Targeting neuronal nitric oxide synthase with gene transfer to modulate cardiac autonomic function.

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Abstract

Microdomains of neuronal nitric oxide synthase (nNOS) are spatially localised within both autonomic neurons innervating the heart and post-junctional myocytes. This review examines the use of gene transfer to investigate the role of nNOS in cardiac autonomic control. Furthermore, it explores techniques that may be used to improve upon gene delivery to the cardiac autonomic nervous system, potentially allowing more specific delivery of genes to the target neurons/myocytes. This may involve modification of the tropism of the adenoviral vector, or the use of alternative viral and non-viral gene delivery mechanisms to minimise potential immune responses in the host.

Here we show that adenoviral vectors provide an efficient method of gene delivery to cardiac-neural tissue. Functionally, adenovirus-nNOS can increase cardiac vagal responsiveness by facilitating cholinergic neurotransmission and decrease β-adrenergic excitability. Whether gene transfer remains the preferred strategy for targeting cardiac autonomic impairment will depend on site-specific promoters eliciting sustained gene expression that results in restoration of physiological function.

(158 words)

Keywords.

Gene Transfer, Nitric Oxide Synthase, Heart, Autonomic Nervous System.

I. Introduction

Following elucidation of its biological action (Furchgott, Ignaro and Murad, Nobel Prize in Physiology or Medicine, 1998), nitric oxide (NO) is now recognised as one of the most important inter/intracellular signalling molecules in the cardiovascular system, where it is involved in the endothelial control of vascular tone (Ignarro et al., 1987) and regulation of ATP production (Takehara et al., 1995). Until relatively recently, little was known about the physiological significance of NO produced in the heart itself. Early reports that cardiac NO could modulate cardiac contractile performance (Kelly et al., 1996) and ion currents (Balligand et al., 1993) were later greeted with contradictory evidence surrounding the precise mechanisms of action of NO (Han et al., 1994; Vandecasteele et al., 1999). However, part of this controversy may have arisen from the nature in which NO is synthesised. Emerging evidence now suggests that cellular microdomains of nitric oxide synthase (NOS) produce NO that then acts in a highly site-specific and differential fashion depending on the proximity of the isoform relative to its target (Barouch et al., 2002; Paton et al., 2002) (see Fig 1). In addition, the actions of NO on the heart are intimately related to the regulatory role played by the autonomic nervous system (Herring et al., 2002). This review examines how NOS affects cardiac autonomic excitability, and addresses how gene transfer of neuronal NOS can be used to modulate cardiac neural function. It also highlights the advantages and limitations of various gene transfer strategies.

[insert figure 1 near here]

II. Biochemistry of Nitric Oxide

NO biosynthesis from the substrate L-arginine is performed by NOS isoforms found in a variety of tissues (eg liver, brain, heart) and cell types (eg, macrophage, endothelial, microglial) (Stuehr and Griffith, 1992). The three NOS isoforms characterised to date all require a number of co-

factors or prosthetic groups: NOS is a heme-containing protein (White and Marletta, 1992) which utilizes oxygen and NADPH (Palmer and Moncada, 1989; Stuehr and Griffith, 1992) and requires flavin nucleotide reductases and tetrahydrobioterin (BH_4) (Hevel et al., 1991) for activity. The structural similarity of NOS isoforms suggests that the mechanism by which they synthesise NO is likely to be similar.

Two of the three distinct NOS isoforms are constitutively expressed (neuronal NOS, nNOS; endothelial NOS, eNOS), while expression of the third isoform (inducible NOS, iNOS) is induced by an inflammatory immune response. Each isoform has a distinct gene loci while sharing approximately 60% nucleotide sequence identity (Forstermann et al., 1994; Michel and Feron, 1997). Their overall enzymatic activity is regulated by phosphorylation and by the Ca^{2+} binding protein calmodulin, although iNOS carries a permanently bound molecule of calmodulin that allows this isoform to function at low cytoplasmic Ca^{2+} levels.

Because NO itself is highly membrane permeant it can potentially affect cells adjacent to its site of synthesis. NO is also a free radical and is reactive with other species, notably oxygen and superoxide and iron-containing heme groups which act as NO-scavengers. For this reason, the half life of NO in haemoglobin or myoglobin is reduced to seconds. Consequently, the functional effects of NO are localised close to its site of synthesis. NO binds to the second messenger soluble guanylate cyclase (GC) and increases cGMP production (Friebe and Koesling, 2003; Ignarro, 1991; Murad, 1994; Schmidt et al., 1993). The intracellular actions of cGMP are mediated by various cGMP receptor proteins such as cGMP-gated ion channels (DiFrancesco and Tortora, 1991; Lincoln and Cornwell, 1993), cGMP-regulated phosphodiesterases (Trong et al., 1990) and cGMP-dependent protein kinases (Lincoln and Cornwell, 1993). cGMP also

stimulates the formation of cyclic ADP ribose (Galione et al., 1993), and if levels are high enough, it can cross activate protein kinase A (Lincoln et al., 1995).

NO may also regulate cardiac function through mechanisms independent from cGMP. An excess NO concentration can be toxic if combined with the free radical, superoxide (O_2^-), to produce highly reactive peroxynitrite ($ONOO^-$). The reason for $ONOO^-$ formation may be due in part to a reduced ability to scavenge O_2^- when intracellular anti-oxidants are depleted (Ferdinandy and Schulz, 2003). The physiological consequences of $ONOO^-$ on the autonomic nervous system are, however, not well established.

III. Functional Localization of NOS in the Brainstem

In the central nervous system, NO is synthesized (i) by spatially distributed nNOS in neurons and (ii) eNOS in the vasculature; although eNOS may also be present in neurons (Paton et al., 2001b; Teichert et al., 2000). Cardiac autonomic function is regulated at the level of the nucleus tractus solarius (NTS) by central integration of afferent neurons from baroreceptors and decending pathways from the cerebellum. Microinjection of L-arginine or NO donors into the nucleus tractus solitarius (NTS) elicits both a bradycardia and hypotension (Lin et al. 1999). Moreover, similar effects can be produced by adenoviral vector-mediated overexpression of eNOS within the NTS (Sakai et al., 2000). However, low concentrations of L-arginine or NO donors powerfully depress activity of cardiac vagal afferent neurons (Paton et al., 2001b). This suggests that a more sensitive mechanism responding to lower levels of NO may operate to activate inhibitory transmission in the NTS and depress the afferent side of the baroreflex. The current proposal is that eNOS-derived NO increases soluble guanylate cyclase via a paracrine mechanism to enhance GABAergic-mediated inhibition of baroreflex afferents (Kasparov and

Paton, 1999; Paton et al., 2001a; Paton et al., 2001b). In contrast, on the efferent side of the baroreflex, microinjection of NO donors facilitates activity in the nucleus ambiguous to cause vagal bradycardia (Ruggeri et al., 2000). Therefore, specificity of action of NO in the brainstem may depend on the NOS isoforms involved and their spatial localisation to the target autonomic cells.

IV. Functional Localization of NOS in the Heart

Within the heart itself, NO may have a number of specialised roles because discreet microdomains containing specialised NOS-containing compartments co-express a diverse set of regulators of NO-production.

For instance, neuronal tissue in the heart is concentrated in the epicardial layers of the inter-atrial region. Within this cardiac plexus of nerve fibres and cell bodies, neurons contained within parasympathetic ganglia produce NO, and this is almost exclusively derived from nNOS. Moreover choline acetyltransferase (ChAT)-containing ganglionic cell bodies express nNOS (Klimaschewski et al., 1992; Mawe et al., 1996). In addition, fibres from pre-ganglionic parasympathetic neurons extending into the heart from cardiac branches of the vagus nerve also express nNOS (Tanaka and Chiba, 1995). Whilst some groups report the presence of nNOS in tyrosine hydroylase (TH)-positive, post-ganglionic sympathetic neurons of the cardiac plexus (Kaye et al., 1995; Schwarz et al., 1995), others have failed to detect this (Calupca et al., 2000). However, nNOS is detected at low levels in the stellate ganglion (Mohan et al., 2000), from where the majority of cardiac sympathetic fibres branch.

Endothelial cells in the coronary vasculature (Griffith et al., 1984) and endocardium lining the heart's interior (Schulz et al., 1991) both contain large amounts of eNOS. Vascular shear stress

sensed at the luminal surface of endothelial cells triggers the activation of eNOS via Akt-mediated phosphorylation (Dimmeler et al., 1999); this mechanism is important for the autoregulation of cardiac blood flow. Furthermore, NO produced from the endocardium may aid diastolic relaxation of the myocardium on a beat-by-beat basis (Pinsky et al., 1997).

Both eNOS and nNOS are expressed in cardiomyocytes, although their subcellular organisation is discreet (Barouch et al., 2002). Whilst eNOS is located within membrane caveolae via caveolin-1 and -3 which act as both scaffold and regulatory proteins (Feron et al., 1996), nNOS is located on the sarcoplasmic reticulum (Xu et al., 1999). NOS is also expressed in mitochondria in cardiomyocytes, although controversy exists over whether this is the eNOS (Bates et al., 1996) or nNOS isoform (Kanai et al., 2001).

1. NO and Cardiac Sympathetic Function

NO acts presynaptically to inhibit cardiac sympathetic neurotransmission, both *in vivo* and *in vitro*. NOS inhibition enhances overflow of noradrenaline during cardiac sympathetic nerve stimulation in the isolated perfused rat heart (Schwarz et al., 1995), and increases the inotropic effect of stellate ganglion stimulation (Takita et al., 1998) and β-agonists (Elvan et al., 1997) in vagotomised dogs. Inhibition of NOS also increases chronotropic responsiveness to stimulation of the stellate ganglion, both in rabbits *in vivo* (Sears et al., 1998) and guinea pigs *in vitro* (Choate and Paterson, 1999), although chronotropic responsiveness to β-agonists is unaffected. This suggests that endogenous NO may exert differential effects on cardiac contraction and rate under conditions of β-adrenergic stimulation; acting pre-junctionally to inhibit release of noradrenaline from the sympathetic varicosity, but also post-junctionally to inhibit the inotropic responsiveness of target myocytes.

