

# Overexpression of Sarcoendoplasmic Reticulum Calcium ATPase 2a Promotes Cardiac Sympathetic Neurotransmission via Abnormal Endoplasmic Reticulum and Mitochondria Ca<sup>2+</sup> Regulation

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**Abstract**—Reduced cardiomyocyte excitation–contraction coupling and downregulation of the SERCA2a (sarcoendoplasmic reticulum calcium ATPase 2a) is associated with heart failure. This has led to viral transgene upregulation of SERCA2a in cardiomyocytes as a treatment. We hypothesized that SERCA2a gene therapy expressed under a similar promiscuous cytomegalovirus promoter could also affect the cardiac sympathetic neural axis and promote sympathoexcitation. Stellate neurons were isolated from 90 to 120 g male, Sprague–Dawley, Wistar Kyoto, and spontaneously hypertensive rats. Neurons were infected with Ad-mCherry or Ad-mCherry-hATP2Aa (SERCA2a). Intracellular Ca<sup>2+</sup> changes were measured using fura-2AM in response to KCl, caffeine, thapsigargin, and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazine to mobilize intracellular Ca<sup>2+</sup> stores. The effect of SERCA2a on neurotransmitter release was measured using [<sup>3</sup>H]-norepinephrine overflow from 340 to 360 g Sprague–Dawley rat atria in response to right stellate ganglia stimulation. Upregulation of SERCA2a resulted in greater neurotransmitter release in response to stellate stimulation compared with control (empty: 98.7±20.5 cpm, n=7; SERCA: 186.5±28.41 cpm, n=8; *P*<0.05). In isolated Sprague–Dawley rat stellate neurons, SERCA2a overexpression facilitated greater depolarization-induced Ca<sup>2+</sup> transients (empty: 0.64±0.03 au, n=57; SERCA: 0.75±0.03 au, n=68; *P*<0.05), along with increased endoplasmic reticulum and mitochondria Ca<sup>2+</sup> load. Similar results were observed in Wistar Kyoto and age-matched spontaneously hypertensive rats, despite no further increase in endoplasmic reticulum load being observed in the spontaneously hypertensive rat (spontaneously hypertensive rats: empty, 0.16±0.04 au, n=18; SERCA: 0.17±0.02 au, n=25). In conclusion, SERCA2a upregulation in cardiac sympathetic neurons resulted in increased neurotransmission and increased Ca<sup>2+</sup> loading into intracellular stores. Whether the increased Ca<sup>2+</sup> transient and neurotransmission after SERCA2A overexpression contributes to enhanced sympathoexcitation in heart failure patients remains to be determined. (*Hypertension*. 2017;69:625–632. DOI: 10.1161/HYPERTENSIONAHA.116.08507.) • [Online Data Supplement](#)

**Key Words:** hypertension ■ rats, inbred SHR ■ sarcoplasmic reticulum calcium-transporting ATPases ■ stellate ganglion ■ sympathetic nervous system

Heart failure remains a predominant cause of mortality and morbidity globally and is characterized by a loss in efficient excitation–contraction coupling<sup>1,2</sup> that leads to reduced inotropy. Downregulation of the SERCA2a (sarcoendoplasmic reticulum Ca<sup>2+</sup> ATPase 2a), a key protein in cardiomyocyte excitation–contraction coupling, has been identified as a therapeutic target in both clinical<sup>1,3</sup> and animal models of heart failure.<sup>4</sup> Increasing myocyte SERCA2a levels by gene transfer in isolated human myocytes<sup>5</sup> and preclinical animal models with heart failure<sup>6,7</sup> restores cardiac inotropy and myocyte Ca<sup>2+</sup> handling, without proarrhythmic side effects.<sup>4</sup> Indeed, early small-scale clinical trials for the treatment of heart failure demonstrated positive results

for outcome and biological safety after intracoronary injection of adeno-associated virus (AAV) type 1 SERCA2a. Prespecified clinical end points, including the 6-minute walk test, peak oxygen consumption, and left ventricular end-systolic pressure all improved.<sup>8,9</sup> However, recent results from a larger phase 2 double-blind, placebo-controlled trial (CUPID2 [Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease]) failed to meet primary clinical end points.<sup>10,11</sup>

Adeno viruses (Ad) and AAV are powerful tools for altering gene expression because of their high transfection efficiency and low risk of pathogenicity.<sup>12</sup> They also have increased efficiency at infecting multiple cell types, including

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myocytes, neurons, and retinal cells,<sup>13</sup> if broad-spectrum promoters are used (eg, cytomegalovirus).<sup>10,14,15</sup> Therefore, it is conceivable that overexpression of AAV SERCA2a when given into the coronary circulation might also transduce the neural cardiac axis, resulting in a deleterious performance. In particular, SERCA and impairment of its regulatory protein phospholamban have been implicated in modulating depolarization-induced  $\text{Ca}^{2+}$  transients in sympathetic neurons,<sup>16</sup> thus promoting neurotransmission.<sup>17</sup> This neural phenotype is a well-established negative prognostic indicator in patients with heart failure.<sup>18–21</sup>

We therefore tested the hypothesis that enhancing SERCA2a gene expression with a cytomegalovirus promoter facilitates cardiac sympathetic neurotransmission via abnormal endoplasmic reticulum (ER) and mitochondrial intracellular  $\text{Ca}^{2+}$  handling in normal stellate neurons. Furthermore, we tested whether dysregulation of SERCA contributes to  $\text{Ca}^{2+}$  impairment in a model of cardiac sympathetic dysautonomia.

## Methods

### Animals

Age- and weight-matched male 4- to 5-week (90–120 g), Sprague–Dawley (SD,  $n=46$ ), spontaneously hypertensive rat (SHR,  $n=22$ ) and normotensive Wistar Kyoto (WKY,  $n=20$ ) rats, in addition adult 16- to 18-week (350–380 g) male SD rats ( $n=20$ ), and 9- to 10-month SHR ( $n=3$ ) and WKY rats ( $n=3$ ), were purchased from Envigo (Harlan, Bicester, United Kingdom) and housed under standard laboratory conditions. 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 (United Kingdom). Procedures were performed under British Home Office license requirements (PPL 30/3131).

### Viral Constructs

Viral constructs were manufactured commercially (Vector BioLabs, Malvern, PA). Viruses were constructed under a nonspecific cell type cytomegalovirus promoter to the same construct of human ATP2Aa as used in the CUPID trials. Ad-mCherry used was for control experiments (stock:  $1 \times 10^{10}$  PFU/mL), and Ad-mCherry-hATP2Aa used to up regulate SERCA2a expression (human ATP2Aa, with mCherry driven under its own cytomegalovirus promoter; stock:  $1.6 \times 10^{10}$  PFU/mL).

### Statistics

All statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data are presented as means  $\pm$  SEM. Analysis was performed using paired or unpaired Student *t* test as appropriate after testing and confirming all data sets were normally distributed. For all experiments, statistical significance was accepted at  $P < 0.05$ .

An expanded materials and methods section is available in the [online-only Data Supplement](#).

