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

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Vanterpool, Conwin K., Elaine A. Vanterpool, William J. Pearce, and John N. Buchholz. Advancing age alters the expression of the ryanodine receptor 3 isoform in adult rat superior cervical ganglia. J Appl Physiol 101: 392–400, 2006. First published April 27, 2006; doi:10.1152/japplphysiol.00167.2006.—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 protein 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 F344 rats aged 6, 12, and 24 mo. 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 mo and declined from 12 to 24 mo. Similarly, RyR3 receptor protein levels also increased from 6 to 12 mo and declined from 12 to 24 mo. 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 to 12 mo followed by decline from 12 to 24 mo. 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 provide some insight into the possible changes in the function of RyRs that may occur with the normal aging process.

aging; cerebrovasculature

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 both in F344 rats and in humans, suggesting that the cerebrovasculature is subjected to increased pressure and risk of hyperemia and stroke (14, 68). The superior cervical ganglion (SCG) provides sympathetic innervations to the cerebrovasculature and dampens 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 (RyR) 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 RyRs 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 neuronal NO synthase (nNOS)-containing neurons. Indeed our studies and others have shown that the SCG and the cerebrovasculature are 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 smooth endoplasmic reticulum (SER) Ca^{2+} stores after depletion (60). These data suggest that advancing age alters the capacity of SCG cells to release Ca^{2+} from SER stores and that the levels of Ca^{2+}...
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) and 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 central nervous system from rupture of blood vessels at higher systemic pressures. Because 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 RyRs by nNOS with late maturation or advancing age has not previously been studied in the rat SCG model. Because the function of multiple subtypes of RyRs is important to the overall function of numerous excitable cells, investigation of the genetic expression and protein levels of RyR channels in models that have not been previously studied is 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, is altered during late maturation and advancing age in F344 rats aged 6, 12, and 24 mo.

**MATERIALS AND METHODS**

**Experimental animals.** Male Fischer 344 (F344) 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 F344 rats is about 12–24 mo with a mean age of about 18–20 mo at euthanasia. The age range designation comes from other studies showing the median life span in F344 rats is about 12–24 mo. The age range designation comes from other studies showing the median life span in F344 rats is about 12–24 mo. The age range designation comes from other studies showing the median life span in F344 rats is about 12–24 mo. The age range designation comes from other studies showing the median life span in F344 rats is about 12–24 mo.

**SCG isolation and sample preparation.** Rats were anesthetized with CO\(_2\) (45 s) followed by decapitation. Both carotid arteries were dissected from each male F344 rat. SCG were dissected from the carotid artery bifurcation as previously described (60). Tissue was preserved in liquid nitrogen into a fine powder using a metal mortar and pestle, then placed in cold lysis buffer (8.77 g NaCl, 7.88 g KCl, 0.1 M Tris-HCl, 3.29 g EDTA, 5 ml 0.5% Tween-20), and broad-spectrum protease inhibitors and sonicated until homogenous.

**RT-PCR.** The primers used for 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 1 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.

**SDS-PAGE and immunoblot analysis.** This study utilized an ELISA assay along with selective antibodies to determine relative RyR2, RyR3, and nNOS levels in SCG. Therefore, we first validated the selectivity of our antibodies for these respective proteins using 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 RyRs using a modified method (Fig. 3A; Ref. 21). Briefly, SDS-PAGE was performed using a 4–12% Bis-Tris polyacrylamide gel (NuPAGE Novex gels, Invitrogen, Carlsbad, CA) in MOPS-SDS running buffer according to the manufacturer’s instructions (Invitrogen). Samples were prepared (65% sample containing 25 μg of total protein; 25% 3X NuPAGE LDS sample buffer; 10% NuPAGE reducing agent), heated at 72°C for 10 min, and then electrophoresed at 200 V for 120 min in the XCell SureLock Mini-Cell system using the Hi-Mark high molecular weight standards (Invitrogen). The separated proteins were then transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The blot was probed with tyrosine-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 is further validated by Chemicon using RyR2 and RyR3 positive and negative antibodies, and assurance of these controls is provided by Chemicon. This information from Chemicon and our independent analysis together suggest that the RyR2- and RyR3-selective antibodies are reliable for quantification of these isoforms by ELISA assay.

