Effects of Advancing Age on ER Calcium Regulation in Rat Superior Cervical Ganglia

Conwin Kodel Vanterpool

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Effects of Advancing Age on ER Calcium Regulation in Rat Superior Cervical Ganglia

by

Conwin Kodel Vanterpool

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

June 2006
Each person whose signature appears below certifies that this dissertation in his opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

John Buchholz, Professor of Physiology/Pharmacology

Sue Duckles, Professor and Interim Chair, Assoc. Dean of Pharmacology

William Pearce, Professor of Physiology/Pharmacology and Biochemistry

Lawrence Sowers, Chair and Professor of Biochemistry/Microbiology

Lubo Zhang, Professor of Pharmacology and Pediatrics
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>[Ca^{2+}]i</td>
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<td>VOCC</td>
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ABSTRACT OF THE DISSERTATION

The Effects of Advancing Age on ER Calcium Regulation in Rat Superior Cervical Ganglia

by

Conwin Kodel Vanterpool

Doctor of Philosophy, Graduate Program in Physiology and Pharmacology
Loma Linda University, June 2006
Dr. John Buchholz

Intracellular calcium ([Ca^{2+}]i) release from endoplasmic reticulum (ER) stores plays an important role in cell signaling. These stores are rapidly refilled via voltage-gated calcium channels or spontaneously via store-operated calcium channels and subsequent pumping by ER Ca^{2+}-ATPases. Calcium release is triggered by the activation of the ryanodine receptor (RyR) channels and their function is in part dependent on their expression. We analyzed the [Ca^{2+}]i transients in fura 2-loaded superior cervical ganglion (SCG) cells from 6, 12, 20, and 24-month-old Fischer 344 rats for their ability to rapidly and spontaneously refill. For rapid refilling, the percent difference between the peak and rate of rise of the first and second caffeine-evoked [Ca^{2+}]i transient significantly declined with age. Spontaneous refilling significantly declined from 12-24 month-old F-344 rats. These data suggest that the ability of SCG cells to sustain release of [Ca^{2+}]i decline with age, but the underlying mechanisms that may be responsible for a decline in caffeine-evoked release of Ca^{2+} remain elusive. Hence, we then focused on molecular characterization of the [Ca^{2+}]i release channel. The pattern of genetic expression of RyR1, 2 and 3, has not been previously characterized with age in this model. In all age
groups ryr 1 does not appear to be expressed in the SCG and ryr2 and ryr3 appear to be the predominantly transcribed isoforms in adult rat SCG. mRNA and protein levels for RyR2 did not change with age, however, the ryr3 mRNA and protein levels significantly increased from 6 to 12 months and then significantly declined from 12 to 24 months. These data alone, cannot account for the dramatic decline in caffeine-evoked Ca$^{2+}$ release observed. However, if along with the changes in gene expression there was altered modulation, this may elucidate more clearly the reasons for the change in function of the RyR. In order to examine how the modulation of the RyR is affected by age, we then focused on modulators such as phosphorylation, and nNOS protein expression levels were analyzed for changes with age. There were no significant changes in the phosphorylation of the total RyRs with age. However, there was a significant increase in nNOS production from 6 months to 12 months and decline in protein expression of nNOS from the 12 months to 24 month-old rat SCG. We have demonstrated that aging significantly impacts the ability of the SCG to sustain calcium release, and exhibits altered genetic and protein expression of the major RyR isoforms, and nNOS protein expression. Taken together, these data suggest that the alteration in release of Ca$^{2+}$ is due to a combination of altered filling of the ER and changes in the modulation of the release mechanism. The compromised ability of these neurons to sustain calcium signaling may possibly alter the overall function of adrenergic neurons innervating the cerebrovasculature.
CHAPTER ONE
INTRODUCTION

Innervation of the cerebro-vasculature by adrenergic neurons arising from the superior cervical ganglia (SCG), curtails increased cerebral blood flow that may arise as a result of hypertension (20; 47; 53; 68; 144). Thus, these neurons may play an increased role in reducing the risk of stroke due to blood brain barrier displacement in the face of hypertension. SCG function depends on the release of calcium from the endoplasmic reticulum (ER). This occurs by a process called calcium induced calcium release (CICR) (11; 12; 26; 96; 142; 150; 154). CICR amplifies increases in intracellular calcium ([Ca^{2+}]_i) caused by influx through calcium channels (148-150). The goal of this project is to understand the mechanism(s) underlying age-related changes in [Ca^{2+}]_i signaling in adrenergic neurons arising from the SCG. Preliminary and published studies with our SCG neuronal model show that age decreases (1) the ability of rat SCG cells to sustain Ca^{2+} release from the ER, (2) the fast refilling and the rate of spontaneous refilling of caffeine-inducible intracellular calcium stores, (3) smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA) function (17; 40; 41; 115-119; 152). These findings suggest that at least two mutually dependent mechanisms contribute to an age-related decline in CICR: (1) decreased filling of ER and (2) possible changes in the function of the ER Ca^{2+} release mechanism.
Intracellular Calcium: A universal second messenger

Calcium is a ubiquitous intracellular second messenger (10). At rest, cells maintain an intracellular calcium concentration at much lower levels than what is found extracellularly. By opening calcium selective pores, calcium is allowed to flow from outside of the cell into the cytosol, or is released from specialized intracellular calcium ([Ca$^{2+}$]$_i$) stores by CICR. Cerebrovascular adrenergic nerves rely on this process to function (Figure 1.1). CICR mobilizes Ca$^{2+}$ from the ER in response to an elevation in [Ca$^{2+}$]$_i$ mediated by voltage-gated Ca$^{2+}$ channels. CICR is relevant in processes such as release of neurotransmitters and hormones, secretion, contraction, and alterations in membrane excitability (55; 121; 149; 150). Since [Ca$^{2+}$]$_i$ is such a universal component for so many cellular processes, deregulation could lead to problems of devastating proportions.
Figure 1.1. Model of CICR in sensory neurons (adapted from 150). Five electrical pulses (blue line) induce influx of Ca\(^{2+}\) via Ca\(^{2+}\) channels. [Ca\(^{2+}\)]\(_i\) is rapidly buffered by Ca\(^{2+}\) binding proteins inducing a small increase in [Ca\(^{2+}\)]\(_i\). With 7 pulses (red line) [Ca\(^{2+}\)]\(_i\) rises to a "threshold" resulting in CICR and the development of a full [Ca\(^{2+}\)]\(_i\) transient. With 11 pulses (green line) a transient similar to 7 pulses (red line) develops with no further increase in [Ca\(^{2+}\)]\(_i\). Thus, seven pulses or greater increases [Ca\(^{2+}\)]\(_i\) to a "threshold" that triggers CICR. CICR to Ca\(^{2+}\) is subject to modulation via the levels of cADPr (81; 140). The levels of cADPr are in turn modulated via the NO/guyanylate cycles/cGMP-PKG\(_1\) pathway (33; 79).
Transmitter release, plasticity and excitability

Calcium plays a crucial role in neuronal excitability and transmitter release (11; 34; 111; 154). Within neurons, an important source of calcium is found within the ER (11). The ER is formed by an elaborate network of endomembranes, and is found in all neurons (154). One of the roles of the ER is to monitor internal signals like Ca$^{2+}$, IP$_3$, cyclic ADP ribose, and cyclic AMP, via receptor protein complexes found on its membrane (10-12; 16; 43; 82). In addition, this organelle can produce signals using calcium released through the IP$_3$ and ryanodine receptors (RyR) located on its membrane. The trigger for exocytosis of neurotransmitters has been classically believed to be due to voltage-dependent calcium entry, but increasing evidence points to the ER’s involvement in some forms of neurotransmission (9-12). Efflux of Ca$^{2+}$ from the ER has also has been implicated in different aspects of excitability. It can stimulate neurotransmitter release and promote membrane excitability, and when in close proximity to the plasma membrane can activate Ca$^{2+}$ activated K$^+$ channels, which hyperpolarizes the cell membrane inhibiting this process. So the ER not only participates in, but also modulates neuronal excitability.

Cell death

There have been debates on the involvement of calcium in the regulation of cell death. Intrinsic apoptosis can occur by contributions of the mitochondria, plasma membrane, or the endoplasmic reticulum. Which pathway predominates depends on the cell type (164). It has been demonstrated that persistent ER stress induces apoptosis. Rao et. al. 2001 demonstrated that procaspase 12, involved in ER-stress induced apoptosis, can become cleaved and activated after calcium is released from the ER (124). The
translocation of caspase-7 from the cytoplasm to the ER also occurs in a calcium-dependent manner. In has also been reported that in breast epithelial cells, the overexpression of Bcl-2, an anti-apoptotic protein, results in an up-regulation of SERCA2 mRNA expression. This overexpression would result in increased Ca$^{2+}$ uptake in the ER, which may prevent Ca$^{2+}$ mediated apoptosis from occurring.

**ER/SR Ca$^{2+}$ homeostasis and intracellular Ca$^{2+}$ handling**

Releasing [Ca$^{2+}$]i from internal stores, and refilling these stores is vital for sustaining many cellular processes. This delicate regulation of [Ca$^{2+}$]i demonstrates the importance of the SERCA pump and the RYR channel's function. This balance suggests that CICR and [Ca$^{2+}$]i buffering are intimately related processes (154; 155). For example in SCG and sensory neurons, ER Ca$^{2+}$ stores can be rapidly refilled by depolarization with high K$^+$ or spontaneously refill within 3-10 min following depletion with caffeine (51; 52; 149; 150; 155); Depletion of ER Ca$^{2+}$ stores with repeated exposure to caffeine will prohibit CICR, and is attenuated by the application of a SERCA antagonist to reduce refilling of ER Ca$^{2+}$ stores (51; 52; 89; 148). In summary, adrenergic nerves play an important role in regulating cerebral blood flow in response to rising blood pressure.

CICR represents a modulator of transmitter release, and modulators of the CICR process such as NO released from NOS-containing, nerves can modulate transmitter release itself (98). This modulation of CICR by NO occurs through the NO guanylate cyclase/cGMP-dependent PKG$_1$/cADPr pathway (33) (**Figure 1.2**).
Figure 1.2. Modulation of RyR by nitric oxide. cADPR has been shown to play a role in the modulation of the RyR. It is modulated by nitric oxide via nNOS, through the nitric oxide (NO), guanylate cyclase, cyclic guanosine monophosphate (cGMP), cGMP-dependent protein kinase-1 (PKG₁), cADPr pathway which can stimulate CICR.
Ca\textsuperscript{2+} channels and pumps of the ER/SR

\textit{Ryanodine receptor calcium release channels}

\textit{Structure and function}

RyR channels are located on the membrane of the ER. These receptors have been shown to be responsible for calcium release from stores found in the ER (11; 32; 48; 49). The RyR monomer is approximately 565 kDa. Cryoelectron microscopy and 3D reconstruction studies demonstrated that the RyRs is an assembly of four monomers forming a tetramer (36; 158). These tetramers have been reported to be either homo or hetero tetramers (36). It has been demonstrated that monomers of RyR2 can form tetramers with monomers of RyR1 or RyR3; however, monomers of RyR3 can only form tetramers with monomers of RyR2, not RyR1.

\textit{Different isoforms}

In mammals, there are three major isoforms of the RyR family (13; 33; 36; 48; 49; 101). The three isoforms (RyR1, RyR2, and RyR3) are encoded by three different chromosomes (126). The expression of the RyRs are different in different cell types (36; 42; 48; 154). In skeletal muscle, RyR1 has been reported to be the major isoform expressed and is referred to as the skeletal muscle isoform (48; 49; 131). RyR2 has been reported to be abundant in cardiac muscle and is therefore referred to as the cardiac isoform [Reviewed in (48; 49)]. RyR3 has been found in skeletal muscles in low levels, but it was first shown to be expressed in neuronal tissue and have been termed the brain type (48; 104; 141). The role of RyR3 is currently being studied in the different tissues. It has been reported that mice lacking RyR1 or RyR2 die during the embryonic stage.
(137; 139), where as the mice lacking RyR3 live, but CICR in the skeletal muscle has been impaired (48), more investigation on its specific role is needed.

**Endogenous modulators of RyRs**

**Ca**\(^{2+}\)

Both cytosolic and luminal calcium has been shown to modulate the function of the RyR channels. The RyR have been shown to be activated by low levels of cytosolic calcium (1-10 \(\mu\)M) and inhibited by high concentrations (1-10 mM) (48). Reports show that RyR2 and RyR3 respond at a greater magnitude to cytosolic calcium than the RyR1 isoform (19; 31; 35). Luminal calcium levels have been demonstrated to affect RyR function by interacting with the luminal binding sites and binding to luminal proteins (48). These findings however are still controversial in relevance to physiological roles.

**ATP and Mg**\(^{2+}\)

Cytosolic ATP has been shown to be very important in the RyR channel activation. \(\sim 300\) \(\mu\)M concentrations of free cytosolic ATP has demonstrated to activate the RyR channels (48). Mg\(^{2+}\) has also been shown to play a role in regulating the receptors. It has been shown to be a potent inhibitor of the channels by competing with calcium at calcium binding sites (48). Reports have indicated that the RyR1 is more sensitive to activation by physiological calcium levels and inhibition by magnesium levels in comparison to RyR2 and RyR3 isoforms (35; 36; 48).

**Redox potential and Nitric Oxide**

The RyR channels have been shown to contain approximately 80-100 cysteine residues indicating that these sites can be modified by oxidants (136; 138). Selected sulfurhydryl groups present on the RyR are sensitive to the redox state. Alteration of the redox state
can inhibit the binding of calmodulin, a calcium binding protein, to the RyR. This disruption can result in the alteration of calmodulin’s ability to activate or inhibit the RyR channel function depending on calcium concentration. (48). It has been documented that nitric oxide (NO), an oxidant, can also regulate the RyR channels (48). Initial release of nitric oxide has been shown to activate the RyR channels and subsequent increases in NO levels can inhibit the activity of the RyR channels (60). Recent studies indicated the biochemistry of this facilitation is through S-nitrosylation of the RyR channel which can affect calmodulin binding to the receptor (45). Earlier studies demonstrated that the cGMP pathway may be involved (46), which can modulate cADPr. However later studies suggest that this occurs via a cGMP-independent pathway (45). More studies are currently being pursued in this area.

Phosphorylation/dephosphorylation

Analysis of the RyR protein identified many consensus phosphorylation sites (48). Kinases such as protein kinase A (PKA) was shown to activate RyR1. Calmodulin kinase (CaMK) was also shown to phosphorylate the RyR1 leading to channel activation, but both PKA, and CaMK also have contradictory reports in the activation of RyR1 (37; 48). Further, studies on PKA, and CaMK on phosphorylating and activating RyR2 were also showed (151). It has been suggested that phosphorylation actually stabilizes the RyR channel to open via the ability to dissociate FKBP, which is a RyR binding protein (discussed below) (93). These studies are also debated, adding further mystery as to the exact roles PKA and CaMK play in modulating the RyRs.
Calmodulin

Calmodulin has been one of the first reported proteins to interact with the RyR [Reviewed in(48)]. Studies have shown that at low calcium levels, calmodulin can activate the RyR1 and RyR3 channels or inhibit the channels at high calcium concentrations [Reviewed in (48; 48; 49)]. Hamilton et al has further demonstrated that calmodulin can also inhibit RyR1 by binding DHPR, disrupting the DHPR-RyR1 complex (48). It has also been reported that calmodulin cannot activate, but only inhibit the RyR2 isoform [Reviewed in (48; 49)]. In addition, recent studies indicate calmodulin can indirectly modulate the RyRs via the modulation of nNOS as calcium levels can regulate calmodulin’s modulation of nNOS. In reviewing the role of calmodulin, it has also been shown that oxidation of selective residues of the RyR can affect the binding of calmodulin to the RyR and other reports suggests that calmodulin can protect the RyRs during increased periods of oxidative stress (59). There are some debates, but it appears that calmodulin has a dual role in modulating the RyR channel, and can influence its modulation through other pathways.

