Targeted Neuronal Nitric Oxide Synthase Transgene Delivery Into Stellate Neurons Reverses Impaired Intracellular Calcium Transients in Prehypertensive Rats

Dan Li, Natalia Nikiforova, Chieh-Ju Lu, Kate Wannop, Mary McMenamin, Chee-wan Lee, Keith J. Buckler, David J. Paterson

Abstract—Hypertension is associated with the early onset of cardiac sympathetic hyperresponsiveness and enhanced intracellular Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_i\) in sympathetic neurons from both prehypertensive and hypertensive, spontaneously hypertensive rats (SHRs). Oxidative stress is a hallmark of hypertension, therefore, we tested the hypothesis that the inhibitory action of the nitric oxide-cGMP pathway on [Ca\(^{2+}\)]\(_i\) transients is impaired in cardiac sympathetic neurons from the SHR. Stellate ganglia were isolated from young prehypertensive SHRs and age-matched normotensive Wistar-Kyoto rats. [Ca\(^{2+}\)]\(_i\) was measured by ratiometric fluorescence imaging. Neurons from the prehypertensive SHR ganglia had a significantly higher depolarization evoked [Ca\(^{2+}\)]\(_i\) transient that was also associated with decreased expression of neuronal nitric oxide synthase (nNOS), \(\beta\)1 subunit of soluble guanylate cyclase and cGMP when compared with the Wistar-Kyoto rat ganglia. Soluble guanylate cyclase inhibition or nNOS inhibition increased [Ca\(^{2+}\)]\(_i\) in the Wistar-Kyoto rats but had no effect in SHR neurons. A nitric oxide donor decreased [Ca\(^{2+}\)]\(_i\) in both sets of neurons, although this was markedly less in the SHR. A novel noradrenergic cell specific vector (Ad.PRSx8-nNOS/Cherry) or its control vector (Ad.PRSx8-Cherry) was expressed in sympathetic neurons. In the SHR, Ad.PRSx8-nNOS/Cherry-treated neurons had a significantly reduced peak [Ca\(^{2+}\)]\(_i\) transient that was associated with increased tissue levels of nNOS protein and cGMP concentration compared with gene transfer of Ad.PRSx8-Cherry alone. nNOS inhibition significantly increased [Ca\(^{2+}\)]\(_i\) after Ad.PRSx8-nNOS/Cherry expression. We conclude that artificial upregulation of stellate sympathetic nNOS via targeted gene transfer can directly attenuate intracellular Ca\(^{2+}\) and may provide a novel method for decreasing enhanced cardiac sympathetic neurotransmission. (Hypertension. 2013;61:000-000.)

Key Words: hypertension ■ calcium transient ■ NO ■ gene transfer ■ sympathetic neuron

Nitric oxide (NO) synthesis catalyzed by neuronal NO synthase (nNOS) acts as a biological messenger involved in events crucial to neuronal cell function, such as neurotransmitter release and gene transcription.\(^1\) Emerging evidence shows that disruption of the NO-cGMP pathway enhances sympathetic function at several sites in the cardiac-neural axis.\(^2\)\(^–\)\(^5\) Hypertension causes enhanced neurohumoral activation\(^6\)\(^–\)\(^8\) and abnormal calcium signaling in cardiac sympathetic neurons,\(^9\) including the pacemaker cells they innervate.\(^10\) The mechanisms responsible for this sympathetic phenotype still remain elusive. One candidate is oxidative stress that is reported to disrupt NO-soluble guanylate cyclase (sGC)-cGMP–dependent pathways that couple to the regulation of intracellular calcium.\(^10\)\(^,\(^11\)\) This pathway appears to play an important role in determining cardiac noradrenergic activity both pre- and postjunctionally at the end organ level during the development of hypertension.\(^2\)\(^,\(^10\)

Gene transfer using adenoviral vectors constructed with a noradrenergic neuron-specific promoter (PRSx8) to drive nNOS (Ad.PRSx8-nNOS) is highly effective in modifying gene expression\(^12\) and decreasing intracellular Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_i\) in cardiac sympathetic postganglionic neurons from normotensive rats.\(^13\) Moreover, it also decreases cardiac sympathetic transmission in adult normotensive\(^12\) and spontaneously hypertensive rats (SHRs).\(^2\) Therefore, we hypothesized that the recently reported impaired calcium handling in postganglionic stellate sympathetic neurons in young (prehypertensive) SHRs was a consequence of impaired NO-cGMP signaling that occurs as an early cellular marker linked to disruption of Ca\(^{2+}\) regulated exocytosis. We further hypothesized that transduction of SHR stellate sympathetic neurons with targeted gene transfer with nNOS marked by mCherry could restore normal NO-cGMP signaling and rescue abnormal calcium handling.
Materials and Methods

Animals
Prehypertensive young (34±3 days), male SHRs (n=64) and normotensive Wistar-Kyoto (WKY; n=33) rats were used in this study. Cells were isolated from the cardiac stellate ganglia for phenotyping. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85–23, revised 1996) and the Animals (Scientific Procedures) Act 1986 (United Kingdom).

