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Original article

Targeted nNOS gene transfer into the cardiac vagus rapidly increases parasympathetic function in the pig

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Abstract

Nitric oxide (NO) derived from neuronal nitric oxide synthase (nNOS) facilitates cardiac vagal neurotransmission and bradycardia in vitro. Here we provide evidence of rapid (within 9 h) protein expression and increased vagal responsiveness in vivo following targeted gene transfer of nNOS into the cardiac vagus of the pig. Right vagi were injected with vector encoding nNOS (Ad.nNOS) or saline, while left vagi received an injection of vector encoding enhanced green fluorescent protein (Ad.eGFP). Enhanced nNOS protein expression was detected exclusively in the right vagus nerve, with no evidence of iNOS expression. This was associated with increased baroreflex sensitivity and greater heart rate responsiveness to right vagal stimulation. In contrast, responsiveness of left vagi, or sham-injected right vagi remained constant over the same time period. Basal heart rate was unchanged following gene transfer, suggesting no change in vagal tone. These results support the pre-/post-ganglionic synapse as a site for NO-mediated facilitation of vagal bradycardia in the pig. In addition they demonstrate in vivo that functional gene expression induced with adenoviral vectors occurs earlier than first thought, and may therefore, provide a novel intervention to acutely modulate the neural control of cardiac excitability.

Keywords: Vagus; nNOS; Nitric oxide; Gene transfer; Autonomic

1. Introduction

Increasing evidence supports a role for neuronal nitric oxide synthase (nNOS)-derived nitric oxide (NO) in the facilitation of cardiac cholinergic neurotransmission, both in experimental animals [1,2] and in human subjects [3]. Pharmacological interventions suggest that the effect of NO occurs via production of cGMP and subsequent inhibition of PDE3, leading to greater phosphorylation of the N-type calcium channel and an increase in the release of ACh [4]. However, the site of action has not been firmly established, and may occur at the level of the pre-/post-ganglionic synapse [5] and/or the post-ganglionic neuroeffector junction [6]. We have previously shown that nNOS gene transfer into the right atrium of the guinea pig increased nNOS protein expression within intracardiac cholinergic ganglia after 5 days, and enhanced vagal function [7]. However, percutaneous injection of adenoviral vectors into the right atrium leads to transgene expression in several cell types, making it difficult to uncouple the autocrine and paracrine actions of NO [6]. In this study we sought to evaluate the effect of direct nNOS gene transfer into the surgically-exposed vagal nerves of the pig, thus allowing highly targeted transgene delivery. We tested the hypothesis that targeted gene transfer of the vagus with Ad.nNOS would rapidly increase nNOS protein expression and enhance parasympathetic function in anesthetised pigs.

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2. Materials and methods

Animal procedures were performed in accordance with national and institutional guidelines. Young domestic pigs of either sex (10 males and one female) were used (mean weight 29 ± 2.6 (S.D.) kg; Institute for Animal Health, Compton, UK).

2.1. Anesthesia and surgery

Animals were anesthetised with halothane (Fluothane, Concord Pharmaceuticals Ltd.; 4–5% for induction and 2–3% for maintenance; in 100% O_2) using an Ayre's T piece with a mask and bag. In addition a marginal ear vein was cannulated (Y-CAN, 19G) for administration of supplementary intravenous anesthetic (1:7 pentobarbitone (60 mg/ml): urethane (25% w/v)) as necessary. A tracheostomy was performed and a cuffed endotracheal tube (9 mm, Portex) was inserted into the trachea and secured in place. Following this the cervical vagi were exposed bilaterally, placed over custom-built bipolar stainless steel hook electrodes, and immersed in mineral oil (Sigma). A femoral artery and vein were cannulated (5FG, Portex) for measurement of arterial blood pressure (via a saline-filled pressure transducer; SensoNor 840) and infusion of drugs respectively, and subcutaneous stainless steel needle electrodes were placed for recording of the ECG. Heart rate was triggered from the blood pressure and ECG records and displayed in real time (MP100, Biopac Systems Inc. with Acqknowledge software). Halothane was removed after completion of surgery, and anesthesia was maintained with α-chloralose (Sigma; 100 mg/kg i.v., repeated approximately every 2 h as required).

