Phosphodiesterase 2A as a therapeutic target to restore cardiac neurotransmission
during sympathetic hyperactivity

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Abstract

Elevated levels of brain natriuretic peptide (BNP) are regarded as an early compensatory response to cardiac myocyte hypertrophy, although exogenously administered BNP shows poor clinical efficacy in heart failure and hypertension. We tested whether phosphodiesterase 2A (PDE2A), which regulates the action of BNP-activated cyclic guanosine monophosphate (cGMP), was directly involved in modulating Ca\(^{2+}\) handling from stellate ganglia (SG) neurons and cardiac norepinephrine (NE) release in rats and humans with an enhanced sympathetic phenotype. SG were also isolated from patients with sympathetic hyperactivity and healthy donor patients. PDE2A activity of the SG was greater in both spontaneously hypertensive rats (SHRs) and patients compared to their respective controls, whereas PDE2A mRNA was only high in SHR SG. BNP significantly reduced the magnitude of the calcium transients and \(I_{CaN}\) in normal Wistar Kyoto (WKY) SG neurons, but not in the SHRs. cGMP levels stimulated by BNP were also attenuated in SHR SG neurons. Overexpression of PDE2A in WKY neurons recapitulated the calcium phenotype seen in SHR neurons. Functionally, BNP significantly reduced \[^3\text{H}\]-NE release in the WKY, but not in the SHR. Blockade of overexpressed PDE2A with Bay60-7550 or overexpression of catalytically inactive PDE2A re-established the modulatory action of BNP in SHR SG neurons. This suggests that PDE2A may be a key target in modulating the action of BNP to reduce sympathetic hyperactivity.

**Key-Words:** BNP; PDE2A; sympathetic nervous system; synaptic transmission; Calcium
Introduction

Elevated levels of brain natriuretic peptide (BNP) may provide compensation in pathologies associated with heart failure, hypertension and cardiac dysautonomia (1). Cyclic nucleotide phosphodiesterases (PDEs) are enzymes that regulate the cellular levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) by controlling their rates of degradation in response to different stimuli such as natriuretic peptides (2) and β-adrenergic receptors (3). Among the PDE superfamily, PDE2A as a dual-specificity enzyme, has received considerable attention because it is markedly upregulated in heart failure and blunts β-adrenergic responses via hydrolysis of cAMP in cardiomyocytes (4). It also affects the action of BNP to stimulate cGMP, reducing protein kinase G (PKG) inhibition of intracellular calcium concentration ([Ca^{2+}]i) and subsequent neurotransmitter release (5). This indicates that manipulation of PDE2A might either be beneficial or deleterious depending on the target cell.

Physiologically, raised BNP is well established to bring about beneficial effects on cardio-renal homeostasis (6) and sympatho-vagal balance (7), therefore infused BNP should theoretically have positive clinical utility in a number of cardiovascular pathologies. However, large-scale, randomized, double-blinded, and placebo-controlled trials such as FUSION II (8) and ASCEND-HF (9) failed to demonstrate efficacy using a recombinant form of human BNP, nesiritide, in the setting of acute decompensated heart failure. These disappointing results were compounded by the meta-analysis (10) suggesting that nesiritide may actually increase mortality (11) where BNP might facilitate norepinephrine (NE) release (in PC12 cells) and provide a trigger for arrhythmia (12). Interestingly, the sympatholytic action of paracrine BNP is substantially attenuated when PDE2A activity is enhanced in cardiac stellate neurons (13), which is observed during dysautonomia associated with hypertension (14) and heart failure (4).

Therefore we tested if PDE2A is present in human stellate ganglia (SG) and hypothesized that its levels are enhanced in neurons from patients with sympathetic overdrive. These SG were derived from patients undergoing stellectomy for treatment of intractable ventricular arrhythmias (15) and were compared to those harvested from organ donors. We also tested in a rat model of sympathetic hyperactivity whether BNP loses its efficacy on post-ganglionic stellate sympathetic neurons due to over-activity of PDE2A, thereby impairing cGMP-PKG via regulation of the neuronal calcium current, intracellular calcium
transient and with resultant effects on cardiac NE release. We identified PDE2A in human and rat SG and found increased PDE2A activity in SG from patients with refractory ventricular arrhythmias and rats that exhibit a sympathetic phenotype. Moreover, we demonstrate that blockade of overexpressed PDE2A or overexpression of catalytically inactive PDE2A in SG neurons reverse-remodeled the modulatory actions of BNP to decrease Ca^{2+}-induced exocytosis and neurotransmission.
Results

Impaired cGMP signaling in SHR neurons following BNP stimulation

Real-time measurements of intracellular cGMP concentration in living cardiac sympathetic neurons were performed using ratiometric fluorescence resonance energy transfer (FRET) imaging in the presence of exogenous BNP (10, 100, 250 nmol/L, Figure 1A). BNP increased cGMP levels in a dose-dependent manner (calculated as a mean of the four values prior to the addition of the subsequent dose). However, the cGMP response to BNP was attenuated in SHR neurons relative to Wistar (W) neurons at 100 & 250 nmol/L (10 nmol/L: W=1.60±0.31%, SHR=1.10±0.30%, P=0.262; 100 nmol/L: W=4.02±0.73%, SHR=2.16±0.46%, P=0.043; 250 nmol/L: W=6.16±0.70%, SHR=3.89±0.61%, P=0.021; W n=14, SHR n=15; Figure 1Bi). In order to investigate the contribution of PDE2A and the total PDE to the different cGMP response between SHR and Wistar rats, a specific PDE2A inhibitor Bay60-7550 (1 µmol/L) and non-specific PDE inhibitor IBMX (100 µmol/L) were added in the presence of 100 nmol/L BNP. Both Bay60-7550 and IBMX abolished the differences of the cGMP generation stimulated by 100 nmol/L BNP (Figure 1Bii). There was also no difference between the Bay60-7550 and IBMX responses, suggesting that PDE2A is the major PDE linked to the reduction of cGMP in the SHR sympathetic neurons.

