the RyR1 in conjunction with two other proteins: junctin and triadin (Zhang et al., 1997). Calsequestrin acts as both a calcium buffer and a calcium sensor, and its structural arrangement is modified by luminal calcium concentration (Beard et al., 2009). In the presence of low levels of luminal calcium, calsequestrin is not bound to calcium, dissociates from the channel complex and de-polymerizes. At physiological levels of luminal calcium, approximately 1 mM, calsequestrin binds calcium, polymerizes and attaches to junctin and triadin. Through its binding to junctin it inhibits RyR1 activity and thus maintains appropriate calcium concentrations within the ER. At higher levels of calcium, calsequestrin polymers become super-compacted and dissociate from junctin and triadin, and the inhibition of RyR1 is removed (Beard et al., 2004).

The RyR is part of a macromolecular complex of proteins many of which attach to the large cytoplasmic scaffolding domain of the RyR. RyR1 is known to associate with
PDE4D3 (bound to the receptor by mAKAP), PKA, FKBP12 (calstabin), CaM, and PP1 (through binding to spinophilin) (Bellinger et al., 2008). When each subunit of the RyR binds a calstabin molecule, the closed state of the channel is stabilized (Lanner et al., 2005). Calstabs also play a role in coordinating the gating of the four subunits of the RyR and in coupling the opening of neighboring channels (Brillantes et al., 1994; Avila et al., 2003). Protein kinase A (PKA) phosphorylation of the channel on Ser 2844 can lower the affinity of calstabin binding and results in activation. Hyperphosphorylation at this residue causes depletion of calstabin binding and can result in calcium “leak” by destabilizing the closed state of the channel (Reiken et al, 2003).

Calmodulin, another protein capable of binding to the RyR, regulates the channel differently dependent upon cytoplasmic calcium concentration. Calmodulin has four calcium binding sites, two on the N-terminus and two on the C-terminus (reviewed in Jiang et al., 2010). The N-terminal sites have a ten-fold lower calcium affinity than the C-terminal sites. When calcium binds the C-terminal calcium binding domains, calmodulin switches from an activator into an inhibitor (Jiang et al., 2010).

The RyR1 itself can act as a regulator of neighboring RyR1s. Calcium released through an individual channel will not affect that same channel since the A-site is inaccessible to calcium feedthrough while in an open configuration (Laver et al., 2004). This calcium does have an effect on neighboring channels, however, and removes Mg$^{2+}$ inhibition by competitively binding at the A-sites.

Even with this extensive regulation of the RyR, however, a single point mutation within the channel can result in severe pathology. Missense mutations in the RyR1 can result in malignant hyperthermia (MH), central core disease, and multi-mini core disorder
(Betzenhauser and Marks, 2010). MH is a disorder characterized by an increased sensitivity of the RyR1 to activation, and mouse models of MH have recently been created and characterized in skeletal muscle (Chelu et al., 2006; Durham et al., 2008).

**Malignant Hyperthermia**

Malignant hyperthermia is a pharmacogenetic disorder, meaning that it is hereditary but the major symptoms are not overtly expressed unless the individual is exposed to volatile anesthetics, depolarizing muscle relaxants, or heat stress. The incidence of diagnosed anesthetic-induced MH is approximately 1 in 14,000 for the pediatric population and 1 in 50,000 for the adult population (MacLennan, 1992). MH susceptibility may actually be closer to 1 in 2,000-3,000 since prevalence is difficult to determine; MH is only apparent after exposure to triggering agents (Monnier et al., 2002). However, diagnosis is possible using the *in vitro* contracture test prior to administering anesthesia. Biopsied skeletal muscle fibers are submerge d in baths containing either halothane or caffeine, and if the muscle cells are hypersensitive to both of these agents, the individual will be diagnosed as malignant hyperthermia susceptible (MHS). When an MHS individual undergoes anesthesia, the RyR1 releases excessive amounts of calcium from the SR, resulting in sustained whole-body muscle contracture, elevated body temperature, and rhabdomyolysis; without treatment this is a fatal response in 80% of cases (reviewed by Lanner et al., 2010). Currently, dantrolene, an RyR antagonist, is the only known treatment for MH (reviewed by Lanner et al., 2010). Although mutations in the DHPR can result in MH (Carpenter et al., 2009), missense mutations in the RyR1 are the main cause of the disorder. There are three different regions within the RyR1 which account for the loci of the vast majority of MH mutations.
These regions include the N-terminus, a myo/cytoplasmic domain, and a small portion of the C-terminus which resides in the lumen of the ER/SR (McCarthy et al., 2000). The RyR1<sup>Y522S/+</sup> mutation is located within the N-terminal domain.

There are two different mechanisms theorized to underlie MH and/or CCD; the “leaky” RyR1 hypothesis, and the excitation-contraction uncoupling hypothesis (Dirksen and Avila, 2002). The Y523S mutation in the RyR1, the human equivalent of the Y522S mutation in mice, has been attributed to the “leaky” hypothesis (Dirksen and Avila, 2002). In most MH mutations, this leak can for the most part be compensated for by the calcium reuptake, extrusion and buffering machinery of the cell (Bellinger et al., 2008; Dirksen and Avila, 2002). Thus, SR calcium stores remain relatively full and basal cytoplasmic calcium levels close to normal. However, uncompensated calcium leak can result in CCD (Bellinger et al., 2008). Thus, calcium store depletion ensues, leaving little calcium for release during stimulation and subsequently only weak muscle contraction.

A number of proposed mechanisms for the cause of leaky RyR1 exist, and leaky RyRs have actually been associated with a number of different disorders. The Y522S-RyR1 mutation, associated with MH in mice, causes a calcium leak through the RyR1 that results in an elevated intracellular calcium concentration triggering the production of reactive nitrogen species (RNS) by nitric oxide synthases (NOS) (Durham et al., 2008). An increased basal level of RNS results in S-nitrosylation of the RyR1, which actually serves to amplify the inherent leak present in mutant channels (Durham et al, 2008). Calcium leak through the RyR1 (due to either an MH or CCD mutation) alters the normal functioning of skeletal muscle cells in multiple ways including a reduced threshold for luminal Ca<sup>2+</sup> activation (Jiang et al., 2008), an altered retrograde signal from RyR1 to the
DHPR which results in a leftward shift in DHPR inactivation and window calcium release (Andronache et al., 2009), enhanced excitation-coupled Ca\(^{2+}\) entry (Cherednichenko et al., 2008), and altered sensitivity to temperature, activators, and redox modifications (Durham et al., 2008). The phenotype of this disorder has been well characterized in skeletal muscle, but RyR1 is also expressed in a number of different tissues including cerebellar PNs (Fig 2; Giannini et al., 1995). PNs are an extensively studied cell type, and calcium signaling within these cells is essential for normal functioning of the entire cerebellum, yet the specific role of RyR1 is not fully understood. An MH mutation in the RyR1 could serve as a unique tool to study altered RyR1 channel function in PNs.