Calsequestrin

Calsequestrin is a calcium binding protein that can bind a high number of calcium molecules (7). It has also been shown to be the major calcium binding protein in the ER, and that changes in calsequestrin due to calcium and pH can affect or modulate the RyR (7; 48). There are however some controversial debates over the role of calsequestrin in RyR modulation, because it has been suggested that calsequestrin has to be bound to triadin in order to modulate the channels function (171). Further studies will have to be performed to determine how exactly calsequestrin regulates the channels.
FKBP

There are great controversies on the mechanisms of how the FKBPs modulate RyR function. It has been shown that the FKBP 12 and FKBP12.6 can associate with all three isoforms of the RyRs (48; 143). Four FKBPs can bind a single RyR channel. Their role in binding the RyR’s has been the topic of debate. One group suggested that the removal of FKBP promotes the subconductance states of the receptors, but others reported no changes in the subconductance states (2; 3). Others have reported that the FKBPs can stabilize the RyRs (48). It has also been reported that the FKBPs may synchronize the function of the surrounding RyRs allowing simultaneous opening and closing of the RyR channels (92). Debates of how these binding proteins could both stabilize and synchronize the action of the RyRs are still current.

Smooth Endoplasmic Reticulum Calcium (SERCA) pumps

Structure and function

SERCA1s have been shown to play a very crucial role in the calcium homeostasis in cells. These calcium pumps maintain homeostasis via helping to return cytosolic calcium levels to normal resting levels after increases in [Ca^{2+}]i, and help to maintain a high level of luminal calcium levels as well as high levels in the Golgi and other components of the secretory pathway to maintain normal cellular processes [Reviewed in (91; 164)]. This will be discussed in more detail below.

Isoforms of SERCA

There are three different isoforms of SERCA, each isoform having variants [Reviewed in (91; 120; 164)]. SERCA1 has two variants, SERCA1a/b. SERCA1 has been shown to be mainly expressed in fast-twitch skeletal muscle fibers. SERCA2 also has two isoforms,
SERCA2a/b. [Reviewed in (91; 120; 164)]. The SERCA2a isoform has been shown to mainly be expressed in cardiac muscle and slow-twitch skeletal muscle. SERCA2b has been found to be expressed in different cell types at various concentrations and it has been said to posses a house keeping function in many tissues [Reviewed in (91; 120; 164)]. SERCA3 is the most recently discovered isoform out of all the isoforms. It has five different variants, SERCA3a-e. This isoform is shown to have both different structures and functions than the other isoforms [Reviewed in (91; 120; 164)]. All of the variants of this isoform has been shown to have a 5-fold less affinity for cytosolic calcium and require a slightly higher pH for function in comparison to the other isoforms (164). In addition, it is not regulated by phospholamban (PBL) (90; 113; 164). It has been suggested that their low affinity for calcium allows them to be activated in the presence of high calcium levels. This isoform has been found in a variety of tissues including: neurons, endothelial and secretory epithelial cells, trachea, salivary glands, endocrine cells and white blood cells. SERCA3 appears to co-exist with SERCA2b which has been suggested to also have house keeping function. In contrast to SERCA2b, SERCA3 has been suggested to perform only a subsidiary role in Ca\(^{2+}\) homeostasis and signaling [Reviewed in (120; 164)].

SERCA in ER stress and apoptosis

Under stressful conditions in the ER, such as disturbances in ER redox states, glucose deprivation, altered calcium regulation, and viral infection (166), a process called the unfolding protein response (UPR) occurs (164; 166). The unfolding of proteins in the ER prevents proper post-translational modifications of vital functional proteins from occurring. This leads to an accumulation of misfolded or damaged proteins in the ER,
which can lead to apoptosis (44). There are debates on how exactly the ER plays a role in apoptosis (12), but previous studies have demonstrated that the luminal calcium in the ER is directly linked to apoptosis in contrast to the cytosolic calcium (164). Studies have implicated the pro-apoptotic members of the Bcl-2 family of proteins in the increase leaking of ER Ca^{2+}, since they have structural similarities to bacterial pore forming proteins. This enables Bcl-2 to assemble in the membrane of the ER acting as an ion channel (166). Other Studies have linked the anti-apoptotic members of the Bcl-2 family to cell survival, by interaction with SERCA2b (39; 164). Though still controversial, it is believed that the anti-apoptotic Bcl-2 members reduce SERCA2b function, preventing complete Ca^{2+} store filling. This essentially reduces the amount of Ca^{2+} that can be released in order to trigger apoptosis. The actions of SERCA2b are thought to be cell-type specific (166). Some studies report increased activity of SERCA2b during ER stress. In this case, it is thought that apoptosis is avoided by maintaining cell proliferative levels of [Ca^{2+}]_{i} (73). This area of research is still controversial and more studies are being done in this area.

SERCA and cell growth

There have been some debates about whether SERCA2 can regulate the growth of cells. Most studies show that there is a correlation between filling states of calcium pool and cell growth rate (164). It has been shown that disruption of the SERCA2 function results in decreased cell proliferation. In other cell types, blocking calcium pumps resulted in cell proliferation (164). From the scientific pool of evidence, it is clear that SERCA function plays a role in cell growth. However, to what degree and in which cell types is still under debate.
Pharmacological modulators of ER/SR associated Ca\textsuperscript{2+} pumps and channels

**SERCA**

*Thapsigargin*

Thapsigargin is an antagonist of SERCA pumps, and is isolated from the roots and fruit of the Mediterranean umbelliferous plants (75). It blocks SERCA pumps by locking them in their Ca\textsuperscript{2+} free conformation by forming a dead-end complex with them (66; 67). Since SERCA function is important for RyR function, Thapsigargin can also indirectly affect RyR function (75).

*Cyclopiazonic acid*

Cyclopiazonic acid (CPA) also antagonizes SERCA pumps, and it is produced by some strains of the molds *Penicillium cyclopuim* and *Aspergillus flavus* (65). CPA decreases SERCA affinity for ATP (75). This inhibits the enzymatic hydrolytic activity of the pump, thus blocking function (75). CPA is not as potent of a blocker as is thapsigargin (75).

*2,5-di (tert-butyl)1,4-benzohydroquinone (tBuBHQ)*

2,5-di (tert-butyl)1,4-benzohydroquinone (tBuBHQ) is a synthetically made phenolic compound, originally thought to be important for its antioxidant properties (161). It was observed that tBuBHQ demonstrated a potent block of SERCA function as well as ATP hydrolysis, the mechanism is not well understood (75).

**RyR**

In order to understand the RyR characteristics and function, pharmacological modulation of [Ca\textsuperscript{2+}]\textsubscript{i} is necessary. The following drugs are commonly used as pharmacological tools
in studying the RyR. These modulators work by either stimulating or inhibiting Ca$^{2+}$ release from the RyR (Figure 1.3).

**Caffeine**

Caffeine is an alkaloid that is found in plants. It is believed to selectively activate the RyR channels by increasing the channels open probability without changing the channels conductance properties (127; 128; 130). This increases the channels sensitivity to Ca$^{2+}$, which leads to the activation of the CICR process.

**Ryanodine**

Ryanodine is an alkaloid found in shrubs and trees of the genus Ryania (75). At submicromolar concentrations, ryanodine increases RyR channel activity, and at micromolar concentrations inhibits channel activity (75). It is still unclear whether or not ryanodine modifies conductance by allosterically stabilizing the channel in a closed formation, or actually interfering with the flow of ions through the pore of the channel (135)

**Procaine**

Procaine is a derivative of cocaine (75). It was synthesized to utilize the anesthetic and vasoconstrictive properties of cocaine, while avoiding its hallucinogenic and euphoric affects (75). Unlike some of the other RyR channel blockers it does not affect channel open time or ion conductance. In contrast, procaine lengthens the duration of the channels closed state (75).
**Ruthenium red**

Ruthenium red is a water-soluble dye that is generated from the reaction of RuCl₃ and NH₃ in solution (75). Ruthenium red selectively blocks RyR channels, but also has some non-selective affects as in blocking voltage-gated Ca²⁺ channels (75).

**Dantrolene**

Dantrolene is a skeletal muscle relaxant that is used to treat human malignant hyperthermia (62). Dantrolene’s antagonistic affects are more selective for RyR1, and less selective for the other isoforms of the RyR channels (57; 62; 72; 167). The mechanism of action is still not fully understood and it is not known whether dantrolene exhibits a direct or indirect inhibition of the ryanodine receptor.
Figure 1.3. Modulation of the RyRs. Endogenous modulators of the RyR receptors play a key role in the CICR process. Intracellular calcium can directly modulate the CICR process once threshold intracellular calcium levels have been reached, and when bound to calmodulin activates nitric oxide synthase. The activation of this enzyme leads to the production of another modulator, cyclic adenosine diphosphate ribose (cADPr), through the nitric oxide (NO), guanylate cyclase, cyclic guanosine monophosphate (cGMP), cGMP-dependent protein kinase-1 (PKG\textsubscript{1}), cADPr pathway which can stimulate CICR. Calmodulin can also inhibit the CICR process by binding free intracellular calcium, preventing threshold calcium levels from being reached. Phosphorylation of the RyRs is another important aspect of their modulation, increasing open probability of the channel. All of these mechanisms in conjunction with one another contribute to the modulation of the CICR process. Pharmacological modulators of RyRs includes: caffeine, exogenous cADP and ryanodine.
Pathology of ER/SR calcium release dysfunction

**Congestive heart failure (CHF)**

Calcium mishandling has been shown to play a crucial role in the development of congestive heart failure (8; 15; 61; 168; 169). A lot of studies have reported the involvement of RyR2 phosphorylation and dephosphorylation in the development of this disease. In fact, an enhanced calcium leak has been proposed to be the cause of this pathogenesis (8). It has been reported that in normal ventricular cells that have been stimulated with a β-adrenergic agonist, the RyR2 undergoes phosphorylation Normally when FKBP is bound to RyR2, the channel is maintained in a closed state. PKA phosphorylation of RyR2 results in the opening of the channel. One group hypothesizes that in heart failure there is a hyper-adrenergic state, which leads to the activation of PKA resulting in hyperphosphorylation of the RyR2. This hyperphosphorylation results in the dissociation of FKBP's which will cause enhanced calcium leak (93). However this remains controversial.

**Malignant hyperthermia (MH)**

This disease is a genetic disease caused by mutation in the RyR1 gene (8; 15). Studies in pigs show that this mutation leads to a hypersensitivity to calcium, resulting in an increase in CICR, and a decrease in Ca$^{2+}$ dependent deactivation. Rapid utilization of ATP by SR calcium pumps and transporters trying to maintain calcium homeostasis leads to an increase in heat generation (8; 15; 62; 69; 85). Certain anesthetics can also cause muscle rigidity and an increase in body temperature, and if this increased rise in temperature is not controlled by dantrolene (blocks RyR1), death will result (6; 72).
Catecholaminergic polymorphic ventricular tachycardia (CPVT)

Mutations in RyR2 have been shown to be associated with catecholaminergic polymorphic ventricular tachycardia (8; 50; 88). CPVT is a malignant, rare exercise-related tachycardia. It usually occurs during intense exercise, and can result in sudden cardiac death. One explanation of the mechanism is that a mutated RyR2 leads to an increase in Ca\(^{2+}\) leak, overloads the Na\(^+\)/Ca\(^{2+}\) exchanger which normally generates an inward current as it extrudes Ca\(^{2+}\). This overload results in proximal tachycardia and arrhythmias. Another is that phosphorylation of the mutated RyR2 leads to increased Ca\(^{2+}\) leakage similar to that in malignant hyperthermia. This leads to hypersensitivity to Ca\(^{2+}\) and decreased ability to inactivate the channel.

Central core disease (CCD)

The mechanisms for this disease remains elusive, however there has been some speculation on the cause of this disease. CCD disease is a result of RyR1 mutations (8; 15; 100). Clinical symptoms usually exhibit muscle weakness, hypotonia, and motor deficiencies. Type I skeletal muscle fibers are usually affected and are usually lacking the central mitochondria and their oxidative enzymes. The reason for the lack of central mitochondria is unknown. Like malignant hyperthermia, this disease causes and excessive Ca\(^{2+}\) leak by an unknown mechanism. It may be possible that the lack of central mitochondria may lead to decreased Ca\(^{2+}\) buffering capacity leading to this pathology.

Brain disorders and neurodegenerative diseases

Changes in intracellular calcium homeostasis have been associated with the pathogenesis of Alzheimer’s disease (AD) (70; 96; 97; 108). RyRs have been shown to be expressed in
abundance in brain regions that are shown to accumulate plaque. Also Querfurth et al has demonstrated that the RyR-regulated pools have been shown to accelerate amyloid beta production(122). Another group has reported that increased RyR levels were detected in brain sections of Alzheimer’s patients, suggesting that the increased expression of the receptors are involved in the development of this disease (70). Age in conjunction with calcium dysregulation has also been implicated in Huntington’s disease, Parkinson’s disease, and stroke (96; 97; 154).

**Sympathetic neurons and SCG**

**Cervical ganglia**

There are three cervical sympathetic ganglia which have three parts: 1) the superior cervical ganglion; 2) the middle cervical ganglion; and 3) the cervicothoracic ganglion. The superior ganglion has been shown to innervate the viscera of the head; the middle and cervicothoracic ganglion innervate the viscera of the neck, thorax and upper limb.

**Thoracic and paravertebral ganglia**

The thoracic sympathetic ganglia, innervates the trunk region, and the lumbar and sacral sympathetic ganglia innervate the pelvic floor and lower limb. The paravertebral ganglia is responsible for sympathetic innervation to blood vessels in muscle and skin, arrector pili muscles attached to hairs, and sweat glands.

**SCG innervation and their physiological roles**

All organs and tissues have nervous innervation to connect the central nervous system to the tissue. Alterations in the function or neuronal synaptic transmission of these neuronal bridges may impact the function of that organ or may also affect other normal bodily functions. The SCG has been suggested to be a peripheral neuroendocrine center (24; 25). It innervates the pineal gland, thyroid and parathyroid gland, eye, cephalic
blood vessels, chorotid plexus, carotid body, salivary gland, salivary glands, parotid gland, thymus, tongue, cervical lymph nodes, eustachian tubes, ophthalmic artery, the brain and other tissues, structures and organs (Table 1.1) (23-25; 27; 71; 76; 77; 94; 95; 103; 109; 123; 125; 132; 134; 153; 157; 159; 160; 162; 163).

The physiological roles of the SCG innervation to specific structures were assessed in many studies. It has been demonstrated that upon stimulation of the CNS, SCG terminals in the heart release NE that binds adrenergic receptors causing strengthening of the heart contractions (162). Altered innervation of sympathetics has been implicated in heart disease (28). Other studies by Chou et al showed that denervation of the SCG resulted in altered choroidal blood flow (29). It has also been demonstrated that the sympathetics, including the SCG plays a role in CSF formation by influencing the function of the carotid plexus (86; 87). Another study by Chou et al also demonstrated that the ophthalmic artery is innervated by the SCG and showed to play a role in regulating the blood flow to the optic nerve (30). The data from these studies suggest that innervation by the SCG is critical for cardiovascular function, and cerebral blood flow. Therefore, the absence of this innervation can possibly lead to increased risk of heart disease and hemorrhagic stroke.

Studies have reported that the SCG neurons tend to innervate organs that have some secretory function (reviewed in (5). Earlier studies showed that removal of SCG resulted in neuroendocrine changes including alteration in pituitary function (25), water balance, and photoperiodic control of reproduction in several animals due to SCG denervation to the pineal (133). In other studies, a decrease in LH and FSH were observed as well as thyroid stimulating hormone and growth hormone release after SCG
removal (22). There has also been suggested that there are associations between the SCG and hypothalamus as a result of decreased NE uptake in rats that had the SCG removed (reviewed in (25; 125)).

There have been numerous reports on altered immune functions after disruption of SCG innervation to certain structures (94; 123; 157). Many studies are pointing to the important role in the nervous system in immune function. The sympathetics have been shown to innervate the cervical lymph nodes and the thymus, both which are involved in the immune response (123). In addition, innervation of the lymphoid structures was shown to play an important role in inflammation. Ramaswamy et al demonstrated that disruption of SCG innervation resulted in reduced pulmonary inflammation after attempting to stimulate a massive immune response (123). It was concluded that the SCG can modulate the inflammation response. Studies have shown that the removal of the SCG resulted in decreased infiltration of white blood cells into the lungs after attempting to stimulate an inflammatory response (94). Neutrophils of rats with removed thymus demonstrated a reduced chemotactic response and was unresponsive to lipopolysaccharides stimulation (94). Another study also confirmed the involvement of SCG in neutrophil function modulation in addition to macrophage responses (94). Removal of the SCG also resulted in reduced mast cell mediated tumor necrosis factor alpha-dependent cytotoxicity, solidifying its importance in immune function (14). It is evident that disruption of SCG function will potentially compromise immune responsiveness.

