Neuronal Calcium Regulation and Cellular Stress in a Malignant Hyperthermia Disease Model

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Neuronal Ca$^{2+}$ regulation and cellular stress in a Malignant Hyperthermia disease model

A Thesis
Presented to
the Faculty of Natural Sciences and Mathematics
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of the Requirements for the Degree
Master of Sciences

by
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Advisor: Nancy M. Lorenzon
Abstract

Calcium ions (Ca\textsuperscript{2+}) are essential signaling molecules and must be carefully regulated to preserve specificity in function. One indispensible Ca\textsuperscript{2+} signaling protein is the ryanodine receptor (RyR) calcium release channel. RyR1 is essential for muscle contraction, and RyR1 mutations can lead to severe muscle disorders such as malignant hyperthermia (MH). Recently, a mouse model of human MH (Y522S-RyR1) has been developed. In skeletal muscle, Y522S-RyR1 exhibits an increased sensitivity to activation resulting in Ca\textsuperscript{2+} leak, mitochondrial disorganization, and cellular stress. Although RyR1 expression is limited in the brain, the cerebellum may be particularly vulnerable to this disease because RyR1 is highly expressed in Purkinje neurons. In this study, Y522S-RyR1 in Purkinje cells exhibits a lower threshold for activation, but does not cause severe cellular stress and damage. Future investigation of compensatory mechanisms for increased Ca\textsuperscript{2+} release in Purkinje cells could be of therapeutic value for many neurological and muscular disorders.
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Introduction

Part I: RyR1, a key regulator of Ca\(^{2+}\) dynamics

Calcium ions (Ca\(^{2+}\)) are involved in an array of cellular activities and intracellular signaling cascades within a diverse group of cell types. Ca\(^{2+}\) can influence many aspects of cellular life including cell growth and proliferation and programmed cell death. Indeed, the importance of Ca\(^{2+}\) to the vitality of an entire organism is difficult to succinctly and thoroughly convey. Thus, one can imagine that if every function that Ca\(^{2+}\) can perform were allowed to take place with every increase in intracellular Ca\(^{2+}\), life would be nothing short of utter chaos. But how can such a ubiquitous molecule elicit highly specific cellular responses? The answer lies in the spatial and temporal regulation of Ca\(^{2+}\) in a cell. The significance of this regulation is clearly illustrated within excitable cells. Excitable cells, such as neurons and muscle fibers, rely on precise control of both the localization and concentration of Ca\(^{2+}\) to carry out their unique cellular functions. This feat is accomplished through the use of an extensive Ca\(^{2+}\) toolkit, which consists of Ca\(^{2+}\) entry and release pathways, mobile and immobile Ca\(^{2+}\) buffers, transient Ca\(^{2+}\) storage sites, and Ca\(^{2+}\) extrusion mechanisms (Berridge et al., 2003). The collaborative work of these regulatory elements is the key to maintaining the dynamic nature of Ca\(^{2+}\) as a signaling molecule and also preventing pathological consequences of Ca\(^{2+}\) dysregulation.

One protein that has a major role in this process is the ryanodine receptor. Ryanodine receptors (RyRs), along with inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs),
are Ca\(^{2+}\) release channels located in the membrane of the sacroplasmic/endoplasmic reticulum (SR/ER). RyRs and IP\(_3\)Rs are homotetramers of subunits with apparent molecular weights of \(~500\) kD and 260 kD, respectively (Walton et al., 1991). Functional RyRs are among the largest proteins known to date; the N-terminal cytoplasmic domains are comprised of roughly 4,000 amino acids (Takeshima et. al., 1989). In fact, RyRs act like enormous macromolecular signaling complexes by scaffolding an array of regulatory proteins such as kinases, phosphatases, and phosphodiesterases that modulate channel activity and integrate signals from second messenger cascades to finely tune the dynamics of Ca\(^{2+}\) release.

Three RyR isoforms are expressed in mammals and are encoded by separate genes (reviewed by Rossi and Sorrentino, 2002). Although the amino acid sequences of these proteins are about 70% homologous, they have considerably different tissue expression and some notable functional differences (Takeshima et. al., 1989). For example, the predominant isoform expressed in skeletal muscle is RyR1, and in cardiac muscle it is RyR2. In brain, RyR2 and RyR3 are expressed most ubiquitously; however, RyR1 is expressed in a limited number of brain regions. RyR1 is particularly interesting because of its specialized role in ‘excitation-contraction’ (EC) coupling within skeletal muscle. Unlike the other RyR isoforms, RyR1 is closely apposed and ‘mechanically-coupled’ to voltage-gated Ca\(^{2+}\) channels (VGCC) in the sarcolemma of skeletal muscle fibers. This physical link allows conformational changes that occur within VGCC during membrane depolarization to elicit conformational changes in RyR in order to facilitate Ca\(^{2+}\) release from lumenal ER stores (reviewed by Avila, 2004).
RyR1 channel activity has been shown to be modulated by numerous ions, protein-protein interactions, and post-translational modifications. Foremost among these modulators is Ca$^{2+}$ itself, which elicits a biphasic response in RyR1 channel activity. At nanomolar to micromolar concentrations Ca$^{2+}$ activates RyR1 but at micromolar to millimolar concentrations Ca$^{2+}$ functions as an inhibitor (Rossi and Sorrentino, 2002). The Ca$^{2+}$-dependence of RyR1 ensures that the release channel opens upon stimulation and quickly closes during prolonged intracellular Ca$^{2+}$ elevation (Betzenhauser and Marks, 2010). The Ca$^{2+}$ binding protein, calmodulin, also has a dual effect on RyR1 channel activity. Upon binding four molecules of Ca$^{2+}$, CaM undergoes a conformational change that affects its ability to bind to and activate other proteins such as Ca$^{2+}$/calmodulin-dependent protein kinase (CaMK). At nanomolar Ca$^{2+}$ concentrations, CaM exists in a Ca$^{2+}$-free state and acts as a partial agonist of RyR1. However, at higher concentrations Ca$^{2+}$-bound CaM functions as an inhibitor of channel activity (Zalk et al., 2008).

Another important regulator of RyR1 channel activity is FKBP12. FKBP12, also known as calstabin, stabilizes the closed state of RyR1 thereby inhibiting channel activity. Calstabin also facilitates cooperative gating between neighboring RyRs to establish synchronous release and thereby shorten the decay phase of Ca$^{2+}$ elevations (Marx et al., 1998). However, the binding affinity of calstabin for RyR1 can be altered through post-translational modifications such as protein kinase A (PKA)-mediated phosphorylation (Bellinger et al., 2008) and cysteine (S)-nitrosylation (Sun et al., 2001). In addition, Mg$^{2+}$, cyclic ADP-ribose, CaMK phosphorylation, and adenine nucleotides
can also modulate RyR1. Since these are just a few of the ways that RyR channel activity is modulated, it is clear that the precise control of RyR1-mediated Ca\(^{2+}\) release is not only of great importance, but is also highly regulated and complex. Moreover, mutations in regulatory regions of RyR1 could greatly affect channel activity and thus drastically influence Ca\(^{2+}\) dynamics and cellular function.

Accordingly, genetic mutations of RyR1 can often lead to serious skeletal muscle disorders. The most prevalent of these disorders are malignant hyperthermia (MH) and central core disease (CCD). MH is a potentially life threatening pharmacogenetic disorder characterized by whole-body skeletal muscle contractions and elevated core body temperature in response to halogenated anesthetics such as halothane. The majority of MH mutations are missense substitutions resulting from single nucleotide errors (Bellinger et al., 2008). For the most part, these mutations result in a gain-of-function by increasing the sensitivity of RyR1 to activation by Ca\(^{2+}\) or other agonists.