Enhanced PDE2A expression and activity in sympathetic ganglia from disease models

Here we show the presence of PDE2A in human SG. cGMP-PDE2A specific activity in patients was higher (134.6±10.1 pmol/mg/min, from 5 left SG) compared to donors (75.6±17.1 pmol/mg/min, from 4 left SG P=0.028, Figure 2Ai). Total PDE activity was not statistically different, although there was a trend for an increase in the diseased group (Figure 2Ai). There was also no significant difference in right SG between patients and the control group (Figure 2Aii). cGMP-PDE2A specific activity was enhanced in 38 week-old SHRs (96.8±6.8 pmol/mg/min, n=6 pairs SG) compared to the age-matched WKY rats (52.8±3.0 pmol/mg/min, n=6 pairs SG) (P=0.04, Figure 2B). This is similar to that previously reported for PDE2A activity in 4 week-old SHRs (13). Total PDE activity and following PDE2A inhibition (1 µmol/L Bay60-7550) were not statistically different (p=0.053 & 0.066 respectively, Figure 2B), there was a trend for an
increase in the diseased group suggesting that other PDEs might also be enhanced in 38-weeks SHRs. For the cAMP-PDE specific activity, no difference was detected in total PDE, with PDE2A inhibition (1 µmol/L Bay60-7550) and relative PDE2A activity (Figure 2C) between 4-weeks SHR and WKY SG.

Quantitative RT–PCR analysis of relative PDE2A mRNA levels showed the presence of PDE2A gene expression in patients and donors (n=4 left and right SG in each group), although there was no difference due to lack of statistical power (Figure 2D) and probably reflects clinical heterogeneity in the human samples (see Table). PDE2A mRNA levels in 4 week WKY was significantly lower than age-matched SHR (n=6 in each group, P=0.04, Figure 2E) suggesting some conservation of this molecular phenotype across species and developmental state.

**Effect of BNP on norepinephrine release from the WKY and the SHR**

To test whether neurotransmitter release is altered in pro-hypertensive rats, we directly measured the level of [3H]-NE release in response to field stimulation of the right atrium. There was no difference between the peak of the first (S1) and second (S2) field stimulation (5-Hz for 1 minute) in both WKY (S1: +0.72±0.09%, S2: +0.69±0.04% n=6, t-test, P=0.724) and SHR group (S1: +1.01±0.12%, S2: +1.03±0.15% n=7, t-test, P=0.946, Figure 3A & B), indicating no significant time-dependent changes. However, the level of [3H]-NE release in the SHR was significantly higher than the WKY rats at both baseline (WKY: 2.392±0.078%, n=6 vs SHR: 3.314±0.19%, n=7, P<0.01 unpaired T-test) and after 5-Hz field stimulation (S1: WKY: 0.71±0.09%, n=6 vs SHR: 1.06±0.10% n=7, P<0.05; S2: WKY: 0.65±0.06%, n=6 vs SHR: 1.1±0.11%, n=7, P< 0.05, one-way ANOVA; Figure 3A & B).

To investigate whether BNP affected [3H]-NE release during 5-Hz field stimulation, a high concentration of 250 nmol/L BNP was added after the first stimulation from 27 minutes. This produced a ~38% reduction of [3H]-NE release in the WKY (S1: +1.07±0.08%, S2: +0.66±0.05% n=9, P=0.04, one-way ANOVA). However, BNP failed to change [3H]-NE release in the SHR (S1: +1.17±0.15%, S2: +1.18±0.14%, n=9, P=0.97, one-way ANOVA Figure 3C & D).
Effect of BNP on calcium current ($I_{\text{CaN}}$) and intracellular free calcium transients in SG neurons in the WKY and the SHR

We next investigated whether BNP would affect calcium signaling in isolated SG neuron from SHR and WKY rats. Anti-tyrosine hydroxylase (TH, sympathetic neuron marker) and anti-PDE2A immunofluorescence staining from fixed cultured SG neuron are shown in Figure 4A, confirming that PDE2A was expressed in sympathetic neurons. The peak $I_{\text{CaN}}$ was significantly reduced by -18.2±7.2% (P<0.05; n=6) after 10-minute exposure to 100 nmol/L BNP in WKY neurons (Figure 4Ba), but BNP failed to affect the SHRs neurons (Figure 4Bb). However, BNP restored its inhibitory action following inhibition of PDE2A with Bay60-7550 (1 μmol/L, reduced by -12.5±6.3%, P<0.05; n=6, Figure 4Bc).

We measured intracellular calcium concentration using ratiometric recordings (with Fura-2-acetoxymethyl ester, Fura-2/AM) in single SG neurons. A protocol for the calcium-transient response to 50 mmol/L KCl is shown in Figure 4Ci&ii. BNP was introduced at 7 minutes after the first KCl application. After 10 minutes of treatment, neurons were stimulated again (S2) in the presence of different concentration of BNP with or without the PDE2A inhibitor Bay60-7550. In the WKY rats, 100 or 250 nmol/L BNP caused a reduction in $[\text{Ca}^{2+}]_i$ evoked by high K+ depolarization by about 22% (n=13) and 20% (n=13) respectively. In the presence of Bay 60-7550 (1 μmol/L), application of BNP reduced the $[\text{Ca}^{2+}]_i$ transient by -23.32 ±5.1% (n=10, P<0.01) at 100 nmol/L, and -21.17 ±3.4% (n=10, P<0.01) at 250 nmol/L (Figure 4Di) in the WKYs. In contrast, BNP alone did not affect evoked $[\text{Ca}^{2+}]_i$ in the SHRs (90.51 ± 2.71% at 100 nmol/L n=10 and 108.91 ±4.22% at 250nmol/L, n=12, Figure 4Dii). Moreover, the inhibitory action of BNP was unmasked by the presence of PDE2A inhibition (100 nmol/L BNP: -24.3 ±3.1%, n=11, P<0.001; 250 nmol/L BNP: -13.6 ±3.6%, n=11, P<0.05, Figure 4Dii). Taken together, these results suggest there is significant cellular impairment in SG neurons from pro-hypertensive rats involving the BNP-cGMP-PDE2A pathway linked to calcium signaling.
Overexpression of PDE2A in stellate neurons from normotensive rats prevents the inhibitory action of BNP

To directly test the hypothesis that increased PDE2A activity abolishes the capacity of BNP to reduce 
\([\text{Ca}^{2+}]_i\), in depolarised neurons, Ad.CMV-mCherry/PDE2A or its control vector (Ad.CMV-mCherry) was 
transduced in the neurons of the WKY rat. Western Blot showed that PDE2A.mCherry expression (135KDa, 
with anti-PDE2A antibody) was significantly enhanced in Ad.PDE2A transduced SG tissue when 
compared with those transduced with the mCherry virus alone (n=4 in each group, Figure 5A).

