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# Abnormal Intracellular Calcium Homeostasis in Sympathetic Neurons from Young Prehypertensive Rats

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## Abstract

Hypertension is associated with cardiac noradrenergic hyperactivity, although it is not clear if this precedes or follows the development of hypertension itself. We hypothesized that $Ca^{2+}$ homeostasis in postganglionic sympathetic neurons is impaired in spontaneously hypertensive rats (SHR), and may occur before the development of hypertension. The depolarization induced rise in intracellular free calcium concentration ($[Ca^{2+}]_i$) (measured using fura-2/AM) was significantly larger in cultured sympathetic neurons from pre-hypertensive SHR's than in age matched normotensive Wistar-Kyoto (WKY) rats. The decay of the $[Ca^{2+}]_i$ transient was also faster in SHRs. The endoplasmic reticulum $Ca^{2+}$ content and caffeine induced $[Ca^{2+}]_i$ amplitude were significantly greater in the young SHR. Lower protein levels of phospholamban and more copies of ryanodine receptor mRNA were also observed in the young SHR. Depleting the endoplasmic reticulum $Ca^{2+}$ store did not alter the difference of the evoked $[Ca^{2+}]_i$ transient and decay time between young SHR and WKY. However, removing mitochondrial $Ca^{2+}$ buffering abolished these differences. A lower mitochondrial membrane potential was also observed in young SHR sympathetic neurons. This resulted in impaired mitochondrial $Ca^{2+}$ uptake and release which might partly be responsible for the increased $[Ca^{2+}]_i$ transient and faster decay in SHR sympathetic neurons. This $Ca^{2+}$ phenotype seen in early development in cardiac stellate and superior cervical ganglion neurons may contribute to the sympathetic hyper-responsiveness that precedes the onset of hypertension.

### Keywords

Hypertension; Sympathetic neuron; Calcium homeostasis; endoplasmic reticulum; mitochondria

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Hypertension is a multi organ disease involving the kidney, vasculature, and autonomic nervous system. In particular, abnormal neurohumoral activation is a hallmark of hypertension and is a negative prognostic indicator for sudden cardiac death and a strong independent predictor of mortality[1, 2]. Much evidence supports the observation that sympathetic hyper-responsiveness is involved in the pathophysiology of human and animal primary hypertension[3-5]. Increases in muscle sympathetic nerve activity in response to mental stress has been documented in normotensive offspring linked to a family history of hypertension[6]. This suggests that dysregulated sympatho-humoral activation may be an early marker of hypertension in those that are genetically predisposed to the disease.

Intracellular calcium concentration ($[Ca^{2+}]_i$) plays a pivotal role in triggering neurotransmitter release from sympathetic neurons[7]. In superior cervical ganglion (SCG) neurons, $Ca^{2+}$ signals govern the release of norepinephrine (NE)[8, 9]. In turn, NE release plays a critical role in the regulation of blood pressure and cerebral blood flow distribution[10, 11]. We have shown in the spontaneously hypertensive rat (SHR), enhanced heart rate responses and evoked NE release from the post-ganglionic neurons innervating the heart when compared to normotensive Wistar Kyoto (WKY) rats[12, 13]. Post synaptically, basal and NE-stimulated L-type calcium current ($I_{CaL}$) in single sino-atrial node cells was also enhanced in the SHR, as was the heart rate response to bath applied NE[14]. Taken together, these results suggest a significant component of the sympathetic hyper-responsiveness in the SHR occurs at the end organ level.

We tested the hypothesis that intracellular $Ca^{2+}$ homeostasis is dysregulated in the SHR, and that this is genetically programmed and precedes the subsequent development of hypertension. To investigate the mechanisms involved in the potential differences between the SHR and WKY rat, we targeted calcium handling by the endoplasmic reticulum (ER) and mitochondria in an attempt to delineate whether disruption of $Ca^{2+}$ handling proteins in intracellular stores might explain the enhanced exocytotic response in the SHR[12, 15].

## Methods

### Animals

Neonatal (4-7 days), pre-hypertensive young (4-6 weeks) and hypertensive adult (15-17 weeks old) male SHRs and WKY rats were used in this study. Cells were isolated from the SCG and cardiac stellate ganglion for phenotyping. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Animals (Scientific Procedures) Act 1986 (UK).

An expanded Materials and Methods section is available in the online data supplement at http://hyper.ahajournals.org.

## Results

### Animal characteristics

The phenotypical characteristics of both young and adult SHRs and WKY rats are summarized in Supplement table S1. No differences in ventricular weight / body weight ratio (an indication of left ventricular hypertrophy), mean arterial blood pressure or heart rate measured *in- vivo* were observed in the young SHRs, whereas these measures were significantly increased in the adult SHRs when compared to the age matched WKY rats.