One autosomal dominant mutation in RyR1, Y522S, is strongly associated with MH. Recently, the first mouse model of human MH was developed to express this mutation. Mice homozygous for the Y522S mutation in RyR1 exhibit severe skeletal muscle defects and die soon after birth. Heterozygous (Hz) mice develop normally although they are more sensitive to heat and anesthetic-induced MH episodes (Chelu et al., 2005). Additional studies revealed that skeletal myotubes derived from heterozygous (Y522S/+) mice exhibit a Ca\(^{2+}\) leak in response to voltage stimulation and heat stress that is due to an increased sensitivity of activation (Durham et al., 2008). Although activation of RyR1 elicits greater Ca\(^{2+}\)-release in Y522S/+ mice, the excess Ca\(^{2+}\) is
buffered in such a way that the Ca\(^{2+}\) levels in the sarcoplasm and SR lumen are unaffected. This contrasts with many RyR1 mutations that cause CCD, which is typically characterized by a rise in resting Ca\(^{2+}\) levels, decreased SR Ca\(^{2+}\) store content, and concomitant muscle damage and weakness. However, skeletal muscle from Y522S/+ mice is not entirely unscathed.

Although MH does not lead to overt muscular pathology, Y522S/+ mice display signs of cellular stress in adulthood because the consequences of the Ca\(^{2+}\) leak are exacerbated over time. In response to heat stress, skeletal myotubes from Y522S/+ mice develop increased levels of reactive oxygen/nitrogen species (ROS/RNS) (Durham et. al., 2008). This leads to nitrosylation of RyR1, which further enhances the activity of the channel for several reasons. S-nitrosylation of a critical cysteine residue (C3635) located in the CaM binding domain decreases the affinity of CaM for RyR1 (Aracena-Parks et. al., 2006). At high Ca\(^{2+}\) concentrations, this modification attenuates the inhibitory influence that CaM has on RyR1, thereby increasing channel activity. In addition, S-nitrosylation of C3635 destabilizes the closed state of RyR1 by decreasing the binding affinity of calstabin. Lastly, nitrosylation of RyR1 is thought to shift the Ca\(^{2+}\)-dependence of inactivation of RyR1 so that it stays open at higher Ca\(^{2+}\) concentrations (Durham et. al., 2008).

The increase in RNS is likely due to enhanced activity of nitric oxide synthase (NOS) since inhibition of NOS reverses temperature-dependent increases in RNS (Durham et. al., 2008). It is not clear which isoform(s) of NOS are responsible for generating additional nitric oxide, but skeletal muscle is rich in a splice variant of
neuronal (n) NOS. Some studies suggest that nNOS can associate with triad junctions, which are membrane complexes that link the plasma membrane to Ca\textsuperscript{2+} release sites on the sacroplasmic reticulum. In this way, elevated Ca\textsuperscript{2+} levels could enhance the activity of nearby nNOS to create microdomains of RNS that could preferentially modify RyR1. Over time, excessive and prolonged exposure to Ca\textsuperscript{2+}, ROS, and RNS can result in irreversible cellular damage. Evidence for this is found in skeletal muscle fibers from adult Y522S/+ mice. Muscle fibers from mutant mice exhibit increased lipid peroxidation, disorganized mitochondrial ultrastructure, and impaired muscle function (Durham et. al., 2008).
Figure 1. Mechanism of cellular damage in skeletal muscle of Y522S mice

1) Y522S mutation causes an increased sensitivity to activation resulting in a Ca\(^{2+}\) leak.  
2) Increased [Ca\(^{2+}\)]\(_i\) levels lead to increased production of RNS/ROS. This is likely due to enhanced enzymatic activity of nNOS, xanthineoxidase (XO), or NADPH oxidases (NOX). Increased mitochondrial Ca\(^{2+}\) sequestration and concomitant oxidative stress is also thought to contribute to ROS.  
3) RyR1 is hypernitrosylated.  
4) S-nitrosylation increases Ca\(^{2+}\) leak by destabilizing closed state of RyR1 and shifting the Ca\(^{2+}\) dependence of inactivation.  
5) Increased [Ca\(^{2+}\)]\(_i\) leads to further production of RNS/ROS.  
6) In response to heat stress, perturbed Ca\(^{2+}\) release can lead to heat stroke.  
7 & 8) Chronically elevated levels of Ca\(^{2+}\) and RNS/ROS lead to disruption of mitochondrial ultrastructure and impaired muscle function (adapted from Durham et. al., 2008).
Part II: RyR1 in cerebellar Purkinje cells

Although the effects of the Y522S-RyR1 mutation on skeletal muscle have been studied, the effects on other tissues in which RyR1 is expressed have not been investigated. RyR1 is enriched in several regions of the brain whose function could also be affected by MH mutations (see Fig. 2). The cerebellum may be particularly vulnerable to the consequences of this mutation because RyR1 is expressed abundantly in Purkinje cells, which are the main output of the cerebellar circuit.

Figure 2. RyR1 is enriched in the cerebellum

Immunoblot showing the protein expression of the three RyR isoforms in muscle and brain. Note that most brain regions express more RyR2 and RyR3 than RyR1, whereas, the cerebellum expresses high levels of RyR1 (Giannini et al., 1995).
Figure 3. Organization of the cerebellar cortex

This figure illustrates the spatial organization of the major cerebellar neurons within a sagittal section of one of the cerebellar folia. The cerebellar cortex is divided into three basic layers. The molecular layer is the outermost layer and it contains most of the dendrites of the cerebellar neurons. The Purkinje cell layer is naturally formed by the series of large Purkinje cell bodies. The innermost layer of the cortex is called the granule cell layer. The white matter of the cerebellar cortex is comprised of axons from efferent (Purkinje cell) and afferent (mossy fiber, climbing fiber) pathways (Martin et. al., 1989)

Purkinje cells are large neurons with prolific dendritic arborizations that integrate multiple signals and synapse onto the deep cerebellar and vestibular nuclei, which provide major inputs to the primary and pre-motor cortices (see Figs. 3& 4). Purkinje cells receive inputs from the two principal cerebellar afferents: climbing fibers and mossy fibers. Climbing fibers (CF) originate from the inferior olivary nuclear complex and form numerous excitatory synapses on the somata and proximal dendrites of Purkinje
cells. Because these synapses are located in close proximity to the axon hillock, CF have a strong influence on Purkinje cell excitability. Mossy fibers (MF) originate from the spinal cord and brain stem and excite Purkinje cells via granule cells. Granule cells have long axons that project deep into the molecular layer of the cerebellar cortex where they bifurcate into long parallel fibers (PF) that form excitatory synapses on the distal dendrites of Purkinje cells and on other interneurons (see Figs. 3 & 4).

Purkinje cells receive inhibitory input from two types of interneurons: stellate and basket cells. The somata of these cells are located within the molecular layer of the cerebellum although the interneurons have somewhat different localizations (see Figs. 3 & 4). Basket cells send axonal projections onto the cell bodies of Purkinje cells and are located closer to the Purkinje cell layer. Stellate cells are located more towards the outer part of the cerebellar cortex and synapse onto Purkinje cell dendrites. The last of the five neurons of the cerebellar cortex is the Golgi cell. This cell forms inhibitory synapses onto granule cells, thus modulating the lone excitatory input within the cerebellar cortex.

Balance, eye muscle control, posture and limb movement, and movement planning are all functions of the cerebellum. Since Purkinje cells are indispensable components of the cerebellar circuit, some or all of these functions are adversely affected when Purkinje cell excitability is altered. Therefore, the primary goal of this study is to determine if Purkinje cells expressing Y522S-RyR1 exhibit signs of cellular stress that could produce acute or chronic cerebellar dysfunction. Even in the absence of basal cellular stress, mutant Purkinje cells may be more sensitive to induced cellular stress and/or demonstrate mechanisms for compensation of increased Ca^{2+} or ROS/RNS levels.
The results of these experiments will help to distinguish between these different possibilities and elucidate the effects of MH mutations in the brain.