We then compared \(I_{\text{CaN}}\) in WKY SG neurons with the mCherry virus and Ad.PDE2A virus. 100 nmol/L 
BNP reduced \(I_{\text{CaN}}\) (-21.7±6.1%, n=9, P <0.05, Figure 5Ba) with the mCherry virus; However, 100 nmol/L 
BNP failed to reduce \(I_{\text{CaN}}\) (n=7, P = 0.79, Figure 5Bb) in the WKY SG neurons with overexpressing PDE2A. Interestingly, 100 nmol/L BNP in the presence of Bay60-7550 decreased the calcium current (-14.4±5.7%, 
n=6, P<0.05, Figure 5Bc) in these neurons over-expressing PDE2A, thus restoring the efficacy of BNP.

For the intracellular calcium transient, after transduction with Ad.PDE2A, 100 nmol/L BNP failed to 
reduce the depolarization-induced \([\text{Ca}^{2+}]_i\) transient (-6.7±4.0%, n=7, P=0.79, Figure 5Cii). Indeed, there 
was a trend for 250 nmol/L BNP to increase \([\text{Ca}^{2+}]_i\), (+7.8±13.9%, n=8, P=0.99 , Figure 5Cii) when 
compared with the control group. In contrast, BNP (100 & 250 nmol/L) reduced \([\text{Ca}^{2+}]_i\) in neurons 
transduced with the mCherry virus in the WKY (Figure 5Ci). Moreover, 100 or 250 nmol/L BNP in the 
presence of Bay60-7550 (1 µM) caused a decrease in peak \([\text{Ca}^{2+}]_i\), (-27.3±2.5%, n=11, P<0.05 or -32±4.1%, 
n=9, P<0.05, Figure 5Cii) in the WKY neurons over-expressing PDE2A. Thus, as in SHR neurons, 
concurrent PDE2A inhibition restores the capacity of BNP to decrease calcium transients. Collectively, 
these data indicate that up-regulation of PDE2A in sympathetic neurons impairs BNP responsiveness, 
inducing an SHR-like calcium phenotype.
Over-expression of dn.PDE2A in cardiac stellate neurons and atria from SHR rescues the inhibitory action of BNP

Overexpression of catalytically inactive PDE2A was previously shown to increase local levels of cAMP by displacement of endogenous active PDE2A from its intracellular anchor sites (16, 17). Therefore, we introduced a dominant-negative PDE2A (a catalytically-dead mutant of PDE2A) using a viral vector (Ad.CMV-mCherry.dnPDE2A) to assess whether inactive PDE2A can recover the BNP effect in the SHR. Reduced PDE2A activity in transduced Ad.dnPDE2A sympathetic ganglia from SHR was confirmed by measuring cGMP-PDE2A specific activity when compare with transduced Ad.mCherry empty virus (Figure 6A). As we expected, 100 nmol/L BNP did not produce any significant change of the calcium current in the SHR with the control mCherry viral vector (Fig. 6Bi). However, following Ad.dnPDE2A, 100 nmol/L BNP caused a significant decrease of $I_{CaN}$ compared to the control ($-18.5\pm7.9\%$, n=6, P<0.05, Fig. 6Bii), indicating that in SHR cells endogenous active PDE2A degrades cGMP generated on BNP stimulation, blocking its effect on $I_{CaN}$.

We then measured the $[Ca^{2+}]_i$ transient in the Ad.dnPDE2A transduced SG neurons in the SHR. Similar to the calcium current, BNP (100 and 250 nmol/L) did not produce any significant change of $[Ca^{2+}]_i$ in SHR neurons with the mCherry vector (Fig. 6Ci). However, in the presence of Bay60-7550, BNP (100 and 250 nmol/L) caused a significant decrease of the $[Ca^{2+}]_i$ transient compared to the control (100 nmol/L: $-17.8\pm3.8\%$ n=10, P<0.05; 250 nmol/L: $-31.1\pm3.1\%$ n=9, P<0.001, Fig. 6Ci). By comparison, treatment with 100 or 250 nmol/L BNP decreased $[Ca^{2+}]_i$ transient in dnPDE2A transduced SHR neurons when compared with control ($-18.1\pm2.2\%$ n=13, P<0.001; $-27.6\pm2.3\%$ n=8, P<0.01, Fig. 6Cii).

For $[^3H]$-NE release, Ad.dn.PDE2 transduction did not change the first field stimulation (S1) on the isolated right atrium from the SHR when compared with the mCherry virus (Figure 7B, Ad.dn.PDE2A S1: $+1.22\pm0.12\%$ n=10, vs mCherry S1: $+1.35\pm0.11\%$, n=8, P=0.72, one-way ANOVA), indicating the gene transfer of Ad.dn.PDE2A did not affect the ability of the neurons to release NE on depolarisation. There was no significant change of $[^3H]$-NE release after application of 250 nmol/L BNP (S2) in the mCherry virus group when compare with S1 (S1: $+1.35\pm0.11\%$, S2: $+1.4\pm0.13\%$, n=8, P=0.74, one-way ANOVA, Figure 7Ai & 7B). However, in the Ad.dn.PDE2A group, BNP (250 nmol/L) produced a ~50% reduction...
in [3H]-NE release (S1: +1.22±0.12%, S2: +0.61±0.1%, n=10, P<0.01, one-way ANOVA, Fig. 7Aii & 7B).

These data support the hypothesis that reduced PDE2A activity by inactive PDE2A catalytic domains can rescue the capacity of BNP to reduce NE release in stimulated right atrium.
Discussion

Three novel findings are presented in this study. First, PDE2A is present in human SG where its activity was higher in the left SG from patients with neuro-hormonal hyperactivation compared to control. This molecular phenotype was also seen in SG from hypertensive and pre-hypertensive rats, in addition PDE2A mRNA levels were higher in the SHR compared to normal SG. Secondly, the ability of BNP to enhance cGMP production and decrease calcium signaling and neurotransmitter release was impaired in SHR sympathetic neurons. Finally, PDE2A inhibition or catalytically inactive PDE2A can restore the efficacy of BNP to decrease sympathetic neurotransmission in diseased neurons as depicted in figure 8.

