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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-1 reduces norepinephrine release; however, this pathway is downregulated in animal models of cardiovascular disease. Because sympathetic hyperactivity can trigger arrhythmia, is this neural phenotype linked to CAPON dysregulation? We hypothesized that CAPON resides in cardiac sympathetic neurons and is a part of the prediseased neuronal phenotype that modulates calcium handling and neurotransmission in dysautonomia. CAPON expression was significantly reduced in the stellate ganglia of spontaneously hypertensive rats before the development of hypertension compared with age-matched Wistar–Kyoto rats. The neuronal calcium current (I_{Ca}; n=8) and intracellular calcium transient ($[Ca^{2+}]_i$; n=16) were significantly larger in the spontaneously hypertensive rat than in Wistar–Kyoto rat ($P<0.05$). A novel noradrenergic specific vector (Ad.PRSx8-mCherry/CAPON) significantly upregulated CAPON expression, NO synthase-1 activity, and cGMP in spontaneously hypertensive rat neurons without altering NO synthase-1 levels. Neuronal I_{Ca} and $[Ca^{2+}]_i$ were significantly reduced after CAPON transduction compared with the empty vector. In addition, Ad.PRSx8-mCherry/CAPON also reduced ^{3}H-norepinephrine release from spontaneously hypertensive rat atria (n=7). NO synthase-1 inhibition (AAAN, 10 μmol/L; n=6) reversed these effects compared with the empty virus alone. In conclusion, targeted upregulation

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None.

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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.

Keywords

calcium; CAPON; hypertension; primary dysautonomias; sympathetic nervous system; synaptic transmission

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Large genome-wide association studies have implicated the neuronal nitric oxide synthase adaptor protein (NOS1-AP or CAPON) as a potential molecular marker of both corrected QT interval on the ECG and increased risk of sudden cardiac death.[1–5] Single-nucleotide polymorphisms in the CAPON gene have also been shown to be an important risk modifier in patients with inherited long-QT syndrome[6–8] where sympathetic drive further exacerbates the electrophysiological phenotype.[9]

In ventricular myocytes, CAPON is colocalized 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, suggesting the interaction with CAPON is required for nNOS redistribution in injured myocardium.[12]

CAPON was first identified in neuronal tissue, where it interacts with the N-terminal PDZ domain of nNOS via C-terminal competition with PSD95[13]. CAPON escorts nNOS to specific target proteins, such as synapsin[14] and the small monomeric G protein, Dexras1[15], 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 2[16] to reduce cAMP-protein kinase A–dependent neuronal calcium-handling and cardiac norepinephrine 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 it is reduced in cardiac sympathetic neurons from an animal model of dysautonomia.[19] As a consequence, this contributes to a predisease neuronal phenotype that enhances 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 norepinephrine release through an nNOS-cGMP–dependent pathway.

Methods

Age- and weight-matched prehypertensive young male spontaneously hypertensive rats (SHRs; n=51) and normotensive Wistar–Kyoto (WKY; n=45) rats were purchased from Harlan (Bicester, United Kingdom) 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 (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 30/2360). Further methodological detail is available in the online-only Data Supplement.

Sympathetic Stellate Neuron Isolation

Sympathetic neurons were isolated and cultured using a previously published method,[20] and the media used for isolation were based on modification of those previously described.[21] Briefly, cardiac stellate ganglia were dissected, desheathed, and enzymatically digested. After 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 cm^2/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 previously.[22] 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 (mouse mAb, 1:200; Sigma) in 1% BSA overnight at 4°C. Signals were visualized with antirabbit antibody conjugated to Alexa-488 (1:1000; Molecular Probes) and antimouse 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 described (details are available in the online-only Data Supplement).[20]

