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Calcium Regulation in Neuronal Function with Advancing Age: Limits of Homeostasis

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1. Introduction

Neurons are specialized excitable cells that transmit information to other neurons or to end effector cells such as skeletal muscle, heart muscle and blood vessel smooth muscle cells, maintaining posture, locomotion and cardiovascular function. Transmission of information from neurons to effector cells usually involves secretion of neurotransmitters contained in clustered pools of synaptic vesicles. This unique anatomical arrangement allows for rapid response to the stimulus of calcium influx and sustained release of neurotransmitters under low and higher frequency conditions (Peirbone *et al*., 1995; Siksou *et al*., 2011).

Calcium is hypothesized to act as a universal second messenger in excitable cells; a large volume of literature spanning more than 30 years supports this idea. Calcium integrates multiple neuronal cell functions including initiation of neurotransmitter release, regulation of gene expression, proliferation, excitability and plasticity, and activation of cell death pathways in apoptosis (Malenka *et al*., 1989; Choi, 1992; Berridge, 1995, 1998; Clapham, 1995; Ginty, 1997; Wuytack *et al*., 2002; Cavazzini *et al*., 2005).

In resting neurons a large electrochemical gradient of approximately 10,000-fold exists between the extracellular environment and the cytosol; this physical arrangement allows for rapid activation of transmitter release via mobilization of release vesicles. In peripheral neurons including sympathetic neurons, calcium signaling begins with rapid opening of voltage-gated L, N and some R type calcium channels allowing calcium to enter the cytosol (Kostyuk, 1989; Kostyuk *et al*., 1993; Trouslard *et al*., 1993; Vanterpool *et al*., 2005). More recently the nomenclature of L, N and R type channels has taken on increased complexity due to definition of differences in their activation voltages, coding genes and hence amino acid sequences of the α_1-subunits that comprise the channel pore (Catterall *et al*., 2005). For

example L and N type channels have been divided into five groups: $Ca_v1.1$. Ca_v 1.2, Ca_v 1.3 and Ca_v 1.4 (L-type) and Ca_v 2.2 (N-type). R type channels, originally termed "residual" because they are resistant to dihidropyridines, which block L-type channels and omega conotoxin, which blocks N-type channels (Catteral *et al*., 2005), are now denoted as Ca_v 2.3. Given the importance of precise neuronal signaling and the need for regulatory mechanisms to sustain efficient signaling, a number of additional cellular mechanisms play a critical role in modulating cellular calcium. These complex calcium regulatory mechanisms are illustrated in figure 1(A). Following the activation of calcium influx through voltage-gated calcium channels, much of the calcium increase is immediately dampened by multiple calcium buffering proteins (Dove *et al*., 2000). Despite this rapid calcium buffering, functional signaling is sustained by rapid release of calcium from smooth endoplasmic reticulum (SER) stores. This process has been termed calcium-induced calcium release (CICR) and is mediated by calcium activation of ryanodine receptor (RyR) channels (Belan *et al*., 1993; Verkhratsky & Shmigol, 1996; Usachev & Thayer, 1997, 1999a,b; Verkhratsky & Petersen, 1998; Akita & Kuba, 2000; Berridge 2002; Behringer et al., 2009[a]).

A variety of additional calcium buffering systems can be activated by a rise in intracellular calcium ($[Ca^{2+}]i$), depending on both the magnitude and duration of the $[Ca^{2+}]i$ transient. Buffering systems include calcium-buffering proteins, energy dependent SER Ca^{2+} ATPases (SERCA), plasmalemmal Ca^{2+}-ATPases (PMCA), mitochondrial Ca^{2+}/H^{+} symporters and Na^{+}/Ca^{2+}-exchangers. All of these calcium buffering systems act to modulate the shape and magnitude of stimulus-evoked $[Ca^{2+}]i$ transients and contribute to restoration of $[Ca^{2+}]i$ levels (Werth & Thayer, 1994; Buchholz *et al*., 1996; Werth*et al*., 1996; Usachev & Thayer, 1999a; Pottorf *et al* ., 2000[a,c], 2002; Wuytack *et al*., 2002). Furthermore, the SERCA function and the CICR process are interdependent, as SERCA pumps not only buffer $[Ca^{2+}]i$ transients but also refill SER calcium stores. This interdependence maintains CICR during repeated neuronal activation (Vanterpool *et al*., 2005). Disruption of any of these mechanisms with advancing age may result in altered function and health of peripheral neurons. This chapter will focus on the impact of advancing age, from young adult to senescence, on the function of adrenergic nerves and the mechanisms by which alterations in function may be related to age-related changes in modulation of $[Ca^{2+}]i$ with ongoing neuronal activity. Data from seminal studies on function of peripheral sensory and central neurons will also be included as important background for understanding the impact of age on adrenergic nerves.

2. Adrenergic neurons arising from the Superior Cervical Ganglion as an important study model

The superior cervical ganglion (SCG) is a peripheral neuroendocrine center, as many tissues receive adrenergic input from the SCG (Cardinali et al., 1981). For example, cardiac output, a function of heart rate and stroke volume, is modulated via the SCG. The SCG relays cardioregulatory signals from the central nervous system (CNS) via the SCG axons terminating in the heart. These nerves release noradrenaline (NA) and increase heart rate and contractile tension through β_1-adrenergic receptors (Wingerd *et al*., 2004). Furthermore, these nerves are implicated in sudden cardiac death following myocardial infarction (MI), as the density of adrenergic innervation increases after MI resulting in severe arrhythmias (Chen *et al*., 2001). Adrenergic nerve density within the SCG is maintained by neuronal

growth factor (NGF); thus this trophic factor ensures proper function of these nerves in target tissues innervated by the SCG. Indeed, there is an age-related decline in NGF; however, the trophic response of aged peripheral adrenergic nerves to added NGF is maintained (Cowen & Gavazzi, 1998; Isaacson & Crutcher, 1998; Dickason & Isaacson, 2002). Loss of NGF during the aging process may have implications for the function of target organs innervated by the SCG. Nevertheless, peripheral neurons can still respond to trophic influences later in life.

Within the rat cerebrovasculature, sensory and adrenergic innervation of blood vessels is complete within the first 30 days of life (Tsai et al., 1989). In addition, adrenergic innervation of cerebral blood vessels influences vascular development and motor function, and the presence of a functional innervation is critical for maintenance of angiogenesis and the modulation of contractile function. For example, in rabbits aged 3–20 weeks, removal of the SCG results in loss of vascular smooth muscle mass, reduced wall thickness and attenuated contractile responses to NA (Bevan & Tsuru, 1981).

Hemorrhagic stroke is a major issue in human health in terms of morbidity, mortality, and health care costs, accounting for about 10% of cerebrovascular disease. Risk factors include low birth weight, premature birth, maternal hypertension and age over 60 years. (Martyn *et. al.,* 1996; Abbott *et. al.,* 2003; Zhang *et. al.,* 2003; Lawes *et. al*., 2004; Hermes-Desantis and Clyman, 2006; Barker 2008). Constriction or dilation of cerebral blood vessels in response to changing pressure is termed autoregulation and serves to maintain cerebral blood flow (CBF) constant over a normal systolic pressure range of 60-120 mmHg (Faraci and Heisted, 1998; Zhang *et al*., 2003). Sympathetic nerves arising from the SCG provide a short-term mechanism to increase the upper limit of autoregulation of cerebral blood flow. When systolic pressures exceed ~140 mmHg, activation of sympathetic nerves is important in activating further constriction of cerebral blood vessels to further protect from forced dilation and rupture (Faraci and Heisted 1998; Furuichi *et al.,* 1999; vanLieshout and Secher, 2008). In humans, acute hypercapnia elevates arterial pressure and CBF, which is attenuated by sympathetic nerve activity (Jordan *et al*., 2000). In lambs with elevated systolic pressures above 140 mmHg or elevated systolic pressure during REM sleep, sympathetic discharge from the SCG significantly increases (Cassaglia *et. al*., 2008, 2009). Thus, increased activity of the SCG in response to elevated blood pressure helps to protect cerebral blood vessels from rupture.