An expanded Materials and Methods section is available in the online-only Data Supplement.

Results
Cardiac-Neural Phenotype of the Prehypertensive SHRs
Western Blotting and cGMP Production
Hearts from the young SHRs were not hypertrophied compared with the age-matched WKY (ventricular weight [g]; body weight [g] ratios of 3.73±0.43×10⁻³ [SHR, n=28] versus 3.64±0.45×10⁻³ [WKY, n=16]; P=0.15, unpaired t test). Protein level of β-actin that was taken from stellate ganglia did not differ between SHRs and WKY rats (intensity of bands, 2.76±0.21×10⁵ in SHR versus 2.71±0.21×10⁵ in WKY, n=16). Expression of nNOS and β1-sGC normalized to β-actin was significantly lower in the SHR (P<0.05, t test; Figure 1A and 1C). However, the α1 subunit of sGC protein was not different between the 2 strains (Figure 1B). cGMP concentration in stellate ganglia was significantly lower in the young SHRs compared with the WKY rats (Figure 1D).

Intracellular Free Calcium Transients in Cardiac Sympathetic Neurons
Intracellular calcium concentration was measured by ratiometric imaging of Fura-4F in single cardiac sympathetic neurons. Two to 4 separate cultures were used in each experiment. Each coverslip was only imaged once because of different pharmacological interventions. A typical calcium transient response to 100 µmol/L of KCl is shown in Figure 2A. Baseline [Ca²⁺], was not different between the 2 strains (SHR, 0.48±0.05 µmol/L, n=45; WKY, 0.43±0.05 µmol/L, n=48; P=0.56, unpaired t test). The high K⁺ evoked [Ca²⁺], transient was significantly enhanced in the stellate ganglia of the SHRs when compared with age-matched WKY rats (Figure 2B).

Pharmacological Manipulation of NO-cGMP Pathway
To determine whether the NO-cGMP pathway was impaired in the SHR, the exogenous NO donor SIN-1 (10 µmol/L), guanylyl cyclase inhibitor 1H-(1,2,4)-oxadiazolo-(4,3-a)-quinoxalin-1-one (10 µmol/L), and the nNOS-specific inhibitor N-(4S)-4-amino-5-[(2-aminooethyl)aminopentyl]-N'-nitroguanidine (AAAN; 10 µmol/L) were introduced separately at 6 minutes from the start of the experiment after the first high K⁺ stimulation (S1; Figure 3A). After 10 minutes of incubation, neurons were stimulated again (S2 at 16 minutes) in the presence of drugs. In the control experiments, no significant changes were observed between S1 and S2 (Figure S2) in both SHRs and WKY rats. SIN-1 significantly decreased high K⁺ evoked [Ca²⁺], in both strains (31.07±7.07% in the WKY rats, P<0.01; Figure 3B), although this was markedly less in the SHRs (10.89±3.88%, P<0.05; Figure 3C). Conversely, oxadiazolo-(4,3-a)-quinoxalin-1 and AAAN increased high K⁺ evoked [Ca²⁺], by 14.06±4.22% (P<0.01) and 17.64±6.86% (P<0.05) in the WKY rats, respectively (Figure 3B), but they did not affect high K⁺ evoked [Ca²⁺], in the SHRs (Figure 3C). Taken together, these results show there is a significant molecular and cellular impairment in prehypertensive SHR stellate sympathetic neurons involving the nNOS-sGC-cGMP pathway linked to [Ca²⁺], signaling.

Effect of nNOS Gene Transfer
Western Blotting and cGMP Production
Western blotting in SHRs showed increased nNOS expression in Ad.PR5x8-nNOS/Cherry-transduced stellate ganglia compared with those transduced with Ad.PR5x8-Cherry (Figure 4A). nNOS expression in nNOS virus-transduced SHR stellate ganglia was not different compared with the WKY (Figure S3A). The α1 and β1 subunits of sGC proteins were not affected by overexpression of nNOS (Figure 4B and 4C). However, cGMP concentration from infected SHR stellate ganglia was significantly increased in the transduced with Ad.PR5x8-nNOS/Cherry when compared with the transduced with Ad.PR5x8-Cherry (Figure 4D).