**Figure 2: RyR1 is expressed in the cerebellum**

Adapted from Giannini et al., 1995

**Purkinje neurons**

Purkinje neurons are the integrator and sole-output of the cerebellar cortex (Ito, 1984). Four types of cells provide input to PNs (Schilling et al., 2008). Two inputs are inhibitory, GABA-ergic synapses: stellate and basket cells. The other two of these inputs
are excitatory, glutamatergic synapses: parallel fibers, originating from granule neurons, and climbing fibers, originating from the inferior olive.

The first input, parallel fibers, synapse onto the dendrites of PNs and elicit two different types of responses. A single stimulus from the parallel fiber produces only small and local Ca\(^{2+}\) signals in PNs (Eilers et al., 1995). Repetitive parallel fiber stimuli create a biphasic Ca\(^{2+}\) response “the simple spike” (Fig 3; reviewed by Hartmann and Konnerth, 2005). The first phase is the result of Na\(^{+}\) currents through the AMPAR that depolarize the membrane and trigger Ca\(^{2+}\) entry through voltage gated P-type calcium channels. The second phase of the response is mediated by mGluR1 activation which in turn triggers the release of the G-proteins G\(_{\alpha q}\) and G\(_{\alpha_{11}}\). Phospholipase C\(_{\beta}\), which is activated by the G-proteins, cleaves IP\(_3\) from PIP\(_2\) and thus triggers calcium release through the IP\(_3\)R (Hartmann et al., 2005). Even this second type of response is local and remains in small dendritic regions and sometimes even single spines (Hartmann et al., 2005). Recent evidence suggests that RyRs may play a role in amplifying the second phase of this response through CICR. If a dendritic spine receives a strong enough repetitive stimulus, calcium fluxed into the cell through VGCCs and released through the IP\(_3\)R can diffuse out of the spine and into the dendritic shaft where RyRs are located. Resultant CICR through the RyRs is thought to be responsible for spreading the signal to other parts of the dendritic tree (Eilers et al., 1996).
Figure 3: Calcium signaling in cerebellar Purkinje neurons

Adapted from Hartmann and Konnerth 2005,

*Left:* Schematic of the calcium response to AMPAR activation.

*Right:* Schematic of the calcium response to mGluR1 activation.

The second input, climbing fiber activation or “the complex spike”, on the other hand, results in a widespread signal that originates in the proximal dendrites (where climbing fibers synapse) and can be carried into the soma and the distal portions of the dendritic tree (Eilers et al., 1996). Presynaptic release of glutamate initiates the complex spike by activation of AMPARs and mGluR1s located on the postsynaptic PN plasma membrane. This triggers fast, inactivating sodium currents which are responsible for the initial fast components of the spike. Slow, non-inactivating sodium currents, P/Q-type
currents, and T-type currents shape the slower complex spike components. Finally, $K^+$ currents are mainly responsible for the repolarization of the complex spike, but have been implicated in a number of other roles (Schmolesky et al., 2002). Approximately 50% of the $K^+$ current is the result of calcium-dependent potassium current activation (Schmolesky et al., 2002), and it has recently been suggested that RyR-mediated calcium stores might be a contributing source of calcium to these channels (Edgerton and Reinhart, 2003; Gruol et al., 1996; Brorson et al., 1991). There is also evidence that RyRs contribute to calcium signals evoked by both mGluR and AMPAR activation (Gruol et al, 1996).

There are two major GABA-ergic inhibitory synapses on Purkinje neurons; stellate cells, which synapse onto dendritic regions of the PNs and are located in the molecular layer of the cerebellum, and basket cells, which synapse onto the soma and proximal dendrite and are located in the molecular layer as well, but are closer to the Purkinje cell layer than stellate cells (Schilling et al., 2008). Signals from these four regulators of PNs are integrated and sent to the deep cerebellar nuclei where PN GABA-ergic axonal projections synapse.

Release of calcium from intracellular stores is immensely important for the calcium dynamics of PNs. The ER stores themselves form a continuous network throughout PNs, and are present even within dendritic spines (Terasaki et al., 1994). The IP$_3$R is also expressed throughout the cell and into the spines, while the RyR1 is expressed everywhere except the spines (Martone et al., 1993). Another indication of the importance of calcium signaling within these cells is the great capability of these cells to regulate free calcium. Calbindin D28K and parvalbumin, endogenous, cytosolic calcium
buffering proteins, are very highly expressed in Purkinje neurons (Celio, 1990), and these two proteins along with the contribution of immobile calcium buffering machinery account for an enormously high calcium buffering capacity within Purkinje neurons (Fiero et al., 1996). The calcium binding ratio of PNs is about an order of magnitude higher than other neuronal cell types. Calcium signaling can also be important for nuclear functions. Calcium signals originating from depolarization of the plasma membrane can reach the nucleus; RyRs amplify these somatic calcium signals and set the gain of nuclear calcium signals (Gruol et al., 2010).

The RyR plays a diverse number of roles in different cells, but the specific contribution of the RyR1 within PNs has yet to be elucidated. Since RyR2 and RyR3 are much more prevalent in other neuronal cell types, this begs the question: why are RyR1s expressed in PNs? Using the recently created mouse model of MH, Y522S-RyR1, we hope to learn more about the function of this specific channel in the brain. This study shows that the MH mutant form of RyR1 exhibits an enhanced leak through the RyR1 at elevated temperatures, and a decreased threshold for activation by caffeine. RyR1<sup>Y522S/+</sup> mutant mice are, therefore, a unique tool that can be used in future studies to examine the functional effects of altered RyR1 activity on PN excitability and firing behavior.
Chapter Two: Methods

Genotyping of Y522S-RyR1 mice

The protocol for genotyping of Y522S-RyR1 mice was modified from the procedures described by Chelu et al., 2006. Genomic DNA was isolated from ear punches using the Promega Wizard SV Genomic DNA Purification System (Promega Corporation) and subjected to PCR with primers (Invitrogen). Amplified DNA was cleaned using an E.Z.N.A Cycle Pure Kit (Omega bio-tek), and digested with B1p1. Digested samples were run on a 1.5% agarose gel containing ethidium bromide (110 mV for 30 minutes). Imaging of the gel was accomplished using a Bio-Rad chemi-doc system.

