Impaired regulation of neuronal nitric oxide synthase and heart rate during exercise in mice lacking one *nNOS* allele

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We tested the hypothesis that a single allele deletion of neuronal nitric oxide synthase (nNOS) would impair the neural control of heart rate following physical training, and that this phenotype could be restored following targeted gene transfer of nNOS. Voluntary wheel-running (+EX) in heterozygous *nNOS* knockout mice ($nNOS^{+/-}$, +EX; $n = 52$; peak performance 9.1 ± 1.8 km day^{-1}) was undertaken and compared to wild-type mice ($n = 38$; 9.5 ± 0.8 km day^{-1}). In anaesthetized wild-type mice, exercise increased phenylephrine-induced bradycardia by 67% (measured as heart rate change, in beats per minute, divided by the change in arterial blood pressure, in mmHg) or pulse interval response to phenylephrine by 52% (measured as interbeat interval change, in milliseconds, divided by the change in blood pressure). Heart rate changes or interbeat interval changes in response to right vagal nerve stimulation were also enhanced by exercise in wild-type atria ($P < 0.05$), whereas both *in vivo* and *in vitro* responses to exercise were absent in $nNOS^{+/-}$ mice. nNOS inhibition attenuated heart rate responses to vagal nerve stimulation in all atria ($P < 0.05$) and normalized the responses in wild-type, +EX with respect to wild-type with no exercise (−EX) atria. Atrial nNOS mRNA and protein were increased in wild-type, +EX compared to wild-type, −EX ($P < 0.05$), although exercise failed to have any effect in $nNOS^{+/-}$ atria. *In vivo* nNOS gene transfer using adenoviruses targeted to atrial ganglia enhanced choline acetyltransferase–nNOS co-localization ($P < 0.05$) and increased phenylephrine-induced bradycardia *in vivo* and heart rate responses to vagal nerve stimulation *in vitro* compared to gene transfer of enhanced green fluorescent protein (eGFP, $P < 0.01$). This difference was abolished by nNOS inhibition ($P < 0.05$). In conclusion, genomic regulation of NO bioavailability from nNOS in cardiac autonomic ganglia in response to training is dependent on both alleles of the gene. Although basal expression of *nNOS* is normal, polymorphisms of nNOS may interfere with neural regulation of heart rate following training. Targeted gene transfer of nNOS can restore this impairment.

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Exercise has been implicated as an important factor in the up-regulation of both endothelial nitric oxide (NO) synthase (eNOS) (Sessa *et al.* 1994) and neuronal NOS (nNOS) (Mohan *et al.* 2000). The molecular mechanisms responsible for this in cardiac tissues are not established but may involve an interplay between haemodynamic elements (Ziegler *et al.* 1998) and oxidative stress (Drummond *et al.* 2000). The physiological significance of enhanced NO bioavailability may also be complex as NOS-containing cellular microdomains may be involved in site-specific and differential actions depending on the isoform relative to its target (Barouch *et al.* 2002; Paton *et al.* 2002). In general, NO derived from eNOS is important in establishing endothelium-dependent vasorelaxation (Huang *et al.* 1995) and myocardial perfusion (Gielen & Hambrecht, 2001), whereas NO derived from increased nNOS expression in intracardiac cholinergic ganglia enhances the release of acetylcholine (Herring & Paterson, 2001) and cardiac vagal responses to direct nerve stimulation following training (Danson & Paterson, 2003). Although the role of peripheral nNOS in cardiac responses to centrally mediated vagal activation remains

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to be demonstrated, this effect could be important in facilitating parasympathetic regulation of heart rate, which dramatically reduces mortality in cardiac disease (Cole *et al.* 1999; Li *et al.* 2004).

