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Cyclic nucleotide regulation of cardiac sympatho-vagal responsiveness

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Abstract

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are now recognized as important intracellular signalling molecules that modulate cardiac sympatho-vagal balance in the progression of heart disease. Recent studies have identified that a significant component of autonomic dysfunction associated with several cardiovascular pathologies resides at the end organ, and is coupled to impairment of cyclic nucleotide targeted pathways linked to abnormal intracellular calcium handling and cardiac neurotransmission. Emerging evidence also suggests that cyclic nucleotide coupled phosphodiesterases (PDEs) play a key role limiting the hydrolysis of cAMP and cGMP in disease, and as a consequence this influences the action of the nucleotide on its downstream biological target. In this review, we illustrate the action of nitric oxide-CAPON signalling, and brain natriuretic peptide, on cGMP/cAMP regulation of cardiac sympatho-vagal transmission in hypertension and ischaemic heart disease. Moreover, we address how PDE2A is now emerging as a major target that effects the efficacy of soluble/particulate guanylate cyclase coupling to cGMP in cardiac dysautonomia.

Abbreviations cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; PDE, phosphodiesterases; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; $[Ca^{2+}]_i$, intracellular calcium concentration; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; NOS1-AP/CAPON: neuronal nitric oxide synthase adaptor protein; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; sGC, soluble guanylate cyclase; pGC, particulate guanylyl cyclase; NA, noradrenaline; ACh, acetylcholine; NP, natriuretic peptide; ANP, atrial natriuretic peptide; BNP, brain or B-type natriuretic peptide; CNP, C-type natriuretic peptide; DNP, D-type or dendroaspis natriuretic peptide; NPR, natriuretic peptide receptor.

Introduction

The cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), were identified more than five decades ago (Rall & Sutherland, 1958; Ashman *et al.*, 1963). Since then they have been recognized as important intracellular signalling molecules, acting as second messengers between hormones, neurotransmitters or cytokines. These extracellular signals regulate the concentration of cyclic nucleotides, but their levels are ultimately determined by the balance between their production and degradation by 3',5'-cyclic nucleotide phosphodiesterases (PDEs) (Francis *et al.*, 2001; Omori & Kotera, 2007; Maurice *et al.*, 2014). The downstream effector proteins of cAMP and cGMP are cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), cyclic nucleotide-gated ion channels, and the exchange protein activated by cAMP (Epacs) (Zaccolo & Movsesian, 2007).

It is well established that a significant component of autonomic dysfunction associated with several cardiovascular pathologies resides at the end organ and is coupled to impaired cyclic nucleotide pathways linked to abnormal intracellular calcium handling (Wang *et al.*, 2007; Danson *et al.*, 2009; Herring & Paterson, 2009). This neuromodulation is dependent on the site-specific action of PDEs effecting the hydrolysis of cGMP and cAMP in a differential way, that in turn alters the intracellular calcium concentration ($[Ca^{2+}]_i$) regulating exocytosis (Paton *et al.*, 2002; Li *et al.*, 2015). Specifically, when cGMP is activated by nitric oxide (NO) in sympathetic neurons, it inhibits cAMP by increasing its hydrolysis by stimulating the main phosphodiesterase PDE2A. This causes a decrease in cAMP dependent phosphorylation of calcium channels and a decrease in neurotransmission, whereas in parasympathetic neurons NO inhibits PDE3, resulting in a decrease hydrolysis of cAMP. This leads to more cAMP dependent phosphorylation of calcium channels and facilitated the release of

acetylcholine. The ability of cyclic nucleotides to manipulate cardiac sympatho-vagal responsiveness is of immediate interest, since disruption of cardiac sympatho-vagal balance plays an important role in the progression of heart disease.

In hypertension, cardiac sympathetic hyperactivity, tachycardia, pathological left ventricular hypertrophy (Burns *et al.*, 2007; Malpas, 2010; Shen & Zipes, 2014; Grassi *et al.*, 2015) are all hallmarks of the disease that are also associated with decreased cardiac parasympathetic responsiveness (Langewitz *et al.*, 1994). Interestingly, raised sympathetic activity in borderline and white coat hypertensive patients (Grassi & Mancia, 2004; Smith *et al.*, 2004) has been implicated as a major contributing component of hypertension itself and end organ disease (Burns *et al.*, 2007). This has led to the suggestion that reducing sympathetic drive in the early stages of hypertension may be therapeutically beneficial. This review focuses on recent insights into the regulation of the cGMP/cAMP signalling network in cardiac sympatho-vagal function. We also examine the emerging role of PDEs in autonomic imbalance in various models of hypertension and ischaemic heart disease as a prelude to identifying potential therapeutic targets.

Nitric oxide/soluble guanylate cyclase/cGMP signalling in cardiac sympatho-vagal impairment

Emerging evidence now suggests that as cardiovascular disease progresses from the pre-diseased state to the overt clinical signs (hypertension and heart failure) (Fig 1), dysregulation of neuronal nitric oxide synthase (nNOS) is associated with disease progression (Zhang *et al.*, 2014).

Impaired sympathetic functioning of the nitric oxide /cGMP pathway. In 1995, Schwarz et al first showed that NO could reduce cardiac noradrenaline release during sympathetic stimulation (Schwarz *et al.*, 1995). This translated into a reduction in the positive chronotropic and inotropic response in the isolated guinea-pig atrial / right stellate ganglion preparation (Choate & Paterson, 1999) via PDE2A hydrolysis reducing cAMP-PKA-dependent modulation of the intracellular calcium transient reducing exocytosis (Wang *et al.*, 2007). Similarly, Zanzinger et al (Zanzinger *et al.*, 1997) also reported that inhibition of nNOS in the paraventricular nucleus could drive central sympathetic outflow, suggesting an important role of NO in the cardiac sympathetic neural axis (reviewed by Paton *et al* 2002 and Danson *et al* 2009) . Since then, it gradually became recognized that NO dysregulation may play a major role in the aetiology of sympathetic hyperactivity in hypertension and other cardiovascular pathologies (Hamilton *et al.*, 2001; Gamboa *et al.*, 2012; Zhang *et al.*, 2014; Khan *et al.*, 2015). However, a detailed understanding of the cellular pathways underpinning the action of cyclic nucleotides and protein kinase regulation on cardiac neurotransmission in disease is still relatively poorly understood.