Studies on how neural input regulates cerebrovascular tone and blood flow have underscored the importance of the "neurovascular unit" in brain function. The combination of neuronal input to vascular smooth muscle and the inherent contractile properties of smooth muscle provide for the optimum function of cerebral blood vessels (reviewed by Hamel, 2006). Given that adrenergic nerves arising from the SCG innervate numerous organs including cerebral blood vessels and the importance of the homeostatic modulatory function of blood vessels in the brain, our group has chosen to focus our aging studies on this neuronal model.

3. General overview of calcium regulation in aging neurons

The 20th century brought extraordinary gains in public health, medicine and food production. These gains have resulted in increased life span with a progressive decline in

the ratio of people less than 20 years old to those aged 65 and older (Gems, 2011). Aging is a natural and progressive process and has been suggested to involve a combination of factors including genetic and environmental influences, altered hormonal levels and an inborn aging process. Exquisite studies have shown that the rate of the aging process in animal models can be altered by genetic manipulations resulting in reduction of insulin growth factor 1 signaling and reduced oxidative stress. (Harman, 1998; Guarente & Kenyon, 2000; Clancy *et al*., 2001; Tatar *et al*., 2001; Troen, 2003). More recently androgen replacement has been shown to reverse age-related cognitive declines in male rats (Frye *et al*., 2010). These data suggest that androgenic hormones play a role in modulating neuronal function, and hormone replacement may attenuate and/or restore changes in cognition that occur with advancing age. Caloric restriction in animal models has also been shown to prolong lifespan and reduce age-related morbidity and organ pathology via reduced oxidative stress that accompanies metabolism (Bodkin *et al*., 2003; Forster *et al*., 2003). However, these outstanding studies on caloric restriction, hormone replacement, reduced oxidative stress, and lifespan do not fully explain age-related changes in the function of critical organ and neuronal systems or the vulnerability of particular physiological processes to advancing age.

As discussed above, ionized free calcium is a ubiquitous second messenger in neurons serving as both a charge carrier and chemical intermediate linking physiological stimuli to intracellular effectors (Friel and Chiel, 2008). Subtle age-related declines in mechanisms that modulate stimulation-evoked increases in $[Ca^{2+}]i$ have been hypothesized to contribute to age-related neuronal dysfunction and degeneration; this has became known as the "calcium hypothesis" of neuronal aging (Khachaturian, 1987; Landfield, 1987). Indeed subtle declines in mechanisms that modulate $[Ca^{2+}]i$ levels may be responsible for the elevated resting cytosolic $[Ca^{2+}]i$ that has been seen in several types of peripheral and central neurons in tissue culture, as well as in acutely isolated tissue slices or neurons (Kirischuk & Verkhratsky, 1996; Verkhratsky & Toescu, 1998; Raza *et al*., 2007). One potential consequence of an age-related decline in regulation of intracellular calcium is that calcium overload increases mitochondrial calcium uptake, in turn activating caspases that mediate neuronal cell apoptosis, reducing neuronal survival with age (Ichas & Mazat, 1998; Thibault *et al* ., 1998; Begley *et al* ., 1999; Toescu, and Verkhratsky, 2007).

As the population ages there is an increased incidence of age-related pathology, leading to a tendency to assume a general age-related deterioration in cellular function, including calcium regulatory processes, leading to increased susceptibility to pathology and cell death (Porter *et al*., 1997). However, this common assumption does not take into account that human populations in developed countries are living longer and healthier than at any point within history (Gems, 2011). Thus, with regards to the function of cellular mechanisms that regulate $[Ca^{2+}]i$ homeostasis, a change in the function of one system may actuate compensatory calcium regulatory mechanisms maintaining some degree of neuronal function in senescent neurons or during acute insults such as stroke (Murchinson & Griffith, 1998; Verkhratsky & Toescu, 1998; Lee *et al*., 1999; Griffith *et al*., 2000; Pottorf *et al*., 2000[a], 2002; Buchholz *et al*., 2007). Studying normal aging in the absence of pathology, healthy aging, offers the most promise in trying to understand fundamental aging processes.

4. Aging alters function of adrenergic neurons via multiple mechanisms

There is a correlation between risk of stroke and advancing age, which is associated with an age-related increase in systolic blood pressure (Faraci & Heisted, 1998; Abbott *et al*., 2003;

Zhang *et al*., 2003; Lawes *et al*., 2004). Systolic blood pressure rises with age in both F344-rats and humans; this is correlated with rising plasma catecholamine levels. Thus there may be fundamental age-related changes in the function of peripheral adrenergic nerves, vascular responses to NA and ability to modulate circulating levels of NA (Palmer *et al*., 1978; Esler *et al*., 1981[a,b]; Barnes *et al*., 1982; Yu *et al*., 1985; Insel, 1993). The concept of altered function of peripheral adrenergic nerves with advancing age is supported by our *in vitro* studies showing that stimulation-evoked fractional NA release increases with age in the rat tail and superior mesenteric arteries (Buchholz & Duckles, 1990; Buchholz *et al*., 1998). In addition, this age-related increase in NA release occurs over a wide range of stimulation frequencies from 0.5 - 8 Hz (Tsai *et al*., 1995). An explanation for the observed age-related change in function of vascular adrenergic neurons is complex, involving multiple mechanisms such as changes in density of adrenergic neurons, NA content, re-uptake, function of prejunctional inhibitory α_2-autoreceptors, and calcium regulation (Pottorf *et al*., 2000[b]). In addition, other mechanisms may also modulate the function of adrenergic nerves, and these complicating factors must also be addressed. For example, our studies and others have shown that the SCG (Dun *et al*., 1995; Wu *et al*., 1997) and the cerebral vasculature contain adrenergic and neuronal nitric oxide (nNOS) containing nerves; NO released from nNOS neurons facilitates stimulation-evoked NA release in both blood vessels and the CNS (Montague *et al*., 1994; Yamamoto *et al*., 1997; Zhang *et al*., 1998; Lee *et al*., 2000; Mbaku *et al*., 2000, 2003). Facilitated function of adrenergic nerves via nNOS nerves may occur through enhancement of Ca^{2+} influx and/or Ca^{2+}release from internal stores. These mechanisms may also be altered with age and cannot be discounted in future discussions on the impact of age on function of adrenergic nerves.

4.1 Neurotransmitter content and density of adrenergic nerves with advancing age

In peripheral organs and blood vessels NA content serves as an index of adrenergic density. In the rat heart, NA content decreases with age (Martinez *et al*., 1981; Dawson & Meldrum, 1992), while in blood vessels the story is more opaque. NA content in rat renal, femoral, and saphenous arteries increases with age, while in renal, femoral, and saphenous veins there is no change, and a decline in tail arteries (Handa & Duckles, 1987). Consistent with measurements of NA content in rat arteries, catecholamine histofluorescence, another measure of adrenergic nerve density, increases with age in rat superior mesenteric and renal arteries and portal vein (Mione *et al*., 1988). In contrast spinal cord blood vessels show no age-related change in adrenergic nerve density (Amenta *et al*., 1990). In two studies by the same group sympathetic innervation in the internal carotid artery and anterior cerebral arteries was found to decline with age. Interestingly after intracerebral infusion of NGF, the number of sympathetic axons, NA content and numbers of tyrosine hydroxylase-containing nerve fibers increased in these same arteries from aged animals (Isaacson & Crutcher, 1998; Dickason & Isaacson, 2002). These studies would suggest that there is an innate age-related ability to maintain a critical number of functioning sympathetic neurons. The NGF studies cited above open up possibilities for therapeutic interventions to maintain cardiovascular homeostasis with advancing age. However, there is no clear relationship between age-related changes in adrenergic nerve density, increases in circulating plasma NA levels reported by others and increased stimulation-evoked NA release in our earlier studies; age-related changes in NA content appear to be species or vascular bed dependent.

