

**CAPON MODULATES NEURONAL CALCIUM HANDLING AND CARDIAC
SYMPATHETIC NEUROTRANSMISSION DURING DYSAUTONOMIA IN
HYPERTENSION**

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Abstract

Genome wide association studies implicate a variant in the neuronal nitric oxide synthase adaptor protein (CAPON) in electrocardiographic QT variation and sudden cardiac death. Interestingly, nitric oxide generated by neuronal NO synthase (NOS-1) reduces norepinephrine release, however, this pathway is downregulated in animal models of cardiovascular disease. Since sympathetic hyperactivity can trigger arrhythmia, is this neural phenotype linked to CAPON dysregulation? We hypothesized that CAPON resides in cardiac sympathetic neurons and is part of the pre-diseased neuronal phenotype that modulates calcium handling and neurotransmission in dysautonomia. CAPON expression was significantly reduced in the stellate ganglia of Spontaneously Hypertensive rats (SHRs) at 4 weeks of age before the development of hypertension compared to age-matched Wistar-Kyoto (WKY) rats. The neuronal calcium current (I_{Ca}) (n=8) and intracellular calcium transient ($[Ca^{2+}]_i$) (n=16) were significantly larger in the SHR compared to WKY ($P<0.05$). A novel noradrenergic specific vector (Ad.PRSx8-mCherry/CAPON) significantly upregulated CAPON expression, NOS1 activity and cGMP in SHR neurons without altering NOS1 levels. Neuronal I_{Ca} and $[Ca^{2+}]_i$ were significantly reduced following CAPON transduction compared to the empty vector. In addition, Ad.PRSx8-mCherry/CAPON also reduced 3H -norepinephrine release from SHR atria (n=7). NOS1 inhibition (AAAN, 10 μ mol/L; n=6) reversed these effects compared with the empty virus alone. In conclusion, targeted up-regulation of CAPON decreases cardiac sympathetic hyperactivity. Moreover, dysregulation of this adaptor protein in sympathetic neurons might further amplify the negative cardiac electrophysiological properties seen with CAPON mutations.

Word count: 231 (limit 250) Key words: Hypertension, CAPON, Dysautonomia, Calcium, neurotransmission, Sympathetic nervous system

Introduction

Large genome wide association (GWAS) studies have implicated the neuronal nitric oxide synthase adaptor protein (NOS1-AP) CAPON as a potential molecular marker of both corrected QT interval on the electrocardiogram (ECG), and increased risk of sudden cardiac death¹⁻⁵.

Single nucleotide polymorphisms (SNPs) in the CAPON gene have also been shown to be an important risk modifier in patients with inherited long QT syndrome⁶⁻⁸ where sympathetic drive further exacerbates the electrophysiological phenotype⁹.

In ventricular myocytes CAPON is co-localised with neuronal nitric oxide synthase (nNOS/NOS1)^{10, 11}. Increasing CAPON expression using adenoviral gene transfer accelerates cardiac repolarization (shortening action potential duration) by suppressing the L-type calcium current $I_{Ca,L}$ and enhancing the delayed rectifier potassium current I_{Kr} ¹⁰. Furthermore, CAPON facilitates nNOS translocation to caveolae post myocardial infarction (MI), suggesting the interaction with CAPON is required for nNOS redistribution in injured myocardium¹².

CAPON was first identified in neuronal tissue, where it interacts with the N-terminal PDZ domain of nNOS via C-terminus competition with PSD95¹³. CAPON escorts nNOS to specific target proteins, such as synapsin¹⁴ and the small monomeric G protein, Dexas1¹⁵, and thus may play an important role in calcium dependent exocytosis. Taken together with the observation that nNOS generated NO acts via cGMP and phosphodiesterase 2¹⁶ to reduce cAMP-protein kinase A dependent neuronal calcium handling and cardiac norepinephrine (NE) release^{17, 18}, it is therefore conceivable that an impaired neuronal CAPON-nNOS interaction might augment cardiac sympathetic neurotransmission.

We hypothesized that CAPON is present, but is reduced in cardiac sympathetic neurons from an animal model of dysautonomia¹⁹. As a consequence this contributes to a pre-disease neuronal phenotype that modulates enhanced calcium handling and neurotransmission. By developing an adenoviral vector with a noradrenergic neuron specific promoter to increase CAPON expression, we also tested the hypothesis that CAPON reduces the neuronal calcium current, intracellular calcium transient and cardiac NE release through an nNOS-cGMP dependent pathway.

Methods

Age- and weight-matched pre-hypertensive young male SHR (n=51) and normotensive Wistar-Kyoto (WKY; n=45) rats were purchased from Harlan (Bicester, UK) and housed under standard laboratory conditions. This investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Animals Scientific Procedures Act 1986 (UK). Procedures were performed under British Home Office license requirements (PPL 30/2360). Further methodological detail is available in the online supplement.

Sympathetic stellate neuron isolation

Sympathetic neurons were isolated and cultured using a previously published method²⁰ and the media used for isolation were based on modification of those previously described²¹. Briefly, cardiac stellate ganglia were dissected, de-sheathed and enzymatically digested. Following a sequential mechanical trituration, cell suspension containing stellate neurons was plated onto poly-D/lysine/laminin coated coverslips.

Adenovirus vector transduction

A novel adenoviral vector expressing CAPON fused in frame at the C-terminal end to red fluorescent protein mCherry with either a CMV promoter (Ad.CMV-mCherry/CAPON) or a noradrenergic cell specific promoter (Ad.PRSx8-mCherry/CAPON) were transferred to isolated cardiac sympathetic neurons or whole stellate ganglia tissue. An adenoviral vector expressing only mCherry (Ad.CMV-mCherry or Ad.PRSx8-mCherry) served as a control. 2×10^9 pfu of adenoviral vector was used to infect neurons or ganglia in a 4-well-pate ($1.9 \text{ cm}^2/\text{well}$, Nunc, Denmark). The virus-containing medium was left in the well for a maximum of 12 hours before replacing with fresh plating medium. The experiments were performed after 3 days post gene transfer for calcium transient measurements, and after 12 hours for measuring the calcium current, because of the necessity to minimize space clamp error caused by dendritic growth.