Polymorphisms of the nNOS gene are associated with a number of neurological diseases in humans (Levecque *et al.* 2003; Yu *et al.* 2003). However, the way in which these polymorphisms interfere with the regulation of gene expression during exercise, and whether they affect the cardiac vagal response to training are not known. Endothelial NOS (eNOS) gene polymorphisms accelerate coronary artery disease (Rossi *et al.* 2003), and an eNOS knockout mutation carried on one allele abrogates the eNOS training response in vascular endothelium (Kojda *et al.* 2001). Our aims were therefore: (1) to test whether the enhanced peripheral and central vagal control of heart rate were affected by a single nNOS allele knockout mutation; and (2) whether nNOS gene transfer could substitute for the enhanced intracardiac ganglionic nNOS expression seen following training where exercise alone was an ineffective promoter of increased vagal function.

Methods

Animals

Mice homozygous for targeted disruption of the *nNOS* gene (B6,129-*nNOS*^{tm1plh}, *nNOS*$^{-/-}$) were purchased from Jackson Laboratories (Bar Harbour, ME, USA) and a colony was established by backcrossing the *nNOS*$^{-/-}$ on a C57BL/6 background. N4 littermate mice heterozygous for the *nNOS* gene (*nNOS*$^{+/-}$, $n = 98$) were used as test animals whilst homozygous wild-type (WT, $n = 76$) were used as controls. The treatment of all animals was in accordance with the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986* and National Institutes of Health Guide for the Care and Use of Laboratory Animals and experiments were approved by the Physiology and Psychology local Ethics Review Committee, University of Oxford.

Procedures

Training protocol. All mice were housed singly in cages (+EX, $n = 90$; of which 52 were *nNOS*$^{+/-}$ and 38 were WT) and an equal number of each genotype were housed in cages without wheels (−EX, $n = 84$; of which 46 were *nNOS*$^{+/-}$ and 38 were WT). Voluntary running distances covered by +EX mice over a 24 h period for a training period of 5 weeks were measured as previously described (Danson & Paterson, 2003). Ventricular weights were measured post mortem and ventricle : body weight ratios were calculated as an index of training.

Cardiac gene transfer. Whilst experiments were commenced on some mice without gene transfer (WT, −EX, $n = 6$; WT, +EX, $n = 6$; *nNOS*$^{+/-}$, −EX, $n = 10$; *nNOS*$^{+/-}$, +EX, $n = 10$), other mice received percutaneous injections of adenovirus (5×10^9 to 5×10^{10} virus particles of Ad.*eGFP* (WT, −EX, $n = 16$; WT, +EX, $n = 16$; *nNOS*$^{+/-}$, −EX, $n = 18$; *nNOS*$^{+/-}$, +EX, $n = 21$) or Ad.*nNOS* ($n = 71$, same distribution of groups) in 100 μl phosphate-buffered saline (PBS)) directed to the right atrial wall, 5 days before the 5 week training period had elapsed, under halothane anaesthesia.

Replication-deficient adenoviral vectors encoding recombinant enhanced green fluorescent protein (Ad.*eGFP*) or neuronal NOS (Ad.*nNOS*) were generated and purified as previously described (Channon *et al.* 1996). This technique is described fully in Mohan *et al.* (2002).

Experimental protocols

***In vitro* measurement of direct vagal bradycardia.** In order to test the effect of training on vagal bradycardia in WT and *nNOS*$^{+/-}$ mice, preparations of both right and left atria with intact right vagal innervation were isolated *in vitro* from mice from trained and untrained mice of both genotypes without gene transfer (WT, −EX, $n = 6$; WT, +EX, $n = 6$; *nNOS*$^{+/-}$, −EX, $n = 10$; *nNOS*$^{+/-}$, +EX, $n = 10$). Heart rate (HR; computed as spontaneous beating rate in beats per minute or pulse interval duration in milliseconds) responses to vagal nerve stimulation (VNS, 3 and 5 Hz; 10 V, 1 ms pulse interval) or bath-applied carbachol (CCh; Sigma, UK) *in vitro* (10^{-7} and 3×10^{-7} mol l^{-1}) were measured. After control responses were repeated three times and a mean taken, atria from all groups were treated with the NOS inhibitor, vinyl-L-*N*-5-(1-imino-3-butenyl)-L-ornithine (L-VNIO (Amersham Biosciences, Little Chalfont, UK), 10^{-4} mol l^{-1}, equilibration period 20 min) and the protocol was repeated.