Figure 4. Simplified schematic of the cerebellar circuit

The neuron organization and their synaptic connections within the cerebellar cortex are illustrated in this cartoon. Grey arrows highlight the basic flow of synaptic transmission into and out of the cerebellar cortex. Note that the Purkinje cell is the only efferent of the cerebellar cortex. Sizes and spatial distribution of the cells are not drawn to scale.
Materials and Methods

Acute Dissociation

Cerebella were dissected from WT and Y522S/+ mice and placed into plastic culture dishes containing sterile Ca\(^{2+}\) and Mg\(^{2+}\)-free (CMF) PBS. Pieces of cerebella were incubated in a papain-containing solution for 45 minutes at 37\(^{\circ}\)C and agitated every 10-15 minutes. Dishes were rinsed twice with fresh CMF-PBS. Tissue was transferred to clinical centrifuge tubes containing 2 mL of serum-containing media. Cells were mechanically dissociated by trituration with three increasingly smaller diameter fire-polished Pasteur pipettes. Cell suspensions were passed thru a 50-micron cell filter and collected. Cells were counted in the filtrate and diluted, if necessary, with serum-containing media to a density of 3 x 10\(^6\) cells/mL. Two hundred microliters of cell suspension was plated on polyethyleneimine-coated glass culture dishes and flooded with an additional 2 mL of media 2 hours later.

Live Calcium Imaging

Purkinje cells were acutely dissociated 18-24 hours prior to experimentation. Cells were loaded with 5 \(\mu\)M of the ratiometric calcium indicator dye Fura-2-AM, for 30 minutes at room temperature in the dark. Dishes were rinsed 3 times with sterile Rodent Ringer’s plus glucose before imaging to clear residual indicator dye. Composition of Rodent Ringer’s used is as follows: 145.5 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, and 11.1 mM glucose. Average resting intracellular Ca\(^{2+}\) levels were recorded for 2-3 minutes. Then, a sterile solution of 100 \(\mu\)M glutamate + 10 \(\mu\)M
glycine dissolved in Rodent Ringer’s plus glucose was applied to the bath. Changes in intracellular Ca$^{2+}$ levels were recorded for 5-25 minutes. After which, a new intracellular Ca$^{2+}$ baseline was measured, and an 80 mM KCl (high K$^+$) solution was applied to the bath. Changes in intracellular Ca$^{2+}$ levels were recorded for an additional 5-10 minutes. All recordings were made using a 40x dry objective with a Hamamatsu CCD camera. Slidebook imaging software (Intelligent Imaging Innovations, Inc) was used to collect and analyze data. Images were collected every 2.5 seconds with 100 msec exposure to 340/380 excitation. Images were binned 4x4.

**Measuring RNS Production**

Purkinje cells were acutely dissociated as described previously. The production of nitric oxide (NO) was measured using 4-amino-5-methylamino-2',7'-difluorofluoresceindiacetate (DAF-DA) purchased from Invitrogen. Cells were loaded with 1 µM of a DAF-DA for 30 minutes at room temperature in the dark. Dishes were rinsed as described previously. Baseline levels of RNS were recorded for 2-3 minutes. Then, the glutamate and high K$^+$ stimulation protocol previously described was elicited to determine if evoked increases in intracellular Ca$^{2+}$ result in changes in RNS production. Images were taken every 5 seconds, with 50 msec exposure to 480 nm excitation. Images were binned 2x2.

**Tissue Homogenization**

Whole cerebella and gastrocnemius/solei were dissected from wild type and Y522S/+ mice. Cerebella were dounce homogenized in a 1% Triton-Wahl lysis buffer containing 1 µL/mL of the protease inhibitors aprotinin and leupeptin. Muscle was
homogenized in the same solution with a motorized Tissue Ruptor (Qiagen) over ice on med-hi for 30 seconds. Homogenates were collected and centrifuged at 10,000 rpm for 5 min to pellet debris. Supernatants were decanted and stored at -20°C until ready for use. Total protein concentration for each whole cell lysate was determined using a BioRad RC/DC protein assay kit.

**Mitochondrial Subcellular Fractionation**

Whole cerebella and gastrocnemius/solei were dissected from wild type and Y522S/+ mice. Cerebella were dounce homogenized in a cytosolic extraction buffer supplemented with a proprietary cocktail of protease inhibitors and DTT (BioVision: Mountain View, CA). Muscle was homogenized with a motorized Tissue Ruptor on med-hi in cytosolic extraction buffer on ice for 30 seconds. Homogenates were collected and centrifuged at 700 x g for 10 min at 4°C to pellet debris. Supernatants were collected and re-centrifuged at 10,000 x g for 30 min at 4°C. Next, supernatants were collected and saved as cytosolic fractions. The remaining pellet was resuspended in mitochondrial extraction buffer (BioVision) and assayed for protein content using a BioRad RC DC protein assay kit.

**Western Blotting**

Sixty micrograms of total protein from each cerebellar lysate and 40 micrograms of each muscle lysate were individually added to Lammeli sample buffer containing 2-mercaptoethanol and heated on aluminum block at 95°C for 10 min to denature and reduce proteins. Samples were loaded on a 4-20% gradient Tris-HCl pre-cast gel from BioRad. Electrophoresis began at 75 volts while proteins passed through the stacking
gel, then ramped to 100 volts through the resolving gel until the dye front nearly reached the bottom of the gel. PVDF membranes were prepared for electro-blotting by soaking them in methanol for 1-2 min and then incubating them with 20% MeOH in PBS for 5 min. Membranes, filter papers and pads, and transfer cassettes were allowed to soak in transfer buffer at 4°C until ready for transfer. Transfer of proteins was completed at 100 V for 1 hour over ice with stirring. Non-specific binding was blocked by incubating all blots with 5% milk in PBST for 1 hour at room temperature on a rocker. Proteins were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies and imaged with a Bio-Rad ChemiDoc system. Additionally, anti-DNP blots (Cosmo Bio, Japan) were incubated with 10 mM 2, 4-dinitrophenylhydrazine (DNPH) dissolved in 2N HCl for 5 min to derivitize oxidatively-modified proteins prior to the blocking step. Extensive rinses were done to ensure the preservation of the membrane. Antibodies were washed from the membranes by incubation in a low pH stripping buffer containing 1.5% glycine, 0.1% SDS, and 1% Tween-20 for 30 minutes on a rocking platform. Membranes were then washed with PBST, blocked as previously described, and re-probed with a GAPDH antibody (Sigma) as a loading control.

**Lipid Peroxidation**

As a measure of lipid peroxidation, MDA levels were assayed using a colorimetric microplate assay obtained from Oxford Biomedical Research (Oxford, MI). An MDA standard curve was made enabling a relationship between absorbance and concentration. An equal volume of mitochondrial fractions from each tissue sample was incubated with the non-chromomeric substrate and absorbance of the chromogenic
product was measured at 586 nm. MDA concentrations were predicted from the linear transgression derived from the standard curve and normalized to overall protein content for a final unit of nmol of MDA/mg of total protein. Each sample was plated in triplicate, and each experiment was repeated two times.

**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 1 hour on a rocking platform. For nitrotyrosine experiments, cells were incubated with 100 µM sodium nitroprusside (SNP), a nitric oxide donor, for 6 hours at room temperature on a rocking platform prior to fixation. Cells were then washed repeatedly with PBS over 30 minutes. Non-specific binding was blocked by incubating cells with a 5% BSA in 0.2% Triton X-100 solution for 1 hour on a rocking platform. Cells were incubated with a calbindin D28k (Millipore) antibody that was diluted in a 2% BSA/0.2% Triton X-100/PBS solution at concentration of 1:1000 overnight at 4°C. Proteins were visualized with fluorescently conjugated secondary antibodies and imaged with a 40x dry objective and a Hammatsu CCD camera. Data were collected and analyzed with Slidebook software (Intelligence Imaging Innovations).

**Immunohistochemistry**

Cerebella from wild type and Y522S/+ mice were dissected, rinsed with calcium and magnesium free (CMF) -PBS, and fixed in Methacarn (methanol-chloroform-glacial acetic acid 6:3:1 by volume) for 2 hours at room temperature on a rocking platform. Tissue was dehydrated with 100% EtOH and embedded into paraffin blocks. Sagittal sections were cut at a thickness of 10 microns and mounted on glass slides. Tissue
sections were rehydrated with decreasing concentrations of ethanol. DNPH derivitization was accomplished by incubating sections with 10 mM DNPH in 2N HCl for 1 hour at room temperature. DNPH selectively reacts with proteins covalently modified by reactive aldehydes like MDA. Tissue was washed and acid-quenched before initiating tissue permeabilization with a 0.2% Triton solution. Non-specific binding was blocked with a 2% BSA/2% NGS/PBS solution prior to antibody incubation. Reactive carbonyls were labeled by incubating sections with an anti-DNP antibody. A fluorescent secondary antibody and confocal microscopy were used to visualize protein carbonylation.