It is clear from the studies that have been performed, and the vast amount of scientific evidence in literature, that the SCG plays an important multi-faceted role in the
maintenance and proper function of many different organs and systems. For example, cardiovascular health, cerebral blood flow, immune responsiveness, and reproductive health are all reliant on proper SCG function.
Table 1.1. Innervation of the SCG and consequences of altered SCG function

<table>
<thead>
<tr>
<th>SCG innervates:</th>
<th>Removal or impaired SCG function results in:</th>
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<tbody>
<tr>
<td>- Lacrimal glands</td>
<td>- Heart disease</td>
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<tr>
<td>- Thymus</td>
<td>- Impaired immune response and function</td>
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<tr>
<td>- Salivary glands</td>
<td>- Altered carotid blood flow</td>
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<tr>
<td>- Parotid gland</td>
<td>- Localized hypertension</td>
</tr>
<tr>
<td>- Eye</td>
<td>- Retinal degeneration</td>
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<tr>
<td>- Pineal gland</td>
<td>- Thickening of eye basement membrane (compromises retinal capillaries)</td>
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<tr>
<td>- Cerebrovasculature</td>
<td>- Altered function of the hypothalamus (via pineal)</td>
</tr>
<tr>
<td>- Eustachian tubes</td>
<td>- Altered anterior pituitary function (via pineal)</td>
</tr>
<tr>
<td>- Tongue</td>
<td>- Inability to reproduce</td>
</tr>
<tr>
<td>- Thyroid/parathyroid</td>
<td>- Increased CSF production</td>
</tr>
<tr>
<td>- Cervical lymph nodes</td>
<td>- Altered calcium homeostasis</td>
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<tr>
<td>- Heart (important in “fight or flight” response)</td>
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Superior cervical ganglia (SCG) regulation on cerebral blood flow/pressure and the role of SCG hypertension and stroke

Rising systolic blood pressure in both F344-Rats and in humans increases the risk of stroke by the disruption of blood vessels (170). Thus, the risk of hyperemia and stroke in humans impinges on whether or not the cerebrovasculature is subjected to increased pressures. Adrenergic nerves from the SCG (Fig 1.4) depress increased cerebral blood flow, which is triggered by hypertension or increased intracranial pressures following simulated subarachnoid hemorrhage (53). This increase in sympathetic nerve activity reduces the risk of blood brain barrier disruption (4; 20; 21; 63; 64). Thus, any changes in adrenergic nerve function with advancing age can decrease their protective function. This topic is emerging as an important area in biomedical research (68; 144).

The animal model that we use is the Fischer-344 rat. This model is a well-established model of aging with relevance to humans (170).

The effect of altered cerebral blood pressure: hemorrhagic stroke

Hemorrhagic stroke has been one of the major clinical issues that affect the morbidity, mortality and health care costs of our population today (1; 78). About 72% of the people who suffer a stroke are 65 years of age and older. It is the third leading cause of death.
behind heart disease and cancer. The most important factor responsible for increased risk of stroke with increasing age is hypertension. Among its many harmful effects high blood pressure puts unnecessary stress on blood vessel walls which can cause the blood vessels to thicken and deteriorate, eventually leading to a stroke. The aging process may also accelerate several common forms of heart disease which can also lead to stroke.

**Regulation of intracellular calcium (mechanisms) and its regulation with age**

**Aging in neurons**

Our laboratory focuses on elucidating the functional changes in Ca\(^{2+}\) regulation that occur during advancing age in vascular adrenergic nerves (117; 119; 142). As adrenergic nerves play an important role in regulating cerebral blood flow especially in cases of hypertension, we believe that elucidating these mechanisms may have clinical significance in the future.

By measuring norepinephrine (NE) release, our early work demonstrated that advancing age alters the function of adrenergic nerves (17; 18; 40; 41). These studies suggested that adrenergic nerve endings become more reliant on mitochondrial Ca\(^{2+}\) uptake to control NE release (146). Other studies have suggested that there is an age-related decline in the function of [Ca\(^{2+}\)]\(_i\) buffering mechanisms in some central neuronal models (38; 102; 129). Thus, we investigated the impact of age on the effect of a synthetic [Ca\(^{2+}\)]\(_i\) chelator to inhibit stimulation-evoked NE release. The chelator was more effective at reducing stimulation-evoked NE release in arteries from old as compared to young animals (145). In addition, we found that the effect of the SERCA antagonists, thapsigargin or cyclopiazonic acid, to increase stimulation-evoked NE release declined with age (147). Taken together these data suggested that an age-related
decline in some \([\text{Ca}^{2+}]_\text{i}\) buffering systems may cause the previously observed increased reliance on mitochondria to control NE release (146).

Despite the complexity of the calcium buffering systems, our ongoing research has made strides in determining how aging affects their function. By using Fura-2 to directly measure \([\text{Ca}^{2+}]_\text{i}\) in isolated SCG, we have shown that there is an increased reliance on mitochondria and plasmalemma \(\text{Ca}^{2+}\)-ATPases (PMCAs) to control high \(K^+\)-evoked \([\text{Ca}^{2+}]_\text{i}\) transients with advancing age (17; 117; 118). We have shown that SERCA function in SCG cells declines with age (116-118; 147). Despite an age-related decline in SERCA function, these sympathetic nerves rely on compensatory mechanisms to continue normal function (116; 117).

We have now expanded our studies to include the consequences of altered SERCA function in terms of CICR signaling, and how this decline affects the refilling of ER stores ((106; 107; 150). In addition, NO modulates CICR in neurons via the NO/guanylate cyclase/cGMP-dependent PKG\(_1\)/cADPr signaling pathway (33; 79; 83). In our studies we have shown that NO released from nNOS- containing neurons augments the function of adrenergic neurons (17; 98; 99). This may be a vital signaling pathway whereby the impact of age on \(\text{Ca}^{2+}\) signaling in adrenergic neurons can be successfully studied.

\textbf{Ca}^{2+} \textit{homeostasis and aging}

A general age-related decline in the regulation of \(\text{Ca}^{2+}\) has been suggested to be a cause of some pathology and cell death (Porter et. al. 1997). Even though there may be a decline in regulatory function, we cannot rule out possible compensatory feedback mechanisms that can work to maintain homeostatic \([\text{Ca}^{2+}]_\text{i}\) levels, therefore maintaining
neuronal function (58; 80; 84; 106; 119; 156). It is imperative to understand how [Ca\textsuperscript{2+}]i is regulated under normal conditions. This understanding may give us insights into what may be occurring in pathological situations, such as stroke and hypertension.

It is thought that alterations in [Ca\textsuperscript{2+}]i regulation may be a key player in neurodegenerative diseases (110; 155). There is evidence that Ca\textsuperscript{2+} influx in central neurons increases with age (74; 105; 112; 114). Recent studies on CNS neurons have shown that age-related increases in Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} channels may be compensated by increased buffering (58; 106). In understanding the interplay between buffering mechanisms to control [Ca\textsuperscript{2+}]i, it seems logical that if an age-related change in function of one buffering system occurs, a corresponding compensatory response by other buffering systems may also occur (116; 117). For example, the ability of calmodulin to activate plasma membrane Ca\textsuperscript{2+}-ATPases (PMCA's) declines with age (56). However, in rat basal forebrain neurons, mitochondria play an increased role in buffering access [Ca\textsuperscript{2+}]i (107). Thus, increased mitochondrial buffering can compensate for an age-related decline in PMCA function.

SERCA function like PMCA function has also been shown to decline with age in other excitable cells; however the mechanism for the observed age-related decline in SERCA buffering remains elusive. Although SERCA-mediated \textsuperscript{45}Ca\textsuperscript{2+} uptake in skeletal muscle and myocardium appears to decline with age, SERCA protein levels remain stable (54; 165). This may suggest that changes are occurring posttranslationally, such as decreased SERCA phosphorylation. Decreased SERCA phosphorylation would not affect protein levels may possibly lead to a decreased buffering capacity.
Hypothesis and significance

In this study, we examined how aging alters the dynamics of CICR and how declined refilling of the calcium stores by SERCA pumps may change in isolated SCG cells from 6, 12, 20, and/or 24 month-old Fischer-344 rats. Studies on the effects of aging on intracellular calcium regulation in SCG may have implications in the decrease of neuronal plasticity and loss of axons and dendrites during aging. The disruption of their connection and function may in return affect their innervation and function to their organ targets resulting in altered function of their target organs.  Thus our governing hypothesis is: aging can alter the mechanisms of intracellular calcium refilling (rapid and spontaneous) and release in rat superior cervical ganglia.

The specific aims for the proposed research are:

1) To measure rapid and spontaneous refilling of caffeine-sensitive ER, intracellular calcium stores in Fura-2 loaded SCG cells, and to determine if there are any changes with age.  Rationale. For rapid refilling, we have shown that there is an age-related decline in high K⁺-evoked [Ca²⁺]i which suggests that influx of Ca²⁺ through voltage gated Ca²⁺ channels may decline with age. However, high K⁺ evoked [Ca²⁺]i are consistent between successive applications of high K⁺. Caffeine-evoked [Ca²⁺]i also decline with age. However, there is a further age-related decline in the release of Ca²⁺ with successive applications of caffeine. These data suggest that the ability to sustain Ca²⁺ release may decline with age. For spontaneous refilling we have demonstrated that advancing age results in a significant slowing of the rate of refilling of ER Ca²⁺ stores in SCG cells following caffeine-evoked depletion of [Ca²⁺]i stores.
2) To identify the major isoform(s) of the RyR in the rat SCG and to quantify the age-related changes in the genetic and protein expression levels of the major isoforms. Rationale. Preliminary studies have shown that SERCA function declines with age which may affect the level of \([\text{Ca}^{2+}]_i\) that is necessary to cause \(\text{Ca}^{2+}\) release via the ryanodine channel pending further stimulation. By using molecular techniques, we can detect changes in the message levels for the ryanodine channel, and also detect channel density by western analysis or ELISA.

3) To measure the levels of selective endogenous agonists and/or modulators of the ryanodine channel. Rationale. The ryanodine channel is modulated by many mechanisms, including phosphorylation, and by the NO guanylate cyclase/cGMP-dependent PKG\(_1\)/cADPr pathway which modulates the endogenous agonist of the channel, cADPr. By measuring nNOS levels with the ELISA technique, and phosphorylation state of the channels, we may gain some insight on how modulation of these channels may change with age.
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CHAPTER TWO

Advancing age alters rapid and spontaneous refilling of caffeine sensitive calcium stores in sympathetic superior cervical ganglion cells

Conwin K. Vanterpool, William J. Pearce and John N. Buchholz

Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, Loma Linda, California, 92354

Running Head: Aging and refilling of caffeine sensitive calcium stores

Contact information: Address for correspondence and reprint requests: John N. Buchholz, Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, Loma Linda, CA, 92350 (E-mail: jbuchholz@som.llu.edu)

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A. Abstract

Intracellular calcium ([Ca\textsuperscript{2+}]i) release from SER stores plays an important role in cell signaling. These stores are rapidly refilled via influx through voltage gated calcium channels or spontaneously via store-operated calcium channels (SOCC), and subsequent pumping by smooth endoplasmic reticulum Ca\textsuperscript{2+} -ATPases (SERCA). We measured [Ca\textsuperscript{2+}]i transients in isolated Fura-2 loaded superior cervical ganglion (SCG) cells from 6, 12, 20 and 24 month-old F-344 rats. For rapid refilling, [Ca\textsuperscript{2+}]i transients were elicited by a 5 sec exposure to K\textsuperscript{+} (S\textsubscript{1}), caffeine to release Ca\textsuperscript{2+} from SER stores (S\textsubscript{2}), K\textsuperscript{+} to refill SER Ca\textsuperscript{2+} stores (S\textsubscript{3}), and caffeine (S\textsubscript{4}). The % difference between the peak and rate of rise of the first and second caffeine-evoked [Ca\textsuperscript{2+}]i transient significantly declined over the age range of 12-24 months. To estimate spontaneous refilling, cells were depolarized for 5 sec with 68 mM K\textsuperscript{+} (control), followed by a 10 sec exposure to 10 mM caffeine "conditioning stimulus" to deplete [Ca\textsuperscript{2+}]i stores. Caffeine was then rapidly applied for 5 sec at defined intervals from 60 to 300 sec. Integrated caffeine-evoked [Ca\textsuperscript{2+}]i transients were measured and plotted as a percentage of the K\textsuperscript{+} response vs. time. The derivative of the refilling time curves significantly declined over the age range from 12-24 months. Overall, these data suggest that the ability of SCG cells to sustain release of [Ca\textsuperscript{2+}]i, following rapid or spontaneous refilling declines with advancing age. In addition, as sympathetic neurons arising from the SCG serve to protect the cerebrovasculature from hyperemia in the face of elevated blood pressure, these data have implications as to protective function of these neurons during the normal aging process.
**Key words:** Store operated calcium channels, aging and calcium release, aging and refilling of neuronal calcium stores, aging and function of superior cervical ganglia.

**B. Introduction**

A prominent factor for increased risk of stroke during aging is rising systolic blood pressure (1, 24). Adrenergic nerves arising from the superior cervical ganglia (SCG) serve to dampen increased cerebral blood flow in response to hypertension or increased intracranial pressure and reduces the risk of blood brain barrier disruption (3, 6, 7, 12, 18). Thus, age-related changes in adrenergic nerve function is emerging as an important area in the physiology of aging (20, 41).

The function of neurons depends in part on the release of calcium from the smooth endoplasmic reticulum (SER) in response to an elevation in intracellular calcium ([Ca^{2+}]_i) mediated by voltage-gated Ca^{2+} channels (46-48). This process has been termed calcium induced calcium release (CICR) and is relevant in processes such as release of neurotransmitters and hormones (14, 31, 38, 47).

To sustain calcium release during neuronal activity requires refilling of the SER calcium through calcium influx and subsequent uptake into the SER via smooth endoplasmic reticulum calcium ATPase (SERCA) pumps (22, 37, 49). Thus, buffering of [Ca^{2+}]_i transients and refilling [Ca^{2+}]_i stores by SERCA suggest that calcium release and [Ca^{2+}]_i buffering are intimately related processes. In SCG and sensory neurons, SER Ca^{2+} stores can be rapidly refilled by activation of voltage gated calcium channels with high K^+ or they can spontaneously refill within 3-10 min following depletion with caffeine via activation of store operated calcium channels (SOCC) (2, 10, 11, 36, 37, 47).
Aging in all creatures is inexorable and the "why" of aging has been suggested to be a combination of developmental changes, genetic defects, environmental influences and an inborn aging process (8, 16, 17, 39, 42). However, these studies render little explanation in terms of "how" normal aging alters function of critical organ and neuronal systems or the vulnerability of particular physiological processes to advancing age. We have shown that there is an age-related decline in SERCA function with a subsequent increased reliance on mitochondria and plasmalemma Ca^{2+}-ATPases (PMCA) to control high K^+-evoked [Ca^{2+}]_i transients with advancing age (4, 32, 34, 44). Overall these data suggest that despite an age-related decline in the function of SERCA cells may adapt to this loss by increased function of remaining [Ca^{2+}]_i buffering mechanisms. Consistent with our studies in SCG cells others have shown that the function of SERCA declines with age in skeletal and heart muscle cells (13, 50). Thus, in peripheral excitable cells a subtle decline in overall SERCA function may be a common feature of the aging process.

Given that SERCA function declines with age in the SCG we studied how aging may alter the refilling and release of Ca^{2+} from the SER. We tested two hypotheses in this study as illustrated in (Figure 2.1). The first hypothesis is that an age-related decline in SERCA mediated Ca^{2+} uptake alters rapid depolarization induced refilling of Ca^{2+} into the SER following caffeine-evoked depletion of SER Ca^{2+} stores. The second hypothesis is that an age-related decline in SERCA mediated Ca^{2+} uptake alters the spontaneous refilling of SER Ca^{2+} stores following caffeine-evoked depletion.
Hypothesis 2: An age-related decline in SERCA-mediated \( \text{Ca}^{2+} \) uptake alters spontaneous refilling after caffeine depletion.

Hypothesis 1: An age-related decline in SERCA-mediated \( \text{Ca}^{2+} \) uptake alters voltage-dependent rapid refilling after caffeine depletion.