2.2. Intensive care

Animals were artificially ventilated with a hyperoxic gas mixture (Oxford Mark II ventilator, Penlon), and blood samples were regularly taken into pre-heparinised syringes to measure blood gases, pH, and electrolytes (725 M Blood Gas Analyser, Radiometer Copenhagen). Arterial pH was controlled by adjustment of the frequency of ventilation and/or infusion of 8.4% sodium bicarbonate (in 0.9% NaCl) as appropriate. Body temperature was measured using a rectal temperature probe, and heating lamps were used to maintain this within the range 38 ± 1 °C. In addition a sterile saline drip (Baxter Healthcare Ltd.) was provided via the ear vein cannula for fluid replacement (~100 ml/h). Animals were allowed to stabilise for at least 1 h before control recordings were obtained.

2.3. Adenoviral vectors

Replication deficient adenoviral vectors encoding eGFP or nNOS under the control of the cytomegalovirus (CMV) promoter were prepared on an Ad5 backbone as described previously [8,9]. The virus particle: plaque forming unit (vp/pfu) ratio for both vectors was ~ 83:1.

2.4. Experimental protocol

Electrical stimulation of left and right vagi was performed at 3, 5, 7 and 10 Hz (20 V, 1 ms pulse duration; order of stimulations randomised). Baroreflex activation was performed by injection of phenylephrine (5 μg/kg i.v., Sovereign Medical). Sensitivity of the parasympathetic component of the baroreflex (ms/mmHg) was assessed as the lengthening of R–R interval per unit increase in systolic blood pressure, as described previously [10]. Following completion of control stimulations vagi were injected with 10^{11} particles of Ad.nNOS (right) or Ad.eGFP (left) in 600 μl of phosphate-buffered saline (PBS); sham treated vagi received an injection of an equal volume of PBS alone. Injections were performed (using an insulin syringe with a 30G needle; BD Micro-Fine, BD Consumer Healthcare, UK) at ~6 sites (~100 μl at each site) along the exposed cervical region of the nerve, close to its point of entry into the thorax. Electrical and baroreflex-mediated vagal activations were then repeated at regular intervals, up to a maximum of 9 h post-injection (mean time 7.4 ± 1.1 (S.D.) hours post-injection). For clarity end-point results are referred to as being taken at 7 h.

2.5. Molecular phenotyping

Animals were euthanised by intravenous pentobarbitone overdose (200 mg/kg; Pentoject, Animalcare Ltd.) at the end of the experiment and samples of bilateral vagi, atria, and ventricles were removed and snap-frozen in liquid nitrogen. Immunoblotting for nNOS and eGFP was performed as described previously [6]. In addition blots were probed for iNOS expression using rabbit polyclonal anti-mouse iNOS antisera (Upstate Biotechnologies, UK) that has previously been shown to detect porcine iNOS [11]; a TNFα/γ-IFN stimulated macrophage lysate (BD Biosciences, UK) was used as a positive control for iNOS expression. Blots were stained with MemCode reversible protein stain (Perbio, UK) to ensure equal protein loading.

2.6. Data analysis

Data were subjected to a normality test and analysed with the paired *t*-test or Mann–Whitney Rank Sum test as appropriate, using SigmaStat software (Systat Software Inc.). Statistical significance was accepted at $P < 0.05$. Results are presented as mean ± S.E. unless otherwise stated.

3. Results

3.1. Physiological values over time

Physiological variables were well controlled throughout the duration of the experiments (arterial pH: 7.38 ± 0.02; arterial P_{O2}: 281 ± 14 Torr; arterial P_{CO2}: 43.6 ± 2.5 Torr; bicarbonate: 25.2 ± 0.9 mmol/l; body temperature: 38.9 ± 0.2 °C).