Confocal Imaging

Protein carbonylation in Purkinje cells was visualized using a 40x oil objective on an Olympus Fluoview 1000 scanning confocal microscope. Fluorescently conjugated secondary antibodies were excited with 480 nm light using a laser aperture of 75 µm at 20% laser power. Image sections were taken at 1 µM intervals and Z-stacked. The provided Fluoview 1000 software was used to analyze images.

Densitometry for quantification of Western Blots

Western blot lanes were framed and bands created across the center of the protein of interest. Protein levels were quantified by calculating the area under the intensity profile curve of the created band to give units of INT x mm.
Results

To begin characterizing the effects of Y522S-RyR1 in Purkinje cells, we determined the sensitivity of the channel to the RyR1 agonist, caffeine. Using the fluorescent Ca$^{2+}$ indicator dye Fura-2 AM, a cell permeable form of Fura-2, we measured the changes in intracellular Ca$^{2+}$ levels in Wt and Hz Purkinje cells in response to focal application of increasing concentrations of caffeine. Fura-2 is a high affinity, mobile Ca$^{2+}$ buffer that can be used to accurately estimate changes in intracellular Ca$^{2+}$ levels. These changes are reported as increases or decreases in the F340/F380 ratio. The reason for this is due to the spectral properties of Fura-2. In its Ca$^{2+}$-free conformation, Fura-2 only fluoresces weakly when excited by 380 nm wavelengths of light. However, upon binding to Ca$^{2+}$, Fura-2 undergoes an absorption shift that enhances its fluorescence when excited by 340 nm wavelengths of light. Thus, the ratio of the fluorescent emissions produced by excitation at these two wavelengths of light can be used to measure changes in Ca$^{2+}$ levels within a cell. Similar to results found in skeletal muscle, results conducted by others within the lab found that Y522S-RyR1 displayed an increased sensitivity to activation in acutely dissociated cerebellar Purkinje cells (see Fig. 6; Talbott & Lorenzon, unpublished data).

In skeletal muscle, the enhanced sensitivity of Y522S-RyR1 is compensated such that resting sarcoplasmic Ca$^{2+}$ levels and SR Ca$^{2+}$ store content are unchanged. To determine if similar compensation exists in Purkinje cells, we maximally activated RyR1 by applying a saturating dose of caffeine and measured the change in cytoplasmic Ca$^{2+}$. 
This method is often used to estimate ER Ca\(^{2+}\) store content. As hypothesized, there is no significant difference in the lumenal Ca\(^{2+}\) store content of Y522S/+ mice compared to WT (p > 0.05) (see Fig. 7).

To determine if the Ca\(^{2+}\) leak associated with Y522S-RyR1 is fully compensated in Purkinje cells, we also measured resting cytoplasmic Ca\(^{2+}\) levels. Similar to skeletal muscle, Purkinje cells expressing Y522S-RyR1 do not exhibit elevated cytoplasmic Ca\(^{2+}\) levels compared to WT controls (t\(_{c}\) = -0.85355, df = 21, p > 0.05)(see Fig. 8 pg. 27). Immunocytochemistry was performed using the Purkinje cell marker, calbindin D28k, to ensure that Purkinje cells were accurately selected during Ca\(^{2+}\) imaging experiments (see Fig. 5).

In previous studies, skeletal muscle from mice harboring the Y522S mutation in RyR1 displayed increased levels of lipid peroxidation, a form of cellular stress that can be caused by increased levels of ROS. We repeated these experiments using mitochondrial fractions derived from gastrocnemius and solei of Y522S/+ and WT mice at two different ages as a means of generating a positive control. As expected, skeletal muscle from adult Y522S mice demonstrated nearly a 2-fold increase lipid peroxidation as measured by malondialdehyde (MDA) equivalents, a major end product of lipid peroxidation (t\(_{c}\) = 2.9817, df = 4, p < 0.05)(see Fig. 9). However, no significant differences were observed in the extent of lipid peroxidation in the cerebella of Y522S mice at either the juvenile or adult stage.
Figure 5. Purkinje cells from WT and Y522S-RyR1 mice 1 day after acute dissociation

Purkinje cells were acutely dissociated and fixed with 4% paraformaldehyde 24 hours after plating. They were labeled with a primary antibody against the calcium binding protein calbindin D28k. Cells were visualized with a fluorescent secondary antibody. Scale bar is equal to 10 microns. High and low pixel intensities were adjusted to normalize the visual dynamic range.
Figure 6. Y522S-RyR1 in cerebellar Purkinje cells is more sensitive to caffeine

Acutely dissociated Purkinje cells were loaded with 5 μM Fura-2 AM. Increasing concentrations of caffeine were applied focally to cells using a multi-barrel fast perfusion system. Between agonist applications, drug was washed by 30 sec application of normal extracellular solution. A) Ca\(^{2+}\) release of Wt and Hz Purkinje cells in response to various concentrations of caffeine. B) Representative traces of Ca\(^{2+}\) transients from Wt and Hz Purkinje cells in response to increasing doses of caffeine. WT cells N = 9. Hz cells N = 14. Error bars = SE of means. (Talbott & Lorenzon, unpublished data).
Figure 7. Lumenal Ca\textsuperscript{2+} store content of ER is not different between Y522S/+ and WT Purkinje cells

Purkinje cells were loaded with 5 μM Fura-2 AM. Ca\textsuperscript{2+} release was maximally stimulated through the application of 30 mM caffeine. Inset is a representative trace of a Ca\textsuperscript{2+} response from a Hz Purkinje cell. Error bars represent SE of means. For Wt cells, n = 25. For Hz cells, n= 37 (unpublished data from Talbott & Lorenzon).
Figure 8. Average resting cytosolic Ca\(^{2+}\) levels in acutely dissociated Purkinje cells from WT and Y522S/+ mice

Cells were loaded with 5 µM Fura-2 AM, a cell permeant form of Fura-2. Baseline 340/380 ratios were recorded for 2-3 minutes prior to applying 100 µM glutamate to elicit excitatory Ca\(^{2+}\) influx. Only cells that responded positively to glutamate were included in this figure. For WT cells, N=13. For Y522S/+ cells, N=10. There is no significant difference in the average resting cytosolic calcium levels between Y522S/+ and WT Purkinje cells (t\(_s\) = -0.85355, df = 21, p >> 0.05).
Figure 9. Lipid peroxidation in WT and Y522S/+ mice at different ages.

Mitochondrial subcellular fractions were subjected to a colorimetric assay for MDA concentration to assess the lipid peroxidation in WT and Y522S/+ mice. Estimated MDA values were normalized to overall protein content to correct for protein loading. **A)** Lipid peroxidation in juvenile (< 4 mo) mice. **B)** Lipid peroxidation in adult (> 10 mo) mice. Lipid peroxidation is significantly increased in adult Y522S/+ muscle compared to age-matched WT control (t_s= 2.9817, df = 4, p < 0.05). N = 5 for adult mice. N = 3 for juvenile mice.
End products of lipid peroxidation, such as MDA, are often highly reactive aldehydes or ketones that can cause further oxidative damage in a cell by covalently modifying proteins. This process is known as protein carbonylation and is a marker of cellular stress because structural modification of proteins can lead to alteration of function. We used immunohistochemistry to determine if Purkinje cells from adult Y522S/+ mice exhibit increased levels of protein carbonylation compared to WT mice. To do this, thin tissue sections were cut from Y522S and WT cerebella and incubated with 2,4-dinitrophenylhydrazine (DNPH) to derivatize carbonylated proteins. Sections were then incubated with an anti-DNP antibody to selectively label carbonylated proteins. Then, a fluorescent secondary antibody was used to visualize DNP moieties. After normalizing images to the same dynamic range, P cells from Y522S mice appear to exhibit increased levels of protein carbonylation (see middle panel of fig. 10).