Fig. 2.1. Model illustrating overall experimental design of this study. The measured variable is relative cytosolic calcium concentration in response to various protocols used within the study. The overall governing hypothesis is that an age-related decline in SERCA function alters the SER calcium levels and their refilling following depletion. Abbreviations: Smooth endoplasmic reticulum (SER). Smooth endoplasmic reticulum calcium ATP-ase (SERCA). Voltage gated calcium channels (VOCC). Store operated calcium channels (SOCC).
C. Methods

C.1. Experimental animals

Male Fischer-344 (F-344) rats aged 6 mo (young adult), 12 mo (mature adult), 20 mo (old) and 24 mo (senescent) were obtained from NIH-NIA breeding colony (Harlan Sprague-Dawley Incorporated, Indianapolis, IN, USA). The age-range designation comes from other studies showing the median life span in F-344 rats is approx 24 months (26). The animals were allowed to eat and drink at will and were maintained on a 12 hr light/dark cycle under controlled temperature (72–77 °F). All procedures used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Loma Linda University and the approved guidelines were adhered to throughout the study.

C.2. Superior cervical ganglion preparation

Rats were anaesthetized with CO₂ (45 sec) followed by decapitation. The dissection of the superior cervical ganglia and preparation of isolated cells has been described previously (34). Briefly SCG were dissected from the carotid artery bifurcation and placed in cold Tyrode's solution which contained 150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), and 10 mM glucose. The ganglia were then acutely dissociated in 5 ml of Earle's balanced salt solution (EBSS) containing: trypsin (6000 U/ml), collagenase D (1 mg/ml), DNase-1 type IV (0.1 mg/ml), HEPES (20 mM), glucose (10 mM), NaHCO₃ (10 mM) and adjusted to pH 7.4 with NaOH (1M). After incubation in a shaking water bath for 45 min at 34 °C, the digestion reaction was stopped by the addition of 5 ml of
modified Hank's balanced salt solution (HBSS) with 10% fetal calf serum, 1.3 mM CaCl₂, 5 mM HEPES and adjusted to pH 7.4 with NaOH (1M). Dissociated cells were centrifuged at 600 rpm for 5 min and re-suspended in 5 ml of fresh HBSS. Cells were centrifuged again at 600 rpm for 5 min and dispersed in 0.5 ml of HBSS with 10% fetal calf serum, 5 mM HEPES adjusted to pH 7.4 with NaOH (1M) onto Cell-Tak (BD Bioscience, Bedford, MA,) coated glass cover slips, (3.5 μg/cm²). Cover slips were modified by attaching a 2-cm Teflon ring to the surface with Sylgard adhesive (Dow Corning, Inc. MI). Dissociated cells on the cover-slips were incubated for 12-14 hrs at room temperature to allow cells to attach to the Cell-Tak protein coat before they were used in the experiments.

C.3. Measurement of intracellular calcium

SCG cells were loaded with 10 μM fura-2 acetoxymethylester (fura-2/AM) for 20 min at room temperature, then washed with low K⁺ tyrodes buffer containing: 138 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM KCl, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.4 with NaOH (1M). Incubation was continued for an additional 20 min to allow intracellular esterases to convert the fura-2/AM dye into the free acid form (40). A potential problem with loading procedures is that there may be an age-related difference in the amount of Fura-2 taken up by the SCG cells or a difference in the activity of non-specific esterase’s that convert Fura-2/AM to the free salt. To assess this possibility, the intensity of the fluorescence signal at 510 nM when the dye is activated at 380 nM is proportional to the amount of loaded dye (30). In this study we monitored the 510 nM emission fluorescence signal when fura-2 is excited at 380 nm (F₃₈₀) in resting
SCG cells in each age group. The F380 was not significantly different in SCG cells from 6-24 month-old animals. The specific values for F380 following loading were, 160.04 ± 5.6, 169.80 ± 5.0, 164.57 ± 11, 169.90 ± 7.0 in SCG cells from 6, 12, 20 and 24 month-old animals respectively. These data are consistent with our previous studies showing that the F380 is does not change with age suggesting that dye loading is equivalent in SCG cells from each age group (32, 33).

Cover slips were mounted into a superfusion chamber, which was attached to the stage of a Nikon inverted microscope (Nikon Instruments, Tokyo, Japan). The microscope was attached to a Universal Imaging System running MetaFluor version 6.2 (Universal Imaging Corporation, a subsidiary of Molecular Devices, West Chester, PA, USA). The perfusion system allowed the chamber volume (~250μl) to be exchanged at the rate of 500 μL (i.e. 2 times per second). A xenon lamp illuminated the fura-2 probe, and fluorescence was excited alternately at wavelengths 340 and 380 nm by a Lamda DG-4 (Sutter Instruments, Novato, CA, USA) hyper switch. The fura-2 emission fluorescence was measured at 510 nm and recorded by a Photometric Cool Snap 12-bit digital camera (Roper Scientific, AZ, USA). Adjusting the microscope stage to a point with no cells in the field of view and capturing a background image before the start of the experiment corrected for background light levels. Prior to fura-2/AM loading, cellular autofluorescence was examined in SCG cells. Autofluorescence was found to be undetectable by our imaging system and did not significantly alter our [Ca²⁺]i measurements. During the experiment, 340 and 380 fluorometric signal were collected, corrected for background fluorescence, calcium concentration calculated and the data logged to an exel file at a rate of ~300 msec. During dye loading and data collection,
ambient light levels were minimized and SCG cells were only illuminated during data acquisition to minimize bleaching and potential photo damage of the dye.

Intracellular calcium was estimated by both in vitro and in vivo calibration methods. The in vitro method was performed using a calcium calibration kit (Molecular Probes, Eugene, OR, USA) with known [Ca$^{2+}$] ranging from 0 to 40 μM. Each prepared calcium solution was loaded with 4 μM fura-2 pentapotassium salt. A droplet of each [Ca$^{2+}$] was placed onto a glass slide and the fluorescent intensities from 340 and 380 excitation were measured and a curve of 340/380 ratio ($R$) versus [Ca$^{2+}$] was plotted. The in vivo method was performed on SCG cells by decreasing extracellular calcium concentration to 0 mM and fluorescence from 380 ($F_{\text{min}}$), and 340/380 ratio ($R_{\text{min}}$) was recorded for 1 min. The extracellular medium was then replaced with 10 mM [Ca$^{2+}$] with ionomycin (1 μM) and depolarized with high potassium (68 mM). Values were then recorded for 1 min to obtain $F_{\text{max}}$ and $R_{\text{max}}$. The values for $F_{\text{min}}$, $R_{\text{min}}$, $F_{\text{max}}$ and $R_{\text{max}}$ were remarkably similar between the in vitro and in vivo methods. Our in vitro calibration for $K_d$ is comparable with in vivo values in neuronal cells (28, 31) Furthermore, we applied the same $K_d$ for both young and old SCG neurons, since previous reports have shown no significant change in fura-2 $K_d$ values for young and old neurons(28). Since our in vitro method correlated well with in vivo measurements, the values obtained from the in vitro calibration were used to convert the experimental fluorescent intensity ratios ($R$) to [Ca$^{2+}$] over the physiological range of [Ca$^{2+}$] by iterative fit to the equation: [Ca$^{2+}$]$_i$ = $K_d$ ($R - R_{\text{min}}$)/$R_{\text{max}} - R$ $S_f$. $R_{\text{min}}$ is the 340/380 ratio at zero [Ca$^{2+}$] and $R_{\text{max}}$ is the 340/380 ratio at 40 μM [Ca$^{2+}$]. The value of $K_d$ is the dissociation constant of fura-2, while $S_f$ is a correction factor relating the ratio $F_{\text{min}}/F_{\text{max}}$, which is the emission intensity at 380 nm
when fura-2 is in the free ($F_{\text{min}}$, 0 µM [Ca$^{2+}$]) or bound ($F_{\text{max}}$, 40 µM [Ca$^{2+}$]) form (15).

For this report we used multiple calibrations and the [Ca$^{2+}$]i was estimated using averaged in vitro values for $Sf$ (11.3), $R_{\text{min}}$ (0.39), $R_{\text{max}}$ (2.5) and $K_d$ (270 nM).


Figure 2.2A shows representative data in a single SCG cell from a 6 month-old animal demonstrating the response to sequential additions of high K$^+$ and caffeine to release and refill
Fig. 2.2. (A) Representative data of protocol 1 demonstrating caffeine-evoked release of $[\text{Ca}^{2+}]_i$ from SER stores and fast K⁺-evoked refilling of $[\text{Ca}^{2+}]_i$ stores in a single Fura-2 loaded SCG cell from a 6-month animal. An $[\text{Ca}^{2+}]_i$ transient (S₁) was evoked by 5 sec exposure to 68 mM K⁺ followed by 2 min equilibration. A second $[\text{Ca}^{2+}]_i$ transient (S₂) was evoked by 5 sec exposure to 10 mM caffeine to release calcium from $[\text{Ca}^{2+}]_i$ stores followed by 2 min equilibration. The third $[\text{Ca}^{2+}]_i$ transient (S₃) was evoked by 5 sec exposure to 68 mM K⁺ to refill $[\text{Ca}^{2+}]_i$ stores followed by two min equilibration. A final $[\text{Ca}^{2+}]_i$ transient (S₄) was evoked by 5 sec exposure to 10 mM caffeine followed by 2 min equilibration. (B) Data derived from a single Fura-2 loaded SCG cell from a 6 month animal demonstrating selective caffeine evoked Ca²⁺ release from $[\text{Ca}^{2+}]_i$ stores. An $[\text{Ca}^{2+}]_i$ transient was evoked by 5 sec exposure to buffer containing 68 mM K⁺ and 2 mM extracellular Ca²⁺ followed by 2 min equilibration. A second $[\text{Ca}^{2+}]_i$ transient was evoked by 5 sec exposure to buffer containing 10 mM caffeine and zero extracellular Ca²⁺ (3 mM EGTA) followed by 2 min equilibration. Next the cell was exposed for 5 sec to buffer containing 68 mM K⁺ and zero extracellular Ca²⁺ (3 mM EGTA) followed by two min equilibration. A final $[\text{Ca}^{2+}]_i$ transient was evoked by 5 sec exposure to buffer containing 68 mM K⁺ and 2 mM extracellular Ca²⁺.

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SER [Ca$^{2+}$]i stores. Cells were exposed for 5 sec to 68 mM K$^+$ (S$_1$) to insure uniform loading of SER [Ca$^{2+}$]i stores (27). Next cells were exposed for 5 sec to 10 mM supermaximal, caffeine, (S$_2$), to release calcium from SER [Ca$^{2+}$]i stores. Next a 5 sec exposure to high K$^+$ to rapidly refill the SER [Ca$^{2+}$]i stores (S$_3$) and caffeine to once again release calcium from the SER (S$_4$). These data show that we can reproducibly generate [Ca$^{2+}$]i transients with different treatments and that in young cells high K$^+$ is able to refill the SER Ca$^{2+}$ stores. **Figure 2.2B** illustrates that caffeine selectively releases calcium from SER calcium stores in SCG cells. Note that caffeine still evokes an [Ca$^{2+}$]i transient in the absence of extracellular calcium. These data are consistent with other studies demonstrating that caffeine selectively evokes release of calcium from SER [Ca$^{2+}$]i stores in SCG cells (11).

**C.5. Protocol 2: Measurement of Spontaneous Refilling of [Ca$^{2+}$]i stores**

**Figure 2.3** illustrates the protocol in a single SCG cell used to measure how aging may alter the spontaneous refilling of [Ca$^{2+}$]i stores following caffeine-evoked depletion. This protocol was derived from a previous study in acutely dissociated dorsal root ganglion cells (45). Previous studies have shown that the initial responses of isolated neurons to caffeine can be quite variable suggesting variability in the loading of [Ca$^{2+}$]i stores (2, 27). This variability to caffeine can be markedly reduced by initially depolarizing the cells to insure equal loading of [Ca$^{2+}$]i stores. Thus, the cells were exposed for 5 sec to high K$^+$ (S$_1$) as a normalization control (data not shown) and to insure a more uniform loading of [Ca$^{2+}$]i stores (2, 27). Next caffeine sensitive SER [Ca$^{2+}$]i stores were depleted by a 10 sec exposure to 10 mM caffeine (S$_2$) called the
"conditioning response" (data not shown). Following the conditioning response, the SER calcium stores were allowed to spontaneously refill and cells were briefly exposed to 10 mM caffeine for 5 sec at the intervals indicated until the maximal response to caffeine following the "conditioning response" was obtained.

C.6. Data analysis

Calcium transients were analyzed using Origin 6.1 software in all age groups. In protocol 1 peak [Ca\textsuperscript{2+}]i was determined by subtracting basal [Ca\textsuperscript{2+}]i from the maximum K\textsuperscript{+} or caffeine-evoked [Ca\textsuperscript{2+}]i transients. Rate of rise of [Ca\textsuperscript{2+}]i was determined by linear fit (r = 0.99 ± 0.07) from basal [Ca\textsuperscript{2+}]i to the maximum K\textsuperscript{+} or caffeine-evoked [Ca\textsuperscript{2+}]i. Rate of recovery of [Ca\textsuperscript{2+}]i transients was determined using a first order exponential fit (r = 0.99 ± 0.06). In protocol 2 caffeine-evoked [Ca\textsuperscript{2+}]i transients following the conditioning response for were analyzed by taking the total area under the curve and normalization to the area under the curve of the K\textsuperscript{+} control. Thus, data is expressed as integrated caffeine-evoked [Ca\textsuperscript{2+}]i as a percentage of the K\textsuperscript{+} control. These data were plotted vs time after the conditioning response to estimate the spontaneous refilling of [Ca\textsuperscript{2+}]i stores as shown in Figure 2.8A. The rate of spontaneous refilling following caffeine-evoked release of calcium from [Ca\textsuperscript{2+}]i stores was estimated by taking the derivative of the curves in Figure 2.8A using Origin 6.1 and is expressed in Figure 2.8B.

C.7. Statistics

The impact of age on all measured parameters was determined using ANOVA and Fischer PLSD test. All data in each age group were analyzed for heterogeneity of variance using the Cochran's test. If the variances were significantly different then values were log transformed and statistical tests were repeated (51).
D. Results

D.1. Properties of high [K⁺] and caffeine-evoked [Ca²⁺]i transients

Using the protocol in figure 2.2A we calculated the peak and rate of rise of the first and second high K⁺-evoked [Ca²⁺]i transients in each age group as shown in Figure 2.4. There is a clear age related effect on the peak and rate of rise of the first and second high K⁺-evoked [Ca²⁺]i transients. These parameters increased in SCG cells from 6-12 month-old animals and then progressively declined from 12-24 months. Despite the decline in these parameters from 12-24 months the consistency of the dynamic response between the first and second high K⁺-evoked [Ca²⁺]i transients appears to be maintained with advancing age. Again using the protocol in figure 2.2A we calculated the peak and rate of rise of the first and second caffeine-evoked [Ca²⁺]i transients in each age group as shown in Figure 2.5. In a similar fashion to high K⁺, these parameters increased in SCG cells from 6-12 month-old animals and significantly declined from 12-24 months. In contrast to the high K⁺-evoked [Ca²⁺]i transients, the peak and rate of rise of caffeine-evoked [Ca²⁺]i transients appears decline from the first and second exposure to caffeine in SCG cells from 12-24 month-old animals.

To clarify how advancing age may alter the consistency of the peak and rate of rise of high K⁺ and caffeine-evoked [Ca²⁺]i transients, we analyzed the percentage difference between the first and second high K⁺ or caffeine-evoked [Ca²⁺]i transients. When the data were analyzed in this manner, a clearer pattern emerges as shown in figure 2.6. There is no significant age-related decline in the percentage difference in the peak and rate of rise of the first and second high K⁺-evoked [Ca²⁺]i transients. In contrast
to high K\textsuperscript{+}, there is a significant age-related decline in the percentage difference in the peak and rate of rise of the first and second caffeine-evoked [Ca\textsuperscript{2+}]i transient.

