Maturation and Long-Term Hypoxia Alters Calcium-Induced Calcium Release in Sheep Cerebrovascular Sympathetic Neurons

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Running Head
Postnatal Maturation and Hypoxia and Modulators of CICR

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The contribution of sympathetic nerves arising from the superior cervical ganglia (SCG) towards the growth and function of cerebral blood vessels is pertinent throughout maturation as well as in response to cardiovascular stress imposed by high-altitude long-term hypoxia (LTH). The function of SCG sympathetic neurons is dependent upon intracellular calcium [Ca$^{2+}$], signaling, strongly influenced by a process known as calcium-induced calcium release (CICR) from the smooth endoplasmic reticulum (SER). In this study, we used the sheep SCG neuronal model to test the hypotheses that maturation decreases CICR and high-altitude LTH depresses CICR in fetal SCG neurons but not in those of adult. We found that the contribution of CICR to electric field stimulation (EFS)-evoked [Ca$^{2+}$], transients is greatest in SCG cells from normoxic fetuses and is abolished by LTH. The decline in CICR is associated with a reduction in SER Ca$^{2+}$-ATPase (SERCA) function in fetal SCG cells during LTH, reducing SER Ca$^{2+}$ levels below the threshold needed for the coupling of calcium influx and CICR. With respect to maturation from the fetus to adult, the decrease in CICR may reflect both a reduction in the levels of ryanodine receptor (RyR) isoforms RyR2 and RyR3 and SERCA function. In response to LTH and in contrast to the fetus, CICR function in adult SCG cells is maintained and may reflect alterations in other mechanisms that modulate the CICR process. As CICR is instrumental in the function of sympathetic neurons within the cerebrovasculature, the loss of this signaling mechanism in the fetus may have consequences for adaptation to LTH in terms of fetal susceptibility to vascular insults.
Introduction

Calcium signaling controls numerous processes including contraction, secretion, and gene expression (30, 36, 38). In superior cervical ganglia (SCG) neurons, calcium signals govern the release of norepinephrine (NE) (7, 42, 48). In turn, NE release plays a critical role in the regulation of blood pressure and cerebral blood flow distribution (9, 17, 24, 54). Thus, the study of intracellular calcium ([Ca^{2+}]_i) dynamics in peripheral and central neurons has become a useful tool in the investigation of many physiological functions including the neurovascular contribution to the function of cerebral blood vessels (7, 47).

In SCG neurons, [Ca^{2+}]_i signaling and neurotransmitter release begins with the opening of voltage-gated calcium channels, whereby much of the influx intensity of the [Ca^{2+}]_i signal is dampened by calcium buffering proteins (35, 52). However, signaling as defined by increases in [Ca^{2+}]_i, is sustained and amplified by calcium-induced calcium release (CICR) through the activation of ryanodine receptors (RyRs) (38, 49, 50, 51). The RyRs are Ca^{2+}-permeable ion channels that can exist as three isoforms (RyR1, RyR2, and RyR3) on the smooth endoplasmic reticulum (SER) (14). In order to sustain repetitive CICR with ongoing neuronal function, the SER calcium stores must be continually refilled. This function is fulfilled by SER Ca^{2+}-ATPases (SERCAs), which simultaneously buffer [Ca^{2+}]_i transients and refill SER Ca^{2+} stores so that CICR can continue (48, 52). Another component in the refilling of SER calcium stores are the plasma membrane storage-operated calcium channels (SOCCs). These channels open in response to the depletion of SER calcium stores and provide for extracellular Ca^{2+} influx (4, 52).

As CICR is a prominent mode of [Ca^{2+}]_i signaling for processes such as release of neurotransmitters, hormones and smooth muscle tone, it is under tight regulation by intracellular modulators acting on the RyRs (18, 45, 51). These modulators include calmodulin, phosphorylation, SER Ca^{2+} levels, neuronal nitric oxide synthase (nNOS), cyclic adenosine diphosphate ribose (cADPr) and the protein modulators FKBP 12 and 12.6 (14, 26, 30, 50, 57).
Given that sympathetic nerves play an important role in cerebrovascular responses when exposed to stressors such as high-altitude long-term hypoxia (LTH), our research group has studied the function of these neurons in fetal and adult sheep cerebral arteries in response to LTH (28). We have shown the function of cerebrovascular sympathetic nerves to be greater in near-term fetuses as compared to adults (5, 39). During adaptation to high-altitude LTH, the capacity of the nerves to release NE declines in fetal but not adult arteries (6). Overall, the mechanism(s) for this alteration in stimulation-evoked NE release are unknown. However, as CICR is critical to neurotransmitter release, LTH may well alter [Ca^{2+}]_{i} signaling (18, 36, 38, 45).

Using the sheep SCG neuronal model, we tested the hypotheses that [1] maturation decreases CICR and [2] high-altitude LTH depresses CICR in fetal SCG neurons but not in those of adult. Furthermore, the observed alterations in CICR may possibly be accounted for by RyR abundance, cellular levels of CICR modulators nNOS and cADPr, and/or changes in the SER calcium filling levels. We carried out this study in isolated SCG cells and SCG tissue homogenates within four treatment groups: fetal normoxic, adult normoxic, fetal LTH, and adult LTH. To determine the contribution of CICR to electrical field stimulation (EFS) evoked [Ca^{2+}]_{i} transients we used fura-2 microfluorometry and the RyR agonist, caffeine, and antagonist, ryanodine. As refilling of SER calcium stores is necessary to maintain CICR and is mediated in part by the function of SERCA, we used the SERCA blocker cyclopiazonic acid (CPA) to estimate the function of SERCA in SCG cells. CICR is, in part, determined by the levels of the RyRs and their endogenous modulators nNOS and cADPr, thus, we also measured their cellular levels in whole SCG homogenates. Understanding the [Ca^{2+}]_{i} dynamics within these SCG neurons with respect to CICR provides insight as to their function and role in the regulation of the maturing cerebral vasculature and the impact of stressors such as LTH on the function of these neurons.
Materials and Methods

All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Loma Linda University. Forty pregnant and forty nonpregnant ewes of mixed breed were obtained from a single supplier (Nebeker Ranch, Lancaster, CA). These groups were randomly assigned to two groups: control normoxic (20 pregnant and 20 nonpregnant) and LTH (20 pregnant and 20 nonpregnant). The animals in the LTH group were transported to the White Mountain Research Station (Bishop, CA; altitude 3,280 m) where they were maintained for ~100 days. Ewes in the normoxic group remained at Nebeker ranch (718 m). Pregnant animals were studied at 138-142 days of gestation. At the appropriate time, LTH animals were transported to the Center for Perinatal Biology at Loma Linda University where they underwent immediate study. To maintain arterial PO$_2$ at ~60 Torr in the LTH animals, tracheal tubes were surgically implanted to allow for the administration of N$_2$ gas (19). Arterial PO$_2$ values were measured in 0.5-ml blood samples from near-term fetuses and adults (ABL300, Radiometer, Copenhagen, Denmark). The mean PO$_2$ for normoxic and LTH adults was 101 ± 2 and 61 ± 3 Torr respectively. The mean PO$_2$ for normoxic and LTH fetuses was 22.4 ± 0.8 and 19.1 ± 0.6 Torr respectively. These measurements indicate a significant decline in the PO$_2$ for both the fetus and the adult with LTH treatment (P<0.01). On the day of tissue harvest, ewes were euthanized by an intravenous injection of pentobarbital sodium (100 mg/kg) and the fetuses were delivered by cesarean section. Fetal weights were not significantly altered (P>0.05) by LTH. The weights were 4187± 183 g and 4148 ± 255 g for normoxic and LTH-treated fetal sheep respectively (n=30 for fetal normoxic and n=25 for fetal LTH).