Dissociation of cerebellar cells

Two to three week old wild-type and Y522S-RyR1 mice were cervically dislocated, and cerebella were quickly dissected and submerged in ice-cold calcium and magnesium free phosphate-buffered saline (CMF-PBS). Meninges were removed and cerebella were transferred to a fresh culture dish containing a 0.1% papain solution and minced. These dishes were then placed into a 37°C incubator for 45 minutes, with agitation every 10-15 minutes. After the incubation period, the cerebellar pieces were rinsed twice with CMF-PBS and transferred to clinical centrifuge tubes containing 2 mL of dissociation media. Cells were mechanically separated by trituration with three increasingly small diameter fire-polished Pasteur pipettes. Cerebellar cell suspensions
were then filtered through a 50 µm BD Falcon cell filter and collected. Cells were then plated on poly-ethylene imine coated glass cover-slip culture dishes (density of 3x10^6 cells/mL), and incubated at 37°C overnight.

**Identification of dissociated Purkinje neurons**

PNs were visually identified by their large soma and apical dendritic stump. Visual identification of PNs was confirmed by immunocytochemistry (Fig 4). PNs are the only cerebellar cell to express the calcium buffering protein calbindin D28K, and antibodies to this PN specific protein can be utilized to determine cellular identity. Cerebella were dissociated and plated on glass-insert cover-slip dishes as described previously and fixed in 4% paraformaldehyde. These dishes were then rinsed repeatedly over a thirty minute period with PBS. Non-specific binding was prevented by incubation in a blocking solution (5% BSA/0.2% Triton X-100 in PBS). Following the blocking step, dishes were incubated overnight at 4°C with anti-calbindin D28K primary antibodies (Millipore; 1:1000 in blocking solution, rabbit-α-mouse). Fluorescence from conjugated secondary antibodies (Alexa-fluor 488; 1:1000 in blocking solution, goat-α-rabbit) was detected with a Hamamatsu CCD camera and analyzed using Slidebook software (Intelligent Imaging Innovations).
Figure 4: Visual identification of dissociated Purkinje neurons

Cerebellar cells isolated from a 3 week old Y522S-RyR1 mouse and cultured for 18 hours. Standard immunochemical procedures were used to stain Purkinje cells with a calbindin-D28K antibody. Scale bar = 20 µm.

Immunohistochemistry

Cerebella were fixed in 4% paraformaldehyde, paraffin-embedded, and sliced sagittally (10 µm) on a microtome. Non-specific binding was prevented by incubation in a blocking solution (5% BSA/0.2% Triton X-100 in PBS). Cerebellar slices were incubated with anti-calbindin D28K (Millipore; 1:1000 in blocking solution, rabbit-α-mouse), a well-established PN marker (Jande et al., 1981). Following conjugation with secondary antibodies (Alexa-fluor 488; 1:1000 in blocking solution, goat-α-rabbit), the cerebellar slices were imaged on a confocal microscope using Olympus Fluo-view software.
Calcium Imaging Experiments

Cerebellar Purkinje cells were dissociated and plated as previously described. Twelve to twenty four hours after plating, neurons were loaded with the membrane-permeable Ca\(^{2+}\) indicator dye, Fura-2 AM (5 \(\mu\)M; 30 minutes at RT). Cells were then rinsed three times with rodent ringer’s solution (in mM: 145 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 10 glucose, pH 7.4) and incubated at room temperature for at least another 15 minutes to allow for de-esterification of the Fura-2 AM. Following this incubation, dishes were imaged on an Olympus microscope with a Hamamatsu CCD camera using Slidebook software (Intelligent Imaging Innovations). Changes of intracellular calcium levels in Purkinje cell somata were measured with dual excitation wavelength ratiometric imaging (340/380 nm excitation ratio) and expressed either as the raw F340/380 ratio or the change in F340/380 ratio (\(\Delta\)Ratio = \(R_{\text{test}} - R_{\text{baseline}}\)). All measured responses were below the saturation point of Fura-2. All recordings were made using a 40x dry objective and images were taken every second with a 100 millisecond exposure to 340/380 excitation. Images were binned 4x4. For experiments using a 37°C bath, temperatures were controlled with an automatic temperature controller (Warner Instrument Corporation). All solutions used in calcium imaging were prepared in regular rodent ringer’s (RR) solution containing (in mM) 145 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 10 glucose, pH 7.4. Nominally calcium-free RR solutions were prepared with equimolar substitutions of MgCl\(_2\) for CaCl\(_2\) and the addition of 1 mM EGTA.
Dose response to caffeine

Caffeine solutions were prepared in regular rodent ringer’s solution at concentrations of (in mM) 1, 5, 10, 30, and 50, and applied to dissociated PNs that were loaded with Fura-2 AM as previously described. Caffeine solutions were applied with a fast multi-barrel perfusion system. PNs were either sequentially exposed to increasing concentrations of caffeine or exposed to an individual application of one of the caffeine concentrations. The duration of caffeine application was 60 seconds followed by at least 60 seconds of wash-out with regular rodent ringer’s solution.

Depolarization-induced calcium release

Dissociated PNs loaded with Fura-2 AM were depolarized by a 10 second puff-application of a high K⁺ (80 mM) rodent ringer’s solution. PNs were depolarized at room temperature and then again at 37°C.

Store content and SOCE

Dissociated PNs were loaded with Fura-2 AM as described previously, but were rinsed with nominally calcium-free rodent ringer’s solution prior to imaging. Baseline F340/380 ratios were recorded for at least a minute prior to application of 1 µM thapsigargin (in calcium-free RR). For store-operated calcium entry (SOCE), after the F340/380 ratio had returned to baseline, a 2 mM calcium RR solution was applied for 90 seconds.