**Figure 10. Purkinje cells of Y522S/+ mice show an increased protein carbonylation**

Tissue sections were incubated with 10 mM DNPH to derivatize protein carbonyls. Anti-DNP bodies were then used to label carbonylated proteins and fluorescent secondary antibodies were used to visualize the DNP moieties. Optical image sections were taken with an Olympus Fluoview 1000 confocal microscope at 1-2 micron intervals and analyzed using FV1000 software. Red arrows indicate Purkinje cell layer. Scale bar = 40 µm.
Proteins from cerebellar whole cell lysates were separated by SDS-PAGE. After proteins transfer to PVDF membranes, protein carbonyls were derivitized and blots were probed with an anti-DNP antibody. HRP-conjugated secondary antibodies were used to visualize protein carbonyls. After normalizing the intensity levels of protein bands from Wt and Y522S mice to their loading control (see Methods), a nearly two-fold increase was observed in a protein band (arrowhead) with an apparent molecular weight of ~60 kDa in Y522S mice.

Immunoblotting was performed to see if proteins exhibiting increased carbonylation in Y522S/+ mice could be specifically identified. Whole cell lysates from the cerebella of Y522S/+ and WT mice were separated by SDS-PAGE using gradient gels. After proteins were transferred to PVDF membranes, blots were incubated with a 10 mM DNPH solution to derivatize carbonylated proteins. Blots were probed with the same anti-DNP antibody used in the previous experiment. An unknown protein in the cerebella of Y522S mice having an apparent molecular weight of ~60 kDa exhibited a 2-fold increase in carbonylation relative to WT control (see Fig. 11). After examining several markers of cellular stress such as lipid peroxidation and protein carbonylation
within the cerebella of Y522S mice and finding little or no significant differences compared to WT controls, we investigated changes in Y522S mice that might constitute potential compensatory mechanisms for the increased Ca\(^{2+}\) release from stores. Therefore, we posed the question whether mutant mice upregulate the expression of endogenous Ca\(^{2+}\) binding proteins. We assessed calbindin D28k expression levels using western blot analyses on whole cerebellar lysates from Y522S mice at different ages. Adult Y522S mice express significantly less calbindin than WT mice (t stat = -4.54343, df = 2, p < 0.05). Currently, the causes or consequences of such a change in calbindin expression are unclear (see Fig. 12).

![Figure 12](image)

**Figure 12. Expression of the Ca\(^{2+}\) binding protein calbindin D28k in WT and Y522S-RyR1 mice at different ages**

Cerebella from juvenile (~ 2 mo) and adult (~ 12 mo) were dissected and homogenized as previously described. Protein blots were probed with an anti-calbindin antibody, and then stripped and reprobed for GAPDH as a loading control. Densitometry was performed, and all calbindin bands were normalized to their respective loading controls. Adult Y522S/+ mice express less calbindin relative to GAPDH than WT mice (t stat = 4.54343, df = 2, p < 0.05). Error bars represent SE of means.
Since skeletal muscle expressing Y522S-RyR1 exhibit increased levels of ROS/RNS, we sought to determine if the compensatory mechanisms within cerebellar Purkinje cells include increased anti-oxidant potential. Upregulation of anti-oxidant enzymes like glutathione peroxidase, or superoxide dismutase (SOD), could be partly responsible for the lack of gross cellular defects in adult cerebellar Purkinje cells from Y522S mice. Therefore, we examined the protein levels of SOD 1 & 2 in cerebellum to determine if mutant RyR1 leads to SOD protein upregulation. No significant differences were found in the cerebella of Y522S and WT mice. We also examined the levels of SOD1 and SOD2 in the muscles of Y522S and WT mice. Previous studies using the Y522S/+ mice have not investigated whether the expression of these proteins may be increased in skeletal muscle.

We hypothesized that muscle would show increases in SOD2 protein levels because mitochondrial ultrastructure and function is significantly impaired in Y522S/+ mice (Durham et al. 2008). Disorganization and swelling of the inner mitochondrial membrane can have a dramatic effect on the function of proteins associated with the process of oxidative phosphorylation. This could lead to increased levels of ROS, which can oxidize susceptible lipids and proteins. However, we observed no significant differences in the expression of SOD1 or SOD2 in muscle from Y522S mice (see Fig. 13).
Figure 13. Expression of the anti-oxidant enzymes SOD1 and SOD2 in Y522S/+ mice at different ages.

Whole cell lysates were generated and blotted as previously described. Blots were stripped and reprobed with GAPDH as a loading control as shown under SOD blot. After normalization, intensity values of protein bands from Y522S/+ mice were normalized by WT controls. N = 4 mice for each age and genotype. Experiments were duplicated and intensity values averaged. Each SOD blot was loaded with lysate from adult mice as follows: lane 1 is WT muscle; lane 2 is Hz muscle; lane 3 is WT cerebellum; lane 4 is Hz cerebellum.
Although Purkinje cells from Y522S/+ mice do not exhibit gross cellular defects, frequent exposure to elevated levels of Ca$^{2+}$, ROS, or RNS could make them more sensitive to cellular stress. This could be especially true if oxidative damage occurs to proteins that regulate Ca$^{2+}$ homeostasis or have anti-oxidant function. We tested this idea by exposing acutely dissociated Purkinje cells to an excitotoxic dose of glutamate and measured production of RNS.

Prolonged glutamatergic stimulation should cause a strong increase in intracellular Ca$^{2+}$ levels by allowing Ca$^{2+}$ to influx through the ionotropic glutamate receptor, AMPAR, and through the metabotropic glutamate receptors which can release Ca$^{2+}$ through the second messenger, IP$_3$. This increase could lead to increased production of RNS through activation of Ca$^{2+}$-sensitive enzymes like NOS, XO, NADPH oxidase. All of these pathways could lead to increased levels of nitric oxide. To determine the sensitivity of Purkinje cells from Y522S/+ mice to excitotoxicity, we loaded Purkinje cells with the fluorescent NO indicator dye, 4-amino-5-methylamino-2′,7′-difluorofluoresceindiacetate (DAF-DA). We applied 100 µM glutamate plus 10 µM of the glutamate receptor co-agonist glycine to the bath solution and collected fluorescence emission of excited DAF molecules (see Fig. 14). Although Purkinje cells exhibited robust Ca$^{2+}$ responses to this stimulation protocol (Fig. 14, panel D), there was usually little or no change in the fluorescence emission of the nitric oxide indicator, DAF-DA (see Fig. 15).
Figure 14. Intracellular Ca\textsuperscript{2+} levels increase in Purkinje cells from Y522S/+ mouse during glutamate exposure

A) Bright field image of a Hz Purkinje cell prior to glutamate stimulation.  B) Purkinje cells were loaded with 1 µM of the fluorescent NO indicator, DAF-DA.  C) Fluorescence emission of DAF in response to continuous glutamate exposure.  D) Purkinje cells were loaded with 5 µM Fura-2 AM as previously described. Changes in intracellular Ca\textsuperscript{2+} in response to continuous glutamate exposure were recorded by monitoring F340/F380 ratio (see methods).
Figure 15. RNS production in acutely dissociated Purkinje cells from Y522S and WT mice exposed to glutamate.

Cells were loaded with the RNS indicator dye DAF-FM. Baseline fluorescence was recorded and then glutamate (100 µM) was applied. RNS production was recorded for up to 40 min. Error bars = SE of means. N = 10 cells for each genotype. There is no significant difference between Y522S and WT Purkinje cells with respect to RNS production in response to continuous glutamate exposure (t = 1.4357, df = 9, p < 0.05).