## Results

### Confirmation of SERCA2a Gene Transfer Into the Right Atria by Western Blot

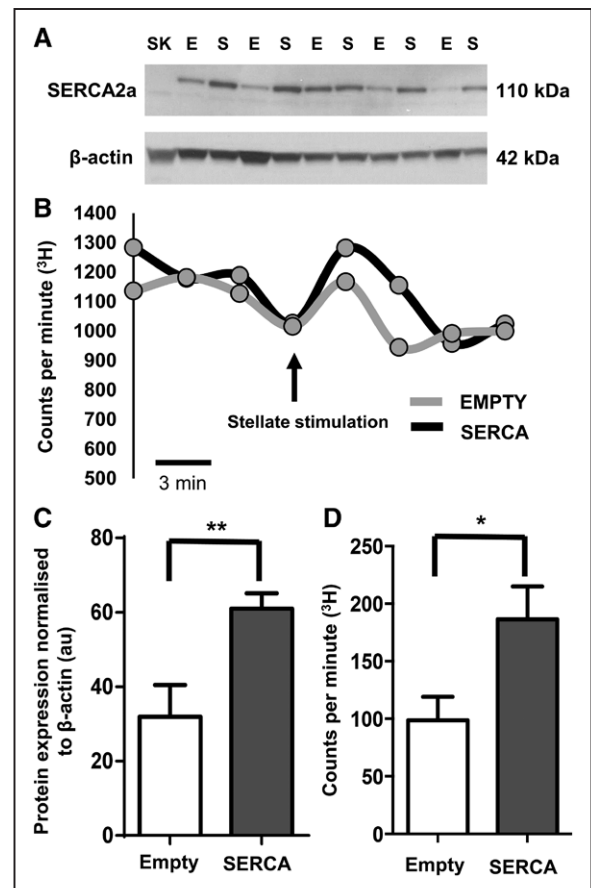
Percutaneous right atrial injection of SERCA2a or mCherry empty ( $3 \times 10^9$  PFU/mL) was confirmed by Western blot analysis (Figure 1A). Atrial myocytes endogenously express SERCA2a, and this expression level was significantly enhanced with atrial transfection with Ad-SERCA2a, when normalized to loading control (Figure 1C) (empty:  $31.9 \pm 8.5\%$ ,  $n=6$ ; SERCA:  $60.9 \pm 4.1\%$ ,  $n=7$ ;  $**P < 0.01$ ).

### Effect of Right Stellate Stimulation on [ $^3\text{H}$ ]-NE Release After Gene Transfer

Right atrial injection of the SERCA2a viral vector transgene significantly increased [ $^3\text{H}$ ]-norepinephrine [ $^3\text{H}$ ]-NE release in response to right stellate stimulation compared with atria that received injection of mCherry empty vector (Figure 1B and 1D; empty:  $98.7 \pm 20.5$  cpm,  $n=7$ ; SERCA:  $186.5 \pm 28.41$  cpm,  $n=8$ ;  $*P < 0.05$ ). This demonstrates that overexpression of SERCA2a can directly increase sympathetic neurotransmission.

### Intracellular Free $\text{Ca}^{2+}$ Transients in Ad-SERCA2a–Transduced Stellate Neurons of the SD Rat

Isolated stellate ganglia neurons from 4-week-old normotensive SD rats were transfected with either Ad-mCherry



**Figure 1.** A, Western blot of right atrial tissue from adult (16 to 18 wk, 350–380 g) Sprague–Dawley (SD) rats who received right atrial percutaneous injection and viral gene transfer 5 d before dissection. B, Representative raw data traces showing [ $^3\text{H}$ ]-norepinephrine (NE) release from 350 to 380 g SD rat right atria in response to stellate stimulation (5 Hz, 1 minute), samples taken every 3 minutes, arrow indicates the time point at which the right stellate was stimulated, data point after stimulation taken as the peak in counts per minute (cpm). C, SERCA2a (sarcoendoplasmic reticulum calcium ATPase 2a) expression is significantly higher in atria receiving Ad-mCherry-SERCA2a gene transfer (S) than those receiving Ad-mCherry empty gene transfer (E) in which only endogenous SERCA2a is seen. No SERCA2a expression in skeletal muscle (SK) negative control,  $\beta$ -actin loading control expressed in all lanes.  $**P < 0.01$ . D, Group mean data of delta CPM of [ $^3\text{H}$ ]-NE release (empty;  $n=7$ ; SERCA;  $n=8$ ).  $*P < 0.05$ .

(empty) or Ad-mCherry-hATP2Aa (SERCA2a). Transfection of the desired gene was confirmed by only selecting cells for further experiments, which expressed the mCherry tag under 587nm excitation (Figure 2A) because gene transfer is not homogeneous. An example of the evoked intracellular free  $\text{Ca}^{2+}$  concentration change  $[\text{Ca}^{2+}]_i$  is shown in Figure 2B with group mean data (Figure 2C). SD stellate ganglion neurons overexpressing SERCA2a exhibit a significantly greater depolarization-induced  $\text{Ca}^{2+}$  transient than those infected with the mCherry empty vector (empty:  $0.64 \pm 0.03$  au,  $n=57$ ; SERCA:  $0.75 \pm 0.03$  au,  $n=68$ ;  $*P < 0.05$ ). The time taken for the peak of the  $[\text{Ca}^{2+}]_i$  to fall by 50% was also significantly shorter in the SERCA2a-treated neurons (empty:  $0.88 \pm 0.06$  s,  $n=37$ ; SERCA:  $0.73 \pm 0.04$  s,  $n=68$ ;  $*P < 0.05$ ; Figure 2D).

### ER $\text{Ca}^{2+}$ Handling Within SD Stellate Neurons

$\text{Ca}^{2+}$  concentrations from the ER were measured by monitoring  $[\text{Ca}^{2+}]_i$  change in response to caffeine (10 mmol/L for 30 seconds) to deplete ER  $\text{Ca}^{2+}$  stores and thapsigargin (1  $\mu\text{mol/L}$ ) to block ER  $\text{Ca}^{2+}$  reuptake. SERCA2a-treated cells had a significantly greater increases in  $[\text{Ca}^{2+}]_i$  in response to caffeine (Figure 3A and 3B; empty:  $0.03 \pm 0.01$  au,  $n=35$ ; SERCA:  $0.15 \pm 0.01$  au,  $n=45$ ) and thapsigargin (empty:  $0.03 \pm 0.001$  au,  $n=33$ ; SERCA:  $0.12 \pm 0.01$  au,  $n=42$ ;  $**P < 0.01$ ). This would support the idea that the increased depolarization-induced  $\text{Ca}^{2+}$  transients observed in the SERCA2a-treated neurons are likely because of greater SERCA2a expression, resulting in greater  $\text{Ca}^{2+}$  load in the ER which is in turn mobilized by calcium-induced calcium release. Not all neurons in wells incubated with the virus expressed the mCherry tag (efficiency  $\approx 60\text{--}70\%$ ). In some experiments within one field of view, separate neurons with varying expression levels could be seen. Within dishes infected with the SERCA2a transgene, cells not expressing mCherry had caffeine and thapsigargin responses similar to empty vector-treated neurons.