***In vivo* measurement of phenylephrine-induced bradycardia.** To test the effect of training on the vagal regulation of HR *in vivo*, the HR response to an I.V. bolus of phenylephrine was assessed in a group of trained and a group of untrained mice of each genotype following gene transfer of eGFP (WT, −EX, $n = 12$; WT, +EX, $n = 6$; *nNOS*$^{+/-}$, −EX, $n = 10$; *nNOS*$^{+/-}$, +EX, $n = 10$). In addition, to test the effect of cardiac gene transfer of nNOS on the vagal regulation of HR *in vivo*, Ad. eGFP

treated tissues were compared with trained mice of each genotype following gene transfer of nNOS (WT, +EX, $n = 6$; $nNOS^{+/-}$, +EX, $n = 10$). Spontaneously breathing mice were anaesthetized with continuous isoflurane (3%) via a nose cone and the abdominal aorta and vena cava were cannulated distal to the renal vessels after pedal reflexes were lost from the animals. Isoflurane was chosen as anaesthetic as it preserves cardiovascular reflexes relatively well. Temperature was measured by means of a rectal thermometer and maintained at $37 \pm 0.5^{\circ}$C using an infrared lamp. HR was triggered from blood pressure pulses in the aorta. A bolus of propranolol (1 mg kg^{-1} I.V.) was given to block the sympathetic component of the baroreflex response, since different mechanisms may be involved in the regulation of sympathetic and parasympathetic limbs of the baroreflex. After a steady-state response to propranolol had been reached, a single bolus of phenylephrine (3 mg kg^{-1} I.V.) was then administered and the peak change in systolic blood pressure, HR and interbeat interval were recorded in order to test the vagally mediated cardioinhibitory limb of the baroreflex response. This was repeated a total of three times after mean arterial blood pressure (MAP) had returned to baseline level. After this, mice were humanely killed by cervical dislocation and atria were removed for *in vitro* experiments. The *in vivo* baroreceptor-mediated effects were expressed as bradycardia (ratio of change in HR to change in MAP; beats min^{-1} mmHg^{-1}) or pulse interval response (ratio of change in interbeat (peak-to-peak) interval to change in MAP; ms mmHg^{-1}).

In order to test the effects of gene transfer on vagal bradycardia *in vitro*, atria from completed *in vivo* experiments and some additional atria from humanely killed mice treated with Ad.*eGFP* (WT, −EX, $n = 4$; WT, +EX, $n = 10$; $nNOS^{+/-}$, −EX, $n = 8$; $nNOS^{+/-}$, +EX, $n = 8$) or Ad.*nNOS* (WT, −EX, $n = 16$; WT, +EX, $n = 10$; $nNOS^{+/-}$, −EX, $n = 18$; $nNOS^{+/-}$, +EX, $n = 8$) were isolated *in vitro* and HR responses to vagal/cholinergic activation were measured as has been described.

Immunohistochemical localization of nNOS in cholinergic ganglia. The remaining mice ($nNOS^{+/-}$, +EX, $n = 6$) were treated with Ad.*eGFP* ($n = 3$) or Ad.*nNOS* ($n = 3$) as has been described, and were terminally anaesthetized (pentobarbitone I.P.) and then fixed by perfusion of the left ventricle with 4% paraformaldehyde–0.1% glutaraldehyde in 0.1 mol l^{-1} PBS (pH 7.1, 20 min). The atria were dissected free and treated for immunohistochemistry as previously described (Wang & Morris, 1996). After processing, the tissue was incubated in primary antisera against nNOS and choline acetyltransferase (ChAT) for 12 h. Tissue was then washed in 1% chicken egg albumin PBS solution and incubated in fluorescently labelled secondary antisera (rhodamine-conjugated anti-goat (1 : 200) and fluorescein-conjugated anti-sheep (1 : 200) antisera. Immunoreactivity was viewed using confocal microscopy (Leica).