Superior cervical ganglion preparation. After death, the SCG were dissected by making incisions in the neck along the trachea, tracing superior and posterior to expose the carotid artery, vagal nerve, and the medial angle of the mandible. The vagal nerve was traced superior and posterior towards the medial angle of the mandible to the cervical sympathetic trunk to
expose the ganglion body. Following dissection, the SCG were placed in ice-cold Krebs solution (bubbled with a 95% O₂/ 5% CO₂ gas mixture, pH 7.4) containing (in mM) 118 NaCl, 4.8 KCl, 1.6 CaCl₂, 1.2 KH₂PO₄, 25 NaHCO₃, 1.2 MgSO₄, 0.3 ascorbic acid, and 11.5 glucose. Tissues were then transported to the laboratory within fifteen minutes of dissection to be immediately processed as described below or snap frozen in liquid nitrogen until molecular analysis.

For calcium imaging, the ganglia were acutely dissociated in 5 mL of Earle’s balanced salt solution (EBSS) containing: trypsin (6000 U/mL), collagenase D (1 mg/mL), DNAse-1 type IV (0.1 mg/m), HEPES (20mM), glucose (10mM), NaHCO₃ (10mM) and adjusted to pH 7.4 with NaOH (1M). After incubation at 4°C overnight, the digestion was continued at 34°C for 40 min and was subsequently stopped by the addition of 5 mL of modified Hank’s balanced salt solution (HBSS) with 10% fetal calf serum, 1.3 mM CaCl₂, 5 mM HEPES and adjusted to pH 7.4 with NaOH (1M).

Acutely dissociated cells were centrifuged at 60 g for 5 min and re-suspended in 5 ml of fresh HBSS. Cells were centrifuged again at 60 g for 5 min and re-dispersed in 5 ml of HBSS (containing 10% fetal calf serum, 5 mM HEPES adjusted to pH 7.4 with NaOH (1M)). Cells were centrifuged again and HBSS was decanted to 1 mL. The cells were dispersed in sterile Pasteur pipettes and a 0.5 mL volume of the dispersed cells was placed onto Cell-Tak (3.5 mg/cm²; BD Biosciences, Bedford, MA) coated glass cover slips. The cover slips were modified by attaching an oval 2-cm ring to the surface with Sylgard adhesive (Dow Corning, Inc. MI). To facilitate cell attachment, the cover slips were placed in 35 mm culture dishes, and centrifuged at 60 g for 5 min (Beckman S4180). Measurements of intracellular calcium were completed within 6 hrs.

**Measurement of intracellular calcium.** Our methods for measuring intracellular calcium have been previously described. As we are studying the cumulative effects of LTH the SCG cells are derived from acute dissociation and the data reflect acquisition of global cytosolic 

[Ca²⁺]ᵢ transients from the soma of the SCG neuron (1, 41, 52). Cells were loaded with 10 µM fura-2 acetoxy methyl ester (fura-2/AM) for 20 min at room temperature, then washed with low K⁺
Tyrode's buffer containing (in mM): 138 NaCl, 2 CaCl₂, 1 MgCl₂, 5 KCl, 10 HEPES, an 10 glucose, adjusted to pH 7.4 with NaOH (1M). Incubation was continued for an additional 20 min to allow intracellular esterases to convert the fura-2/AM dye into the free acid form. We addressed the potential problem of an age-related difference in the amount of fura-2 uptake or a difference in the activity of the non-specific esterases that convert fura-2/AM to the free salt which is assessed by monitoring the 510 nm emission fluorescence signal when fura-2 is excited at 380 nm (F₃₈₀) in resting SCG cells in each age and treatment group (37). We have consistently observed equivalent dye loading in SCG cells from all animal treatment groups in this present study (near-term fetus: 75.50 ± 2.18; adult: 76.30 ± 4.46; fetal LTH: 76.36 ± 4.57; adult LTH: 76.71 ± 4.37) as well as in adult rats from previous studies (1, 41, 52). These data suggest that there are no significant differences in dye loading in SCG cells isolated from fetal and adult normoxic and LTH animals. Cover slips were mounted into a superfusion chamber attached to the stage of Nikon inverted microscope (Nikon Instruments, Melville, NY, USA). The microscope was attached to a Universal Imaging System running MetaFluor version 6.2 (Molecular Devices, Sunnyvale CA, USA). The perfusion system allowed the chamber volume (~250 µl) to be exchanged at the rate of two times per second (500 µL/sec). Fura-2 was illuminated by a xenon lamp and the fluorescence was excited alternately at wavelengths 340 and 380 nm by a Lambda DG-4 (Sutter Instruments, Novato, CA, USA). A Photometric Cool Snap 12-bit digital camera (Roper Scientific, AZ, USA) was used to measure the emission fluorescence at 510 nm. Adjusting the microscope stage where no cells were in the field of view corrected for background light levels. Before loading fura-2/AM, cellular autofluorescence was examined in SCG cells as previously described (52). Autofluorescence was below the limits of detection by our imaging system and did not significantly alter our [Ca²⁺]ᵢ measurements. During the experiment, background fluorescence was subtracted, the 340 and 380 nm fluorometric signals were collected and the calcium concentration was calculated and logged to a Dynamic
Data Exchange Excel file every ~300 msec. Ambient light levels were minimized and SCG cells were illuminated only during data acquisition to minimize dye photo bleaching.

Intracellular calcium was estimated by both in vitro and in vivo calibration methods as we have previously reported (1, 41, 52). We used the experimental fluorescent intensity ratios (R) to calculate $[Ca^{2+}]$ over the physiological range by iterative fit to the equation: $[Ca^{2+}] = K_d \frac{[R-R_min][R_{max}-R]}{S_f}$, where $R_{min}$ is the 340/380 ratio at zero $[Ca^{2+}]$ and $R_{max}$ is the 340/380 ratio at 40 mM $[Ca^{2+}]$. The value of the $K_d$ is the dissociation constant of fura-2, while $S_f$ is a correction factor relating the ratio $F_{min}/F_{max}$, which is the emission intensity at 380 nm when fura-2 is in the free ($F_{min}$, 0 nM $[Ca^{2+}]$) or bound ($F_{max}$, 40mM $[Ca^{2+}]$) form (22). We routinely carry out calibrations and have kept a running average of the $R_{min}$, $R_{max}$ $S_f$ and $K_d$ values for Fura-2 for over 10 years. For this report we used multiple calibrations and the $[Ca^{2+}]$ was estimated using averaged in vitro values for $S_f$ (15.60), $R_{min}$ (0.35), $R_{max}$ (3.13), and $K_d$ (266 nM).

Electric-field stimulation apparatus. Electric-field stimulation (EFS) was delivered to isolated cells through platinum electrodes as previously described (1). EFS-evoked $[Ca^{2+}]$ transients were elicited by square wave pulses via a Grass S48 Stimulator (Grass Medical Instruments, Quincy, MA, USA). A 10 ohm resistor was placed in series with the parallel platinum electrodes and the delivered current was monitored with a Tektronix TDS 2024B oscilloscope (Tektronix, Wilsonville, OR, USA). Subsequently, graded stimulation trains from a minimum of 3 to a maximum of 27 pulses at 3Hz were delivered at 300 mA in the presence and absence of either the RyR agonist 5 mM caffeine (Sigma, St. Louis, MO, USA) or 100 μM of the RyR antagonist, 9, 21-dehydro-ryanodine (Calbiochem, La Jolla, CA, USA), to analyze the enhancement and blockade of $Ca^{2+}$ release from SER stores respectively. Given the differential sensitivity between the near-term fetus and adult (i.e. the number of pulses to reach one-half the maximum of the $\Delta[Ca^{2+}]$ response), fetal cells were typically stimulated up to 24 pulses and the adult, 27 pulses. A 3 Hz frequency and graded trains were observed to be physiologically
sufficient with respect to the application of repetitive stimulation and the prevention of the induction of toxic [Ca\textsuperscript{2+}] levels and cellular stress (1, 50, 51). In the case of SERCA blockade, two to three trains of stimulation (50 pulses, 5Hz, 300 mA) were applied in the absence and presence of 10 µM cyclopiazonic acid (Sigma, St. Louis, MO, USA).