Impaired response to BNP in the SHR

The physiological actions of the natriuretic peptides in cardiorenal homeostasis and endothelial permeability are well known(18), however there has been little attention to their role in the autonomic nervous system, considering some reports suggest that the BNP analog nesiritide facilitates NE release (12). It is well established that the inhibitory action of BNP on ventricular myocyte calcium signaling is modulated by the NPR-A/cGMP/PKG pathway (19), where PDE2A regulates the hydrolysis of cGMP (5) and decreases the calcium transient (19). We have also reported that BNP produces a direct depressant action on sympathetic nerve function by decreasing NE release and heart rate during sympathetic stimulation in vitro (13). This is the first time that aberrant cGMP signaling has been demonstrated in cardiac sympathetic neurons of SHRs in response to BNP indicating possible impairment of PDE regulation of cGMP.

In this study, we questioned whether an aspect of the lack of clinical efficacy of BNP in the treatment of essential hypertension (2, 3) and HF (9, 20) may in part be caused by the aberrations in the sympatholytic effects of natriuretic peptides (13) at the level of the postganglionic sympathetic neuron. SHRs have many similarities with human hypertension, including the level of plasma BNP (21) and NE concentrations (22), and chronic progression to cardiac hypertrophy and heart failure (23). They also have a sympatho-vagal phenotype similar to that observed in human hypertension and heart failure (24, 25). Results presented here on the SHR model support the hypothesis that BNP not only failed to significantly reduce the calcium
transient and the calcium current in depolarised SHR neurons, but it also failed to inhibit NE release. Moreover, higher concentration of BNP actually increased the magnitude of Ca transient in diseased neurons indicating that BNP coupling to intracellular Ca\(^{2+}\) signaling is impaired. Although, the concentrations of BNP used here need to be confirmed as being pathophysiological, natriuretic peptides reach similar concentrations at the postganglionic sympathetic nerve ending in advanced HF (12). Nevertheless, we confirm the intimate link between [Ca\(^{2+}\)] and neurotransmitter release. We also show that impaired calcium responses to BNP in SHR neurons abrogates the BNP-mediated reduction in NE release and HR (26) that is coupled to over-activity of PDE2A. Overexpressing PDE2A in healthy neurons could mimic the disease phenotype and recapitulate the loss of BNP efficacy in SHR neurons.

The role of PDE2-mediated cGMP hydrolysis in cardiac sympathetic neurons

cAMP and cGMP cross-talk is regulated by the PDE family which is considered important in maintaining cell-signaling processes and cyclic nucleotide balance within specific subcellular microdomains (5). PDEs are involved in different stages of advanced cardiac diseases. PDE1-3 hydrolyse cAMP and cGMP, and both PDE2A and PDE3A have been implicated in the pathogenesis of hypertension (13, 27). Some report that the activity of PDE2A towards the hydrolysis of cAMP is relatively low compared to cGMP (28), although it still contributes to the regulation of cardiac L-type calcium channel (\(I_{CaL}\)) activity, where it inhibits the activation of \(I_{CaL}\) by reducing cAMP concentration after being stimulated by cGMP (29). Our findings support the idea that the activity of neuronal PDE2A is favored towards the hydrolysis of cGMP since PDE2A induced cAMP activity in the presence of 1µmol/L cGMP resulted in no difference in cAMP levels. Moreover, overexpressed PDE2A reduced cGMP and not cAMP levels in SHR sympathetic neurons. Previously we have reported that in normal stellate neurons PDE2A inhibition can increase the neuronal calcium current, presumably by slowing cGMP hydrolysis as a consequence of cGMP acting to inhibit PDE3 leading to greater levels of cAMP–PKA activity (14). However, in the present study PDE2A inhibition or IBMX restores BNP activated cGMP to normal levels (Fig 1B) resulting in a decrease in intracellular calcium handling and neurotransmission.

Although PDE2A is up-regulated in cardiomyocytes in human heart failure (4), and angiotension II induced cardiac hypertrophy from rats (28), data are lacking for its role in sympathetic neurons and in cardiac
autonomic pathophysiology. Chan et al. have reported a proexocytotic effect of BNP that involves an increase in intraneuronal cAMP resulting from a cGMP-PKG-mediated inhibition of PDE3 in PC12 cells (12). This is in contrast to previous work (13) and the present findings where raised PDE2A is directly linked enhanced sympathetic transmission in the SHR. Of interest, single nucleotide polymorphisms are present in PDE2A in the SHR/ola strain such as rs197163010 (30), and therefore this could provide a genetic basis for the molecular phenotype seen in our SHR neurons. The mechanism underpinning up-regulation of PDE2A in disease is not fully understood, but might be related to local neural inflammatory pathways, since TNF alpha and IL-6 have been reported to increase PDE2A expression (31, 32).

**Limitation and Clinical Perspectives**

There is evidence that NPR-A receptor is down-regulated in animal models of heart failure (33, 34), and that cGMP production by NPR-A is also reduced in the failing heart (35). However, our results have confirmed that NPR-A protein expression is not altered in SHR neurons (data not shown), indicating the NPR-A receptor itself is unlikely to be responsible for the impaired response to BNP in SHR neurons. Stellate ganglion neurons from the SHR may only provide a qualitative cellular surrogate to study the neurobiology of sympathetic impairment in human dysautonomia, even though stellate ganglion neuronal responses in the SHR and the molecular profile of PDE2A activity closely resembles that seen in human sympathetic pathophysiology. Because of the limited amount of human tissue (disease or donor), we were only able to ascertain a relatively semi-quantitative molecular profile. The variance in these data are influenced by the varied pathology of each patient and the extent of their sympathoexcitation. However, we provide the first evidence for the expression of PDE2A in human SG neurons. Whether these levels of neuronal PDE2A negate the sympathetic action of synthetic natriuretic peptide like nesiritide to treat cardiac failure remains to be established. Interestingly, we found that stellate ganglion neuronal cGMP-PDE specific activity is significantly increased in both sympathetic hyperactive patients and SHR neurons. In particular this was observed only in the left SG stellate from the patient group where sympathetic innervation is predominately to the left ventricle where NE spillover is increased in patients with heart failure (36). Moreover, overexpression of PDE2A in healthy neurons mimics the lack of BNP efficacy seen
in diseased neurons. Elevated PDE2A activity leads to excessive excitability and increased NE release. The specific inhibitor of PDE2A, Bay 60-7550, and the catalytically inactive PDE2A (dnPDE2A) targeted at cardiac sympathetic neurons rescued the BNP responsiveness in SHR stellate neurons. Therefore lowering PDE2A activity is consistent with the hypothesis that neuronal PDE2A targeting might be a putative therapeutic strategy to reduce sympathetic activity. Our data are also consistent with the literature, showing increased intraneuronal cGMP upon inhibition of PDE2A (11, 37-40). Thus, activation of PDE2A by pGC-derived cGMP in response to NPR-A-binding may form a specific regulatory mechanism, limiting the sympatholytic effects of BNP when PDE2A levels are abnormally high.
Methods