Figure 1. Representative Western blot and group mean data showing a significant reduction in stellate ganglia neuronal NO synthase (nNOS) expression in the young spontaneously hypertensive rats (SHRs) compared with the Wistar-Kyoto (WKY) rats (A), with no significant change in α₁ subunit of guanylate cyclase (α₁-sGC) protein expression (B), and significant reduction in β₁-sGC (C). Western blot band optical density was normalized to that of β-actin as a loading control. D, Tissue levels of cGMP in the stellate ganglia from the young SHRs were significantly lower than that measured in the WKY rats. *P<0.05, **P<0.01, unpaired t test.
Intracellular Free Calcium Transients in Cardiac Sympathetic Neurons

Fluorescence microscopy detected mCherry expression in stellate sympathetic neurons after transduction with Ad.PRSx8-cherry (Figure 5A) or Ad.PRSx8-nROS/cherry (Figure 5B). However, not all neurons expressed mCherry (Figure 5Aii and 6A). We compared the high K+ evoked increased [Ca2+]i transient from mCherry-expressing and nonexpressing cardiac sympathetic neurons after gene transfer of Ad.PRSx8-nROS/cherry in SHRs and WKY rats. [Ca2+]i transient was significantly decreased in mCherry-expressing neurons when compared with the nonexpressing neurons in both strains (Table S1, available in the online-only Data Supplement). This indicates the importance of having an independent fluorescent marker to confirm a transgene expression. We did not systematically look across the whole coverslip quantitatively, but we estimated that our transduction efficiency was ≈60% to 70%. We only chose the mCherry-expressing neurons to measure the calcium transient for subsequent comparison between WKY and SHR cells.

Baseline [Ca2+]i was not altered after transfer of Ad.PRSx8-cherry in either strain compared with the nontransduced neurons in both strains (Table S1, available in the online-only Data Supplement). This indicates the importance of having an independent fluorescent marker to confirm a transgene expression. We did not systematically look across the whole coverslip quantitatively, but we estimated that our transduction efficiency was ≈60% to 70%. We only chose the mCherry-expressing neurons to measure the calcium transient for subsequent comparison between WKY and SHR cells.

Figure 2. A. An example recording from young spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) cardiac sympathetic neuron taken from stellate ganglion exposed to 100 mmol/L of KCl for 30 s to depolarize the neuron and evoke voltage-gated Ca2+ entry. B. Statistical data showing the difference in peak evoked [Ca2+]i increase between young SHRs and WKY rats. *P<0.05, unpaired t test.

Figure 3. A. General experimental protocol presenting the effect of the nitric oxide (NO)-cGMP pathway drugs on the changing of intracellular free calcium concentration [Ca2+]i on high K+ exposure (30 s) in the cardiac sympathetic neuron. Drugs were introduced at 6 minutes after first high K+ stimulation (S1). After 10 minutes of incubation, neurons were stimulated again in the presence of drugs (S2). Statistical data showing the NO-cGMP pathway drugs affected the percentage changes of the difference in peak evoked [Ca2+]i (S2) compared with the control (S1) in the Wistar-Kyoto (WKY) rats (B) and the spontaneously hypertensive rats (SHRs) (C). *P<0.05, **P<0.01, compared with control, paired t test. Numbers showed in the bars indicated the number of the neurons. ODQ indicates oxadiazolo[4,3-a]quinoxalin-1-; AAAN, N-(4S)-4-amino-5-[(2-aminomethyl)aminopentyl]-N'-nitroguanidine.