**ELISA.** There are two issues with regard to Western analysis of RyRs. First, RyRs are greater than 500 kDa and because of their high molecular weight may not transfer well. Second, 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 RyRs and nNOS in rat SCG (Fig. 3B). The serial dilutions of total proteins, ranging from 15.6 ng to 2 μg, were incubated in high-binding 96-well plates (Corning, Corning, NY) for 16–24 h at 4°C. The unbound proteins were washed away with PBS-T (138 mM NaCl, 2.7 mM KCl, pH 7.4, 0.1% Tween-20) and

<table>
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<tr>
<th>Primer Sequences</th>
<th>Amplificon Size</th>
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<tr>
<td>RyR1 FWD</td>
<td>5’CCA GGC GAG GAT GAC ATA GAA GC 3’</td>
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<tr>
<td>RyR1 REV</td>
<td>5’GAG GCT CTT GGC GAA ACT TG 3’</td>
</tr>
<tr>
<td>RyR2 FWD</td>
<td>5’ACA CCG TGC TCT ACT CT 3’</td>
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<tr>
<td>RyR2 REV</td>
<td>5’GGG TGA TGC TCT TGG TCT CC 3’</td>
</tr>
<tr>
<td>RyR3 FWD</td>
<td>5’GAA CCG AGA TGT TGC GTC TG 3’</td>
</tr>
<tr>
<td>RyR3 REV</td>
<td>5’GTC CTG CCG ACC AAA AAT G 3’</td>
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<tr>
<td>GAPDH FWD</td>
<td>5’ACC ACA GTC CAT GCC ATC ACT 3’</td>
</tr>
<tr>
<td>GAPDH REV</td>
<td>5’ACT TGG GAT GAG CCG GCC TCT 3’</td>
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Table 1. Primers used in this study

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blocked with 1% BSA for 1 h 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 h at 4°C. The antigen-coated plates were then washed three times with PBS-T and incubated with the horseradish peroxidase-conjugated antibodies for 1 h at 37°C. The unbound antibodies were washed away with PBS-T and then incubated with hydrogen peroxide and 2,2'-azino-di-(3-ethylbenz-thiazoline) sulfonic acid (ABTS) (Zymed, Invitrogen) for 15–20 min. ABTS is oxidized to yield a green chromophore, and absorbance was measured at 405 nm via 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 RyRs were normalized to GAPDH.

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). Briefly, 25 μg determined by Bradford assay (Bio-Rad) of total SCG protein from 6-, 12-, and 24-mo-old animals were used to establish a curve by 1 h at 37°C. The unbound antibodies were washed away with PBS-T and incubated with hydrogen peroxide and 2,2'-azino-di-(3-ethylbenz-thiazoline) sulfonic acid (ABTS) (Zymed, Invitrogen) for 15–20 min. ABTS is oxidized to yield a green chromophore, and absorbance was measured at 405 nm via 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 RyRs were normalized to GAPDH.

RESULTS

Saturation curve for RT-PCR analysis and validation of primer specificity. RT-PCR is a powerful tool and has commonly been used to semiquantitate 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 does 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 10 to 120 ng were used and amplified for 50 cycles; 60–75 ng 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 1). To ensure that the amplified products were the desired products and not nonspecific priming of the oligonucleotides, the amplified products were sequenced by the UC Davis sequencing core laboratory and were shown to be ryr2, ryr3, or GAPDH.

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 have 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-mo, 12-mo, and 24-mo animals. Concentrations RNA up to 1 μg did not yield any amplified fragment consistent with the ryr1 (Fig. 1). Because ryr1 was shown to be present in the brain, we utilized RNA isolated from the cerebral cortex of a 6-mo-old rat as a positive control to ensure that primer conditions were appropriate and primers were specific. The predicted size fragment was amplified from brain RNA, but not from the SCG, confirming that proper primer conditions were used. The ry3 and ry3 were transcribed in rat SCG, demonstrating that these two isoforms are the predominant ryr transcripts in rat SCG cells (Fig. 2, A and C). To ensure that no contaminating DNA was present, the reaction was also performed in the absence of reverse transcriptase (data not shown). No amplified fragment was observed.

Semiquantitative RT-PCR analysis of ry2 normalized to GAPDH from the 6-, 12-, and 24-mo animals showed no significant alteration in the ry2 transcript with advancing age (Fig. 2, A and B). In contrast to ry2, ry3 normalized to GAPDH message significantly increased in the 12-mo compared with the 6-mo rats, followed by a significant decline in 24-mo-old animals (Fig. 2, C and D).

Saturation curve analysis for ELISA, and validation of antibody specificity. Because RyRs are very high molecular weight proteins, quantification by standard Western blotting procedure can be very difficult owing 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 polyvinylidene difluoride membrane, blocked, and then probed with RyR2 and RyR3 specific antibodies. Immunoreactive bands larger than 500 kDa, consistent with the size of the ryanodine receptors, were detected (Fig. 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 that saturation does not occur with the ELISA procedure, a serial dilution of the SCG sample ranging from 15.6 ng to 2 μg of total protein was used; in addition, various antibody dilutions ranging from 1:500 to 1:2,000 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. 3B).