Patch-Clamp Recordings

Cells were patched on freshly isolated neurons for nongene transferred experiments. Calcium current was recorded using conventional whole cell techniques. Pipette resistance varied from 1.5 to 2 2MΩ when filled with the internal solution containing (in mmol/L) 140 CsCl, 10 HEPES, 0.1 $CaCl_2$, 4 MgATP, 1 $MgCl_2$, and 1 EGTA, adjusted to pH 7.4 with CsOH. The isolated neurons were superfused in a 36±0.5°C bath with external solution containing (in mmol/L) 145 TEACl, 10 HEPES, 4.5 KCl, 1 $MgCl_2$ and 11 glucose, 1 $NaHCO_3$, 2 $BaCl_2$, and 0.001 TTX, adjusted to pH 7.4 with Sigma base 7 to 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) relationships 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 described.[19] The specific nNOS inhibitor, N-[(4S)-4-Amino-5-[(2-aminoetyl) amino]pentyl]-N′-nitroguanidine (AAAN; 10 μmol/L) was introduced separately after the first high K^+ (50 mmol/L) stimulation (S1) to depolarize cell as previously described.[20] 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% CO_2. 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 was performed using cGMP Direct Immunoassay Kit (Abcam) according to the manufacturer's instructions.

Measurement of Atrial [^{3}H]-Norepinephrine Release

Spontaneously beating atria were isolated and transferred to a preheated (37±0.2°C), constantly oxygenated (carbogen: 95% oxygen, 5% CO_2), 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 ^{3}H- norepinephrine to field stimulation (5 Hz, 20 V. 1-ms pulse width, for 1 minute) was identical to that which we have previously described.[18]

Data Analysis

Data are expressed as mean±SEM. All data passed a normality test (Shapiro–Wilk). Comparison within groups was performed using the paired Student *t* test and between groups using the unpaired Student *t* test, or ANOVA with Newman–Keuls post hoc analysis for multiple comparisons. A value of $P<0.05$ were considered statistically significant.

Results

Identification of Endogenous CAPON Protein in Cardiac Sympathetic Neurons

Immunostaining demonstrated endogenous CAPON protein colocalized in tyrosine hydroxylase positive neurons (Figure 1A). The endogenous expression of CAPON determined by Western blotting was significantly lower in the prehypertensive SHR ($P<0.05$; n=6) than in WKY controls (n=6) when normalized to β-actin (Figure 1B).

Neuronal Calcium Current and Calcium Transient Are Enhanced in Sympathetic Stellate Neurons From Young Prehypertensive Spontaneous Rat

The neuronal calcium current was recorded using the whole cell configuration of the patch-clamp technique. Currents were evoked by test pulses from a holding potential of −90 mV. The peak calcium current density was significantly increased by 25.04±0.01% 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–1E). Averaged peak current density–voltage relationships showed significant enhancement of I_{Ca} density from −20 to +20 mV ($P<0.05$) in the SHR (Figure 1D). As we have observed previously,[25] the depolarization-induced $[Ca^{2+}]_i$ transient was also significantly larger in SHR (n=16) than in WKY (n=13) stellate neurons (Figure 1F and 1G).

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 tyrosine hydroxylase positive neurons from the stellate ganglia throughout the cytosol (Figure 2B).

Although the level of nNOS expression in SHR stellate ganglia was unchanged after 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 after 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 versus 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—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±1.57% (Ad.CMV-mCherry/CAPON, −20.29±0.78 pA/pF; n=6 and Ad.CMV-mCherry, −25.20±1.59 pA/pF; n=8; $P<0.05$) and by 22.69±0.09% in WKY (Ad.CMV-mCherry/CAPON, −17.92±0.84 pA/pF; n=7 and 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 used 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-overexpressing sympathetic neurons, the depolarization-induced $[Ca^{2+}]_i$ transient was significantly reduced by 33.0±1.34% in the WKY rat (Ad.PRSx8-mCherry/CAPON, 0.62±0.06 μmol/L, n=11 and Ad.PRSx8-mCherry, 0.92±0.11 μmol/L; n=14; $P<0.05$) and 37.8±2.62%.in the SHRs (Ad. PRSx8-mCherry/CAPON, 0.74±0.07 μmol/L; n=13 and Ad.PRSx8-mCherry, 1.19±0.06 μmol/L; n=11; $P<0.05$) when compared with neurons transduced with empty viral vector (Figure 5B and 5C). 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 5C).