## Intracellular free calcium transients in sympathetic neurons

Sympathetic neurons were confirmed by the catecholamine neuronal marker anti-Tyrosine Hydroxylase stain (Fig. 1A). Intracellular calcium concentration was performed by ratiometric recordings in single sympathetic neurons (Fig. 1B). A typical calcium intensity profile of a sympathetic neuron responding to 100 mmol/L KCl was shown in Fig. 1C. In the SHR, high $K^+$ evoked $[Ca^{2+}]_i$ was significantly enhanced in both stellate ganglion (Fig. 2) and SCG neurons (Fig. 3A,B) when compared with age matched WKY rats. In depth profiling of the neural phenotype was performed on SCG cells because of the higher yield of neurons and ease of dissection.

Baseline $[Ca^{2+}]_i$ was significantly higher in SCG neurons from the SHRs when compared with age matched WKY rats in all three age groups (Supplement table S2). The baseline $[Ca^{2+}]_i$ in the SHR decreased with age, whereas this was not seen in the WKY. During depolarization with high $K^+$, SCG neurons from the neonatal SHR had a greater increase in $[Ca^{2+}]_i$ when compared to those from the WKY (Fig. 3A,B). This effect was present in animals of all age groups from neonatal to adult rats. There was a significant increase in evoked $[Ca^{2+}]_i$ between the young and adult SHRs (Fig. 3B). SCG neurons from the WKY also showed an increase in depolarization evoked $[Ca^{2+}]_i$ with age, but the response remained smaller than the SHR (eg. at adult, SHR +32.10%, WKY +13.92% compared with young rats, Fig. 3B). We also calculated the area under the curve of the $Ca^{2+}$ response to high $K^+$ (Supplement Figure S1), and observed that this was significantly greater in the SHR from neonatal to the fully developed hypertensive rat when compared with the age matched WKY. Analyzing the responses as a % change from baseline also resulted in a greater $[Ca^{2+}]_i$ responses in the young SHR, although this was not observed in the neonatal or adult rats (Supplement table 3).

## Increased rate and decay time of the calcium transient in SCG neurons

In both SHR and WKY rats, the $[Ca^{2+}]_i$ increased rapidly in response to high $K^+$ and fell slowly upon high $K^+$ removal (Fig. 3A). The rate of rise ($\Delta$ratio/$\Delta$time) increased with age (Supplement table S4), but there was no significant difference between SHR and WKY rats in any age group. The WKY group demonstrated a significantly longer (50% and 90%) decay time in comparison to the SHR group from young and adult rats (Fig. 3C). Since clear differences in both the amplitude and decay time of the $[Ca^{2+}]_i$ were apparent in young prehypertensive SHR and WKY rats, further experiments to investigate the role of the endoplasmic reticulum and mitochondria focused on young rats (4 weeks old).

## Endoplasmic reticulum calcium handling in SCG neurons

**Monitoring of ER $Ca^{2+}$ concentration—**$Ca^{2+}$ concentration in the ER can be directly measured by monitoring the $Ca^{2+}$ within the organelles after loading with a low affinity $Ca^{2+}$ indicator mag-fura-2/AM (Supplement Figure S2). Baseline mag-fura-2/AM fluorescence ratios were significantly higher in sympathetic neurons of young SHR when compared with WKY (Fig. 4A left group bar). This difference persisted when the suffusate had zero calcium present (Fig. 4A right group bar & 4B). Activation of ryanodine receptors (RyRs) and depletion of $Ca^{2+}$ from ER stores with 10 mmol/L caffeine produced a larger drop in the mag-fura-2 fluorescence ratio in the SHR (−7.05 ± 1.14%) compared with the WKY (−2.58 ± 0.68%) ($p<0.01$, Fig. 4B). Subsequent introduction of the cell-permeant intraluminal $Ca^{2+}$/heavy metal chelator, *N,N,N′,N′*-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN), caused a further reduction in the mag-fura-2 fluorescence ratio in both groups (Fig. 4B). Fluorescence ratio normalized by baseline showed that the caffeine evoked changes in ER $Ca^{2+}$ was significantly greater in the SHR than the WKY rats (to 0.92 ± 0.01 vs 0.96 ± 0.01, $p<0.01$, Fig. 4C), whilst the TPEN response was similar (to 0.82 ±

0.03 vs 0.85 ± 0.03, p=0.51, Fig. 4C). Releasable ER $Ca^{2+}$ load was also estimated based on the $[Ca^{2+}]_i$ responses to caffeine (10 mmol/L, 30 sec, Fig. 4D).