The role of post-junctional eNOS-derived NO has been extensively studied (Balligand, 1999). Inhibition of eNOS augments inotropic responsiveness to β-agonists at the cellular (Goldberg and Haddox, 1977), tissue (Sterin-Borda et al., 1998), and whole animal (Keaney et al., 1996) level, while exogenous NO donors exert the opposite effect (Choate and Paterson, 1999). The role of post-junctional nNOS is, however, more controversial. Microdomains of nNOS are localised within the sarcoplasmic reticulum (Xu et al., 1999) and may modulate Ca^{2+} signalling in ventricular myocytes. It is suggested that nNOS may exert a tonic inhibitory effect in the basal regulation of the L-type Ca^{2+} current and intracellular calcium transients (Sears et al., 2003), although this role has been disputed (Khan et al., 2003). Contradictory results have also been seen under conditions of β-stimulation in the nNOS knockout mouse, with some studies reporting an increase in responsiveness to low doses of β-agonists (Ashley et al., 2002) whilst others report the opposite effect with higher doses (Barouch et al., 2002).

The intracellular mechanism through which NO modulates β-adrenergic function may be similar at both pre- and post-junctional sites. Within sympathetic neurons and cardiac myocytes, nNOS and eNOS, respectively, activate guanylate cyclase (GC) to synthesize cGMP. Within presynaptic sympathetic neurons, neuronal Ca^{2+} channels and subsequent NA release can be inhibited either indirectly (by cGMP-mediated activation of cGMP-phosphodiesterase II) or directly (by cGMP activation of PKG), both of which lead to a reduced activity of the L-type Ca^{2+} current (I_{CaL}). The mechanism of inhibition of contraction by SR nNOS-derived NO is not precisely known, but is speculated to involve an altered phosphorylation state of L-type Ca^{2+} channels or ryanodine receptors (Sears et al., 2003).

2. NO and Cardiac Parasympathetic Function

At the postsynaptic level, early reports implicated NO as an obligatory intracellular molecule in the parasympathetic control of heart rate. Balligand et al (Balligand et al., 1993) first reported that NOS inhibitors abolished the negative chronotropic effects of ACh receptor agonists, and ACh-receptor-mediated inhibition of I_{CaL} in pacemaking cells (Han et al., 1996; Han et al., 1994). Furthermore, cholinergic inhibition of I_{CaL} was absent in eNOS knockout mice (Han et al., 1998), although this finding has been disputed (Vandecasteele et al., 1999). Some of the reasons for these differences have been discussed in detail elsewhere (Balligand, 1999; Herring et al., 2002). Further complicating interpretation of experimental results, NO donors and low doses of cGMP can increase the hyperpolarisation-activated inward current, I_f (Musialek et al., 1997), illustrating the differential regulation of two pacemaking currents by the NO-cGMP pathway.

Of interest, the direct stimulatory effect of NO on heart rate occurs even in cardiac denervated and β-blocked animals. The overall functional significance of this is best illustrated by recent work showing that NO donors can increase heart rate in heart transplant patients devoid of significant sympathetic re-innervation (Chowdhary et al., 2002). This calls into question the use of nitrovasodilating drugs to test the sympathetic component of the baroreflex because they can act directly on the heart (Casadei and Paterson, 2000).

At the presynaptic level, it is now well established that the NO-cGMP pathway facilitates vagal bradycardia. nNOS inhibitors attenuate vagally-induced bradycardia *in vivo* (Conlon and Kidd, 1999; Herring et al., 2000). Moreover, in humans, NO donors can increase the high frequency component of heart rate variability, an index of cardiac vagal tone (Chowdhary and Townend, 1999). Additionally, NO donors and the cGMP analogue 8-bromo-cGMP enhance the heart rate response to vagal nerve stimulation in rabbits and guinea pigs (Sears et al., 1999). However, the

bradycardic response to carbamylcholine (the stable analogue of acetylcholine) is unaffected by NOS or guanylate cyclase inhibitors, consistent with the idea that NO is a presynaptic modulator of vagal neurotransmission (Herring et al., 2000; Sears et al., 1999) (see Figure 2A and 2C). In the mouse, a significant fraction of this effect occurs at the postganglionic-neuroeffector junction, since NOS inhibitors attenuate the heart rate response to nicotinic agonists which stimulate postganglionic neurons directly (Fig 2B). In the dog, neurotransmission appears to be predominantly modulated by NO at the pre/post ganglionic neuronal synapse (Markos et al., 2002).

[insert figure 2 near here]

NO or cGMP can cause an increase in acetylcholine release during field stimulation of the right atrium (Herring and Paterson, 2001). This is thought to result from increased cGMP-mediated inhibition of PDE3, leading to increased PKA-dependent phosphorylation of N-type calcium channels and calcium mediated exocytosis. The likely source of NO is from nNOS, since conscious nNOS knockout mice have a higher baseline heart rate and blunted HR response to atropine compared to wild-type controls (Jumrussirikul et al., 1998). In the isolated atria-vagal preparation from the $nNOS^{-/-}$ mouse, the HR response to vagal nerve stimulation, but not to carbamylcholine, is reduced compared to wild-type litter mates (Choate et al., 2001).

Fig 3 illustrates where NOS isoforms have been implicated in the site-specific modulation of peripheral neuronal cardiac regulation. Although basal expression of nNOS in peripheral autonomic nerves innervating the heart is modest, exercise training in both mice and guinea pigs markedly upregulates nNOS (Danson and Paterson, 2003; Mohan et al., 2000, respectively). Functionally, this upregulation results in increased vagal responsiveness in trained animals

compared to sedentary controls, which is a feature of the althletic phenotype (Danson and Paterson, 2003). Conversely, in several pathophysiological states (eg heart failure, post MI, hypertension), cardiac autonomic balance is impaired and results in sympathetic hyper-responsiveness and decreased vagal responsiveness (Lampert et al., 2003). Impaired NOS signalling has recently been postulated as a contributing factor underlying cardiac autonomic dysfunction, increasing the motivation to develop a strategy to target this signalling system (Heaton et al., 2003).

[insert figure 3 near here]

V. Molecular and genetic strategies to target the role of NOS in the autonomic control of cardiac excitability

Modulating key proteins involved in NOS signalling has recently been made possible by the development of gene knockout techniques to either decrease expression (such as inhibitory RNA) or to initiate/enhance expression of the gene of interest.

1. Adenovirus-mediated Gene Transfer

Recombinant adenovirus has proven to be a popular vector for gene transfer. The first generation of these vectors were based on serotype 5 (Ad5) with engineered deletions in their E1 (early gene) region, thus rendering them replication deficient while at the same time providing a cloning site for gene insertion. These vectors were found to be extremely efficient in delivering genes to vascular tissue following even short exposure times (Baek and March, 1998; Baker et al., 1997; French et al., 1994; Lemarchand et al., 1992; Lemarchand et al., 1993). However, on the negative side these vectors promote only transient expression and at the same time are found

to cause so called “leaky” expression of viral genes, leading to an unwelcome stimulation of the host’s immune response (Einfeld and Roelvink, 2002). This situation has now improved with the development of the second and third generations of these vectors with further viral gene deletions or mutations causing reduced levels of toxicity, which promote less transient expression of the inserted gene, and which in the case of the third generation or “gutless” vectors, possess a larger cloning capacity (up to 36 Kb). However, the gutless vectors, in particular, have their problems as more of the missing viral genes have to be provided *in trans* during virus packaging *in vitro,* necessitating stringent purification procedures (Ng et al., 2001).

2. Mechanism of Gene Delivery

The mechanism of adenovirus-mediated delivery is key to understanding the tropism of the adenovirus vector and possible ways of modifying this tropism. Initial work in this area has related tropism to the binding of the Ad5 fibre knob domain to a target cell surface receptor (coxsackie and adenovirus receptor or CAR), followed by a secondary interaction between integrins on the cell surface and a RGD peptide motif in the penton base of the virus (Fig 4). However, more recent studies have demonstrated the importance of the binding of the Ad5 shaft to heparin sulphate proteoglycans to the hepatic uptake of Ad5. These events ultimately culminate in viral entry to the cell via receptor-mediated endocytosis. Once inside the target cell the internalised endosome is lysed and the virus then traffics to the nucleus for episomal processing of its genomic DNA (Henry et al., 1994; Santis et al., 1999; Tomko et al., 1997).

[insert figure 4 near here]

3. Adenoviral gene transfer to the cardiac autonomic nervous system

In the cardiac autonomic nervous system, we have recently shown that adenoviral mediated gene transfer of the nNOS gene, under the control of the cytomegalovirus (CMV) promoter (Channon

et al., 1996), to the guinea pig right atrium results in nNOS immunolocalisation in cholinergic intracardiac ganglia, increased nNOS protein expression and enhanced vagal responsiveness (Mohan et al., 2002). Importantly, in the nNOS gene transfer treated group, NADPH-diaphorase staining and immunohistochemical analysis of tissue cryosections indicated greater expression of nNOS in atria compared to those treated with Ad.eGFP, and this was confirmed quantitatively by Western blotting (Fig 5a and 5B).

[insert figure 5 near here]

Furthermore, Ad.nNOS gene transfer enhanced vagally mediated bradycardia *in vitro* (Fig 6a) and *in vivo* (Fig 6b), whereas the heart rate response to carbamylcholine was unaffected. Release of ^{3}H-ACh from field stimulated right atria was enhanced in the Ad.nNOS group (Fig 7), confirming a presynaptic action of nNOS to enhance cardiac vagal neurotransmission. Functionally, the enhanced bradycardia in the nNOS treated group was normalised to levels similar to that in the eGFP and sham groups during NOS inhibition (Mohan et al., 2002). In contrast, Ad.nNOS gene transfer decreased the tachycardia caused by bath applied β-adrenergic stimulation (Fig 8). This suggests that NO generated from nNOS during autonomic activation acts predominantly to reduce heart rate due to presynaptic actions within intra-cardiac cholinergic ganglia to enhance vagal function, as well as postsynaptically by decreasing β-adrenergic responsiveness.