Cardiac Norepinephrine Release—Percutaneous gene transfer targeted to the right atrium of SHRs in vivo also increased right atrial CAPON protein expression as assessed by Western blot (Figure 6A). [^{3}H]-norepinephrine release from isolated atrial preparations in response to 5 Hz field stimulation was significantly decreased by Ad.PRSx8-mCherry/CAPON by 22.1±0.97% when compared with the empty virus (Ad.PRSx8-mCherry/CAPON, 2.7±0.15%; n=6 versus Ad.PRSx8-mCherry, 3.5±0.24%; n=6; $P<0.05$; Figure 6B and 6C). The specific nNOS inhibitor AAAN reversed the effect of CAPON gene transfer on [^{3}H]-norepinephrine 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 neurons.[13] Second, the expression of CAPON is reduced in the prohypertensive SHR compared with the WKY rat at 4 weeks, suggesting that it may be linked to the predisease neuronal phenotype. Third, 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 norepinephrine 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 neurons[26] as a binding protein for nNOS.[13] CAPON has been further discovered to be localized in ventricular myocytes,[10] rat neural tissues including the facial nerve,[27] sciatic nerve,[28,29] dorsal root ganglion, and lumbar spinal cord.[29] It has been implicated in neuronal pathogenesis, including peripheral nerve regeneration,[28] neuron loss and survival,[29] schizophrenia,[30] pain,[29] and inflammation.[26] Here, we observed significant CAPON expression in both WKY and SHR stellate neurons; however, levels were significantly reduced in prehypertensive SHR neurons. Interestingly, CAPON is also present in choline acetyltransferase–positive intracardiac neurons (C.-J. Lu, N. Herring, D.J. Paterson, unpublished data, 2014) although it is unknown whether it affects the sympathetic phenotype reported here.

nNOS-CAPON Signaling in the Prehypertensive SHR

Sympathetic hyperactivity and parasympathetic insufficiency are correlated with mortality in patients with and without cardiovascular disease.[31–33] In particular, overactivity of the sympathetic nervous system is implicated in the pathogenesis of human essential hypertension.[34–40] This has also been observed in an animal of model of genetic hypertension, the SHR, as early as 4 weeks where arterial blood pressure and ventricular weight: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 reduced norepinephrine reuptake transporter activity.[43] This translates into an enhanced tachycardia during right stellate ganglia simulation in vitro[19] and elevated heart rates in vivo under anesthesia,[19] as well as in telemetered animals.[42] 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 regulation.[44]

The augmented neuronal calcium current and impaired nNOS/CAPON signaling observed here may account for a major part of altered calcium homeostasis,[25] although defective mitochondrial buffering of $[Ca^{2+}]_i$ may also contribute[25] to this calcium impairment, and excessive adrenergic neurotransmission in the SHR.[18] Dysregulated neuronal Ca^{2+} signaling has recently been reported in both stellate neurons and parasympathetic neurons (intracardiac) in animals with heart failure.[45] Specifically, the N type Ca^{2+} current was enhanced in the sympathetic neuron, but impaired in the cholinergic neuron.[45] These observations provide an electrophysiological basis for the cardiac sympatho-vagal

phenotype seen in heart failure and hypertension. Of interest, we see that dyregulation of both the neuronal Ca^{2+} current and the 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-protein kinase A–dependent regulation of neuronal calcium transients[16] and norepinephrine release[17] in sympathetic neurons. Because prehypertensive SHRs have reduced cardiac stellate expression of nNOS, the β_1 subunit of soluble guanylate cyclase, cGMP,[20] 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 norepinephrine release in cardiac sympathetic neurons. Although the role of CAPON as an inhibitor or mediator of nNOS function in human disease has been widely debated,[46] in our study, overexpression of CAPON increased neuronal nNOS activity and cGMP levels in the SHR, while stabilizing nNOS protein expression. This is similar to that seen in isolated ventricular myocytes where CAPON enhanced NOS enzymatic activity and NO release.[10] 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 suggested.[47] For example, CAPON plays an important role in directing nNOS to specific target proteins such as synpasin.[14] However, like Chang et al,[10] 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 norepinephrine release was reversed by nNOS inhibition, suggesting the involvement of an NO-dependent pathway. Interestingly, CAPON is colocalized not only with nNOS but also with L-type calcium channels ($LTCa^{2+}$) and potassium channels (Kir3.1) in cardiomyocytes,[11] suggesting that 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 junctions.[48]

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 genome-wide association studies have identified a common genetic variant in CAPON that might contribute to QT interval abnormalities.[1–7] Although sympathetic stimulation can shorten the APD, it also stimulates myocyte calcium loading, and therefore it is conceivable that abnormal sympathetic activation because of CAPON impairment superimposed on a long-QT phenotype would further exacerbate the likelihood of afterdepolarizations.