### Effect on Mitochondrial $\text{Ca}^{2+}$ Handling Within SD Stellate Neurons

The effect of SERCA2a overexpression on mitochondrial  $\text{Ca}^{2+}$  handling was observed by using the proton uncoupler carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazine (FCCP; 1  $\mu\text{mol/L}$ ) that causes depolarization of the mitochondrial membrane. This results in depletion of  $\text{Ca}^{2+}$  stores and inhibition of further mitochondrial  $\text{Ca}^{2+}$  uptake.<sup>16,22</sup> Application of FCCP produced a transient increase in  $[\text{Ca}^{2+}]_i$  (Figure 3C). This change was significantly higher in the SERCA2a-transduced neurons compared with empty treated cells (Figure 3D; empty:  $0.05 \pm 0.005$  au,  $n=22$ ; SERCA:  $0.13 \pm 0.009$  au,  $n=22$ ;  $**P < 0.01$ ). This indicates that not only is ER  $\text{Ca}^{2+}$  loading increased by upregulating SERCA2a expression but that the concentration of whole cell bound intracellular  $\text{Ca}^{2+}$  had also increased.

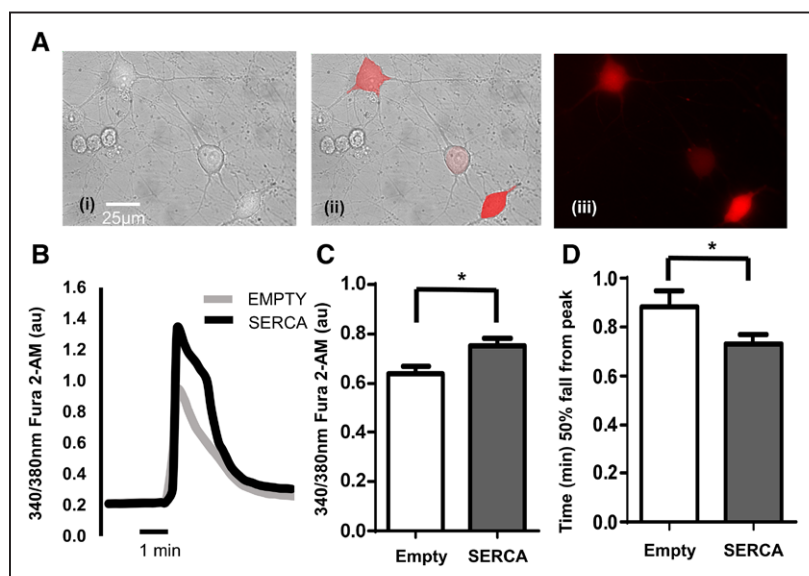
### Intracellular Free $\text{Ca}^{2+}$ Transients in Ad-SERCA2a-Transduced Stellate Neurons of the SHR and WKY

SHR have previously been shown to exhibit high sympathetic drive, even before the onset of hypertension,<sup>16,17,23</sup> and develop heart failure with increasing age,<sup>24</sup> compared with the normotensive WKY. Therefore, the effect of SERCA2a upregulation was studied in these neurons to better reflect the disease model.

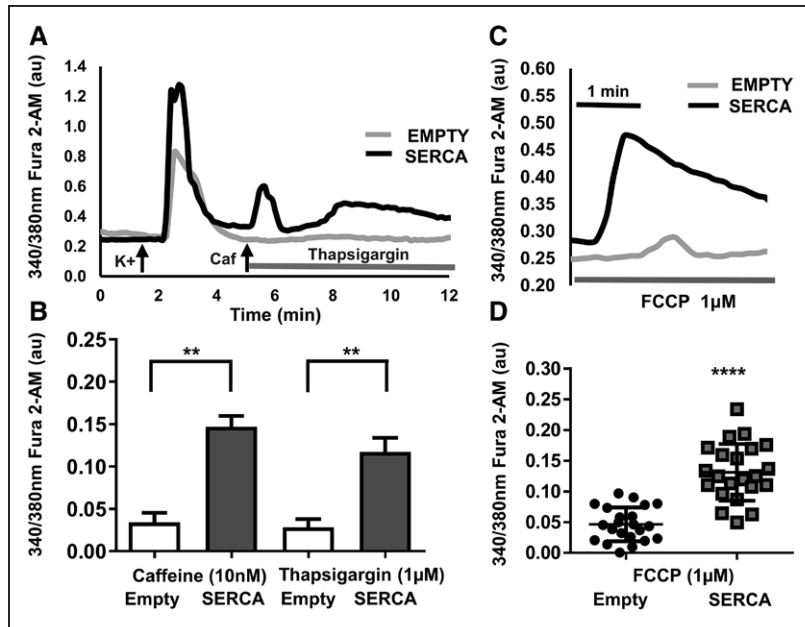
Representative raw data traces (Figure 4A) illustrate that  $[\text{Ca}^{2+}]_i$  transients were significantly greater in neurons of the SHR compared with the WKY rat (as previously reported<sup>17</sup>) in both experimental conditions, when the 2 cell types were carrying either the (i) empty or (ii) SERCA2a transgene ( $*P < 0.05$ ). Moreover, in concordance with the results seen in the SD stellate neurons, SERCA2a overexpression increased  $[\text{Ca}^{2+}]_i$  transients compared with empty control cells in both the WKY and the SHR, group mean data (Figure 4B; WKY, empty:  $0.45 \pm 0.05$  au,  $n=17$ ; SERCA:  $0.66 \pm 0.09$  au,  $n=13$ ; SHR, empty:  $0.65 \pm 0.06$  au,  $n=18$ ; SERCA:  $0.80 \pm 0.04$  au,  $n=25$ ;  $*P < 0.05$ ).

### Transmission Electron Microscopy of ER of Young 4-Week SHR and WKY

Interestingly, transmission electron microscopy images of the ER from stellate ganglia of 4-week-old SHR and WKY rats



**Figure 2.** A, Ad-mCherry-hATP2Aa transfected stellate ganglia neurons from (4 to 5 wk, 90–120 g) Sprague–Dawley (SD) rat. (i) Bright field image, (ii) composite, and (iii) excitation at 587 nm to excite mCherry fluorescent tag. Only cells expressing mCherry fluorescence were used for experiments. B, Example raw data trace from isolated stellate ganglia neurons of the young SD rat (gray line, Ad-mCherry [empty]; black line, Ad-mCherry-hATP2Aa [SERCA (sarcoendoplasmic reticulum calcium ATPase)]) exposed to 50 mmol/L of KCl (30 s) to depolarize the neuron resulting in an increase in intracellular free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ). C, Group mean data showing peak depolarization-evoked intracellular free  $\text{Ca}^{2+}$  increase between Ad-mCherry (gray;  $n=57$ ) and Ad-mCherry-hATP2Aa (black;  $n=68$ ) transfected stellate neurons. D, Group mean data of 50% fall time of ( $[\text{Ca}^{2+}]_i$ ) from the peak (Ad-mCherry, gray;  $n=37$ ; Ad-mCherry-hATP2Aa, black;  $n=42$ ).  $*P < 0.05$ .