**The ryanodine receptor—**Studies have shown that advancing age can selectively change the genetic expression and protein levels of RyR3, but not RyR2 and RyR1 isoforms in the SCG aged 6, 12 and 24 months rats[16]. Therefore, we investigated whether the RyR3 receptor is altered in young SHR. Western blots were not sensitive enough to detect RyR3 receptor protein in the SCG dissected from young rats. However, SHRs were found to have significantly more RyR3 mRNA copies than WKY rats using RT-PCR (Fig. 4E).

**Protein expression of sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) and Phospholamban (PLN)—**To understand the mechanism underlying the faster $[Ca^{2+}]_i$ decay in the SHR, western blot analyses were performed to assess the expression levels of SERCA2a and PLN in SCG homogenates from young WKY and SHRs (Fig. 5). SERCA pump is under the regulatory control of the phosphoprotein phospholamban, which inhibits the apparent affinity of SERCA for $Ca^{2+}$ in its non phosphorylated form[17]. There was no difference in protein level of SERCA2a from young SHRs and WKY rats (p=0.63, Fig. 5B). However, both the total as well as the phosphorylated portion (PLN-$Ser^{16}$) of the PLN were significantly lower in the SHR group (Fig. 5C, D). There was no difference in the PLN-$Thr^{17}$ between the two groups (data not shown).

**ER contribution to the changes $[Ca^{2+}]_i$ in the SHR—**To evaluate the contribution of the ER to the differences in peak $[Ca^{2+}]_i$ rise and recovery time, ER $Ca^{2+}$ stores were depleted by using caffeine (10 mmol/L, 30 sec), and $Ca^{2+}$ reuptake from SERCA pumps were blocked with 1 μmol/L Thapsigargin. Caffeine was re-introduced at 8.5min and 13.5min to confirm ER $Ca^{2+}$ depletion (Fig. 6A). Under these conditions, the SHR group continued to have a significantly higher elevation of $[Ca^{2+}]_i$ during high $K^+$ depolarization when compared with the WKY group (Fig 6B). The baseline $[Ca^{2+}]_i$ was increased by 0.157 ± 0.028 μmol/L in the SHR and 0.136 ± 0.021 μmol/L in the WKY after ER $Ca^{2+}$ depletion ($p$=0.56, $t$-test). Because 90% decay time for the second high $K^+$ depolarization was not reached in some experiments, only 50% decay time was measured and this remained significantly shorter in the SHR when compared with the WKY group (p<0.01, Fig. 6C).

## Mitochondrial calcium in SCG neurons

**Mitochondria contribution to the $[Ca^{2+}]_i$—**The proton uncoupler FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone, 1 μmol/L) causes an immediate depolarization of the inner mitochondrial membrane, resulting in depletion of stored mitochondrial $Ca^{2+}$ and inhibition of any further mitochondrial $Ca^{2+}$-uptake[18]. Application of FCCP produced a small transient rise in $[Ca^{2+}]_i$ (Fig. 7A, center). This increase was not different between the two strains (SHR: 189 ± 10 vs WKY: 198 ± 10 nmol/L, $p$=0.55, $t$-test, n=17 and 23 respectively). Subsequent exposure to high $K^+$ in the continued presence of FCCP resulted in an increase in the amplitude of the $[Ca^{2+}]_i$ transient of 23.15 ± 1.78 % in the WKY and 12.74 ± 2.48% in the SHR ($p$<0.01, $t$-test, Fig. 7B), abolishing the difference in depolarization induced $[Ca^{2+}]_i$ increase between the two groups. The 50% & 90% decay time of the $[Ca^{2+}]_i$ transient were significantly shortened by FCCP in both WKY and SHR neurons ($p$<0.001, ANOVA), and the difference in 50% & 90% decay time between SHR and WKY were abolished (Fig. 7C).

**Measurement of the mitochondria membrane potential in sympathetic neurons—**We then measured the membrane potential of mitochondria ($\Delta \Psi_m$) using a fluorescent dye tetra-methylrhodamine ethyl ester (TMRE, 20 nmol/L, 5min). We confirmed that TMRE uptake was indeed due to accumulation within the mitochondria by

demonstrating that it was rapidly released upon mitochondrial depolarization with FCCP (Fig 8B). TMRE-fluorescence intensity was significantly higher in the WKY than in the SHR (Fig. 8A&B, $p<0.001$, *t*-test).