D.2. Spontaneous refilling of [Ca\textsuperscript{2+}]i stores following caffeine-evoked depletion

To enhance the rigor of our study on the impact of age on spontaneous refilling of [Ca\textsuperscript{2+}]i stores (Figure 2.3) we performed a series of validation controls as shown in figure 2.7. These controls were done to demonstrate that spontaneous refilling of [Ca\textsuperscript{2+}]i stores following caffeine-evoked release occurred independently of voltage-gated calcium channels and appears to be dependent on the activation of SOCC and SERCA as shown in previous studies (2, 45, 47). In figure 2.7A we demonstrate that [Ca\textsuperscript{2+}]i stores in resting SCG cells will refill in the presence of the L and N-type voltage gated calcium channel antagonists nifedipine and \(\omega\)-conotoxin respectively, following caffeine-evoked depletion of [Ca\textsuperscript{2+}]i stores. Note that in the presence of the voltage-gated calcium channel antagonists, cells still exhibit robust caffeine-evoked release of [Ca\textsuperscript{2+}]i. In figure 2.7B we show the efficacy of the L and N-type channel calcium antagonists on high K\textsuperscript{+}-evoked peak [Ca\textsuperscript{2+}]i transients. Note that both nifedipine and \(\omega\)-conotoxin block approximately 97\% of the K\textsuperscript{+}-evoked Ca\textsuperscript{2+} response suggesting that L and N-type channel subtypes predominate in the SCG as previously shown (23). Figure 2.7C demonstrates the activation of SOCC channels following caffeine-evoked depletion of and blockade of the SERCA mediated refilling of [Ca\textsuperscript{2+}]i stores. Note that under these conditions [Ca\textsuperscript{2+}]i begins to rise reflecting the opening of SOCC as shown in previous studies (2, 47). However, in the continued presence of the SERCA blocker THAPS, caffeine no longer elicits a response. These data suggest that in addition to calcium influx via SOCC,
SERCA are also necessary for spontaneous refilling of \([\text{Ca}^{2+}]_{\text{i}}\) stores in resting SCG cells.

An interesting observation is that the time for activation of SOCC in our preparation...
Fig. 2.3. Example of protocol 2 demonstrating the spontaneous refilling of SER calcium stores following caffeine-evoked emptying in a single SCG cell. Cells were exposed for 5 sec to 68 mM K⁺ and then for 10 sec to 10 mM caffeine representing the control and conditioning response respectively (data not shown). At the time points indicated following the conditioning response, 10 mM caffeine was applied for 5 sec until the maximum response to caffeine was achieved.
Fig. 2.7. Validation controls to determine that SOCC channels and SERCA mediate spontaneous refilling of SER calcium stores following caffeine-evoked release. (A) SER calcium stores refill in the presence of calcium channel blockers, nifedipine and ω-conotoxin. Cells were exposed for 5 sec to high K+ buffer (S1) followed by a 10 sec exposure to a buffer containing 10 mM caffeine to release [Ca\(^{2+}\)]i stores (S2). Next the cells were continually exposed to a buffer containing 10 µM nifedipine and 1 µM ω-conotoxin to block L and N-type calcium channels respectively. As indicated the cells were exposed to again exposed for 5 sec to a buffer containing 10 mM caffeine. Data represent the average for 5 cells from a 6 month-old animal. (B) L and N-type channel antagonists, nifedipine and ω-conotoxin block K\(^+\)-evoked [Ca\(^{2+}\)]i transients in SCG cells. Cells were exposed for 5 sec to high K+ buffer (control). Next cells were exposed for 5 sec to high K+ buffer containing 10 µM nifedipine and then for 5 sec to high K+ buffer containing 10 µM nifedipine and 1 µM ω-conotoxin. Data represent the mean ± S.E for 6 cells from a 6 month-old animal. (C) Activation of SOCC channels occur following the caffeine-evoked depletion of SER calcium stores and blockade of SERCA with thapsigargin (THAPS). Cells were exposed for 5 sec to high K+ (S1) and then to 10 mM caffeine for 10 sec (S2). Next cells were continually exposed to a buffer containing the SERCA antagonist THAPS, 1 µM. At the times indicated the cells were exposed for 5 sec to a buffer containing 10 mM caffeine. Data represent the average of 4 cells from a 6 month-old animal. (D) Activation of SOCC channels is blocked with La\(^{3+}\) following caffeine-evoked depletion of SER. Cells were exposed for 5 sec to a buffer containing high K+ (S1). Next cells were exposed for 10 sec to a buffer containing 10 mM caffeine (S2). Following S2 cells were continually exposed to a buffer containing THAPS (1 µM) and La\(^{3+}\) (100 µM). At the times indicated cells were exposed for 5 sec to a buffer containing 10 mM caffeine. Data represent the average for 5 cells from a 6 month-old animal.
appears slow relative to activation of SOCC in other cell models (19). Figure 2.7D demonstrates that La$^{3+}$ blocks the activation of SOCC following caffeine-evoked depletion of [Ca$^{2+}$]i and blockade of SERCA by THAPS. Under these conditions [Ca$^{2+}$]i no longer rises after depletion of [Ca$^{2+}$]i stores and subsequent responses of SCG cells to caffeine are abolished. Overall these data demonstrate that spontaneous refilling of [Ca$^{2+}$]i stores in resting SCG cells following the release of [Ca$^{2+}$]i appears to be mediated by both SOCC and SERCA activity (2, 47).

Figure 2.8 demonstrates that advancing age alters the spontaneous refilling of SER Ca$^{2+}$ stores via SOCC and SERCA activity following caffeine-evoked depletion of [Ca$^{2+}$]i stores. After caffeine evoked depletion (S2), the cells were exposed for 5 sec to caffeine at the time intervals shown in figure 2.3. The data in figure 2.8A show that there is a significant age-related decline in the response to caffeine at each time point after the conditioning response. However, at the 240 sec time interval, there is no longer any significant difference in the response to caffeine in any age group. To estimate the rate of spontaneous refilling of [Ca$^{2+}$]i stores following caffeine-evoked depletion we calculated the derivative of the curves in figure 2.8A, and plotted these values as a function of age (Figure 2.8B). The derivatives were found to be significantly lower in SCG cells from 12, 20, and 24 month-old animals as compared to 6-months (Figure 2.8B).

D.3. Measurement of Basal [Ca$^{2+}$]i and rate of recovery of high K$^+$ and caffeine-evoked [Ca$^{2+}$]i transients

Table 2.1 shows the impact of age on basal [Ca$^{2+}$]i following successive exposures to high K$^+$ and caffeine using the protocol in figure 2A. Under all treatment conditions basal [Ca$^{2+}$]i significantly increases in SCG cells from 6-12 month-old animals and then
significantly declines from 12-24 months. **Table 2.2** shows the impact of age on the recovery rate constant following exposure to high K\(^+\) or caffeine as shown in the protocol in **figure 2.2A**. There is no significant age-related change in the magnitude of the recovery rate constants with any of the successive exposures to high K\(^+\) or caffeine.
Fig. 2.8. (A) Aging alters the spontaneous refilling of SER calcium stores following caffeine-evoked depletion. Using protocol 2 (Figure 2.3), data were plotted as integrated caffeine-evoked release of $[Ca^{2+}]_i$ as a percentage of the $K^+$ control vs time after conditioning response. Data represent the mean ± S.E. n = 13-29 cells from 6-24 month-old animals. ** = significantly different from two other age groups, $P<0.01$. *** = significantly different from three other age groups, $P<0.01$. (B) Aging alters the derivative of caffeine-evoked $[Ca^{2+}]_i$ transients as a percentage of $K^+$ control. Data derived for each individual cell in (A) were plotted and fitted by a Boltzman fit and the derivatives calculated using Origin 6.1. Data represent the mean ± S.E. n = 13-29 cells from 6-24 month-old animals. *** = significantly different from three other age groups, $P<0.001$. ** = significantly different from two other age groups, $P<0.01$. 

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Table 2.1: Basal $[\text{Ca}^{2+}]_i$ significantly declines from 12-24 months following each treatment in protocol 1. Data represent the mean ± S.E. n = 21-42 cells from each age group. P<0.05.

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Table 2.2. Rate of recovery of $[\text{Ca}^{2+}]_i$ transients with each treatment in protocol 1 in isolated SCG cells from 6-24 month-old animals. Rate of recovery was calculated by obtaining the first order time constant (Tc) using Origin 6.1. The reciprocal of Tc yields the rate constant. N= 21-42 cells from 12-20 animals.

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<td>2nd KCl</td>
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<td>0.16 ±0.01</td>
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<td>0.12 ±0.01</td>
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</table>
E. Discussion

The most important and straightforward findings in this study is that release of calcium from [Ca\(^{2+}\)]\(_i\) stores and high K\(^+\)-evoked and spontaneous refilling of these stores declines with advancing age in SCG cells. The magnitude and shape of stimulation-evoked increases in [Ca\(^{2+}\)]\(_i\) are modulated by both influx of and release of calcium in neurons (25, 46-48). Sustaining the release of calcium from intracellular stores during ongoing activity requires the refilling of these stores following the release. Overall, the data in this study suggest that the ability to sustain release of calcium from intracellular stores declines with age and may have implications as to the function of sympathetic neurons during the aging process. As refilling of [Ca\(^{2+}\)]\(_i\) stores is dependent on both influx and uptake into the SER via SERCA the decline in refilling may reflect and age related decline in the function of both mechanisms. Indeed, we have shown that SERCA function declines with age in sympathetic neurons (32, 33, 43, 44). Thus, at least one mechanism that may account for the decline in refilling of [Ca\(^{2+}\)]\(_i\) stores is an age-related decline in the function of SERCA pumps. As adrenergic nerves from the SCG serve to protect the CNS from blood brain barrier disruption (3, 5, 7, 12, 18), these data suggest that the ability of the SCG to sustain its protective function may possibly be altered with advancing age.

E.1. Aging and fast refilling of [Ca\(^{2+}\)]\(_i\) stores

The data reported in this study (Fig. 2.2B) as well as published data suggest that selective release of calcium from intracellular stores in dorsal root ganglion (DRG) and SCG cells can be accomplished with caffeine (11, 25, 35, 47). In addition, these stores are rapidly refilled by depolarization of the neurons following exposure to caffeine (11, 25,
Thus, we utilized a protocol to determine if advancing age alters repeated caffeine-evoked release of [Ca$^{2+}$]i after high K$^+$-evoked refilling (Fig 2.2A). There was no age-related difference in the [Ca$^{2+}$]i dynamics between the first and second high K$^+$-evoked [Ca$^{2+}$]i transient (Fig. 2.6A,B). Thus, although the dynamics of K$^+$-evoked [Ca$^{2+}$]i transients decline with age, they remain constant within each age group. In contrast to K$^+$-evoked [Ca$^{2+}$]i transients, advancing age caused a significant reduction in the [Ca$^{2+}$]i dynamics between the first and second caffeine exposure within the oldest age groups (Fig. 2.6C,D). There are numerous studies demonstrating the impact of age on depolarization-evoked [Ca$^{2+}$]i transients (4, 33, 34, 44, 49). However, to our knowledge this is the first study demonstrating that the ability of peripheral neurons to sustain the release of calcium following rapid depolarization-evoked refilling, declines with age.

Interpretation of the overall age-related decline of K$^+$-evoked [Ca$^{2+}$]i transients is complex (Fig. 2.4) as depolarization-evoked [Ca$^{2+}$]i transients reflects both influx and release of calcium (46). Using patch clamp methods coupled with measurement of [Ca$^{2+}$]i with fura-2, one study showed that stimulation-evoked calcium influx increases but the measured [Ca$^{2+}$]i by fura-2 declines with age (28). The measurement of K$^+$-evoked [Ca$^{2+}$]i in this study does not directly distinguish between the contributions made to the calcium signal by influx, and release of calcium from intracellular stores. Thus, it is possible that at least one mechanism that may account for the decline in K$^+$-evoked [Ca$^{2+}$]i dynamics is reduced release. However, these data do not rule out changes in calcium influx mediated through voltage gated calcium channels.
Fig. 2.4. (A, B) Peak [Ca\(^{2+}\)]\(_i\) evoked by the first and second exposure to high K\(^+\) in isolated SCG cells from animals aged 6-24 months. (C,D) Rate of rise of [Ca\(^{2+}\)]\(_i\) evoked by the first and second exposure to high K\(^+\) in isolated SCG cells from animals aged 6-24 months. These data were derived from the protocol shown in figure 2A. Peak [Ca\(^{2+}\)]\(_i\) was measured by subtracting basal [Ca\(^{2+}\)]\(_i\) from the maximum K\(^+\)-evoked [Ca\(^{2+}\)]\(_i\) transient. Rate of rise of [Ca\(^{2+}\)]\(_i\) from baseline to maximum was determined by linear fit using Origin 6.1. Data represent the mean ± S.E. n = 21-42 cells from each age group. ** = significantly different from two other age groups, P <0.05. *** = significantly different from three other age groups, P<0.05.
Fig. 2.6. (A,B) Impact of age on the percentage difference between the first and second K⁺-evoked peak and rate of rise of [Ca²⁺]ᵢ transients. (C,D) Impact of age on the percentage difference between the first and second caffeine-evoked [Ca²⁺]ᵢ transients. The percentage difference was calculated as the difference between the first and second exposure to K⁺ or caffeine divided by the first exposure to K⁺ or caffeine times 100. Data represent the mean ± S.E. n = 21-42 cells from each age group. * = significantly different from one other age group, P<0.05. ** = significantly different from two other age groups, P<0.05. *** = significantly different from three other age groups, P<0.05.
E.2. Aging and spontaneous refilling of \([Ca^{2+}]_i\) stores

There are studies demonstrating that in SCG and DRG cells the \([Ca^{2+}]_i\) stores can spontaneously refill within 3-10 min following depletion with caffeine (11, 25, 45, 47). This spontaneous refilling of \([Ca^{2+}]_i\) stores requires two interdependent mechanisms. These are the spontaneous influx of calcium through SOCC and subsequent pumping of calcium into the SER via SERCA (2, 47). The function of SOCC channels can be blocked with ions such as La\(^{3+}\) and SERCA function is blocked by THAPS (2, 47). Since we have previously shown that SERCA function declines with advancing age in isolated SCG cells (32, 34, 44), we developed an experimental protocol to study the impact of age on the spontaneous refilling of \([Ca^{2+}]_i\) stores following caffeine-evoked depletion (Fig. 2.3). In order to validate that the spontaneous refilling of \([Ca^{2+}]_i\) stores is independent of VOCC and mediated by SOCC and SERCA activity we performed a series of validation experiments (Fig. 2.7). These control data suggest that in isolated resting SCG cells spontaneous refilling of \([Ca^{2+}]_i\) stores appears to be mediated by influx of calcium via SOCC and subsequent uptake into the SER via SERCA pumps. These data are consistent with previous studies demonstrating that spontaneous refilling of \([Ca^{2+}]_i\) store is mediated by SOCC and SERCA function (2, 2, 47).

To our knowledge this is the first study to demonstrate that spontaneous refilling of \([Ca^{2+}]_i\) stores slows with age in isolated SCG cells (Fig. 2.8). Interestingly, it appears that SCG cells from senescent animals will eventually refill to similar levels if given an appropriate amount of time. Thus, SCG cells from healthy senescent animals maintain some ability to release and spontaneously refill \([Ca^{2+}]_i\) stores, implying that some level of function of sympathetic neurons is maintained with age. Using various antagonists of
SERCA we have shown that SERCA function declines in both SCG cells and sympathetic nerve endings (32, 34, 43, 44). We propose that at least one mechanism that may account for a decline in spontaneous refilling of \([Ca^{2+}]_i\) stores is reduced SERCA function. However, these data do not rule out age-related changes in SOCC function. We are currently developing protocols to study how aging may alter SOCC function.

E.3.Aging and basal \([Ca^{2+}]_i\) levels and rate of recovery of \([Ca^{2+}]_i\)

Surprisingly we found that basal \([Ca^{2+}]_i\) levels rose from 6-12 months and then steadily declined with age (Table 2.1). The increase in basal \([Ca^{2+}]_i\) from 6-12 months may reflect late maturational changes. In our previous studies using only two age-groups we found no significant difference in basal \([Ca^{2+}]_i\) levels in SCG cells from 6 and 20 month-old animals (32, 34). Comparison of the current data with our previous studies succinctly demonstrates the necessity of using more than two age groups in aging studies. Using multiple age groups appears to be essential as the range from maturity to senescence provides a clearer overview of how advancing age affects the parameters of interest (9). Since we have shown that SERCA function declines with age in SCG cells one might predict higher resting levels of \([Ca^{2+}]_i\). Indeed our data stand in contrast to age-related increases in basal \([Ca^{2+}]_i\) in DRG neurons (21). These data may suggest that the aging process may not have uniform effects on all neuronal models. Since SERCA function depends on both the level of cytosolic calcium and modulation by other factors such as phosphorylation (50), reduced levels of cytosolic calcium may possibly contribute to lower loading levels of \([Ca^{2+}]_i\) stores.