For the local evoked norepinephrine release experiment, targeted percutaneous gene transfer to the right atrium was performed under isoflurane (Isocare, Animalcare Ltd) anesthesia (4% for induction and 2-3% for maintenance in 100% O_2), using a technique similar to that described previously²². Animals received an injection of 1.6×10^{10} pfu of Ad.PRSx8-mCherry/CAPON or Ad.PRSx8-mCherry in 300 μl of PBS. Molecular and physiological phenotyping were investigated 5 days after gene transfer.

Immunofluorescence

Cultured primary neurons were fixed in cold acetone/methanol for 10 minutes. After permeabilization and blocking with 0.1% Triton X100 in PBS containing 1% BSA for 1 hour, the neurons were then incubated with primary antibody against CAPON (rabbit pAb, 1:200, abcam) and tyrosine hydroxylase (TH, mouse mAb, 1:200, Sigma) in 1% BSA overnight at 4°C .

Signals were visualized with anti-rabbit antibody conjugated to Alexa-488 (1:1000, Molecular Probes) and anti-mouse antibody conjugated to Alexa-594 (1:1000, Molecular Probes). Nuclear staining was performed with 4',6-Diamidino-2-Phenylindole (DAPI, 1:1000, Sigma). Imaging was performed on a Nikon Ti-U fluorescent microscope.

Western blotting

Protein extraction and Western blotting were performed as previously described²⁰. Details in Supplement.

Patch-clamp recordings

Cells were patched on freshly isolated neurons for non gene transferred experiments. Calcium current was recorded using conventional whole cell techniques. Pipette resistance varied from 1.5-2M Ω when filled with the internal solution containing (in mmol/L) 140 CsCl, 10 HEPES, 0.1 CaCl₂, 4 MgATP, 1 MgCl₂ and 1 EGTA, adjusted to pH7.4 with CsOH. The isolated neurons were superfused in a 36 \pm 0.5 °C bath with external solution containing (in mmol/L) 145TEACl, 10 HEPES, 4.5 KCl, 1 MgCl₂ and 11 Glucose, 1 NaHCO₃, 2 BaCl₂ and 0.001 TTX, adjusted to pH7.4 with Sigma base 7-9. The bath was grounded by a Ag/AgCl electrode connected via a 3M KCl/agar salt bridge. Calcium currents were acquired using Clampex software via an Axopatch 200B amplifier. Series resistance was compensated between 75% and 90%. Current-voltage (I-V) relations were elicited from a holding potential of -90 mV using 50 ms steps (5 s between steps) to test potentials over the range of -50 to +50 mV in 10 mV increments.

Measurement of intracellular calcium concentration

Intracellular free calcium concentration $[Ca^{2+}]_i$ of individual cultured stellate neurons was determined using Fura-2 acetoxymethyl ester (Fura-2/AM) fluorescence ratio imaging as previously described¹⁹. The specific nNOS inhibitor, N-[(4S)-4-Amino-5-[(2-aminoethyl)amino]pentyl]-N'-nitroguanidine (AAAN, 10 μ mol/L) was introduced separately after the first high K^+ (50 mmol/L) stimulation (S1) to depolarize cell as previously described²⁰. After 10 minutes of incubation, neurons were stimulated again in the presence of the drug (S2).

Measurement of tissue NOS activity

The activity of NOS was assessed by measuring the conversion of $[^{14}C]$ L-Arginine to $[^{14}C]$ L-Citrulline as described previously^{23, 24}. Stellates from 2 animals were pooled to provide protein for each NOS activity measure.

Measurement of tissue cGMP levels

Stellate ganglia tissue were isolated and transduced in 4 well plates which contained 2×10^9 pfu of adenoviral vector in 1 ml plating medium that was kept at 37 °C in 5% CO₂. The virus containing medium was left in the well for a maximum of 12 hours before replacing with fresh plating medium. After 5 days of gene transfer, stellate ganglia were rapidly frozen in liquid nitrogen. Measurement of cGMP levels were performed using cGMP Direct Immunoassay Kit (Abcam) according to the manufacturer's instructions.

Measurement of atrial $[^3H]$ -norepinephrine release

Spontaneously beating atria were isolated and transferred to a preheated (37 \pm 0.2°C), constantly oxygenated (carbogen: 95% oxygen, 5% CO₂), water-jacketed organ bath containing 3 ml

Tyrode solution where they were pinned flat on a silver stimulating electrode. The method for determining the local release of ^3H -NE to field stimulation (5Hz, 20V, 1ms pulse width, for 1 min) was identical to that which we have previously described¹⁸.

Data analysis

Data are expressed as mean \pm standard error of the mean. All data passed a normality test (Shapiro-Wilk). Comparison within groups was performed using the paired Student's *t*-test and between groups using the unpaired Student's *t* test, or ANOVA with Newman-Keuls post hoc analysis for multiple comparisons. *P* values <0.05 were considered statistically significant.

Results

Identification of endogenous CAPON protein in cardiac sympathetic neurons

Immunostaining demonstrated endogenous CAPON protein co-localised in tyrosine hydroxylase (TH) positive neurons (**Figure 1A**). The endogenous expression of CAPON determined by Western blotting was significantly lower in the pre-hypertensive SHR ($P<0.05$, $n=6$) compared to WKY controls ($n=6$) when normalized to β -actin (**Figure 1B**).

The neuronal calcium current and calcium transient are enhanced in sympathetic stellate neurons from young pre-hypertensive spontaneous rat

The neuronal calcium current was recorded using whole cell configuration of the patch-clamp technique. Currents were evoked by test pulses from a holding potential of -90mV. The peak calcium current density was significantly increased by $25.04\pm0.01\%$ in the cardiac stellate neurons of the SHR (-27.41 ± 1.41 pA/pF, $n=8$) when compared with age-matched WKY (-

21.92±1.13 pA/pF, n=8, $P<0.05$) (**Figure 1C-E**). Averaged peak current density-voltage relationships showed significant enhancement of I_{Ca} density from -20mV to +20mV ($P<0.05$) in the SHR (**Figure 1D**). As we have observed previously²⁵, the depolarization induced $[Ca^{2+}]_i$ transient was also significantly larger in SHR (n=16) compared to WKY (n=13) stellate neurons (**Figure 1F and G**).