Determination of nNOS mRNA levels. Atrial samples were taken after finishing *in vitro* experiments from tissue used for *in vitro* study only and expression of nNOS mRNA was measured using relative quantitative RT-PCR (RQ-RT-PCR) with QuantumRNA 18S rRNA internal standards from Ambion (WT, −EX, $n = 5$; WT, +EX, $n = 5$; $nNOS^{+/-}$, −EX, $n = 6$; $nNOS^{+/-}$, +EX, $n = 6$). Total RNA was purified from atria using TRI Reagent (Sigma), quantified spectrophotometrically and 200 μg reverse transcribed into cDNA using random DNA

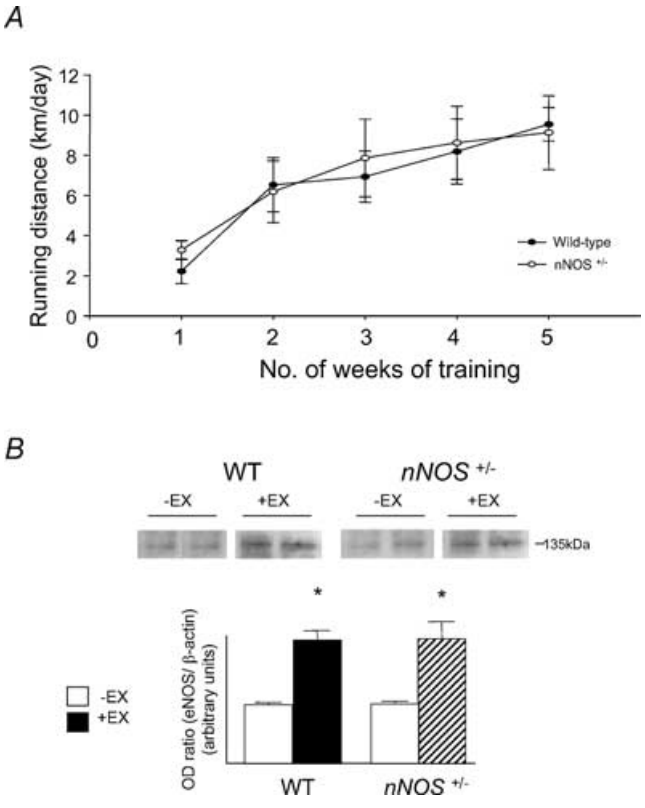

Figure 1. Running distances and aortic eNOS protein levels in mice

A, daily running distances for wild-type (WT) and $nNOS^{+/-}$ mice. *B*, Western Blot analysis showed elevated aortic eNOS protein levels in vessels from WT, +EX compared to WT, −EX and in vessels from $nNOS^{+/-}$, +EX compared to $nNOS^{+/-}$, −EX (*$P < 0.01$, unpaired *t* test).

decamers and the Reverse-IT First Strand Synthesis kit (ABgene). These were then diluted 1/5 v/v with ultrapure water. RT-PCR was performed using 2 μl of each diluted cDNA with SuperTaq reagents (Enzyme Technologies) and the following primers: *nNOS* sense primer: 5′-AGCACCTTTGGCAATGGAGA-3′, *nNOS* antisense primer: 5′-ATCACAGGCTGCCTTGAAGA-3′ and universal 18S primers and competimers (Ambion). The expected amplicon sizes were 402 bp and 315 bp, respectively. Initial experiments were performed to ascertain the linear range of each PCR reaction and the optimal 18S primer : competimer ratio to bring the 18S amplicon to a similar level as the *nNOS* amplicon. This was to ensure that there was no unequal competition for reagents in the multiplex PCR due to the more abundant 18S rRNA target. Reaction products were separated by agarose gel electrophoresis. The optical density of each PCR fragment was established (Syngene Chemigenius Gel Documentation System) and the signal obtained for the *nNOS* amplicon divided by that for the 18S amplicon to give a corrected relative value for the *nNOS* product in each sample. Values were then compared between samples for an estimate of the relative changes in gene expression.