Depolarization-evoked \([Ca^{2+}]_i\) transients depend on influx and release of \([Ca^{2+}]_i\) (46, 47), and caffeine-evoked \([Ca^{2+}]_i\) transients reflect calcium released from \([Ca^{2+}]_i\)
stores. Thus, lower loading levels of \([\text{Ca}^{2+}]_i\) stores may possibly contribute to an age-related decline in both high \(K^+\) and caffeine-evoked release of \([\text{Ca}^{2+}]_i\) observed in these studies (Fig. 2.4,2.5). In future studies we will use calcium indicators such as furaptra to determine the impact of age on the levels of SER \([\text{Ca}^{2+}]_i\) stores similar to studies accomplished in smooth muscle cells (29).

Since SERCA function declines with age in SCG cells (32, 44) it is remarkable that there is no age-related change rate of recovery of high \(K^+\)- or caffeine-evoked \([\text{Ca}^{2+}]_i\) transients. However, rate of recovery of high \([\text{Ca}^{2+}]_i\) transients in SCG cells is complex as we have shown that SERCA, mitochondrial calcium uptake and PMCA all contribute to the rate of recovery of high \([\text{Ca}^{2+}]_i\) transients in SCG cells (4, 32, 34, 44). In addition, we have shown that the age-related decline in SERCA function is accompanied by an apparent increased reliance on mitochondrial calcium uptake and PMCA function (4, 32, 34). Taken together our current and past studies suggest that in the face of an age-related decline in SERCA function, the rate of recovery is maintained by increased activity of other calcium uptake and extrusion systems. These data suggest that with advancing age buffering systems do not necessarily "fall apart" as a decline in one system may be compensated for by increased function of another system. Thus old SCG cells appear to have inherent adaptive responses to maintain some vitality in the face of age related declines the function of particular calcium regulatory mechanisms.

In conclusion, the data presented in this study suggest that with advancing age there is an age-related decline in the ability of SCG cells to sustain release of calcium from \([\text{Ca}^{2+}]_i\) stores. These data may have implications for the function of sympathetic autonomic neurons as animals undergo the normal aging process.
Fig. 2.5. (A,B) Peak [Ca\(^{2+}\)]\(_i\) evoked by the first and second exposure to 10 mM caffeine in isolated SCG cells from animals aged 6-24 months. (C,D) Rate of rise of [Ca\(^{2+}\)]\(_i\) evoked by the first and second exposure to 10 mM caffeine in isolated SCG cells from animals aged 6-24 months. These data were derived from the protocol shown in figure 2.2A. Peak [Ca\(^{2+}\)]\(_i\) was measured by subtracting basal [Ca\(^{2+}\)]\(_i\) from the maximum caffeine-evoked [Ca\(^{2+}\)]\(_i\) transient. Rate of rise of [Ca\(^{2+}\)]\(_i\) from baseline to maximum was determined by linear fit using Origin 6.1. Data represent the mean ± S.E. n = 21-42 cells from each age group. ** = significantly different from two other age groups, P<0.05. *** = significantly different from three other age groups, P<0.05.
F. Acknowledgements

The authors wish to acknowledge the technical expertise of Mr. Charles Hewitt in the development and execution of measurement of $[\text{Ca}^{2+}]_i$ with our imaging system. This work was supported in part by grants from the American Heart Association, National Center (#0040021N) and NIH P01 31226.
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CHAPTER 3

Advancing age alters the expression of the ryanodine receptor 3 isoform in adult rat superior cervical ganglia

Conwin K. Vanterpool¹, Elaine A. Vanterpool², William J. Pearce¹ and John N. Buchholz¹*.

¹Department of Physiology and Pharmacology
²Department of Microbiology and Molecular Genetics
Loma Linda University, School of Medicine
Loma Linda, CA 92350, USA.

Running title: expression of RyRs with advancing age

*Corresponding author: John Buchholz

Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, Loma Linda, CA 92350

E-mail: jbuchholz@llu.edu

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A. ABSTRACT

Sympathetic nerves arising from the superior cervical ganglion (SCG) protect the cerebrovasculature during periods of acute hypertension and may play a role in homeostasis of target organs. The functions of these nerves depend on calcium release triggered by activation of ryanodine receptor (RyR) channels. The function of RyR channels is in part dependent on genetic expression and regulation by numerous proteins modulators such as neuronal nitric oxide synthase (nNOS) neurons also found in the SCG. We have shown that release of calcium in SCG cells is altered during late maturation and advancing age. However, the underlying molecular mechanisms that may in part account for these data are elusive. Therefore we used molecular techniques to test the hypothesis that advancing age alters the pattern of genetic expression and/or protein levels of RyRs and their modulation by nNOS in the SCG in F-344 rats aged 6, 12 and 24 months. Surprisingly, ryr1 expression was undetectable in all age groups and ryr2 and ryr3 are the predominantly transcribed isoforms in the adult rat SCG. mRNA and protein levels for RyR2 isoform did not change with advancing age. However, ryr3 mRNA levels increased from 6 to 12 months and declined from 12 to 24 months. Similarly, RyR3 receptor protein levels also increased from 6 to 12 months and declined from 12 to 24 months. Because nNOS and the phosphorylation of the RyRs have been shown to modulate the function of RyRs, total phosphorylation and nNOS protein levels were analyzed in all age groups. Phosphorylation levels of the RyRs were similar in all age groups. However, nNOS protein levels increased from 6 months to 12 months followed by a decline from 12 months to 24 months. These data suggest that advancing age selectively impacts the genetic expression and protein levels of RyR3 as well as
modulatory nNOS protein levels. In addition, these data may part provide some insight into the possible changes in the function of RyRs that may occur with the normal aging process.
B. INTRODUCTION

Clinical studies show that risk of stroke increases with age, and the single most important factor is rising systolic blood pressure (1, 14, 32, 69). Thus, a more comprehensive understanding of aging and modulation of the cerebrovasculature is necessary. Systolic blood pressure rises in both F344-Rats and in humans suggesting that the cerebrovasculature is subjected to increased pressure and risk of hyperemia and stroke (14, 68). The SCG provides sympathetic innervation to the cerebrovasculature, and dampen increased cerebral blood flow in response to hypertension or increased intracranial pressures (19). Therefore, sympathetic nerves arising from the SCG reduce the risk of blood brain barrier disruption and are becoming an increasingly important area in biomedical research (6, 14, 25, 55).

In addition to the importance of the SCG in the cerebrovasculature, the SCG innervates and impacts the function of many organs including the heart (29, 41, 61, 65, 66), the eye (23, 53, 62, 64), and secretory glands, such as the pineal gland (30, 31) thyroid/parathyroid and salivary glands (7, 9, 10, 26, 49). It has also been suggested that the SCG and other sympathetic neurons function as a peripheral neuroendocrine center (8, 9) serving as the communication bridge between the central nervous system and the endocrine system (9, 52). The SCG has also been shown to play an important role in the immune response (36, 48, 63).

The function of peripheral sympathetic and sensory neurons has been shown to be dependent on release of calcium from intracellular stores in response to stimulation-evoked increases in intracellular calcium ([Ca^{2+}]_i) (34, 44, 56-58). This process is known as calcium induced calcium release (CICR) and occurs through the activation of
ryanodine receptor channels. The function of RyR channels depends in part on density and their regulation. The regulation of the function of RyRs is complex and overall this regulation serves to modulate the sensitivity of RyR’s to cellular Ca\(^{2+}\) levels. These modulators include FKBP proteins, which serve to activate or inhibit channel state depending on its binding status, and activators such as phosphorylation and intracellular molecules including cADP-ribose (cADPr) (24, 35, 45). In the case of cADPr these levels are modulated by nitric oxide (NO) released from nNOS containing neurons. Indeed our studies and others have shown that the SCG and the cerebrovasculature is innervated by nNOS neurons, which function to modulate stimulation-evoked norepinephrine release (12, 33, 37, 38).

To maintain the CICR process the [Ca\(^{2+}\)]i stores must be refilled. Refilling depends on the amplification of [Ca\(^{2+}\)]i, which occurs through such channels as voltage gated calcium channels, and store operated calcium channels, and by pumping Ca\(^{2+}\) back into the stores by smooth endoplasmic reticulum calcium ATP-ases (SERCA). We have previously demonstrated that there is an age related decline in SERCA-mediated Ca\(^{2+}\) uptake in the SCG (47). This decline in SERCA function influences caffeine-evoked release of Ca\(^{2+}\) and both rapid depolarization-induced and spontaneous refilling of SER Ca\(^{2+}\) stores after depletion (60). These data suggested that advancing age alters the capacity of SCG cells to release Ca\(^{2+}\) from SER stores and that the levels of the SER Ca\(^{2+}\) may also decline with age, which may possibly alter the function of the SCG.

The three isoforms of the ryanodine receptor family are RyR1 (found in skeletal muscle), RyR2 (found in cardiac muscle), and RyR3 (found in neurons, and other tissue types) have been shown to be responsible for CICR (45). As discussed above, these
receptors are important in the function of sympathetic neurons and sympathetic neurons arising from the SCG may serve to protect the CNS from rupture of blood vessels at higher systemic pressures. As maturation and aging are normal and inevitable processes it is necessary to study underlying mechanisms that may account for how animals develop and age. Thus, molecular characterization of the RyRs and/or selective modulators may provide information on calcium release during the aging process. The expression, overall phosphorylation, and modulation of RyR’s by nNOS with late maturation or advancing age has not previously been studied in the rat SCG model. As the function of multiple subtypes of RyR’s are important to the overall function of numerous excitable cells the genetic expression and protein levels of RyR channels in models that have not been previously studied are warranted. Using molecular techniques of RT-PCR and ELISA analysis we tested the hypothesis that the genetic and protein expression of the predominant RyR isoform(s) in adult rat SCG, along with selective modulators, are altered during late maturation and advancing age in F-344 rats aged 6, 12 and 24 months.
C. MATERIALS AND METHODS

C.1. Experimental animals

Male Fischer 344 (F-344) rats, aged 6 mo (young adult), 12 mo (mature adult), and 24 mo (senescent), were obtained from National Institutes of Health-National Institute on Aging breeding colony (Harlan Sprague-Dawley, Indianapolis, IN). The age range designation comes from other studies showing the median life span in F-344 rats is ~24 months. The animals were allowed to eat and drink at will and were maintained on a 12:12-h light-dark cycle under controlled temperature (72–77°F). All procedures used in this study were approved by the Institutional Animal Care and Use Committee at Loma Linda University, and the approved guidelines were adhered to throughout the study.

C.2. SCG isolation and sample preparation

Rats were anesthetized with CO₂ (45 s) followed by decapitation. Both carotid arteries were dissected from each male F-344 rat. SCG were dissected from the carotid artery bifurcation as previously described (60). Tissue was pulverized in liquid nitrogen into a fine powder using a metal mortar and pestle, then placed in cold lysis buffer (8.77g NaCl, 7.88g Tris HCL, 3.29g EDTA, 5ml 0.5% Tween-20, and broad spectrum protease inhibitors and sonicated until homogenous.

C.3. Isolation of SCG total RNA

Total RNA from 6, 12 and 24 month old animals were isolated using the Ambion RiboPure RNA isolation kit (Ambion, Austin, TX) according to the manufacturer’s recommendations. Briefly, the rat SCG was dissected out of the 6, 12 or 24 month old animals and pulverized using liquid nitrogen. The pulverized tissue was then re-suspended in RNAwiz and the RNA was separated from SCG proteins by the addition of
chloroform and centrifugation. The RNA was precipitated and treated with DNAse 1 to eliminate genomic DNA. The integrity of the RNA was determined by assessing the 260/280 ratio. RNA samples with ratios between 1.8-2.0 indicate high purity of RNA and samples yielding these values were used for all RT-PCR reactions.

C.4. Reverse-transcriptase PCR

The primers used for reverse transcription-PCR (RT-PCR) analysis were specific for the ryr1, ryr2, ryr3 and GAPDH determined by using the NCBI blast database for Rattus norvegicus (Table 1). RT-PCR reactions (50 µl) using RT-PCR reaction mix (Promega, Madison, WI) were performed with samples containing RNA ranging from 10 ng to 1 µg for optimization. To test for DNA contamination we performed negative controls, which consisted of the same RT-PCR reaction mix contained in the biological samples minus reverse transcriptase.

C.5. SDS-PAGE and Immunoblot analysis

This study utilized an ELISA assay along with selective antibodies to determine relative RyR2, RyR3 levels and nNOS levels in SCG. Therefore, we first validated the selectivity of our antibodies for these respective proteins using sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis and Western blot analysis. We have shown in our previous studies that the antibodies for nNOS are highly selective and yield single bands (37).

Similarly for RyR2 and RyR3 levels we determined the selectivity of polyclonal rabbit anti-rat antibodies for the respective RyR’s using a modified method [Figure 3.3, (21)]. Briefly, SDS-PAGE was performed using a 4 to 12% Bis-Tris polyacrylamide gel (NuPAGE Novex gels, Invitrogen, Carlsbad CA) in MOPS (morpholinepropanesulfonic
acid)-SDS running buffer according to the manufacturer's instructions (Invitrogen, Carlsbad, CA.). Samples were prepared [(65% sample containing 25 μg of total protein), (25% 4x NuPAGE LDS sample buffer), (10% NuPAGE reducing agent)], heated at 72°C for 10 min, and then electrophoresed at 200 V for 120 minutes in the XCell SureLock Mini-Cell system using the Hi-Mark high molecular weight standards (Invitrogen, Carlsbad, CA.). The separated proteins were then transferred to PVDF membrane (Millipore, Bedford, MA). The blots were probed with ryanodine-specific antibodies (Chemicon, Temecula CA). Immunoreactive proteins were detected by the procedure described in the Western Lightning chemiluminescence reagent plus kit (Perkin-Elmer Life Sciences. Boston, MA.). Selectivity of the RyR2 and RyR3 are further validated by Chemicon using RyR2 and RyR3 positive and negative tissues and assurance of these controls are provided by Chemicon. Thus, with information provided by Chemicon and our independent analysis in this study suggest that the RyR2 and RyR3 selective antibodies are reliable antibodies for quantification of these isoforms using ELISA assay.

C.6. ELISA

There are two issues with regards to Western analysis of RyRs. First, RyRs are greater than 500 kDa and due to its high molecular weight may not transfer well. Secondly, incomplete transfer will affect the accuracy of the quantitation of RyR protein levels. Thus, ELISA assays were developed to measure their levels eliminating the need for transfer of proteins from separation gels to blotting membranes (21). We used a modified ELISA assay to measure RyR2 and RyR3 protein levels relative to GAPDH levels. Similarly, we used the ELISA to measure nNOS levels relative to GAPDH. We first performed a saturation curve to determine the optimal amount of protein to use in
quantification of RyR’s and nNOS in rat SCG (Figure 3.6). The serial dilutions of total proteins, ranging from 15.6ng-2µg, were incubated in high binding 96-well plates (Corning, Corning NY) for 16-24 hours at 4°C. The unbounded proteins were washed away using PBS-T (138 mM NaCl, 2.7mM KCl, pH 7.4, 0.1% Tween-20) and blocked using 1% BSA for 1 hour at room temperature. The antigen-coated wells were washed twice with PBS-T and then incubated with the RyR2, RyR3, GAPDH or nNOS antibodies for 16 hours at 4°C. The antigen-coated plates were then washed three times with PBS-T and incubated with the HRP-conjugated antibodies for 1 hour at 37°C. The unbounded antibodies were washed away using PBS-T and then incubated with hydrogen peroxide and 2,2’-Azino-di-(3-ethylbenz-thiazoline) Sulfonic Acid (ABTS) (Zymed, Invitrogen, Carlsbad, CA) for 15-20 minutes ABTS is oxidized to yield a green chromophore and absorbance was measured at 405 nm using a microplate reader. The optimal concentrations of protein to be used for experiments were assessed and experiments were followed as described above. Negative controls were performed in the absence of primary antibodies. GAPDH was also determined in each age group to serve as a loading control. In addition, all colorimetric intensity values obtained for RyR’s were normalized to GAPDH.