## Discussion

There are three novel findings from this study. First, sympathetic neurons from the SHR have a significantly greater depolarization evoked $[Ca^{2+}]_i$ transient when compared with the age matched WKY. This difference persisted from neonates through to young prehypertensive and fully developed adult hypertensive animals. Furthermore, the rate of decay of $[Ca^{2+}]_i$ was faster in young and adult SHR. Secondly, increased ER $Ca^{2+}$ content, upregulated SERCA (due to reduced PLN inhibition) and ryanodine receptor activity were seen in the young SHR. Thirdly, mitochondrial membrane potential was reduced in the young SHR sympathetic neurons, and this may contribute to the differences in depolarization evoked $[Ca^{2+}]_i$ transients and the rate of decay between the two groups. When all results are taken together, they suggest that alteration of $[Ca^{2+}]_i$ in sympathetic neurons from the SCG cells (and by extrapolation, stellate ganglion cells), precedes the actual onset of hypertension, and thereby maybe responsible for the enhanced sympathetic responsiveness seen at this developmental stage.

### Intracellular free calcium transients in the SHR

Calcium is an important ubiquitous secondary signaling messenger that is involved in both short term as well as long term regulation of cell function, metabolism and growth[19, 20]. In neurons, an increase in $[Ca^{2+}]_i$ forms the pivotal link between the action potential and neurotransmitter release[7, 21]. It is well established that hypertension is strongly associated with noradrenergic hyperactivity, with increased central sympathetic output as well as elevated plasma epinephrine and NE levels[22, 23]. This dysregulation occurs at several levels of the cardiovascular neural axis, where peripheral sympathetic hyper-responsiveness in the adult hypertensive rat results in enhanced evoked cardiac NE release[12, 15] and also increased β adrenergic responses in myocytes[24]. Abnormal calcium handling properties have been reported in SHR vascular smooth muscle cells[25] and endothelial cells[26], and it is conceivable that this also occurs in sympathetic neurons, thus providing a possible molecular link to enhanced noradrenergic neurotransmission.

The resting $[Ca^{2+}]_i$ level in the SCG neurons from the neonates through to fully developed hypertensive animals was higher than that observed in the age matched WKY rat. Similarly the $[Ca^{2+}]_i$ handling expressed as a percentage change from baseline was also greater in the young SHR. However, this was not evident in the neonatal or adult cells from the SHR, and may be related to poor statistical power. Nevertheless, when taken together with the absolute $[Ca^{2+}]_i$ responses and the area under the curve measurement for $[Ca^{2+}]_i$ (Fig. S1), the data are consistent with the hypothesis that pre-hypertensive SHRs have abnormal $[Ca^{2+}]_i$ handling properties compared to age matched WKY animals. The overall magnitude of the evoked $[Ca^{2+}]_i$ transient increased with age in both groups, and the increase in the SHR group was larger, thus maintaining a difference between the two groups. This supports the idea that abnormal calcium regulation occurs early before hypertension takes place, but that age alone does not fully explain the development of the calcium phenotype in the SHR when compared to the WKY.

The higher depolarization evoked $[Ca^{2+}]_i$ was not selective for SCG which predominately provide sympathetic innervation to the neck and cranial tissue[27], but it was also seen in neurons from the stellate ganglion that have a significant innervation into the heart. These data may at least partially explain why there is an increased local cardiac noradrenergic neurotransmission in the adult SHR when compared with the WKY[12, 15]. Moreover,

emerging evidence has demonstrated enhanced NE release and heart rate responses to cardiac sympathetic activation in young pre-hypertensive SHR's in-vitro[28].

Evoked $[Ca^{2+}]_i$ responses are influenced by multiple factors, including $Ca^{2+}$ entry, extrusion across the plasma membrane, $Ca^{2+}$ uptake and release from internal stores, and endogenous and exogenous $Ca^{2+}$ buffering. From our results, there was no significant difference in the rate of rise of the $[Ca^{2+}]_i$ transient in response to $K^+$ induced depolarization between SHR and WKY rats in all age groups. This suggests that the depolarization induced $Ca^{2+}$ entry through the plasma membrane was not different in sympathetic neurons from the SHR compared to the WKY at any age. However, we did not measure the membrane calcium current directly, so cannot completely exclude some involvement of plasma membrane bound $Ca^{2+}$ channels.

## Endoplasmic reticulum calcium signaling in the SHR

Increasing evidence suggests that altered ER $Ca^{2+}$ uptake, storage, and release plays a central role in cardiac hypertrophy and heart failure[29]. In the present study, before neurons were re-exposed to high KCl, ER $Ca^{2+}$ stores were depleted by caffeine and $Ca^{2+}$ reuptake from SERCA pumps were prevented by thapsigargin. Under these conditions, the increased $[Ca^{2+}]_i$ should represent a non-ER contribution. We found that the SHR group continued to have a higher increase in depolarization evoked $[Ca^{2+}]_i$ even after ER $Ca^{2+}$ depletion. This result suggests that the non-ER sources may underlie the significantly higher depolarization evoked $[Ca^{2+}]_i$ transients seen in the SHR.