Effect of CAPON gene transfer

Western blotting and cGMP measurement

Western blotting revealed that Ad.PRSx8-mCherry/CAPON increased the expression of CAPON in SHR stellate ganglia *in vitro* compared with Ad.PRSx8-mCherry, however, it did not affect the level of nNOS protein (**Figure 2C**). Fluorescence microscopy demonstrated that Ad.PRSx8-mCherry/CAPON localized in TH positive neurons from the stellate ganglia throughout the cytosol (**Figure 2B**).

Although the level of nNOS expression in SHR stellate ganglia was unchanged following Ad.PRSx8-mCherry/CAPON transduction, NOS activity was significantly enhanced (n=3 measures, with each measure using pooled stellate protein from 2 animals) compared with Ad.PRSx8-mCherry (n=3) (**Figure 3A**). The specific nNOS inhibitor AAAN normalized the difference in NOS activity following transduction with Ad.PRSx8-mCherry/CAPON (n=3) or Ad.PRSx8-mCherry (n=3). Furthermore, the level of cGMP in stellate ganglia from the SHRs was increased by ~52% post CAPON adenoviral gene transfer compared with the empty vector (empty:0.209±0.040 pmol/mg protein, n=10 v's Ad.CAPON:0.317±0.030 pmol/mg protein, n=10, $P<0.05$) (**Figure 3B**).

Calcium current and intracellular free calcium transients in cardiac sympathetic neurons.

In order to explore whether CAPON overexpression also modulates the neuronal calcium current (I_{Ca}), this was recorded using the whole cell configuration of the patch-clamp technique on neurons 12 hours post viral transduction (**Figure 4A**). In CAPON gene transfer neurons from the SHR, the peak calcium current (I_{Ca}) density was significantly reduced by $19.48 \pm 1.57\%$ (Ad.CMV-mCherry/CAPON: -20.29 ± 0.78 pA/pF, $n=6$; Ad.CMV-mCherry: -25.20 ± 1.59 pA/pF, $n=8$, $P<0.05$) and by $22.69 \pm 0.09\%$ in WKY (Ad.CMV-mCherry/CAPON: -17.92 ± 0.84 pA/pF, $n=7$; Ad.CMV-mCherry: -23.18 ± 1.06 pA/pF, $n=7$, $P<0.05$) (**Figure 4C**). In both strains, the averaged peak current density-voltage relationships showed significant attenuation of I_{Ca} from -20 mV to +20 mV ($P<0.05$) in neurons overexpressing CAPON when compared with empty vector controls (**Figure 4B**). The magnitude of the baseline current in cells transduced with the empty virus was similar to that measured in freshly isolated SHR or WKY neurons.

To determine whether the reduction in calcium current from CAPON gene transfer leads to a reduced intracellular calcium ($[Ca^{2+}]_i$) transient, fluorescence microscopy was employed in cells that displayed both mCherry expression and Fura-2 AM loading, after transduction with Ad.PRSx8-mCherry/CAPON (**Figure 5A**) or its control vector (Ad.PRSx8-mCherry). In CAPON over-expressing sympathetic neurons, the depolarization induced $[Ca^{2+}]_i$ transient was significantly reduced by $33.0 \pm 1.34\%$ in the WKY rat (Ad.PRSx8-mCherry/CAPON: 0.62 ± 0.06 μ M, $n=11$; Ad.PRSx8-mCherry: 0.92 ± 0.11 μ M, $n=14$, $P<0.05$) and $37.8 \pm 2.62\%$ in the SHRs (Ad.PRSx8-mCherry/CAPON: 0.74 ± 0.07 μ M, $n=13$; Ad.PRSx8-mCherry: 1.19 ± 0.06 μ M, $n=11$, $P<0.05$) when compared with neurons transduced with empty viral vector (**Figure 5B and C**). The specific nNOS inhibitor AAAN reversed the effect of CAPON gene transfer on the

depolarization induced $[Ca^{2+}]_i$ transient and also normalized the differences between SHR and WKY neurons (**Figure 5B and C**).

Cardiac norepinephrine release

Percutaneous gene transfer targeted to the right atrium of SHR *in vivo* also increased CAPON protein expression from right atria as assessed by Western blot (**Figure 6A**). $[^3H]$ -NE release from isolated atrial preparations in response to 5Hz field stimulation was significantly decreased by Ad.PRSx8-mCherry/CAPON by $22.1 \pm 0.97\%$ when compared with the empty virus (Ad.PRSx8-mCherry/CAPON: $2.7 \pm 0.15\%$, $n=6$, v's Ad.PRSx8-mCherry: $3.5 \pm 0.24\%$, $n=6$, $P<0.05$) (**Figure 6B and C**). The specific nNOS inhibitor AAAN reversed the effect of CAPON gene transfer on $[^3H]$ -NE release.

Discussion

The novel findings of our study are as follows. First, we demonstrate that CAPON is expressed in cardiac sympathetic neurons and is of a similar molecular mass to that first reported in hypothalamic neurons¹³. Secondly, the expression of CAPON is reduced in the pro-hypertensive SHR compared to the WKY rat at 4 weeks of age, suggesting it may be linked to the pre-disease neuronal phenotype. Thirdly, upregulation of CAPON in the SHR restored its expression to similar levels seen in the WKY neurons. It also increased nNOS activity and the concentration of neuronal cGMP without changing the expression of nNOS itself. As a consequence, the neuronal calcium current and intracellular calcium transient was reduced in the SHR to levels observed in the WKY. This translated into reduced atrial NE release. These

effects were reversed by nNOS inhibition, suggesting that CAPON modulation of sympathetic neurotransmission is coupled to an NO dependent pathway.