Hypertension causes enhanced neurohumoral activation (Ely *et al.*, 1997; Yemane *et al.*, 2010), resulting in increased cardiac noradrenaline (NA) release (Rumantir *et al.*, 2000; Zugck *et al.*, 2003), impaired neuronal NA reuptake (Rumantir *et al.*, 2000; Shanks *et al.*, 2013) and abnormal calcium signalling in cardiac pacemaker cells (Heaton *et al.*, 2006). Recent findings by our group demonstrated that sympathetic neurons from spontaneously hypertensive rats (SHR) have a significantly greater depolarization-evoked intracellular calcium transient when compared with age-matched normal rats (Li *et al.*, 2012). The difference persisted from neonates through to young prehypertensive and fully developed adult hypertensive animals (Fig.2). Moreover the neuronal Ca^{2+} current was significantly larger in stellate neurons from the SHR, suggesting that dysregulation of the ion channel also contributes to altered Ca^{2+} transients (Lu *et al.*, 2015). This suggests that disruption

in $[Ca^{2+}]_i$ is an important cellular switch in the evolution of the sympathetic phenotype in this genetic based model of hypertension. What causes the impaired $[Ca^{2+}]_i$ in sympathetic neurons? Metabolic and oxidative stress has been implicated as a potential trigger for this Ca^{2+} phenotype. There is some evidence that oxidative stress exists in blood vessels (Garcia-Redondo *et al.*, 2009; Li *et al.*, 2011) and myocytes (Lu *et al.*, 2009; Okamura *et al.*, 2009) in hypertensive animals, although this has not been established in stellate ganglion neurons. However, oxidative stress has been reported in central autonomic areas (Zimmerman & Davisson, 2004). Interestingly, intermittent hypoxia decreases cardiac sympathetic nNOS expression and enhances heart rate responses to sympathetic activation (Mohan *et al.*, 2001). In addition, application of the proton uncoupler carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 1 μ mol/L) to deplete stored mitochondrial Ca^{2+} and inhibit any further mitochondrial Ca^{2+} uptake, abolished the difference in depolarization-induced $[Ca^{2+}]_i$ transients between young SHR and normotensive Wistar-Kyoto (WKY) rat sympathetic neurons (Li *et al.*, 2012). Moreover, down regulation of nNOS, nNOS adaptor protein (NOS1-AP/CAPON) and the $\beta 1$ sub-unit of soluble guanylate cyclase (sGC) results in decreased cGMP in cardiac stellate ganglia from young prehypertensive SHR (Fig. 3). These molecular results are consistent with earlier observations in the aorta (Ruetten *et al.*, 1999) and the atria (Heaton *et al.*, 2006) of the SHR.

Targeting cardiac sympathetic neurons using gene transfer of nNOS and CAPON. Modulating key proteins involved in NOS signalling has been made possible by the development of gene knockout techniques to either decrease expression or to initiate/enhance expression of the gene of interest. Gene transfer with adeno viral vectors expressing nNOS, under the control of the cytomegalovirus (CMV) promoter, decreases central sympathetic outflow (Li *et al.*, 2002) and suppresses carotid body chemoreceptor activity in heart failure rabbits (Li *et al.*, 2005). It also reduces the heart rate response to sympathetic nerve stimulation (Choate & Paterson, 1999) and normalizes β -adrenergic hyper-responsiveness of sinoatrial node cells in hypertensive rats by decreasing the L-type Ca^{2+} current

(Heaton *et al.*, 2006). However, a limitation of using viral vectors is their potential off target effect (Kootstra & Verma, 2003) that can result in the gene of interest being transferred into cells that may not constitutively express it. This can result in unwanted side effects that confound the interpretation of data. To help address this problem, we developed an adenoviral vector with a promoter selective for noradrenergic neurons that resulted in no detectable expression or leakage into non-tyrosine hydroxylase cells (Wang *et al.*, 2006). This noradrenergic neuron-specific promoter encoding nNOS (Ad.PRS×8-nNOS) reduced the abnormal calcium transient, inhibited sympathetic neurotransmission (Wang *et al.*, 2006), and restored the function of the NO-cGMP pathway in cardiac sympathetic ganglia of the SHR (Li *et al.*, 2013). Indeed long term over expression with a lentivirus encoding nNOS, as well as L-arginine supplementation (Lee *et al.*, 2009), also resulted in decreased sympathetic transmission (Wang *et al.*, 2009).

Emerging evidence now suggests that the efficacy and movement of cytosolic nNOS is tightly regulated by its own shuttle protein. The nNOS adaptor protein (CAPON) was first identified in neuronal tissue where it is involved in the formation of polarity complexes, new synapses, and axonal guidance (Jaffrey *et al.*, 1998). CAPON also facilitates nNOS translocation to caveolae post myocardial infarction (MI), suggesting the interaction with CAPON is required for nNOS redistribution in injured myocardium (Beigi *et al.*, 2009). Interestingly CAPON is highly expressed in cardiac autonomic tissue and is down regulated in SHR stellate tissue (Lu *et al.*, 2015). Upregulation of CAPON with a noradrenergic specific promoters in the SHR increases nNOS activity and the concentration of neuronal cGMP without changing the expression of nNOS itself (Lu *et al.*, 2015). Moreover, gene transfer of CAPON also reduces the neuronal calcium current and intracellular calcium transient in the SHR to levels observed in the WKY. This translated into reduced atrial NA release, suggesting that CAPON modulation of neurotransmission is coupled to an NO dependent pathway since the effects were reversed by nNOS inhibition (Fig. 4). Taken together, these results

suggest that upregulating CAPON could provide a rationale for therapeutic targeting to turn down the gain of heightened sympathetic traffic that can trigger arrhythmia, especially in patients with long QT syndrome. Of interest, Chang *et al.* (Chang *et al.*, 2008) have shown that overexpression of CAPON in ventricular myocytes decreases the action potential duration (Fig. 5).

Impaired cardiac parasympathetic signalling of the nitric oxide/cGMP pathway. Balligand *et al.* (1993) first reported that NOS inhibitors abolished the negative chronotropic effect of acetylcholine (ACh) receptor agonists (Balligand *et al.*, 1993). Subsequently it was demonstrated that NO facilitates the actions of vagal inhibition of heart rate, predominantly via a presynaptic mechanism augmenting the release of ACh at the nerve terminal (Sears *et al.*, 1998; Conlon & Kidd, 1999; Sears *et al.*, 1999; Herring & Paterson, 2001; Massion *et al.*, 2003). The site of action of NO on ACh release is likely to come from nNOS within the cholinergic fibres (Paton *et al.*, 2002), that stimulate presynaptic sGC to produce cGMP dependent inhibition of PDE3. This elevates cAMP levels and increases PKA-dependent phosphorylation of N-type Ca^{2+} channels that controls exocytotic release of ACh, thereby facilitating vagal slowing of heart rate (Paton *et al.*, 2002). Genetic knockout of nNOS (Choate *et al.*, 2001; Danson *et al.*, 2004) or pharmacologic inhibition (Herring *et al.*, 2000), both attenuate heart rate responses to vagal nerve stimulation. Moreover, artificial upregulation of nNOS in the guinea pig right atrium (Mohan *et al.*, 2002) or direct gene transfer into the pig cardiac vagus rapidly enhances parasympathetic function (Heaton *et al.*, 2005). When all data are taken together it highlights a putative role for NO derived from nNOS in cholinergic transmission via a cGMP-dependent pathway.