The larger ER $Ca^{2+}$ store in the SHR was confirmed by directly measuring ER free $Ca^{2+}$ concentration using mag-fura-2/AM, followed by ER $Ca^{2+}$ release/depletion with caffeine and the ER-$Ca^{2+}$ chelator, TPEN. This finding itself does not necessarily indicate a larger ER contribution towards depolarization evoked $[Ca^{2+}]_i$ in the SHR, since the net ER contribution depends on the relative release and re-uptake of $Ca^{2+}$ by the ER[30, 31]. It does however, imply a larger ER $Ca^{2+}$ store that is potentially available for release upon appropriate stimulation.

The rate at which SERCA moves $Ca^{2+}$ across the ER membrane can be reduced by PLN, whereas phosphorylation of the PLN relieves its inhibition[32, 33]. Our results showed that the difference in total PLN was much larger than the difference in PLN-Ser[16], suggesting that there was less non-phosphorylated PLN in the SHR than WKY, and therefore less PLN dependent inhibition of SERCA activity in the pre-hypertensive SHR. This is in keeping with the functional data showing shorter 50% and 90% decay times in $[Ca^{2+}]_i$.

Ryanodine receptors are $Ca^{2+}$ permeable channels that open in responses to increase in $[Ca^{2+}]_i$[34]. We introduced the RyR activator caffeine to deplete $Ca^{2+}$ from ER stores. Prehypertensive SHRs had a significantly higher caffeine induced $[Ca^{2+}]_i$ amplitude when compared with the age matched WKY, indicating that RyRs in sympathetic neurons were up regulated in the young SHR. This was confirmed by more copies of RyR3 mRNA in the SHR. These data are consistent with observations by others in cardiac myocytes[35]. Taken together, these findings suggest that sympathetic neurons from prehypertensive SHR have more active/dynamic ER $Ca^{2+}$ handling machinery.

## Mitochondrial calcium signaling in the SHR

Mitochondria take up $Ca^{2+}$ primarily through the mitochondrial calcium uniporter[36], which is modulated by both $[Ca^{2+}]_i$ and the mitochondrial membrane potential ($\Delta \Psi_m$).[37] The uniporter transports $Ca^{2+}$ down the electrochemical gradient and this gradient is maintained across the mitochondrial inner membrane without direct coupling to ATP hydrolysis or transport of other ions. In this study we found that TMRE uptake was reduced in the SHR

neurons which suggests that the membrane potential of mitochondria ($\Delta \Psi_m$) was more depolarized in the SHR. Depolarization of $\Delta \Psi_m$ could lead to a reduced $Ca^{2+}$ uptake by the mitochondria in the SHR.

A significant association of hypertension with mitochondrial uncoupling proteins (UCPs) has been reported both in experimental[38] and human hypertensive states[39]. Moreover, in experimental hypertension, a decreased activity of complex IV has been observed in the hypertrophied myocardium from the SHRs[40]. However, we could not find any differences in protein levels of mitochondrial UCP2, citrate synthase (used as a quantitative enzyme marker for the presence of intact mitochondria) and complexes I-V in SCG homogenates from young SHRs and WKY rats (Supplement Figure S3). This indicates that mitochondrial number and the electron transport chain were not changed in young SHRs. The differences in depolarization evoked $[Ca^{2+}]_i$ transient and the 50% & 90% decay time between SHR and WKY were abolished by application of the proton uncoupler FCCP. These results suggest that mitochondria play a major role in the depolarization evoked $[Ca^{2+}]_i$ difference observed between the SHR and the WKY groups. Although we have no direct proof to show whether the ER and mitochondria contributed to the differences with age in $[Ca^{2+}]_i$ transients in the SHR, our results are supportive of this idea when taken together with others[41, 42] that showed an age-related decline in SERCA function with a subsequent increased reliance on mitochondria to control high $K^+$-evoked $[Ca^{2+}]_i$ transients.