**Human Subjects.** Use of tissues from human subjects (see Table) was approved by the Institutional Review Board at the University of California Los Angeles (#12-000701) and a materials transfer agreement into the University of Oxford Bio-Bank (MAT2015-705). Bilateral SG were obtained from patients with cardiomyopathy and refractory ventricular arrhythmias undergoing stellate ganglionectomy (6 left and 6 right SG). Control SG were obtained from heart and/or lung donors (8 left and 7 right SG). Patients undergoing elective surgery provided written informed consent. In addition to consent for organ donation, consent to utilize tissues for research was also provided for tissues collected from organ donors. The tissues were immediately frozen in liquid nitrogen and subsequently stored in -80°C for PDE activity measurement, or immediately stored with RNAlater (Ambion, Invitrogen/Thermo Fisher Scientific, Canoga Park, CA, USA) for gene expression analysis.

**Animals.** Four-week old male pre-hypertensive SHRs and normotensive Wistar-Kyoto (WKY) rats (sourced from Harlan, UK) that have a well-established cellular sympathetic phenotype were used in this study (41). In addition, some experiments were performed on older rats (38 weeks) that have an established systemic phenotype. The investigation conformed to the Guide for the National Institutes of Health (Publication No. 85-23, revised 1996) and Animals Scientific Procedures (ASP) Act 1986 (UK). Procedures were performed under British Home Office license requirements (PPL 30/3131).

**PDE2A Activity Assay.** SG from WKY and SHRs or from human were dissected and rapidly frozen in liquid nitrogen. PDE activity was measured using the PDE Activity Assay Kit (Colorimetric) (Abcam, ab139460) according to the manufacturer's instructions. Briefly, tissues were homogenized in lysis buffer and protease inhibitor cocktail (Sigma), centrifuged at 10,000 rpm (4°C, 10 min) in a microfuge. Protein concentration was quantified by Bradford assay; 7–15 μg of protein was used per sample. Samples were desalted using 0.5 mL Zeba Spin Desalting Columns (Thermo Scientific, 89882) to remove endogenous free phosphate. Samples were assayed in a reaction mixture (total volume, 50 μl/well) containing 200 μmol/L cGMP substrate, 5’-nucleotidase (50 kU/well), PDE enzyme (20 mU/well) for 17 min at 30 °C. The reaction was terminated by addition of Green Assay Reagent (100 μl/well). After incubation at room temperature for 25 min, the phosphate-dependent color reaction was measured by reading OD₆₂₀nm in a
microplate-reading spectrophotometer. Individual PDE activities were measured in the absence of the inhibitor as well as in the presence of 1 µmol/L Bay 60-7550, a highly specific PDE2A inhibitor. PDE2A activity is shown as pmol 5’-GMP produced per mg of protein per min inhibited by Bay 60-7550.

To judge the contribution of PDE2A to cAMP hydrolysis in SG tissues of 4 weeks WKY and SHRs, same protocol as above was performed except used 4-7 µg of protein per sample, assayed in 200 µmol/L cAMP (instead of cGMP) substrate plus 1 µM cGMP as an allosteric activator, incubated for 10 min at 30 °C.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total RNA from SG from WKY and SHRs rats or human tissue was extracted using a RNeasy Protect Mini Kit (Qiagen) according to the manufacturer’s instructions. For reverse transcription, first-strand cDNA was synthesized from 1 µg of total RNA with the iScript™ cDNA Synthesis Kit (Biorad) according to the manufacturer’s protocol. qRT-PCR was conducted in a total of 20 µl containing 10 µl of Taqman Universal PCR Master mix (Applied Biosystems), 4 µl of cDNA (10ng/µl), 1 µl of 20X specific primers for Taqman Gene Expression Assays (Rn01648917_m1 for PDE2A of rat, Hs00159935_m1 for PDE2A of human, Rn00667869_m1 for β-Actin of rat, Hs01060665_g1 for β-Actin of human, Thermo Fisher Scientific) and 5 µl of DNase-free water. Quantitative real-time RT-PCR was performed in a 96-well clear optical reaction plate 7000 apparatus (Applied Biosystems), and the thermal cycling conditions were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All samples and standards were run as triplicates. Results were analysed with the ABI Prism 7000 Sequence Detection System software (Applied Biosystems). Gene expression was normalized to β-Actin that was used as an internal control.

[^H]-Norepinephrine Release Experiments on the Isolated Right Atrium. The spontaneously beating rat atrium was isolated and transferred to a preheated (37±0.2°C), continuously oxygenated (carbogen: 95% oxygen, 5% CO2), water-jacketed organ bath containing 3ml Tyrode solution where the atrium was pinned flat on a silver stimulating electrode. The method for determining the local release of ^3H-NE to field stimulation 5 Hz (15 V, 1 ms pulse width, for 1 min) was identical to that which we have previously described (42).
**Primary Cultures of Dissociated Sympathetic Neurons.** Sympathetic neurons were isolated from SHRs and WKY rats using a previously described method (41). Briefly, the SG were dissected from the rats and neurons enzymatically dissociated with collagenase and trypsin on 37°C. After a sequential mechanical trituration, cell suspension containing SG neurons was plated onto poly-D-lysine/laminin-coated coverslips and incubated at 37°C in a humidified atmosphere of 95% air- 5% CO₂. Experiments were performed 2-3 days after plating.