**EFS protocol 1:** Progressive pulse stimulation at maximal current in the absence and presence of 100µM ryanodine (Figure 1A). At relatively low (nM) concentrations, ryanodine activates the channel, locking it into an open low conductance state while high (µM to mM) concentrations block the channel (14). We initially performed dose-response experiments and found that a 100 µM concentration resulted in maximal RyR blockade in both fetal and adult SCG cells. A priming supermaximal stimulus (50 pulses, 10 sec, 5 Hz, 300 mA) was delivered to cells to load SER calcium stores and to ensure uniform SER loading from cell to cell (33). Only cells that responded and recovered from this stimulus were considered for further stimulation. A two-minute equilibration separated each stimulation train. During the last control supermaximal stimulation, Tyrode’s buffer containing 100 µM ryanodine was rapidly superfused onto the cells followed by a 30 min incubation period. This insured the RyRs were fully blocked as ryanodine has greater efficacy when the RyR is activated (36). EFS was repeated again after the drug was applied. Note in Figure 1A that the addition of 5 mM caffeine after the last EFS [Ca\textsuperscript{2+}] transient in the presence of ryanodine did not evoke any further rises in [Ca\textsuperscript{2+}]. These data establish that this protocol blocks RyR activity.

**EFS Protocol 2:** Progressive pulse stimulation at maximal current in the presence and absence of 5 mM caffeine (Figure 1B). This protocol was developed on the basis that caffeine can enhance the contribution of CICR to EFS-evoked [Ca\textsuperscript{2+}] transients (50, 51). After the last train (~24 pulses), Tyrode’s buffer containing 5 mM caffeine was rapidly superfused onto the cells. The application of caffeine caused a rapid increase of [Ca\textsuperscript{2+}], due to release from [Ca\textsuperscript{2+}] stores, which declined to baseline. These data are consistent with our previous study (1) and with data obtained from dorsal root ganglia (DRG) cells (50). The SCG cells were then allowed
to incubate in the presence of caffeine for 10 min to sensitize RyR receptors and to allow
SERCA to refill the SER calcium stores. While still in the presence of 5 mM caffeine, the cells
were exposed to the series of pulses (3-24 pulses, 3 Hz) again.

EFS protocol 3: Measurement of SERCA function using the SERCA antagonist
cyclopiazonic acid (CPA, Figure 6). In this protocol two to three EFS trains (5 Hz, 50 pulses,
300 mA) were applied followed by introduction of 10 µM CPA to the bathing solution. The initial
increase in [Ca^{2+}]_{i} induced by the presence of CPA demonstrates that SERCA were blocked,
and that the SER were depleted of Ca^{2+}. This is consistent with our previous data in rodent
SCG cells (48). The [Ca^{2+}]_{i} transient that resulted from SERCA blockade was allowed to return
back to baseline [Ca^{2+}]_{i} before EFS was repeated. As the recovery rate constant is in part
determined by SERCA function, we analyzed the first order recovery rate constant (τ) in the
absence and presence of CPA using Origin 6.1 software. The difference in τ in the absence and
presence of the SERCA antagonist provides a surrogate measure for SERCA function.

Validation of EFS protocol. In a previous study, we demonstrated that EFS-evoked
increases in [Ca^{2+}]_{i} are lost if calcium influx is blocked with 100 µM La^{3+} (1). Similarly, in this
study we used the same control protocol whereby EFS-evoked increases in [Ca^{2+}]_{i} were lost
when calcium influx was blocked with 100 µM La^{3+}, (data not shown). These data from the
former and present study suggest that calcium influx is necessary to activate CICR and that
release of Ca^{2+} from the SER is not occurring by some other mechanism.

ELISA assays for RyR1, RyR2, RyR3 and nNOS. We have developed a very selective
and sensitive ELISA assay used in this study to quantify the relative levels of RyR1, RyR2,
RyR3 and nNOS in the SCG (53). We validated the selectivity of our antibodies for the RyR
subtypes and nNOS using western analysis to insure that under appropriate conditions the
antibodies would yield single bands (32, 53). The ELISA has advantages over traditional
western analysis especially when attempting to quantify proteins with relatively high molecular
weights. RyRs have a molecular weight of greater than 500 kDa and thus, do not transfer well to blotting membranes and incomplete transfer will affect the quantification of RyR protein levels (21).

To isolate cell protein, SCG were snap frozen in liquid nitrogen and pulverized to a fine powder using a metal mortar and pestle, then placed in 200 µL ice cold lysis buffer containing (in mM) 107 NaCl, 50 Tris-HCl, 10 EDTA, 0.5% Tween-20. 1 mL of a protease inhibitor cocktail (10 mL cocktail stock containing bestatin, E-64, leupeptin, aprotinin; Sigma, St. Louis MO) was added to the lysis buffer to inhibit degradation of proteins of interest. In all protocols, 10 µL of the sample was removed for protein analysis and proteins solubilized in 25 µL 1N NaOH. A bicinchoninic acid protein assay kit and bovine serum albumin (BSA) standards obtained from Pierce (Rockford, IL), was used to quantify total protein content in the SCG (46).

ELISA analysis of RyRs and nNOS was performed as previously developed and validated by our laboratory (53). Briefly, 500 ng (RyR assays) or 250 ng (nNOS assays) of protein were placed in high affinity 96-well binding plates (Corning, Corning NY) for 16-24 hrs at 4°C. Unbound proteins were washed away in PBS-T (mM) 138 NaCl, 2.7 KCl, pH 7.4, 0.1% Tween-20, Sigma, St. Louis MO). Proteins were blocked for 1 hr at room temperature using 1% BSA in physiological buffered saline (PBS, Sigma, St Louis MO). Antigen coated plates were then incubated for 16 hr, 4°C, with selective primary antibodies (1:500 dilution) for RyR1, RyR2 and RyR3 (Chemicon, Temecula CA) or nNOS (Biomol, Plymouth Meeting PA). Plates were washed three times with PBS-T and incubated for 1 hr, 37°C with horseradish peroxidase (HRP) conjugated secondary antibodies (Zymed, San Francisco CA, 1:2000 dilution). The unbound antibodies were washed away three times with PBS-T and plates were incubated in a mixture of hydrogen peroxide and 2,2'-azino-di-(ethylbenz-thiazoline) sulfonic acid (ABTS, Zymed) for 15-20 min. ABTS is oxidized in the reaction yielding a green chromophore, and the absorbance was measured at 405 nm via a microplate reader. To determine relative levels of RyRs the
absorbance at 405 nm was normalized to GAPDH levels also determined by ELISA assay (53; Fig. 5 inset).

The use of GAPDH as a normalizing protein has been a source of controversy in hypoxic studies. The gene promoter of GAPDH has been reported to have an inducible hypoxic responsive element using prostate adenocarcinoma cell line models with acute hypoxic exposure (48hr) (29). However, another report using human glioblastoma indicated no alteration in GAPDH expression with acute hypoxic exposure (48hr) and concluded that GAPDH was an ideal “housekeeping gene” for their study (43). Although our particular study employed a chronic hypoxic approach, we found no significant alteration in the expression of the GAPDH protein with development or LTH.

The abundance of nNOS was determined using a modification of our previously published method (32). A purified recombinant nNOS standard (2-15 ng) (Zymed) was added to the high affinity binding plates and ELISA assay performed in the same manner as with the samples in all experiments. This method yields a linear relation (r=0.99; data not shown) which allows absorbance to be converted to mass of nNOS in each sample.

Tissue preparation for total DNA measurements. In this study, cADPr measurements were normalized to total DNA content as the quantity of soluble protein is variable with development and chronic hypoxia (27). The DNA assay has been previously described and this technique is packaged into DNeasy tissue kits from Qiagen (Qiagen, Valencia CA) (56). Briefly 10-15 mg of snap frozen pulverized SCG were placed in a 1.5 mL microcentrifuge tube lysed and treated with RNAse. DNA was isolated using DNeasy minispin columns and pre-made buffers according to the manufactures instructions. The integrity of the DNA was determined by the 260 nm/280 nm ratio and samples yielding a ratio of 1.8 - 2.0 indicate high purity DNA.