Figure 4. A. Representative Western blot and group mean data showing neuronal nitric oxide synthase (nNOS; A), α1 and β1 subunits of guanylate cyclase (α1-sGC, B; β1-sGC, C) expression in stellate ganglia transduced by Ad.PRSx8-nROS/cherry (nNOS) compared with Ad.PRSx8-cherry (empty) from spontaneously hypertensive rats (SHRs). Western blot band optical density was normalized to that of β-actin as a loading control. D. Tissue levels of cGMP in the SHR stellate ganglia were significantly increased in the transduced by Ad.PRSx8-nROS/cherry than that measured in the transduced by Ad.PRSx8-cherry. *P<0.05, iP<0.001, unpaired t test. n indicates the number of rats used.

versus 0.54±0.07 μmol/L, n=19; SHR, 0.72±0.17 μmol/L, n=11; versus 0.66±0.09 μmol/L, n=17, P=0.49 and 0.77, respectively). Gene transfer of Ad.PRSx8-nROS/cherry reduced high K+ evoked [Ca2+]i by 39.17±4.16% in WKY rats (P<0.01) and 40.55±8.10% in SHRs (P<0.001) compared with neurons transduced with just Ad.PRSx8-cherry (Figure 6B and 6C). Moreover, the nNOS inhibitor AAAN (10 μmol/L) increased high K+ evoked [Ca2+]i to a greater extent after gene transfer with nNOS (39.81% versus 27.31%) in the WKY and partially reversed the effect of Ad.PRSx8-nROS/cherry on the calcium transient in the SHR.
The main new findings presented here are as follows: first, that the enhanced depolarization evoked [Ca\textsuperscript{2+}], transient seen in stellate sympathetic neurons from the prehypertensive SHR is associated with impaired NO-cGMP signaling; second, that nNOS expression by adrenoviruses in these neurons restores function of the NO-cGMP pathway and brings calcium handling back to near normal levels during stimulation.

**Discussion**

The main new findings presented here are as follows: first, that the enhanced depolarization evoked [Ca\textsuperscript{2+}], transient seen in stellate sympathetic neurons from the prehypertensive SHR is associated with impaired NO-cGMP signaling; second, that nNOS expression by adrenoviruses in these neurons restores function of the NO-cGMP pathway and brings calcium handling back to near normal levels during stimulation.

**NO-cGMP Signaling in the Cardiac Sympathetic Ganglia of the SHR**

Abundant evidence supports the concept of sympathetic hyperactivity and parasympathetic insufficiency as a significant component in the pathogenesis of not only early and borderline hypertension, but also in the maintenance of sustained essential hypertension. Moreover, defective NO synthase signaling via nNOS leads to increased norepinephrine release from the right atria in response to field stimulation in the adult SHR. We now show that this defect is also present in young spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats. Gene transfer of nNOS decreased [Ca\textsuperscript{2+}] transient in both strains and reversed the response of nNOS inhibitor N-[4S]-4-amino-5-[2-aminoethyl]aminopentyl]-N-nitroguanidine (AAAN; 10 \textmu mol/L) affected in the SHR. *P<0.05, paired t test, S1 compared with S2 within same group. ††P<0.01, †††P<0.001, 1-way ANOVA. Numbers showed in the bars indicated the number of neurons.

Link to enhanced noradrenergic neurotransmission. In this study, normal NO-cGMP modulated [Ca\textsuperscript{2+}] signaling was demonstrated in young WKY rats, with increased calcium transients after preincubation with nNOS or sGC inhibitors, and reduced transients with an NO donor. This indicates that the NO signaling system was intact in the WKY neurons. In contrast, experiments with cardiac sympathetic neurons from young SHR animals revealed abnormal NO-cGMP signaling, in which NO-cGMP inhibition failed to augment calcium signaling. Bypassing the enzymatically activated pathway with SIN-1 decreased high K\textsuperscript{+} evoked [Ca\textsuperscript{2+}], indicating that pathways downstream to NOS-sGC were still operating, albeit with a markedly reduced responsiveness compared with that seen in the WKY rat. This is in agreement with others who report the NO-cGMP regulatory cascade for Ca\textsuperscript{2+} handling is suppressed in SHR cardiomyocytes and endothelial cells.
nNOS Gene Transfer Restore Abnormal Calcium Transient in the SHR

Gene transfer of nNOS by an adenoviral vector (Ad.CMV. nNOS) has been shown to improve vascular function,20 decrease central sympathetic outflow from the paraventricular nucleus,21 reverse the enhanced arterial chemoreceptor function seen in rabbits with heart failure,22 increase cardiac parasympathetic function,15,23 and decrease β-adrenergic responsiveness of l-type calcium current in pacemaker cells from the SHR.10 However, one of the challenges with viral vectors is to ensure a degree of cell-specific gene transfer to avoid establishing expression of the enzyme in cells that do not constitutively express it, thereby avoiding abnormal paracrine signaling of NO. In a previous study, we have shown that Ad.PRSh8-nNOS is highly specific and effective in modifying gene expression in sympathetic neurons.9 Some of the physiological and patho-