**Adenovirus Vector Transduction.** An adenoviral vector expressing PDE2A or catalytically inactive PDE2A (dominant negative PDE2A, dnPDE2A) tagged with red fluorescent protein mCherry (Ad.CMV.mCherry- PDE2A or Ad.CMV.mCherry-dnPDE2A) (43) was transduced to cultured cardiac sympathetic neurons (for calcium current and transient measurements) or isolated stellate ganglia tissue (for western blot). An adenoviral vector expressing only mCherry (Ad.CMV-mCherry) was used as a control for comparing the effect of viral transduction. 5×10⁷ pfu of adenoviral vector was used to infect neurons or ganglia in a 4 well plate (1.9 cm²/well, Nunc, Denmark). The virus containing medium was left in the well overnight before changing to fresh medium. Experiments were performed after 3 days following gene transfer, except that the patch clamp recordings were performed at 12–22 hours to minimize problems with space clamp (14).

For the evoked NE release experiment, targeted percutaneous gene transfer to the right atrium of rats was performed under isoflurane anesthesia (4% for induction and 2-3% for maintenance in 100% O₂), using a technique similar to that described previously for the guinea-pig (44). WKY and SHRs received an injection of 5×10⁸ PFU/ml of Ad.dn.PDE2A or Ad.PDE2A in 300μL of sterile phosphate-buffered saline. Physiological phenotyping was performed 4 days after the injection.

**Real-time analysis of cGMP dynamics.** Fluorescence resonance energy transfer (FRET) imaging was used to measure the intracellular cGMP. An adenoviral vector expressing the reporter cGi500 (kindly provided by Dr. Michael Russwurm) (45) was used as the cGMP biosensor under the control of the human CMV promoter. A Nikon microscope was connected to an OptoLED fluorescence imaging system (Cairn Research Ltd.) equipped with a 40x oil-immersion objective, a CoolSNAP HQ2 digital CCD camera (Photometrics), and a beam-splitter (DV2, Photometrics) including the specific set of emission filters for
donor and acceptor fluorophore acquisition (dichroic mirror 505DCXR, donor emission of 480nm, and acceptor emission of 535nm; Chroma Technology Corp). Images were obtained every 15 seconds. FRET values were measured as changes in the ratio between 480/535-nm fluorescent emission intensities after background subtraction upon excitation at 430nm. The 480/535nm emission intensity ratio was plotted against time, and the mean FRET response was expressed as the percentage of \( \Delta R/R_0 \), in which \( \Delta R = R - R_0 \). \( R_0 \) is the mean of the FRET ratio values calculated from the four images acquired before the addition of the first drug, and R is the ratio at time = t seconds.

**Patch-Clamp Recordings.** Calcium currents were recorded using conventional whole cell techniques (14). Pipette resistance varied from 1.5 to 2 MΩ 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 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 MgCl₂ and 11 Glucose, 1 NaHCO₃, 2 BaCl₂, and 0.001 TTX, adjusted to pH 7.4 with Sigma base 7 to 9. Barium was chosen to isolate Calcium currents and avoid Ca-dependent current inactivation (46) \( I_{Ba} \) refers to \( I_{Ca} \), unless otherwise specified. The bath was grounded by an Ag/AgCl electrode connected via a 3 mol/L 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. Current amplitude data of each cell was normalized to its capacitance and expressed as current density (pA/pF).

**Measurement of Intracellular Calcium Concentration.** Intracellular free calcium concentration ([Ca²⁺]ᵢ) was determined in single neurons using Fura-2 acetoxy methyl ester (Fura-2/AM, 2 μmol/L). Loaded neurons were imaged with a QICLICK digital CCD camera (Photometrics) connected to an OptoLED fluorescence imaging system (Cairn Research Ltd) housed on an inverted Nikon microscope equipped with a 40x, oil-immersion objective. The cover slip containing the neurons was placed into a temperature-controlled (36±0.5°C), gravity fed, perfusion chamber (volume: 100 μl), perfused with Tyrode solution at a flow rate of 2 ml/min. The evoked [Ca²⁺] transient was evaluated by 30 s exposure to 50 mmol/L KCl (with equimolar reduction in NaCl) in the Tyrode solution. Fura-2AM was excited alternately at 355 nm
and 380 nm and the emitted at 510 nm. Fluorescence excitation ratios were transformed into \([\text{Ca}^{2+}]_i\) concentrations using the equation derived by Grynkiewicz (47).

\[
[\text{Ca}^{2+}]_i = K_d \times \frac{(Sf2/Sb2) \times (R - R_{\text{min}})}{(R_{\text{max}} - R)}
\]

**Western Blot.** Cultured tissue were homogenized in iced lysis buffer with proteinase inhibitors and quantified by Bradford Protein Assay Kit. A total of 30 μg protein was separated in denaturing acrylamide gels (4–12% Criterion™ XT Bis-Tris Precast Gels, Bio-Rad). After transfer to PVDF membranes (Sequiblot PVDF Membrane Roll, Bio-Rad), then blocking the membranes with 0.5% skimmed milk for 1h. Primary antibody PDE2A (Proteintech, 1: 450) was carried out over night at 4°C. After incubation with secondary antibody (Goat anti-Rabbit IgG (H+L), HRP, Thermo Fisher, 1:4000) for 1h, proteins were visualized by enhanced chemo-luminescence and quantified with Quantity one software. The results were normalised to GAPDH that served as a loading control (Proteintech, 1:5000). Brain lysate was used as a positive control throughout the experiments.

**Immunofluorescence.** Cultured primary neurons were fixed in acetone/methanol for 10 min, followed by antigen blocking in 1% BSA and with primary antibody against tyrosine hydroxylase (TH, mouse mAb, 1:1000, Sigma) or anti- PDE2A (rabbit pAb, 1:500, Thermo Scientific) in 1% BSA overnight at -4°C. Signals were visualized with Alexa Fluor 594 anti-mouse IgG (H+L) (1:1000, Invitrogen) and Alexa Fluor 488 anti-rabbit IgG (H+L) (1:1000, Invitrogen) respectively. Nuclear staining was performed with 4’,6-Diamidino-2-Phenylindole (DAPI, Sigma).