4.2 Transmitter uptake and function of inhibitory prejunctional α_2-adrenoceptors

NA concentration in the synaptic cleft is modulated by NA re-uptake and activation of prejunctional α_2-adrenceptors. The latter mechanism has been shown to attenuate NA release by decreasing stimulation-evoked calcium influx in adrenergic neurons by reducing the open probability of voltage-gated calcium channels (Schofield, 1990; Delcour & Tsien, 1993). These mechanisms control the moment-to-moment biophase concentrations of NA with ongoing vascular adrenergic nerve activity (Illes, 1986; Langer & Arbilla, 1990; Buchholz *et al*., 1992; Insel, 1993; Esler *et al*., 1995).

Chemical agents such as cocaine and deoxycorticosterone that block the neuronal and extraneuronal uptake of NA, respectively, are used to estimate the function of these systems. Neuronal and extraneuronal uptake of NA has been shown to be reduced with age in the atria and vas deferens in the pithed rat (Borton & Docherty, 1989; De Avellar *et al*., 1990). These studies suggest that NA uptake declines with age in peripheral adrenergic nerves. In contrast, direct measurement of ^{3}H-NA uptake supports the idea that the function of NA uptake transporters does not change with age in peripheral adrenergic nerves (Duckles *et al*., 1985). In other studies in the rat heart (Limas, 1975) and tail artery (Buchholz & Duckles, 1990), the effect of cocaine and deoxycorticosterone on NA uptake increased with advancing age. Reconciling these contrasting studies would appear difficult. However, in the tail artery model, when the measured release of NA in the presence of uptake blockers was corrected for total NA release in the absence of uptake blockers, there was no change with age (Buchholz & Duckles, 1990). Thus, age-related changes in the effectiveness of uptake blockers on NA may merely reflect age-related differences in NA concentrations within the junctional cleft of the model under study. If there is less or more NA released with age in a particular model, then the observed effectiveness of the uptake blockers may only reflect the concentration of NA in the synaptic cleft. Therefore, data in the tail artery model would suggest that NA uptake with advancing age remains as a constant fraction of the amount of NA released.

If the age-related increase in stimulation-evoked fractional NA release in peripheral adrenergic nerves cannot be explained by an age-related change in the function of NA transporters, an explanation must lie elsewhere, such as possible age-related changes in presynaptic inhibition of NA release. There are a number of studies aimed at measuring changes in the feedback function of prejunctional α_2-adrenoceptors. Measurements of NA overflow have shown an age-related decline in the effect of prejunctional α_2 -adrenoceptor antagonists in pithed rats, rat vas deferens, heart, and tail artery (Hyland & Docherty, 1985; Docherty & Hyland, 1986; Daly *et al*., 1989; Buchholz & Duckles, 1990). On the surface these studies seem to offer support for the idea of a general age-related decline in the function of prejunctional α_2-adrenoceptors. However, the issue appears to be more complicated as the studies cited above used only single concentrations of competitive α_2-antagonists. In the rat tail artery, we measured NA overflow over a full concentration range of the competitive α_2-antagonist, idazoxan. We found that the sensitivity of the prejunctional α_2-adrenoceptors to idazoxan declined with advancing age; however, the maximal response to this drug was not altered with advancing age (Buchholz *et al*., 1992). As fractional stimulation-evoked NA release increases with age, there would be more NA in the junctional cleft interacting with the α_2-adrenoceptor and increased competition between higher NA levels and an antagonist. Chemical competition between NA and the α_2-adrenoceptor antagonists may possibly

reduce the apparent sensitivity of the applied antagonist and account for the decrease in the potency of α_2-adrenoceptor antagonists with age (Pottorf *et al.*, 2000b). In light of the studies cited above, age-related changes in NA release are not adequately explained in terms of age-related alterations in the function of NA uptake mechanisms or α_2-adrenoceptor function. Therefore, we looked at other mechanisms that may account for age-related changes in adrenergic nerve activity, including studies of stimulation-evoked NA release with altered extracellular calcium, calcium influx and altered calcium buffering capacity.

4.3 Role of calcium influx in transmitter release and the effect of altering buffering capacity on transmitter release in aged peripheral neurons

Our studies of the rat tail artery cited above showed that stimulation-evoked NA release increased with advancing age. However, altered function of NA uptake or prejunctional α_2-adrenoceptors appears to reflect increased concentrations of NA in the junctional cleft as opposed to real changes in function of these two systems. Thus, our attention was directed toward the possibility that age-related changes in stimulation-evoked NA release are due to changes in calcium regulation in the nerve endings. Calcium entry through voltage-gated calcium channels provides the secretory potential that initiates NA release in sympathetic neurons. Thus we carried out experiments to examine the effects of increased or lowered extracellular calcium, which alters the chemical driving force of calcium, on stimulation-evoked NA release in tail arteries of young and old rats using short or long stimulation protocols (Buchholz *et al.*, 1994). When extracellular calcium was reduced or elevated, stimulation-evoked fractional NA release declined or increased, respectively in tail arteries from both young and old animals. However, in senescent animals the reduction in extracellular calcium reduced stimulation-evoked NA release to a lesser degree while elevated extracellular calcium increased NA release to a greater degree than in the young animals. In addition, at the highest elevated extracellular calcium concentration, 7.5 mM, the increase in stimulation-evoked NA release was maintained in tail arteries from young animals but declined in tail arteries from senescent animals. The explanation for these results may be attributable a variety of possible functional changes, including altered calcium influx, buffering capacity, or sensitivity of the NA release mechanisms to stimulation-evoked increases in $[Ca^{2+}]i$. Indeed higher extracellular calcium significantly elevated NA release by a greater magnitude in old as compared to young tail arteries suggesting that stimulation-evoked $[Ca^{2+}]i$ transients may be greater and more sustained in adrenergic nerves in senescent animals. The significant decline in stimulation-evoked NA release from adrenergic nerves in tail arteries from old animals at the highest extracellular calcium concentrations suggests that older nerves no longer possess the ability to modulate high $[Ca^{2+}]i$ loads which may then become toxic.

Studies on the impact of age on calcium influx are mixed; calcium influx has been reported to increase with age in central neurons (Landfield & Pitler, 1984; Pitler & Landfield, 1990), decrease in peripheral neurons (Kostyuk *et al.*, 1993), or remain unchanged in central neurons (Murchinson & Griffith, 1996). Thus, we measured the impact of age on stimulation-evoked NA release employing three protocols, two of which involved calcium influx through calcium channels and a third where calcium channels were bypassed using the calcium ionophore, ionomycin (Tsai *et al.*, 1997). When we measured NA release from rat-tail arteries via transmural nerve stimulation or depolarization of nerves with high KCl,

NA release was greater in tail arteries from old as compared to young animals. These data could be explained in part by an age-related increase in calcium influx with age. However, when we bypassed voltage-gated calcium channels using the calcium ionophore, ionomycin, we found that the age-related increase in NA release persisted (Tsai *et al*., 1997). In these experiments $[Ca^{2+}]i$ is elevated via an ionophore, so that $[Ca^{2+}]i$ buffering systems would be the primary mechanisms modulating the magnitude and duration of $[Ca^{2+}]i$ transients and hence transmitter release. While these data do not rule out the possibility that stimulation-evoked calcium increases with age in the rat tail artery model, these data also suggest that other mechanisms, such as $[Ca^{2+}]i$ buffering systems and/or the sensitivity of NA release to calcium, may also be altered with age.

We continued to test the hypothesis that an alteration in the function of $[Ca^{2+}]i$ buffering systems may contribute to the increase in NA release from rat tail arteries (Tsai *et al*., 1997). In this set of experiments we measured stimulation-evoked NA release before and after the addition of a membrane permeant, non-endogenous $[Ca^{2+}]i$ chelator, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetomethoxy ester (BAPTA). In the presence of BAPTA, stimulation-evoked NA release was attenuated to a greater extent in old arteries as compared to young, suggesting that age-related changes in NA release are in part due to altered calcium-buffering capacity. Overall, these studies suggest that age-related changes in NA release from peripheral adrenergic neurons appear to be due in part to altered $[Ca^{2+}]i$ handling mechanisms. However, age-related alterations in the sensitivity of NA release mechanisms per se have not been ruled out.