Myocardial infarction, heart failure and hypertension are associated with impaired cardiac vagal responsiveness, both in patients (Petretta *et al.*, 1995a; Petretta *et al.*, 1995b) and in animal models of the disease (Friberg *et al.*, 1988; Murphy *et al.*, 1991). Similar vagal responses are also observed in young normotensive subjects with a family history of hypertension (Piccirillo *et al.*, 2000) suggesting dysautonomia may be an early trait of the pathology. It is well established that reduced bioavailability

of NO, secondary to oxidative stress, is important in the pathophysiology of hypertension as well as other cardiovascular diseases (Nakazono *et al.*, 1991; Hamilton *et al.*, 2001). At the level of peripheral cardiac autonomic ganglia, neural dysregulation may be related to impaired NO-cGMP signalling since there is an associated down regulation of guanylate cyclase in the aorta from the SHR (Kloss *et al.*, 2000; Kagota *et al.*, 2001) and in the atria where the post ganglionic parasympathetic neurons are located (Heaton *et al.*, 2007). In addition, many groups have shown that decreased NO bioavailability and abnormal superoxide/peroxynitrite production (behaves like a nNOS inhibitor in endothelial dysfunction) (Hamilton *et al.*, 2001; Wiemer *et al.*, 2001) is associated with the inhibition of ACh release from synaptosomes, thus disrupting NO-cGMP cholinergic signalling (Morot Gaudry-Talarmain *et al.*, 1997). Conversely, overexpressing nNOS in intracardiac vagal ganglia restores the HR responsiveness to vagal activation in the SHR (Heaton *et al.*, 2007) and in the guinea pig following myocardial infarction (Dawson *et al.*, 2008). Moreover, improved vagal responsiveness post gene transfer of nNOS was also associated with improved mortality post myocardial infarction compared to those infarcted animals that were gene transferred with only eGFP (Dawson *et al.*, 2008).

Natriuretic peptides/particulate guanylyl cyclase/cGMP signalling in cardiac sympatho-vagal balance

Natriuretic peptides (NP) have a well-established antihypertensive action by reducing plasma renin-aldosterone concentrations and causing natriuresis (Volpe, 2014). This family includes atrial natriuretic peptide (ANP), brain or B-type natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and D-type natriuretic peptide (DNP). NPs, like NO, stimulate cGMP synthesis, but they elicit their response by binding to the transmembrane particulate guanylyl cyclase (pGC, rather than sGC) complex that is coupled to natriuretic peptide receptors (NPRs) (Chinkers *et al.*, 1989), resulting in

intracellular cGMP formation (Potter *et al.*, 2006). This in turn modulates PKG (Castro *et al.*, 2010) and cyclic nucleotide-coupled phosphodiesterases (Omori & Kotera, 2007) leading to the regulation of $[Ca^{2+}]_i$ (Sodi *et al.*, 2008). Each peptide is distributed and regulated in a tissue-specific fashion (Levin *et al.*, 1998). Even though the role of NPs in regulating effective circulation volume is well characterized (Potter *et al.*, 2006; Zois *et al.*, 2014), their role in modulation of the autonomic nervous system is still controversial.

Atrial natriuretic peptide was discovered in the early 1980s (de Bold *et al.*, 1981) and its protective role in cardiovascular disease is well-documented (eg. (Suzuki *et al.*, 2001; Song *et al.*, 2015). ANP is produced primarily in the cardiac atria in response to atrial stretch. It produces a direct depressant action on sympathetic nerve function that is associated with a reduction in both cardiac output and arterial blood pressure in dogs (Bergey *et al.*, 1989), and heart failure patients (Abramson *et al.*, 1999; Kasama *et al.*, 2004). ANP does not appear to alter the heart rate response to peripheral vagal stimulation *in vitro* (Herring *et al.*, 2001) or release of acetylcholine *in vitro* (Hiwatari *et al.*, 1986). ANP can however, augment the bradycardia in response to efferent vagal nerve stimulation in humans (Zeuzem *et al.*, 1990) and rats (Ackermann *et al.*, 1988) *in vivo*. This might be due to cross talk between adrenergic-cholinergic receptors on parasympathetic ganglia, since the effect of ANP can be blocked by an alpha 1 antagonist (Atchison & Ackermann, 1993).

B-type natriuretic peptide is secreted largely by ventricular myocytes in a setting of volume expansion or pressure overload (Daniels & Maisel, 2007). Thus, the plasma concentration of BNP is significantly raised in a subset of patients with essential hypertension, and correlates with hypertensive severity (Kohnno *et al.*, 1992; Cheung & Brown, 1994). BNP is generally regarded as cardio-protective (Levin *et al.*, 1998; Daniels & Maisel, 2007) and an early compensatory response to the disease itself. BNP suppresses cardiac hypertrophy (Rosenkranz *et al.*, 2003), interstitial fibrosis (Glenn *et al.*, 2009), the sympathetic nervous system (Brunner-La Rocca *et al.*, 2001), and the renin–

angiotensin–aldosterone axis (Richards, 1996). It also accentuates the positive action of the cardiac vagus in humans (Zeuzem *et al.*, 1990), guinea pigs (Herring *et al.*, 2001) and rats (Ackermann *et al.*, 1988). In contrast, Chan *et al.* (2012) reported that BNP can increase NA release in PC12 cells suggesting this could underpin the lack of clinical efficacy of intravenous BNP in patients with heart failure (Chan *et al.*, 2012). Although, recent work calls into question this latter result. Li *et al.* (2015) found that BNP actually reduces $[Ca^{2+}]_i$ transients evoked by high K^+ depolarization in a concentration-dependent manner and also decreases the calcium current in primary cultured cardiac sympathetic neurons. This translated into a reduction in neurotransmitter release in response to field stimulation of right atrial preparations, and heart rate responsiveness to direct sympathetic nerve stimulation *in vitro* (Fig. 6). The inhibitory action of BNP on the $[Ca^{2+}]_i$ transient was shown to be regulated by the NPR-A/cGMP/PKG pathway, where PDE2A modulates the hydrolysis of BNP stimulated cGMP (Li *et al.*, 2015). This is consistent with the proposal that BNP activates a PKG signalling pathway involved in the inhibition of neurotransmission in the dorsal root ganglion (Zhang *et al.*, 2010). The reasons for the above discrepant findings are not very clear, but may reflect the use of different tissue types, cell models, and variations in dose and duration of BNP administration. Nevertheless, the latter results are consistent with the literature that shows a beneficial action of BNP in healthy tissue. Interestingly, recent work shows that BNP knockout rats demonstrated adult-onset hypertension, increased left ventricular mass with hypertrophy and substantially augmented hypertrophy signalling pathway genes. Systemic BNP overexpression reversed the phenotype of genetic BNP deletion (Holditch *et al.*, 2015).

C-type natriuretic peptide was first isolated from porcine brain in 1990 (Sudoh *et al.*, 1990) and is the most widely expressed NP. CNP produced by the endothelium and the heart plays a prominent role in cardiovascular function (Kalra *et al.*, 2001; Lumsden *et al.*, 2010). In contrast to BNP, CNP levels in plasma are not elevated in hypertensive patients (Cheung & Brown, 1994) or chronic heart failure

(Cargill *et al.*, 1994; Totsune *et al.*, 1994). However, urinary excretion of CNP is significantly increased in patients with congestive cardiac failure (Mattingly *et al.*, 1994). Exogenous CNP has been shown to facilitate bradycardia and ACh release (Herring *et al.*, 2001). Furthermore, exogenous CNP also found to inhibits the neuromodulatory effect on induced release of the sympathetic cotransmitters NA and ATP from rat tail arteries (Mutafova-Yambolieva & Westfall, 1998). Whether CNP alters NA release and calcium transient from cardiac sympathetic ganglia has not been established.