Fluorimetric assay for cADPr. Quantification of tissue levels of cADPr, a modulator of the CICR process was done by a previously developed fluorometric assay (20). This cycling assay works on the principle that ribosyl cyclase will work in reverse in the presence of excess
nicotinamide, which then converts all tissue cADPr to NAD. NAD is consumed in a cycling assay by alcohol dehydrogenase, reducing NAD to NADH, which is subsequently utilized by diaphorase to reduce resazurin to resorufin (20). The final product resorufin fluoresces at 590 nm when activated at 544 nm. Briefly, snap frozen SCG (1 SCG per assay for adult, 2 SCG per assay for fetuses) were pulverized and placed in 500 µL of 0.6 M perchloric acid (PCA), homogenized, sonicated and extracted with 500 µL of 3:1 chloroform and tri-n-octylamine. The samples were centrifuged for 10 min at 1500 g and the aqueous top layer containing cADPr was decanted. Samples were adjusted to pH 8.0 with 20 mM sodium phosphate buffer. The samples were then treated with an enzyme solution to remove contaminating nucleotides. This buffer solution contained 0.44 units/mL nucleotide pyrophosphatase, 12.5 units/mL alkaline phosphatase, 0.0625 units/ml NADase, 2.5 mM MgCl$_2$, 20 mM sodium phosphate, pH 8.0. Samples were incubated for 3 hrs at 37°C and then centrifuged at 3000 g for 30 min in Centricon-3 tubes (Bedford, MA). Next, 100 µL of the samples were added to 96 well microliter plates and then 50 µL of ribosyl cyclase solution (containing 0.3 µg/mL purified ribosyl cyclase and 30 mM nicotinamide) was added and samples were incubated for 15 min at room temperature to convert cADPr to NAD. Following the conversion of cADPr to NAD, 100 µL of cycling reagent (containing 0.1 mg/ml BSA, 10 mM nicotinamide, 100 µg/mL alcohol dehydrogenase, 2% ethanol, 10 µg/mL diaphorase, 10 µM flavin mononucleotide, resazurin 5 µg/mL) was added to each sample and incubated for 4 hrs at room temperature. Plates were placed on a Biotec FLX800 fluorometer and illuminated at 544 nm with emitted light recorded at 590 nm. A series of pure cADPr standards (0.2 to 100 nM) were prepared and treated with ribosyl cyclase and cycling reagent solutions in the same manner as the samples. The 590 nm intensity versus concentration of cADPr standards yields a linear relation, which was used to convert emission intensity at 590 nm in each sample to cADPr concentration ($r=0.99$; data not shown). The lower limit of detection in this assay is approximately 0.5 nM. All assays in this study were run in triplicate and the standard error was less than 3% of the mean for each triplicate.
Data analysis. All calcium transients were analyzed by using customized algorithms in Origin 6.1 (Origin Lab Inc, Northampton, MA). Peak $[Ca^{2+}]_i$, ($\Delta[Ca^{2+}]_i$), was determined by subtracting basal $[Ca^{2+}]_i$ from maximum stimulation-evoked $[Ca^{2+}]_i$ for all transients. Plots of pulse number versus percentage of maximal $\Delta[Ca^{2+}]_i$ were generated and fit by a Boltzmann sigmoid function using Origin 6.1. The $\tau$ constant for recovery was obtained for all $[Ca^{2+}]_i$ transients as a first-order exponential decay fit from the peak $[Ca^{2+}]_i$ to basal $[Ca^{2+}]_i$ using Origin 6.1.

Statistics. The impact of development and hypoxia on all parameters was determined by ANOVA and Fisher-protected least significant differences (PLSD) for comparative analysis between treatment groups. Within-groups analysis for $[Ca^{2+}]_i$ parameters before and after drug treatments in the single cell experiments were compared using a Student’s paired $t$-test. Statistical analysis was done using StatView 5.0 software (Abacus Concepts, Berkeley, CA).
RESULTS

Impact of maturation and LTH on the contribution of CICR to EFS-evoked $[Ca^{2+}]_i$ transients in single SCG neurons. To measure the contribution of CICR to EFS-evoked $[Ca^{2+}]_i$ transients we used the RyR antagonist, ryanodine, to block CICR and a RyR agonist, caffeine, to sensitize CICR to EFS-evoked increases in $[Ca^{2+}]_i$. Figure 1A is representative of EFS-evoked $Ca^{2+}$ transients for a single SCG cell from a normoxic fetus in the absence and presence of ryanodine. Ryanodine clearly decreases the magnitude of EFS-evoked $[Ca^{2+}]_i$ transients. Figure 1B is a representative $[Ca^{2+}]_i$ recording of a SCG cell from a normoxic fetus in the absence and presence of caffeine. When cells were exposed to 5 mM caffeine there was a clear caffeine-evoked $[Ca^{2+}]_i$ transient which returns to baseline. This effect is consistent with previous studies in isolated sensory and sympathetic neurons (1, 50). While in the continued presence of caffeine, EFS-evoked $[Ca^{2+}]_i$ transients clearly increase as compared to the controls.

Figure 2 summarizes the impact of maturation and LTH on the contribution of CICR to EFS-evoked $[Ca^{2+}]_i$ transients in isolated SCG cells. In SCG cells from normoxic fetuses, (Fig. 2A) ryanodine significantly decreased EFS-evoked $[Ca^{2+}]_i$ transients, from 6 to 24 pulses. In SCG cells from normoxic adults, ryanodine also decreased the efficacy of EFS to evoke $[Ca^{2+}]_i$ transients, but in contrast to the normoxic fetus, over a higher range of 21 - 27 pulses (Fig. 2B). Thus, development from the near-term fetus to the adult appears to decrease the CICR mechanism in ovine SCG neurons.

In comparison to SCG cells from the normoxic fetus, in cells from LTH fetuses, ryanodine fails to decrease EFS-evoked $[Ca^{2+}]_i$ transients over the entire stimulation range (Fig. 2C). However, in SCG cells from LTH adults ryanodine decreases EFS-evoked $[Ca^{2+}]_i$ transients beginning at 15 pulses (Fig. 2D), as compared to 21 pulses in the normoxic group (Fig. 2B). Overall, the impact of LTH treatment on the CICR is as follows: [1] a decrease in
CICR in SCG neurons from the fetus and [2] maintained CICR in SCG neurons in the adult as compared to their respective normoxic counterparts.

Caffeine has been noted to sensitize CICR to EFS in sensory and sympathetic neurons (1, 50). Thus, we tested the hypothesis that the application of caffeine may reclaim some CICR in fetal LTH SCG cells. The application of caffeine significantly enhanced EFS-evoked \([Ca^{2+}]\) transients at all stimulation trains in normoxic fetal and adult SCG cells and in SCG cells from LTH adults (Fig. 3 A,B,D). In stark contrast, caffeine failed to enhance EFS-evoked \([Ca^{2+}]\) transients in SCG cells from LTH near-term fetuses (Fig. 3C).

**Effect of maturation and LTH on maximal EFS-evoked \([Ca^{2+}]\) transients in the absence and presence of ryanodine and on caffeine-evoked \([Ca^{2+}]\) transients.** We compared the magnitude (\(\Delta[Ca^{2+}]\)) of maximal EFS-evoked \([Ca^{2+}]\) transients at 24 pulses between all four treatment groups (Fig. 4A). Maximal control EFS-evoked \([Ca^{2+}]\) transients from all cells that preceded either caffeine or ryanodine treatment were greatest in SCG cells from normoxic near-term fetuses and they significantly declined with postnatal maturation from fetus to adult. Furthermore, after acclimatization to LTH, maximal EFS-evoked \([Ca^{2+}]\) transients significantly declined in fetal SCG cells but not in adult.

Maximal EFS-evoked \([Ca^{2+}]\) transients are due to the combined influence of extracellular calcium influx and CICR (15). When the contribution of CICR to EFS-evoked \([Ca^{2+}]\) is blocked with ryanodine, the remaining \([Ca^{2+}]\) transient reflects calcium influx, which we presume to be mainly due to the activation of voltage-gated calcium channels. In the presence of ryanodine, maximal EFS-evoked \([Ca^{2+}]\) transients were not significantly different in SCG cells from all four treatment groups (Fig. 4B).