Statistical Analysis

All statistical analyses were performed using the unpaired Student’s t test, unless otherwise stated. Area under the curve was determined by calculating ΔF340/380 ratios.
(ΔRatio = R_{test} - R_{baseline}) for every data point recorded and summing all appropriate values for each trial. Full-duration at half maximal amplitude was determined by counting the number of data points for a given trace that were larger than the half maximal response. Since data were recorded once every second this value was reported in seconds. The same analysis was performed for full-duration at one-tenth maximal amplitude, except that the data points that were counted corresponded to values larger than one-tenth of the maximal response.
Chapter Three: Results

Cerebellar structure and Purkinje cell morphology is not grossly altered in Y522S-RyR1/+ mice

The first aim of our study was to determine if the morphology of Purkinje neurons was altered in Y522S-RyR1 mice compared to wild-type as a result of altered RyR1 channel function. We did not observe any gross morphological defects in Y522S-RyR1 PNs, as we expected (Fig 5). This is consistent with findings in skeletal muscle. There are no major structural deficits in RYR$^{Y522S/+}$ myotubes, although this mutation is associated with development of abnormal mitochondrial morphology in adult cells (Durham et al., 2008).
Figure 5: Morphology of RyR1-Y522S/+ Purkinje neurons

Confocal microscopy of cerebellar section stained with antibody to the ‘Purkinje cell marker’ calbindin-D28K (blue staining is nuclear staining with DAPI). Images are z-compressions of several confocal optical sections from a wild-type (Wt) or Y522S-RyR1/+ (Hz) cerebellum. Scale bar = 50 µm (top) and = 30 µm (bottom).

Decreased threshold for activation in RyR1-Y522S/+ channels

One of the hallmark traits of MH is that the RyR1 in skeletal muscle cells exhibits an increased sensitivity to caffeine (Chelu et al., 2006), and, as mentioned previously, this increased sensitivity can be used as a measure to diagnose MHS (Allen et al., 1998).
Whether the RyR1<sup>Y522S/+</sup> mutation exhibits similarly altered function within the central nervous system has not, to our knowledge, been addressed.

In order to ascertain the functional effects of this mutation on channel activity within PNs, we exposed dissociated cells to the RyR1 agonist caffeine and recorded the size of the associated calcium transient as the change in F340/380 ratios from baseline. Specifically, regular rodent ringer’s (RR) solutions containing increasing concentrations of caffeine (in mM: 1, 5, 10 and 30) were sequentially perfused onto PNs from wild-type and RyR1<sup>Y522S/+</sup> mice for 60 seconds followed by a 60 second wash period with RR. To ensure that sequential (seq) applications of caffeine were not depleting the ER store, we also perfused cells with an individual (ind) 60 second application of only one of the caffeine containing solutions (Fig 6). We compared the responses from both of these experimental paradigms in RyR1<sup>Y522S/+</sup> neurons and found no significant differences in the size of the response (change in F340/380 ratio from baseline), or the sensitivity of the channel to caffeine (EC-50) (Fig 6). Interestingly, while the size of the responses were also unchanged between the ind and seq trials in wild-type neurons, the average EC-50 of wild-type cells perfused with only an individual caffeine solution is less than in the sequential trials (ind=12.8±5.8, seq=30.3±4.3). However, the EC-50 from either wild-type trial was larger than the EC-50 from the RyR1<sup>Y522S/+</sup> trials (Fig 6). We decided to pool the data from both the seq trial and the ind because the responses were not significantly different in size and sequential applications did not appear to be depleting the ER store of calcium.
Figure 6: Individual versus sequential caffeine dose response

(A, B) Dissociated Purkinje neurons were perfused with a single concentration of caffeine (ind) or with sequentially applied (seq) increasing concentrations of caffeine (1, 5, 10, 30 mM) and the change in fluorescence was measured using the ratiometric calcium indicator dye Fura-2 in wild-type (ind, n=25, 14, 17, 19; seq, n=20) (A), and Y522S (ind, n=27, 16, 22, 34; seq, n=27) (B) neurons. (C) Curves were fit to the data using the “Michaelis-Menton” function in Kaleidagraph. To show the leftward shift in the Y522S trials more clearly, the data was normalized to the response to 30 mM caffeine, which was set to 1. The responses to all other concentrations of caffeine were expressed as a fraction of the maximal change in
F340/380 ratio. The data from the ind and seq trials were pooled (pool) and shown on this graph as well. (D) Summary graph showing the calculated EC-50 values ± parameter error generated by Kaleidagraph.

We compared the responses from wild-type and RyR\textsuperscript{Y522S/+} neurons and although the size of the calcium transient in response to each the four caffeine solutions is not significantly different between RyR\textsuperscript{Y522S/+} neurons and wild-type neurons, the dose that invokes maximal release is reduced in RyR\textsuperscript{Y522S/+} cells. The maximal response to caffeine (30 mM) is not significantly larger than the response to 5 mM caffeine in RyR\textsuperscript{Y522S/+} neurons (\(t=0.841\), df=106, p>0.05), indicating that at 5 mM caffeine RyR\textsuperscript{Y522S/+} neurons are maximally activated. However, in wild-type cells, the response to 5 mM caffeine is significantly lower than the response to 30 mM caffeine (\(t=2.871\), df=51, p<.01), indicating that maximal activation of wild-type cells is not achieved until the application of 30 mM caffeine. The fact that RyR\textsuperscript{Y522S/+} neurons are maximally activated by lower concentrations of caffeine supports the apparent leftward shift in EC-50 (Wt=21.1±5.5, Y522S=3.8±1.7) exhibited by RyR\textsuperscript{Y522S/+} cells (Fig 7).