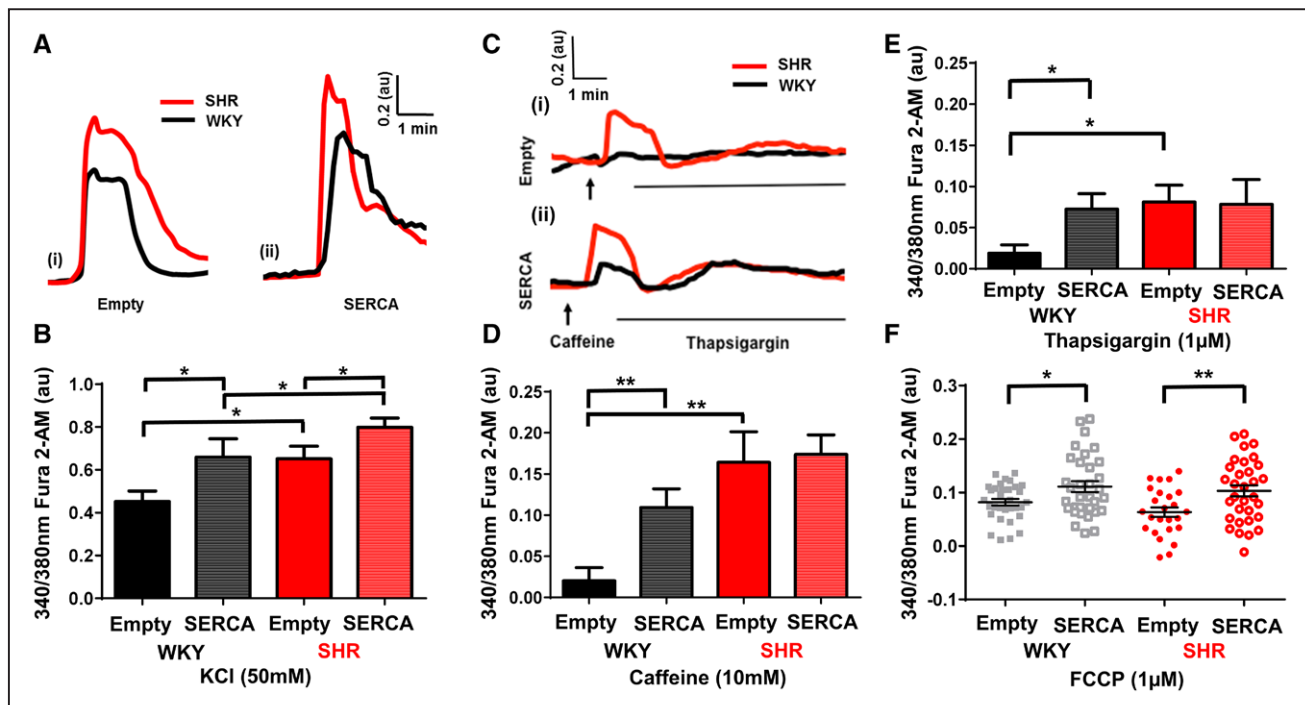


**Figure 3.** **A**, Representative raw data trace showing fluorescence ratio of fura-2/AM to assess the effect of SERCA (sarco-endoplasmic reticulum calcium ATPase) gene transfection on endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  handling. The effect of caffeine (10 mmol/L for 30 s at 5 min; to empty ER  $\text{Ca}^{2+}$  store) and thapsigargin (1  $\mu\text{mol/L}$  thapsigargin from 5 min; to prevent ER  $\text{Ca}^{2+}$  reuptake) evoked intracellular  $\text{Ca}^{2+}$  changes in isolated Sprague–Dawley (SD) stellate neurons transfected with mCherry empty or SERCA. **B**, Group mean data of  $[\text{Ca}^{2+}]_i$  in response to caffeine (empty:  $n=35$ ; SERCA:  $n=45$ ) and thapsigargin (empty:  $n=33$ ; SERCA:  $n=42$ ).  $**P<0.01$ . **C**, Representative raw data trace showing the effect mCherry empty or SERCA2a gene transfection had on mitochondrial  $\text{Ca}^{2+}$  uncoupling by carbonylcyanide- $p$ -trifluoromethoxyphenylhydrazine (FCCP; 1  $\mu\text{mol/L}$ ) in isolated SD rat stellate ganglia neurons. **D**, Group mean data of  $[\text{Ca}^{2+}]_i$  in response to FCCP (empty:  $n=22$ ; SERCA:  $n=22$ ).  $****P<0.0001$ .

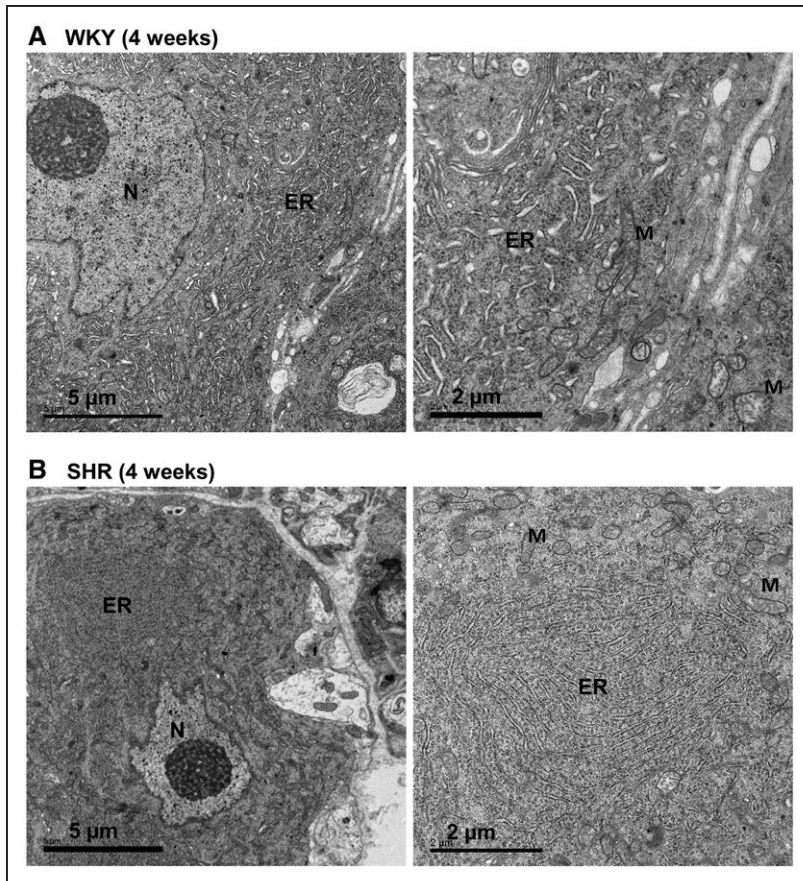
shows a striking difference in ER structure and organization (Figure 5). In SHR rats, the ER is organized into spatially compact sheets compared with the more disperse and varied ER form observed in WKY (Figure 5), suggesting that structural changes might underpin ER  $\text{Ca}^{2+}$  handling differences observed in the SHR compared with the WKY.