[insert figures 6-8 near here]

Cardiac-ganglionic gene transfer of nNOS provides a tool for proof of principle concerning the role of nNOS in the vagal control of heart rate, although there are limitations with this approach.

One of the main disadvantages with the adenovirus system for gene delivery *in vivo* is the promiscuity of the virus, due mainly to the widespread expression of CAR. This has lead to a great deal of research aimed at achieving expression of the gene of interest in target tissues alone, and without the major trafficking to the liver and other vital organs that is often observed in *in vivo* experiments when the adenovirus is given intravenously. Such research can be subdivided into two broad approaches: genetic and non-genetic.

VI. Genetic Strategies for Targeted Adenovirus-mediated Gene Expression *in vivo*

1. Insert tissue-specific promoters

Gene expression in the target tissue has traditionally been initiated by the inclusion of a viral promoter, such as the CMV promoter, in the virus-gene construct. A characteristically strong and promiscuous promoter, CMV ensures a high level expression of the gene of interest in the target tissue. Unfortunately, these properties also contribute to the high levels of expression observed in other tissues. A way of overcoming this is to switch the CMV promoter for one which would direct gene expression in only a limited number of tissues or, if possible, in the target tissue alone.

Clearly, it would be desirable to more tightly control the expression of the AD5-nNOS construct in order to delineate more specifically the affect of nNOS expression in specific tissues and/or cell types. This may be achieved by replacing the CMV promoter with, for example, a cholinergic neuron-specific promoter, such as the choline acetyltransferase/vesicular acetycholine transporter promoter (Naciff et al., 1999), an atrial myocyte specific promoter such as the myosin light chain 1A promoter (Catala et al., 1995), or a noradrenergic promoter (Hwang et al., 2001). In each case tissue-specificity has been demonstrated. However, it will be important

to demonstrate the specificity conferred by each of these promoters on the nNOS gene expression *in vitro* before moving on to *in vivo* experiments.

2. Choose a different serotype

While most studies have focused upon using Ad5 this is not the only serotype identified. Data are now emerging regarding the wide tissue and species tropisms expressed by some of the other adenoviral serotypes. These differences are now being exploited for more targeted gene delivery since there are at least 50 human serotypes of adenovirus. By identifying fibres from these serotypes that are more efficient at binding to the target tissue under investigation it has been possible to modify Ad5 particles to contain such fibres – a technique known as pseudotyping. For example, fibres from Ad3 are able to enhance gene transfer to smooth muscle cells *in vitro* by as much as 15-fold compared to the native vector expressing the native Ad5 fibre (Su et al., 2001). Ad4 and Ad11 fibres bind with a higher affinity to vascular endothelial cells, with a consequent higher infectivity compared to Ad5 vectors (Zhang et al., 2003).

Fibre length has also been shown to influence tropism in addition to any fibre-receptor interaction. Ad40 has both long and short fibres that do and do not recognise CAR respectively. Nakamura *et al.,* (Nakamura et al., 2003) have prepared several Ad5 mutants containing either long or short Ad40-derived fibres, together with either CAR -binding or non-binding knob domains, and found that the simultaneous ablation of the CAR interaction and the expression of a short fibre dramatically reduced gene transduction in the liver and spleen following intravenous injection.

3. Modify the Ad5 fibre knob domain by inserting a new ligand

As well as pseudotyping to alter the fibre-cellular interaction another avenue of research has focused on re-engineering the structure of the native Ad5 fibre. This presents the opportunity to

create a greater range of vectors tailored to individual targeting requirements. Modification of the Ad5 fibre has been made possible following publication of the Ad5 fibre crystal structure, revealing sites on the fibre protein that would be open to peptide insertion (Xia et al., 1994). Since then various peptides have been successfully inserted into different regions of the virus resulting in incorporation of the peptides into the virus proteins following assembly. Consequently, the externally expressed HI loop that links the β strands of the fibre knob has been identified as the most efficient site for foreign ligand insertion (Krasnykh et al., 1998) particularly as it does not affect fibre trimerisation (which is an essential stage in virion development). In one example the SIGYPLP peptide has been inserted to target Ad5 virus specifically to human venous endothelial cells *in vitro.* This was coupled with a mutation that prevented binding to CAR, resulting in no transduction into non-endothelial cells. This type of viral re-engineering has been coupled to the phage display technique to identify suitable ligands that will target the virus to the cells of choice. There are various refinements to this technique, but in its simplest form phage expressing peptides which are ligands for receptors on the cells to be infected by virus are isolated from phage libraries by several rounds of panning against cultures containing the target cells. By discarding unbound phage and then eluting bound phage for repeated panning the pool is enriched for phage containing specific target cell ligands (Barry et al., 1996; Doorbar and Winter, 1994; Goodson et al., 1994). Peptides can then be purified from these phage for insertion into the HI loop of the Ad5 fibre (Nicklin et al., 2001; Nicklin et al., 2000). It is also possible to perform panning *in vivo*; extracting the target tissue following systemic application of the phage library and purifying any phage that has been internalised (Pasqualini and Ruoslahti, 1996; Rajotte et al., 1998). This latter modification may prove advantageous to virus-mediated gene delivery as it will allow selection for diseased tissue - specific ligands.

4. Modify the Ad5 fibre by knob domain stripping

Genetic modification of the fibre has its limitations, particularly since only short ligands can be added to or substituted into the fibre before altering its trimeric structure and thus affecting its uptake efficiency into cells. Several groups have recently shown that the native trimerisation domain can be replaced by a foreign trimeric structure to produce trimeric fibres that lack the knob domain (Krasnykh et al., 2001; Magnusson et al., 2001; van Beusechem et al., 2000). Unfortunately, this vector now requires a cell line expressing a receptor for the new targeting ligand in order to propagate and produce useful virus, which makes the method more cumbersome. A subsequent refinement designed to overcome this involves re-constructing the fibre protein to carry a foreign trimerisation domain, a target cell-specific ligand and a single Factor Xa proteolytic cleavage site between this ligand and the terminal knob domain. Thus, the native knob domain can be cleaved from the virus following viral production in the normal way using the HEK-293 cell line (Hong et al., 2003) leaving a structurally intact and targeted gene delivery system.

VII. Non-Genetic Strategies for Targeted Ad-mediated Gene Expression

1. Antibody-coated viruses.

Non-genetic approaches represent the first attempts to improve upon targeting gene delivery by adenovirus. Initial studies used a bi-specific antibody with specificity for an epitope in the Ad5 penton base and containing a RGD peptide motif for interaction with cell-surface integrins. On incubation with virus, the antibody effectively blocked the fibre knob interaction with CAR and redirected transduction entirely to integrin expressing cells. While a significant achievement this still targeted the adenovirus to a variety of integrin-expressing cell types. This approach has subsequently been combined with the phage display technique described earlier in order to

identify a target cell-specific peptide which can then be used to create a fusion protein with an antibody that binds and inhibits the CAR interaction. Although initial used *in vitro* (Watkins et al., 1997), similar success has now been achieved *in vivo* to direct gene delivery to pulmonary endothelial cells, utilising the preferential expression of angiotensin-converting enzyme (ACE) in this tissue (Reynolds et al., 2000).

2. Polymer -coated viruses.

A different approach, which relies on relatively simple technology, is the concept of coating the already prepared viral construct with a polymer. This idea has several advantages in that it would allow evasion of an inappropriate immune response to the virus, which would be important for any future clinical applications; it would prevent indiscriminate CAR-mediated viral entry into cells by obscuring the uptake machinery; and it would, at the same time, provide a medium for the ready incorporation of almost any reactive motif of choice. Initial experiments have demonstrated that coating of Ad5 with a hydrophilic polymer containing fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) can restore viral uptake by cells expressing FGF receptors and VEGF receptors respectively in both mixed cell culture and *in vivo* (Fisher et al., 2001). Although much of this virus still traffics to the liver, the amount is reduced compared to non-coated alternatives; anionisation of the virus using the polymer coat may be one way of further reducing hepatic uptake. In addition, this technique also raises the possibility of altering the species specificity of the virus, allowing a greater choice of delivery systems.

VIII. Alternatives to Adenovirus-mediated Gene Expression in vivo

In addition to modifying the tropism of adenovirus, there remains the possibility of using an alternative gene delivery system, either viral or non-viral in nature.

1. Use of other viruses

Adeno-associated viruses (AAV) are defective human Parvoviruses that are non-pathogenic and rely on helper virus co-transfection for growth (Bueler, 1999). These viruses are difficult to prepare as vectors and have a very limited insert DNA capacity (< 5Kb) which is potentially prohibitive. However, they do facilitate stable, long-term gene expression, albeit at lower levels compared to Ad5, and they also lack much of the immunogenicity associated with adenovirus. Many of the modifications described previously for adenovirus have now also been applied to AAV in order to improve targeting, although capacity still remains a major limitation.

Alternatively, Herpes viruses are able to accommodate very large transgenes. Herpes Simplex-1 (HSV-1) has a capacity in excess of 35Kb and has been used in cardiovascular gene transfer to deliver very large proteins such as ryanodine receptors (Goins et al., 2002). It also has the added advantage of a natural tropism towards nerve cells, which would make it useful for targeting parasympathetic and sympathetic neurons individually if used in combination with specific promoters.