CAPON is expressed in cardiac sympathetic neurons

CAPON, a highly conserved protein, was first identified in rat brain neurons²⁶ as a binding protein for nNOS¹³. CAPON has been further discovered to be localized in ventricular myocytes¹⁰, rat neural tissues including the facial nerve²⁷, sciatic nerve^{28, 29}, dorsal root ganglion and lumbar spinal cord²⁹. It has been implicated in neuronal pathogenesis including peripheral nerve regeneration²⁸, neuron loss and survival²⁹, schizophrenia³⁰, pain²⁹ and inflammation²⁶. Here we observed significant CAPON expression in both WKY and SHR stellate neurons, however levels were significantly reduced in pre-hypertensive SHR neurons. Interestingly, CAPON is also present in ChAT positive intracardiac neurons (unpublished), although it is unknown whether it impacts on the sympathetic phenotype reported here.

nNOS-CAPON signaling in the pre-hypertensive SHR

Sympathetic hyperactivity and parasympathetic insufficiency is correlated with mortality in patients with and without cardiovascular disease³¹⁻³³. In particular, over-activity of the sympathetic nervous system is implicated in the etiology of human essential hypertension³⁴⁻⁴⁰. This has also been observed in an animal model of genetic hypertension, the SHR, as early as 4 weeks of age where arterial blood pressure and ventricular weight-to-body weight ratio^{19, 20} is not different from age- and weight-matched WKY^{19, 41, 42}. At this age however, these animals have a distinct autonomic phenotype of heightened cardiac sympathetic neurotransmission driven by enhanced calcium transients^{20, 25} and a reduced norepinephrine re-uptake transporter⁴³. This translates into an enhanced tachycardia during right stellate ganglia simulation *in vitro*¹⁹ and

elevated hearts rates *in vivo* under anesthesia¹⁹, as well as in telemetered animals⁴². How sympathetic impairment occurs during cardiovascular disease is not fully understood, but it clearly involves changes at different sites in the neural-cardiac axis, from brain nuclei down to alterations in local neuronal circuits at the end organ that further add to the complexity of this regulation⁴⁴.

The augmented neuronal calcium current and impaired nNOS/CAPON signaling observed here may account for a major part of altered calcium homeostasis²⁵, although defective mitochondrial buffering of $[Ca^{2+}]_i$ may also contribute²⁵ to this calcium impairment, and excessive adrenergic neurotransmission in the SHR¹⁸. Dysregulated neuronal Ca^{2+} signaling has recently been reported in both stellate neurons and parasympathetic neurons (intracardiac) in animals with heart failure⁴⁵. Specifically, the N type Ca^{2+} current was enhanced in the sympathetic neuron, but impaired in the cholinergic neuron⁴⁵. These observations provide an electrophysiological basis for the cardiac sympatho-vagal phenotype seen in heart failure and hypertension. Of interest, we see that dysregulation of both the neuronal Ca^{2+} current and intracellular Ca^{2+} transient are early cellular markers in the evolution of sympathetic hyperactivity. What is the cellular link to impaired intracellular Ca^{2+} handling and is it related oxidative stress as some have suggested?^{20, 26}.

nNOS acts through modulation of cGMP and PDE2 to reduce cAMP-PKA dependent regulation of neuronal calcium transients¹⁶ and NE release¹⁷ in sympathetic neurons. Since pre-hypertensive SHR's have reduced cardiac stellate expression of nNOS, the β_1 sub unit of soluble guanylate cyclase, cGMP²⁰, and given that CAPON acts as a modifier of nNOS in brain neurons, we suspected that CAPON might also play a role in calcium handling and NE release in cardiac sympathetic neurons. Although the role of CAPON as an inhibitor or mediator of nNOS function

in human disease has been widely debated⁴⁶, in our study, overexpression of CAPON increased neuronal nNOS activity and cGMP levels in the SHR, whilst stabilizing nNOS protein expression. This is similar to that seen in isolated ventricular myocytes where CAPON enhanced NOS enzymatic activity and NO release¹⁰. Considering the brief half-life of NO and its high reactivity as a free radical gas, the biological and cellular effects of nNOS-NO signaling may be highly localized and dependent on the subcellular translocation of nNOS between membrane and cytosolic compartments as previously suggested⁴⁷. For example, CAPON plays an important role in directing nNOS to specific target proteins such as synapsin¹⁴. However, like Chang et al¹⁰ we cannot rule out the possibility that CAPON also acts through nNOS independent pathways given that CAPON may compete with other PDZ-binding proteins through interaction via its C terminus.

Our electrophysiological experiments could not determine whether CAPON modulation was more important in the neuronal soma or axonal terminal that is more applicable to localized release. Nevertheless, increased CAPON/NOS-cGMP signaling restored the neuronal calcium current and calcium transient in the SHR to the levels seen in WKY neurons. The reduction in the neuronal calcium transient and NE release was reversed by nNOS inhibition, suggesting the involvement of a NO dependent pathway. Interestingly, CAPON is co-localised not only with nNOS but also with L-type calcium channels ($LTCa^{2+}$) and potassium channels (Kir3.1) in cardiomyocytes¹¹, suggesting it may play a more widespread role in the modulation of ion channels. Moreover, given the localization of CAPON to the intercalated disc in human cardiomyocytes, it has been suggested that CAPON regulates ion flow through the cardiomyocyte gap junctions⁴⁸.

Overexpression of CAPON in ventricular myocytes significantly shortens the APD by inhibiting $I_{Ca,L}$ and activation of I_{Kr} ¹⁰. This may have implications in long QT syndrome where GWAS studies have identified a common genetic variant in CAPON that might contribute to QT interval abnormalities¹⁻⁷. Although sympathetic stimulation can shorten the APD, it also stimulates myocyte calcium loading, and therefore it is conceivable that abnormal sympathetic activation due to CAPON impairment superimposed on a long QT phenotype would further exacerbate the likelihood of afterdepolarizations.