Table 1

	Heart rate (bpm)		Systolic ABP (mmHg)		Diastolic ABP (mmHg)		Core body temp (°C)	
	Control	7 h	Control	7 h	Control	7 h	Control	7 h
Gene transfer ($n = 8$)	117 ± 6	109 ± 7	105 ± 7	104 ± 7	70 ± 6	66 ± 8	39.2 ± 0.3	39.1 ± 0.2
Sham injection ($n = 2$)	124 ± 5	120 ± 20	115 ± 15	95 ± 5	75 ± 15	50 ± 10	38.8 ± 0.7	39.8 ± 0.5

Baseline and end-point values for heart rate, blood pressure and core body temperature in gene transferred and sham pigs. No significant changes in any of these variables were observed over time.

In addition, no significant changes in heart rate, arterial blood pressure, or body temperature were observed over time in either gene transferred or sham-injected pigs (Table 1).

3.2. Molecular phenotype

Gene transfer was performed in eight animals, while two animals received bilateral sham injections (saline only). In addition, vagal biopsies were removed from one untreated animal to assess native nNOS expression (grouped with data from sham-injected vagi in Fig. 1). Expression of nNOS and eGFP protein in tissue samples was measured by western blotting, and the ratio of nNOS expression between left and right vagi was determined in an attempt to normalise inter-animal variations in expression. This ratio was significantly increased from 0.45 ± 0.24 (sham/untreated; $n = 3$) to 3.96 ± 0.74 ($n = 8$) following injection of Ad.nNOS into the right vagus (Fig. 1; $P < 0.05$). In contrast, eGFP was detected exclusively in the Ad.eGFP-injected left vagus (Fig. 1). There was no evidence for increased nNOS expression or expression of eGFP in either the atria or ventricles following vagal gene transfer (data not shown), indicating that gene transfer was localised to pre-ganglionic vagal neurons. Furthermore, we could not detect expression of iNOS in any of the tissue samples studied (Fig. 1).

3.3. Baroreflex sensitivity

The parasympathetic component of the baroreflex was assessed before and after gene transfer or sham injection using an intravenous bolus of phenylephrine that increased peak systolic pressure by 66 ± 9 mmHg (mean ± S.D.; $n = 39$ observations in eight pigs). Under control conditions phenylephrine induced a bradycardia of slope 1.04 ± 0.17 ms/mmHg ($n = 6$); following nNOS gene transfer this was increased to 3.51 ± 0.82 ms/mmHg 7 h post-injection (Fig. 2B; $P < 0.01$, $n = 4$). In two pigs baroreflex responsiveness was lost during the course of the experiment. However, responses to direct electrical stimulation of the vagus were preserved, indicating a failure of the baroreflex pathway (e.g. at the level of the carotid sinus baroreceptors, the carotid sinus nerve, or central integration in autonomic nuclei). In contrast two sham-injected pigs displayed baroreflex slopes of 0.99 ± 0.31 and 1.3 ± 0.4 pre- and 7 h post-injection respectively