## Perspectives

Results from this study suggest that the difference in $Ca^{2+}$ homeostasis between sympathetic neurons of SHR and WKY occurs early in the development, and before the actual onset of hypertension itself. Impairment of $[Ca^{2+}]_i$ handling was observed at two neural sites in the sympathetic nervous system, suggesting that this impairment may be wide spread. A close link between faulty mitochondria $Ca^{2+}$ release and re-uptake appears to be central to the enhanced $[Ca^{2+}]_i$ transients observed in pre-hypertensive SHR's. The precise molecular pathway underpinning this is not firmly established, but warrants further investigation. Moreover, it would be desirable to see whether the changes we report here are also seen in other animal models of hypertension. When all observations are viewed together with the current data, there is compelling evidence to suggest that alterations of $Ca^{2+}$ homeostasis are central to sympathetic hyperactivity in the SHR, resulting in enhanced sympathetic neurotransmission at the end organ. The resultant chronotropic and inotropic action of NE will contribute to raising cardiac output and arterial blood pressure. These pathways may be important targets to prevent sympathetic dysregulation that occur before the onset of hypertension

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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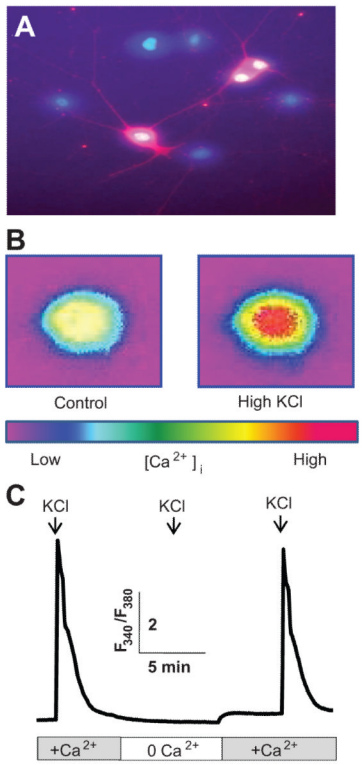


**Figure 1.**

**A**: Fluorescence images of sympathetic neurons derived from SCG which were stained with the adrenergic marker anti-Tyrosine Hydroxylase (Texas-red) and the nuclear marker DAPI (blue). **B**: Pseudo-color-coded ratio-images of Fura-2 loaded SCG neuron were obtained by conventional fluorescence microscopy. $Ca^{2+}$ concentrations were color-coded with a basal $Ca^{2+}$ concentration in blue and a high $Ca^{2+}$ concentration in red. **C**: An example recording from young WKY single SCG neuron exposed to 100 mmol/L KCl (30 sec) to depolarize the neuron resulting in $[Ca^{2+}]_i$ increase. The peak evoked $[Ca^{2+}]_i$ was absent in the extracellular $Ca^{2+}$ free solution.

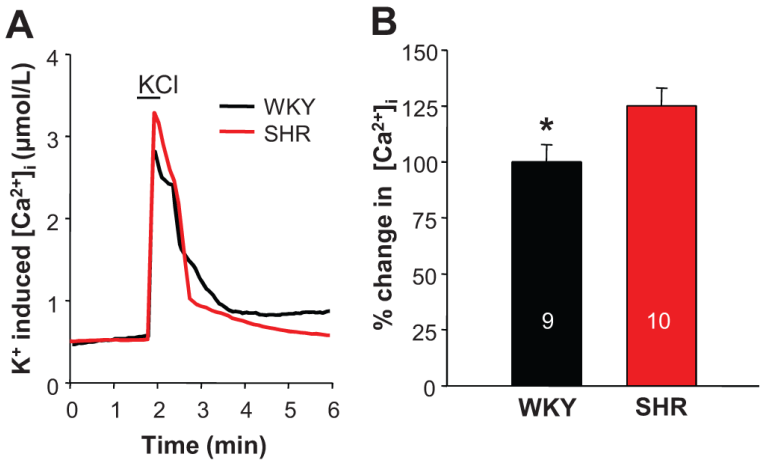


**Figure 2.**

**A**: An example recording from young SHR & WKY cardiac sympathetic neuron taken from stellate ganglion exposed to 100 mmol/L KCl for 30 sec to depolarize the neuron and evoke voltage-gated $Ca^{2+}$ entry. **B**: Statistical data showing the difference in peak evoked $[Ca^{2+}]_i$ increase between young SHR & WKY. *p<0.05, *t*-test.