Perspective

We present evidence that CAPON-nNOS modulated sympathetic neurotransmission and that this is dysregulated in the early stages of an animal model of sympathetic hyperactivity. Whether polymorphisms in the CAPON gene are associated with sympathetic hyperactivity remains to be established. However, sympathetic drive can modulate the QT interval and trigger life threatening ventricular arrhythmias. Differences in CAPON expression or activity related to SNPs in GWAS studies are associated with altered QT interval. It is therefore conceivable that abnormal sympathetic neurotransmission due to the same CAPON SNPs could potentially further amplify the electrophysiological phenotype. Strategies that up-regulate CAPON will shorten the APD¹⁰, and also decrease sympathetic drive, thereby providing a rationale for therapeutic targeting.

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Disclosures

None

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Novelty and Significance

What Is New?

- The neuronal calcium current and intracellular calcium transient are larger in the pro-hypertensive SHR compared to the WKY rat and this is associated with reduced expression of CAPON.
- Targeted up-regulation of CAPON reduced the intracellular neuronal calcium current and calcium transient by increasing nNOS activity and cGMP production, resulting in decreased adrenergic neurotransmission.

What Is Relevant?

Single nucleotide polymorphisms (SNPs) in the CAPON gene have also been shown to be an important risk modifier for sudden cardiac death and QT variability in patients.

Summary

Artificial up-regulation of CAPON decreases cardiac sympathetic hyperactivity. Dysregulation of this adaptor protein in sympathetic neurons might exacerbate the electrophysiological phenotype seen in patients with CAPON mutations.

Figure legends

Figure 1. A, Co-immunostaining of stellate neurons with anti-tyrosine hydroxylase (TH, red) and anti-CAPON (green) antibodies showed CAPON expression in sympathetic neurons. Nuclear staining with DAPI is in blue. Scale bar represents 25 μm . B, Representative Western blot and group mean data showing a significant reduction in CAPON protein expression relative to β -actin in stellate ganglia from 4-week-old spontaneously hypertensive rats (SHR, $n=6$) compared to age matched Wistar Kyoto controls (WKY, $n=6$, * $P<0.05$, unpaired t -test). C, Representative traces elicited by depolarizing voltage steps (50 ms) from -50 to +50 mV in 10-mV increments from a holding potential of -90 mV in stellate neurons from 4 week old SHR and WKY rats. D, Current density-voltage relationship curve of the neuronal calcium current (I_{Ca}) demonstrating significantly larger I_{Ca} at multiple voltages in stellate neurons from SHRs ($n=8$) when compared to WKY controls ($n=8$). E, The peak calcium current density in SHR stellate neurons ($n=8$) was larger than in WKY controls ($n=8$, * $P<0.05$, unpaired t -test). F, Representative calcium fluorescence traces from 4-week old SHR and WKY cardiac stellate neurons loaded with Fura2-AM and depolarized to evoke voltage-gated Ca^{2+} entry. G, Group data showing the difference peak evoked $[\text{Ca}^{2+}]_i$ transients in 4-week old SHR and WKY stellate neurons ($n=16$ v's $n=13$, ** $P<0.01$, unpaired t test).

Figure 2. A, Map of adenoviral vector construct containing a noradrenergic neuron-specific promoter, (PRScx8), NOS1-AP (CAPON) gene and red mCherry fluorescent protein (Ad.PRScx8-mCherry/CAPON). As a control, the same construct without NOS1-AP (CAPON) gene insert was used (Ad.PRScx8-mCherry). B, Co-immunostaining of stellate neurons with anti-tyrosine

hydroxylase (TH, green) and mCherry (red) tagged viral construct showed viral transduction in sympathetic neurons. Nuclear staining with DAPI is in blue. Scale bar represents 25 μm . C, Representative Western blot and group mean data showing CAPON expression (74kDa) and nNOS expression in stellate ganglia from 4 week old SHR three days after transduction with Ad.PRSx8-mCherry/CAPON (Ad.CAPON: n=6) and Ad.PRSx8- mCherry (Empty: n=6, $**P<0.01$, unpaired *t*-test).

Figure 3. A, Gene transfer of CAPON (Ad.PRSx8-mCherry/CAPON) significantly increased NOS activity in stellate ganglia from SHR when compared with the empty vector (Ad.PRSx8-mCherry) control (n=3 measures, with each measure from tissue pooled from 2 animals). The specific nNOS inhibitor, S2, N-[(4S)-4-Amino-5-[(2-aminoethyl) amino] pentyl]-N'-nitroguanidine (AAAN, 10 $\mu\text{mol/L}$) normalized the difference in NOS activity following transduction with CAPON (n=3 measures) or the empty vector control (n=3 measures, $*P<0.05$, ANOVA). B, cGMP concentration in SHR stellate ganglia tissue was significantly enhanced by Ad.PRSx8-mCherry/CAPON transduction (n=10) when compared to empty controls (Ad.PRSx8-mCherry, n=10, $*P<0.05$, unpaired *t*-test).

Figure 4. A, Representative fluorescence (mCherry) and bright field images of single stellate neuron transduced with Ad.CMV-mCherry/CAPON (Ad.CAPON) with a patch pipette (pointed by a white arrow). Scale bar represents 20 μm . B, Current density-voltage relationship curve of the neuronal calcium current (I_{Ca}) demonstrating attenuation of I_{Ca} at multiple voltages in CAPON-overexpressing stellate neurons from both 4 weeks old SHR and age matched WKY

controls when compared to cells transduced with empty virus (Ad.CMV-mCherry). C, The peak I_{Ca} density in CAPON-overexpressing stellate neurons from both SHR (n=6) and WKY (n=7) was significantly less than in empty controls (SHR: n=8, WKY: n=7, * $P<0.05$, unpaired t -test).