Lentiviruses, which are derived from a family of retroviruses and include Human Immunodeficiency Virus -HIV, are, however, able to infect both dividing and non-dividing cells leading to stable integration into the host genome and potentially life-long expression. This makes them potentially very useful for gene transfer to cardiovascular and other non-proliferating tissue. They have a slightly higher insert capacity than AAV (up to 12Kb, Coleman et al., 2003) and the absence of viral genes precludes any potent humoral immune response, making them suitable for repeated intervention. Although they have been complex to prepare, this situation has improved and is still evolving with the development of better packaging and purification methods (Coleman et al., 2003; Farson et al., 2001) and advanced attenuated HIV

vectors (Dull et al., 1998), which have also removed some of the safety issues associated with lentiviruses such as HIV.

2. Non-viral Mediated Gene Delivery

Due to the many problems that can be associated with viral delivery of genes, notably the immunological side-effects and widespread tropism, and the often technical complexity of the methodology employed to overcome these problems, some considerable attention has been focused on naked DNA gene transfer as an alternative approach.

Plasmid-liposome complexes represent a relatively non-immunogenic alternative to viral gene delivery due to their lack of antigenic protein moieties. They generally consist of synthetic cationic lipid bilayers complexed to the negatively charged plasmid thus facilitating cell transfection. Furthermore, by incorporating specific ligands or antibodies into the liposome (as with viral coat modifications) it has also been possible to improve the tissue targeting of this lipofection technique. Alternatively, the electroporation approach relies on high-voltage pulses to produce transient pores in cell membranes through which DNA can enter (Gehl, 2003). This has, for example, been used to enhance the delivery of plasmids and plasmid-liposome complexes to mammary tumours (Wells et al., 2000). It has also been useful for enhancing other delivery methods and for improving gene delivery to difficult tissues, including muscle (Li and Benninger, 2002) and articular cartilage (Grossin et al., 2003). The more novel “Gene Gun” particle bombardment technique has also been developed to transfer microparticles coated with DNA into the target tissue. The most common ballistic delivery systems use helium gas pressure pulses for particle acceleration (Biewenga et al., 1997). It has found some success in gene transfer to mammalian tissues including rat brain (Jiao et al., 1993). Finally, therapeutic doses of ultrasound have been applied in the vicinity of the target tissue as a way of increasing cell

membrane permeability to previously injected naked DNA - often complexed to lipid microspheres (Bao et al., 1997). This sonoporation method has been successfully used for DNA delivery to tumour cells (eg Manome et al., 2000) and muscle (Danialou et al., 2002).

One of the main attractions with these alternative, non-viral, approaches has been the increased capacity for DNA delivery when compared to some viral methods which are limited to transferring DNA of less than 10Kb in size. Additionally, the lack of viral sequences and, in some cases any antigenic protein moieties associated with the DNA, also results in reduced or even absent immunogenicity. Unfortunately, however, the amount of DNA that is often required for efficient delivery and expression may preclude more widespread use of these techniques and deny easy applicability to a more clinical setting. Furthermore, the advantages of long-lived expression associated with DNA integration into the target cell genome, found with some viral-DNA constructs, eludes these non-viral delivery methods. It is envisaged that continuing development of plasmid expression vectors, which will allow more sustained DNA expression *in vivo*, will help in the development of these alternative delivery systems.

[insert figure 9 near here]

IX. The Future of Gene Transfer to the Cardiac Autonomic Nervous System

Abnormalities in sympathetic and parasympathetic signalling are key components in the aetiology and progression of several cardiovascular diseases, yet the most effective way to target these systems remains elusive. As research in this area continues to intensify, new methods are rapidly developing (gene transfer and neonatal stem cell transfer) and may serve as viable alternatives to the surgical and pharmacological interventions which have been the historical mainstay of treating cardiovascular disease. Preliminary work (see Fig 9 for a summary) has

successfully employed cardiac/neural gene transfer to show the role of nNOS in the neural control of cardiac excitability. Whether gene transfer remains the preferred strategy will depend on the successful development of site-specific promoters eliciting sustained gene expression that result in restoration of autonomic function in pathophysiological states.

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FIGURE LEGENDS

Fig 1. Site-specific and differential modulation of neuronal activity affecting cardiac function by NO The nucleus tractus solitarii (NTS) receives input from barorecptors that is conveyed by glossopharyngeal (IX) afferents. It connects with the nucleus ambiguus (NA), which projects via the vagus to the sinoatrial (SA) node of the heart. The connection from NTS to NA might not be direct, as indicated by the dashed projection. NO has unknown effects (green arrows, labelled with `?') on baroreceptors and IX neurons; it is inhibitory (black arrows, labelled with -) for the NTS and excitatory (red arrows, labelled with +) for the NA (Ruggeri et al., 2000) and SA node. NO also acts on the rostral ventrolateral medulla (RVL) (Kishi et al., 2001; Krukoff, 1999; Zanzinger, 1999) and the intermediolateral cell column (ICC) of the spinal cord (Arnolda et al., 2000; Hakim et al., 1995; Koga et al., 1999; Wu and Dun, 1995; Wu and Dun, 1996), although the reported actions are inconsistent (indicated by a broken black arrow, labelled with `+ and -'). The sympathetic neurons innervated by the ICC are affected by NO in an unknown way, and the cardiac cells these innervate can be inhibited by NO. NO can also activate the pace-making current (If) in cardiac myocytes. The effect of NO on the NTS relates only to its modulation of the baroreceptor reflex. The cerebellum is shown for orientation. *(from Paton et al., 2002, used with permission)*

Fig 2. (a) nNOS inhibition with 100μM L-VNIO attenuates bradycardia in response to 3Hz vagal nerve stimulation in the *in vitro* mouse double atrial preparation ($n=15$ $p<0.05$). Addition of excess L-arginine (1mM) reverses this effect. (b) The nicotinic agonist Anatoxin Fumarate (40μM) was used to selectively stimulate postganglionic vagal neurons. Again, the resulting bradycardia was attenuated by 100μM L-VNIO ($n=15$ $p<0.05$), suggesting that nNOS-derived NO acts to facilitate vagal neurotransmission at the postganglionic-neuroeffector junction. Reversal of the effect of nNOS inhibition was again seen on addition of L-arginine (1mM). (c) Bradycardia in response to 0.1μM carbamylcholine is unaffected by nNOS inhibition ($n=15$), indicating that the facilitatory effect of NO on vagal neurotransmission occurs at the pre-synaptic level.

Fig 3. Action of NO in the regulation of cardiac sympatho–vagal activity in the PNS. Proposed model in which NO generated by neuronal nitric oxide synthase (nNOS) increases the heart-rate response to vagal nerve stimulation. NO stimulates presynaptic soluble guanylate cyclase (sGC) to produce cGMP, which inhibits phosphodiesterase (PDE) 3. This elevates cAMP levels and increases protein kinase A (PKA)-dependent phosphorylation of N-type Ca2+ channels. Both N- and P-type Ca2+ channels (responsible for currents ICaN and ICaP, respectively) control exocytotic release of ACh (triangles), to activate M2 muscarinic ACh receptors and inhibitory G proteins (Gi) of cardiac myocytes. When the sympathetic varicosity is activated, NO-dependent stimulation of cGMP might act to decrease neurotransmission, via PDE2-mediated inhibition of cAMP-dependent phosphorylation of the neuronal Ca2+channel to modulate the release of noradrenaline (circles), which activates $\beta_1$adrenoceptors and stimulatory G proteins (Gs) of cardiac myocytes. The source of NO is not known but it might be either an autocrine or a paracrine site (or both). *(from Paton et al., 2002, used with permission)*

Fig 4. The Adenovirus particle is icosahedral, with an outer capsid, consisting mainly of hexon proteins, and an inner core of 36Kb linear double-stranded DNA (modified for gene delivery). A pentameric penton base and protruding trimeric fibres form the vertices of the capsid. These fibres form a linear shaft with a carboxy-terminal globular head domain of about 200 amino acids which contains the receptor (eg CAR) binding determinant. Potential sites for tropism modification are shown (see review text for details). This image was adapted, by kind permission, from the web page of Prof. Urs Greber, Department of Zoology, University of Zurich (http://www.unizh.ch/~cellbio/).

Fig 5. (a) Confocal imaging of guinea pig atria treated with Ad.eGFP (top row) and Ad.nNOS (bottom row). Immunofluorescence was performed in whole atria, using sheep α-nNOS and goat α-ChAT, with fluorescein isothiocyanate or texas red conjugated secondary antisera. Ad.nNOS treatment increased nNOS expression and co-localisation of nNOS with ChAT, indicating up-regulated nNOS expression in cholinergic ganglia. (b) Quantitative analysis of nNOS protein expression by Western blotting shows a 110% increase in nNOS expression in the Ad.nNOS group ($p \leq 0.01$, unpaired t-test). Expression of eGFP was seen only in Ad.eGFP treated atria (data not shown). nNOS expression was normalised using β-actin. *(adapted from Mohan et al., 2002)*

Fig. 6. (a) Raw data traces showing heart rate responses to 3Hz right vagal nerve stimulation *in vitro*. Treatment with Ad.nNOS significantly enhanced ($p<0.001$, unpaired t-test) the vagal bradycardia, compared to both Ad.eGFP and sham groups. (b) Raw data traces showing heart rate and blood pressure responses to 5Hz vagal stimulation. Again, the vagal bradycardia was significantly increased in the Ad.nNOS group ($p<0.05$, unpaired t-test). *(adapated from Mohan et al., 2002)*

Fig 7. Treatment with Ad.nNOS significantly increased ($p<0.001$, unpaired t-test) release of [^{3}H]ACh in response to 10Hz field stimulation. Release of transmitter was normalised following inhibition of soluble guanylate cyclase with 10μM 1H-(1,2,4)oxadiazolo(4,3-a)quinoxaline-1-one (data not shown), supporting an action of nNOS-derived NO via stimulation of cGMP production. Bar indicates 1min of 10Hz field stimulation. *(adapted from Mohan et al., 2002)*

Fig 8. Heart rate responses to noradrenaline were measured using isolated guinea pig atria, ~5 days after right atrial injection of $5x10^{10}$ particles of Ad.nNOS or Ad.eGFP. Isolated double atrial preparations were mounted vertically in Ringer's solution, continuously bubbled with 95% O_2/5% CO_2, and maintained at 37°C. An isometric force transducer was used to trigger heart rate from contraction. Chronotropic responses to 0.3-2.0μM noradrenaline were significantly attenuated relative to the Ad.eGFP group (n=19) by treatment with Ad.nNOS (n=22, $p<0.05$, unpaired t-test).