### ER $\text{Ca}^{2+}$ Handling in Prehypertensive SHR and Age-Matched WKY Stellate Neurons

SERCA2a-treated cells of WKY neurons showed increased  $[\text{Ca}^{2+}]_i$  in response to mobilization of ER load with caffeine (WKY; empty:  $0.02\pm0.02$  au,  $n=17$ ; SERCA:  $0.11\pm0.02$  au,  $n=13$ ; Figure 4C and 4D) and thapsigargin (empty:  $0.02\pm0.01$



**Figure 4.** **A**, An example raw data traces showing free intracellular  $\text{Ca}^{2+}$  change in response to KCl (30 s; 50 mmol/L) depolarization of isolated stellate ganglia neurons from Wistar Kyoto (WKY; black) and spontaneously hypertensive rats (SHR; red), transfected with mCherry empty (i) or SERCA (sarco-endoplasmic reticulum calcium ATPase; ii). **B**, Group mean data of peak depolarization-induced free intracellular  $\text{Ca}^{2+}$  change (WKY: empty;  $n=17$ ; SERCA;  $n=13$ ; SHR: empty;  $n=18$ ; SERCA;  $n=25$ ).  $*P<0.05$ . **C**, Representative raw data trace showing the effect of SERCA gene transfection on endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  handling, caffeine (10 mmol/L) and thapsigargin (1  $\mu\text{mol/L}$ ), in isolated WKY (black) and SHR (red) stellate neurons transfected with mCherry empty (i) or SERCA (ii). Group mean data of  $[\text{Ca}^{2+}]_i$  in response to caffeine (**D**) and thapsigargin (**E**; WKY: empty;  $n=17$ ; SERCA;  $n=13$ ; SHR: empty;  $n=18$ ; SERCA;  $n=25$ ). **F**, Effect on mitochondrial  $\text{Ca}^{2+}$  uncoupling by carbonylcyanide- $p$ -trifluoromethoxyphenylhydrazine (FCCP; 1  $\mu\text{mol/L}$ ) in isolated WKY and SHR rat stellate ganglia neurons with either mCherry empty or SERCA gene transfection. Group mean data of WKY: empty;  $n=32$ ; SERCA;  $n=33$ ; And SHR: empty;  $n=25$ ; SERCA;  $n=32$ .  $**P<0.01$ .



**Figure 5.** Transmission electron micrographs of stellate neurons in young Wistar Kyoto (WKY) rat (**A**) and spontaneously hypertensive rats (SHR; **B**). Endoplasmic reticulum (ER) morphology is affected in stellate neurons from SHRs. WKY rat shows a dispersed ER network, whereas SHR rats exhibit regions of highly enriched sheet-like ER. M indicates mitochondria; and N, nucleus.

au,  $n=17$ ; SERCA:  $0.07 \pm 0.02$  au,  $n=13$ ;  $*P < 0.05$ ;  $**P < 0.01$ ; Figure 4E). Although mCherry empty-treated SHR neurons had a greater increase in  $[Ca^{2+}]_i$  in response to caffeine and thapsigargin compared with mCherry empty-treated WKY neurons, no further increase was observed after treatment with SERCA2a (SHR caffeine, empty:  $0.16 \pm 0.04$  au,  $n=18$ ; SERCA:  $0.17 \pm 0.02$  au,  $n=25$ ; thapsigargin, empty:  $0.08 \pm 0.02$  au,  $n=18$ ; SERCA:  $0.08 \pm 0.03$  au,  $n=25$ ; Figure 4C–4E). These data suggest that stellate neurons of the prehypertensive SHR present altered ER  $Ca^{2+}$  handling compared with normotensive WKY controls, even before upregulation of the SERCA2a transporter, in keeping with the observation that there is more ER per unit of cell volume in SHR neurons. Moreover, under basal conditions, upregulation of SERCA2a seems to have no further compounding effect on ER loading in the SHR, suggesting that the ER may already be working at full  $Ca^{2+}$  load.

#### Effect of Mitochondrial $Ca^{2+}$ Handling in SHR and Age-Matched WKY Stellate Neurons

In SHR stellate neurons, transfection with SERCA2a was still able to increase the mitochondrial  $Ca^{2+}$  store as measured using the mitochondrial membrane uncoupler FCCP (Figure 4F), despite not increasing ER  $Ca^{2+}$  load. This may explain why SERCA2a transfection still leads to an increased overall depolarization-induced  $[Ca^{2+}]_i$  transient in these neurons. WKY neurons overexpressing SERCA2a also had a greater increase in  $[Ca^{2+}]_i$  in response to FCCP compared with mCherry empty controls (Figure 4F; WKY, empty:  $0.08 \pm 0.006$  au,  $n=32$ ;

SERCA:  $0.11 \pm 0.01$  au,  $n=33$ ; SHR, empty:  $0.06 \pm 0.009$  au,  $n=25$ ; SERCA:  $0.10 \pm 0.01$  au,  $n=32$ ;  $**P < 0.01$ ).

#### Discussion

The key findings of this study are as follows: (1) The SERCA2a protein (predominantly thought to be the cardiomyocyte isoform of SERCA<sup>1,2</sup>) can be transduced into rat stellate neurons. (2) Upregulating SERCA2a in normal rat stellate neurons leads to greater depolarization-induced  $Ca^{2+}$  transients, as well as greater ER and mitochondrial  $Ca^{2+}$  load. (3) Right atrial percutaneous injection of the SERCA2a virus results in increased  $[^3H]$ -NE release in response to right stellate stimulation. (4) Stellate neurons from SHRs have a greater ER calcium load than the WKY and have a greater abundance of ER per unit cell volume compared with WKY neurons. (5) SERCA2a overexpression does not increase ER load further in SHR neurons, but still increases the depolarization-induced  $Ca^{2+}$  transient, potentially through increased mitochondrial calcium loading.