Figure 5. A, Stellate neurons demonstrating loading of Fura-2 AM by green fluorescence and viral transduction with mCherry (red). Scale bar represents 25 μ m. Representative ratio data trace (B) and group mean data (C) showing that the depolarization induced intracellular calcium ($[Ca^{2+}]_i$) transient was significantly less in stellate neurons transduced with Ad.PRSx8-mCherry from 4 week old WKY (n=14) rats compared to age matched with SHR n=11, * $P<0.05$, unpaired t -test; # $P<0.05$, paired t -test). Gene transfer with CAPON (Ad.PRSx8-mCherry/CAPON) significantly reduced the calcium transient in both WKY (n=11) and SHR (n=13) neurons to a similar levels. The effect of CAPON gene transfer in both strains can be reversed with a specific nNOS inhibitor (S2, N-[(4S)-4-Amino-5-[(2-aminoethyl) amino] pentyl]-N'-nitroguanidine, AAAN, 10 μ mol/L).

Figure 6. Representative Western blot showing the up-regulation of CAPON expression in right atria by *in vivo* percutaneous injection with Ad.PRSx8-mCherry/CAPON (Ad.CAPON, n=6) when compared with Ad.PRSx8-mCherry (Empty: n=6, * $P<0.05$, unpaired t -test) in the 4 week old SHR. Representative raw data (B) and group mean data (C) showing that *in vivo* percutaneous transduction with Ad.PRSx8-mCherry/CAPON (Ad.CAPON: n=6) reduced 3H -NE release from isolated double atrial preparations in response to electrical field stimulation (S1, 5Hz) when compared to Ad.PRSx8-mCherry (Empty: n=6, * $P<0.05$, unpaired t -test; # $P<0.05$,

paired *t*-test). The effect of CAPON gene transfer can be abolished with a specific nNOS inhibitor (S2, AAAN, 10 μ mol/L).