Caffeine (5 mM) application induces a global \([Ca^{2+}]\) transient due to uniform activation of all RyRs in the cell, and thus this transient reflects the capacity of SER to release calcium (14, 52). The \([Ca^{2+}]\) transient evoked by the application of 5 mM caffeine in SCG cells from all four treatment groups is summarized in Figure 4C. Caffeine-evoked \([Ca^{2+}]\) transients in SCG cells
from near-term fetuses tended to be greater as compared to SCG cells from normoxic adult. In addition, caffeine-evoked [Ca\textsuperscript{2+}]\textsubscript{i} transients in SCG cells from LTH fetuses were similar in magnitude to those of adult SCG cells, and significantly less than their normoxic counterparts. In SCG cells from adult animals, LTH did not alter the magnitude of caffeine-evoked [Ca\textsuperscript{2+}]\textsubscript{i} transients.

**Impact of maturation and LTH on cellular levels of RyRs.** RyRs are the channels that mediate CICR, a process that functionally alters with LTH and maturation. Therefore, we quantified relative RyR levels in SCG from each of the treatment groups (Fig. 5). RyR levels were normalized to GAPDH levels in each ELISA assay. GAPDH protein levels in SCG from normoxic and LTH fetuses and adults were virtually identical, which validates the normalization of relative RyR isoform levels to this marker (Fig. 5, inset). RyR1 was the dominant isoform in normoxic and LTH near-term fetal and adult SCG and LTH did not alter the levels of RyR1 in either group. RyR2 and RyR3 levels significantly declined with maturation from near term fetus to adult and LTH did not alter the levels of these receptors in either group.

**Impact of maturation and LTH on cellular levels of nNOS and cADPr.** Our studies and others have shown that the SCG as well as the peripheral and cerebral vasculature contains adrenergic and nNOS containing nerves. In addition, nNOS neurons augment the function of adrenergic neurons and these effects may be mediated through modulation of cADPr levels (10, 12, 31, 32, 53). Table 1 summarizes the impact of development and LTH on nNOS and cADPr levels normalized to cellular DNA levels. The abundance of nNOS in normoxic and LTH adult SCG was significantly greater than in the fetal normoxic and LTH SCG. Acclimatization to high-altitude LTH did not alter nNOS abundance in adult SCG. However, LTH significantly increased the abundance of nNOS in fetal SCG.

nNOS modulates the activity of ribosyl cyclase which synthesizes cADPr, which in turn modulates the sensitivity of RyR's to elevations in [Ca\textsuperscript{2+}]\textsubscript{i} (12, 26). Therefore, we quantified the impact of development and LTH on tissue levels of cADPr. The tissue levels of cADPr...
normalized to total DNA content were not significantly different in fetal or adult normoxic or LTH SCG (Table 1). Furthermore, DNA content per mass of tissue was not significantly different in SCG from any of the study groups (Table 1).

**Impact of maturation and LTH on SERCA function in SCG cells.** In order to maintain repetitive CICR during normal neuronal function, the SER calcium stores must be refilled. The refilling of the SER calcium stores is in part maintained by the function of SERCA. SERCA function can be assessed by the application of SERCA antagonists, which block the uptake of calcium into the SER (38, 48). Two key features of SERCA antagonists allow for this type of surrogate measure; First, there is a small transient reflecting a progressive release of calcium from the SER and second, there is a prolonged rate of recovery of EFS-evoked [Ca$^{2+}$]$_i$ transients as reflected by a broadening of the EFS-evoked [Ca$^{2+}$]$_i$ transient and an increase in first order recovery time constant, $\tau$. Thus, by measuring $\tau$ we evaluated the role of SERCA during EFS-evoked (5 Hz, 50 pulses, 300 mA) [Ca$^{2+}$]$_i$ transients before and after the application of a SERCA blocker, cyclopiazonic acid (CPA, 10 $\mu$M) in SCG cells from each treatment group (Fig. 6). Figure 6A, B are representative EFS-evoked [Ca$^{2+}$]$_i$ transients in SCG cells from a fetal normoxic and fetal LTH animal respectively. In the fetal normoxic SCG cell, CPA application led to a distinct broadening of the EFS-evoked [Ca$^{2+}$]$_i$ transient (Fig. 6A), an effect that was compromised in an SCG cell from a LTH fetus (Fig. 6B).

Figure 7 summarizes the data obtained from the protocol as shown in Figure 6. In the presence of CPA, there is a significant increase in $\tau$ in all four treatment groups (Fig. 7A). However, this increase is greatest in SCG cells from normoxic near-term fetuses. The increase in the $\tau$ constant declines with maturation from normoxic near-term fetus to normoxic adult. Furthermore, during LTH the $\tau$ constant declines in fetal SCG cells only.
Discussion

The ovine model and high-altitude LTH treatment. The ovine model has been recognized as an ideal model in translational research from animal to human studies with respect to many topics including reproductive physiology, development, and chronic hypoxia (44). The temperament and size of the ovine model allows for chronic instrumentation and monitoring of the vasculature within the fetus, mother, or non-pregnant adult to obtain data regarding blood gases, pH, and hemoglobin levels. With respect to development alone, the gestational period of the ovine fetus (138-142 days) is more similar to that of the human than most other animal models. The sequence of development of the ovine fetus may better reflect that of a human than the commonly used rodent or mouse models.

Our group has also observed that the ovine model is ideal for investigating the acclimatization of animals to LTH in combination with the process of development. Adaptation to LTH at higher altitudes is, in part, dependent on proper functioning of the sympathetic nervous system (23). The model of maternal and fetal hypoxia in this study (sheep maintained at 3,820 m) is thought to be one of moderate and well-adapted hypoxia. There is a threshold level of oxygen and duration of exposure that, if exceeded, detrimental effects can occur (27). In this study, adult and fetal arterial PO2 values decline significantly while arterial pH remains unchanged and hemoglobin rises to increase oxygen carrying capacity (25). The large decrease in the adult PO2 (~40%) in comparison to the decrease in fetal PO2 (~15%) in response to LTH is a reflection of the differential capacity of oxygen-hemoglobin binding kinetics and saturation between each age group (2, 25). Near-term fetal LTH weights are comparable to control fetuses maintained at 718 m and fetal mortality and morbidity do not increase during LTH exposure. Thus, this ovine model is ideal for observing adaptive responses to high-altitude LTH.

SCG innervation targets: While the SCG mostly innervates the cerebrovasculature, it also provides sympathetic neural input into other organs such as the eye and heart to modulate
pupillary diameter and heart rate respectively (3, 8). The SCG provides sympathetic innervation to cerebral blood vessels whereby, these nerves are recognized as playing a vital role in modulating cerebral blood flow and protection of the blood vessels that may occur with environmental stress such as high altitude LTH (24).

**Contribution of CICR to EFS-evoked \([Ca^{2+}]_i\) transients during maturation and LTH.** Changes in \([Ca^{2+}]_i\) levels in sympathetic nerves are a necessary signaling mechanism for proper neuronal function and CICR contributes to stimulation-evoked increases in \([Ca^{2+}]_i\) (16, 18, 45). Therefore, we have assessed for the first time, the contribution of CICR to EFS-evoked \([Ca^{2+}]_i\) transients in the ovine SCG cell model. We have also assessed the combined impact of maturation and LTH on the function of CICR. The most significant findings in this study are that CICR contributes to EFS-evoked \([Ca^{2+}]_i\) transients in fetal and adult SCG cells, and that CICR is greater in normoxic fetuses as compared to normoxic adults (Fig. 2 A,B). Furthermore, CICR appears to be completely abolished in SCG cells from fetuses during LTH (Fig. 2C). These data suggest that CICR is a greater component in calcium signaling in normoxic sympathetic neurons and may ultimately aid in the regulation of the cardiovascular system before birth. Furthermore, adaptation to LTH results in the loss of CICR as a signaling mechanism in fetal SCG cells, which may possibly reduce the protective function of these neurons during LTH stress.