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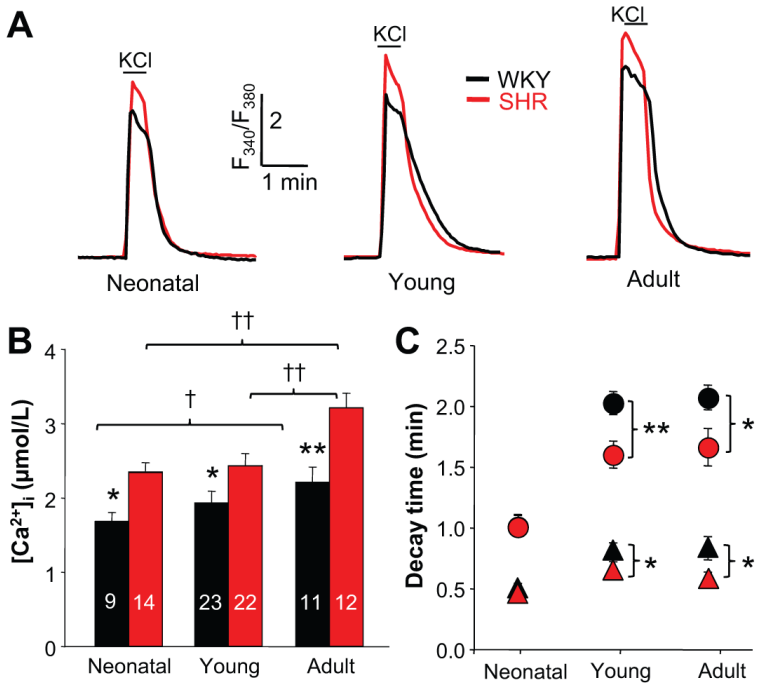


**Figure 3.**

**A**: Typical fluorescence ratio profile of single SCG neurons following exposure to high KCl. **B**: Statistical data showed peak increase in $[Ca^{2+}]_i$ upon high $K^+$ exposure from SHRs compared with WKY rats of the same age. **C**: Time taken for the $[Ca^{2+}]_i$ from peak to 50% & 10% of the increase (triangle, 50%& circles, 90%decay time) following high $K^+$ challenge. * $p$<0.05, ** $p$<0.01; $t$-test. † $p$ < 0.05, †† $p$<0.01; one way ANOVA.




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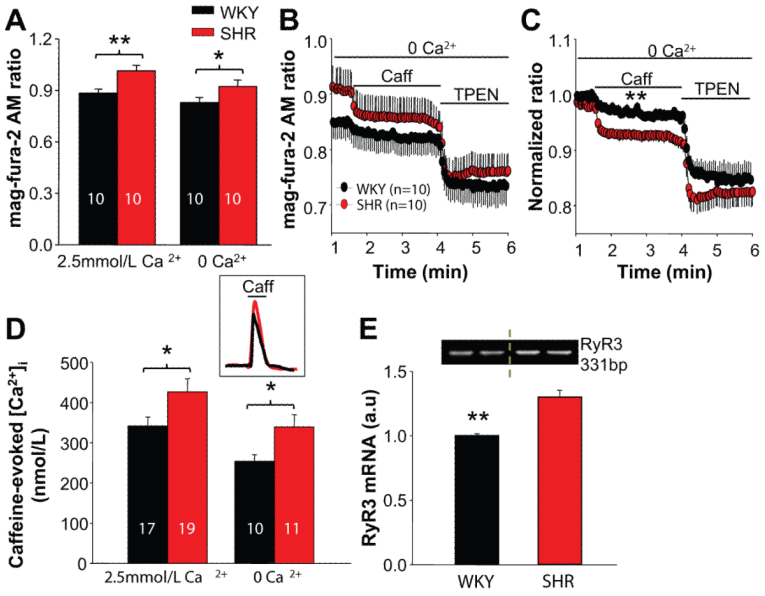


**Figure 4. Enhanced ER calcium load in SCG neurons in the young prehypertensive SHR**

**A:** Direct measurement of resting ER $Ca^{2+}$ concentration using mag-fura-2/AM. **B:** ER $Ca^{2+}$ depleted with caffeine followed by TPEN. **C:** Normalization of ratio by baseline. **D**: Caffeine evoked $Ca^{2+}$ release from the ER (measured using fura-2/AM) was significantly higher in SHRs either in normal or zero calcium solution. The inset is an example of the fluorescence ratio in the SCG neurons in response to 10 mmol/L caffeine for 30 seconds. **E**: The SCG from the SHR has significantly more copies of ryanodine receptor (RyR3) mRNA after 46 cycles of semi-quantitative RT-PCR when compared with the WKY (n=8 SCGs per group). *$p$<0.05, **$p$<0.01, $t$- test, SHR compared with WKY.

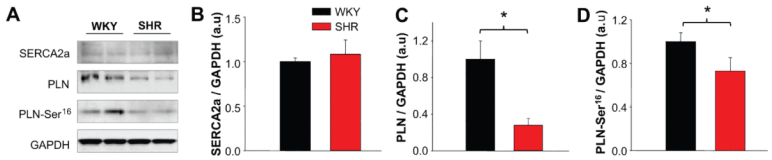


**Figure 5.**

**A**: Representative immunoblots using antibodies specific for SERCA2a, total PLN, Ser$^{16}$-phosphorylated PLN and GAPDH (as a loading control), using SCG homogenates from young SHRs and WKY rats. Equal amounts of protein were loaded in each line. **B, C, D:** Bar graphs showing protein level of SERCA2a, total PLN and Ser$^{16}$-phosphorylated PLN contents from the young SHRs and WKY rats (n=10 SCGs per group, *$p$<0.05, $t$-test).