Figure 1

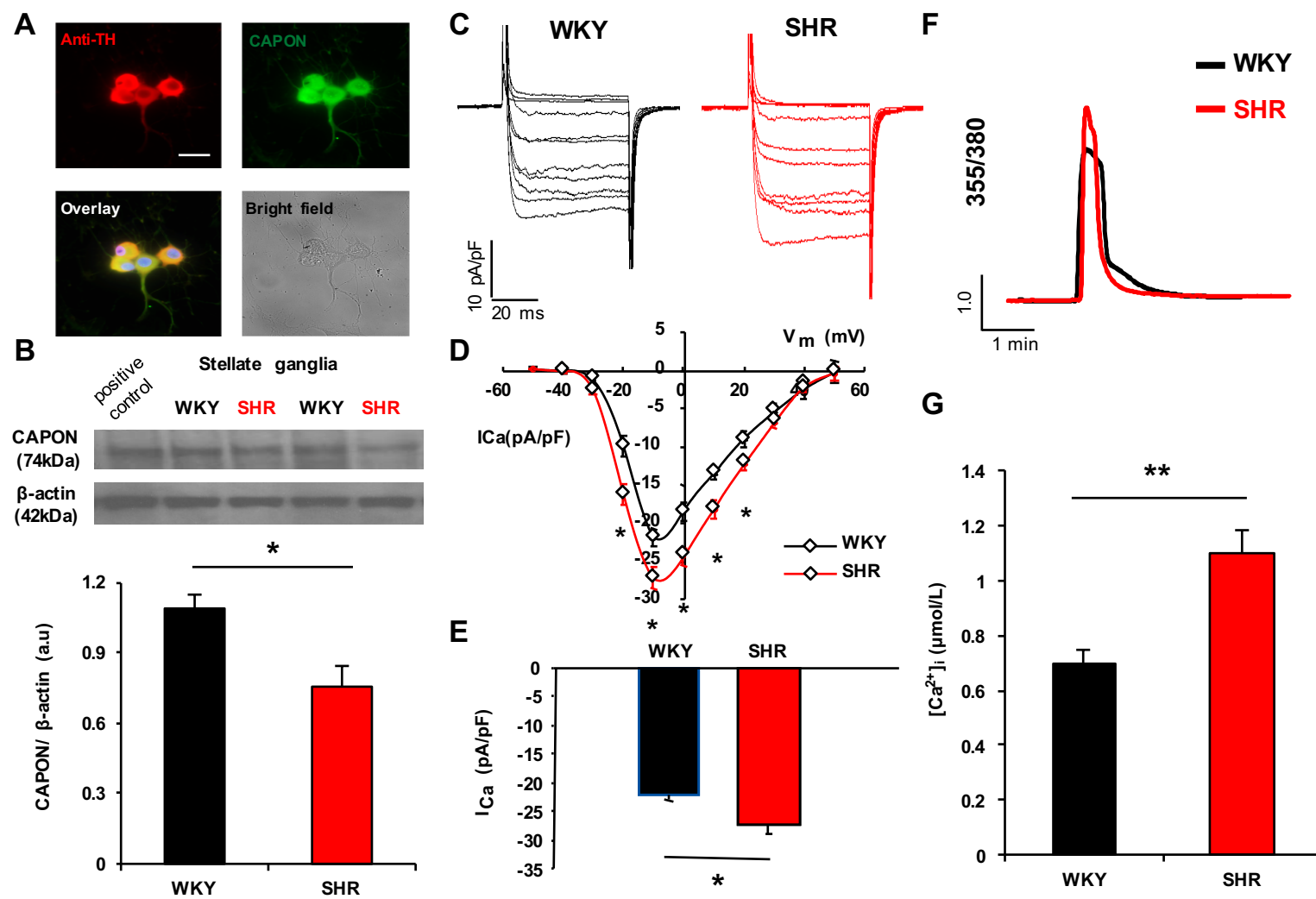


Figure 2

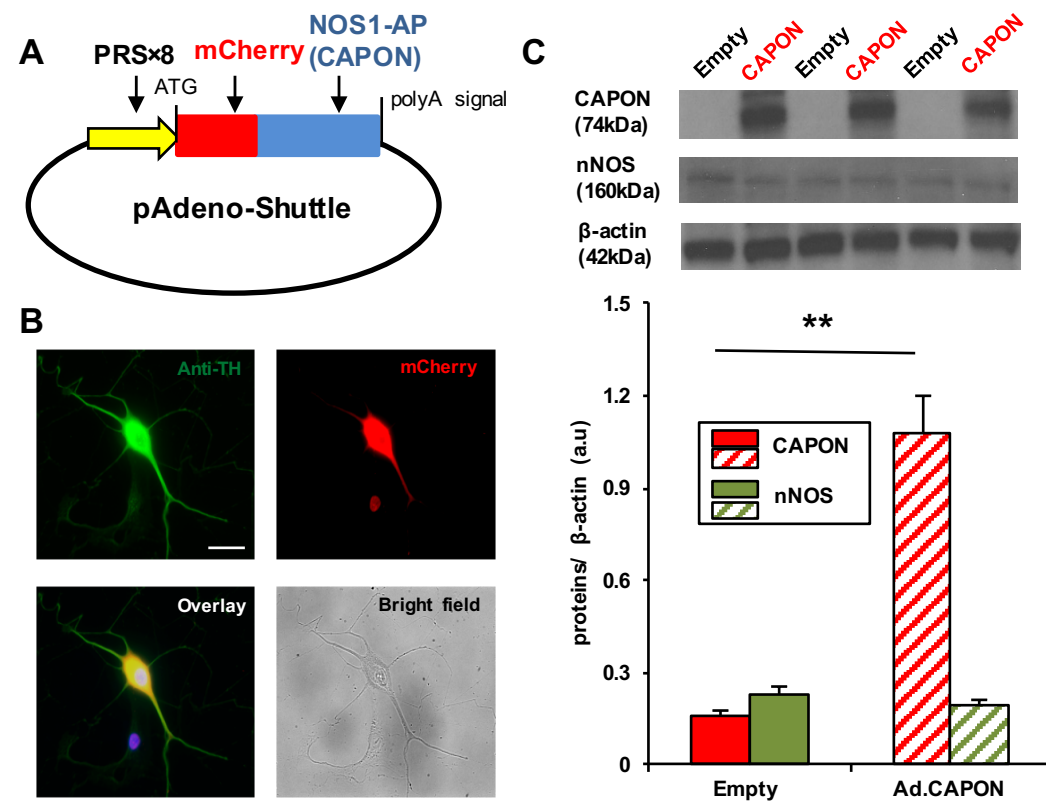


Figure 3

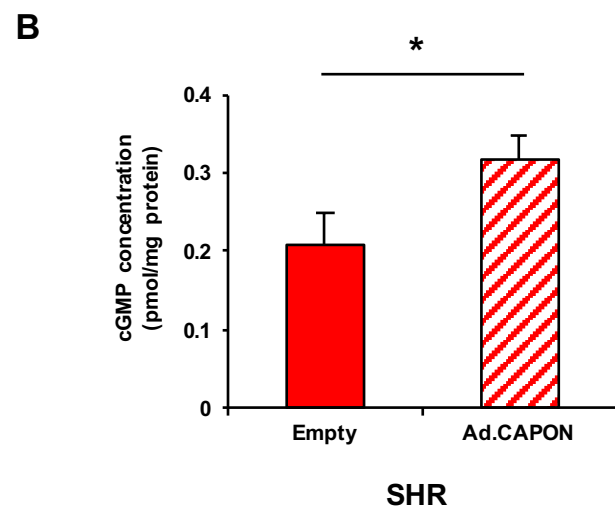
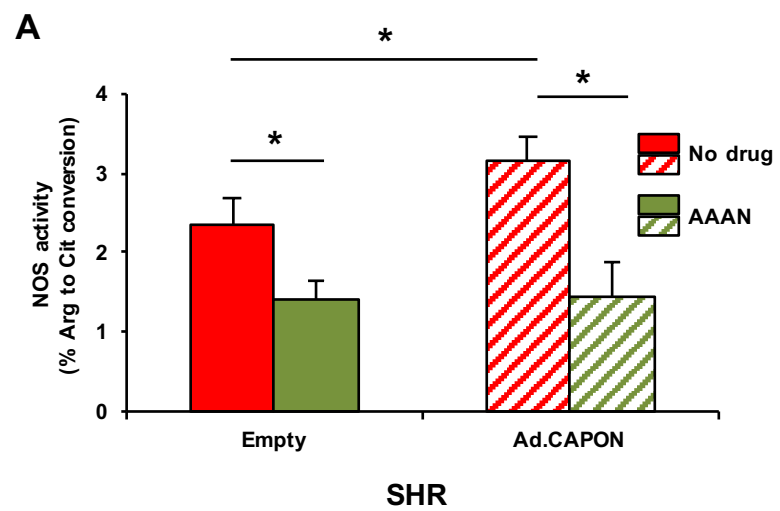