In a final experiment, the sensitivity of mutant Purkinje cells to induced nitrosative stress was tested. We incubated acutely dissociated Purkinje cells with 100 µM sodium nitro-prusside (SNP), a highly unstable molecule that readily dissociates into nitric oxide molecules. We used immunocytochemistry to assess the levels of nitrotyrosine residues in Purkinje cells. Tyrosine residues are particularly susceptible to nitration by RNS like peroxynitrite. Peroxynitrite can form when both RNS and ROS are present, mainly through the interaction of superoxide anion and nitric oxide. After a
6-hour incubation with SNP, we measured protein nitration in Y522S and WT mice. After analyzing the images, we did not find any significant differences in the sensitivity of Y522S mice to nitrosative stress compared to Wt mice (see Fig. 16). However, a high degree of protein nitration was observed in the unstressed controls, and the degree to which further increases in protein nitration could be detected is unclear. It is possible that acute dissociation alone causes significant nitrosative stress and that our nitric oxide donor could not elicit increased levels of protein nitration beyond this.
Figure 16. Acutely dissociated Purkinje cells from WT and Y522S/+ mice following short term nitrosative stress

Acutely dissociated Purkinje cells from WT and Y522S mice were challenged with the nitric oxide donor SNP. Cells were fixed and probed with an anti-nitrotyrosine antibody to assess the levels of protein nitration. Unstressed controls are shown to demonstrate the degree of basal nitrosative stress. Scale bars = 10 µm.
Discussion

Part 1: Basal cellular stress within cerebellar Purkinje cells from Y522S mice

Preliminary studies conducted within our lab demonstrate that Y522S-RyR1 in cerebellar Purkinje cells is more sensitive to channel activators such as caffeine (see Fig. 6). These observations correlate with previous findings using skeletal muscle harvested from the Y522S/+ mice (Durham et. al., 2008). Thus, both muscle and neuronal cell types exhibit enhanced Ca$^{2+}$ release channel activation. In skeletal muscle, the consequences of this enhanced sensitivity include: increased production of ROS/RNS, increased lipid peroxidation, disorganization of mitochondrial ultrastructure, and impaired muscle function. However, the effect of enhanced RyR1 sensitivity has not been investigated in Purkinje cells. Therefore, we examined several markers of cellular stress to determine if similar pathological consequences exist in Purkinje cells.

In skeletal muscle, the Y522S-RyR1 mutation causes cellular stress despite the fact that resting cytosolic Ca$^{2+}$ levels and SR Ca$^{2+}$ store content are unaffected compared to normal muscle (Durham et. al., 2008). We report that ER Ca$^{2+}$ store content and resting cytosolic Ca$^{2+}$ levels in Purkinje cells are not significantly different between Y522S and WT mice (see Figs. 7 & 8). These findings support the hypothesis that MH mutations in RyR1 typically affect the sensitivity of channel activation and enhance channel activity, but may not necessarily cause major global perturbations in Ca$^{2+}$ homeostasis.
One caveat of our experimental design that could have influenced these results pertains to the age of the mice used in these experiments. Cerebellar tissue for single cell experiments was harvested from mice no older than 4 weeks of age. This age of mice was chosen for all of our Ca$^{2+}$ imaging experiments to ensure that a high percentage of cells would be physiologically active; that is, capable of releasing Ca$^{2+}$ in response to RyR1 activation. It is unclear whether Purkinje cells from adult Y522S/+ mice would exhibit similar resting cytoplasmic Ca$^{2+}$ levels and ER Ca$^{2+}$ store content compared to WT mice if they were as responsive in short term culture as they are in their youth. It is possible that frequent and excessive exposure to elevated levels of Ca$^{2+}$, ROS, and RNS could undermine the regulatory function of certain proteins that would affect Ca$^{2+}$ levels in these compartments. For example, the activity of SERCA pumps is known to be endogenously regulated by redox modifications. While mild oxidative conditions can increase pump activity through S-glutathionylation of a key cysteine residue (674), a highly oxidative environment can lead to sulphonylation of Cys674, which irreversibly inhibits pump activity (Csordas and Hajnoczky, 2009).

The Ca$^{2+}$ leak associated with enhanced RyR1 channel sensitivity in skeletal muscle of Y522S/+ mice causes cellular stress through a feed-forward mechanism. Elevated Ca$^{2+}$ levels in this tissue lead to increased production of ROS, which leads to increased lipid peroxidation (Durham et al., 2008). Polyunsaturated acyl chains of phospholipids and polyunsaturated fatty acids (PUFA) are particularly sensitive to oxidation by ROS. Susceptible lipids can form lipid hydroperoxides when exposed to free radicals like superoxide anion (see Fig. 17).
Figure 17. Lipid peroxidation leads to formation of reactive aldehydes that can covalently modify proteins

Polyunsaturated fatty acids (PUFA) can be oxidized by ROS resulting in lipid hydroperoxides (PUFA-OOH). These oxidatively modified lipids can undergo cleavage that leads to the formation of alpha, beta-unsaturated aldehydes like 4-HNE and dialdehydes like MDA. These reactive aldehydes can covalently modify proteins on certain electrophilic amino acids like histidine, cysteine, and lysine through Michael addition in a process called protein carbonylation (adapted from Grimsrud et al., 2008).
Lipid peroxidation is a particularly perilous consequence of ROS because it can potentiate cellular stress. This can happen in one of two ways. First, oxidation of critical lipids can directly affect the fluidity, permeability, passive electrical properties, and enzymatic activity of cellular membranes, which can drastically affect their structure and function (reviewed by Paradies et al., 2009). Lipid peroxidation can be especially harmful to a cell if ROS oxidatively modify lipids that are involved in oxidative phosphorylation. For example, peroxidation of a key mitochondrial phospholipid, cardiolipin, has been shown to cause decreased activity of complexes I, III, and IV of the electron transport chain, resulting in reduced efficiency of electron transport (Paradies et al., 2009). In this way, ROS impairs the production of ATP, which may be needed to support antioxidant activity or rectify Ca\(^{2+}\) dysregulation.

The second way that lipid peroxidation causes cellular stress is more indirect. Lipid hydroperoxides readily undergo non-enzymatic Hock cleavage, which generates fragments of lipid-derived aldehydes and ketones that are highly reactive and can cause cellular damage at distant sites (Grimsrud et al., 2008). These “reactive carbonyls” can covalently modify proteins on susceptible amino acids and thereby alter their function. Protein carbonylation is the name given to this process and it is another marker of cellular stress that can occur in response to prolonged exposure to ROS. Protein carbonylation simply means the addition of reactive carbonyl functional groups to susceptible amino acid side chains. Direct carbonylation can occur through oxidative modification of lysine, arginine, and proline residues in the presence of H\(_2\)O\(_2\) and Fe\(^{2+}\) (Houtkooper and Vaz, 2008). More commonly however, protein carbonylation occurs through Michael
addition of reactive aldehydes, formed from the cleavage of lipid hydroperoxides, to lysine, histidine, and cysteine residues (see Fig. 18).

**Figure 18. Protein carbonylation through 4-HNE and MDA**

Oxidation of polyunsaturated fatty acids (PUFA) leads to reactive aldehyde fragments like 4-HNE, which can covalently modify proteins on His, Cys, or Lys residues through Michael addition. Dialdehydes like MDA can also be formed from cleavage of lipid hydroperoxides and covalently modify amino groups to carbonylated proteins (modified from Berlett and Stadtman, 1997).

Quantification of one of these lipid-derived molecules, malondialdehyde (MDA), is a method often used to indirectly measure the extent of lipid peroxidation in a cell (see Fig. 9). Previous studies (Durham et al. 2008) have shown that skeletal muscle from adult Y522S/+ mice exhibit enhanced lipid peroxidation. We repeated these experiments by measuring the levels of MDA in mitochondrial subcellular fractions from skeletal muscle and cerebella. Since mitochondria generate large quantities of ROS in comparison to other subcellular compartments, lipid peroxidation is likely to be observed
in this fraction. A two-fold increase in lipid peroxidation of skeletal muscle from adult Y522S mice (p = 0.04) was observed. However, the same assay performed on cerebellar mitochondrial fractions did not reveal any significant differences between Y522S and WT mice although a trend appeared to exist (see Fig. 9).

Our results do not rule out the possibility that individual Purkinje cells experience increased lipid peroxidation, only that a significant increase in peroxidation is not detected in the whole cerebellar fractions. Although Purkinje cells are vitally important to overall cerebellar function, they are greatly outnumbered by other cells of the cerebellum. In this regard, subtle changes in lipid peroxidation in Purkinje cells could easily be masked by lack of changes in other cerebellar cell types. Therefore, in order to determine if changes in lipid peroxidation specifically occur in Purkinje cells, a separation/enrichment procedure should be employed. However, it is technically more challenging to separate Purkinje cells from other cerebellar neurons in mice older than a few weeks of age due to increases in similarity in cell size and surface antigens; differences that are often exploited by separation techniques in embryonic or early postnatal mice.