Since sensitivity to activation is affected by temperature in RyR\textsuperscript{Y522S/+} myotubes (Durham et al., 2008), we also performed this experiment at more physiological temperatures (37°C). At this temperature, there were no significant differences between wild-type and RyR\textsuperscript{Y522S/+} neurons. The presence of any difference might have been obscured by the leftward shift in the EC-50 of wild-type cells (Wt 25=21.1±5.5, Wt 37=3.1±1.9) as a result of the temperature increase. Furthermore, the EC-50 for both
wild-type and Y522S cells at 37°C (Wt=3.1±1.9, Y522S=2.1±1.3) both fell between the two lowest concentrations of caffeine applied (1 mM and 5 mM) to the cells. Future experiments need to be performed with lower concentrations of caffeine, to determine whether there is a difference in activation at this temperature. Although the EC-50 at 37°C is not significantly shifted, RyR1^{Y522S/+} PNs exhibited an increase in the amplitude of their response to 1 mM caffeine ($t_s=1.939$, df=54, $p<.05$) when compared to the amplitude of the response in wild-type cells. These data do suggest, however, that at 25°C there is a decreased threshold for activation.
Figure 7: Decreased threshold for activation in RyR1<sup>Y522S/+</sup> channels

(A) Dissociated Purkinje neurons were perfused with increasing concentrations of caffeine (1, 5, 10, and 30 mM) at 25 degrees Celsius and the change in fluorescence was measured using the ratiometric calcium indicator dye Fura-2 (Wild-type, n=24, 34, 37, 39; Y522S-RyR1, n= 27, 43, 49, 61). (B) Dissociated Purkinje neurons were also perfused with increasing concentrations of caffeine (1,
5, 10, and 30 mM) at 37 degrees Celsius. The response to 1 mM caffeine was significantly larger in Y522S neurons (unpaired t test, $t_s=2.266$, df=57, $P<0.05$). (Wild-type, n=31; Y522S-RyR1, n=38) (C) Summary graph comparing the EC-50 of curves fit to the data in Kaleidagraph using the “Michaelis-Menten” function. Data are reported as the EC-50 ± the parameter error generated by Kaleidagraph. (D) Representative traces of the responses at both temperatures for both wild-type and Y522S neurons. Axis labels indicate the change in F340/380 ratio (ΔF) and caffeine concentration ([Caff]). Data in (A) and (B) are presented as the mean ± SEM.

**Increased intracellular calcium concentration in RyR1-Y522S/+ Purkinje neurons**

The RyR1<sup>Y522S/+</sup> mutation causes a decrease in the threshold for activation of the RyR1 in PNs (Fig 7), suggesting that basal RyR1 activity might also be increased. If the calcium buffering, extrusion, and sequestration machinery of the cell is not able to compensate for enhanced activity of the RyR channel, this could result in an increase in basal cytoplasmic calcium concentrations. In order to investigate whether altered RyR1 activation affects cytoplasmic calcium concentrations in PNs, we measured baseline F340/380 ratios in wild-type and RyR1<sup>Y522S/+</sup> neurons. Baselines were recorded for 30 seconds at room temperature (25°C) and more physiological temperatures (37°C). There were no significant differences in F340/380 ratios at room temperature, but at elevated temperature, the RyR1<sup>Y522S/+</sup> neurons exhibited increased intracellular calcium concentrations (Fig 8). PNs, therefore, are either capable of compensating for a slight
increase in basal RyR1 activity at lower temperatures or RyR1 activity itself is not enhanced without the challenge of elevated temperature.

**Figure 8: Increased intracellular calcium concentration in RyR-Y522S/+ Purkinje neurons**

Baseline fluorescence ratios, measured using Fura-2-AM, were recorded at room temperature and more physiological temperatures. There is a significant difference in intracellular calcium levels between wild-type (n=19) and Y522S-RyR1 (n=22) cells at 37°C (unpaired t test; t_s=1.828, df=23, P<0.05). Data are presented as the mean ± SEM.
Intracellular calcium stores are unaffected by the RyR1-Y522S/+ mutation

The ER serves as a source of calcium for a number of different vital cellular functions, and calcium release from the ER can be mediated by RyR calcium channels (Simpson et al., 1995). Chronically hyperactive RyR1 channels could potentially deplete the ER of calcium, and indirectly affect a diversity of cellular functions. Since RyR1\textsuperscript{Y522S/+} PNs appear to exhibit a decreased threshold for activation and an increased cytoplasmic calcium concentration at higher temperatures, there is a possibility that the ER calcium store content could be affected by this mutation. To address this issue, we took a two-fold approach. First, we examined whether maximal calcium release from RyR1-mediated calcium stores was altered by the RyR1\textsuperscript{Y522S/+} mutation. Dissociated wild-type and RyR1\textsuperscript{Y522S/+} PNs were perfused with a RR solution containing 30 mM caffeine for 60 seconds, and F340/380 ratios were recorded at both 25°C and 37°C. The amplitude of the response was measured as the change in F340/380 ratio from baseline, and the amount of calcium released was indirectly measured as the area under the curve. There were no significant differences between the responses of wild-type and RyR1\textsuperscript{Y522S/+} neurons at either temperature (Fig 9).
Figure 9: ER calcium store content is unaffected by the RyR1^{Y522S/+} mutation as assessed by maximal doses of caffeine

(A) Representative traces from wild-type (25, n=23; 37, n=31) and Y522S (25, n=39; 37, n=38) Purkinje neurons that were loaded with Fura-2-AM and perfused with maximally activating concentrations of caffeine (30 mM) for 60 seconds. This experiment was performed at room temperature (25) and more physiological temperatures (37). Bars indicate duration of caffeine application. (B) The change in F340/380 ratio from baseline. There was no significant difference in the amplitude of the response in any condition (p>0.05). (C) Area under the curve of the response. There were also no significant differences in the overall size of the response (p>0.05). Data are presented as the mean ± SEM.

Our second approach involved blocking the sarcoplasmic/endoplasmic reticular calcium ATPase (SERCA) pharmacologically with the SERCA inhibitor, thapsigargin. SERCA acts as a calcium pump that sequesters calcium into the ER store. Without a functioning SERCA, calcium content in the ER cannot be replenished. Therefore, activity of the RyR1 and/or IP\textsubscript{3}R will eventually completely deplete the ER of calcium. As mentioned previously, intracellular calcium originates from either influx across the plasma membrane, or release from internal stores. To ensure that measured changes in F340/380 ratios were a function of release from internal stores, we applied 1 µM thapsigargin to dissociated PNs bathed in a nominally calcium-free extracellular solution.
There were no significant differences in either the amplitude, duration at half maximal activation, or the area under the curve of the response to thapsigargin at either 25°C or 37°C (Fig 10). Although none of the differences were significant, it does appear that a trend toward larger store content in RyR1$^{Y522S/+}$ neurons exists. This is surprising given the apparent hyperactive nature of mutant RyR1, but might be a result of compensatory mechanisms.
Figure 10: Store content assessed by thapsigargin

Dissociated wild-type and Y522S Purkinje cells bathed in a nominally calcium-free extracellular solution were perfused with 1 µM thapsigargin and the resulting change in fluorescence was recorded at 25°C (A) and 37°C (B). (C) The
∆F340/380 was not significantly different between Y522S (25, n=22; 37, n=12) or wild-type (25, n=24; 37, n=12) cells at either temperature. (D) The area under the curve was also not significantly different between Y522S-RyR1 and wild-type cells. (E) The duration of the response at half maximal activation was also unaffected. Data are presented as mean ± SEM.