Transmitter release is determined by the magnitude and duration of \([Ca^{2+}]_i\) transients as well as the sensitivity of the release mechanism to changes in \([Ca^{2+}]_i\) (38). CICR mediated by RyRs, in part, determines the size and duration of neuronal \([Ca^{2+}]_i\) transients (49, 51), and is important to the magnitude of stimulation-evoked neurotransmitter release (18, 36, 45). The robust CICR in normoxic fetal as compared to adult SCG correlates well with our previous findings demonstrating that stimulation-evoked NE release from sympathetic nerve endings in fetal middle cerebral artery (MCA) is two-fold greater than the adult (5, 39). These studies are consistent with others that show sympathetic nerve activity rises before birth in both sheep and rat models (11, 55). One possible mechanism that is supported by our evidence is that the
rapid rise in sympathetic nerve activity before birth may be due to a greater contribution of CICR
to transmitter release.

The capacity of stimulation-evoked NE release declines with LTH in fetal but not adult
sheep MCA (6). However, the mechanism(s) underlying this difference is unknown. The loss of
the contribution of CICR to EFS-evoked $[Ca^{2+}]_i$ in fetal sympathetic neurons due to LTH (Fig.
2C), may in part, account for the decline in NE release in our previous study (6). As this study
focused on CICR in the SCG which is the cell body, the implications for function at the level of
NE release in the nerve ending are correlative. However, Smith and Cunnane showed that
ryanodine applied pre-synaptically decreased the excitatory post-synaptic response using an
intact sympathetic neuronal model (45). These data suggest that altered CICR pre-synaptically,
alters a post-synaptic response. Thus, the data presented in this study showing that CICR is
abolished in fetal SCG neurons obtained from LTH animals may indeed have functional
consequences of sympathetic neurons innervating the cerebrovasculature that originate in the
SCG.

Contrasting the fetus, in adult SCG cells, LTH appears to sensitize CICR to EFS, and
CICR is maintained (Fig. 2D). Thus, it is possible that stable contribution of CICR to nerve
function in the adult may possibly provide for maintained function of these sympathetic nerves
during LTH stress. Indeed, in our previous study the capacity of stimulation-evoked NE release
in adult MCA during LTH is maintained (6). Our previous and current studies are entirely
congruent with one another and illustrate that fetal and adult sympathetic nerves adapt
differently to LTH stress.

Caffeine-sensitization of EFS-evoked $[Ca^{2+}]_i$ transients during maturation and LTH.
Caffeine, a RyR agonist, is a well known CICR sensitizing agent, potentiating EFS-evoked
$[Ca^{2+}]_i$ transients in sensory and sympathetic neurons (1, 50, 51). In a recent study, we have
shown that the contribution of CICR to EFS-evoked $[Ca^{2+}]_i$ transients significantly declines in
SCG cells from senescent rats and that caffeine can reclaim a portion of the EFS-induced CICR
response to that of the middle-aged adult (1). As LTH abolishes CICR in fetal sheep SCG cells, much like aging leads to reduced CICR in rat SCG neurons, we measured EFS-evoked [Ca\textsuperscript{2+}]i in the presence of caffeine to see if CICR could be reclaimed. Caffeine clearly enhanced EFS-evoked [Ca\textsuperscript{2+}]i transients in SCG cells from normoxic fetuses (Fig. 1B, Fig. 3A) and in neurons from normoxic and LTH adults (Fig. 3B, D). However, caffeine failed to enhance EFS-evoked [Ca\textsuperscript{2+}]i transients in SCG cells from LTH fetuses. These data suggest that there may be a fundamental alteration in the regulation of RyR in fetal neurons during LTH, which completely decouples CICR from membrane depolarization, something that does not occur in adult SCG neurons. Furthermore, as RyR abundance does not decline with LTH in fetal sympathetic neurons, this suggests that there is a fundamental alteration in the interaction between RyR and the known agonist caffeine.

**Magnitude of EFS and caffeine-evoked [Ca\textsuperscript{2+}]i transients and coupling of calcium influx and CICR during maturation and LTH.** The magnitude and duration of stimulation-evoked [Ca\textsuperscript{2+}]i transients are dependent on the coupling of calcium influx to CICR (14, 49, 52). The decline of EFS-evoked [Ca\textsuperscript{2+}]i transients that occurs in SCG cells from normoxic fetus to adult and in the fetus exposed to LTH (Fig. 4A), appears to be mostly due to loss of CICR as opposed to a decline in calcium influx through voltage-gated calcium channels. This conclusion is supported by the comparison of EFS-evoked [Ca\textsuperscript{2+}]i transients in the presence of ryanodine (Fig. 4B). When ryanodine is present, CICR is blocked and the remaining EFS-evoked [Ca\textsuperscript{2+}]i transient reflects calcium influx and we found there were not any significant differences between the groups examined. These data suggest that calcium influx in SCG neurons is maintained during LTH in both the fetus and the adult.

The RyR agonist caffeine has been used to estimate the capacity of SCG neurons to release calcium from SER stores (52). The application of 5 mM caffeine to SCG cells evoked robust Ca\textsuperscript{2+} responses in the neurons we studied. However, LTH depressed caffeine-evoked [Ca\textsuperscript{2+}]i transients in fetal SCG neurons (Fig. 4C). Interestingly, while the contribution of CICR to
EFS-evoked [Ca\textsuperscript{2+}]\textsubscript{i} transients is lost in SCG cells from fetuses exposed to LTH, the response to caffeine significantly declines but is not eliminated suggesting that SER stores still contain releasable calcium. These data reinforce the hypothesis that the coupling between calcium influx and CICR is lost in fetal SCG cells during LTH possibly as a result of the decline in the sensitivity of RyR to changes in [Ca\textsuperscript{2+}]. In contrast, CICR in adult SCG cells appears to be sustained in response to EFS, suggesting that the coupling between calcium influx and CICR is maintained during LTH in the adult.

**Impact of maturation and LTH on RyR levels.** The RyRs are the prime mediators of CICR, thus, we measured the RyR levels in SCG cells in all study groups (Fig. 5). In this study RyR1 is the dominant subtype of RyR in SCG from fetal and adult normoxic and LTH sheep. There is a significant post-maturational decrease in the levels of RyR2 and RyR3, however, LTH does not influence RyR expression in fetal or adult SCG neurons. These data are consistent with a long-term hypoxic study (56 days) using cardiomyocytes from rats (40), which showed that RyRs did not decline over the duration of hypoxia. As RyR1, RyR2 and RyR3 are all expressed in sheep SCG, it is reasonable to conclude that they all contribute to CICR in the ovine SCG. However, these data do not support the hypothesis that the loss of CICR in fetal sympathetic neurons during LTH is due to a change in the levels of RyRs. Thus, the alteration in CICR appears to be due to fundamental changes in the response of RyR channels to EFS-evoked increases in [Ca\textsuperscript{2+}]\textsubscript{i} concentration.

The impact of postnatal maturation on the levels of the three RyR subtypes we studied is comparable to other animal models with some notable differences. In the mouse brain, the genetic expression of RyR1 mRNA predominates during the embryonic stage with a progressive increase in RyR2 mRNA after postnatal day 7 (P7), and RyR3 mRNA following P7 (34). In addition, immunodetection of RyR2 in mouse cerebral cortex occurs by embryonic day 12 and continues to increase during development, and caffeine elicits [Ca\textsuperscript{2+}]\textsubscript{i} release in developing neurons (13). In near-term fetal sheep SCG, all three RyR subtypes are robustly expressed,
while the levels of RyR2 and RyR3 decrease sometime after birth. These data contrast that of
the mouse model. Overall, the data suggest that RyRs are expressed in CNS and SCG
neurons over the course of development and are important to CICR in maturing neurons.
Furthermore, as CICR contributes to EFS-evoked [Ca\textsuperscript{2+}], in fetal neurons at much lower
numbers of pulses, this suggests that the RyRs in these neurons are more sensitive to changes
in [Ca\textsuperscript{2+}] levels, where fetal neurons have greater coupling between calcium influx and CICR.