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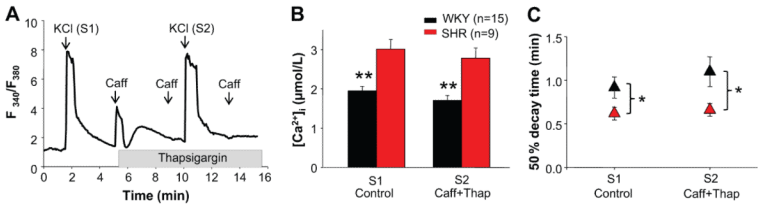


**Figure 6.**

**A**: Typical experimental protocol presenting fluorescence ratio of Fura-2/AM to assess the contribution of the ER to the increase in $[Ca^{2+}]_i$ upon high KCl exposure in the young WKY rats. **B**: Statistical data showing the peak evoked $[Ca^{2+}]_i$ transient both before (S1) and after (S2) ER $Ca^{2+}$ store depletion between young SHR and WKY. **C**: The difference between SHR and WKY in regards to 50% decay time before (S1) and after (S2) ER $Ca^{2+}$ store depletion. Caff – caffeine, Thap – thapsigargin. *$p$<0.05, **$p$<0.01, $t$-test.




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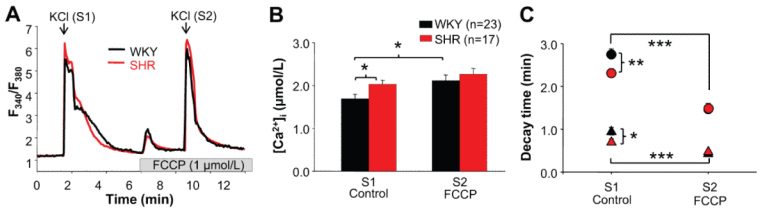


**Figure 7.**

**A**: Typical experimental protocol showing Fura-2/AM fluorescence ratio to assess the contribution of mitochondria to $[Ca^{2+}]_i$ in SCG neurons from young SHR and WKY rats. B: Statistical data showing the peak evoked $[Ca^{2+}]_i$ both before (S1) and after (S2) mitochondrial $Ca^{2+}$ store depletion with FCCP between young SHR & WKY rats. C: Time taken for the $[Ca^{2+}]_i$ from peak to 50% & 10% of the increase (triangle, 50% & circles, 90% decay time) following high $K^+$ challenge. *$p$<0.05, **$p$<0.01, ***$p$<0.001.




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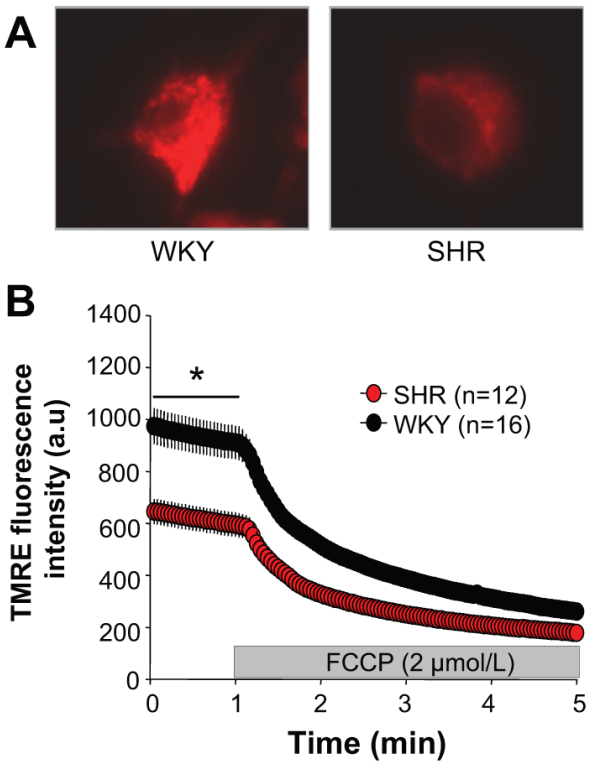


**Figure 8.**

**A:** Comparison of the mitochondria membrane potential ($\Delta \Psi_m$) using the fluorescence indicator TMRE in SCG neurons from young SHR (right) and WKY (left). **B**: Changes in TMRE intensity at resting state and during superfusion of 2 μmol/L FCCP in two groups of neurons. * $p<0.001$, *t*-test.