Determination of nNOS and eNOS protein levels. Atrial samples from WT, −EX ($n = 5$), WT, +EX ($n = 5$), $nNOS^{+/-}$, −EX ($n = 6$) and $nNOS^{+/-}$, +EX ($n = 6$), and the same number of samples of the thoracic aorta were also taken at random after completion of the *in vivo*/*in vitro* experiments for measurement of the expression of nNOS and eNOS protein, respectively. Tissue was immersed in iced CelLytic MT lysis buffer (Sigma) containing a 1/40 v/v dilution of a proprietary mixture of mammalian protease inhibitors: 4-(2-aminoethyl)benzenesulphonyl fluoride, bestatin, pepstatinA, E-64, leupeptin and aprotinin (P8340, Sigma). Tissues were homogenized for 30 s in a Polytron homogenizer and homogenates centrifuged for 10 min at 10 000 *g*. Total protein levels were determined by the Bradford method (Bradford, 1976) and 200 μg protein loaded per well. Western blot analysis was performed as previously described (Danson & Paterson, 2003) using commercially available polyclonal antibodies to nNOS, eNOS and β-actin (BD Biosciences) and the Western

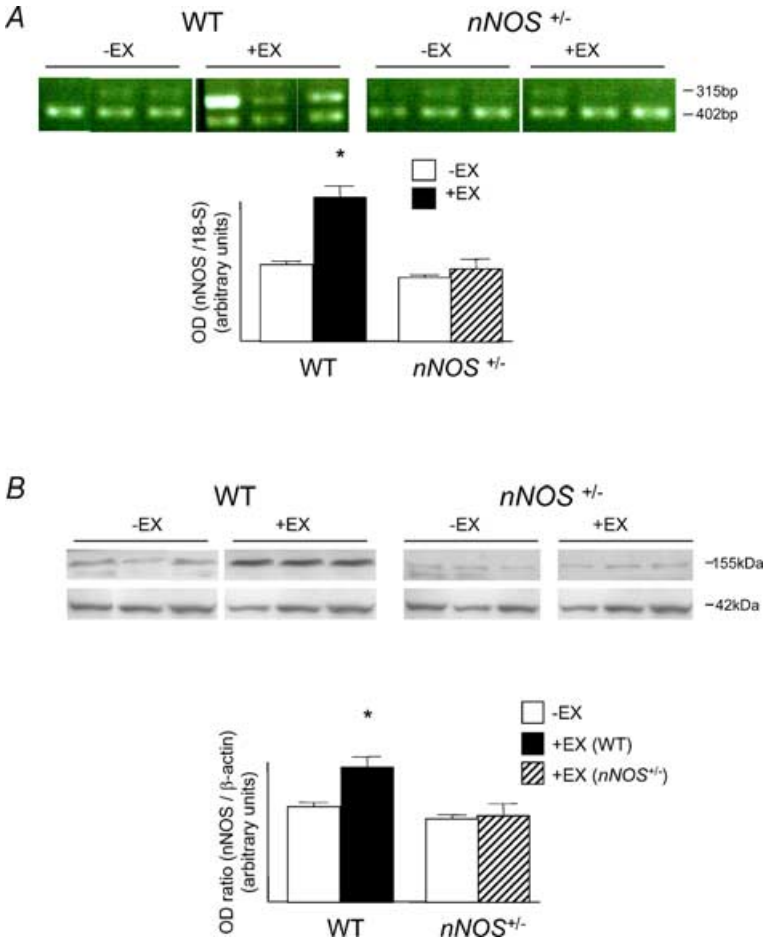

Figure 2. nNOS mRNA and protein levels in atria
A, PCR-amplified nNOS mRNA expression revealed that nNOS mRNA was increased in WT, +EX atria with respect to WT, −EX (*$P < 0.05$, unpaired *t* test), but this effect was not evident in $nNOS^{+/-}$ atria. *B*, Western Blot analysis showed elevated nNOS protein levels in WT, +EX atria compared to WT, −EX (*$P < 0.05$, unpaired *t* test), but no effect in $nNOS^{+/-}$ atria.