5. Alterations in neuronal calcium buffering and extrusion during aging in peripheral neurons

The magnitude and temporal properties of calcium signals are critical, and sustained high levels of $[Ca^{2+}]i$ can be toxic. Thus, calcium is tightly regulated by channels, pumps, exchangers and protein buffers that control the shape and duration of stimulation-evoked $[Ca^{2+}]i$ transients (Fig. 1A; Friel and Chiel 2008). Age-related changes in the function of any one or a combination of the components of this $[Ca^{2+}]i$ control system may alter the function of neurons and/or contribute to neuronal degeneration (Meldrum & Garthwait, 1990; Peterson, 1992; Verkhratsky *et al*., 1994; Pottorf *et al*., 2000b, 2002). Given the complex interplay between calcium influx and numerous buffering systems, declining function of one or more systems may possibly be compensated for by increased function of other mechanisms (Pottorf *et al*., 2000b). Thus, in aging studies investigators cannot assume that a decline in one or more buffering systems automatically leads to cellular dysfunction, as other systems may be able to compensate for decline in a particular calcium buffering system. In this sense, senescent neurons may still maintain a degree of homeostasis even though the limits of that ability are narrower as compared to younger neurons.

The advent in the last 25 years of cell permeable dyes capable of rapid and reversible binding to calcium as $[Ca^{2+}]i$ levels change has contributed greatly to understanding the dynamics of cellular $[Ca^{2+}]i$ signaling. Quantification of dynamic $[Ca^{2+}]i$ transients is possible because the emission fluorescence intensity of these dyes is altered by reversible binding of calcium (Tsien *et al*., 1985; Roe *et al*., 1990; Thayer & Miller, 1990; Neher, 1995; Baylor and Hollingworth, 2000; Friel and Chiel, 2008). Microfluorometry coupled with

calcium sensitive dyes has allowed investigators to measure the impact of advancing age on the mechanisms that modulate stimulation-evoked $[Ca^{2+}]i$ transients and overall calcium homeostasis (Kirischuk *et al*., 1992; Kirischuk & Verkhratsky, 1996; Neher, 1998; Baylor & Hollingworth, 2000; Pottorf *et al*., 2002).

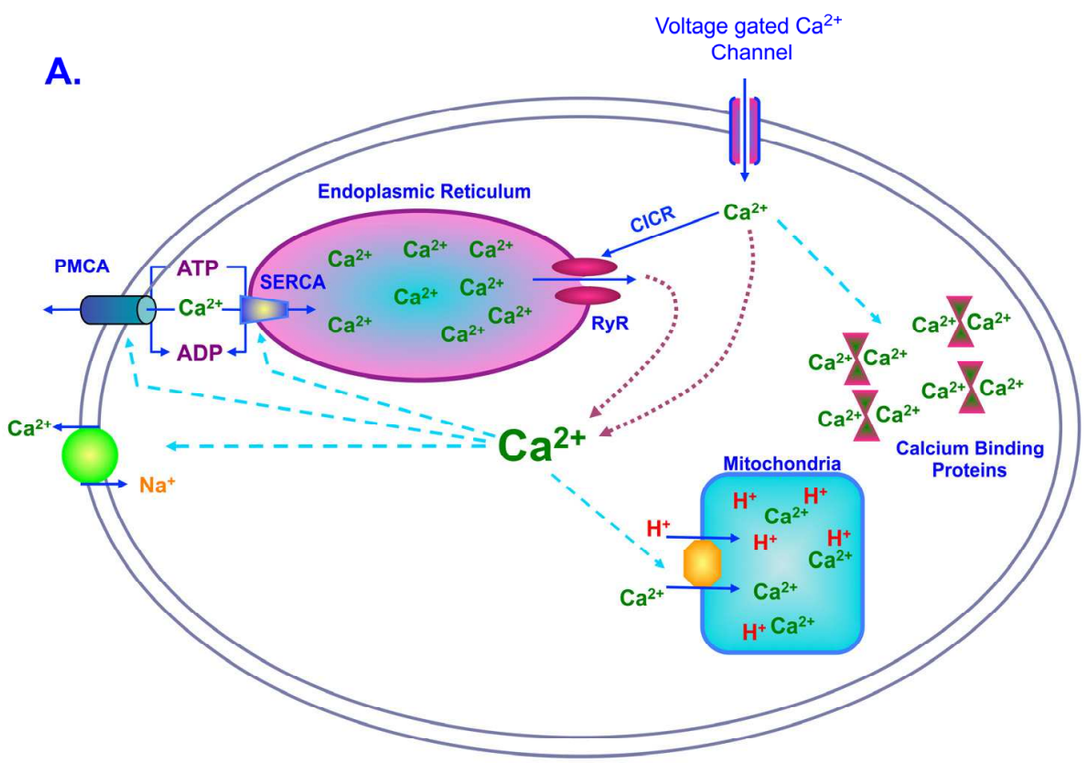

Fig. 1. (A) Representation of mechanisms that modulate stimulation-evoked $[Ca^{2+}]i$ transients in peripheral adrenergic nerves. Depolarization elevates $[Ca^{2+}]i$ via calcium influx through voltage-gated calcium channels. The calcium influx is immediately attenuated via calcium-binding proteins; however, the residual elevated calcium exceeding particular threshold will act on ryanodine receptors (RyR) to evoke "all or none" release of calcium from the endoplasmic reticulum known as calcium-induced calcium release (CICR). The elevation in $[Ca^{2+}]$ is controlled by a dynamic interplay of multiple buffering systems, including: (1) smooth endoplasmic reticulum calcium ATPases (SERCA) sequestration of calcium into the ER that serves to buffer and refill ER calcium stores thus maintaining the ability of the neuron to undergo repetitive CICR; (2) mitochondrial calcium uptake by a H^+/Ca^{2+} uniporter; (3) removal of calcium via plasma membrane calcium ATPase (PMCA) pumps and the Na^+/Ca^{2+} exchanger. Plum dotted lines represent calcium influx and release pathways that elevate $[Ca^{2+}]i$. Light blue dashed lines represent calcium-buffering pathways that control increases in $[Ca^{2+}]i$ and restoration to basal levels.