C.7. Phosphorylation analysis of the RyRs

The relative total RyRs present in adult rat SCG were analyzed for levels of phosphorylation according to manufacturer’s protocol (Molecular Probes, Invitrogen, Carlsbad, CA). Briefly, 25 µg determined by Bradford assay (BioRad) of total SCG protein from 6, 12 and 24 month old animals were subjected to SDS-PAGE for separation. After 1 hour of electrophoresis, the PeppermintStick phosphoprotein
molecular weight standard (Molecular Probes, Invitrogen, Carlsbad CA) was then loaded on the gel and electrophoresed for an additional 1 hours. This standard served as a positive phosphoprotein control. The gel was fixed, washed, stained with the Pro-Q Diamond phosphoprotein stain and destained as described by manufacturer’s protocol. The phosphoproteins were imaged using transillumination at 540 nm. The images were documented and the proteins were then stained using SYPRO Ruby protein gel stain. The total proteins were then documented and ratio of phosphoprotein stain signal to total protein stain signal of the total RyRs were assessed. To ensure that the proper protein band was being assessed, immunoblot analysis was performed using mouse anti-RyR antibodies which detect all three isoforms (Sigma, St. Louis MO). As an additional internal control to insure that the method accurately detected phosphorylated forms of the ryanodine receptors, we prepared gels using two different amounts of total protein (12.5 μg and 25 μg). There was an increase in intensity of phosphorylated ryanodine receptor for the higher total protein, however, the ratio of total ryanodine protein to phosphorylated ryanodine did not change.
D. RESULTS

D.1. Saturation curve for RT-PCR analysis and validation of primer specificity

Reverse-transcriptase PCR is a powerful tool and has commonly been used to semi-quantitate mRNA levels. However, because PCR is an exponential process, if not optimized correctly, PCR type detection can be problematic. To ensure that saturation of the amplified products do not occur, primers for GAPDH, ryr2, and ryr3 were used to establish a curve to determine the optimal concentration of total RNA concentrations to use. Total RNA concentrations ranging from 10ng-120ng were used and amplified for 50 cycles. 60-75ng of total RNA showed to be ideal to use for quantitative purposes (data not shown). The desired molecular weights were determined by electrophoresis of the amplicons (Table 3.1). To ensure that the amplified products were the desired products and not non-specific priming of the oligonucleotides, the amplified products were sequenced by the UC Davis sequencing core facility and were shown to be either ryr2, ryr3 or GAPDH.

D.2. Identification of the major ryanodine receptor isoforms in rat and impact of age on their expression

The predominant isoforms of the ryr in rat SCG has not previously been demonstrated. To identify what ryr isoform transcript(s) are predominantly present in rat SCG, RT-PCR performed on SCG DNase treated total RNA isolated from 6 month, 12 month and 24 month animals. Concentrations RNA up to 1 μg, did not yield any amplified fragment consistent with the ryr1 (Fig. 3.1). Since ryr1 was shown to be present in the brain, we utilized RNA isolated from the cerebral cortex of a 6 month old rat as a positive control to ensure that primer conditions were appropriate and primers were
Table 3.1. Primers used in this study

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<td>RyR1 REV 5'GAG GGT CAG GTT TGC GCT CAT TG 3'</td>
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</table>

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specific. The predicted size fragment was amplified from brain RNA, but not from the SCG, confirming that proper primer conditions were used. ryr2 and ryr3 were transcribed in rat SCG demonstrating that these two isoforms are the predominant ryr transcripts in rat SCG cells (Figs 3.2). To ensure no contaminating DNA was present, the reaction was also performed in the absence of reverse transcriptase (data not shown). No amplified fragment was observed.

Semi-quantitative RT-PCR analysis of ryr2 normalized to GAPDH from the 6, 12 and 24 month showed no significant alteration in the ryr2 transcript with advancing age (Fig 3.2). In contrast to ryr2, ryr3 normalized to GAPDH message significantly increased in the 12 month in comparison to the 6 month, followed by a significant decline in 24 month-old animals.

D.3. Saturation curve analysis for ELISA, and validation of antibody specificity

Since RyRs are very high molecular weight proteins, quantification by standard western blotting procedure can be very difficult due to incomplete transfer. Because of these factors, ELISA assay was used to measure RyR levels as it is commonly used for protein detection and quantification. To determine that the antibodies used in these ELISA are specific for RyR2 and RyR3, SCG proteins were transferred onto a PVDF membrane, blocked and then probed using RyR2 and RyR3 specific antibodies. Immunoreactive bands larger than 500 kDa, consistent with the size of the ryanodine receptors were detected (Fig. 3.3A). These data demonstrate that the antibodies are selective for the ryanodine receptors 2 and 3, which were detected in the SCG samples. To ensure saturation does not occur with the ELISA procedure, a serial dilution of the
Figure 3.1. *The ryr1* transcript is not the predominant *ryr* isoform in adult SCG. 75 ng of DNase treated total RNA isolated from rat SCG ganglia or rat brain was subjected to reverse transcriptase-PCR (RT-PCR) using *ryr1*-specific oligonucleotides. RT-PCR products were then subjected to gel electrophoresis and stained with ethidium bromide and visualized by UV-excitation. No detectable amplified product consistent with the predicted *ryr1* transcript was detected from rat SCG RNA. To ensure primer conditions were optimized and specific, RNA from rat brain was used as a control.
Figure 3.2. Transcriptional profiling of *ryr2* and *ryr3* with advancing age. (A, C) 60 ng of DNase treated total RNA isolated from rat SCG was subjected to RT-PCR using *ryr2* or *ryr3* specific oligonucleotides shown in Table 1. RT-PCR products were amplified then subjected to gel electrophoresis and stained with ethidium bromide and visualized by UV-excitation and optical density was measured. (B, D) Density analysis of the *ryr* messages were normalized to the GAPDH transcript; (A, C). (A, B): There is no detectable age-related alteration of *ryr2* transcript in adult rat SCG. (C, D): *The ryr3* message expression changes with age in rat SCG. Data represent the mean ± S.E. n = 3 replicates from 12 pooled ganglia from each age group, performed in two independent experiments. Data were analyzed by ANOVA and Fischer-PLSD test. * = significantly different from one other age group, P <0.02. ** = significantly different from two other age groups, P<0.02.
SCG sample ranging from 15.6 ng-2ug of total protein was used, in addition various antibody dilutions ranging from 1:500-1:2000 were also used. These data demonstrate 500 ng of total SCG protein/well and a 1:500 dilution of both RyR2 and RyR3 were optimal (Fig. 3.3B).

D.4. Protein expression analysis of RyR2, RyR3 and nNOS by ELISA assay and phosphorylation of total RyR’s with advancing age

ELISA quantification showed that RyR2 levels normalized to GAPDH showed no significant differences with advancing age (Fig. 3.4A). In these studies GAPDH served as a normalization control since there is no change in its levels over the age range 6, 12 and 24 months (Fig. 3.4A inset). In contrast to RyR2, levels of RyR3 normalized to GAPDH, there was a significant increase in RyR3 levels in the 12 month old SCG in comparison to the SCG of the 6 month old animals followed by a significant decrease in the RyR3 levels at 24 months (Fig. 3.4B with inset GAPDH). In addition to changing RyR expression levels, phosphorylation level of the total RyR was assessed. Ratio of phosphostaining to total protein staining showed no significant alterations in the levels of RyR phosphorylation with advancing age (Fig. 3.5). As an added control to insure detection of phosphorylated RyR’s we used a phosphoprotein standard kit containing ovalbumin phosphoprotein, which was postively phosphostained. In contrast the non-phosphophorylated protein controls bovine serum albumin and beta-glactosidase did not stain with the phosphostain (data not shown). We assessed the protein levels of nNOS normalized to GAPDH in the SCG over the age range from 6-24 month-old animals. There was a significant increase in the proteins levels of nNOS 6 month to 12 month, followed by a significant decline of nNOS protein levels in 24 month old animals (Fig. 3.6).
Figure 3.3. Validation of RyR2 and RyR3 antibody specificity, and saturation curve analysis for RyR2 and RyR3 ELISA. (A) 25 μg of total rat SCG protein were subjected to SDS-PAGE, transferred to PVDF membrane and probed with RyR2 or RyR3 antibodies. The ryanodine receptor antibodies immunoreacted with high molecular weight bands approximately 550 kDa, demonstrating the selectivity of the antibodies for RyR2 and RyR3. (B) SCG were isolated from 12 month old rats. ELISA plates were coated with serially diluted SCG protein, concentrations ranging from 23.5 ng-2μg total protein. Saturation curves determined that 500 ng of total SCG protein/well and a 1:500 dilution of both RyR2 and RyR3 were optimal.
Figure 3.4. Ryanodine receptor expression levels with advancing age. High binding ELISA plates were coated with 500ng of rat SCG proteins from all age groups. The RyR2, RyR3 and GAPDH (normalization controls, inset) proteins were probed with rabbit RyR2, RyR3 or GAPDH-selective antibodies. H$_2$O$_2$ and ABTS serves as substrates for the conjugated HRP secondary antibody to RyR2, RyR3 or GAPDH antibody complex. ABTS is oxidized to yield a green chromophore and absorbance was measured at 405 nm. (A): Results demonstrate that RyR2 protein expression does not significantly change with age. (B): RyR3 protein expression levels change with age. Bars represent the mean ± S.E. and were analyzed by ANOVA and Fischer-PLSD test. ** = significantly different from two other age groups, P < 0.03. n=3 experiments using 6 pooled ganglia per experiment.
Figure 3.5. Phosphorylation of total ryanodine receptors do not change with age. 25 μg of total SCG protein from 6, 12 and 24 month old animals were separated and the RyRs were analyzed for phosphate groups stained with the Pro-Q Diamond phosphoprotein stain or stained using SYPRO Ruby protein for total protein. The phosphoproteins and total proteins were imaged using transillumination at 540 nm. The ratio of phosphoprotein stain signal to total protein stain signal of the total RyRs were assessed and plotted. Bars represent the mean ± S.E. and were analyzed by ANOVA and Fischer PLSD test. n= 3 experiments with 6 pooled ganglia per experiment.
Figure 3.6. nNOS levels increase from 6 – 12 months and then decline from 12 – 24 months. High binding ELISA plates were coated with 500ng of rat SCG proteins from all age groups. The nNOS protein was probed with monoclonal nNOS-selective antibodies, and GAPDH (normalization control, inset) was probed with Rabbit GAPDH-selective antibodies. Relative levels of nNOS were normalized to GAPDH, and determined as stated for RyR2 and RyR3. Bars represent the mean ± S.E. and were analyzed by ANOVA and Fischer-PLSD test. ** = significantly different from two other age groups, P < 0.03. n= 3 experiments with 6 pooled ganglia per experiment.
E. DISCUSSION

We have previously demonstrated a decline in caffeine-evoked release of Ca\(^{2+}\) and a decline in the ability of the superior cervical ganglia cells to sustain release of intracellular calcium with age (60). In the present study we attempted to illuminate some of the possible molecular mechanisms underlying our previous functional studies. We have molecularly identified and characterized the ryanodine receptors with advancing age in this study. Surprisingly, we have found that the ryr1 message is not predominantly expressed in the rat SCG contrasting other studies demonstrating ryr1 is present in excitable cells including neurons (Reviews (17, 51). Thus, a novel conclusion from these studies is that independent of late maturation and advancing age RyR1 does not appear to play role in mediating the release of calcium in the SCG. However, RyR2 and RyR3 are the major receptor isoforms that regulate calcium release from RyR sensitive stores in the SCG in all age groups. In addition, late maturation and advancing age from adult to senescent animals only alters the ryr3 mRNA and RyR3 protein levels. Overall phosphorylation of RyR2 and RyR3 did not change in any of the age groups, suggesting that aging does not necessarily alter modulation of RyR’s via phosphorylation. However, nNOS has been shown to play a role in modulation of RyR sensitivity to changes in [Ca\(^{2+}\)]i levels through modulation of cADPr levels (13, 27). Our data demonstrates the presence of nNOS in the SCG and late maturation and senescent aging significantly increase then decrease nNOS levels. Thus, overall the combination of age-related changes in RyR3 levels, altered modulation by nNOS and our previous data suggesting the SER calcium levels may decline with age (47, 60) may combine to significantly alter
the function of RyR3 and possibly RyR2 in the SCG and alter the function of calcium release mechanism.

D.1. Aging and genetic expression ryr1, 2 and 3

It has been demonstrated that the ryr1 is predominantly expressed in skeletal muscle, ryr2 is predominantly expressed in cardiac muscle and ryr3 is expressed in many tissue types, but predominantly in brain and neurons [Reviewed in (17, 18, 35, 39, 45, 50, 51)]. Numerous studies have shown differential genetic expression of ryr 1, 2 and 3 isoforms in various neuronal models (15, 28, 42, 43, 59). However, to our knowledge this is the first report of on the genetic expression of ryr1, 2 and 3 in the SCG model. In this study, transcriptional analysis of adult rat SCG demonstrated the transcription of both ryr2 and ryr3, however, to our surprise no transcription of ryr1 was detected in the SCG using total RNA concentrations up to 1 microgram, yet it was detectable in substantial quantity in our positive control, the cerebral cortex. This data does not necessarily suggest that RyR1 is not expressed in adult rat SCG, but suggests that it is not the major isoform of the adult rat SCG and may be detected using higher concentrations of RNA. Furthermore, these observations may vary among tissues of different developmental age. It has been shown that during the embryonic stage of the mouse brain, ryr1 expression was the highest in the rostral cortical plate, ryr3 expression was the highest in the caudal cortical plate, and low levels of ryr2 were expressed in the diencephalon and brain stem. However, after 7 days postnatal, ryr2 appeared to be the major isoform in many regions of the brain (42). Indeed this observation has also been shown in during embryonic development in the mouse cerebral cortex where the expression of ryr2 progressively increases and becomes the predominant isoform, where the other isoforms remained
lower (15). This may also possibly occur in the embryonic and postnatal development of the rat SCG and is a subject of ongoing investigation in our laboratory.

Transcriptional analysis of the ryanodine receptors in the SCG demonstrates that \textit{ryr2} and \textit{ryr3} are the major \textit{ryr} transcripts present in SCG and their gene products may be responsible for calcium release from the SER stores. The \textit{ryr2} gene expression did not change with age suggesting that \textit{ryr2} levels remain stable during late maturation and senescent aging. However, mRNA coding for the \textit{ryr3} isoform increased from 6 to 12 months and then significantly declined from 12 to 24 months of age. To our knowledge this study is the first to show that there appears to be a late maturational increase in the \textit{ryr3} isoform with a subsequent decline with senescence. These results do not necessarily differentiate between changes in genetic expression or altered stability of the mRNA in the age groups. Thus, both possibilities may explain the results. Overall, the data suggest that the contribution of \textit{ryr3} to the RyR3 protein levels and ultimately calcium signaling in the SCG may change with age while the contributions provided by RyR2 levels remain stable.

\textit{D.2. Aging and protein levels of RyR2, RyR3 and modulatory nNOS}

Changes in genetic expression alone do not necessarily hold a complete explanation for altered RyR protein levels or regulation of the RyR’s. Thus, we measured RyR2 and RyR3 protein levels using an ELISA assay and normalized to the stable marker GAPDH. Following a similar pattern mRNA RyR2 protein levels were not changed in any age groups. The lack of a change in RyR2 protein levels in any age group suggests that this isoform may remain stable and functional throughout the adult life span. On the other hand the turnover rate of the RyR2 in neuronal cells was not evaluated, which may
possibly affect protein function. Indeed protein turnover plays a role in recycling of amino acids and ensures the destruction of proteins that have been damaged by cellular processes or oxidation (54). In addition, numerous proteins turnover rates decline with age (16). For example in muscular tissue, the turnover rate of the RyR was decreased by 25% in the aged rats in comparison to the younger rats (16). RyR turnover has not been evaluated in SCG and may change with age. Any alterations in turnover rate may have an impact on the RyR2 protein function such as increased oxidation and protein damage in the SCG. In contrast to RyR2, RyR3 protein levels increased from 6 to 12 months and then declined at 24 months. However, the decline at 24 months was not significantly smaller than at 6 months. Thus, a straight-forward conclusion as to functional consequences of this age-related decline is difficult to make. We have shown that caffeine-evoked release of calcium declines with age (60). As caffeine-evoked calcium release offers a measurement of the functional capacity of the RyR sensitive stores, it is difficult to interpret a mechanism based on the alterations in RyR3 protein levels alone. However, the function of the RyR’s also depends on SER calcium filling levels as well as the modulation of the RyR’s. Indeed, we have shown that SERCA function declines in the SCG (47) and that basal levels of [Ca$^{2+}$]i also decline with age (60). Thus, filling levels of the SER may also be compromised which may alter the functional capacity of the release mechanism. In addition, protein levels do not necessarily fully correlate with protein function as some may undergo post-translation modification and may require accessory proteins and modulators to regulate their function. It has been reported that RyR function can be influenced by several factors, including phosphorylation, binding
proteins, calcium levels and nNOS which modulates cADPr levels and in turn modulates RyR's [Reviewed in (3, 11, 13, 20, 40)].