Store-operated calcium entry - a compensatory mechanism?

The RyR1<sup>Y522S/+</sup> mutation causes increased RyR1 activity, therefore might be partially depleting ER calcium stores. However, we did not observe any significant alterations in the calcium store content; measured by maximal RyR activation and SERCA inhibition (Fig 9 and 10). One possibility could be activation of an ER calcium-store refilling mechanism and store-operated calcium entry (SOCE) might serve this role. PNs express the principal proteins typically associated with SOCE, STIM 1 and Orai 1 (Klejman et al., 2009). Store depletion results in clustering of these two proteins at discrete puncta on the ER and plasma membranes, respectively (Klejman et al., 2009). The rearranged proteins trigger calcium influx that can replenish the stores. To determine whether RyR1<sup>Y522S/+</sup> neurons exhibited increased SOCE, we depleted the ER stores of RyR1<sup>Y522S/+</sup> and wild-type neurons with 1 µM thapsigargin in a nominally calcium-free extracellular solution and measured the responses. After calcium release from the stores had ceased, indicated by a return of F340/380 ratios to baseline, a 2 mM calcium solution was perfused into the dish and the resultant calcium response was recorded as F340/380 ratios. In wild-type PNs we recorded SOCE in 17 out of the 27 cells tested at 25°C, and 2
out of the 12 cells tested at 37°C. While, in RyR1<sup>Y522S/+</sup> PNs we recorded SOCE in 21 out of the 28 cells tested at 25°C, and 2 out of the 12 cells tested at 37°C. Further analysis on the cells tested at 37°C was not performed, since only 2 out of the 12 cells responded for both wild-type and Y522S PNs. For those tested at 25°C, however, we calculated the amplitude of the response as the change in F340/380 ratio from baseline, and the area under the curve to assess the overall size of the response. Neither the amplitude nor the area of the SOCE response was significantly different between wild-type and Y522S neurons at either temperature (Fig 11). Thus, it does not appear that differences in SOCE explain the normal store content in neurons exhibiting increased RyR activity.

![Graph of SOCE response](image-url)
Figure 11: SOCE—a compensatory mechanism?

Purkinje cells loaded with Fura-2-AM were bathed in nominally calcium free extracellular solution and perfused with 1 µM Thapsigargin (black bar). Once the response F340/380 ratio had returned to baseline, the extracellular solution was
changed to a 2 mM calcium solution for 90 seconds (blue bar). (A) Schematic representation of the experimental paradigm. (B) Traces showing the change in F340/380 ratio from baseline in response to extracellular calcium. At 25°C, SOCE occurred in 9 out of 24 wild-type and 11 out of 22 Y522S cells. At 37°C, SOCE occurred in only 2 out of the 12 cells tested for both wild-type and Y522S Purkinje neurons. Neither the amplitude (C), nor the area under the curve (D) were significantly different at 25°C. Further analysis was not performed on the cells tested at 37°C because so few cells responded at this temperature. Data are presented as the mean ± SEM.

**Effects of altered RyR1 function on cellular function**

The effects of the RyR1<sup>Y522S/+</sup> mutation on RyR1 function in PNs contribute to an apparent lower threshold for activation and, furthermore, increased basal RyR1 activity at physiological temperatures. However, the implications of altered RyR1 function on more global calcium dynamics and cellular function in PNs are not fully understood. Even though PNs express the isoform of the RyR that is typically associated with mechanical coupling, most evidence suggests that CICR is the main pathway for activation in these cells. By depolarizing PNs with a high potassium solution, we triggered calcium influx through voltage gated calcium channels. This calcium signal would then be amplified by calcium-induced calcium release through the RyR1. We measured the global calcium response to depolarization in Y522S and wild-type cells as a means to elucidate the consequences of altered RyR1 function on global calcium dynamics within PNs.
Specifically, F340/380 ratios were recorded at both 25˚C and 37˚C, and the amplitude of the response was calculated using the change in F340/380 ratio from baseline. The overall size of the response was calculated using the area under the curve. When the F340/380 ratio had returned to a baseline, we stopped recording the response. In some cells, this baseline either overshot or undershot the original baseline. The number of wild-type and RyR1\textsuperscript{Y522S/+} cells exhibiting this behavior did not appear to be different (Wt 25: 2/10, Y522S 25: 5/15, Wt 37: 3/10, Y522S 37: 5/15 cells).

Since the initial phase of the response is likely due to calcium influx, and calcium release through the RyR1 primarily contributes in later phases of the response, we measured the full duration of the curve at both one-half maximal values (FDHM) and one-tenth maximal values (FDTM). The amplitude, area, and the durations of the response were not significantly different between wild-type and Y522S neurons (Fig 12). However, some interesting trends in the data exist. The amplitude of the response indicates a trend toward larger initial responses in wild-type neurons than in RyR1\textsuperscript{Y522S/+} neurons, and this trend is reversed when comparing either the area under the curve or the duration of the response.
Figure 12: Effects of the Y522S mutation on calcium dynamics

An 80 mM K$^+$ solution was puff applied for 10 seconds onto dissociated Purkinje that were loaded with Fura-2-AM and the calcium response was recorded as the change in F340/380 ratio from baseline. (A) Traces showing the calcium response to depolarization in each condition. (B) Summary graph showing the ΔF340/380 ratio. There were no significant differences between groups at either temperature. (C) Summary graph displaying the area under the curve that was calculated for each condition. There were no group differences for this measure. (D, E) Graphs displaying the duration of time the size of the response was above either half of
maximal activation (FDHM) or one-tenth of maximal activation (FDTM). There were no significant differences between Y522S-RyR1 (n=15) and wild-type neurons (n=10) for any of the measures. Data are presented as the mean ± SEM.
Chapter Four: Discussion