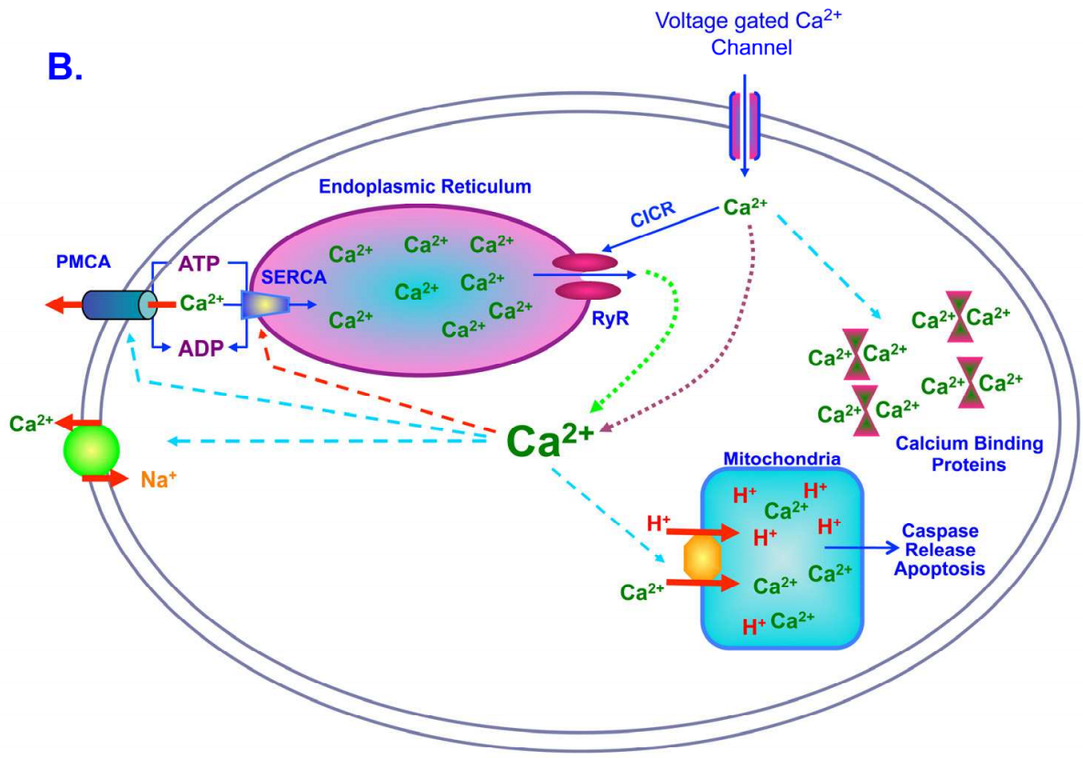

Fig. 1. (B) A model of the current hypothesis that advancing age, in the absence of pathology, results in a subtle decline in the control of [Ca^{2+}]I in peripheral adrenergic neurons. Compensation by other control mechanisms may allow neurons to adapt to an age-related decline in control of [Ca^{2+}]i. This model illustrates the mechanisms that lead to elevated [Ca^{2+}]i in aged peripheral adrenergic neurons. The rise in [Ca^{2+}]i mediated via calcium influx and release from the SER is buffered by SERCA whose function declines with age (broken red line). In response to the decline in SERCA function, mitochondria, PMCA and Na^+/Ca^{2+} exchangers compensate (thick red solid lines) by increasing Ca^{2+} uptake and removal so as to preserve neuronal viability. However, increased mitochondrial calcium uptake may also reduce long-term neuronal survival by activation of caspases leading to apoptosis. Another feature of this model is that the decline in SERCA function in senescent neurons reduces ER Ca^{2+} filling levels and reduces CICR (bright green line).

5.1 Age-related changes in SERCA function in peripheral and central neurons

It has been suggested that an age-related decline in SERCA function contributes to calcium dysregulation in peripheral sympathetic and sensory neurons (Kirischuk *et al.*, 1992; Kirischuk & Verkhratsky, 1996; Pottorf *et al.*, 2002). Such a decline in SERCA function with age may also have additional consequences with regards to calcium signaling in neurons. The SER serves as a source of calcium that can be released by a process called CICR. SERCA buffer [Ca^{2+}]i transients and reload the SER calcium stores with ongoing neuronal activity. Thus an age-related decline in SERCA function may possibly also cause alterations in CICR from the SER (Murchinson & Griffith, 1999; Usachev & Thayer, 1999a; Behringer *et al.*, 2009).

Studies of stimulation-evoked NA release in rat tail artery sympathetic neurons suggested age-related changes in SERCA function (Tsai *et al.*, 1998). When SERCA were blocked with the irreversible or reversible SERCA antagonists, thapsigargin or CPA respectively, stimulation-evoked NA release from sympathetic nerves increased only in tail arteries from young animals with no change in arteries from old animals. These data suggest that in sympathetic neurons from young animals SERCA-mediated calcium buffering modulates depolarization-induced $[Ca^{2+}]i$ transients and hence transmitter release. In contrast, in old animals, SERCA-mediated calcium buffering does not appear to affect transmitter release. To further explore this hypothesis, we measured intracellular calcium in isolated SCG cells and quantified the function of SERCA using reversible or irreversible SERCA blockers, cyclopiazonic acid and thapsigargin, respectively. Each of these agents caused a decline in the rate of recovery of high K^+-evoked $[Ca^{2+}]i$ transients only in cells from young animals with no significant change in old cells (Tsai *et al.*, 1998), supporting our conclusion that SERCA function is lost in sympathetic nerves of old animals.

In a more complex study using SCG cells, we blocked the contribution of PMCA's, mitochondria and the Na^+/Ca^{2+} exchanger and measured $[Ca^{2+}]i$ transients evoked by high K^+. To block PMCA and mitochondrial calcium buffering we utilized low concentrations of vanadate and dinitrophenol (DNP), respectively. To block the Na^+/Ca^{2+} exchanger we replaced Na^+ with the charge carrier tetra-ethyl ammonium. Under these conditions, neurons must rely on SERCA to modulate the rate of recovery of high K^+- evoked transients. Remarkably, with the loss of three calcium buffering systems, SERCA were still able to control the magnitude of $[Ca^{2+}]i$ transients and return elevated $[Ca^{2+}]i$ to resting levels. However, the rate of recovery of high $[Ca^{2+}]i$ transients was significantly slowed in SCG cells from both young and old animals with a significantly greater slowing in SCG cells from old animals (Pottorf *et al.*, 2000c). Thus one important mechanism that can account for a decline in $[Ca^{2+}]i$ homeostasis in aged peripheral neurons is a loss of SERCA function. One possible explanation for a decline in SERCA function is a decline in the genetic expression of SERCA isoforms. Indeed myocardial SERCA-mediated $^{45}Ca^{2+}$ uptake declines with age and there is a decline in genetic expression of SERCA isoforms (Maciel *et al.*, 1990). However, paradoxically, SERCA protein levels remain stable. This study suggests that, even if the genetic expression of SERCA may decline with age, changes in translation of mRNA coding for SERCA or alterations in mRNA stability may be able to compensate for the age-related decline in SERCA gene expression. Alternatively, alterations in mechanisms that modulate on-going SERCA activity, such as phosphorylation, may offer an explanation for the decline in SERCA function (Gafni & Yuh, 1989; Xu & Narayanan, 1998). Indeed, more in-depth studies using a wider range of excitable cell models will be necessary to uncover the mechanisms that account for an age-related alteration in SERCA function.

5.2 Age and mitochondrial calcium buffering

The role of mitochondria in modulation of $[Ca^{2+}]i$ has been an area of intense study. Mitochondria can sequester calcium and act as a buffer using the energy contained in the proton motive force and a Ca^{2+}/H^+ symporter to transport cytosolic calcium into the mitochondria. Early studies suggested that mitochondria only buffer $[Ca^{2+}]i$ transients in excess of 2 μM which may occur only under excessive stimulation or pathological conditions (Nicholls, 1978, 1985). In contrast to this view, however, mitochondrial calcium uptake has

been shown to produce a clear "mitochondrial set point" or shoulder in the shape of depolarization evoked $[Ca^{2+}]i$, transients in central and peripheral sensory neurons (Thayer *et al.*, 1994; Murchison *et al*., 2004). Furthermore, proton motive force disruptors such as dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) demonstrate the role of mitochondria in buffering normal $[Ca^{2+}]i$ transients (Thayer & Miller, 1990; Miller, 1991; Werth and Thayer, 1994; Buchholz *et al*., 1996; Murchinson & Griffith, 1999; Colegrove *et al*., 2000, Murchison *et al*., 2004). Thus, it is clear that mitochondria play a role in shaping stimulation-evoked $[Ca^{2+}]i$ transients in central and peripheral sensory and sympathetic neurons under normal $[Ca^{2+}]i$ concentrations in the mid to high nanomolar ranges. Since mitochondria are an integral part of neuronal cellular calcium buffering mechanisms, either a decrease in mitochondrial calcium uptake or an increased reliance on mitochondria to control $[Ca^{2+}]i$ levels could potentially disrupt mitochondrial function leading to calcium overload and apoptosis.

There are studies in central neurons supporting an age-related decline in mitochondrial calcium uptake (Vitorica & Satrustegui, 1985; Villalba *et al*., 1995; Satrustegui *et al*., 1996). In a more recent study using basal forebrain neurons the addition of CCCP to dissipate the mitochondrial proton motive force significantly elevated both high K^+ and caffeine-evoked $[Ca^{2+}]i$ transients in neurons from 1-7 month-old rats with little effect in neurons from 24-27 month-old (Murchison *et al*., 2004). These studies suggest a general decline in mitochondrial calcium buffering capacity accompanying the process of advancing age in CNS neurons.