**Statistics.** Data were expressed as means ± SEM. All statistical calculations were performed using the SigmaPlot 12.5 software package (Systat Software Inc.). For comparison of two groups, an unpaired two-tailed \(t\)-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 \(P<0.05\).
**Study approval.** The study was approved by the UCLA IRB. Written informed consent was provided by the patient or appropriate designee. For organ donors, consent for research was provided by the donor or appropriate signatory.
Author contributions

KL performed western blots, qRT-PCR, [3H]-NE release, calcium transient measurements, analyzed data and drafted the manuscript. DL participated in design of the study, supervision, performed PDE activity assay, analyzed data, and co-wrote the manuscript. GH performed patch-clamp recordings and analyzed data. DM, ON, LW and DI performed FRET and measured the calcium transient. CJL performed the in vivo injections. MB and MZ provided the reporter and contributed towards the FRET experimental design. HT, OAA, JA and KS provided human tissue and edited the manuscript. DJP oversaw experiments, design, supervision, resources and edited the manuscript. All authors reviewed the manuscript.
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Figure 1. (A) Representative data traces showing dynamic of cytosolic cGMP-induced fluorescence resonance energy transfer (FRET) changes by ratiometric recording of CFP and YFP emission changes in response to increasing concentrations of BNP in SHR & Wistar cardiac sympathetic neurons. Saturation of the sensor was achieved using SIN-1 (10 μmol/L) + IBMX (100 μmol/L). (B) Percent changes in cGMP in response to increasing concentrations of BNP (i, from 10-250 nmol/L) and 100 nmol/L BNP with 1 μmol/L Bay60-7550 or 100 μmol/L IBMX (ii) in SHR & Wistar. (*p<0.05, unpaired t-test). In each case, neurons were derived from ≥ 3 rats. N number indicates SG neurons number.
Figure 2. Representative data showing cGMP-PDE specific activities and relative PDE2A activities in stellate ganglia tissue from sympathetic hyperactive patients (pathological) & normal control (Ai, Aii) and 38 weeks WKY & SHR rats (B). cAMP-PDE specific activities and relative PDE2A activities in stellate ganglia tissue from 4 weeks WKY & SHR rats (C). Quantitative RT–PCR analysis of PDE2A mRNA levels in stellate ganglia tissue of sympathetic hyperactive patients (pathological) & normal control (D) and 4-week WKY & SHR rats (E); data are shown as mean ±SEM. *p<0.05, unpaired t-test. Left: left stellate ganglion; Right: right stellate ganglion in human. N number indicates stellate ganglia number.
Figure 3. Representative group raw data traces showing the time control (A) and with addition of 250 nmol/L BNP (C) on [3H] NE release from isolated atria harvested from 4-week WKY and SHR. The atria were stimulated at 5Hz for 1 minute at the 16th (S1) and 40th (S2) minutes. (B) Group mean data show no significant changes between S1 and S2 within group in response of 5Hz stimulation evoked [3H] NE release of time control. However, both S1 and S2 are significantly enhanced in the SHRs compare to the WKY rats (WKY: n=6, SHR n=7 *P<0.05, one way ANOVA). Group raw data traces (C) and mean data (D) show BNP caused a significant decrease in 5Hz stimulation evoked [3H] NE release (S2) in WKY, but not in SHR (WKY: n=10, SHR n=9, *P<0.05, one way ANOVA). Percentage changes in NE release was expressed as a ratio of increase in NE radioactivity after electrical stimulation over the total radioactivity.
Figure 4

(A) Fluorescent images of a cultured cardiac sympathetic neuron derived from a 4-week old WKY stellate ganglion which was stained with the catecholamine neuronal marker anti-tyrosine hydroxylase (TH, red), PDE2A antibody (Green), co-stained with the nuclear marker DAPI (blue) and overlay. Scale bar represents 20 μm. (B) Representative whole cell calcium current traces (upper graph) obtained before and after exposure to 100 nmol/L BNP (a, b) or 100 nmol/L BNP with 1 μmol/L Bay60-7550 (c) from 4-week old WKY and SHR. Currents were evoked by test pulses to −10 mV from a holding potential of −90 mV. Mean current density–voltage relations (lower graph) in the presence and absence of 100 nmol/L BNP (a, b) or 100 nmol/L BNP with 1 μmol/L Bay60-7550 (c) from 4-week old WKY and SHR. *P<0.05, paired t test. Vm, membrane voltage, mV; I Ca indicates calcium current; pA, picoampere; and pF, picofarad. (C) Pharmacological protocols (i) and raw data trace (ii) recording intracellular calcium transient ([Ca2+]i) in a single cardiac sympathetic neuron from 4-week old WKY and SHR. The Fura-2 acetoxyethyl ester (Fura-2/AM, 2 μmol/L) loaded neuron was stimulated by 50 mmol/L KCl for 30 s to depolarize the neuron and evoke voltage-gated Ca2+ entry. The size of first (S1) and second (S2) KCl stimulation were compared. (D) Group data showing KCl evoked peak [Ca2+]i changes expressed as a ratio (%) of S2 compared with S1 in response to 100, 250 nmol/L BNP with or without phosphodiesterase 2 (PDE2) inhibitor Bay60-7550 (1 μmol/L) in the WKY (i) and the SHRs (ii). *P<0.05, **P<0.01, ***P<0.001, compared with control, †P<0.05, †††P<0.001, one way ANOVA; n numbers indicate the number of the neurons.
**Figure 5.** (A) Representative western blot showing PDE2A.mCherry expression (127KDa) in WKY stellate ganglia tissue (with anti-PDE2A antibody) three days after transduction with mCherry virus or Ad.mCherry-PDE2A. Band optical density was normalized to that of β-actin (42KDa) as a loading control. n=4 in each group, \( P<0.05 \). (B) Representative calcium current traces (upper graph) and mean current density–voltage relations (lower graph) obtained before and after exposure to 100 nmol/L BNP (a, b) or BNP with 1 μmol/L Bay60-7550 (c) in the transduced with mCherry virus (i) or Ad.PDE2A (ii) from cardiac sympathetic neurons. \( *P<0.05 \), paired \( t \) test. Vm membrane voltage, mV; I\(_{Ca}\) indicates calcium current; pA, picoampere; and pF, picofarad. (C) Percentage change in the peak of intracellular calcium transient ([Ca\(^{2+}\)]) in response of 50 mmol/L KCl for 30 s in cardiac sympathetic neurons from WKY gene transferred with mCherry virus (i) or Ad.PDE2A (ii) in the presence of 100, 250 nmol/L BNP with or without PDE2A inhibitor Bay60-7550 (1 μmol/L). \( *P<0.05 \), \( **P<0.01 \), \( ***P<0.001 \), compared with control, \( \dagger P<0.05 \), one way ANOVA; n numbers indicate the number of the neurons.
Figure 6