**Cellular nNOS and cADPr levels during maturation and LTH.** Nitric oxide (NO) in
mammalian biology appears to be ubiquitous and regulates a number of cellular processes
including blood vessel contractility and neuronal excitability (7, 10, 31). We have quantified
nNOS levels within the sympathetic nerve endings in sheep MCA and rat SCG, and the function
of nNOS appears to augment stimulation-evoked NE release from these nerve endings (31, 32).
One proposed mechanism that accounts for the augmentation of NE release via nNOS nerves
is enhancement of Ca\textsuperscript{2+} influx and/or Ca\textsuperscript{2+} release from internal stores. In this case, nNOS
increases the synthesis cADPr via increased activity of ribosyl cyclase, which in turn facilitates
CICR (10, 12). Given the importance of nNOS as a modulator of NE release and cADPr levels,
we quantified the abundance of nNOS and the ribosyl cyclase end product cADPr in fetal and
adult sheep SCG (Table 1). nNOS abundance in the SCG more than doubles with development
from fetus to adult, however, LTH only increased nNOS abundance in fetal SCG. Despite these
changes in nNOS expression cADPr levels were well maintained across the groups we studied.
Thus, as cADPr sensitizes the coupling between calcium influx and CICR in different cellular
models (12, 26, 57), the decline in CICR in SCG cells from fetus to adult, the abolition of CICR
in the LTH fetus, and sensitization of CICR in the LTH adult can not be explained via this
pathway. Furthermore, despite the differences in nNOS levels found between the groups,
overall the data suggest that nNOS function in terms of cADPr synthesis is not altered with
postnatal maturation or LTH in the ovine SCG. Regulation of RyR also occurs by proteins such
as calmodulin, FKBP and phosphorylation (14, 30). Thus, the measurement of the level of these modulators and their function is a logical avenue for future studies.

Our results for nNOS abundance in SCG from each treatment group are in direct contrast to those observed in our previous study in nNOS containing neurons within the MCA (32). In that study, nNOS abundance was nearly identical in fetal and adult MCA and during LTH, nNOS levels declined in both age groups. In addition, the nNOS levels in fetal SCG are approximately 30-fold greater as compared to nNOS levels in the fetal MCA, and in the adult SCG, nNOS levels are approximately 100-fold greater as compared to nNOS levels in adult MCA. These data demonstrate that there is great heterogeneity in nNOS levels between the point of origination of nNOS containing neurons and termination of the neurons in the blood vessels themselves.

Impact of maturation and LTH on SERCA function in the SCG. The molecular data derived in this study do not provide a clear mechanism that can account for the profound changes in CICR that occur with LTH. Another modulator of the coupling between calcium influx and CICR is the luminal SER calcium level. Indeed the magnitude of stimulation-evoked [Ca\(^{2+}\)], can be altered by manipulation of SER calcium levels, demonstrating that the contribution of CICR to these transients is in part dependent on the calcium levels within the SER (15, 16, 51, 52). Furthermore, the refilling of SER calcium stores is dependent on SERCA function which simultaneously buffer [Ca\(^{2+}\)], transients and refill SER calcium stores (48, 52). Thus, in this study we blocked SERCA function with CPA and measured the rate of recovery of EFS-evoked [Ca\(^{2+}\)]i transients as an index of SERCA function (Fig. 6, 7). In fetal SCG cells, SERCA function declines profoundly with LTH. Furthermore, as compared to fetal SCG cells SERCA function is clearly less evident in neurons from adult sheep. These data suggest that the SER filling levels are greatest in SCG cells from near-term normoxic fetuses, consistent with the indication of a robust CICR. The LTH induced depression in SERCA function in fetal SCG neurons may therefore account for a portion of the loss of CICR that occurs. However, LTH fetal SCG cells
can still release some calcium (Fig. 4C). Thus, the loss of CICR appears to be a combination of
lowered luminal SER Ca\textsuperscript{2+} levels and a decoupling between extracellular Ca\textsuperscript{2+} influx and
intracellular Ca\textsuperscript{2+} release.

The maturational decrease in SERCA function in SCG neurons from normoxic animals
may explain why CICR contributes less to EFS-evoked [Ca\textsuperscript{2+}], transients in adult SCG cells.
However, it is important to note that RyR2 and RyR3 levels also decline during postnatal
maturation from fetus to adult (Fig. 5). Thus, the lowered CICR in the adult may be a
combination of lowered SER calcium and RyR levels. In contrast to the fetus, SERCA function
does not significantly change during LTH in adult SCG cells and CICR appears to be sensitized
to EFS as CICR occurs at lower trains of EFS. However, the modulation of CICR is dependent
on other modulators of RyR function such as FKBP proteins (30) and changes in the levels of
these modulators may possibly account for the maintenance of CICR in adult SCG cells during
LTH.

While SERCA function is important to the refilling of SER calcium stores, calcium entry
through SOCC also play an indirect role in maintaining SER calcium levels. The role of SOCC
in the maintenance of SER calcium stores (4) is an immediate topic in progress in our
laboratory. It is possible that differential SOCC activity between the groups in this study may
offer an additional explanation of varying SER filling levels and hence, CICR in these studies.

Summary and Conclusions. To our knowledge, this is the first investigation to use the ovine
SCG model to study the impact of maturation and LTH on calcium signaling, specifically the
contribution of CICR to EFS-evoked [Ca\textsuperscript{2+}], transients. In this study, we found that the
contribution of CICR to EFS-evoked [Ca\textsuperscript{2+}], transients is greatest in SCG cells from normoxic
fetuses and is abolished during LTH. These data cannot be accounted for by alterations in RyR
or cADPr levels, which are critical to CICR. However, a decline in SERCA function in fetal SCG
cells during LTH may reduce SER Ca\textsuperscript{2+} levels to a threshold that reduces the coupling between
calcium influx and CICR. During development from fetus to adult, the decrease in CICR may
reflect both a reduction in RyR2 and RyR3 levels and SERCA function. In contrast to the fetus, CICR function in adult SCG cells is maintained during LTH and may reflect alterations in other mechanisms that modulate the CICR process.

As CICR is necessary for the function of sympathetic neurons in the cerebrovasculature, the loss of this signaling mechanism in the fetus may have consequences for adaptation to LTH and may leave the fetus more susceptible to vascular insults during LTH stress.
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coupling of Ca²⁺ channels to ryanodine receptors at presynaptic terminals.


Figure Legends

Figure 1: Representative EFS-evoked [Ca\(^{2+}\)]\(_i\) transients in fetal normoxic SCG showing the application of EFS protocols 1 (Panel A) and 2 (Panel B). (A) Protocol 1; a priming stimulus of square wave EFS (50 pulses at 5 Hz 1 msec duration, 300 mA) was used to ensure filling of SER stores calcium stores. This was followed by a set of graded pulses (3 to 27 pulses at 3 Hz, 1 msec duration, 300 mA). Ryanodine was added during the last supermaximal transient with a subsequent equilibration period of 30 minutes. To ensure maximal blockade of RyR, caffeine was applied and failed to evoke an [Ca\(^{2+}\)]\(_i\) transient. (Panel B) Protocol 2; the control stimulations were as described in (Panel A) followed by the addition of caffeine indicated by the large transient that returns to baseline [Ca\(^{2+}\)]\(_i\). EFS was then repeated ten minutes after the formation of the caffeine-evoked [Ca\(^{2+}\)]\(_i\) transient. All electrical stimulations were followed by at least two minutes for recovery.

Figure 2: The impact of maturation and LTH on EFS-evoked [Ca\(^{2+}\)]\(_i\) transients in the absence and presence of RyR antagonist ryanodine in isolated ovine SCG cells. Cells were activated by EFS as per protocol 1 in the absence and presence of 100 µM ryanodine (see Figure 1A; methods). (Panel A) fetal normoxic; (Panel B) Adult normoxic; (Panel C) Fetal LTH; (Panel D) Adult LTH. Data represent the mean ± S.E. n=12-19 cells from 6-8 animals in all groups; *=significantly greater than ryanodine-treated; P<0.05 by paired t-test.