Fig 9. Summary of the effects of nNOS gene transfer on the autonomic control of cardiac excitability. Increased NO production from nNOS targeted to cardiac parasympathetic neurons and post-junctional myocytes affects autonomic function at both pre- and post- synaptic sites. Acting pre-junctionally, NO facilitates cholinergic neurotransmission and therefore enhances the heart rate response to vagal nerve stimulation (VNS). Within cardiac myocytes nNOS-derived NO may blunt the heart rate response to noradrenaline (NA). In addition, endogenous NO produced within the sympathetic varicosity may inhibit release of NA and the heart rate response to sympathetic nerve stimulation (SNS). Gene transfer of nNOS to the right atrium therefore results in enhanced vagal and reduced sympathetic responsiveness. This may provide a novel cardioprotective intervention for pathophysiological states.

Figure 1

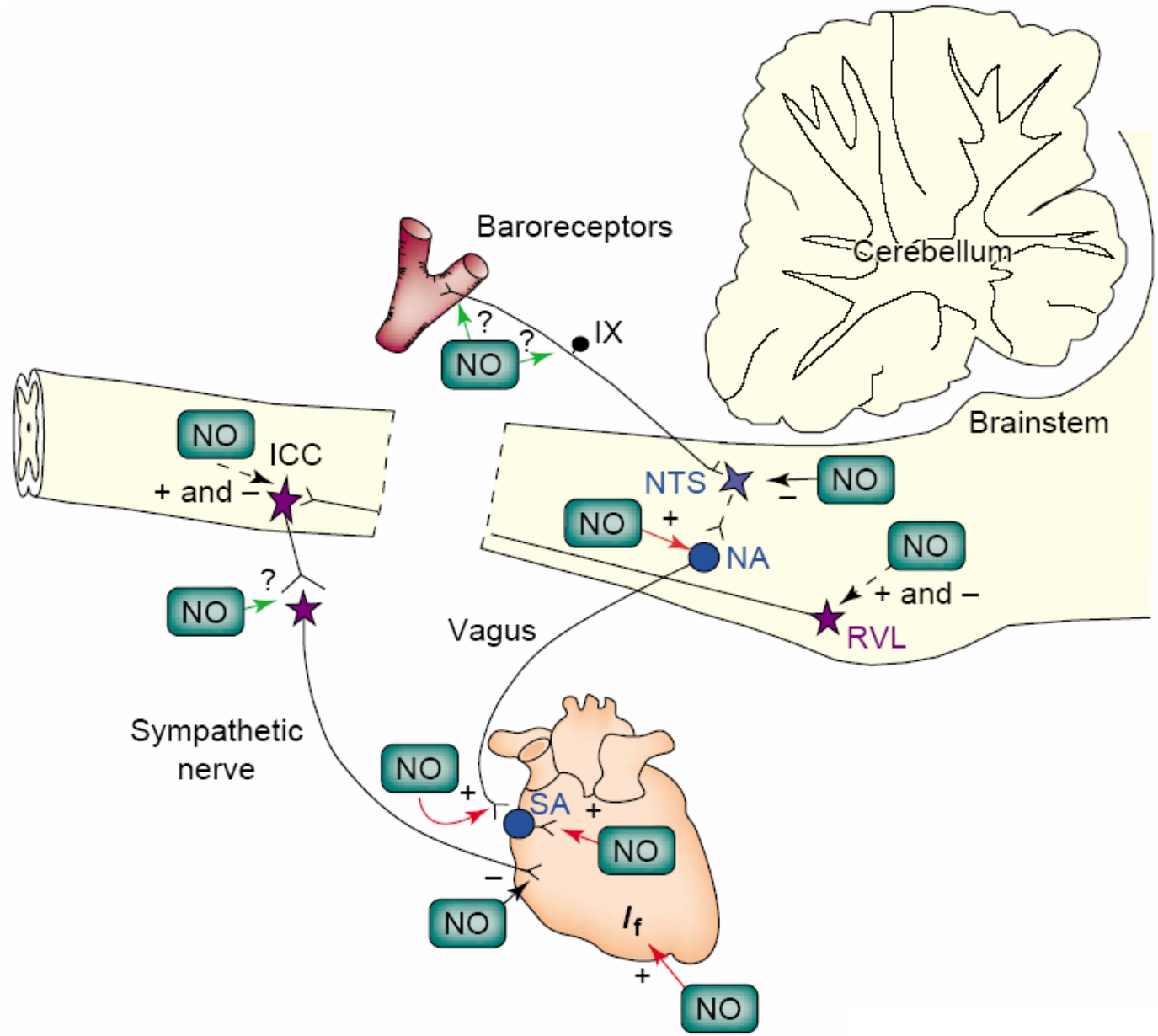

Baroreceptors
Cerebellum
IX
NO
?
?
Brainstem
NO
ICC
+ and –
NTS
NO
–
NO
+
NA
NO
+ and –
RVL
NO
?
Vagus
Sympathetic
nerve
NO
+
SA
+
NO
–
NO
I_f
+
NO

Figure 2

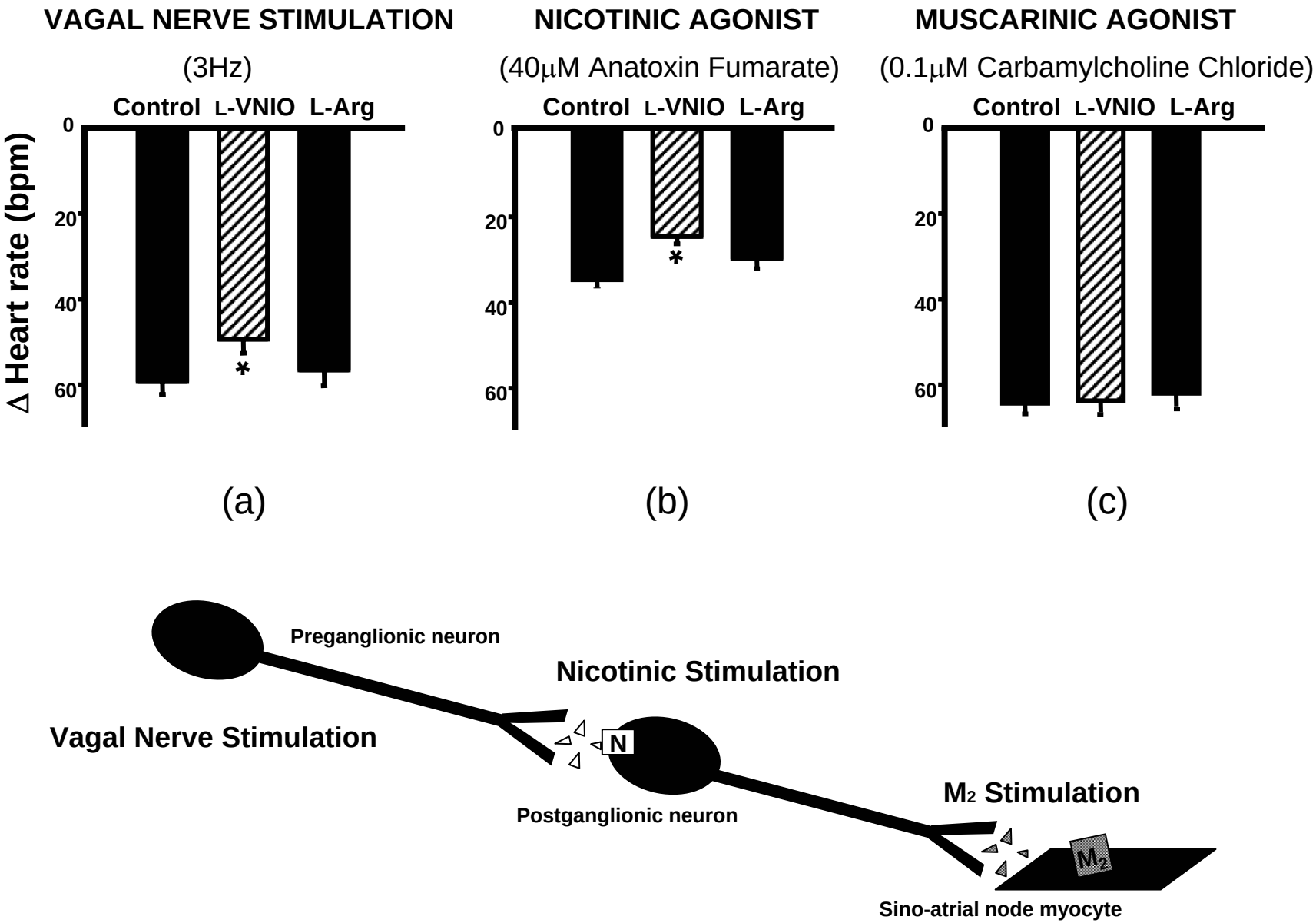

VAGAL NERVE STIMULATION
(3Hz)
NICOTINIC AGONIST
(40μM Anatoxin Fumarate)
MUSCARINIC AGONIST
(0.1μM Carbamylcholine Chloride)
Control
L-VNIO
L-Arg
Δ Heart rate (bpm)
0
20
40
60
*
(a)
(b)
(c)
Preganglionic neuron
Nicotinic Stimulation
Vagal Nerve Stimulation
N
M2 Stimulation
Postganglionic neuron
M2
Sino-atrial node myocyte