D-type or Dendroaspis natriuretic peptide (DNP) has 38 amino acids and a 17-amino acid disulfide ring structure, and was originally isolated from the venom of the green Mamba snake *Dendroaspis angusticeps*, (Schweitz *et al.*, 1992). Studies have reported that DNP is present in human and canine plasma, and atrial myocardium. Its concentration is elevated in plasma of humans with congestive heart failure (CHF) (Lisy *et al.*, 1999; Schirger *et al.*, 1999). Functionally, DNP decreases mean arterial pressure, pulmonary capillary wedge pressure and cardiac output in normal (Lainchbury *et al.*, 2002) and failing (Lisy *et al.*, 2001) canine. It also causes significant natriuresis (Lisy *et al.*, 1999). Moreover, DNP can inhibit the L-type Ca^{2+} channel via PKG activation in rabbit ventricular myocytes (Park *et al.*, 2012). However, little is known about the action of DNP on cardiac autonomic responses.

PDEs as therapeutic targets in cardiac dysautonomia

Cyclic nucleotide phosphodiesterases (PDEs) catalyse the hydrolysis of cAMP and cGMP by controlling their rates of degradation, thereby regulating the intracellular concentrations of the cyclic nucleotide (Maurice *et al.*, 2014). PDEs comprize a superfamily of 11 gene-related isozymes (PDE1 to PDE11), associated with 21 genes that generate more than 100 proteins via alternative splicing of mRNA (Bender & Beavo, 2006; Francis *et al.*, 2011). Some PDEs specifically hydrolyse cAMP

(PDE4, 7 and 8), whereas others specifically hydrolyse cGMP (PDE5, 6 and 9), and some possess dual specificity (PDE1, 2, 3, 10 and 11) (Conti & Beavo, 2007; Keravis & Lugnier, 2012). However, there is relatively little published work on PDE's in autonomic neurons, although emerging evidence suggests PDE2A in sympathetic neurons is important in neurotransmission (see below and table 1).

cAMP-Specific PDEs: PDE4 is one of the main enzymes that specifically hydrolyzes cAMP after stimulation of β -adrenergic receptors and several other G protein-coupled receptors (Manning *et al.*, 1996). Lack of PDE4 responsiveness to acute rises in noradrenaline in the brain, heart and skeletal muscle has been observed in rat models of obesity (Greene *et al.*, 2009), suggesting dysregulation of this PDE is coupled to abnormal sympathetic signalling. In addition, another cAMP-specific phosphodiesterase 7 (PDE7) inhibitor, S14, exerts potent neuroprotective and anti-inflammatory effects in different rodent models of Parkinson's disease by stopping the dopaminergic cell loss that occurs during the progression of the Parkinson's (Morales-Garcia *et al.*, 2015).

cGMP-Specific PDEs: The PDE5 inhibitor sildenafil citrate reduces vagal activation and increases sympathetic modulation in men with chronic heart failure (Piccirillo *et al.*, 2002), although this probably occurs through its reflex vasodilatory action. Moreover, sildenafil significantly increases plasma noradrenaline concentration in healthy middle-aged men (Dopp *et al.*, 2013) and alleviates functional muscle ischemia in boys with Duchenne muscular dystrophy in a dose-dependent manner (Nelson *et al.*, 2014). Lee *et al.* (2015) recently found that PDE9A protein expression is upregulated during hypertrophy and cardiac failure. They also demonstrated that PDE9A inhibition reverses pre-established heart disease independent of NOS activity, whereas PDE5A inhibition requires active NOS (Lee *et al.*, 2015). This highlights the importance of PDE spatial localization to its target, and also illustrates the complexity of PDE signalling in disease states.

Dual-Specificity PDEs: A recent study reported that the PDE3 inhibitor cilostazol significantly

blunted sympathetic hyperinnervation in a rat model of myocardial infarction, possibly by counterbalancing the PDE3 inhibition-induced cAMP and adenosine-related antioxidation pathways (Lee *et al.*, 2014). Moreover, PDE3 also reduces β_1 - and β_2 -adrenoceptor-mediated positive inotropic and lusitropic effects of catecholamines in human failing myocardium, suggesting that treatment with a PDE3-selective inhibitor could potentially facilitate adverse stress-induced adrenaline effects through β_2 adrenoceptors in patients treated with metoprolol (Molenaar *et al.*, 2013).

PDE2A has been shown to play important roles in many signal transduction pathways as a regulator of both cGMP and cAMP levels (Suvarna & O'Donnell, 2002; Reinecke *et al.*, 2011; Hu *et al.*, 2012; Maurice *et al.*, 2014). PDE2A is markedly upregulated in heart failure and blunts β -adrenergic responses by hydrolysis of cAMP in cardiomyocytes (Mongillo *et al.*, 2006; Mehel *et al.*, 2013). This provides a negative crosstalk mechanism between cAMP and cGMP signalling pathways. PDE2A has a slightly higher affinity for cGMP compared to cAMP, where it can metabolize both cyclic nucleotides in a tissue dependent fashion. Moreover, the endogenous substrate of this enzyme may functionally vary across tissues given its subcellular localization and/or splice variant expression (Garcia-Osta *et al.*, 2012). Interestingly, most studies on the role of PDE2A in brain tissue point to its modulation of cGMP levels (Schmidt, 2010). In addition, a recent study from our group observed that PDE2A activity was significantly elevated in stellate neurons from prehypertensive rats compared with the normotensive control. Furthermore, the action of BNP was regulated by PDE2A, which modulates cGMP-PKG coupling to neuronal calcium channels and neurotransmitter release (Li *et al.*, 2015). In healthy cells, overexpression of PDE2A in sympathetic neurons markedly decreases BNP stimulation of cGMP and enhances the intracellular calcium transient resulting in more neurotransmitter release (Fig. 7). However, the inhibitory action of BNP via cGMP on the calcium current/ $[Ca^{2+}]_i$ and NA release was prevented by overexpression of PDE2A. Indeed selective PDE2A inhibition restored the inhibitory effects of BNP on calcium influx and NA release (Li *et al.*, 2015).

This work may have identified a new molecular mechanism, whereby enhanced neuronal PDE2A expression in disease, annihilates the beneficial efficacy of BNP to decrease sympathetic transmission (Seifert, 2015). Whether this translates into meaningful beneficial effects in cardiovascular disease associated with sympathetic dysautonomia remains to be established.

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Additional information

Competing interests

None declared.

Author contributions

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Table

Table 1: Expression of phosphodiesterase in the autonomic nervous system

Characteristics	Isoenzyme family	Sympathetic expression	Parasympathetic expression
cAMP specific	PDE4, 7, 8	?	?
cGMP specific	PDE5, 6, 9	PDE5	PDE5
Dual action	PDE1, 2, 3, 10, 11	PDE2, 3	PDE3

Abstract Figure Legend

Diagram of nitric oxide and natriuretic peptide on cGMP/cAMP regulation of cardiac neurotransmission.