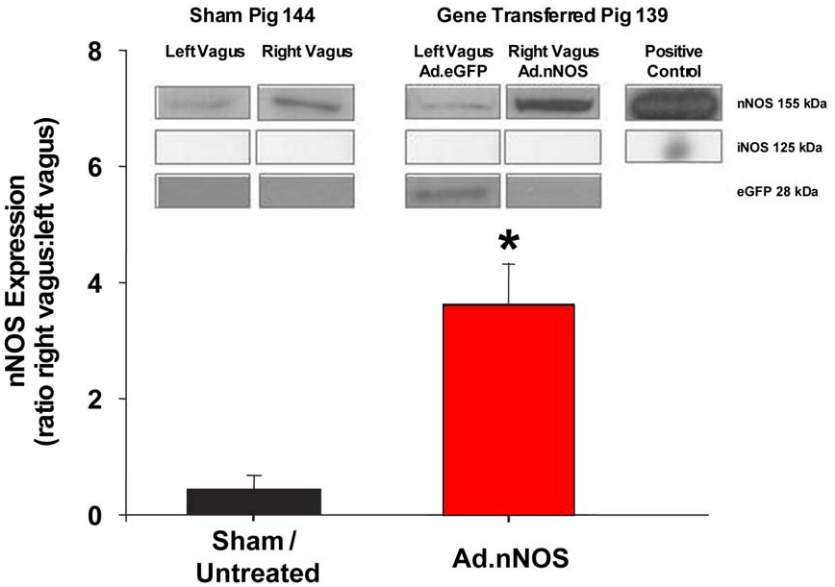

Fig. 1. The ratio of nNOS expression between left and right vagi was significantly increased following right vagal injection with Ad.nNOS ($n = 8$ vs. $n = 3$ sham/untreated; $^*P < 0.05$, Mann–Whitney Rank Sum test). In contrast nNOS expression in the atria was unchanged by vagal gene transfer (data not shown). eGFP was detected exclusively in the Ad.eGFP-injected left vagus. Expression of iNOS was undetectable in all tissue samples studied (positive control shows expression in an activated macrophage lysate). Blots were stained using MemCode reversible protein stain (Perbio) to ensure equal protein loading. Representative blots are shown above the graph.