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Perspective

We present evidence that CAPON-nNOS modulate 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 single-nucleotide polymorphisms in genome-wide association studies are associated with altered QT interval. It is, therefore, conceivable that abnormal sympathetic neurotransmission because of the same CAPON single-nucleotide polymorphisms could potentially further amplify the electrophysiological phenotype. Strategies that upregulate CAPON will shorten the APD[10] and also decrease sympathetic drive, thereby providing a rationale for therapeutic targeting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

What Is New?

- The neuronal calcium current and intracellular calcium transient are larger in the prohypertensive spontaneously hypertensive rat than in the Wistar–Kyoto rat, and this is associated with reduced expression of neuronal nitric oxide synthase adaptor protein (CAPON).
- Targeted upregulation 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 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 upregulation 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.

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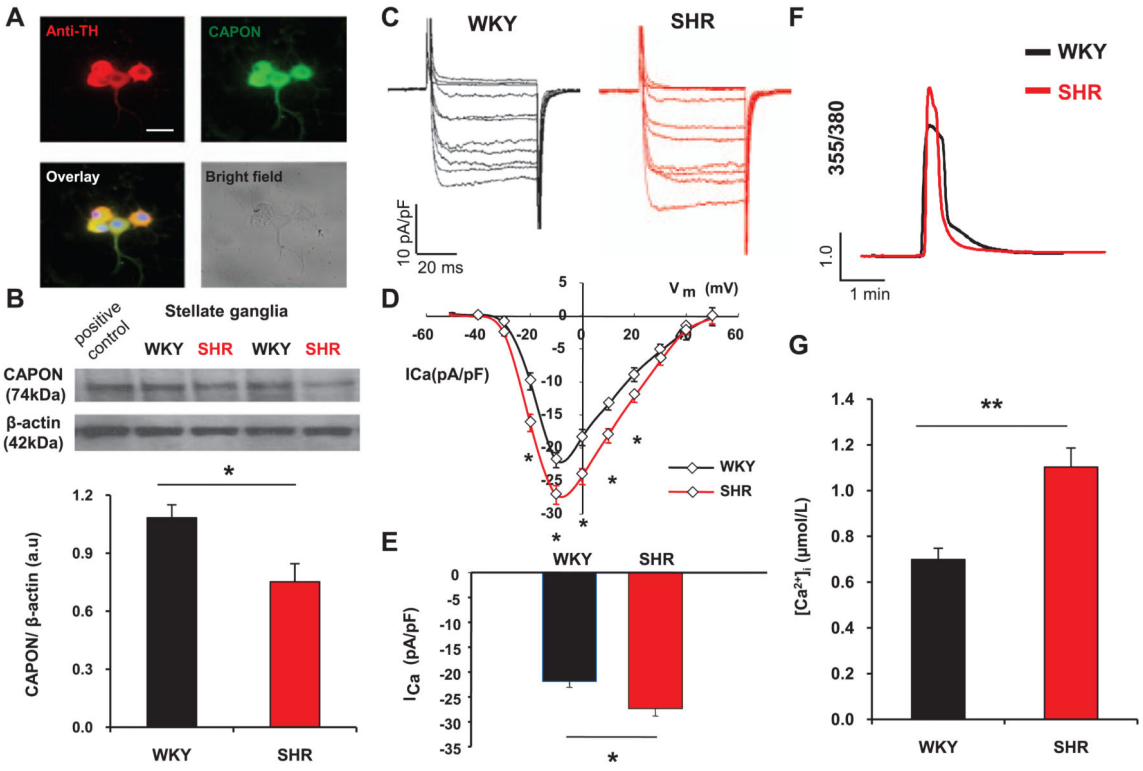

Figure 1.