logical effects of NO result from its actions at the mitochon-
drial level. NO/cGMP stimulates mitochondrial biogenesis,17,25 and this stimulation is associated with increased mitochondrial function.27 Further research is needed to investigate whether overexpression of nNOS activates mitochondrial biogenesis in cardiac postganglionic sympathetic neurons. Although gene transfer with viral vectors is a number of less invasive interventions have proved promising. Feeding hypertensive rats with l-arginine supplements28 or statins29 promotes NO activity and decreases cardiac sympathetic responsiveness. Interestingly, l-arginine supplementation reduces tyrosine hydroxylase levels,28 a key precursor for the production of norepinephrine, although we saw no change in this enzyme after nNOS gene transfer (Figure S3B).

Sources of Funding
This work was supported by a project grant from the British Heart Foundation. D.J.P. acknowledged support from the BHF Centre of Research Excellence, Oxford.

Disclosures
None.

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**Novelty and Significance**

**What Is New?**

- We show that impaired signaling involving the NO-cGMP pathway in stellate sympathetic neurons is directly related to enhance intracellular calcium transients.
- Noradrenergic cell specific gene transfer with nNOS rescues the abnormal calcium phenotype in these neurons.

**What Is Relevant?**

- We provide a critical link among abnormal sympathetic neurotransmission, impaired free radical signaling, and enhanced intracellular calcium levels in animals predisposed to hypertension.

**Summary**

Moreover, this dysregulated signaling in the peripheral nervous system is an early marker in the genesis of the hypertensive phenotype.

nNOS gene transfer may be a novel method to decrease calcium transients resulting in the presynaptic inhibition of cardiac sympathetic hyperresponsiveness in hypertension.
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Hypertension. published online November 19, 2012;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Targeted nNOS transgene delivery into stellate neurons reverses impaired intracellular calcium transients in prehypertensive rats

Dan Li, Natalia Nikiforova, Chieh-Ju Lu, Kate Wannop, Mary McMenamin, Chee-wan Lee, Keith J. Buckler, David J. Paterson

Burdon Sanderson Cardiac Science Centre and BHF Centre of Research Excellence, Department of Physiology, Anatomy & Genetics, Sherrington Building, University of Oxford, Parks Road, Oxford, OX1 3PT, UK

Short title: NO/cGMP and Calcium in SHR stellate neurons

Correspondence:
Dan Li MD, D.Phil & David J. Paterson D.Phil, D.Sc
Department of Physiology, Anatomy and Genetics
University of Oxford
Sherrington Building
Parks Road,
Oxford, OX1 3PT
UK
Fax: +44 1865 272453
Tel: +44 1865 272547
E-mail: dan.li@dpag.ox.ac.uk & david.paterson@dpag.ox.ac.uk
Supplement Methods

Sympathetic neuron isolation and tissue culture

Young SHRs and WKY rats were humanely killed by overdose of pentobarbital (500 mg/kg) followed by exsanguination. The stellate ganglia (which predominately innervates the heart) from both strains were removed under sterile conditions, placed in cold L-15 medium and desheathed carefully under a dissection microscope to remove all surrounding connective tissue. Ganglia were cut into 6-8 pieces and digested with collagenase and trypsin, then rinsed twice in L-15 blocking medium, and rinsed two more times in plating medium. The ganglia were dissociated by sequential mechanical trituration using fire-polished glass pipettes. Dissociated neurons were purified by seeding on a collagen coated dish for 1.5 h to minimize the number of fibroblasts and Schwann cells in the culture. The supernatant containing mostly neurons was plated onto poly-D-lysine/ laminin coated 6 mm cover slips and cultured in plating medium, then kept at 37°C in 5% CO₂. Media were changed every day, and experiments were performed 2-3 days after plating.