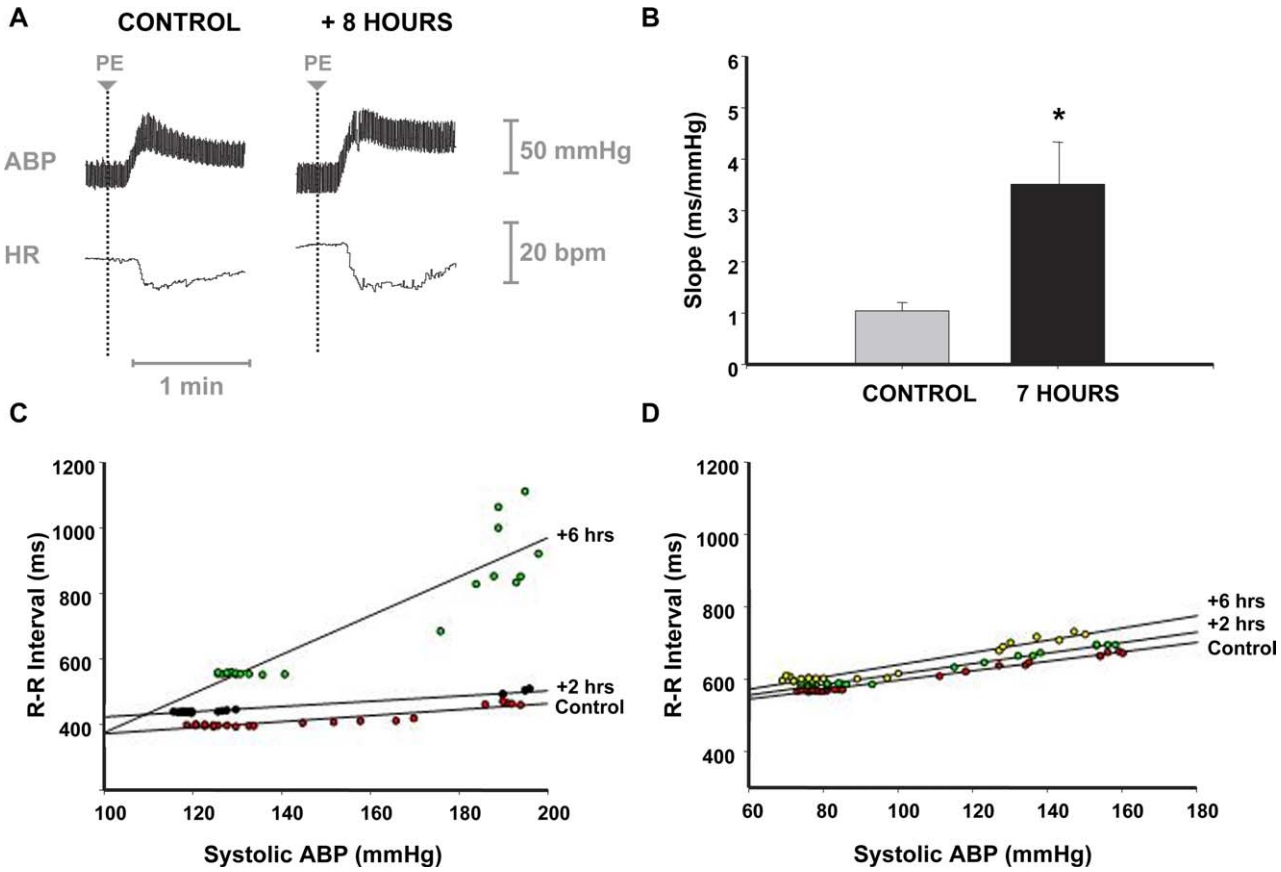

Fig. 2. **A**. Raw data traces obtained from one pig, showing increased baroreflex sensitivity 8 h after injection of the right vagus with Ad.nNOS. **B**. Baroreflex sensitivity was significantly increased following gene transfer of nNOS to the right vagus (*$P < 0.01$, Mann–Whitney Rank Sum test). Graph shows control values for six pigs and values obtained 7 h post-injection for four animals. **C**. Representative results from one animal showing an increase in baroreflex slope after right vagal injection of Ad.nNOS. **D**. Representative results from a sham-injected pig, showing unchanged baroreflex sensitivity over the same time period. The hypertensive response to phenylephrine (PE) remained constant over the time period of the experiments (increase in systolic pressure of 66 ± 4 mmHg ($n = 8$) vs. 70 ± 4 mmHg ($n = 6$) at time zero and 7 h post-injection, respectively). PE-induced bradycardia was abolished following vagotomy (data not shown).

(e.g. Fig. 2D), indicating that the change in baroreflex function observed in gene transferred pigs was not a non-specific response to the injection procedure.

3.4. Responses to vagal stimulation

Direct electrical stimulation of left or right vagi at 3–10 Hz produced a frequency-dependent bradycardia. As expected, responses to right vagal stimulation were greater than those to left vagal stimulation at all frequencies tested, given the dominant right vagal innervation of the sino-atrial node (Fig. 3A, B). Heart rate responses to all frequencies of right vagal stimulation were significantly enhanced 7 h post-transfection with Ad.nNOS (Fig. 3A, $P < 0.01$; $n = 7$). Inhibition of nNOS with Nω-nitro-L-arginine (L-NA; 50 mg/kg i.v.) in two pigs attenuated this enhanced right vagal responsiveness (e.g. 3 Hz: –34 ± 1 (7 h) vs. –22 ± 1 (7 h + L-NA)). In contrast responses to left vagal stimulation remained constant following transfection with Ad.eGFP or sham injection (Fig. 3B; $n = 6$). Furthermore, no increase in right vagal responsiveness was observed following sham injection ($n = 2$; data not shown).

4. Discussion

The results presented here provide novel evidence that adenoviral vectors can induce rapid functional gene expression in vivo, leading to modulation of cardiac autonomic phenotype. We have demonstrated enhanced right vagal function 7 h after gene transfer of nNOS to the right vagus nerve of the pig. Importantly, transgene expression was localised exclusively to the injected nerves, suggesting that increased parasympathetic function can be attributed to an autocrine effect of increased NO bioavailability within pre-ganglionic vagal neurons. Although we were unable to probe for eGFP or nNOS in brainstem vagal nuclei, gene expression must have taken place in the cell nucleus, and therefore, this indicates that the virus was translocated within the neurons. The lack of detectable iNOS protein expression in transfected tissue and stable core body temperature over time suggests that the increase in vagal function is not a consequence of a non-specific inflammatory response to the adenoviral vector.