A, Coimmunostaining of stellate neurons with anti–tyrosine hydroxylase (TH, red) and anti–neuronal nitric oxide synthase adaptor protein (CAPON, green) antibodies showed CAPON expression in sympathetic neurons. Nuclear staining with DAPI (4′,6-diamidino-2-phenylindole) is in blue. Scale bar, 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 with 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 $[Ca2+]_i$ transients in 4-week-old SHR and WKY stellate neurons (n=16 versus n=13; **P<0.01, unpaired t test).

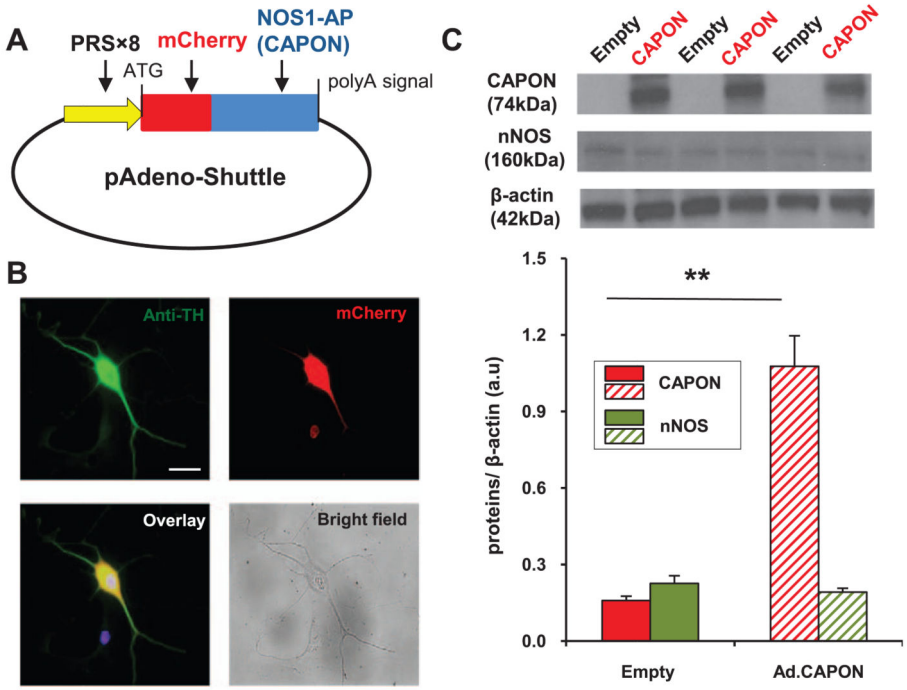

Figure 2.

A, Map of adenoviral vector construct containing a noradrenergic neuron-specific promoter, (PRSx8), NOS1-AP (neuronal nitric oxide synthase adaptor protein [CAPON]) gene, and red mCherry fluorescent protein (Ad.PRSx8-mCherry/CAPON). As a control, the same construct without CAPON gene insert was used (Ad.PRSx8-mCherry). **B**, Coimmunostaining 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 (4′,6-diamidino-2-phenylindole) is in blue. Scale bar, 25 μm. **C**, Representative Western blot and group mean data showing CAPON expression (74 kDa) and nNOS expression in stellate ganglia from 4-week-old SHR 3 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).