Ad.PRSx8-nNOS/Cherry and Ad.PRSx8-Cherry construction

At the first step an intermediate plasmid carrying C-terminal nNOS (kindly provided by Channon K.M., University of Oxford, UK) fused in frame to Cherry cDNA (Clontech, USA) was constructed by PCR amplification using nNOS specific primers: forward 5’-GGCGTCGACAATCCAAGATAGATCATATC-3’; reverse 5’-ATACCGCGGAGCTGAAAACCTCATC-3’ and mCherry specific primers: forward 5’-ATTCCGCAGTTAATTGTGAGCAAGG-3’; reverse 5’-GGAGGATCCACAACTAGAATGCAGTGAA-3’. At the same time PRSx8 promoter (gift from Teschemacher A.G., University of Bristol, UK) was subcloned in adenoviral shuttle vector pShuttle (Stratagene, Agilent Technologies, USA) at NotI blunted by Klenow large fragment/Xhol followed by subcloning of C-terminal nNOS/Cherry-polyA at SacII/BamHI restriction sites and C-terminal nNOS at Sall/SacII restriction sites. At the same time PRSx8 promoter (gift from Teschemacher A.G., University of Bristol, UK) was subcloned in adenoviral shuttle vector pShuttle (Stratagene, Agilent Technologies, USA) at NotI blunted by Klenow large fragment/Xhol followed by subcloning of C-terminal nNOS/Cherry-polyA at SacII/BamHI restriction sites and C-terminal nNOS at Sall/SacII restriction sites. At the same time PRSx8 promoter (gift from Teschemacher A.G., University of Bristol, UK) was subcloned in adenoviral shuttle vector pShuttle (Stratagene, Agilent Technologies, USA) at NotI blunted by Klenow large fragment/Xhol followed by subcloning of C-terminal nNOS/Cherry-polyA at SacII/BglII. At the last step N-terminal nNOS was PCR amplified using nNOS specific primers: forward 5’-GACCTCGAGACCATGGAAGAACAC-3’; reverse 5’-ATTGTCGACACCGGAAGACAGAACAC-3’ and subcloned in the same plasmid at XhoI/Sall completing pShuttle-PRSx8-nNOS/Cherry construct.

mCherry-PolyA PCR product was amplified from pmCherry-N1 (Clontech, USA) using PCR primers: forward 5’-AATCTTCGAATTCTCTGAGTGCAGTGTA-3’; reverse 5’-GGCAGATCTACAACTAGAATGCAGTGAA-3’ and subcloned into pShuttle-PRSx8 at Sall/BglII restriction sites.

Correctness of the resulting plasmids was verified by sequencing analysis.

pShuttle-PRSx8-nNOS/Cherry was subjected to homologues recombination with pAdEasy-1 (Stratagene, Agilent Technologies, USA) encoding human adenovirus serotype 5 proteins by cotransformation into BJ5183 bacterial strain (Stratagene, Agilent Technologies, USA). Positive
clones containing nNOS/Cherry transgene driven by PRSx8 promoter and adenoviral proteins were selected and propagated. Plasmid DNA from these clones was purified and transfected into HEK293 packaging cell line using Lipofectamine 2000 transfection reagent (Invitrogen, USA) for virus construction. Viral stock was amplified to preparative amount by subsequent passaging and purified by ion exchange chromatography (AdenoPure kit; PureSyn, USA). Viral titre was determined by immunocytochemistry using antibody against hexon viral envelop protein (Adeno-X Rapid Titer Kit; Clontech, USA). Ad-PRSx8-nNOS/Cherry titre was 2.8x10e10 iu/ml. Ad-PRSx8-mCherry adenovirus was constructed, propagated, purified and titred according to the same protocols; Ad-PRSx8-mCherry titre was 4.17x10e10 iu/ml.

nNOS/Cherry and mCherry expression driven by PRSx8 promoter was verified by transducing PC12 rat neuronal cell line.

Adenovirus vector transduction

A novel noradrenergic cell specific adenoviral vector expressing nNOS fused in frame at C-terminal end to red fluorescent protein mCherry (Ad.PRSx8-nNOS/Cherry) was transferred to cultured cardiac sympathetic neurons. An adenoviral vector expressing only mCherry (Ad.PRSx8-Cherry) was used as a control for comparing the effect of viral transduction (supplement Figure S1). 2×10^9 pfu of adenoviral vector was used to infect neurons or ganglia in a 4 well plate (1.9 cm^2/well, Nunc, Denmark). The virus containing medium was left in the well a maximum of 12 hours before changing to fresh medium. The experiments were performed after 3-4 days following gene transfer.