Figure 3: The impact of maturation and LTH on EFS-evoked [Ca\(^{2+}\)]\(_i\) transients in the absence and presence of the RyR agonist, caffeine in isolated ovine SCG cells. Cells were activated by EFS as per protocol 2 in the absence and presence of 5mM caffeine (see Figure 1B; Materials and Methods). (Panel A) fetal normoxic; (Panel B) Adult normoxic; (Panel
C) Fetal LTH; (Panel D) Adult LTH. Data represent the mean ± S.E. n=14-24 cells from 6-8 animals in all groups; *=significantly less than caffeine-treated, P<0.05 by paired t-test.

**Figure 4: The impact of maturation and LTH on maximal EFS-evoked [Ca^{2+}]_i transients in the absence and presence of ryanodine in isolated ovine SCG cells and on caffeine-evoked [Ca^{2+}]_i transients in isolated ovine SCG cells.** The maximal response to EFS is defined as the \( \Delta [Ca^{2+}] \), at 24 pulses at 3Hz, 300mA. (Panel A) The magnitude of EFS-evoked [Ca^{2+}]_i transients in SCG cells at maximal response (24 pulses) in the absence of ryanodine. (Panel B) The magnitude of EFS-evoked [Ca^{2+}]_i transients in SCG cells at maximal response (24 pulses) in the presence of 100 µM ryanodine. (Panel C) Measurement of 5 mM caffeine-evoked [Ca^{2+}]_i transients. Data represent the mean ± S.E. (Panel A) n=26-43 cells from 12-16 animals in each treatment group; (Panel B) n=12-19 cells from 6-8 animals in each treatment group; (Panel C) n=14-24 cells from 6-8 animals in each treatment group; **=significantly different from adult normoxic and fetal LTH, P<0.05 by ANOVA PLSD.

**Figure 5: Impact of maturation and LTH on cellular levels of RyR1, RyR2, and RyR3 isoforms in ovine SCG.** RyR isoforms levels were quantified using ELISA and normalized to GAPDH (see Materials and Methods). Inset graphs show GAPDH controls. Data represent the mean ± S.E. n=8 ganglia from 8 normoxic and 8 LTH adults; 16 ganglia (2 pooled for each experiment) from 8 normoxic and 16 ganglia (2 pooled for each experiment) from 8 LTH fetuses. Each experiment was performed in triplicate. *=significantly different from normoxic or hypoxic fetus, P<0.05 by ANOVA and Fisher PLSD test; **=significantly different from two other isoforms, P<0.01 by ANOVA and Fisher PLSD test.

**Table 1: The impact of maturation and LTH on the abundance of nNOS and normalized cADPr levels in ovine SCG.** nNOS was quantified by ELISA assay and using a recombinant
nNOS standard (see Materials and Methods). Data represent the mean ± S.E. n = 10 ganglia from 10 normoxic and 10 ganglia from 10 LTH adults; 20 ganglia (2 pooled for each experiment) from 10 normoxic and 20 ganglia (2 pooled for each experiment) from 10 LTH fetuses. Each experiment was performed in triplicate. **= significantly different from fetus, P<0.005, by ANOVA and Fisher PLSD test. += significantly different from fetal normoxic, P<0.001 by ANOVA and Fisher PLSD test.

cADPr was extracted from SCG homogenates and quantified using a fluorimetric cycling assay and a standard curve (r=0.99; data not shown; see Materials and Methods). cADPr values were normalized to total genomic DNA content. Data represent the mean ± S.E. n= 10 ganglia from 10 normoxic and 10 ganglia from 10 LTH adults. 20 ganglia (2 pooled for each experiment) from 10 normoxic and 20 ganglia (2 pooled for each experiment) from 10 LTH fetuses. Each experiment was performed in triplicate.

Figure 6: Representative EFS-evoked [Ca^{2+}]_i transients in an isolated fetal normoxic (Panel A) and fetal LTH (Panel B) SCG cell in the absence and presence of the SERCA antagonist, CPA. Two to three supramaximal EFS trains (5 Hz, 50 pulses, 300 mA; see Materials and Methods—Protocol 3) were applied in and absence (control) and presence of 10 µM CPA. The initial [Ca^{2+}]_i rise induced by the presence of CPA was allowed to return back to baseline [Ca^{2+}]_i before EFS was repeated. The τ constant for recovery was obtained for all [Ca^{2+}]_i transients as a first-order exponential decay fit from the peak [Ca^{2+}]_i to 95% of basal [Ca^{2+}]_i.

Figure 7: Impact of maturation and LTH on recovery of maximal EFS-evoked [Ca^{2+}]_i transients in isolated ovine SCG cells. Supramaximal trains were applied in the absence and presence of 10 µM CPA (Fig. 6; Materials and Methods). The τ constant for recovery was
obtained for all \([\text{Ca}^{2+}]\) transients as a first-order exponential decay fit from the peak \([\text{Ca}^{2+}]\) to
95% basal \([\text{Ca}^{2+}]\). Data represent the mean ± S.E. \(n=10-14\) cells from 6-8 animals. * =
significantly different from control, \(P<0.01\) by paired \(t\)-test. +++ = significantly different from
three other treatment groups, \(P<0.05\) by ANOVA and Fisher PLSD test.
Figure 1

A

B

[Ca^{2+}]_i (nM)

0 1000 2000 3000 4000 5000 6000

50 pulses, 5 Hz

100 µM ryanodine

5 mM caffeine

0 500 1000 1500 2000 2500 3000

50 pulses, 5 Hz

5 mM caffeine

[Ca^{2+}]_i (nM)

time (sec)
Figure 3

A  Fetal normoxic

B  Adult normoxic

C  Fetal LTH

D  Adult LTH

Δ[Ca\textsuperscript{2+}] (\% of control max) vs. number of pulses.
Figure 4

A

$\Delta [Ca^{2+}]_{i} \text{ (nM)}$

Fetal normoxic  Adult normoxic  Fetal LTH  Adult LTH

B

$\Delta [Ca^{2+}]_{i} \text{ (nM)}$

Fetal normoxic  Adult normoxic  Fetal LTH  Adult LTH

C

$\Delta [Ca^{2+}]_{i} \text{ (nM)}$

Fetal Normoxic  Adult normoxic  Fetal LTH  Adult LTH

$p < .03$

$p < .03$
Figure 5

![Graph showing absorbance at 405nm for RyR1, RyR2, and RyR3 for Fetal normoxic, Adult normoxic, Fetal LTH, and Adult LTH.]
Figure 6

A

10 μM CPA

B

10 μM CPA

[Ca^{2+}]_i (nM)

time (sec)

EFS

EFS

10 µM CPA
Figure 7

![Graph showing recovery constant tau (sec) for different conditions: control, + CPA.](image)

- Fetal normoxic
- Adult normoxic
- Fetal LTH
- Adult LTH

Key:
- Control
- + CPA

* denotes statistically significant difference.
Table 1: Levels of nNOS protein and cADPr with maturation and LTH

<table>
<thead>
<tr>
<th>Group</th>
<th>ng nNOS/ µg protein</th>
<th>pmol cADPr/ mg DNA</th>
<th>mg DNA/ mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal normoxic</td>
<td>2.56 ± 0.18</td>
<td>254.79 ± 29.19</td>
<td>2.45 ± 0.17</td>
</tr>
<tr>
<td>Adult normoxic</td>
<td>4.65 ± 0.30**</td>
<td>267.66 ± 52.18</td>
<td>2.21 ± 0.13</td>
</tr>
<tr>
<td>Fetal LTH</td>
<td>3.30 ± 0.25*</td>
<td>254.79 ± 50.70</td>
<td>2.48 ± 0.16</td>
</tr>
<tr>
<td>Adult LTH</td>
<td>4.51 ± 0.32**</td>
<td>298.33 ± 76.39</td>
<td>2.25 ± 0.17</td>
</tr>
</tbody>
</table>