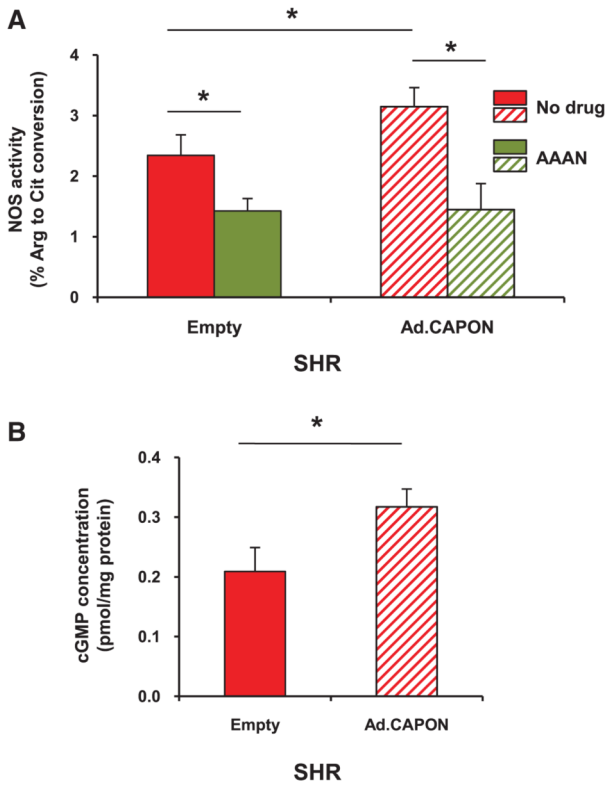

Figure 3.

A, Gene transfer of neuronal nitric oxide synthase adaptor protein (CAPON; Ad.PRSx8-mCherry/CAPON) significantly increased NOS activity in stellate ganglia from spontaneously hypertensive rat (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-aminoetyl) amino] pentyl]-N′-nitroguanidine (AAAN, 10 μmol/L) normalized the difference in NOS activity after 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 with empty controls (Ad.PRSx8-mCherry; n=10; **P*<0.05, unpaired *t* test).

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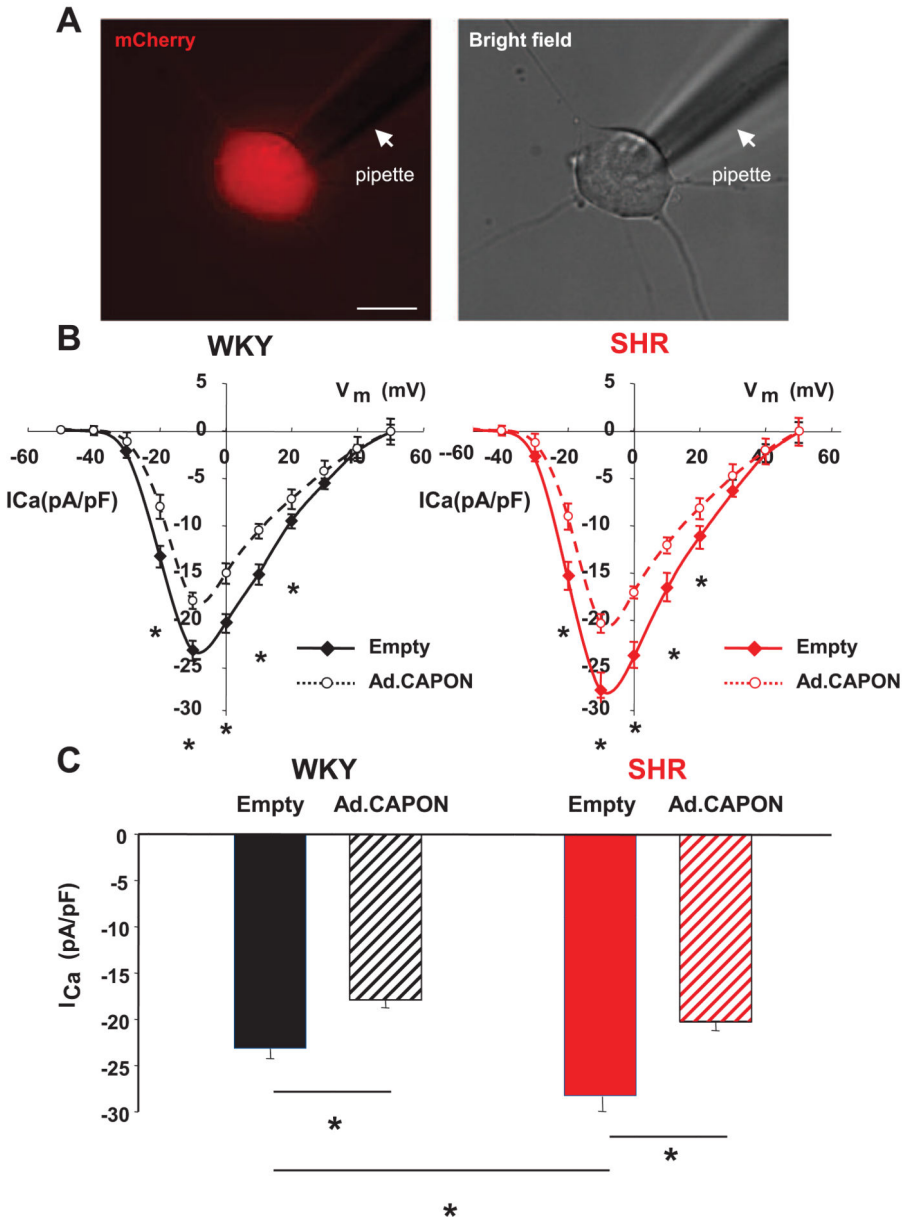