Measurement of free intracellular calcium concentration

[Ca^{2+}]_i, was determined in single neurons using Fura-4F acetoxyethyl ester (Fura-4F/AM) fluorescence ratio imaging. Subconfluent sympathetic neurons were loaded with 2.5 μmol/L Fura-4F/AM at 37 °C for 30 min. Loaded neurons were imaged with a CoolSnap digital CCD camera (Photometrics) connected to a PTI easy ratio pro fluorescence imaging system (Photon Technology International, Inc) housed on an inverted Nikon microscope equipped with a 60x, oil-immersion objective. The cover slip containing the neurons was placed into a temperature-controlled (37 °C), gravity fed, perfusion chamber (volume: 100 µl), perfused with Tyrode solution at a flow rate of 3 ml/min. The evoked [Ca^{2+}]_i transient was evaluated by 30 s exposure to 100 mM KCl (with equimolar reduction in NaCl) in the Tyrode solution. Fura-4F/AM was excited alternately at 340 nm and 380 nm at intervals of 2500 ms, and the emitted fluorescence measured at 510 nm. Fluorescence excitation ratios were transformed into [Ca^{2+}]_i concentrations using the equation derived by Grynkiewicz et al^2

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[Ca^{2+}]_i = K_d \times \frac{S_{b2}}{S_{f2}} \times \frac{(R - R_{min})}{(R_{max} - R)}
\]

\[K_d = \text{dissociation constant of Fura-4F (770 nM under standard conditions)}, S_{f2} = \text{fluorescence signal at 380 nm of Ca}^{2+}\text{ unbound (free) form}, S_{b2} = \text{fluorescence signal at 380 nm in Ca}^{2+}\text{ bound form}, R = \text{actual measured Fura-4F ratio}, R_{min} = \text{minimal Fura-4F ratio in zero [Ca}^{2+}]_i,\]
\( R_{\text{max}} \) = maximum Fura-4F ratio at saturating \([\text{Ca}^{2+}]\). The constants \( R_{\text{min}} \) and \( R_{\text{max}} \) were determined in situ using Ionomycin.

Experiments were carried out for 22 minutes. Three different drugs were used. NO donor SIN-1 (10 µmol/L), guanylyl cyclase inhibitor 1H-(1,2,4)-oxadiazolo-(4,3-a)-quinoxalin-1-one (ODQ, 10 µmol/L)\(^3\) and the nNOS specific inhibitor N-[(4S)-4-Amino-5-[(2-aminoetyl)amino]pentyl]-N'-nitroguanidine (AAAN, 10 µmol/L)\(^4\) were introduced separately at 6 minutes from the start of the experiment after the first high K\(^+\) stimulation (S1). After 10 minutes of incubation, neurons were stimulated again (at 16 minute) in the presence of drugs (S2).

**Immunofluorescence**

Cultured primary neurons were fixed with 2% Paraformaldehyde (in phosphate buffered saline, PBS) and permeabilized with 0.1% Triton X100 and 1% Bovine Serum Albumin (BSA). Cells were then processed for immunoreactivity with mouse anti-tyrosine hydroxylase (TH, 1:200, Sigma) or rabbit anti-nNOS (1:500, Invitrogen) in blocking solution (1% BSA) overnight at -4°C. After washing with PBS for several times, fixed cells were incubated with anti-mouse or anti-rabbit antibody conjugated to Alexa-488 (1:1000, Molecular Probes) in 1% BSA respectively for 1 hour (room temperature). Upon washing with PBS, then nuclear staining was performed with 4’,6-Diamidino-2-Phenylindole (DAPI, 1:1000, Sigma).

**Western blot analysis**

Stellate ganglia were isolated from young SHR and WKY rats and immediately frozen in liquid nitrogen. For preparation of virus infected tissue, stellate ganglia were dissected from young SHR and put in 4 well plate which contain 2×10^9 pfu of adenoviral vector in 1 ml plating medium that was kept at 37°C in 5% CO\(_2\). The virus containing medium was left in the well for a maximum of 12 hours before changing to fresh medium. After 3 days of gene transfer, tissues were frozen in liquid nitrogen.

Sample protein concentrations were quantified according to the Bradford protein assay. 30 µg of total protein was separated by SDS-PAGE. The expression of nNOS, \( \alpha_1 \) and \( \beta_1 \) subunit of sGC, TH proteins in stellate ganglia was compared. Antibodies and their sources were as follow: anti-nNOS 1:1000 (Invitrogen); anti-sGC-\( \alpha_1 \) 1:1000, anti-sGC-\( \beta_1 \) 1:4000, anti-TH (1:1000) and anti \( \beta \)-actin 1:1000 (Sigma). Immunodetection was based on chemiluminescence quantification (Western Lightning Plus, Perkin Elmer Life Science). The results were normalised to \( \beta \)-actin that served as a loading control.