#### SERCA2a Upregulation Results in Enhanced Intracellular $Ca^{2+}$ Handling in Sympathetic Neurons

The predominant neuronal SERCA isoforms are SERCA3 in the cerebral cortex,<sup>25</sup> SERCA2b in hippocampal pyramidal neurons,<sup>26</sup> with low levels of SERCA2a expression in superior cervical ganglia neurons of young SHR and WKY rats, with no observed differences between the 2 strains.<sup>16</sup> Incorporation of the SERCA2a isoform into cardiac stellate neurons resulted in increased depolarization-induced  $Ca^{2+}$  transients in normotensive and prehypertensive animal models and increased ER

Ca<sup>2+</sup> load within stellate neurons isolated from young SD and WKY rats. Increased depolarization-induced Ca<sup>2+</sup> transients have previously been described in sympathetic neurons of the neonatal to adult SHR compared with age-matched WKY rat controls.<sup>17</sup> Isolated neurons from the superior cervical ganglia have alluded to increased ER Ca<sup>2+</sup> load in young prehypertensive SHR,<sup>16</sup> analogous to the data shown here in stellate neurons exposed to the mCherry empty viral vector. Young prehypertensive SHR had larger ER Ca<sup>2+</sup> stores and greater caffeine-evoked ER Ca<sup>2+</sup> release compared with WKY neurons. This may be related to the activity of the SERCA transporter that is under the control of regulatory protein phospholamban. Phospholamban (PLN) is a small phosphoprotein that can regulate the activity of the SERCA. Dephosphorylated PLN is an inhibitor of SERCA, whereas phosphorylation of PLN relieves its inhibition.<sup>27</sup> We have previously reported that expression of phosphorylated (Ser16) compared with total PLN is reduced in prehypertensive SHR superior cervical ganglion neurons. Therefore, less dephosphorylated PLN may increase SERCA activity, resulting in more rapid reuptake of calcium into the ER and faster recovery of the intracellular calcium transient in the prehypertensive SHR.<sup>16</sup> We have now evaluated SERCA2a and PLN expression in stellate ganglia of WKYs and SHRs at 9 to 10 months of age when the SHR develops impaired left ventricular function.<sup>28</sup> At this age, these preliminary data suggest there is no apparent statistical difference in the expression of both SERCA2a (WKY: 1.000±0.0003 au, n=3; SHR: 0.572±0.232 au, n=3) and PLN (WKY: 1.01±0.01 au, n=3; SHR: 1.52±0.52 au, n=3). However, given the small sample size and the difficulty in extracting sufficient levels of protein from this small ganglion, we cannot rule out that physiological reductions in SERCA2a occurred.

Although SERCA2a overexpression increased ER Ca<sup>2+</sup> load in WKY stellate neurons to a level comparable with the SHR, no difference was seen in the ER load of the SHR between SERCA2a overexpression and control. This indicates that part of the faulty and heightened Ca<sup>2+</sup> handling observed in stellate neurons of the SHR may be because of already maximal Ca<sup>2+</sup> loading of the ER. Electron microscopy of the young WKY and prehypertensive SHR indicates that the ER is more densely and structurally organized within stellate neurons of the SHR. Although ER load was not altered in SHR neurons with SERCA2a overexpression, an increase in depolarization-induced Ca<sup>2+</sup> transients in the SHR was still observed that may be because of both a greater ER and a mitochondrial Ca<sup>2+</sup> load and release after subsequent depolarization. It remains to be seen whether sympathetic neuronal ER Ca<sup>2+</sup> is already maximally loaded in a heart failure model, and whether upregulating SERCA2a in these neurons would also increase the depolarization-induced calcium transient and subsequent NE release. However, the fact that there is no difference in the expression of SERCA2a or PLN in 9- to 10-month-old SHR and WKYs and SERCA2a overexpression is still able to increase the depolarization-induced Ca<sup>2+</sup> transient in prehypertensive SHRs when the ER is fully loaded makes potentiation of sympathetic neurotransmission likely.

Mitochondria are fundamentally important for maintaining cellular Ca<sup>2+</sup> homeostasis, as well as energy production. Mitochondrial research has shown them to be necessary for regulating Ca<sup>2+</sup> in many physiological processes, including

vasomotion in blood vessels<sup>29</sup> and accumulation of Ca<sup>2+</sup> when cytosolic levels are low in synaptosomes.<sup>30</sup> Functional or direct coupling of the ER and mitochondria has been suggested in many cell types, including sympathetic neurons,<sup>31,32</sup> and mitochondria have been indicated to be involved in the uptake of ER-released Ca<sup>2+</sup>, regulating neuronal excitability.<sup>32</sup> FCCP depolarization within this study has been used as a means to assess mitochondrial Ca<sup>2+</sup> load, although it does not rule out that part of the Ca<sup>2+</sup> transient observed with FCCP could be because of a coupling between the mitochondria and the ER. The difference observed with FCCP-liberated free Ca<sup>2+</sup> in SHR SERCA2a neurons indicates that FCCP is predominantly releasing Ca<sup>2+</sup> from a non-ER store. The transient time scales of the application of FCCP reduce the chance that the observed Ca<sup>2+</sup> transients are because of changes in energy production of the cell inhibiting SERCA activity by reducing ATP production.

Increased mitochondrial Ca<sup>2+</sup> concentrations alter the mitochondrial membrane potential, with elevated mitochondrial Ca<sup>2+</sup> levels being linked to impaired mitochondrial energetics<sup>33</sup> and increased oxidative stress within cardiomyocytes.<sup>34,35</sup> Previously, SERCA2a transgene in cardiomyocytes has been predicted to be protective at preventing mitochondrial stress by ensuring resting intracellular Ca<sup>2+</sup> levels remain low,<sup>36</sup> thereby protecting the myocardial energetics of the cell.<sup>37,38</sup> We could not directly record the mitochondrial membrane potential in this study because of the mCherry fluorescent tag exhibiting cross fluorescent spectra with tetramethylrhodamine ethyl ester (used to measure mitochondrial membrane potential<sup>16</sup>). Therefore, it remains to be established whether SERCA overexpression protects neuronal energetics.

### SERCA2a Overexpression Results in Increased Neurotransmitter Release in Response to Right Stellate Stimulation

Right atrial injection of adeno- and lentiviral constructs has previously been established as viable tools to upregulate target genes of interest that can modulate neurotransmission in cardiac autonomic nerves.<sup>39–43</sup> We established whether the increased depolarization-induced Ca<sup>2+</sup> transients and elevated intracellular Ca<sup>2+</sup> handling observed in isolated stellate neurons functionally translates. Direct stimulation of the isolated stellate ganglia infected with the SERCA2a gene construct resulted in significantly greater neurotransmitter release from the atria and potentially greater postsynaptic excitability. Although we have highlighted the effect that incorporation of atrial-injected SERCA2a gene transfer on sympathetic neurons may have, we cannot rule out that the viral construct could also be expressed in both cardiac afferent and vagal nerve fibers. This may alter local network processing and subsequent NE release, for example, via the release of other neurotransmitters and neuropeptides.

### Limitations

As has previously been described,<sup>39</sup> transfection rate with adenovirus is not 100% efficient (~70%); therefore, it was vital that before Ca<sup>2+</sup> imaging experiments, only cells expressing the target gene were used. Our mCherry fluorescent tag confirmed cells had integrated the transgene and were expressing the protein of interest. Inefficient transfection rate without a

method of monitoring gene delivery could result in inconsistent or false-negative results.

Although within this study we have highlighted the effects of incorporation of SERCA2a into cardiac sympathetic nerves, the stellate ganglia contain a heterogeneous profile of sympathetic efferent cardiac and noncardiac neurons.<sup>44,45</sup> The promiscuous nature of the cytomegalovirus-Ad viruses used means it is highly likely that all neurons of the stellate ganglia had the potential to overexpress SERCA2a after viral transfection. Overexpression of SERCA2a could have resulted in altered intracellular  $\text{Ca}^{2+}$  handling in neurons innervating noncardiac, as well as cardiac, tissue. Cardiac neurons and noncardiac neurons from the rat stellate ganglia do not have clearly distinct morphologies or resting membrane potentials<sup>45</sup> and have been identified through their electrophysiological responses to cardiac nerve stimulation<sup>45</sup> or through their endogenous activity in relation to the cardiac cycle,<sup>44</sup> which cannot be assessed in isolated cultured neurons during calcium imaging. The inclusion of some noncardiac neurons may have introduced variability within our experimental groups although all sympathetic neurons responded similarly with SERCA2a overexpression.