Figure 4.

A, Representative fluorescence (mCherry) and bright field images of single stellate neuron transduced with Ad.CMV-mCherry/neuronal nitric oxide synthase adaptor protein (CAPON; Ad.CAPON) with a patch pipette (pointed by a white arrow). Scale bar, 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-week-old spontaneously hypertensive rat (SHR) and age-matched Wistar–Kyoto (WKY) controls when compared with 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).

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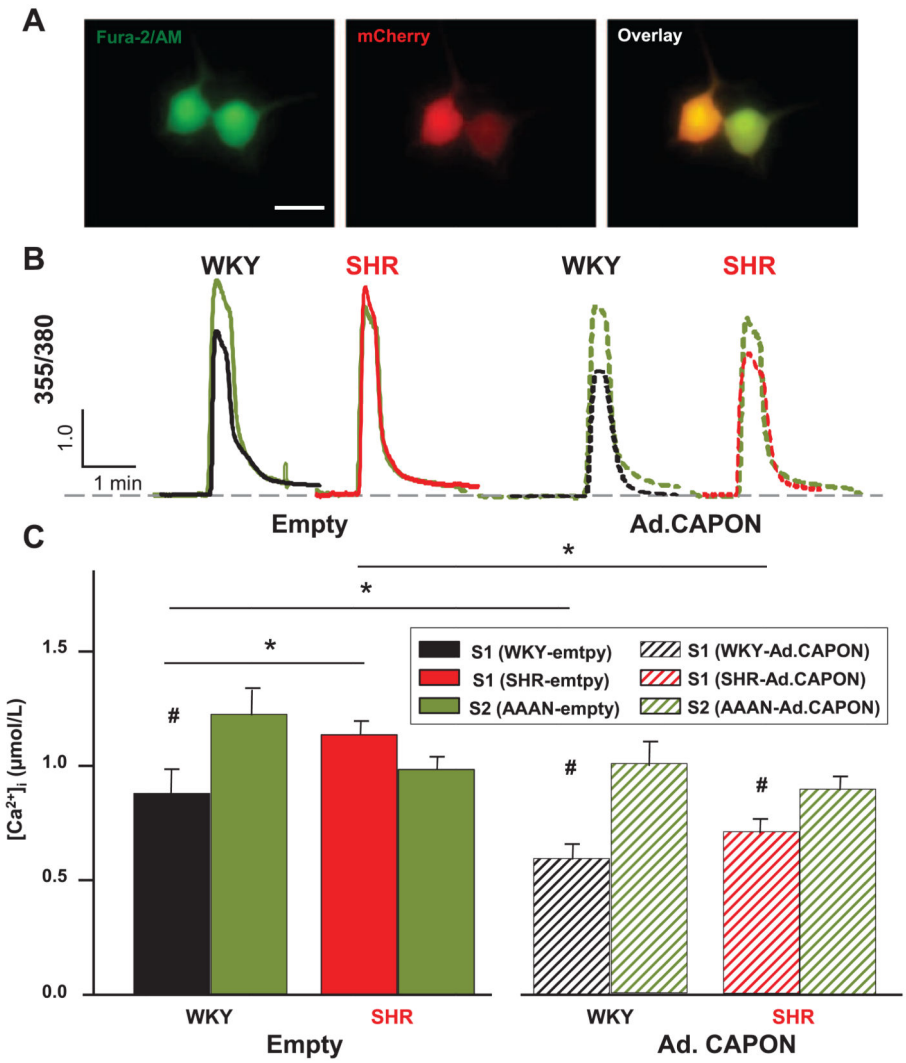

Figure 5.