Augmented vagal function was observed during baroreflex-mediated activation of the parasympathetic nervous system,

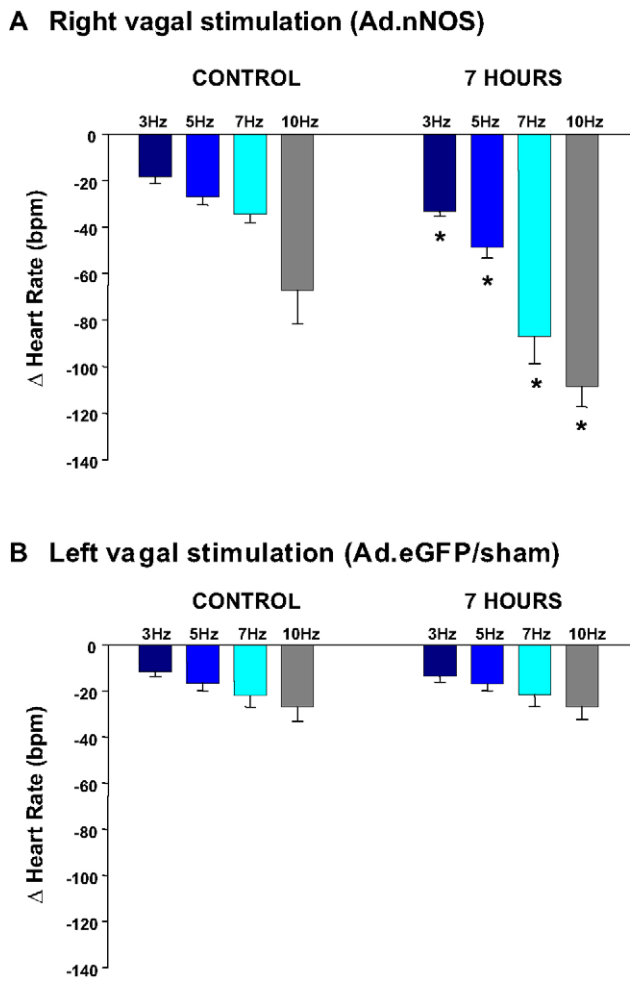

Fig. 3. **A**. Ad.nNOS significantly increased heart rate responsiveness to right vagal stimulation within 7 h (*$P < 0.01$, paired *t*-test; $n = 7$). In contrast, no increase in responsiveness was seen following sham injection ($n = 2$; data not shown). **B**. Left vagal responsiveness was unchanged over the same time period following injection of either Ad.eGFP ($n = 4$) or saline (sham injection; $n = 2$). Graph shows responses of Ad.eGFP and sham treated vagi grouped together ($n = 6$).

indicating that the nNOS transgene can be activated to produce NO under conditions of physiological stimulation. Importantly, this enhancement of baroreflex sensitivity shows that the increase in vagal responsiveness seen during electrical stimulation is due to an enhancement of efferent vagal function, and not due to a block of conduction in vagal afferents. Furthermore, our results suggest that the effect of increased nNOS expression may become most apparent during vagal activation, since there was no significant change in resting heart rate or blood pressure over the time course of the experiments; this confirms our previous observations in the guinea pig [7].

Although the facilitatory effect of nNOS-derived NO on cardiac vagal bradycardia has been broadly established, controversy remains as to its precise site of action. Evidence in the dog suggests that NO may facilitate vagal transmission at the pre-/post-ganglionic junction [5], while previous work in the mouse showed that the bradycardia evoked by nicotinic stimulation of vagal post-ganglionic neurons is sensitive to nNOS inhibition [6]. Data showing nNOS expression in vagal neurons innervating the porcine heart has remained elusive to date, although NOS immunoreactivity in post-ganglionic parasympathetic neurons innervating the submandibular salivary gland is evident [12]. The present study indicates expression of nNOS in vagal pre-ganglionic neurons of the pig under basal conditions (untreated, sham, and Ad.eGFP-injected vagi), and shows that increased pre-ganglionic nNOS expression (following Ad.nNOS injection) can enhance vagal responsiveness. This supports a role for nNOS-derived NO in the facilitation of pre-/post-ganglionic neurotransmission in this species.