**Measurement of tissue cGMP levels**

Stellate ganglia were dissected and immediately frozen in liquid nitrogen for subsequent analysis of cGMP using enzyme-linked immunosorbent assay (ELISA) Kit (NewEast Biosciences, Malvern, PA). For virus infected tissue, this was prepared the same as for the western blot.
Tissues were homogenized in 0.1M HCl. Precipitated proteins were removed by centrifugation, and the supernatant was collected for the cGMP assay according to the manufacturer's instructions. Proteins in the acid precipitate were treated with 1N NaOH and assayed by the method of Lowry.\(^5\)

**Solutions and materials**

Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO) and Invitrogen (Eugene, OR). Experiments were performed with a normal Tyrode solution containing (in mmol/L) 135 NaCl, 4.5 KCl, 20 HEPES, 11 Glucose, 1 MgCl\(_2\), 2 CaCl\(_2\). Fluorescent dye, Fura-4/AM was obtained from Molecular Probes (Eugene, OR). L-15 blocking medium: 96.8% L-15 medium supplemented with 0.6% D-(+)-Glucose solution, 2 mmol/L L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10% Fetal bovine serum). Plating medium: 90% L-15 medium supplemented with 24 mmol/L NaHCO\(_3\), 38 mmol/L Glucose, 50 units/ml penicillin, 50 µg/ml streptomycin, 50 ng/ml Nerve growth factor, 10% Fetal bovine serum.

**Statistical Analysis**

Data were expressed as means ± s.e.m. All statistical calculations were performed using the SigmaPlot 11 software package (Systat Software Inc.). For comparison of two groups, an unpaired \(t\)-test was performed, or a Mann-Whitney Rank Sum Test if the data were not normally distributed. To compare more than two groups, One-Way analysis of variance (ANOVA) was performed and the Holm-Sidak method was used as a post-hoc test. Paired \(t\)-test was performed when compared S1 and S2. For all experiments, statistical significance was accepted at \(P<0.05\).

**References**

Table S1. Comparison of High K⁺ evoked increased [Ca^{2+}]i transient from mCherry-expressing or non-expressing neurons after gene transfer (µmol/L).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Transferred Ad.PRS-nNOS/Cherry</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mCherry-expressing</td>
<td>mCherry non-expressing</td>
</tr>
<tr>
<td>WKY</td>
<td>1.63 ± 0.11 (n=19)*</td>
<td>2.75 ± 0.20 (n=8)</td>
</tr>
<tr>
<td>SHR</td>
<td>2.11 ± 0.29 (n=17)†</td>
<td>3.07 ± 0.80 (n=8)</td>
</tr>
</tbody>
</table>

*P<0.01, compared with WKY mCherry non-expressing neurons; †P<0.05, compared with SHR mCherry non-expressing neurons.
Supplement Figures

Figure S1

A

Human DBH promoter:

Phox2a/2b

PRS

TATA

h DBH

CCGCTAGACAAATGGT5ATTACC PRS x 8

B

PRSx8 nNOS Cherry

ATG PolyA signal

pAdeno-Shuttle

PRSx8 Cherry

ATG PolyA signal

pAdeno-Shuttle

Figure S1. A: Diagram of Phox2a/Phox2b response site (PRS) in the 5' proximal area of hDBH gene. PRSx8 promoter contains 8 multimerized PRS sites and a minimum promoter with the TATA box and transcription start site (arrow). B: pAd.PRSx8-nNOS/Cherry. (left) and pAd.PRSx8-Cherry (right) shuttle vector maps. Following a 240 bp PRS promoter, a 5.0 kb mCherry.nNOS and 0.7kb mCherry genes were inserted in adenoviral pShuttle (Stratagene, Agilent Technologies, USA).
Figure S2

**Figure S2.** A: An example recording from young WKY & SHR cardiac sympathetic neuron taken from stellate ganglion exposed to 100 mmol/L KCl at 1.5 min (S1) and 16 min (S2) for 30 seconds to depolarize the neuron and evoke voltage-gated Ca\(^{2+}\) entry. B: Statistical data showing that no differences in peak evoked [Ca\(^{2+}\)]\(_i\) increase between S1 and S2 in both WKY (n=7, \(P=0.80\), paired t-test) & SHR (n=4, \(P=0.50\), paired t-test).
Figure S3. Representative Western blot and group mean data showing nNOS (A) and Tyrosine Hydroxylase (TH, B) expression in cardiac stellate ganglia in normal WKY and following transduction with Ad.PRSx8-nNOS/Cherry (nNOS) or Ad.PRSx8-Cherry (empty) in the SHRs. Western blot band optical density was normalised to that of β–actin as a loading control. No significant changes were observed between two groups. n=4 in each group.