Abstract Figure

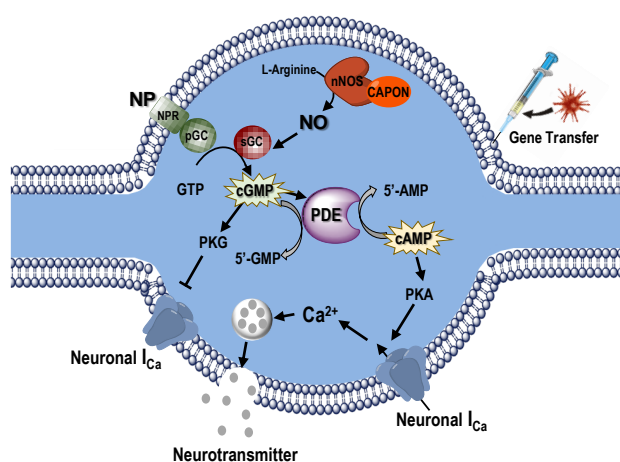


Figure Legends

Figure 1. Distribution of neuronal nitric oxide synthase (nNOS) protein in the heart and the disease progression from healthy state to heart failure where changes in nNOS may contribute to the aetiology of disease. Modified from Zhang et al. (2014) with permission.

Figure 2. **A**, Bright field and immunofluorescence staining image of a cultured cardiac sympathetic neuron derived from a stellate ganglion which was stained with the catecholamine neuronal marker tyrosine hydroxylase (TH, red) and co-stained with the nuclear marker 4',6-diamidino- 2-phenylindole (DAPI, blue). Scale bar represents 20 μ m. **B**, Current density–voltage relationship curve of the neuronal calcium current (I_{Ca}) demonstrating significantly larger I_{Ca} at multiple voltages in stellate neurons from spontaneously hypertensive rats (SHRs, n=8) when compared to Wistar-Kyoto (WKY) rats controls (n=8). **C**, **(i)** Typical fluorescence ratio profile of single sympathetic neurons after exposure to high KCl. **(ii)** Pseudocolor-coded ratio images of Fura-2–loaded neuron were obtained by conventional fluorescence microscopy. Ca^{2+} concentrations were colour-coded with a basal Ca^{2+} concentration in blue and a high Ca^{2+} concentration in red. **(iii)** Statistical data showed peak increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) on high K^+ exposure from SHRs compared with WKY rats of the same age. * $P<0.05$, ** $P<0.01$, t -test. † $P<0.05$, †† $P<0.01$, one-way ANOVA. Modified from Li *et al.* (2012, 2015) and Lu *et al.* (2015) with permission.

Figure 3. Representative Western blot and group mean data showing a significant reduction in CAPON (**A**), nNOS (**B**) and β 1-sGC (**C**) protein expression relative to β -actin in stellate ganglia from 4-week-old spontaneously hypertensive rats (SHR) compared with age matched Wistar–Kyoto (WKY) controls. **D**, Tissue levels of cGMP in the stellate ganglia from the young SHRs were

significantly lower than that measured in the WKY rats. (* $P < 0.05$, ** $P < 0.01$, unpaired t -test). Modified from Li *et al.* (2013) and Lu *et al.* (2015) with permission.

Figure 4. **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 (i)**, Representative Western blot showing CAPON expression (74 kDa) in stellate ganglia from 4-week-old SHR 3 days after transduction with Ad.PRSx8-mCherry/CAPON (Ad.CAPON) and Ad.PRSx8-mCherry (empty). **(ii)**, 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, 25 μ m. **C**, Gene transfer of CAPON (Ad.CAPON) significantly increased NOS activity in stellate ganglia from SHR when compared with the empty vector control. The specific nNOS inhibitor, S2, N-[(4S)-4-Amino-5-[(2-aminoethyl) amino] pentyl]-N'-nitroguanidine (AAAN, 10 μ mol/L) normalized the difference in NOS activity after transduction with CAPON or the empty vector control (* $P < 0.05$, ANOVA). **D**, cGMP concentration in SHR stellate ganglia tissue was significantly enhanced by Ad.CAPON transduction when compared with empty controls (* $P < 0.05$, unpaired t -test). **E**, 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 4-week-old SHR when compared with cells transduced with empty virus. Inserted images representative fluorescence (mCherry) and bright field images of single stellate neuron transduced with Ad.CAPON with a patch pipette (pointed by a white arrow). **(F)** Gene transfer with CAPON in SHRs significantly reduced the calcium transient in sympathetic neurons and **(G)** ^3H -noradrenaline (NA) release from isolated double atrial preparations when compared with the empty vector. The atria were stimulated at 5 Hz for 1 minute at the 16th (S1) and 40th (S2) minutes. Bath solution was

collected by replacing 3 ml Tyrode's solution every 3 minutes and measured using a liquid scintillation counter. The effect of CAPON gene transfer can be reversed with a specific nNOS inhibitor (AAAN, 10 μ mol/L). Modified from Lu *et al.* (2015) with permission.

Figure. 5. Summary diagram illustrating the potential signal transduction pathways mediated by natriuretic peptides (NP) and nitric oxide (NO)/CAPON in depolarized cardiac stellate neurons.

Figure 6. **A, (i)** Representative whole cell calcium current density traces obtained with or without 100 nmol/L B-type natriuretic peptide (BNP, 10 minutes) and after wash out. Currents were evoked by test pulses to -10 mV from a holding potential of -90 mV. **(ii)** Mean current density–voltage relations in the presence and absence of 100 nmol/L BNP. Wash off data were only recorded at -10 mV as the quality of the recordings deteriorates over time. $*P<0.05$, paired *t*-test, $n=6$ neurons. **B, (i)** An example recording calcium transient in a single cardiac sympathetic neuron. Neuron was exposed to KCl for 30 s to depolarize the neuron with (S2) or without (S1) BNP. **(ii)** Statistical data showing that concentration–effect relationship of BNP (1–250 nmol/L) changed KCl evoked increase in $[Ca^{2+}]_i$ expressed as a ratio (%) of S2 compared with S1. $**P<0.01$, compared with control, unpaired *t*-test ($n=8-16$). **C**, Grouped data showing the time control **(i)** and with addition of 250 nmol/L BNP **(ii)** on $[^3H]$ -Noradrenaline (NA) release from isolated atria. The atria were stimulated at 5 Hz for 1 minute at the 16th (S1) and 40th (S2) minutes. ($n=6$ $*P<0.05$). Bath solution was collected by replacing 3 ml Tyrode's solution every 3 minutes and measured in a beta radiation scintillation counter. **D**, Representative raw data traces **(i)** and grouped data **(ii)** showing the heart rate responses to sympathetic nerve stimulation (SNS) at 1, 3, 5 and 7 Hz for 30 s with 250 nmol/L BNP compared with control. $n=10$, $*P<0.05$, $**P<0.01$, paired *t*-test. Modified from Li *et al.* (2015) with permission.

Figure 7. **A (i, ii)**, Western blot showing PDE2A.mCherry expression (127 KDa) in transduced empty and Ad.PDE2A stellate ganglia tissue (with anti-PDE2A antibody). Band optical density was

normalized to that of β -actin (42 KDa). **(iii)**, Effect of 250 nmol/L BNP on cGMP concentration in transduced empty and PDE2A stellate ganglia tissue. **B**, Ratio data traces **(i)** and statistical data **(ii)** showing 50 mmol/L KCl evoked intracellular calcium transient ($[Ca^{2+}]_i$) in the transduced empty and Ad.PDE2A cardiac sympathetic neurons. **(iii)**, Group data showing noradrenaline (NA) release during 5-Hz field stimulation was significantly enhanced in the percutaneous gene transfer to right atria with Ad.PDE2A when compared with the empty. * $P < 0.05$, ** $P < 0.01$, unpaired t -test. **C**, Diagram illustrating the potential signal transduction pathways mediated by B-type natriuretic peptide (BNP) in depolarized stellate neurons. BNP binds to the natriuretic peptide receptor A (NPR-A) and stimulates particulate guanylyl cyclase (pGC). Newly synthesized cGMP activates phosphodiesterase 2 (PDE2) to degrade cGMP, but not cAMP, and thus limits the increase in protein kinase G (PKG) activity. Moreover, PKG inhibits calcium current by phosphorylating voltage-gated calcium channels (VGCCs) and reduces the intracellular calcium transient. This in turn decreases NA release from synaptic vesicles and reduces the heart rate response to sympathetic stimulation. In addition, elevated intracellular calcium triggers the activation of adenylate cyclase (AC) via calmodulin (CaM) and produces cAMP. cAMP activates cAMP-dependent protein kinase (PKA), thus phosphorylation of calcium channel. Modified from Li *et al.* (2015) with permission.