However, the picture is quite different in peripheral neurons, such as those from the SCG or adrenergic nerve endings in blood vessels, where we have shown that mitochondrial calcium uptake actively participates in buffering high-K^+ evoked $[Ca^{2+}]i$ transients in neurons from old animals. Our studies suggest that, in peripheral neurons, the function of mitochondrial calcium uptake is preserved with advancing age. We first measured stimulation-evoked NA release from adrenergic nerve endings in tail arteries and blocked mitochondrial calcium uptake with DNP. In this case, stimulation-evoked NA release increased in arteries from old animals with no significant effect in young arteries (Tsai *et al*., 1995). To follow up, SCG neurons were exposed to DNP to block mitochondrial calcium uptake, and $[Ca^{2+}]i$ transients were measured. The peak and rate of rise of high K^+-evoked $[Ca^{2+}]i$ transients were both increased in neurons from old animals with no significant effect in young neurons (Buchholz *et al*., 1996). In another study we blocked PMCA, SERCA, and Na^+/Ca^{2+} exchangers from participating in calcium buffering in SCG neurons. Under these conditions the neurons must rely primarily on mitochondrial calcium uptake to regulate high K^+-evoked $[Ca^{2+}]i$ transients (Pottorf *et al*., 2000a). A remarkable aspect is that, despite compromising multiple calcium modulatory systems, mitochondria were still capable of controlling $[Ca^{2+}]i$ transients in SCG neurons, and mitochondrial calcium uptake appeared to be preserved with advancing age. Overall, our studies in the SCG and adrenergic nerve endings suggest that mitochondrial calcium buffering is maintained with advancing age, and there is an increased tendency with age to rely on mitochondria to control $[Ca^{2+}]i$ transients. This increased reliance on mitochondria to modulate elevated $[Ca^{2+}]i$ may possibly place an added stress on aged neurons. Indeed in experiments using brain slices from mice, mitochondrial depolarization served as an index of mitochondrial calcium uptake in response to high K^+-evoked $[Ca^{2+}]i$ transients. Mitochondrial depolarization was observed in brain slices from both young and old mice; however, in old animals the rate of

mitochondrial repolarization was significantly slower corresponding to slowed recovery of high K^+-evoked $[Ca^{2+}]i$ transients (Xiong *et al*., 2002). Thus, increased reliance on mitochondrial calcium uptake with advancing age has a downside in the case of high neuronal activity in CNS neurons. As mitochondrial repolarization is slowed with advancing age, over time the ability of the mitochondria to sustain its buffering of repeated $[Ca^{2+}]i$ transients may be compromised.

All of the studies cited above are consistent in suggesting that mitochondrial calcium uptake is essential for modulation of $[Ca^{2+}]i$ transients with advancing age. However, as more stress is placed on mitochondria to control stimulation-evoked increases in $[Ca^{2+}]i$, the 'polarization state' which maintains the ability of the mitochondria to produce vital cellular energy and modulate $[Ca^{2+}]i$ transients may subtly decline over the lifespan (Toescu and Verkhratsky, 2007). Overall, with advancing age mitochondrial calcium uptake may become more central to controlling neuronal $[Ca^{2+}]i$ transients. Maintenance of mitochondrial function is critical to healthy aging as altered mitochondrial function is thought to play a role in the progression of age related diseases (Miller, 2005). Maintaining mitochondrial function with improved diet and exercise as humans age may reduce the burden of health-related costs that comes with an aging population (Duckles *et al*., 2006).

5.3 Aging and the potential for PMCA to compensate for age-related declines in SERCA function

The PMCA and Na^+/Ca^{2+} exchange systems are two vital plasma membrane calcium-pumping mechanisms in peripheral and central neurons; each of these plays a role in buffering $[Ca^{2+}]i$ transients via extrusion of calcium from the cytosol. PMCA, like SERCA, requires energy and pumps calcium against the chemical gradient from the cytosol to the extracellular fluid. The Na^+/Ca^{2+} exchanger utilizes energy stored in the Na^+ gradient to pump calcium out of the cellular cytosol (Werth *et al*., 1996; Blaustein & Lederer, 1999; Pottorf & Thayer, 2002; Pottorf *et al*., 2006). A noted feature of the PMCA is that its activity can be elevated by a priming stimulus which induces prolonged elevated $[Ca^{2+}]i$ transients. Following prolonged elevation of $[Ca^{2+}]i$, the PMCA-mediated rate of recovery of depolarization-evoked $[Ca^{2+}]i$ is accelerated, and this elevated function can be retained for up to 40 min after the priming stimulus (Pottorf and Thayer, 2002). These studies suggest that some buffering systems are flexible and can elevate their function during times of high neuronal activity to protect the cell from $[Ca^{2+}]i$ overload. There are at least two mechanisms that can account for increased function of PMCA with sustained $[Ca^{2+}]i$ loads: induction of PMCA gene expression and interaction with calmodulin. Blocking SERCA function with SERCA antagonists combined with prolonged $[Ca^{2+}]i$ transients has been shown to induce PMCA gene expression (Kuo et al., 1997). Furthermore, accelerated function of PMCA with prolonged $[Ca^{2+}]i$ transients increased the interaction of calmodulin with PMCA (Pottorf & Thayer, 2002). Thus redundant calcium buffering components, along with functional alteration of some components mediated by molecular modulators, can compensate for a reduction in function of one or more calcium buffering components as illustrated by the case of prolonged $[Ca^{2+}]i$ transients. Further support for this proposition comes from studies showing that, in the failing myocardium, Na^+/Ca^{2+} exchanger levels increase as SERCA levels decline (Hasenfuss *et al*., 1991).

Our studies of isolated SCG neurons lend direct support to the idea that multiple components of the calcium buffering system can compensate for a loss of SERCA function. As discussed above we found that, with advancing age, SCG neurons become more reliant on mitochondria to control high K^+-evoked $[Ca^{2+}]i$ transients. In a study with isolated SCG neurons, when both SERCA and mitochondrial calcium uptake were blocked with thapsigargin and DNP respectively, PMCA and Na^+/Ca^{2+} exchangers are required to control high K^+-evoked $[Ca^{2+}]i$ transients. In this case SCG neurons from both young and old animals were able to recover from high K^+-evoked $[Ca^{2+}]i$ transients (Tsai *et al*., 1998). These data suggest that, in peripheral adrenergic neurons, plasma membrane calcium extrusion systems can by themselves control $[Ca^{2+}]i$, and their function is maintained with advancing age. In support of this study, we used a unique ATPase antagonist, vanadate, a compound that, at low concentrations (0.25 μM), selectively blocks PMCA function but does not significantly alter SERCA function. Under these conditions, the rate of recovery of $[Ca^{2+}]i$ transients was diminished to a greater extent in SCG neurons from old as compared to young animals (Pottorf *et al*., 2000a). This shows again that PMCA function may be elevated to meet the calcium buffering demands that occur as SERCA function declines with advancing age. Although PMCA function has been reported to decline with age in synaptosomes derived from neurons in the CNS (Qin *et al*., 1998), our studies suggest that during normal aging PMCA function in peripheral adrenergic neurons is maintained with age.

6. Consequences of age-related changes in SERCA function: Aging and SER calcium levels and the role of CICR in neuronal function

Neuronal function depends in part on release of calcium from the SER in response to $[Ca^{2+}]i$ elevation (Usachev & Thayer, 1997, 1999a,b; Behringer *et al*., 2009). Rapid calcium buffers immediately dampen much of the calcium influx through voltage-gated calcium channels while rapid release of calcium from the SER sustains depolarization-evoked increases in $[Ca^{2+}]i$. This process has been termed CICR, is mediated by RyR channels and has been shown to be relevant in modulating release of neurotransmitters and hormones. For example, when RyR are blocked and hence CICR, excitatory or inhibitory post synaptic currents, indices of presynaptic transmitter release, significantly decline (Emptage *et al*., 2001; Galante and Marty, 2003).

A unique feature of the CICR process, the "all or none" release of calcium from the SER, has been observed in peripheral sensory neurons (Usachev and Thayer, 1997). Using a combination of patch clamp and microfluorometry, $[Ca^{2+}]i$ was elevated with graded step depolarizations of varied time length. This method produces small graded elevations in $[Ca^{2+}]i$ until a "threshold" of $[Ca^{2+}]i$ is reached, and the full $[Ca^{2+}]i$ transient due to combined calcium influx and CICR is achieved. As CICR is mediated via the opening of RyR channels, agents such as caffeine, which sensitize RyR to calcium, reduce the threshold of $[Ca^{2+}]$ necessary to evoke CICR (Usachev and Thayer, 1997; reviewed by Usachev and Thayer, 1999a,b). In addition, ryanodine, which blocks RyR channel function can ablate CICR so that stimulation-evoked $[Ca^{2+}]i$ transients reflect calcium influx through calcium channels only (Usachev and Thayer, 1997; Behringer *et al*., 2009).