A, Stellate neurons demonstrating loading of Fura-2 AM by green fluorescence and viral transduction with mCherry (red). Scale bar, 25 μm. 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 Wistar–Kyoto (WKY; n=14) rats compared with age-matched with SHR n=11, **P*<0.05, unpaired *t* test; #*P*<0.05, paired *t* test. Gene transfer with neuronal nitric oxide synthase adaptor protein (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-aminoetyl) amino] pentyl]-N′-nitroguanidine, AAAN, 10 μmol/L).

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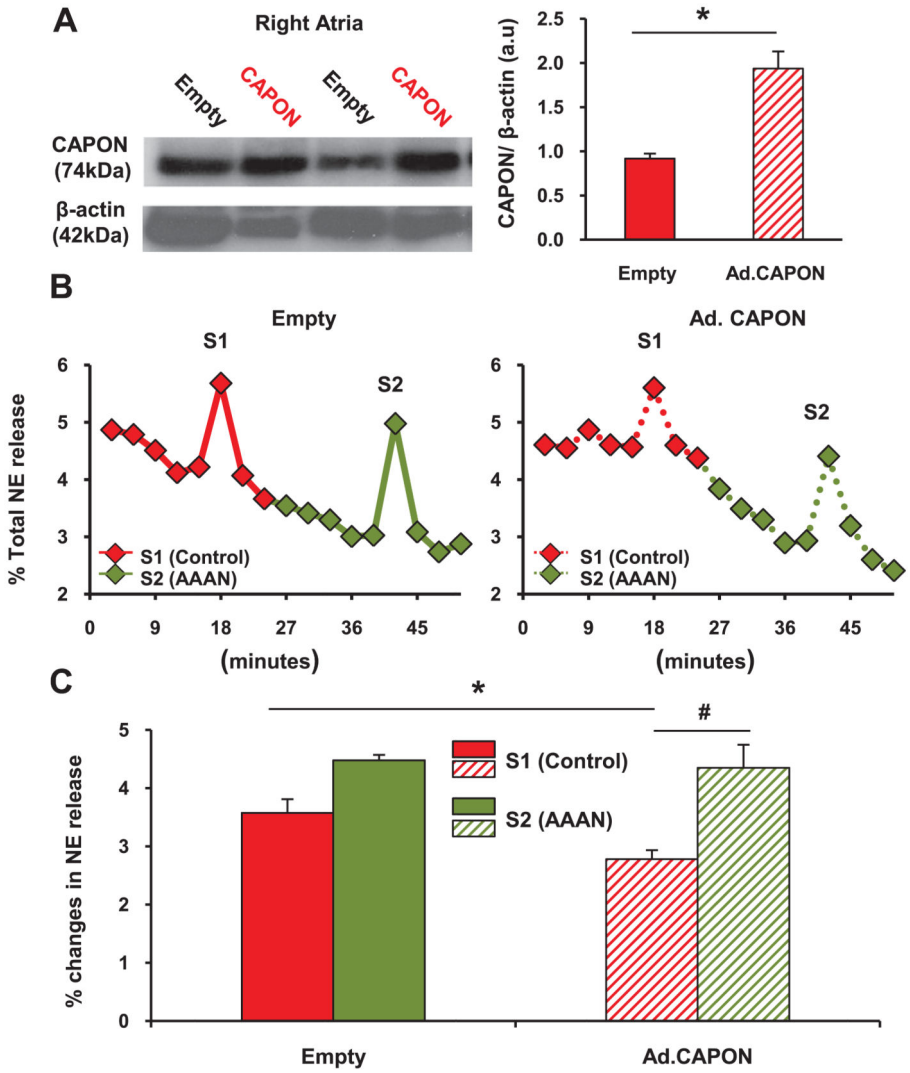

Figure 6.

Representative Western blot showing the upregulation of neuronal nitric oxide synthase adaptor protein (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 spontaneously hypertensive rat. 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 ^{3}H-norepinephrine (NE) release from isolated double atrial preparations in response to electric field stimulation (S1, 5 Hz) when compared with 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 μmol/L).