4.1. Functional significance of cardiac vagal nNOS

Emerging evidence indicates that up-regulation of cardiac vagal nNOS is essential for improved heart rate recovery following exercise training [13]. This enhanced parasympathetic function is thought to be cardioprotective, since the vagus is able to exert powerful antiarrhythmic effects [14]. Furthermore, recent evidence indicates that chronic vagal stimulation increases survival post-myocardial infarction [15]. In contrast, impaired vagal activity is a strong independent predictor of mortality [16], and is characteristic of a number of states of cardiovascular pathophysiology (e.g. hypertension [17], heart failure [18] and post-myocardial infarction [19]). In a dog model of heart failure vagal impairment occurs at the level of the pre-/post-ganglionic synapse [20]. Whether targeted nNOS gene transfer can rescue this phenotype is not known.

In conclusion, the results presented here show that highly targeted gene transfer of nNOS to pre-ganglionic neurons of the right vagus leads to rapid functional gene expression and gain of function (see summary Fig. 4). Since exercise training is often poorly tolerated by patients with cardiovascular pathology, this intervention could in principle be used to acutely increase vagal function in these patient groups if gene transfer was established as a safe therapeutic methodology [21].

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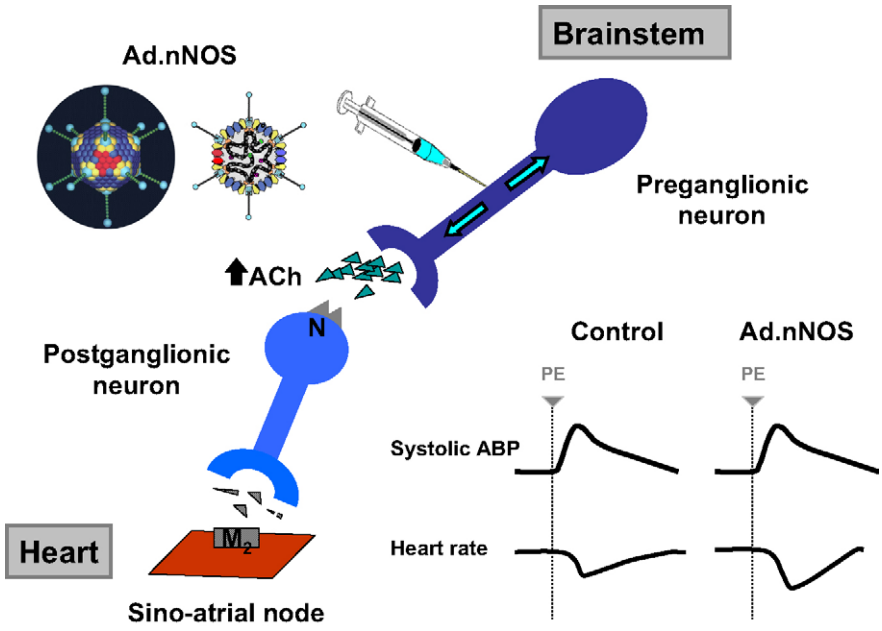

Fig. 4. Summary diagram illustrating gene transfer of nNOS to the vagus nerve of the pig. Increased nNOS expression within the pre-ganglionic vagus enhances baroreflex-induced bradycardia, suggesting enhanced neurotransmission at the pre-/post-ganglionic junction. N represents nicotinic ACh receptors at the ganglionic synapse; M_2 represents muscarinic type 2 receptors at the sino-atrial node. Adenoviral image adapted, by kind permission, from the web page of Professor Urs Greber, Department of Zoology, University of Zurich (http://www.unizh.ch/~cellbio/).

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