The function of the RyR1 in skeletal muscle has been well established; membrane depolarization induces a conformational change in L-type voltage-gated calcium channels that, by mechanical coupling, triggers release of $\text{Ca}^{2+}$ through the RyR1 (Beam and Horowicz, 2004). It is unclear if the RyR1, which is highly expressed in cerebellar Purkinje neurons, is involved in a similar type of mechanical coupling or acts solely through a Ca-induced $\text{Ca}^{2+}$ release mechanism. Furthermore, the role of calcium released through the RyR1 is not clearly defined. The “cardiac”- and “brain”-RyR isoforms (RyR2 and RyR3) are more generally expressed throughout the cerebellum, while the “skeletal”-isoform (RyR1) is limited in expression within the brain (Ledbetter et al., 1994, Giannini et al., 1995). This begs the question: does RyR1 function differently in Purkinje cells than RyR2 or RyR3 does in other neuronal cell types? There are currently no commercially available isoform-specific pharmacologic agents directed against RyR1. Thus, a mutant form of the RyR1 that alters channel function could potentially serve as a unique tool to assess the individual contribution of this RyR isoform to PN function.

The RyR1$^{Y522S/+}$ mutation has been shown to affect RyR1 channel activity in skeletal muscle. Mutant channels are hypersensitive to activation by agonists (caffeine, voltage, and temperature) and exhibit enhanced basal activity contributing to increased intracellular calcium concentrations at higher temperatures (Chelu et al., 2006; Durham et al., 2008). However, to our knowledge, no previous study has examined whether MH
mutations exhibit similar phenotypes in non-muscle cell types. RyR1 expressed in a neuronal environment might behave differently than RyR1 expressed in skeletal muscle, and the etiology of an MH mutation might not be restricted to skeletal muscle cells. Our results indicate that this mutant RyR1\textsuperscript{Y522S/+} in PNs, exhibits a decreased threshold for activation by caffeine, and increased basal intracellular calcium concentrations at physiological temperatures and therefore represents a valid tool to examine altered RyR1 function within PNs.

Calcium release from the ER can be induced by the RyR agonist caffeine, and an increased sensitivity of RyR1\textsuperscript{Y522S/+} to this compound within skeletal muscle cells has been well documented. We used caffeine to determine if the RyR1\textsuperscript{Y522S/+} mutation affects the threshold for channel activation in PNs, and discovered that mutant channels exhibit a decreased threshold for activation. However, at higher temperatures there were no significant differences in the threshold for activation. To fully understand the consequences of altered RyR1 activation as a result of the RyR1\textsuperscript{Y522S/+} mutation in PNs, it is important to distinguish what is known about RyR1 in skeletal muscle from what we know about RyR1 in PNs. The regulatory proteins that are associated with RyR1 in skeletal muscle are not necessarily expressed in PNs. Although, at least some of the key players expressed in skeletal muscle are present in PNs. RyR1 associates with calsequestrin in skeletal muscle, and calsequestrin is expressed in PNs of the chicken (Volpe et al., 1993), but it is unclear if calsequestrin has the same functional interactions in PNs. In addition, it is currently unknown if RyR1 is arranged in PNs in orderly arrays as it is in skeletal muscle. RyR1s, in skeletal muscle, that are not functionally coupled to
neighboring channels do not maintain a stabilized closed state as well as RyR1s that are coupled with their neighbors (Liang et al., 2009), so this could potentially account for observed differences in channel function. Another important question is whether RyR1s are activated by mechanical coupling or CICR in PNs. There is strong evidence that RyRs in PNs are associated with CICR (Kano et al., 1995) and the presence of mechanical coupling is unlikely as there is no calcium release when PNs are depolarized in zero extracellular calcium solution (Eilers et al., 1996). Furthermore, P-type voltage-gated calcium channels carry ~90% of the calcium current in response to depolarization in PN and are likely the main contributors to activation of RyR1 (Mintz et al., 1992).

While in skeletal muscle, L-type voltage-gated calcium channels physically interact with the channel and depolarization induces a conformational change in the DHPR that results in activation (Tanabe, 1990). L-type voltage-gated calcium channels are also expressed in PNs (Ahlijanian et al., 1990), but the function of these channels remains unclear, a recent study suggests a larger role for L-type channels early in development (Gruol et al., 2006).

Whether altered activation of the RyR1 due to the RyR1$^{Y522S/+}$ mutation affects PN morphology is an important question to address. MHS individuals may be suffering from neurological symptoms that may be overlooked, since the characteristic skeletal muscle defects are overshadowing. Thus, the effects of MH mutations on cerebellar cellular organization and neuronal morphology need to be addressed as many processes involved in development are regulated by calcium. Using confocal microscopy, we determined that the RyR1$^{Y522S/+}$ mutation does not appear to grossly alter PN morphology.
(Fig 4). Previous studies of the effects of the RyR1<sup>Y522S/+</sup> mutation on skeletal muscle cell morphology, revealed no major structural differences, but did find swollen and abnormal mitochondria in adult mutant mice due to an enhanced basal leak of calcium through the RyR1 (Durham et al. 2008).

Increased intracellular calcium levels can, over time, result in excessive production of reactive nitrogen species (RNS) by nitric oxide synthases within the cell (Durham et al., 2008). Lingering RNS can enhance S-nitrosylation of the RyR1 on cys 3635, which lies in the calmodulin binding domain (Sun et al., 2001). S-nitrosylation acts to exacerbate calcium leak through the RyR1 through two main mechanisms. The first is that S-nitrosylation increases the K<sub>d</sub> of CaCaM for the RyR1 (the calcium-bound, inhibitory form of calmodulin) which reduces calmodulin’s inhibitory actions on the channel (Boschek et al., 2007). The second is that S-nitrosylation increases the K<sub>d</sub> of calstabin for the RyR1, which destabilizes the closed state of the channel resulting in an enhanced leak (Bellinger et al., 2009). In the RyR1<sup>R615C/+</sup> porcine model of MH, it has been proposed that calstabin depletion interferes with the ability of RyR1 to interact with neighboring channels, thus leading to both enhanced calcium release in response to halothane, and a basal calcium leak through the RyR1 (Liang et al., 2009). Consistent with the notion of an enhanced calcium leak, we observed larger Fura-2 fluorescence ratios at 37˚C in RyR1<sup>Y522S/+</sup> PNs than in wild-type PNs (Fig 8).