(A) cGMP-PDE specific total activities in stellate ganglia tissue from 4 weeks SHR rats transduced with Ad.mCherry and Ad.dnPDE2A. (B) Representative calcium current traces (upper graph) and mean current density–voltage relations (lower graph) obtained before and after exposure to 100 nmol/L BNP in the transduced mCherry (i) and Ad.dnPDE2A (ii) virus in SG neurons. *P<0.05, paired t test. Vm membrane voltage, mV; ICa indicates calcium current; pA, picoampere; and pF, picofarad. (C) Percentage change in the peak of intracellular calcium transient ([Ca^{2+}]i) in cardiac sympathetic neurons from SHR gene transferred with mCherry (i) or Ad. dnPDE2A (ii) virus in the presence of 100 and 250 nmol/L BNP with or without PDE2A inhibitor Bay60-7550 (1 μmol/L). Both 100 and 250 nmol/L BNP failed to decrease [Ca^{2+}]i with mCherry virus, unless in the presence of Bay60-7550 (i). However, 100 and 250 nmol/L BNP decrease [Ca^{2+}]i after transduced with Ad.dnPDE2A (ii). *P<0.05, **P<0.01, ***P<0.001, compare to control, †P<0.05, †††P<0.001, one way ANOVA; n numbers indicate the number of the neurons.
Figure 7

(A) Representative raw data trace showing the effect of 250 nmol/L BNP on $[^3]H$ NE release during 5Hz field stimulation from isolated 4-week old SHR atria that gene transferred with mCherry (i) or Ad.dnPDE2A (ii) virus. (B) Group mean data show percentage changes of NE release expressed as a ratio of increase in NE radioactivity after 5Hz field stimulation over the total radioactivity. 250 nmol/L BNP significantly reduce $[^3]H$ NE release in SHR that transduced with Ad.dnPDE2A (n=10), but not in mCherry group (n=8). **P<0.01, ***P<0.001 one way ANOVA.
Figure 8. Schematic representation depicting the effect of PDE2A on the regulation of BNP on cardiac neurotransmission in normal and diseased neurons. Panel A: BNP can decrease neurotransmission in healthy neurons by activating pGC coupled cGMP pathway to decrease intracellular calcium transients and exocytosis resulting in a decrease in the HR response to sympathetic nerve stimulation. Overexpression of PDE2A in healthy neurons mimics the lack of BNP efficacy seen in diseased neurons. Panel B: In the diseased neuron PDE2A is increased and increases the hydrolysis of cGMP and decreases the sympatholytic action of BNP that results in an increase in neurotransmission and HR responses to sympathetic activation. Blockade of overexpressed PDE2A with Bay60-7550 or overexpression of catalytically inactive PDE2A re-established the modulatory action of BNP in the diseased neuron.
| Sample ID | Group                  | Age  | Gender | LVEF | Clinical characteristics                                                                 |
|-----------|------------------------|------|--------|------|------------------------------------------------------------------------------------------|
| 2         | Control (L+R)          | 52   | Male   | 70%  | CVA with hemorrhage (Normal heart)                                                         |
| 5         | Control (L+R)          | 45   | Female | 70-75% | Right MCA aneurysm rupture, Subarachnoid hemorrhage w/ herniation (Normal heart)           |
| 6         | Control (L)            | 30   | Female | 55-60% | Cardiac arrest - smoker, lung donor, toxic positive for metamphetamines, marijuana (Normal heart) |
| 7         | Control (L+R)          | 37   | Male   | 57%  | ICH (Normal heart)                                                                         |
| 9         | Pathology (L+R)        | 46   | Male   | 30%  | NICM, VC                                                                                 |
| 10        | Pathology (L+R)        | 30   | Male   | 15%  | NICM                                                                                     |
| 11        | Pathology (L+R)        | 45   | Female | 38%  | Sarcoi d cardiomyopathy                                                                     |
| 14        | Pathology (L+R)        | 65   | Male   | 35%  | NICM                                                                                     |
| 15        | Pathology (L+R)        | 69   | Male   | 20%  | NICM, CAD (Mild, Nonobstructive)                                                           |
| 16        | Control (L+R)          | 56   | Male   | > 60% | Normal heart                                                                               |
| 17        | Control (L+R)          | 49   | Male   | > 60% | Normal heart                                                                               |
| 19        | Pathology (L+R)        | 77   | Male   | 30-35% | NICM, VF                                                                                 |
| 20        | Control (L+R)          | 61   | Female | normal presumably | Aortic and carotid dissection -> anoxic brain injury Hypertension, hypothyroidism. No known CAD. Tobacco use x 30-40yrs |
| 21        | Pathology (L+R)        | 34   | Female | Not available | Excessive tachycardia, normal heart                                                        |
| 22        | Pathology (L+R)        | 70   | Male   | 30%  | ICM, VT, low EF                                                                            |
| 23        | Control (L+R)          | 19   | Male   | 55-60% | Normal heart size, and chambers, no lung problems some atelectasis that improved           |
| 24        | Pathology (L+R)        | 62   | Male   | 50%  | NICM, LV EF 30%, improved to 50%, PMVT/VF with high PVC burden                            |

LVEF: Left ventricular ejection fraction  
L: left stellate ganglion  
R: right stellate ganglion  
CVA: Cerebrovascular accident  
MCA: Middle Cerebral Artery  
ICH: Intracranial hemorrhage  
NICM: Nonischemic Cardiomyopathy  
VC: Valvular cardiomyopathy  
CAD: Coronary Artery Disease  
VF: Ventricular Fibrillation  
ICM: Implantable Cardiac Monitor  
VT: Ventricular Tachycardia  
EF: ejection fraction  
PMVT: Polymorphic ventricular tachycardia  
PVC: Premature Ventricular Contraction