**Protein expression analysis of RyR2, RyR3, and nNOS by ELISA assay and phosphorylation of total RyRs with advancing age.** ELISA quantification showed that RyR2 levels normalized to GAPDH showed no significant differences with advancing age (Fig. 4A). In these studies GAPDH served as a normalization control because there is no change in its levels over the age range 6, 12, and 24 mo (Fig. 4A, inset). In contrast to RyR2, levels of RyR3 normalized to GAPDH, and there was a significant increase in RyR3 levels in the 12-mo-old SCG compared with the SCG of the 6-mo-old animals followed by a significant decrease in the RyR3 levels at 24 mo (Fig. 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. 5). As an added control to ensure detection of phosphorylated RyRs we used a phosphoprotein standard kit containing ovalbumin phosphoprotein, which was positively phosphostained. In contrast, the nonphosphorylated protein controls bovine serum albumin and β-galactosidase did not stain with the phosphostain (data not shown).

We assessed the protein levels of nNOS normalized to GAPDH (Fig. 6, inset) in the SCG over the age range from 6- to 24-mo-old animals. There was a significant increase in the proteins levels of nNOS 6 to 12 mo, followed by a significant decline of nNOS protein levels in 24-mo-old animals (Fig. 6).

**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 Ca\(^{2+}\) with age (60). In that study caffeine-evoked calcium transients increased from 6–12 mo and then declined at 24 mo. Thus we chose these age groups for the present study because we anticipated the greatest changes in the genetic expression and RyR protein levels and modulatory nNOS levels to occur at these ages. In the present study we attempted to illuminate some of the possible molecular mechanisms underlying our previous functional studies. We have molecularly identified

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**Fig. 2. Transcriptional profiling of ryr2 and ryr3 with advancing age.** A and C: 60 ng of DNase-treated total RNA isolated from rat SCG were 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 analyses of the ryr messages were normalized to the GAPDH transcript (A, C). A and B: there is no detectable age-related alteration of ryr2 transcript in adult rat SCG. C and D: ryr3 message expression changes with age in rat SCG. RyR, ryanodine receptor. Data represent means ± SE; n = 3 replicates from 12 pooled ganglia from each age group, performed in 2 independent experiments. Data were analyzed by ANOVA and Fisher’s protected least significant difference (PLSD) test. *Significantly different from 1 other age group, P < 0.02; **significantly different from 2 other age groups, P < 0.02.
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 that ryr1 is present in excitable cells including neurons (reviewed in Refs. 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 RyRs via phosphorylation. However, nNOS has been shown to play a role in modulation of RyR sensitivity to changes in [Ca2+]i levels through modulation of cADPr levels (13, 27). Our data demonstrate the presence of nNOS in the SCG, and late maturation and senescent aging significantly increase then decrease nNOS levels.

The nature of the biphasic alteration in RyR3 and nNOS levels is intriguing and suggests that there are important maturational changes in the function of RyRs followed by a decline during senescent aging. In light of the present study and our published functional data (60), there appears to be an
important increase in the function of RyRs during late maturation that is then followed by a decline in function in the senescent animals. 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 the calcium release mechanism.

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 Refs. 17, 18, 35, 39, 45, 50, 51). Numerous studies have shown differential genetic expression of ryr1, 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 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 mo and then significantly declined from 12 to 24 mo of age. In light of the studies mentioned above, demonstrating that there is differential expression of ryr isoforms in development, it is possible that the differential expression of ryr3 in the SCG observed in this study may play a role in the developmental transition from 6–12 mo and in decreases from 12- to 24-mo-old animals. 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. 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 contribution provided by RyR2 levels remains 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 RyRs. 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 compared with 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 mo and then declined at 24 mo. However, the decline at 24 mo was not significantly smaller than at 6 mo. Thus a straightforward 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). Because 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 RyRs also depends on SER calcium filling levels as well as the modulation of the RyRs. Indeed, we have shown that SERCA function declines in the SCG (47) and that basal levels of [Ca^{2+}] 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 because some may undergo posttranslational 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 RyRs (reviewed in Refs. 3, 11, 13, 20, 40).

To evaluate whether 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 RyRs and was used to determine whether further investigation of phosphorylated RyRs was necessary. 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 are not necessarily changing with age. NO synthesized by nNOS has been shown to be implicated in modulating calcium release in neurons (13). It is involved in the production of NO 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 (8, 15). In addition, we have observed that the addition of NO donors increased caffeine-induced calcium release in SCG cells (unpublished data). Given the importance of nNOS in the function of RyRs, 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 mo and significantly declines from 12 to 24 mo. In addition, because nNOS activity modulates cADPr levels, which in turn modulate RyR activity, it may be reasonable to speculate that these data may possibly suggest that cADPr levels may also decline with age. As there appear to only be two RyRs 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 RyRs. We are currently determining whether advancing age alters cADPr levels in the SCG, which may shed light on activity of nNOS during the aging process and regulation of RyRs.

As previously noted, the regulation of the RyRs is very complex and there is not necessarily a consensus on the mechanisms governing the regulation of RyRs. 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 with 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 accumulate with the natural aging process and these mutations may influence calcium regulation in the SER (reviewed in Ref. 4). In addition, there is an increase in reactive oxygen species produced by the mitochondria with advancing age (reviewed in Ref. 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 Ref. 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. It does not appear that 


11. Danila CI and Hamilton SL. 

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