Table 1. Heart rate (HR) and mean arterial blood pressure (MAP) changes in response to phenylephrine in anaesthetized mice

	Wild-type			*nNOS*$^{+/-}$		
	−EX	+EX		−EX	+EX	
	Ad.*eGFP*	Ad.*eGFP*	Ad.*nNOS*	Ad.*eGFP*	Ad.*eGFP*	Ad.*nNOS*
Resting HR (beats min^{-1})	686 ± 12	641 ± 17*	650 ± 16	712 ± 11	707 ± 12	650 ± 16**
ΔHR (PRL) (beats min^{-1})	46 ± 3	33 ± 5	35 ± 4	51 ± 3	35 ± 6*	40 ± 2
ΔMAP (Phe) (mmHg)	61 ± 3	53 ± 2	40 ± 1	58 ± 3	47 ± 4	53 ± 3
ΔHR (Phe) (beats min^{-1})	167 ± 7	263 ± 15	268 ± 14	174 ± 18	176 ± 17	260 ± 9
ΔHR/ΔMAP (beats min^{-1} mmHg^{-1})	2.7 ± 0.1	4.5 ± 0.3*	6.7 ± 0.5**	3.0 ± 0.3	3.8 ± 0.3	5.0 ± 0.4**
ΔRR interval/ΔMAP (ms mmHg^{-1})	2.1 ± 0.1	3.2 ± 0.3*	4.5 ± 0.5**	2.2 ± 0.1	2.7 ± 0.2	3.3 ± 0.3**

Cardiac parameters measured via aortic catheter in isoflurane-anaesthetized mice. Phenylephrine (Phe)-induced bradycardia (HR change (beats min^{-1}) or interbeat interval (RR) change (ms)/aortic blood pressure change (mmHg)) was significantly elevated in response to exercise training in wild-type (WT) mice, whereas resting heart rate before β-adrenoceptor blockade (with propranolol PRL) was significantly reduced (*$P < 0.05$, unpaired *t* test). These effects were absent in mice lacking one allele of neuronal nitric oxide synthase (*nNOS*$^{+/-}$). However, gene transfer of nNOS to the atria significantly enhanced phenylephrine-induced bradycardia in WT and *nNOS*$^{+/-}$ mice (**$P < 0.01$, unpaired *t* test).

Lightening detection system (Perkin Elmer Life Sciences). Protein levels were expressed as a ratio of the optical densities of the nNOS/eNOS bands and the β-actin band to control for any inaccuracies in the protein loading.

Results

Effect of *nNOS* allele deletion on voluntary exercise

Average daily running distances of WT and *nNOS*$^{+/-}$ mice are shown in Fig. 1*A*. Both groups of animals demonstrated a similar running performance. Trained animals showed a significantly lower final body weight (BW) and higher ventricle/body weight ratio (Ve/BW) (WT, +EX: BW 35.4 ± 0.4 g, Ve/BW 5.5 ± 0.2 mg g^{-1}; WT, −EX: BW 37.8 ± 0.6 g, Ve/BW 4.9 ± 0.2 mg g^{-1}, $P < 0.01$, unpaired *t* test; *nNOS*$^{+/-}$, +EX: BW 35.2 ± 0.5 g, Ve/BW 5.8 ± 0.3 mg g^{-1}; *nNOS*$^{+/-}$, −EX: BW 37.5 ± 0.9, Ve/BW 4.9 ± 0.2 mg g^{-1}, $P < 0.01$, unpaired *t* test). There were no differences in the effects of exercise on BW or Ve/BW between genotypes.

Exercise training also increased the expression of eNOS protein in the aortas of both WT and *nNOS*$^{+/-}$ mice suggesting that the *nNOS* allele mutation had no effect on the exercise-related induction of *eNOS* gene expression (see Fig. 1*B*).