As CICR is a regenerative process it is sustained during ongoing neuronal activity via refilling of the SER calcium stores through calcium influx and subsequent uptake into the

SER via SERCA pumps (Kostyuk & Verkhratsky, 1994; Verkhratsky *et al*., 1994; Shmigol *et al*., 1996). Therefore, it is clear that calcium pumps such as SERCA serve at least two functions, buffering of elevated $[Ca^{2+}]i$ and refilling $[Ca^{2+}]i$ stores to maintain calcium signaling via CICR. In CNS, SCG, and sensory neurons, SER calcium stores can be rapidly refilled by activation of voltage gated calcium channels or following depletion with caffeine they can spontaneously refill within 3–10 min via activation of store operated calcium channels (SOCC) (Friel & Tsien, 1992; Shmigol *et al*., 1994, 1996; Usachev & Thayer, 1999b; Baba *et al*., 2003; Vanterpool *et al*., 2005).

SOCC channels have received much attention recently, and their function and modulation is an ongoing topic in excitable cell models (Cahalan, 2009). SOCC channels on the plasmalemma mediate a process called "store operated calcium entry." This is a complex process involving calcium-sensing proteins within the SER, STIM1 and STIM2, that activate SOCC. The STIM proteins contain calcium-binding sites, the "EF-hand domain", within the SER lumen and act as "sensors" of SER calcium levels. When SER calcium levels are high, STIM proteins form dimers, but when SER calcium levels fall calcium dissociates from the EF-hand domain, and the STIM proteins form oligomers and aggregate in puncta in the SER. STIM oligomers then interact with SOCC dimers to form SOCC tetramers, which conduct calcium (Cahalan, 2009). Calcium influx through SOCC coupled with SERCA-mediated calcium uptake allows for refilling of SER calcium stores.

Our work with adrenergic nerve endings and isolated SCG cells has consistently shown that SERCA-mediated calcium uptake is compromised in senescent animals. As SERCA function declines with age in the SCG, we studied the impact of advancing age on refilling and release of calcium from the SER. Two protocols were used to measure rapid depolarization-evoked refilling of SER calcium stores and spontaneous refilling following caffeine-evoked depletion of SER calcium stores in isolated Fura-2 loaded SCG neurons from rats aged 6-24 months. Following caffeine-evoked depletion of SER calcium stores, rapid high K+-evoked refilling of these stores markedly declined in SCG cells from 20 and 24 month-old animals but not in 6 and 12 month-old animals. Next we measured spontaneous refilling of SER via the opening of SOCC channels and subsequent SERCA-mediated uptake of calcium into the SER following selective caffeine-evoked depletion of SER calcium stores. To ensure that the rising $[Ca^{2+}]i$ following caffeine-evoked depletion of SER calcium stores was mediated via activated SOCC, we used the SOCC blocker lanthanum which, indeed, abolished the influx of calcium. Spontaneous refilling of SER calcium stores significantly declined in SCG cells from 20 and 24 month-old animals. Overall, these data suggest that a functional consequence of reduced SERCA activity with advancing age is a compromise in the ability of SCG neurons to sustain release of $[Ca^{2+}]i$ during ongoing neuronal activity (Vanterpool *et al*., 2005).

In a more recent series of experiments we tested the hypothesis that reduced refilling of SER calcium stores with advancing age would reduce the contribution of CICR to electrical field stimulation (EFS)-evoked $[Ca^{2+}]i$ transients in isolated SCG cells from 6, 12 and 24 month-old rats (Behringer et al., 2009). We used small, graded increases of applied EFS current at a constant stimulation train length. With this protocol and measuring the peak and rate of rise of $[Ca^{2+}]i$ we were able to estimate the contribution of CICR to EFS-evoked elevations in $[Ca^{2+}]i$. In the presence of ryanodine to block the contribution of CICR, peak and rate of rise

of EFS-evoked [Ca^{2+}]i transients were significantly reduced in SCG cells from both 6 and 12 month-old animals. In contrast, in SCG cells from 24 month-old animals ryanodine had no effect on peak or rate of rise of EFS-evoked [Ca^{2+}]i transients. These data suggest that the contribution of CICR to calcium signaling in senescent SCG neurons is virtually absent, and function of calcium signaling in senescent SCG neurons is maintained only by calcium influx through voltage gated calcium channels (Behringer *et al*., 2009). There are at least two possible mechanisms accounting for this precipitous loss of CICR in senescent neurons. The first is our demonstration of an age-related decline in SERCA function, which would result in depleted SER calcium levels with age. The second mechanism is that the function of RyR channels may also decline with age. Indeed caffeine is a ryanodine agonist and has been used to sensitize CICR to EFS-evoked [Ca^{2+}]i transients (Usachev and Thayer, 1997). Thus, we tested the hypothesis that CICR may still possibly contribute to EFS-evoked [Ca^{2+}]i transients in senescent neurons in the presence of the RyR agonist caffeine. Indeed, caffeine significantly increased EFS-evoked [Ca^{2+}]i transients with short stimulation train lengths in SCG cells from 6, 12 and 24 month-old animals (Behringer *et al*., 2009). These data suggest that although CICR from the SER may not contribute to calcium signaling in senescent SCG neurons, possibly due to a combination of reduced SERCA and RyR function, senescent neurons still retain some capacity for CICR in the presence of a RyR agonist.

7. Altered expression of ryanodine receptors (RyR) and modulators of CICR with advancing age

We have shown that the contribution of CICR to EFS-evoked [Ca^{2+}]i transients is abolished with advancing age in SCG neurons; this may be a consequence of reduced SERCA and/or RyR function (Behringer et al., 2009). In this section we will discuss RyR function in more depth so as to illuminate possible age-related changes. As discussed above, release of calcium from the SER is mediated via RyR ion channels. RyR function is controlled both by gene expression and by secondary molecular modulators, which regulate the sensitivity of RyR to [Ca^{2+}]i levels. For example, regulators of RyR play an important role in other excitable cells such as in the heart, and altered regulation of RyR appears to be a root cause of cardiac arrhythmias that occur in the elderly as well as in other pathological states (Marx and Marks, 2002). Thus, RyR are an important clinical target for therapeutic intervention in cardiovascular challenges faced by aging populations.

Modulators of RyR include FK506 binding protein, phosphorylation and intracellular molecules including cyclic adenosine diphosphate ribose (cADPr). FK506 inhibits the channel when bound to RyR, while phosphorylation of RyR reduces the binding of FK506, activating RyR by increasing its open probability (Danila and Hamilton, 2004). In the case of cADPr, in excitable cells including sympathetic neurons, this modulator can mobilize calcium from SER calcium stores and sensitize the RyR to elevated [Ca^{2+}]i , thus reducing the threshold of elevated [Ca^{2+}]i necessary to evoke CICR (Galione, 1993; Hua *et al*., 1994; Ogawa *et al*., 2000; Hagashida et al., 2001; Marx & Marks, 2002). Furthermore, there are reports that RyR function is influenced by neuronal nitric oxide synthase (nNOS), via nitosylation of RyR by NO itself and modulation of cADPr levels (Galione, 1993; Eu *et al*., 1999; Hagashida *et al*., 2001; Balshaw *et al*., 2002; Meissner, 2002).

Three isoforms of RyR are known; these are RyR1 (skeletal muscle), RyR2 (cardiac muscle and neurons), and RyR3 (neurons and other tissue types). All three have been shown to

participate as calcium release channels responsible for CICR (Ogawa et al., 2000). Because RyR are integral components in $[Ca^{2+}]i$ signaling in SCG neurons, we tested the hypothesis that, with advancing age, expression of the predominant RyR isoform(s) in adult rat SCG, along with selective modulators, is altered (Vanterpool *et al*., 2006). For this study we used F-344 rats aged 6, 12 and 24 months and molecular techniques of reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assays (ELISA). ELISA was used to measure RyR protein levels as these proteins are larger than 500 kDa, limiting the accuracy of Western blots.