To evaluate if there are changes in selected modulators of the channels, we measured the levels of total phosphorylation. This type of assay provides a general probe as to the relative phosphorylation levels of the RyR’s and was used to determine if further investigation of specific phosphorylations RyR’s with antibodies was warranted. Total phosphorylation of RyR channels was not altered with age. These data suggest that changes in steady state phosphorylation and hence regulation by this mechanism is not necessarily occurring. NO synthesized by nNOS has been shown to be implicated in modulating calcium in neurons (13). It is involved in the production of nitric oxide and can be directly regulated by calmodulin, a calcium binding protein. It is part of the signal transduction pathway responsible for cADPr synthesis, which directly affects the activity of the ryanodine receptors and modulates release of calcium from SER stores. In addition, we have observed that the addition of nitric oxide donors increased caffeine-induced calcium release in SCG cells (unpublished data). Given the importance of nNOS in the function of RyR’s we measured nNOS protein levels in the SCG. Our data demonstrate that nNOS is indeed present in the SCG which is consistent with other studies demonstrating that the SCG contains nNOS neurons in addition to adrenergic neurons (12, 67). Our data demonstrated that with advancing age, nNOS protein expression increases from 6 to 12 months and significantly declines from 12 to 24 months. In addition, as nNOS activity modulates cADPr levels, which in turn modulate RyR activity it may reasonable to speculate that these data may possibly suggest that cADPr levels may also decline with age. As there appear to only be two RyR’s
contributing to calcium release in the SCG, overall, the age-related decline in RyR3, coupled with a decline in nNOS levels may in part provide an explanation for the decrease in RyR mediated calcium release with age noted in our previous study (60). We hypothesize that an age-related reduction in RyR3 receptor levels and cADPr levels may account in part for a decline in the function of RyR’s. We are currently determining if advancing age alters cADPR levels in the SCG, which may shed light on activity of nNOS during the aging process and regulation of RyR’s.

As previously noted the regulation of the RyR’s is very complex and there is not necessarily a consensus on the mechanisms governing the regulation of RyR’s. In addition to the regulatory mechanisms discussed above, we offer some speculative discussion on other processes not evaluated in this study that may also affect the function of the ryanodine receptors, for example, modulation by thiol oxidation (2, 22, 46). It has been reported that oxidizing conditions can affect the calcium sensitivity of the RyR2 and the affinity of the RyR2 for calmodulin (22). The oxidation states and the ability of the RyRs to be regulated by oxidation in the SCG cells has not been evaluated and may be altered in aged SCG compared to younger SCG. In addition, we cannot rule out the possibility that accumulating mutations may arise with natural aging. These mutations may compromise the function of these receptors. It has been shown that the mitochondria play an important role in calcium regulation from buffering of calcium to production of ATP for promoting proper cellular processes to maintain homeostasis (4, 5). It has been reported that defects and mutations of the mitochondrial DNA and genes accumulates with the natural aging process and these mutations may influence calcium regulation in the ER [Reviewed in (4)]. In addition, there is an increase in reactive oxygen species
produced by the mitochondria with advancing age [Reviewed in (4)], which may result in increased DNA or protein damage in the SER. This increase in oxidative stress in the aging brain and neurons may be due to the reported decrease in antioxidant enzyme activities [Reviewed in (4)].

In this study, we have identified the major ryanodine isoforms of the adult rat SCG and molecularly characterized their expression levels and at least one protein modulator, nNOS with advancing age. *ryr1* does not appear to be expressed in the adult SCG suggesting that RyR1 does not contribute to calcium release in the adult SCG. However, RyR2 and RyR3 appear to be the major contributors to calcium release function in the SCG. Advancing age only altered RyR3 levels but to a small extent. Thus, based on results of this study and our previous work, the combination of a decline in RyR3, reduced nNOS levels, reduced function of calcium pumps that fill SER calcium stores, may possibly act in concert to alter modulation of RyR’s in the SCG and in part may play a role in the age-related decline in intracellular calcium release in adult rat SCG. Overall these data may have implications for the protective function that the SCG provides to the cerebrovasculature in the face of age-related elevations in blood pressure.
F. Acknowledgements

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G. Reference


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CHAPTER FOUR

FINAL DISCUSSION/CONCLUSION

The aging process does not always result in the dramatic deterioration in all individuals, hence the coined phrase "aging gracefully". However, there are those individuals who appear to deteriorate rapidly, showing marked changes over the course of their battle with time. The differences that make some people evade the effects of the aging process, and the succumbing of others is not well understood. This study focuses on the dynamics of intracellular calcium regulation and its role in autonomic nerve function.

We have evaluated the effects of aging on the dynamics of intracellular calcium (both refilling and release), and expression of the ryanodine channels in rat SCG. Studies on the effects of aging on intracellular calcium regulation in the SCG may have implications in decreased neuronal plasticity and loss of axons and dendrites during the aging process. The disruption of their connections and function may in return affect innervation, and function to their target organs resulting in organ pathology. Thus, the central hypothesis of this study is aging can alter the mechanisms of intracellular calcium refilling (rapid and spontaneous) and release in rat superior cervical ganglia. Several questions were posed to address the central hypothesis (Figure 4.1) and are addressed in the following specific aims:

1) To measure rapid and spontaneous refilling of caffeine-sensitive ER, intracellular calcium stores in Fura-2 loaded SCG cells, and to determine if there are any changes with age.

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Findings of this study

Clinical implications

The most important and straightforward findings in this study is that the release of calcium from $[\text{Ca}^{2+}]_i$ stores, and both rapid and spontaneous refilling of these stores declines with advancing age in SCG cells. Sustaining the release of calcium from intracellular stores during ongoing activity requires the refilling of these stores following the release. Overall, the data in this study suggest that the ability to sustain release of calcium from intracellular stores declines with age and may have implications as to the function of sympathetic neurons during the aging process. Since refilling of $[\text{Ca}^{2+}]_i$ stores is dependent on both influx and uptake into the SER via SERCA, the decline in refilling may reflect an age related decline in the function of both mechanisms. We have demonstrated in previous studies that SERCA function declines with age in sympathetic neurons (7; 24; 25; 29), and this may be at least one mechanism that could account for the decline in refilling of $[\text{Ca}^{2+}]_i$ stores with age. As adrenergic nerves from the SCG serve to protect the CNS from blood brain barrier disruption (4; 5; 13; 15), these data suggest that the ability of the SCG to sustain its protective function may possibly be altered with advancing age.

Aging and fast refilling of $[\text{Ca}^{2+}]_i$ stores in aging SCG

In this study, there was no age-related difference in the $[\text{Ca}^{2+}]_i$ dynamics between the first and second high $K^+$-evoked $[\text{Ca}^{2+}]_i$ transients (Chapter. 2.6A,B). Although the dynamics of $K^+$-evoked $[\text{Ca}^{2+}]_i$ transients decline with age, they remain constant within each age group. In contrast to $K^+$-evoked $[\text{Ca}^{2+}]_i$ transients, advancing age caused a significant reduction in the $[\text{Ca}^{2+}]_i$ dynamics between the first and second caffeine
exposure within the oldest age groups (Chapter. 2.6C,D), which directly implicates changes in the dynamics of release. There are numerous studies demonstrating the impact of age on depolarization-evoked [Ca\textsuperscript{2+}]\textsubscript{i} transients (3; 25; 26; 29; 34). However, to our knowledge this is the first study demonstrating that the ability of peripheral neurons to sustain release of calcium following rapid depolarization-evoked refilling, declines with age.

Interpretation of the overall age-related decline of K\textsuperscript{+}-evoked [Ca\textsuperscript{2+}]\textsubscript{i} transients is complex (Chapter. 2.4) as depolarization-evoked [Ca\textsuperscript{2+}]\textsubscript{i} transients reflects both influx and release of calcium (30). It does not distinguish between the contributions made to the calcium signal by influx, and release of calcium from intracellular stores. However, it is possible that at least one mechanism that may account for the decline in K\textsuperscript{+}-evoked [Ca\textsuperscript{2+}]\textsubscript{i} dynamics is reduced release.

**Aging and spontaneous refilling of [Ca\textsuperscript{2+}]\textsubscript{i} stores in rat SCG**

In this part of the study, we developed an experimental protocol to study the impact of age on the spontaneous refilling of [Ca\textsuperscript{2+}]\textsubscript{i} stores following caffeine-evoked depletion (Chapter. 2.3). In order to validate that the spontaneous refilling of [Ca\textsuperscript{2+}]\textsubscript{i} stores is independent of VOCC and predominantly mediated by SOCC and SERCA activity, we performed a series of validation experiments (Chapter. 2.7). These control data suggest that in isolated resting SCG cells spontaneous refilling of [Ca\textsuperscript{2+}]\textsubscript{i} stores appears to be mediated by influx of calcium via SOCC and subsequent uptake into the SER via SERCA pumps. These data are consistent with other studies demonstrating that spontaneous refilling of [Ca\textsuperscript{2+}]\textsubscript{i} stores is mediated by SOCC and SERCA function (1; 31).
Molecular characterization of RyRs and modulators

**Identifying mechanisms of aberrant calcium release with age in aging rat SCG**

In light of the observable alteration in rapid and spontaneous refilling in chapter 2, and previous studies demonstrating a decline in SERCA function, it is possible that calcium release can also be altered as a result. In chapter 3 we have molecularly characterized the putative mechanisms contributing to our previous functional studies.

**Aging and genetic expression ryr1, 2 and 3**

It has been demonstrated that the *ryr1* is predominantly expressed in skeletal muscle, *ryr2* is predominantly expressed in cardiac muscle and *ryr3* is expressed in many tissue types, but predominantly in brain and neurons [Reviewed in (11; 12; 18; 19; 23; 27; 28)]. Numerous studies have shown differential genetic expression of *ryr* 1, 2 and 3 isoforms in various neuronal models (9; 16; 21; 22; 32). However, to our knowledge this is the first report of on the genetic expression of *ryr1*, 2 and 3 in the SCG model. In this study, transcriptional analysis of adult rat SCG demonstrated the transcription of both *ryr2* and *ryr3*. However, there was no transcription of *ryr1* detected in the SCG using total RNA concentrations up to 1 microgram, yet it was detectable in substantial quantity in our positive control, the cerebral cortex. This data does not necessarily suggest that RyR1 is not expressed in adult rat SCG, but suggests that it is not the major isoform of the adult rat SCG and may be detected using higher concentrations of RNA.

Transcriptional analysis of the ryanodine receptors in the SCG demonstrates that *ryr2* and *ryr3* are the major *ryr* transcripts present in SCG and their gene products may be responsible for calcium release from the SER stores. The *ryr2* gene expression did not change with age suggesting that *ryr2* levels remain stable during late maturation and
senescent aging. However, mRNA coding for the ryr3 isoform increased from 6 to 12 months and then significantly declined from 12 to 24 months of age. To our knowledge this study is the first to show that there appears to be a late maturational increase in the ryr3 isoform with a subsequent decline with senescence (Figure 4.2). These results do not necessarily differentiate between changes in genetic expression or altered stability of the mRNA in the age groups. Thus, both possibilities may explain the results. Overall, the data suggest that the contribution of ryr3 to the RyR3 protein levels and ultimately calcium signaling in the SCG may change with age while the contributions provided by RyR2 levels remain stable.

Aging and protein levels of RyR2, RyR3 and modulatory nNOS

Changes in genetic expression alone do not necessarily hold a complete explanation for altered RyR protein levels or regulation of the RyR’s. Thus, we measured RyR2 and RyR3 protein levels using an ELISA assay and normalized to the stable marker GAPDH. Following a similar pattern mRNA RyR2 protein levels were not changed in any age groups. The lack of a change in RyR2 protein levels in any age group suggests that this isoform may remain stable and functional throughout the adult life span. On the other hand the turnover rate of the RyR2 in neuronal cells was not evaluated, which may possibly affect protein function. For example in muscular tissue, the turnover rate of the RyR was decreased by 25% in the aged rats in comparison to the younger rats (10). RyR turnover has not been evaluated in SCG and may change with age. Any alterations in turnover rate may have an impact on the RyR2 protein function such as increased oxidation and protein damage in the SCG.
In contrast to RyR2, RyR3 protein levels increased from 6 to 12 months and then declined at 24 months (Figure 4.2). However, the decline at 24 months was not significantly smaller than at 6 months. Thus, a straight-forward conclusion as to functional consequences of this age-related decline is difficult to make. We have shown that caffeine-evoked release of calcium declines with age (33). As caffeine-evoked calcium release offers a measurement of the functional capacity of the RyR sensitive stores, it is difficult to interpret a mechanism based on the alterations in RyR3 protein levels alone. However, the function of the RyR’s also depends on SER calcium filling levels as well as the modulation of the RyR’s. We have shown that SERCA function declines in the SCG (26) and that basal levels of [Ca\(^{2+}\)]\(_i\) also decline with age (33). Thus, filling levels of the SER may also be compromised which may alter the functional capacity of the release mechanism. In addition, protein levels do not necessarily fully correlate with protein function as some may undergo post-translation modification and may require accessory proteins and modulators to regulate their function. It has been reported that RyR function can be influenced by several factors, including phosphorylation, binding proteins, calcium levels, and nNOS which modulates cADPr levels and in turn modulates RyR’s [Reviewed in (2; 6; 8; 14; 20)].
1) RyR2 and RyR3 are the predominant RyR isoforms in rat superior cervical ganglia

2) mRNA transcript of ryr3 increases at 12 months and then declines at 24 months. No alteration in ryr2 message expression was detected with advancing age

3) RyR2 protein expression did not change with advancing age, however RyR3 protein expression changes with age

Figure 4.2. Transcriptional summary of ryr profile in aging SCG
To evaluate if there are changes in selected modulators of the channels, we measured the levels of total phosphorylation. Total phosphorylation of RyR channels was not altered with age (Figure 4.3). These data suggest that changes in steady state phosphorylation and hence alteration in the regulation by this mechanism is not necessarily occurring.

NO synthesized by nNOS has been shown to be implicated in modulating calcium in neurons (8). It is involved in the production of nitric oxide and can be directly regulated by calmodulin, a calcium binding protein. It is part of the signal transduction pathway responsible for cADPr synthesis, which directly affects the activity of the ryanodine receptors and modulates release of calcium from SER stores. We have observed that the addition of nitric oxide donors increased caffeine-induced calcium release in SCG cells. Our data demonstrated that with advancing age, nNOS protein expression increases from 6 to 12 months and significantly declines from 12 to 24 months (Figure 4.3). In addition, as nNOS activity modulates cADPr levels, which in turn modulate RyR activity it may reasonable to speculate that these data may possibly suggest that cADPr levels may also decline with age. As there appear to only be two RyR’s contributing to calcium release in the SCG, overall, the age-related decline in RyR3, coupled with a decline in nNOS levels may in part provide an explanation for the decrease in RyR mediated calcium release with age as noted in our previous study (33).
1) No alteration in phosphorylation levels of total RyRs were detected with advancing age

2) nNOS protein expression increases from 6 month old to 12 month old SCGs, but drastically declines at 24 months.

$P = \text{phosphate groups}$

$\text{RyR3} = \text{RyR3}$

$\text{RyR2} = \text{RyR2}$

$\text{yr}2 = \text{yr}2$

$\text{yr}3 = \text{yr}3$

Figure 4.3. Summary of RyR regulators with advancing age
1. The ability to rapidly refill the caffeine-inducible calcium stores in rat SCG is altered with age.

2. There is an age-related decline in the rate of rise of K+-evoked [Ca^{2+}]i transients.

3. The efficiency of K+ to refill [Ca^{2+}]i stores declines with age.

4. The ability to spontaneously refill the caffeine-inducible calcium stores in rat SCG is altered with age.

5. RyR1 is not the predominantly expressed isoform in adult rat SCG.

6. RyR2 and RyR3 are the predominantly expressed RyR isoforms in adult rat SCG.

7. RyR2 genetic and protein levels remain constant throughout the aging process.

8. RyR3 genetic and protein levels changes with advancing age.

9. nNOS levels which modulate ryr2 and ryr3 in adult rat SCG changes with advancing age.

10. Phosphorylation of the total RyRs does not change with age.

Figure 4.4. Findings of this study (the big picture)
Reference List


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