Figure 4

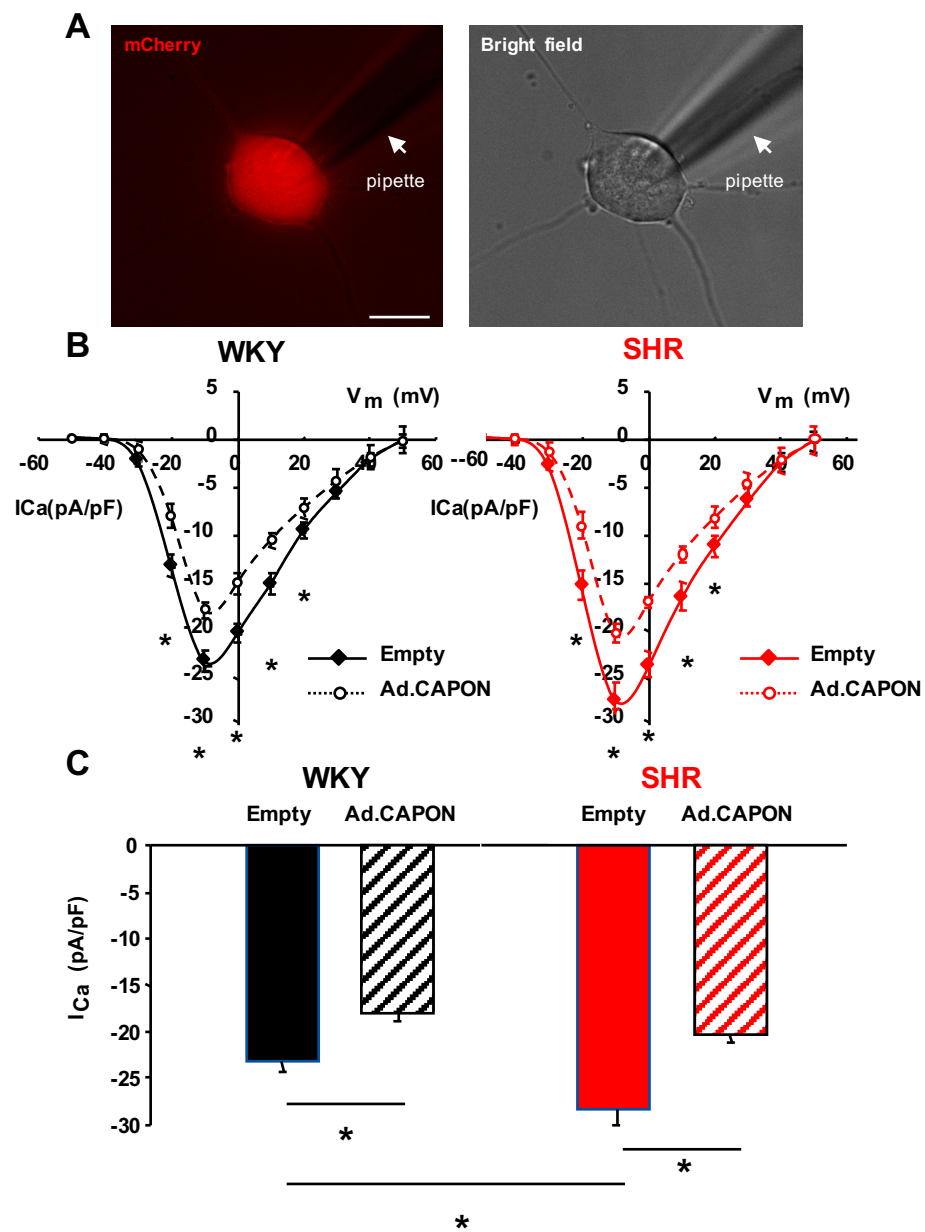


Figure 5

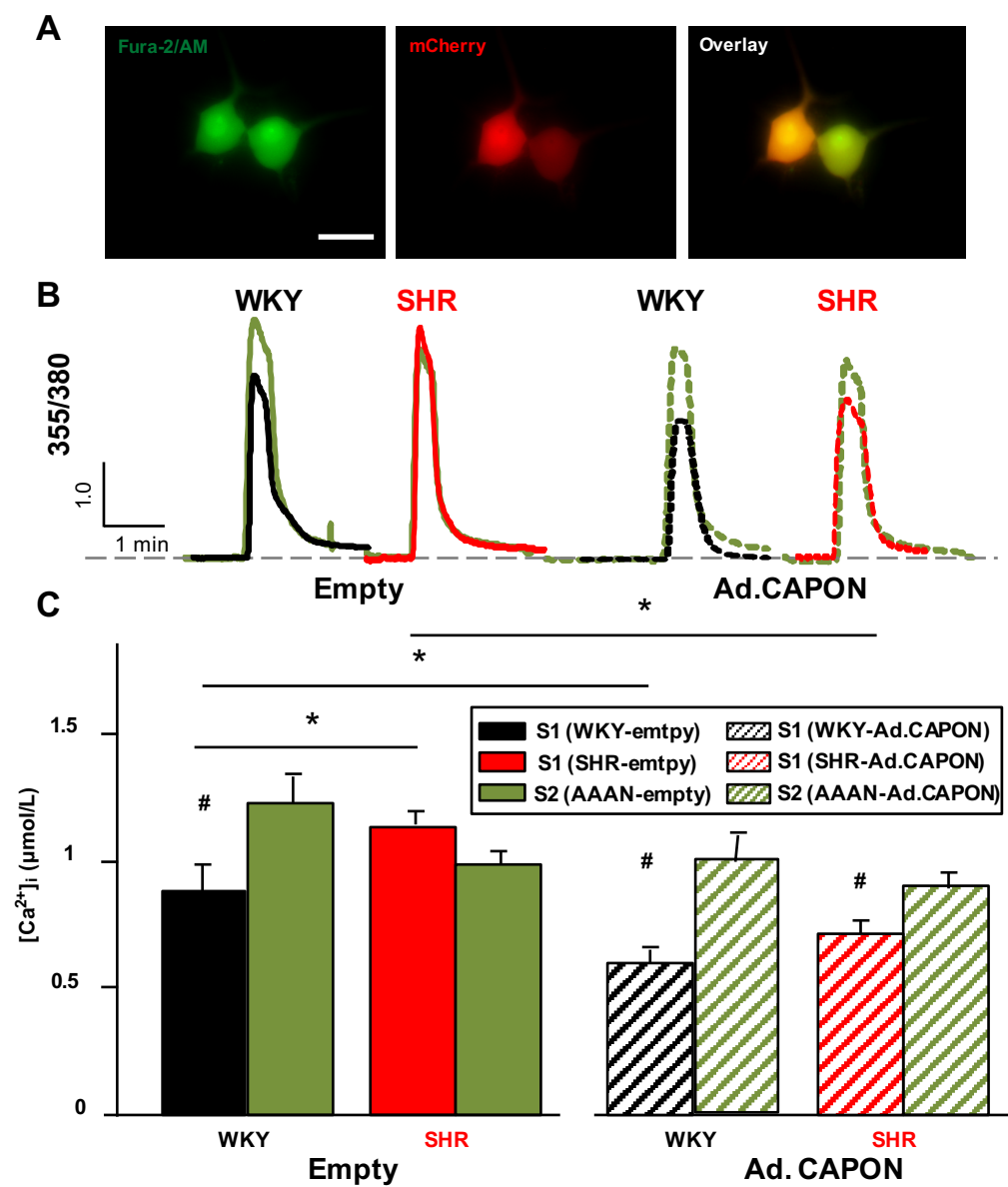


Figure 6