Figure 3

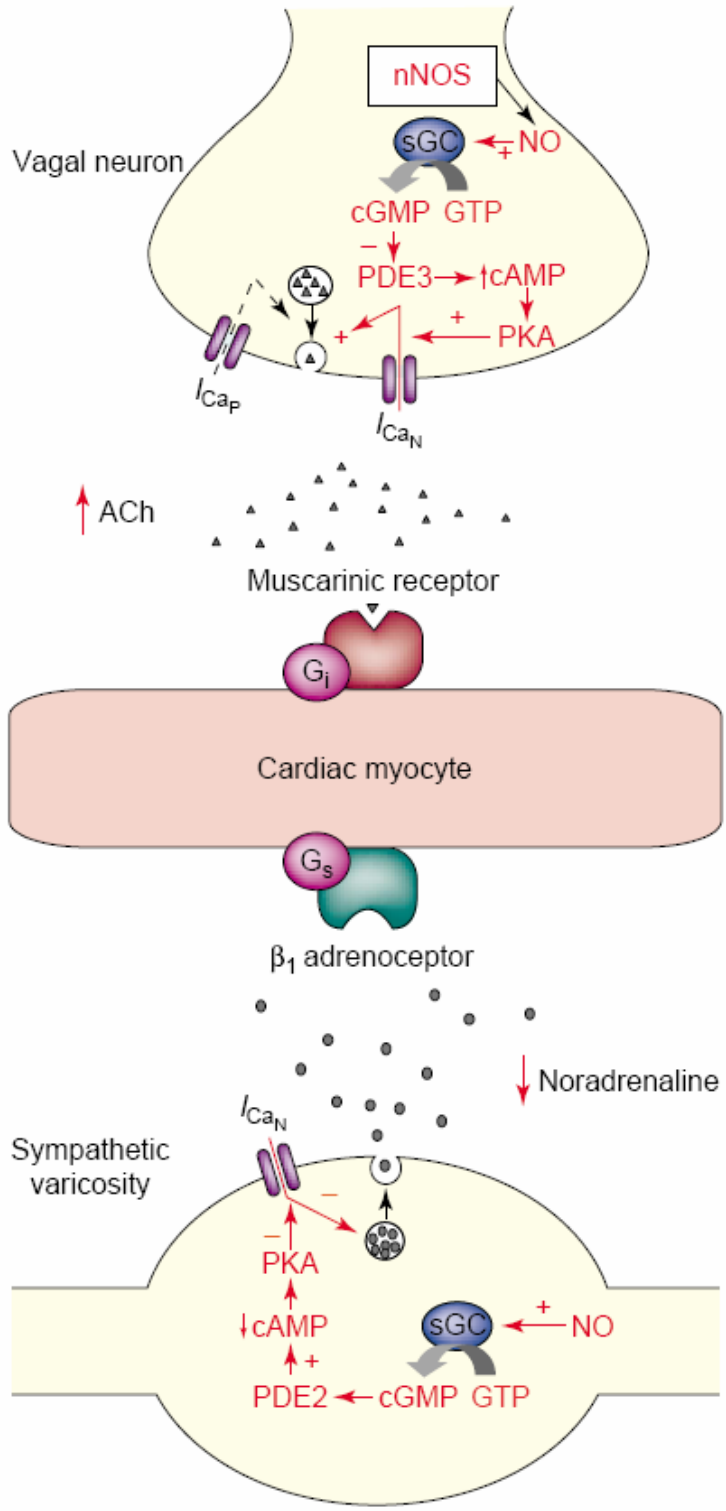

nNOS
Vagal neuron
sGC
NO
cGMP GTP
PDE3
cAMP
PKA
I_{Ca_P}
I_{Ca_N}
ACh
Muscarinic receptor
G_i
Cardiac myocyte
G_s
β_1 adrenoceptor
Noradrenaline
I_{Ca_N}
Sympathetic varicosity
PKA
cAMP
sGC
NO
PDE2
cGMP GTP

Figure 4

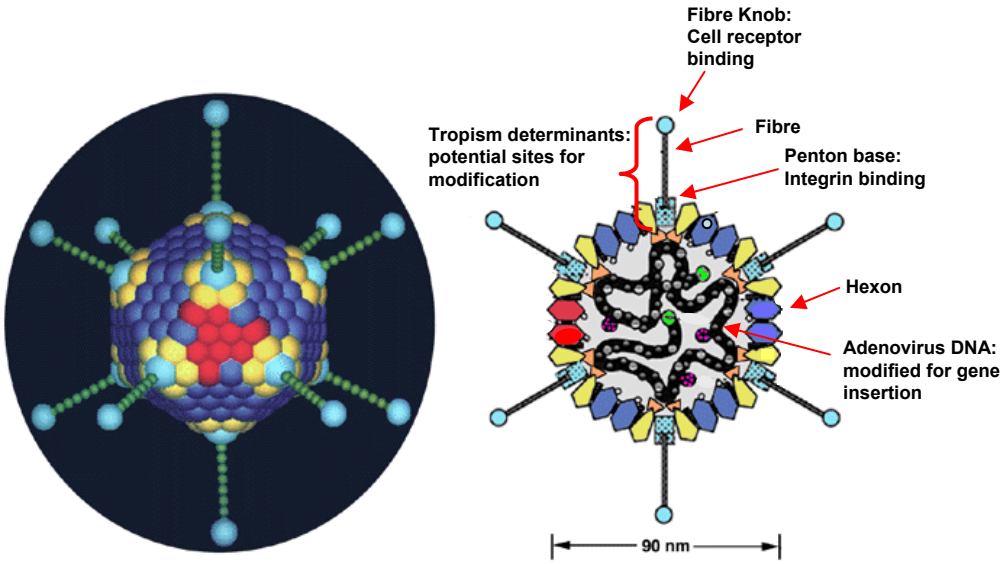

Fibre Knob:
Cell receptor
binding
Tropism determinants:
potential sites for
modification
Fibre
Penton base:
Integrin binding
Hexon
Adenovirus DNA:
modified for gene
insertion
90 nm