To circumvent this issue, immunohistochemical techniques were used to measure Purkinje cell-specific changes in protein carbonylation, a marker of oxidative stress that is correlated with lipid peroxidation. In several experiments, the Purkinje cell layer of Y522S/+ mutant cerebella appeared to exhibit increased protein carbonylation compared to WT controls (see Fig. 10).
As a second method to assess the extent of protein carbonylation, we performed western blots using whole cerebellar lysates from mutant and WT mice. There were few differences in the carbonylation of proteins between Y522S/+ and WT mice. However, one protein band ~ 60 kDa in size had a two-fold increase in carbonylation in Y522S/+ mice. As a quick attempt to identify the modified protein, we re-probed the blot with antibodies against several proteins that we thought might be oxidatively modified in the mutant mice. However, the specific protein(s) responsible for the increase was not determined. One concern with this experiment is that our method of derivatizing the protein carbonyls was not fully optimized. For the experiment shown in Fig. 11, PVDF membranes were incubated with DNPH after proteins were transferred. Other protocols for derivatization of protein carbonyls suggest incubating the cell lysates themselves with DNPH. While both immunoblotting methods have been used to study protein carbonylation, it is unclear whether one method is more effective.

Additional measures of cellular stress in the cerebella of Y522S/+ and WT mice were attempted; however, reproducible results were not obtained in these experiments. Experiments to detect changes in DNA damage were completed using immunohistochemical methods with an antibody against 8-oxo-2’-deoxyguanosine, a marker for a certain type of oxidative DNA damage. We also measured levels of protein tyrosine nitration using immunohistochemical and immunoblotting techniques. A more thorough account of basal cellular stress levels should be conducted to be certain of the effects of the Y522S mutation in RyR1 in cerebellar Purkinje cells.
Part II: Compensatory mechanisms involved in regulating Ca\textsuperscript{2+} release and preventing cellular stress

Our results suggest that Purkinje cells from Y522S/+ mice do not exhibit gross cellular defects. This is not surprising because cerebellar Purkinje cells are known to have a considerable Ca\textsuperscript{2+} buffering capacity. This is due in part to the expression of several high-affinity Ca\textsuperscript{2+} binding proteins, especially parvalbumin and calbindin. Calbindin is commonly utilized as a Purkinje cell marker since it is highly expressed in Purkinje cells and only weakly expressed in other cerebellar neurons. Thus, if RyR1 mutation causes a change in calbindin expression in Purkinje cells, this change is less likely to be masked by the calbindin expression in other cerebellar cells. No compensatory upregulation of calbindin expression was observed in Y522S mice relative to WT. On the contrary, adult Y522S/+ cerebellum express less calbindin protein than WT controls, although the significance is marginal (p = 0.045).

Besides changes in Ca\textsuperscript{2+} buffering protein expression, mutant Purkinje cells could also compensate for hypersensitive RyR1 by sequestering excess Ca\textsuperscript{2+} into subcellular compartments. Mitochondria play a major role in Ca\textsuperscript{2+} uptake. In neurons, where the resting cytosolic Ca\textsuperscript{2+} concentration is ~ 50-150 nM, mitochondria can accumulate Ca\textsuperscript{2+} as soon as cytosolic levels reach 500 nM (Nicholls, 2008). Interestingly, the mitochondrial Ca\textsuperscript{2+} import machinery has a relatively weak affinity for Ca\textsuperscript{2+} (Graier et. al., 2007). This perplexity has not been entirely resolved, but is best explained by mitochondria that are exposed to microdomains of high Ca\textsuperscript{2+} concentration. Indeed, numerous imaging studies have observed protein “tethers” that exist between
mitochondria and SR/ER membranes that likely couple Ca\(^{2+}\)-release sites to mitochondrial Ca\(^{2+}\) entry channels (reviewed by Franzini-Armstrong, 2007).

Although mitochondria have an incredible capacity to store Ca\(^{2+}\), they do not solely function as Ca\(^{2+}\) sinks. It is now widely accepted that mitochondrial Ca\(^{2+}\) uptake and release are important events that can affect numerous cellular processes and contribute to the spatial and temporal regulation of Ca\(^{2+}\) within a cell. For example, mitochondrial Ca\(^{2+}\) uptake is known to stimulate oxidative phosphorylation by enhancing the activity of three dehydrogenases coupled to the citric acid cycle: pyruvate dehydrogenase, isocitrate dehydrogenase, and alpha-ketoglutarate dehydrogenase (reviewed in Gunter et. al., 2000). Ca\(^{2+}\) sensitivity has also been reported for the F\(_0\)F\(_1\)-ATPase (Harris and Das, 1991) and the adenine nucleotide transporter (ANT) (Moreno-Sanchez, 1985). In this way, Ca\(^{2+}\) can act as a positive modulator of ATP synthesis. The ability of Ca\(^{2+}\) to respond to energy demands is certainly valuable within excitable cells, where rapid and substantial increases in cytoplasmic Ca\(^{2+}\) levels occur frequently. Thus, mitochondrial Ca\(^{2+}\) uptake functions not only to help buffer the transient elevations but also to provide energy for enzymes in order to store or extrude Ca\(^{2+}\) after a signaling event has transpired.

Unfortunately, mitochondrial Ca\(^{2+}\) uptake is known for its more nefarious roles in generating ROS and inducing cell death. Ironically, moderate Ca\(^{2+}\) uptake is considered in some cases to be a protective mechanism against oxidative stress. By stimulating oxidative phosphorylation as previously described, Ca\(^{2+}\) increases O\(_2\) consumption, lowers O\(_2\) tension in the mitochondrial microenvironment, and thereby decreases the risk
of ROS formation (Kowaltowski et. al., 2009). In addition, an increased electron transport rate typically results in a lower inner mitochondrial membrane potential ($\Delta \Psi_m$), a condition that thermodynamically disfavors reverse electron transfers, which can sometimes lead to the production of superoxide at complex I (Turrens, 2003). Furthermore, additional NADPH produced by Ca$^{2+}$-sensitive activation of matrix dehydrogenases provides antioxidant enzymes with electrons to prevent ROS-mediated damage. However, when the mitochondrial Ca$^{2+}$ load becomes too great, the detrimental effects of Ca$^{2+}$ uptake prevail.

Prolonged elevation of cytosolic Ca$^{2+}$ levels and consequent mitochondrial Ca$^{2+}$ overload can quickly lead to cellular demise. In the cytoplasm, excessive Ca$^{2+}$ can cause increased production of ROS and/or RNS through Ca$^{2+}$-dependent activation of NADPH oxidases (NOX) and nitric oxide synthases (NOS) (reviewed by Gu et. al., 2010). Ca$^{2+}$ has also been shown to affect mitochondrial motility and morphology. In fact, mitochondrial motility was observed to be completely arrested by high (1-2 µM) cytoplasmic Ca$^{2+}$ concentrations (Yi et. al., 2004). Limiting the movement of mitochondria may prevent them from being able to access and buffer microdomains of high Ca$^{2+}$ concentrations.

High cytoplasmic Ca$^{2+}$ levels can also disrupt mitochondrial structure, which often exacerbates Ca$^{2+}$ dysregulation and oxidative stress. For instance, Ca$^{2+}$-induced translocation of the cytosolic dynamin related protein, DRP-1, to the outer mitochondrial membrane promotes mitochondrial fission (Graier et. al., 2007). Excessive mitochondrial fission has been shown to facilitate release of ROS thereby potentiating cellular stress.
(Yu et al., 2006). At the inner mitochondrial membrane, Ca\(^{2+}\) has been shown to bind to cardiolipin and induce the formation of immobilized lipid clusters (Grijalba et al., 1999). Since cardiolipin is believed to interact with components of the ETC, lipid/protein alterations could lead to aberrant electron transfers and increased ROS production. Furthermore, Ca\(^{2+}\)-dependent activation of mitochondrial phospholipase A\(_2\) leads to the release of fatty acids, resulting in the swelling of the mitochondrial matrix and release of pro-apoptotic factors (Waite et al., 1969).