Much to our surprise we found that RyR1 mRNA was undetectable in the rat SCG, in direct contrast with other studies demonstrating that RyR1 mRNA is expressed in excitable cells, including neurons (Fill & Copello, 2002). Thus, RyR1 does not appear to play a role in mediating CICR from SER calcium stores in the SCG, regardless of animal age. However, RyR2 and RyR3 mRNA are amply present in rat SCG, appearing to be the major receptor isoforms regulating calcium release from RyR-sensitive stores in all age groups. Furthermore, RyR3 mRNA and protein levels increased from 6 to 12 months and then decreased in senescent (24 month) animals, while RyR2 mRNA and protein levels remained constant with advancing age (Vanterpool *et al*., 2006). From these data showing the impact of age on mRNA and protein levels of RyR3 it is difficult to make a straightforward conclusion. However, as discussed above, protein levels are not the only important measure of RyR function, as activity of these channels is be regulated by several factors.

Therefore, we extended our studies to investigate possible age-related changes in selected modulators of the RyR, including phosphorylation and nNOS levels which are known to modulate levels of cADPr (Vanterpool *et al*., 2006). We found that total phosphorylation of RyR channels was not altered with age, suggesting that steady state phosphorylation of RyR and hence regulation by this mechanism does not necessarily change with advancing age. In contrast, however, nNOS protein levels increased from 6 to 12 months and significantly declined from 12 to 24 months. Since nNOS function modulates cADPr levels a reasonable inference is that cADPr levels may also decline with age. In the SCG, as there appear to be only two RyR contributing to calcium release, it is possible that the decline in RyR3 coupled with a decline in nNOS levels resulting in reduced cADPr may account in part for a decline in RyR function with age.

We have shown that caffeine-evoked release of calcium declines with age suggesting that the capacity of RyR to release calcium from the SER declines with advancing age. In addition, the contribution of CICR to EFS-evoked $[Ca^{2+}]i$ transients is abolished in SCG cells from senescent animals (Vanterpool *et al*., 2005; Behringer et al., 2009). Both agonist-mediated release of calcium from the SER and CICR activated via calcium influx depend on SER calcium filling levels, as well as modulation of RYR. Indeed, we have shown that SERCA function declines in the SCG (Tsai *et al*., 1998; Pottorf *et al*., 2000c). In light of these former studies a reasonable inference is that filling levels of the SER may be compromised, altering both agonist-mediated capacity of calcium release and activation of CICR from the SER.

8. Proposed model for age-related changes in [Ca2+]i regulation in peripheral adrenergic neurons

We have cited various reports and reviews suggesting that advancing age leads to $[Ca^{2+}]i$ dysregulation and neuronal loss (Choi, 1992; Kirischuk & Verkhratsky, 1996; Ichas & Mazat,

1998; Thibault *et al*., 1998; Begley *et al*., 1999; Toescu and Verkhratsky, 2007). However, this hypothesis may be more applicable to pathological conditions as opposed to normal aging. For example, in our studies of peripheral adrenergic neurons, despite age-related changes in $[Ca^{2+}]i$ buffering which in turn alter $[Ca^{2+}]i$ signaling, the cells remain both viable and functional within reasonable limits. Thus, we pose another hypothesis: In the absence of any visible pathology, advancing age leads to subtle changes in the control of $[Ca^{2+}]i$ that may lead to altered neuronal function, but adjustments of other $[Ca^{2+}]i$ control mechanisms may allow neurons to adapt. This model is summarized in Fig. 1(B), emphasizing that loss of SERCA function may be compensated for by increased mitochondrial calcium uptake and plasma membrane calcium extrusion, so as to preserve some degree of cell viability in the face of advancing age. However, increased reliance on mitochondrial calcium buffering may have consequences for long-term viability as increased mitochondrial calcium may increase the chance of mitochondrial dysfunction and release of the apoptotic signals, caspases. This model also suggests that decline with age in SERCA function reduces SER calcium levels, thus reducing the contribution of CICR to overall calcium signaling in aging peripheral neurons.

9. Summary and conclusions

These data from our laboratory and supporting studies from others suggest that, in peripheral autonomic neurons in the absence of recognizable pathology, advancing age is a subtle and complex process that does necessarily lead to dramatic deterioration. Moreover, it is clear that advancing age does not alter the function of excitable neurons in a uniform manner, as we point out differences in the function of peripheral and central neurons with advancing age. With regards to age-related changes in $[Ca^{2+}]i$ regulation, we presented the idea that cell viability of peripheral neurons is maintained as they compensate for an age related decline in the function of at least one calcium-buffering system, SERCA, by increased function of other calcium-buffering systems, namely, the mitochondria and plasmalemmal calcium extrusion. Increased mitochondrial calcium uptake as a compensatory mechanism may leave the cell more susceptible to apoptosis which contributes to cell death (Ichas & Mazat, 1998; Thibault *et al*.,1998; Begley *et al*., 1999; Toescu and Verkhratsky, 2007). This review summarizes the major findings in our work on the dynamics of $[Ca^{2+}]i$ regulation and possible consequences for autonomic nerve function with advancing age.

Based on results of our most current studies and reviews (Vanterpool *et al*., 2005, 2006; Buchholz *et al*., 2007; Behringer *et al*., 2009) and our previous work and that of others we propose the following: With advancing age an alteration in $[Ca^{2+}]i$ signaling and function of peripheral adrenergic neurons results from a complex interplay of mechanisms, including increased sensitivity of the NA release mechanism to calcium, decline in SERCA function that alters calcium buffering and refilling of SER calcium stores, reduced RyR3 and decline in nNOS levels, which in turn modulates cADPr levels. This combination ultimately reduces the capacity of the SER to release calcium and abolishes the CICR process. With respect to the loss of CICR, this apparently leaves calcium influx as the primary mechanism to elevate $[Ca^{2+}]i$ in response to nerve activation, allowing some function to continue in senescent neurons. In addition, despite loss in the contribution of CICR to EFS-evoked $[Ca^{2+}]i$ transients, some residual CICR capacity is still retained if RyR are sensitized to elevated $[Ca^{2+}]i$ with an agonist. This latter observation offers some intriguing clinical possibilities to treat an age-related alteration in calcium signaling and hence peripheral neuronal function.

10. Future directions

Given advances in molecular techniques, including the explosion of knowledge of the function of micro RNA as inhibitors of mRNA translation and hence gene function (Saugstad, 2010), future studies are limitless. These may include investigations on the impact of advancing age on the genetic expression and protein levels of multiple buffering systems, including soluble calcium-buffering proteins, SERCA, mitochondrial calcium pumps, PMCA, and the Na^+/Ca^{2+} exchanger. Our work also strongly suggests that calcium influx through store operated calcium channels is compromised with age. Thus, genetic expression and function with advancing age of store operated channels may provide another fruitful avenue of research. Finally, all of our studies suggest that SER calcium levels decline with age, compromising CICR. New calcium indicators are now available to directly study SER calcium levels; this approach may allow more in depth studies of the impact of advancing age on SER calcium levels. These types of future studies and many more may be expected to provide more direct insight on how CICR may be altered with advancing age, potentially leading to novel therapeutic modalities to prevent and/or treat age- and disease-related alterations in neuronal and cardiovascular function.

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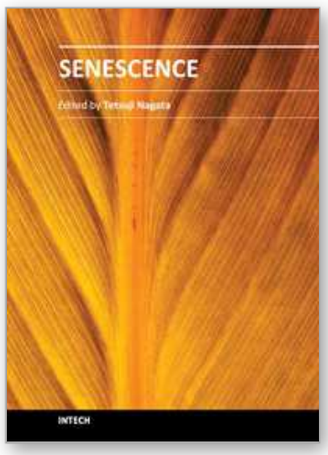

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The book "Senescence" is aimed to describe all the phenomena related to aging and senescence of all forms of life on Earth, i.e. plants, animals and the human beings. The book contains 36 carefully reviewed chapters written by different authors, aiming to describe the aging and senescent changes of living creatures, i.e. plants and animals.

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