Figure 5A

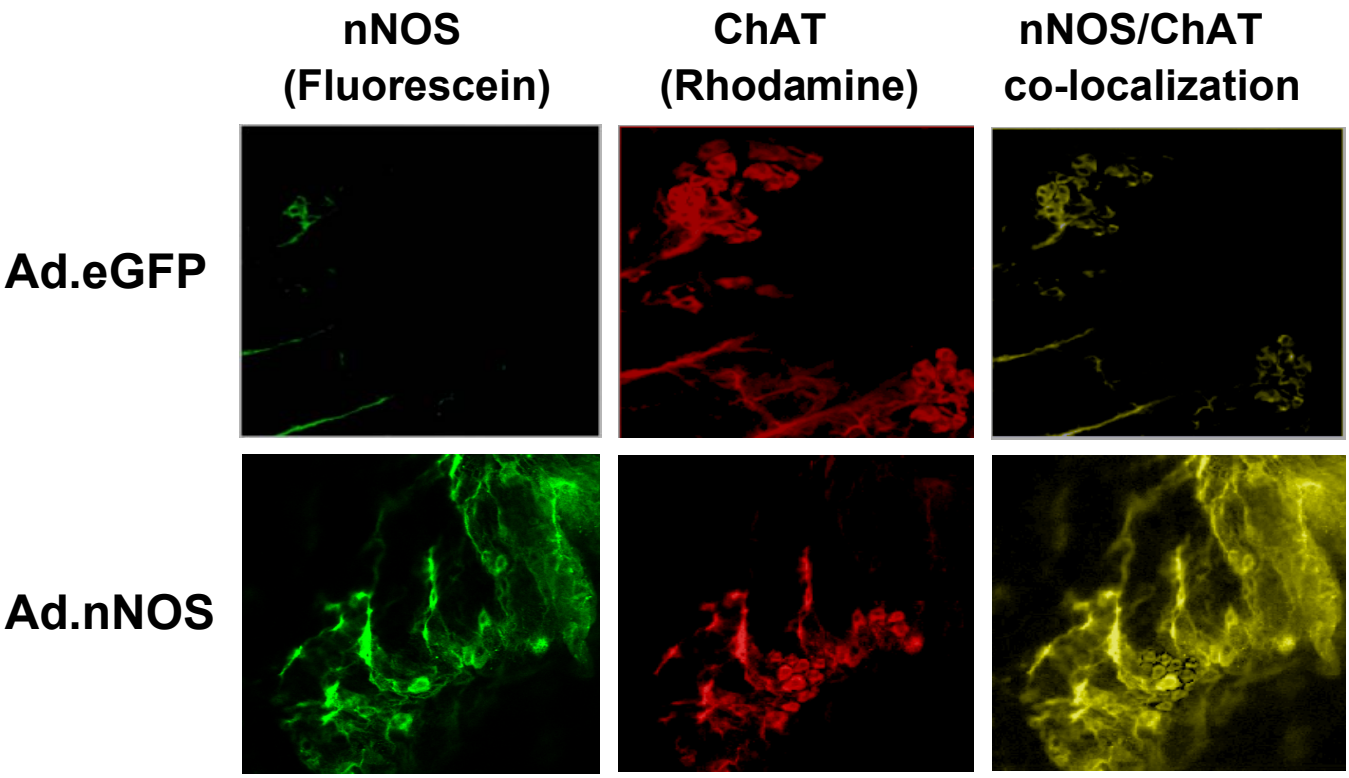


Figure 5b

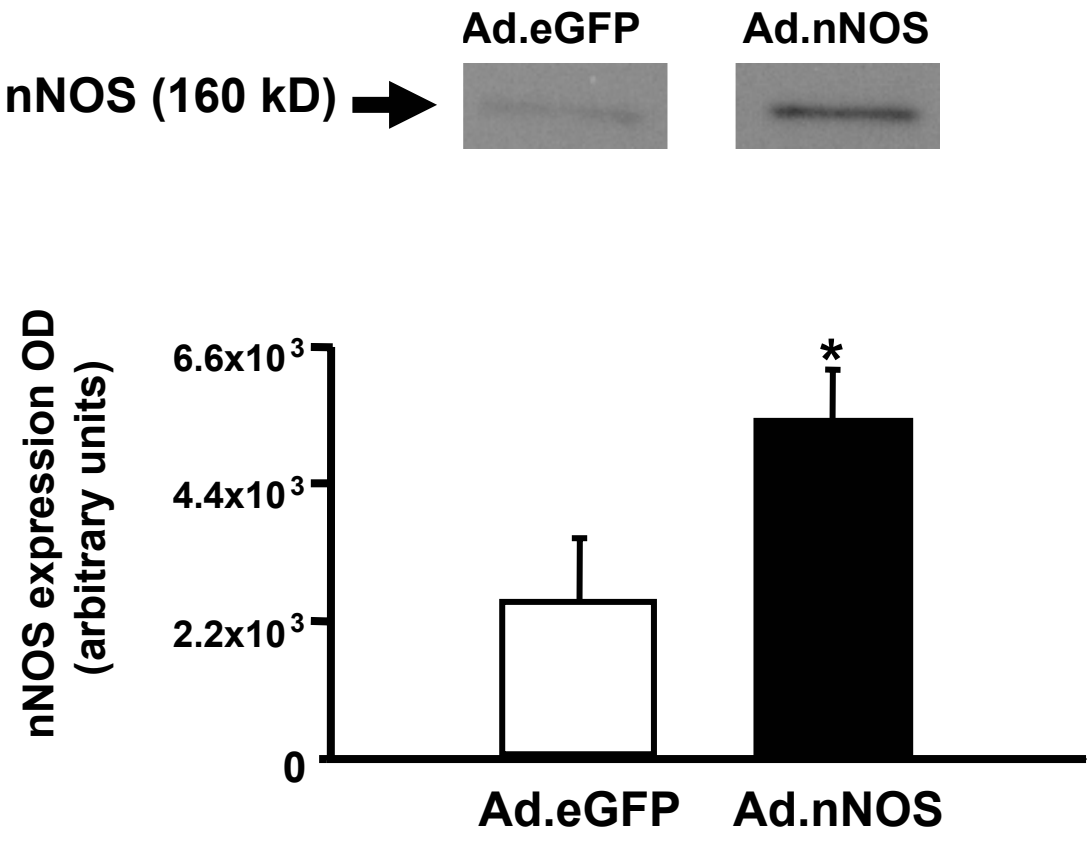


Figure 6a

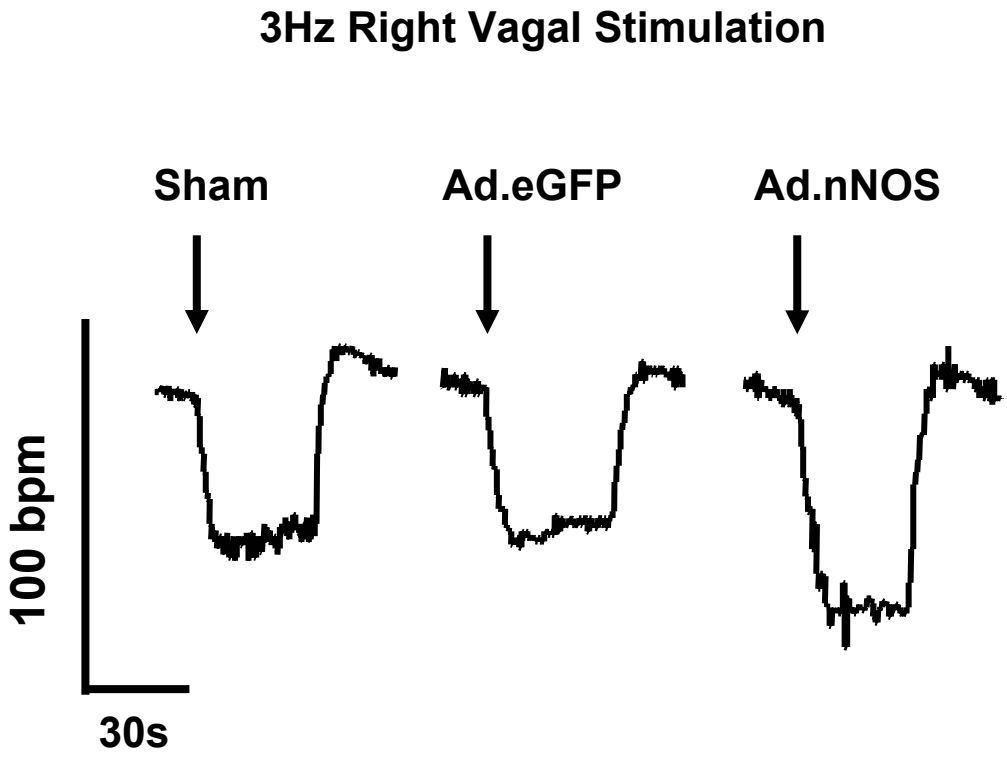


Figure 6b

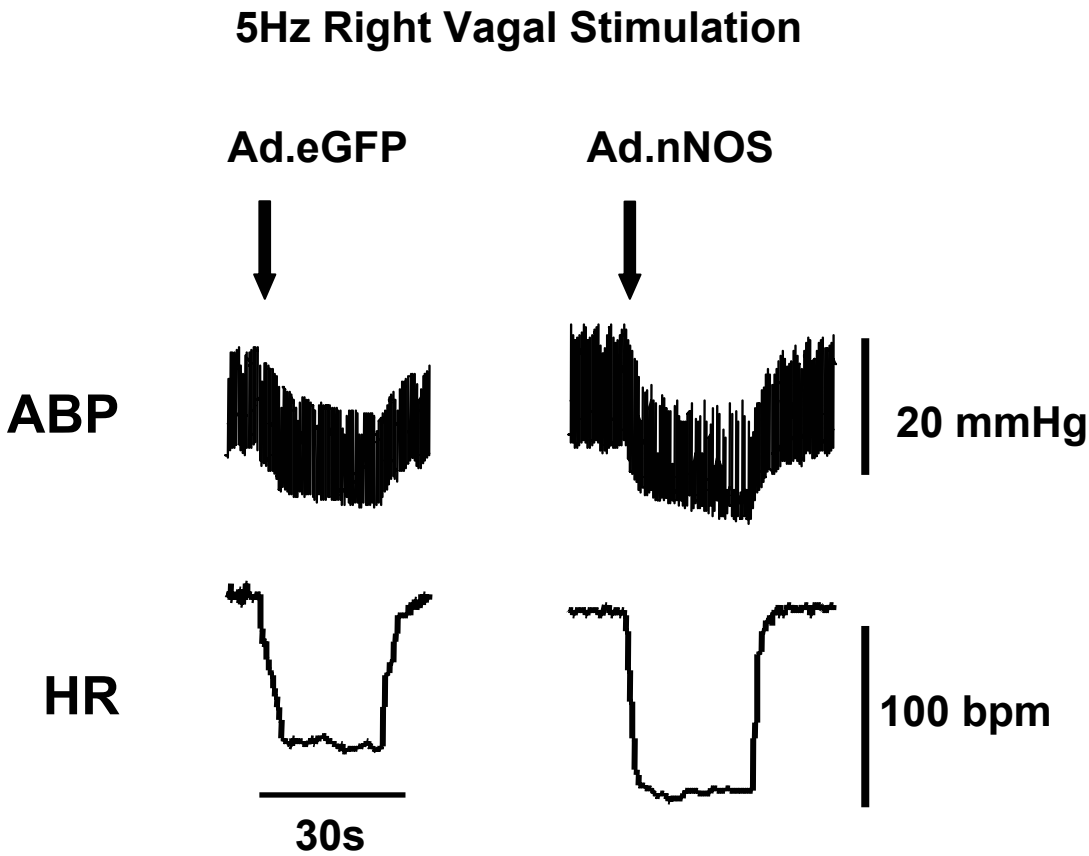


Figure 7

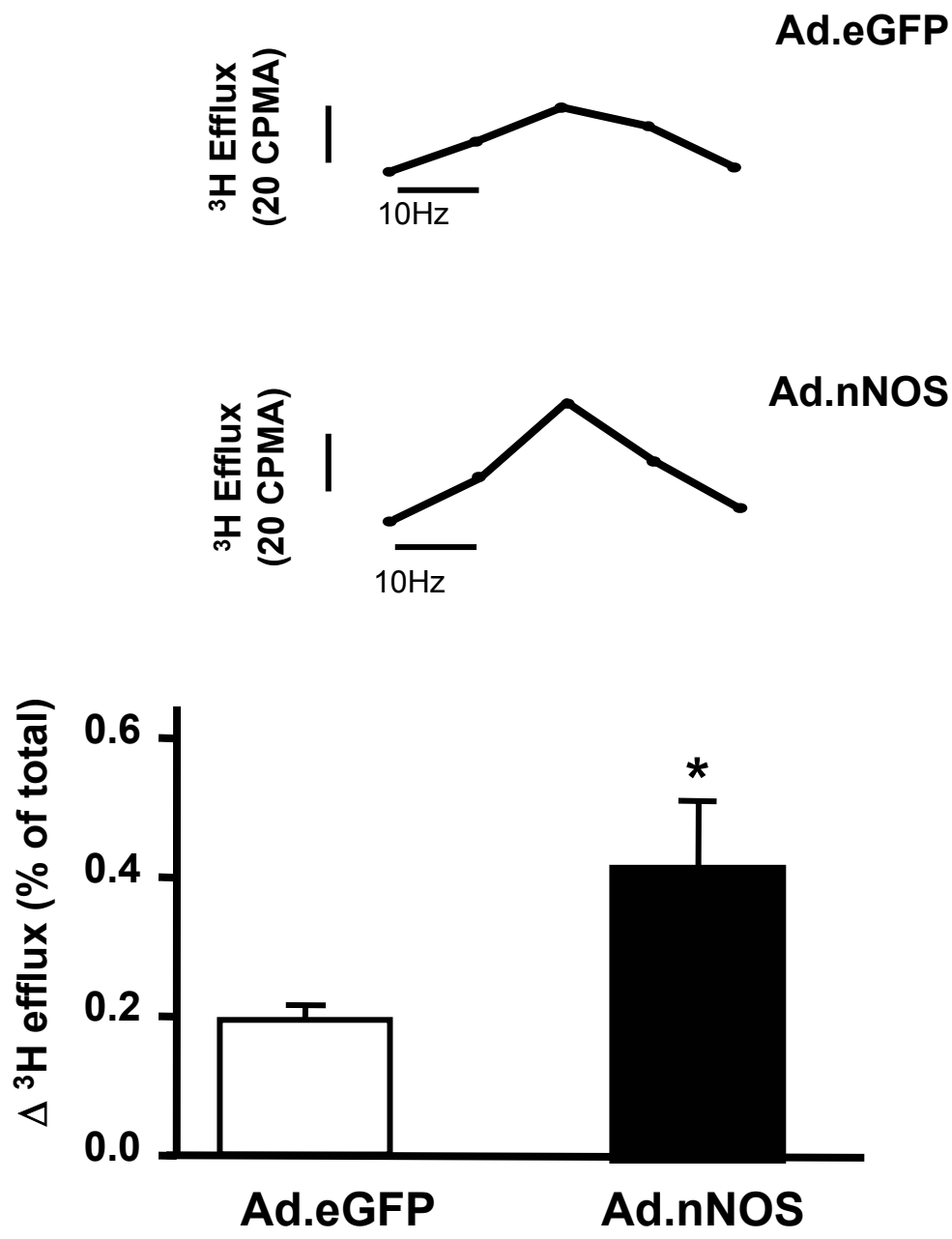

Ad.eGFP
3H Efflux
(20 CPMA)
10Hz
Ad.nNOS
3H Efflux
(20 CPMA)
10Hz
Δ 3H efflux (% of total)
0.6
0.4
0.2
0.0
*