Even though RyR1<sup>Y522S/+</sup> PNs exhibit a calcium leak, work in our lab has not detected any changes in the amount of cellular and mitochondrial stress present in PNs. The levels of lipid peroxidation in RyR1<sup>Y522S/+</sup> cerebellar neurons, as measured by MDA
equivalents, are not significantly different than the levels of lipid peroxidation in wild-
type neurons (Santiago and Lorenzon, unpublished data). This suggests that the
phenotype of MH might be more severe in skeletal muscle cells, however whole
cerebellar lysates were used in these experiments, and since PNs only make up about
10% of the cerebellum, actual differences in cellular stress may have been masked by the
nature of the experiment. Future studies will be performed on Purkinje neuron enriched
preparations to further investigate this phenomenon.

Lipid peroxidation is a measure of stress directly resulting from action of reactive
oxygen species (ROS), but the negative effects of the RyR1<sup>Y522S/+</sup> mutation within
skeletal muscle appears to be more directly related to the action of reactive nitrogen
species (RNS). In order to determine if the production of RNS is enhanced in Y522S-
RyR1 PNs in response to elevated levels of calcium, isolated PNs were loaded with DAF-
FM (a NO indicator dye) and exposed to high levels of glutamatergic stimulation. Even
though glutamate increased the intracellular concentration of calcium, preliminary
experiments demonstrated that the production of NO was not significantly increased in
either Y522S-RyR1 or wild-type neurons (Although there appeared to be a trend toward
an increase in NO production: Santiago and Lorenzon, unpublished).

Interestingly, it has been found that the RyR has essentially two separate release
pathways, one that is insensitive to inhibitory concentrations of ryanodine, and one that is
blocked by ryanodine (reviewed by Eltit et al., 2010). The ryanodine insensitive
conformation of the RyR1, thought to be the calcium “leak” pathway, is active at rest and
can be blocked by bastadin 5 (Eltit et al., 2010). This pathway is the main source for
maintaining resting levels of intracellular calcium, contributing approximately 50% (Eltit et al., 2010). It is unclear which of these two proposed pathways are responsible for the MH phenotype, or whether both are involved. Since the ryanodine-insensitive pathway seems to be mainly responsible for setting cytoplasmic calcium levels, it seems that the presence of increased intracellular calcium concentrations in RyR1^{Y522S/+} neurons is likely the result of the “leak” pathway. The classical form of calcium release, or the ryanodine-sensitive pathway, might also be affected by the RyR1^{Y522S/+} mutation in PNs, because caffeine activates the RyR by increasing A-site affinity to the endogenous ligand, calcium (Meissner et al., 1997). Theoretically, increases in activity of either of these pathways could result in increased calcium signaling, increased cytoplasmic calcium levels and/or a depleted ER calcium store.

ER calcium store content does not appear to be significantly affected in PNs with the RyR1^{Y522S/+} mutation. Even though RyR1^{Y522S/+} mutants exhibit hypersensitivity to activation, the amount of calcium released in response to maximal activation, was not different. Consistent with our results, RyR1^{Y522S/+} skeletal myotubes do not show depletion of SR calcium stores (Chelu et al., 2006). Furthermore, the porcine model of MH, RyR1^{R615C/+}, exhibits a calcium leak and an increased sensitivity to activation, but no depletion of calcium stores (Liang et al., 2009). Furthermore, assessment of the content of the ER stores with the SERCA poison, thapsigargin, did not yield any significant differences between wild-type and RyR1^{Y522S/+} neurons.

One possible mechanism that could contribute to refilling the stores and compensation for excessive calcium leak is store-operated calcium entry (SOCE). SOCE
is a pathway of calcium influx that is triggered by store depletion, and the calcium that enters the cytoplasm can be sequestered into the ER by SERCA. STIM1, which resides in the ER membrane, senses intra-luminal calcium levels and clusters to form discrete puncta when calcium stores are depleted (Klejman et al., 2009). Orai1, a protein residing in the plasma membrane, also forms clusters that create overlapping puncta with STIM1. Following protein clustering, calcium flows inward through store-operated calcium channels, the exact molecular make-up of these channels is still unclear, but it is thought that Orai1 and possibly TRPC channels can form pores in the plasma membrane that allows calcium flux into the cytosol (reviewed by Hewavitharana et al., 2007). SOCE has not, to our knowledge, been directly measured in PNs, although STIM1 expression has been demonstrated in these cells (Klejman et al., 2009). Thus, we investigated if SOCE is present in PNs and if SOCE is altered in RyR1\textsuperscript{Y522S/+} PNs. PNs do express SOCE, but the size of the response is unchanged by altered RyR1 activity in RyR1\textsuperscript{Y522S/+} cells. Furthermore, since SOCE is unchanged in RyR1\textsuperscript{Y522S/+} neurons, it is unlikely that this phenomenon serves as a compensatory mechanism for an enhanced leak.

After characterizing how the RyR1\textsuperscript{Y522S/+} mutation affects channel function, we hoped to investigate the affects of this mutation on cellular calcium signaling by depolarizing RyR1\textsuperscript{Y522S/+} and wild-type PNs with a high potassium solution. While no significant differences were found between F340/380 responses, there were some interesting trends in the data. Wild-type responses appeared slightly larger in amplitude, but when examining the duration of the response this trend reverses. Differences in global calcium responses might be revealed using inhibitory concentrations of ryanodine.
during periods of depolarization; pharmacological subtraction of RyR-mediated calcium release might reveal more subtle differences in calcium dynamics.

Although the functional consequences of the RyR1\textsuperscript{Y522S/+} mutation might differ between cell types, the presence of an enhanced leak and an increased sensitivity to caffeine in PNs suggests that perhaps the MH-phenotype is not constrained solely to skeletal muscle. Furthermore, this mutation represents a valuable tool to further investigate the role of RyR1 in PNs.
Chapter Five: Summary

Our study represents the first examination of the consequences of an MH-inducing mutation in a non-muscle environment. We have found that Y522S-RyR1 PNs exhibit an enhanced basal leak at physiological temperatures, and an enhanced sensitivity to agonists without affecting store content. In addition, SOCE does not appear to be a compensatory mechanism for enhanced calcium leak through the RyR1, but further investigation of this phenomenon is warranted. Now that we have determined that this mouse model of MH is a valid tool to examine altered RyR1 function within PNs, we hope to determine how this mutation affects the excitability and firing behavior of the cell.
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