Fig.1

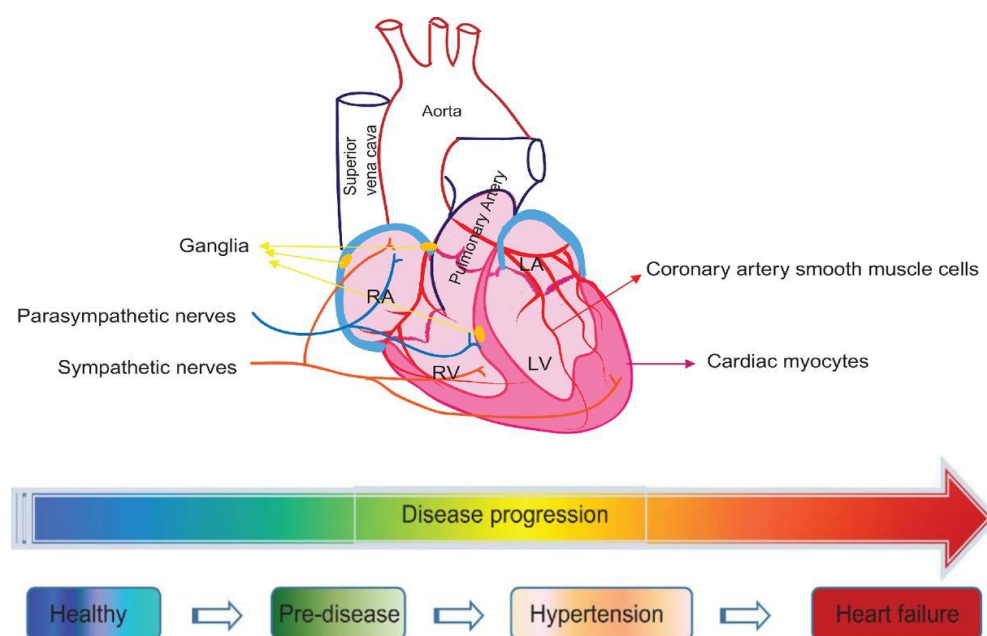


Fig.2

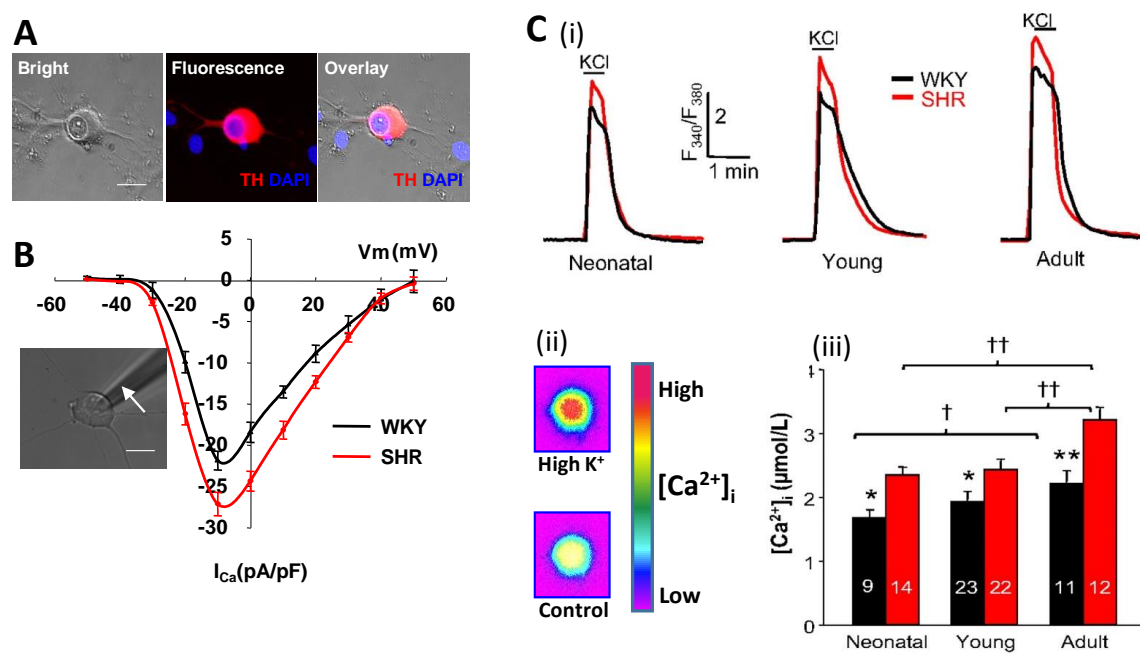


Fig.3

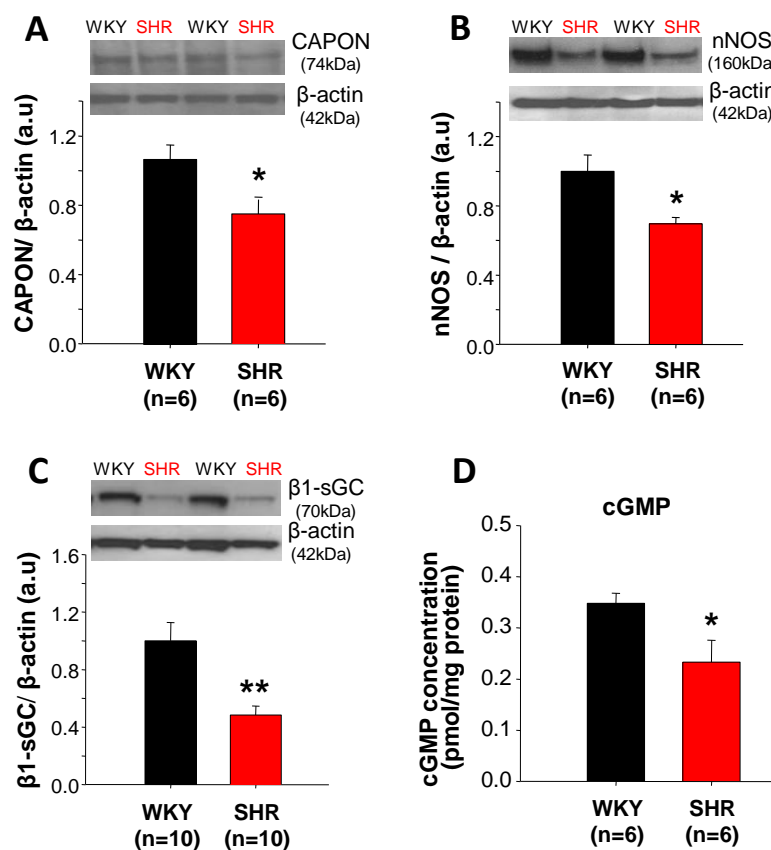


Fig.4

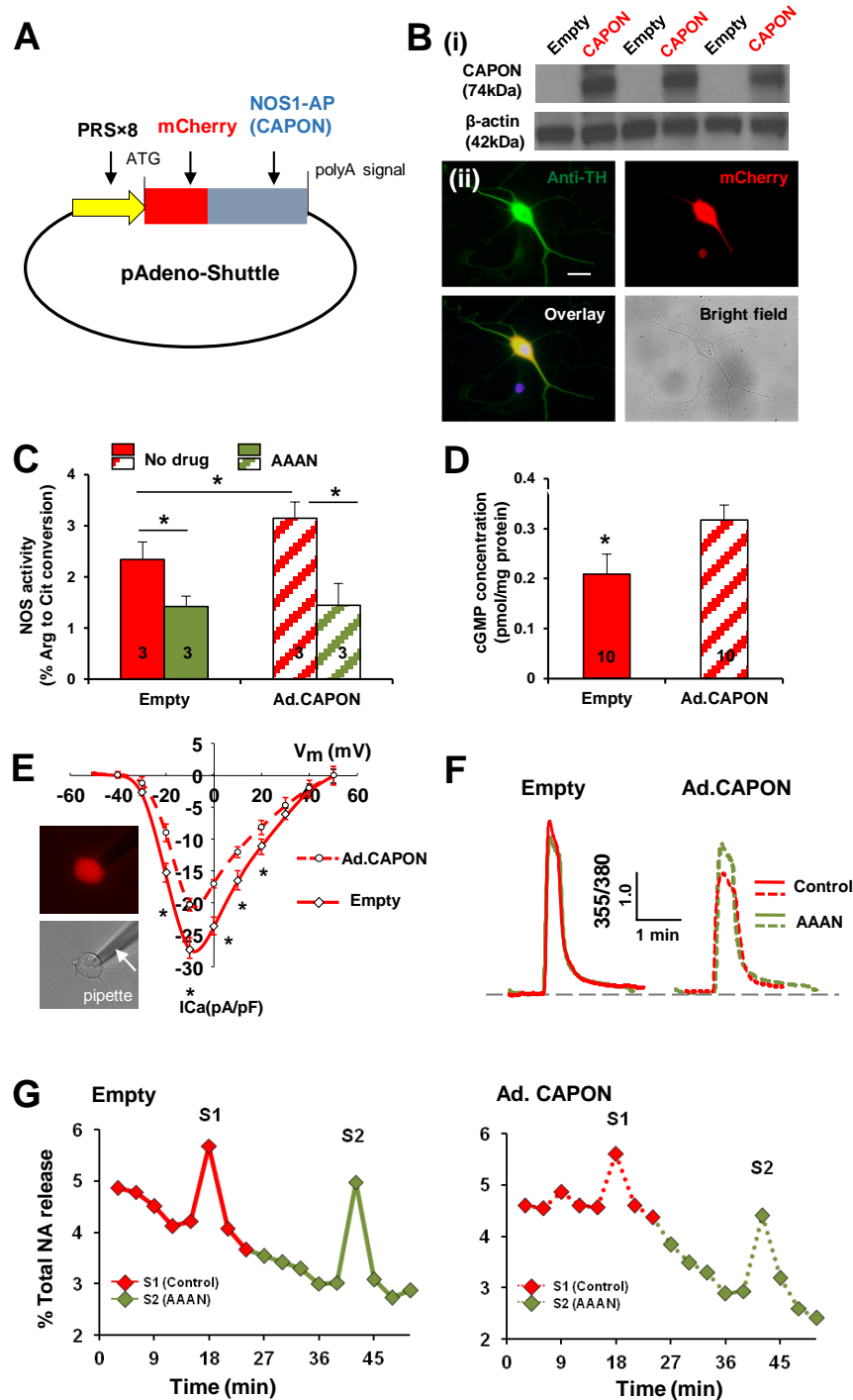


Fig.5

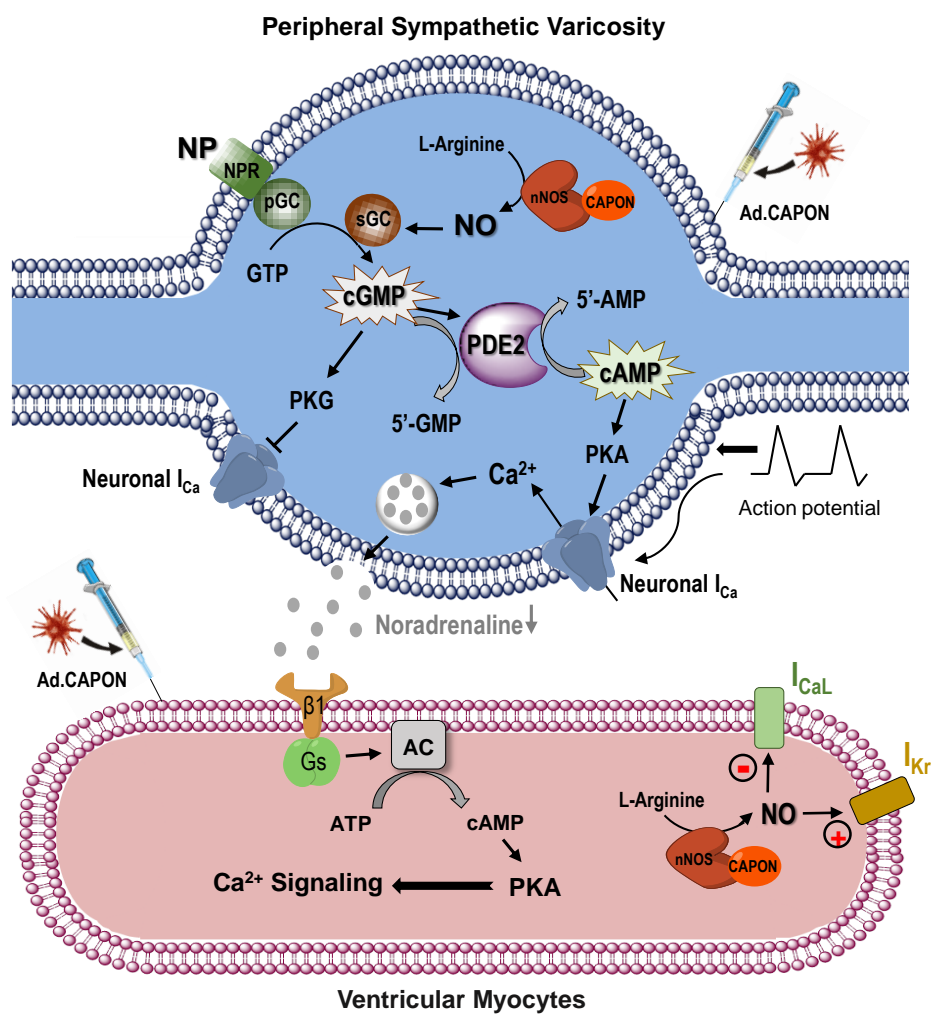


Fig.6

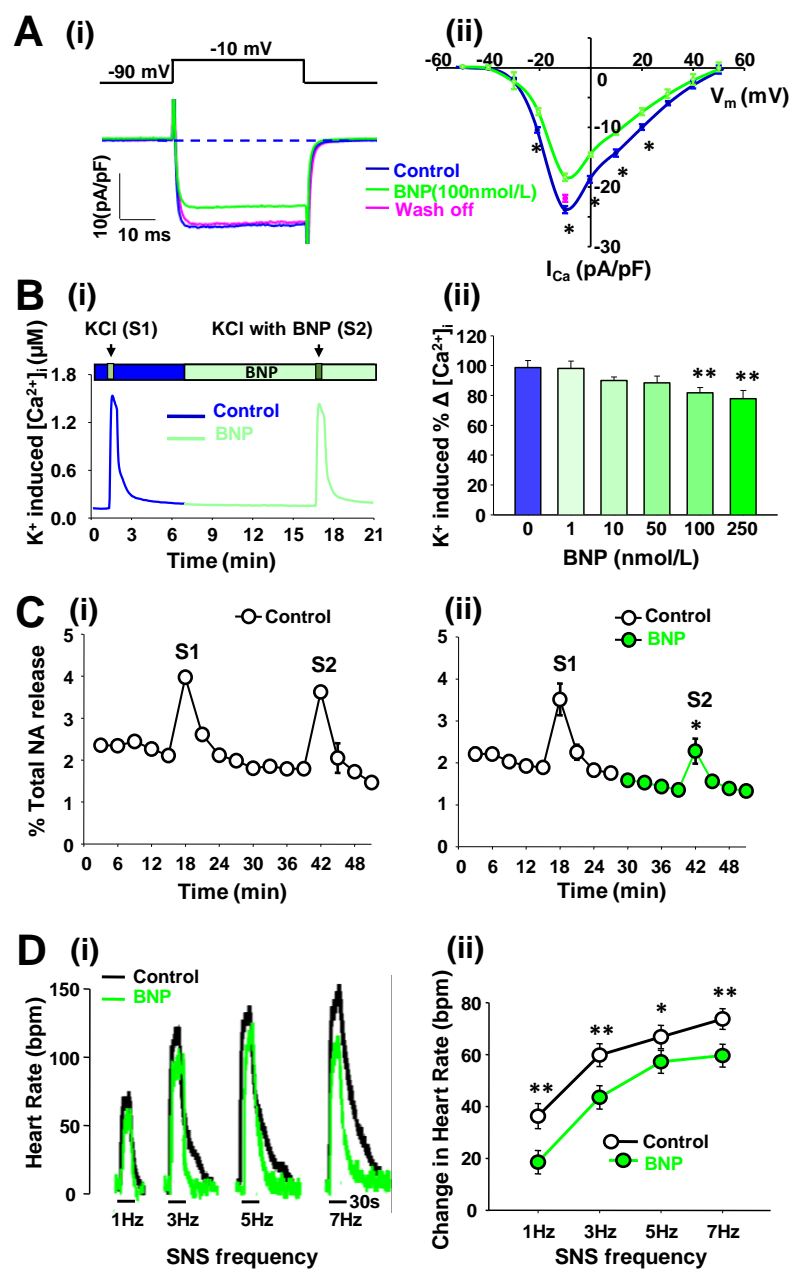
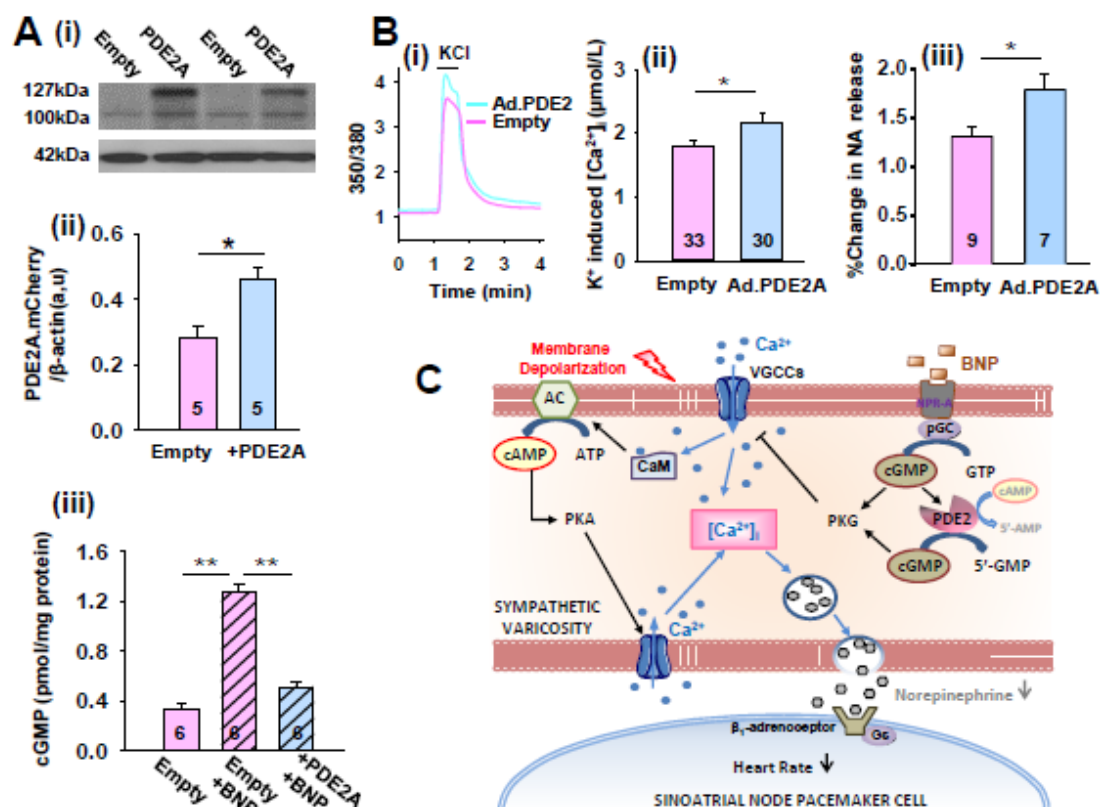


Fig.7



Biography

David J. Paterson is Professor of Physiology in the Department of Physiology, Anatomy & Genetics at the University of Oxford. He graduated from the Universities of Otago (NZ), Western Australia and Oxford, gaining his DPhil from Oxford and DSc from the University of Western Australia. He is a group leader in the British Heart Foundation Centre of Research Excellence at Oxford, and is Joint Director of the Burdon Sanderson Cardiac Science Centre. As a cardiac neurobiologist, his research focuses on the neural control of the cardiorespiratory system in normal and diseased states. In 2014 he was made an Honorary Fellow of The Royal Society of New Zealand.

Dan Li is Senior Research Associate in the Department of Physiology, Anatomy & Genetics at the University of Oxford. She received her PhD from Chonbuk National University, South Korea in 2003, and then moved to the Oxford to pursue post-doctoral research in Professor David Paterson's laboratory. Her main research interest is using cellular and molecular approaches to investigate how second messengers impact on neuronal and cardiac function in health and disease.