Although the effects of high Ca\(^{2+}\) concentrations on the structure and function of mitochondria can be severe, the major source of Ca\(^{2+}\)-mediated oxidative stress comes from the generation of ROS in the electron transport chain (ETC). An estimated 1-4% of all O\(_2\) that enters ETC experience single electron reductions, which result in the formation of the highly unstable superoxide anion (\(\cdot\)O\(_2\)) (reviewed by Csordas and Hajnoczky, 2009). As mentioned previously, Ca\(^{2+}\) uptake stimulates mitochondrial respiratory rate through activation of Ca\(^{2+}\)-sensitive matrix dehydrogenases. To an extent, this protects mitochondria from oxidative stress by lowering \(\Delta\Psi_m\). However, Ca\(^{2+}\) overload increases the respiratory rate in such a way that single electron reductions of O\(_2\) become more frequent. Thus, during prolonged Ca\(^{2+}\) elevations, large quantities of superoxide can be generated.

Fortunately, mitochondria are not defenseless to Ca\(^{2+}\)-mediated insults. Mitochondria house a variety of enzymatic and non-enzymatic antioxidants that function to maintain ROS/RNS at low levels. One of these proteins, superoxide dismutase (SOD) is specifically responsible for reducing superoxide anion to hydrogen peroxide so it can
be further reduced to H$_2$O. There are two isoforms of SOD expressed in mammals. SOD-2 has a manganese center and resides in the mitochondrial matrix. SOD-1 has a copper-zinc center and resides in the inner membrane space and cytoplasm. Both isoforms reduce superoxide anion to hydrogen peroxide so it can be reduced to H$_2$O by enzymes like glutathione peroxidase, or thioredoxin peroxidase. Considering that there are numerous ways that Ca$^{2+}$ elevations can lead to increased ROS production, upregulation of these proteins could be a means of preventing oxidative stress during Ca$^{2+}$ overload. Therefore, protein levels of SOD-1/2 in the muscle and cerebella of Y522S/+ were measured. However, no significant differences were observed in either of the SOD isoforms within cerebella or muscle of Y522S/+ mice compared to WT mice (see Fig. 13).

The fact that neither isoform of SOD is upregulated in skeletal muscle of Y522S/+ mice does not rule out the possibility that antioxidant activity could still be enhanced. In fact, several studies have demonstrated that the activity of SOD-2 is enhanced by Ca$^{2+}$-CaM (Yan et. al., 2006). In this way, Ca$^{2+}$ can attenuate its own deleterious effects on ROS production during prolonged Ca$^{2+}$ elevation. It is also possible that new $\Delta \Psi_m$ are established in the mitochondria of mutant mice, which attenuate the production of ROS while still permitting respiration to occur, albeit at a slower rate. In a similar way, Purkinje cells expressing Y522S-RyR1 may compensate for excessive Ca$^{2+}$ release by modulating the activity of existing regulatory proteins, as opposed to inducing expression of new ones. For instance, plasma membrane Ca$^{2+}$ ATPases (PMCA) that participate in Ca$^{2+}$ homeostasis by extruding Ca$^{2+}$ from the
cytoplasm as well as SERCA pumps that re-fill ER Ca\textsuperscript{2+} stores, could be potential targets of regulation. In future studies, the expression levels and activities of these proteins should be investigated.
Part III: Mutant Purkinje cells susceptibility to induced cellular stress

The final experiments conducted were aimed at determining whether Purkinje cells from Y522S are more sensitive to induced cellular stress. Even though basal stress levels were not elevated in Y522S Purkinje cells, these neurons may be more sensitive to induced stress. Purkinje cells receive glutamatergic synaptic input from PF, and CF. Therefore, we imposed a glutamate challenge on acutely dissociated Purkinje cells from Y522S and WT mice and recorded changes in intracellular Ca\(^{2+}\) levels and nitric oxide production. We assessed RNS production because Y522S-RyR1 has been shown to be hypernitrosylated in skeletal muscle (Durham et. al., 2008). We postulated that during continuous exposure to an excitotoxic dose of glutamate, mutant RyR1 would exhibit excessive Ca\(^{2+}\) release that would enhance the activity of nNOS, resulting in increased nitrosylation of RyR1. As discussed previously, this modification enhances RyR1 channel activity by destabilizing inhibitory regulators (CaM, calstabin) and enhancing the Ca\(^{2+}\)-dependence of release.

During RNS imaging experiments, healthy cells responded robustly to 100 µM glutamate by increasing cytosolic Ca\(^{2+}\) levels about two-fold, often within 2 or 3 minutes (see Fig. 14, panel D). Purkinje cells usually established a new Ca\(^{2+}\) set point that is higher than the initial resting level. Sometimes, another rapid increase in cytosolic Ca\(^{2+}\) occurs spontaneously but the second baseline is usually steady. Ca\(^{2+}\) levels often remained elevated for over 20 min. Although acutely dissociated Purkinje cells often exhibited robust Ca\(^{2+}\) responses to the glutamate we applied, very little changes occurred in the production of RNS as measured by the fluorescent NO indicator dye (see Fig. 15).
This does not rule out the possibility that changes in RNS and redox status of RyR1 occur in response to this type of stress, but they may simply occur during a time frame that we were not able to observe.

In our final experiment we induced nitrosative stress through use of the nitric oxide donor, sodium nitroprusside (SNP). To determine if Purkinje cells from Y522S/+ mice are more sensitive to nitrosative modifications, we measured changes in protein tyrosine nitration. We did not observe any differences between Y522S/+ and WT mice (see Fig. 16). We would like to repeat this experiment several more times while changing the concentration and time period of the induced nitrosative stress. It should be noted that unstressed control plates for each genotype also expressed high levels of nitrated proteins. It is possible that our dissociation process or culture conditions induce nitrosative stress on their own and thus changes in protein nitration would be harder to observe in response to induced stress.

This study is the first attempt to characterize the effects of an MH mutation in RyR1 in the brain. Although the findings presented in this report are mostly negative, they represent notable steps forward on the path to understanding how Ca\(^{2+}\) dysregulation contributes to cellular pathology. One of the most talked-about ways that Ca\(^{2+}\) can lead to cellular demise is through mitochondrial oxidative stress. Oxidative stress is postulated to be a contributing factor, if not the main one, in an ever-increasing number of pathological conditions. Foremost among these are disorders of the musculoskeletal and central nervous system. For example, many neurodegenerative disorders such as
ALS, Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease are thought to be largely caused by mitochondrial dysfunction.

Although mitochondria are able to sequester large amounts of Ca$^{2+}$, prolonged exposure to high levels of Ca$^{2+}$ can cause irrevocable cellular damage. As discussed in this report, Ca$^{2+}$ overload can lead to energy crisis, lipid peroxidation, protein carbonylation, DNA damage and other forms of cellular stress. This study highlights the importance of remedying problems associated with Ca$^{2+}$ regulation so that, if at all possible, the induction of oxidative stress and the cellular damage that often accompanies it, can be avoided altogether.

Countless groups have attempted to attenuate oxidative stress by targeting the mitochondria. This makes perfect sense because mitochondria are the principal sources of oxidative stress in a cell. But much fewer studies have looked at RyR1 as a potential therapeutic target. As we have seen, RyR1 is an important regulator of Ca$^{2+}$ dynamics within numerous cell types. A host of different proteins, ions, and post-translational modifications can alter RyR1 channel activity. Interestingly, many disorders that are postulated to be caused by oxidative stress also exhibit signs of Ca$^{2+}$ dysregulation. Therefore, therapeutics could be designed to enhance the activity of RyR1 stabilizers or inhibit the activity of channel activators. For instance, synthetic versions of calstabin could be created so that the binding affinity of calstabin for RyR1 is not reduced by post-translational modifications. On the other hand, competitive inhibitors of RyR1 agonists could be developed to prevent cellular stress during hyperactivated conditions. These are just a few of the ways that RyR1 may be targeted in order to rectify Ca$^{2+}$ dysregulation.
Hopefully, future studies will continue to elucidate the effects of MH mutations in the brain and promote the importance of Ca\(^{2+}\) homeostasis in the prevention of oxidative stress and pathological states associated with Ca\(^{2+}\)-dysregulation.
References