Ad.eGFP
Ad.nNOS

Figure 8

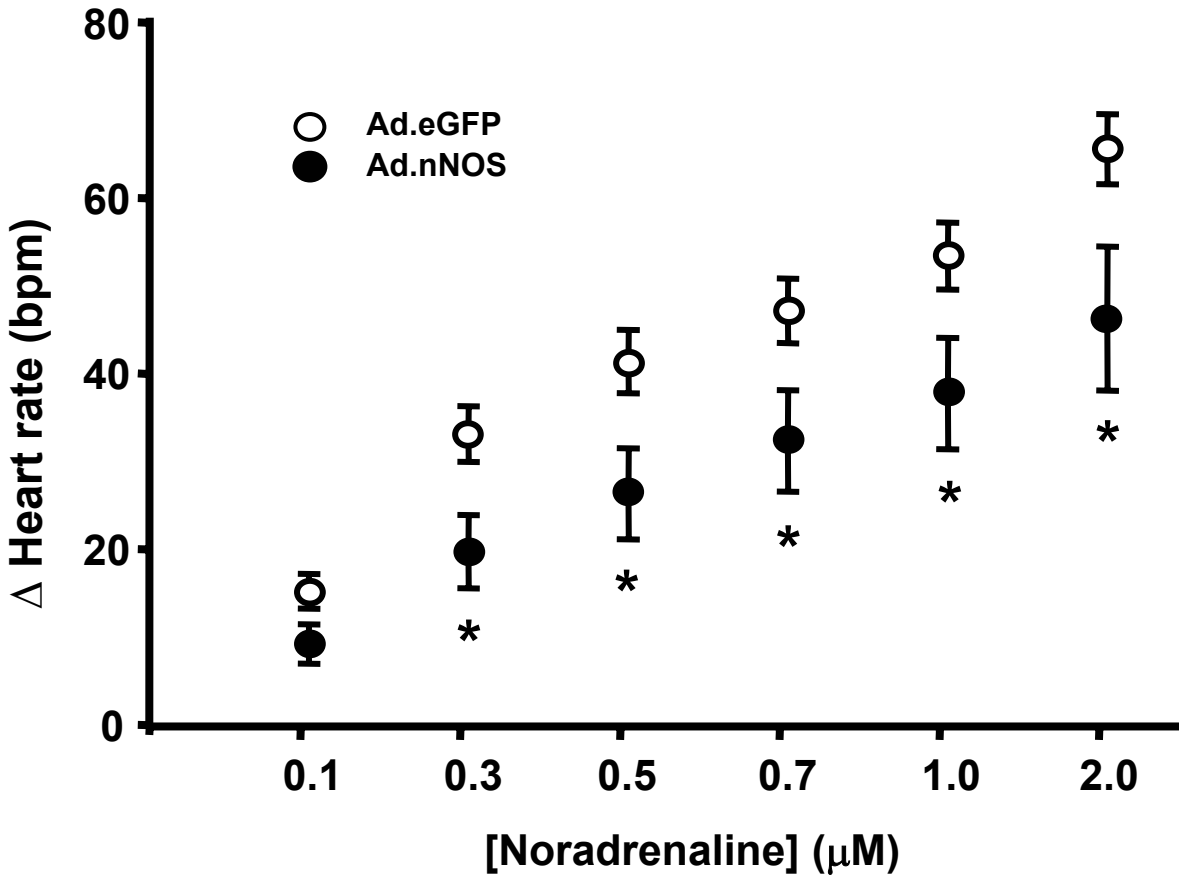
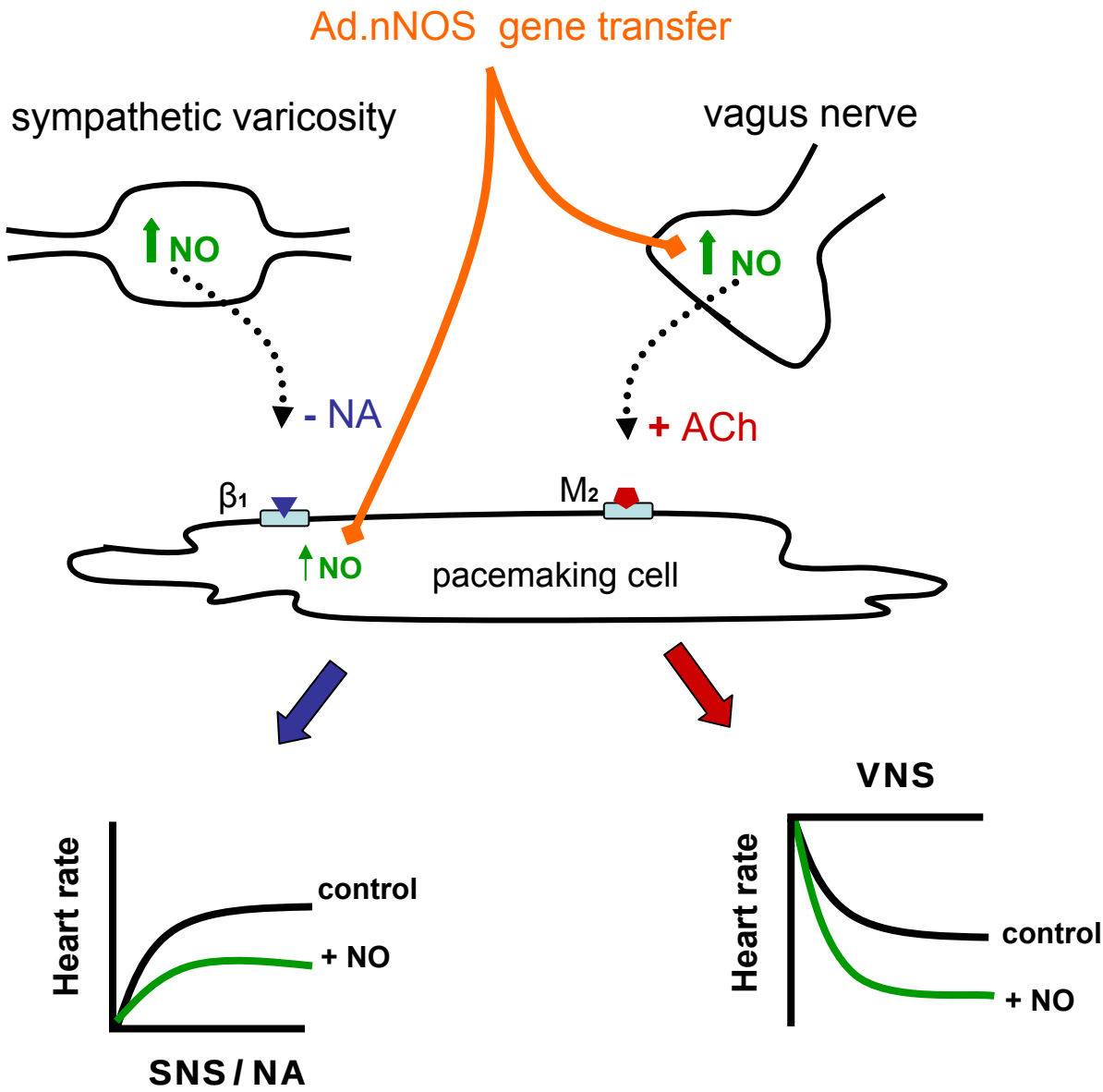

80
60
40
20
0
Δ Heart rate (bpm)
Ad.eGFP
Ad.nNOS
*
*
*
*
*
0.1
0.3
0.5
0.7
1.0
2.0
[Noradrenaline] (μM)

Figure 9


Ad.nNOS gene transfer
sympathetic varicosity
vagus nerve
NO
NO
- NA
+ ACh
β1
M2
NO
pacemaking cell
Heart rate
control
+ NO
SNS / NA
VNS
Heart rate
control
+ NO