To assess the functional significance of SERCA2a gene therapy coinfecting the cardiac autonomic axis, in vivo large mammal models would have to be studied to establish whether this gene transfer approach translated into more cardiac excitability. Specifically, this would need to be performed in an established heart failure model. Because adenovirus and AAV have high specificity of transfection for both myocytes and nerve cells,<sup>13</sup> it is plausible that the intracoronary perfusion of SERCA2a gene constructs within the CUPID trials could have resulted in gene transfection into cardiac sympathetic neurons, as well as cardiomyocytes.<sup>9</sup> Studies in spinal cord injury have shown that AAV–green fluorescent protein transduction close to the site of injury can result in the spread of a green fluorescent protein–tagged fluorescence throughout the spinal cord and into the central nervous system.<sup>46</sup> This suggests the need for cell specific targeting in gene therapy.

## Perspectives

Overexpression of SERCA2a using a promiscuous cytomegalovirus viral promoter resulted in increased neurotransmission and altered intracellular  $\text{Ca}^{2+}$  handling within neurons isolated from the stellate ganglia of normotensive rats. Recent use of gene therapy in clinical trials of heart failure failed to show a beneficial effect of SERCA2a overexpression targeted at myocytes, constructed under a similar promiscuous promoter. The potential for off target expression of the SERCA2a transgene in other cell types, including sympathetic neurons, may have compounded these results. Whether SERCA2a overexpression has a similar effect on the cardiac sympathetic neural axis in heart failure remains to be established.

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## Disclosures

None.

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## Novelty and Significance

### What Is New?

- The first study to demonstrate that upregulating a myocyte isoform of SERCA (sarcoendoplasmic reticulum calcium ATPase) into isolated sympathetic neurons affects intracellular  $\text{Ca}^{2+}$  handling dynamics.

### What Is Relevant?

- Dysregulation of SERCA2a in heart failure has led to the development of therapeutic strategies to upregulate its activity in myocytes. It is feasible possible these viruses may also infect sympathetic neurons altering their  $\text{Ca}^{2+}$  handling phenotypes.
- An observed increase in endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  capacity within the prehypertensive spontaneously hypertensive rats may contribute to the sympathetic hyperactivity.

### Summary

An adenoviral gene transfer technique to upregulate SERCA2a in isolated sympathetic neurons resulted in altered intracellular  $\text{Ca}^{2+}$  handling, greater ER  $\text{Ca}^{2+}$  load, and greater depolarization-induced  $\text{Ca}^{2+}$  transients within 4- to 5-week Sprague–Dawley and Wistar Kyoto rats. Upregulation of SERCA2a expression had no effect on the ER  $\text{Ca}^{2+}$  load within 4- to 5-week prehypertensive spontaneously hypertensive rats, and transmission electron microscopy revealed a more disperse and varied ER appearance compared with the Wistar Kyoto, indicating that increased ER  $\text{Ca}^{2+}$  capacity and altered intracellular  $\text{Ca}^{2+}$  handling within the prehypertensive spontaneously hypertensive rats may contribute to the observed sympathetic hyperactivity. Therapeutic targets aimed at reducing rather than increasing ER load within sympathetic neurons in hypertension may be beneficial.

## Overexpression of Sarcoendoplasmic Reticulum Calcium ATPase 2a Promotes Cardiac Sympathetic Neurotransmission via Abnormal Endoplasmic Reticulum and Mitochondria $\text{Ca}^{2+}$ Regulation

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## Online methods supplement

### **Overexpression of SERCA2a promotes cardiac sympathetic neurotransmission via abnormal endoplasmic reticulum and mitochondria Ca<sup>2+</sup> regulation.**

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## **Animals**

SD rats were used as a proof of principle study in a normotensive model. We also assessed the effect that upregulating SERCA2a expression has on  $\text{Ca}^{2+}$  handling in stellate neurons from young SHR. These animals express cardiac sympathetic hyperactivity compared to age matched WKY controls prior to the development of hypertension<sup>1</sup>, and progress to develop heart failure with age<sup>2</sup>. Cardiac sympathetic hyperactivity is a negative prognostic indicator in the development of hypertension and heart failure in clinical and animal models. The majority of experiments and all cellular imaging were performed in young animals (90-120 g). This allowed for maximal cell yield and maximum success rate of cell culture, whilst still using animal models that displayed sympathetic phenotypes concordant with their adult counterparts. Older rats (350-380g) were used for the percutaneous viral gene transfer experiments to better reflect the anatomical adult model.

## **Isolation and preparation of sympathetic post-ganglionic neurons**

Sympathetic neurons were isolated from the stellate ganglion that predominantly innervate the heart<sup>3</sup>, from 90g-120 g rats as previously described<sup>4</sup>. Rats were rendered unconscious using general anesthesia (3% isoflurane and 97% oxygen) then humanely killed by an approved Home Office schedule 1 method: cervical dislocation followed by exsanguination. Dissociated neurons were plated onto poly-D-lysine/ laminin substrate coated cover slips (5mm), placed at 37°C 5%  $\text{CO}_2$  for 3 days before use. The medium used for the isolation and culture of the isolated postganglionic sympathetic neurons were based on modifications of those previously described<sup>25</sup>. In brief, dissected and cleaned ganglion were placed in a 10ml falcon tube containing collagenase type 4 (1 mg/ml in  $\text{Ca}^{2+}$  and  $\text{Mg}^{+2}$ -free phosphate-buffered saline, PBS) for 14 min at 37 °C in a shaking water bath. The collagenase was removed and replaced with trypsin TRL (2mg/ml in PBS) for 14 min 37°C. The neurons were then washed for 2 x 3 min washes in an L-15 based blocking medium, followed by 2 x 3 min washes in L-15 based plating medium to stop the action of the digestion enzymes.

Ganglia were titrated into a single-cell suspension with a narrow opening fire polished Pasteur pipette 5 times. After the trituration the solution was left to rest for 1.5 minutes to allow any large tissue fragments to settle to the base of tube whilst leaving the single cells in suspension. The top 0.5 ml of solution was then removed and placed in a plastic petri dish (2.5 mm diameter). 0.5 ml of fresh L-15 based plating medium was added to the falcon tube and this process was repeated 5 times. The cell suspension solution was then added to wells containing Poly-D lysine and laminin coated cover slips (6 mm), and incubated at 37°C 5%  $\text{CO}_2$  for 3 days before use.