Effect of exercise on atrial nNOS mRNA and protein levels

RT-PCR showed that although nNOS mRNA levels were unchanged in WT, −EX compared to *nNOS*$^{+/-}$, −EX groups, training significantly enhanced levels in the WT group ($P < 0.05$, unpaired *t* test) but failed to have any effect in the *nNOS*$^{+/-}$ group (see Fig. 2). Western blot appeared to reveal bands at both 155 kDa and 120 kDa in some tissues although we decided not to include the 120 kDa bands in the analysis of nNOS expression since these may represent cross-reactivity of the nNOS antibody with eNOS protein, and were only picked up as faint bands in some samples. Importantly, the 155 kDa bands revealed the same effect of exercise on nNOS protein as was demonstrated by RQ-RT-PCR ($P < 0.05$, unpaired *t* test).

Effect of exercise on phenylephrine-induced bradycardia and vagal bradycardia

In WT mice, phenylephrine-induced bradycardia (in beats min^{-1}) and pulse interval response (in ms) were significantly augmented by exercise training (see Table 1; from 2.1 ± 0.1 to 3.2 ± 0.3 ms mmHg^{-1}, $P < 0.05$, unpaired *t* test). However, this effect was absent in the *nNOS*$^{+/-}$ mice (from 2.2 ± 0.1 to 2.7 ± 0.2 ms mmHg^{-1}, despite there being no difference between animals without training.

Similarly, HR responses to VNS were significantly enhanced in +EX atria from WT mice *in vitro* compared to −EX at both 3 Hz (see Table 2, $P < 0.01$, unpaired *t* test) and 5 Hz stimulation frequencies (see Fig. 3, $P < 0.01$, unpaired *t* test). However, HR responses were not different in *nNOS*$^{+/-}$, +EX atria compared to *nNOS*$^{+/-}$, −EX at either 3 Hz (see Table 2, $P < 0.01$, unpaired *t* test) or 5 Hz (see Fig. 3), despite there being no impairment in *nNOS*$^{+/-}$, −EX responses compared to WT, −EX responses. Statistical differences between pulse rate

Table 2. Effects of nNOS inhibition on HR responses (A, beats min^{-1}) or interbeat interval changes (B, ms) to vagal nerve stimulation in isolated atria *in vitro*

A.	Wild-type						*nNOS*$^{+/-}$					
	−EX			+EX			−EX			+EX		
	Con	Ad.e*GFP*	Ad.*nNOS*	Con	Ad.e*GFP*	Ad.*nNOS*	Con	Ad.e*GFP*	Ad.*nNOS*	Con	Ad.e*GFP*	Ad.*nNOS*
Control												
3 Hz	50	44	123	99	76	126	58	60	139	48	55	137
	± 5	± 4	± 12*	± 11**	± 3**	± 8*	± 8	± 10	± 36*	± 8	± 3	± 10*
5 Hz	89	82	159	112	125	192	86	92	153	85	92	202
	± 6	± 5	± 20*	± 11**	± 8**	± 13*	± 7	± 10	± 35*	± 16	± 3	± 12*
L-VNIO												
3 Hz	44	31	59	53	51	59	51	40	40	39	50	57
	± 5†	± 8†	± 9†	± 17†	± 4†	± 8†	± 7†	± 5†	± 10†	± 7†	± 6†	± 5†
5 Hz	66	53	78	67	81	86	78	84	75	70	76	91
	± 12†	± 17†	± 17†	± 10†	± 4†	± 7†	± 4†	± 11†	± 23†	± 13†	± 5†	± 7†
L-Arg												
3 Hz	48	62	107	96	82	98	55	59	108	53	58	96
	± 3	± 11	± 5*	± 13**	± 11**	± 6*	± 4	± 6	± 24*	± 10†	± 5	± 17*
5 Hz	83	103	140	103	107	132	83	98	116	80	89	147
	± 5	± 10	± 40*	± 8**	± 11**	± 11*	± 7	± 8	± 20*	± 12†	± 4	± 22*
Baseline HR (beats min^{-1})												
	376	371	378	350	349	367	383	398	388	354	379	347
	± 25	± 18	± 29	± 19	± 33	± 30	± 20	± 29	± 25	± 23	± 34	± 32