## **Measurement of free intracellular $\text{Ca}^{2+}$ concentration**

### *Virus infection*

Isolated stellate ganglion neurons were incubated with the virus, either Ad-mCherry ( $2 \times 10^7$  PFU/ml), or Ad-SERCA2a-mCherry ( $6.4 \times 10^7$  PFU/ml) for 24 hours post isolation. Cells were then changed to fresh virus free media for 48 hours prior to imaging. Cells were checked for mCherry fluorescence to confirm expression of the viral gene transferred protein before each experiment.

## *Protocol*

Neurons were loaded with 2.5  $\mu\text{M}$  Fura-2/AM for 40 min at room temperature in Tyrodes solution, then washed for 10min with room temperature Tyrodes solution to allow the intracellular Fura-2/AM to de-esterify. Loaded neurons were transferred to a temperature controlled (37 °C) gravity fed perfusion chamber (volume 500  $\mu\text{l}$ ); flow rate 3 ml/min, visualised on a Nikon Eclipse TE200-U microscope with a 40X oil immersion lens. Cells expressing fluorescence of the mCherry tag (excitation 587 nm; emission 610 nm) were selected for study.

Images were acquired every 3 seconds using a photometrics CoolSNAP HQ<sup>2</sup> camera. Neurons were excited alternately at 340 nm and 380 nm and detecting emission at 510 nm. The ratio of 380/340 nm gave a measure of free intracellular  $\text{Ca}^{2+}$  concentration change.

Application of KCl (50 mM; 30 s), caffeine (10 mM; 30 s), thapsigargin (1  $\mu\text{M}$ ; 10 min), and FCCP (1  $\mu\text{M}$ ; 3 min), were used to evoked transient intracellular  $\text{Ca}^{2+}$  changes.

### **Measurement of local 3H-norepinephrine release from isolated double atria.**

350-380 g SD rats were given a percutaneous right atrial injection (under 3% isoflurane) of either Ad-mCherry or Ad-SERCA2a-mCherry ( $3 \times 10^9$  PFU/ml) in 300  $\mu\text{l}$  PBS. Experiments were performed 5-6 days post gene transfer.

Spontaneously beating right atria with intact sympathetic innervations and right stellate ganglion were isolated from 350-380 g SD rats, and transferred to a pre-heated ( $37 \pm 0.2^\circ\text{C}$ ), water jacketed, carbogen-aerated water bath containing 3 ml Tyrode solution. The atria were pinned flat over a silver stimulating electrode, and the right stellate ganglion was threaded over a two prong stimulating electrode and fixed in place. The method for determining local <sup>3</sup>H-norepinephrine (NE) release was based on one previously described<sup>26</sup>. Briefly, following a 20min equilibration period, the double atria preparation was incubated with 5  $\mu\text{M}$  <sup>3</sup>H-NE (0.185 MBq, Perkin Elmer) and ascorbic acid (30  $\mu\text{M}$ , Sigma). The atria were field stimulated at 5Hz (15 V, 1 ms pulse width) for 10 s every 30 s, for 30 min to facilitate uptake of 3H-NE into the transmitter stores of the pre-synaptic terminal. Following <sup>3</sup>H-NE incubation excess radioactivity was washed from the preparation with Tyrode's solution superfusion for 45 min, at a rate of 3 ml/min. Bath solution was then replaced every 3 min for 51 min. A 0.5 ml sample from each solution change was added to 4.5 ml scintillation liquid (Ecoscint A, National Diagnostics) and the amount of radioactivity measured (counts per minute, CPM) using a liquid scintillation counter (Tri-Carb 2800TR, Perkin Elmers Life Science). At 16 min the atria were field stimulated at 5 Hz for 1 min, at 41 min the right stellate were stimulated at 5 Hz for 1 min. The <sup>3</sup>H-NE outflow from the right atria was presented as change in counts per minute in response to stimulation of the stellate compared to the data point taken immediately before the peak. Post experiment right atria were snap frozen to be used in western blot and confirm transgene expression.

### **Western blot**

Right atria from 350-380 g SD rats post <sup>3</sup>H-NE overflow measurements were immediately snap frozen in liquid nitrogen. Atria were lysed in the presence of proteases inhibitors to prevent target protein breakdown. Sample protein concentrations were quantified according to the

Bradford protein assay. 20 µg of total protein was separated by SDS-PAGE. The expression of SERCA2a (abcam 1:1000) and β-actin (Sigma 1:1000) were detected. Immunodetection was based on chemiluminescence quantification (Western Lightning Plus, Perkin Elmer Life Science). The results were normalized to β-actin that served as a loading control, and antibody specificity tested using rat thigh skeletal muscle lysate as a negative control to SERCA2a.

### Electron microscopy

Excised ganglion tissue was immediately immersed in pre-warmed fixative (2.5% glutaraldehyde, 2% PFA in 0.1 M sodium cacodylate buffer, pH 7.2) for approximately 2 h at room temperature, then stored at 4 °C for two weeks before further processing. Samples were then washed on a rotor in 0.1 M sodium cacodylate buffer pH 7.2 for 4x 30 min, then in 100 mM glycine in the same buffer for 60 min and finally for 2x 30 min in sodium cacodylate buffer. Samples were then incubated in 1% osmium tetroxide in the same buffer for 2 h at 4°C, washed with water for 3x 20 min and incubated in 0.5% uranyl acetate (aqueous) overnight at 4°C. Samples were washed briefly with water, then taken through a graded ethanol series (30%, 50%, 70%, 80%, 90% and 95% ethanol for 15 min each, then 100% ethanol for 2 h, with 3 solution changes during this time). Samples were gradually infiltrated with TAAB TLV epoxy resin, starting with 25% resin for 1 hr, 50% resin for 2 h, 75% resin for 1.5 h and 100% resin overnight. Samples were transferred to a fresh 2ml tube of resin, spun for ~30s in a mini centrifuge then placed on a rotator at room temperature for several hours. This step was repeated four more times, with another overnight incubation in between. Individual tissue pieces were transferred to Beem capsules filled with fresh resin, then polymerised for 48 hrs at 60 °C. Blocks were sectioned using a Leica UC7 ultramicrotome with a diamond knife (Diatome). Ultrathin sections (90nm) were placed on 200 mesh Copper grids, post-stained with Reynold's lead citrate for 5 min, and imaged on a FEI Tecnai 12 Transmission Electron Microscope (TEM)EM operated at 120 kV using a Gatan OneView CMOS camera.

### Solutions and Drugs

Tyrode solution for isolated postganglionic sympathetic neuron experiments contained (in mmol/L: NaCl 145, KCl 5, HEPES 10, glucose 10, CaCl<sub>2</sub> 2 and MgCl<sub>2</sub> 1) (pH 7.38-7.42, 37±0.5°C). All drugs were stored as stock solutions and made to the desired concentration on the day. All drugs underwent no more than one freeze thaw cycle. Tyrode solution for isolated right atria/right stellate ganglia experiments contained (mmol/L NaCl 120, KCl 4.7, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, CaCl<sub>2</sub> 2, KH<sub>2</sub>PO<sub>4</sub> 1.2 and glucose 11) and was aerated with 95 % O<sub>2</sub>/5 % CO<sub>2</sub> (pH 7.4).

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