B.	Wild-type						*nNOS*$^{+/-}$					
	−EX			+EX			−EX			+EX		
	Con	Ad.e*GFP*	Ad.*nNOS*	Con	Ad.e*GFP*	Ad.*nNOS*	Con	Ad.e*GFP*	Ad.*nNOS*	Con	Ad.e*GFP*	Ad.*nNOS*
Control												
3 Hz	34	31	87	69	49	99	28	29	89	27	32	108
	± 2	± 1	± 5*	± 7**	± 3**	± 5*	± 3	± 4	± 16*	± 4	± 2	± 5*
5 Hz	59	54	127	82	97	210	45	49	104	54	60	226
	± 2	± 2	± 9*	± 7**	± 5**	± 8*	± 3	± 4	± 15*	± 9	± 2	± 6*
L-VNIO												
3 Hz	31	24	39	32	31	36	24	18	18	21	28	33
	± 12	± 13	± 13†	± 10†	± 3†	± 5†	± 3	± 2†	± 4†	± 4†	± 3†	± 3†
5 Hz	44	36	51	42	53	57	40	44	38	42	47	59
	± 15	± 17	± 17†	± 6†	± 3†	± 5†	± 1†	± 4	± 10†	± 7†	± 3†	± 4†
L-Arg												
3 Hz	32	41	73	66	54	68	26	28	61	30	34	64
	± 1	± 4	± 2*	± 8**	± 7**	± 4*	± 1	± 2	± 10*	± 5†	± 3	± 9*
5 Hz	55	70	104	73	77	105	43	54	68	50	57	121
	± 2	± 4	± 2*	± 5**	± 7**	± 7*	± 3	± 3	± 8*	± 6†	± 2	± 12*

In vitro atrial beating rate responses to vagal nerve stimulation (VNS) at 3 and 5 Hz stimulation frequencies. Part A shows rate responses calculated as beats per minute change and part B shows responses expressed as change in contraction interval (ms). Control responses were significantly increased in trained (+EX) compared to untrained (−EX) in wild-type tissue (**$P < 0.05$, unpaired *t* test), although this effect was not present in atria from mice lacking one allele of neuronal nitric oxide synthase (*nNOS*$^{+/-}$). Gene transfer using adenoviruses containing *nNOS* (Ad.*nNOS*) increased responses to VNS in all atria compared to gene transfer using adenoviruses containing enhanced green fluorescent protein (Ad.e*GFP*) and control (Con, no gene transfer) atria (*$P < 0.01$, unpaired *t* test). Selective nNOS inhibition with vinyl-L-*N*-5-(1-imino-3-butenyl)-L-ornithine (L-VNIO, 100 μmol l^{-1}) attenuated the response to nerve stimulation in all atria, and abolished the effects of training and Ad.*nNOS* treatment. This effect was reversed in all cases with L-arginine (L-Arg, 1 mmol l^{-1}, †$P < 0.05$, repeated measures ANOVA).

responses to VNS were also apparent between the same comparisons made when data were expressed as pulse interval change.

Responses to CCh were not affected by exercise training or allele ablation (data not shown), although this is not surprising as we have previously shown that *nNOS* gene knockout (Choate *et al.* 2001), *nNOS* gene transfer (Mohan *et al.* 2002) or exercise training (Danson & Paterson, 2003) have no independent effect on the IC_{50} dose of CCh.

The intrinsic rates of atria *in vitro* did not reveal any statistical differences between groups, although there was a trend for training to reduce intrinsic sinus rate in both WT (from 376 ± 25 to 350 ± 19 beats min^{-1}) and *nNOS*$^{+/-}$ atria (from 383 ± 20 to 354 ± 23 beats min^{-1}) (see Table 2).

Effect of nNOS inhibition on vagal HR responses *in vitro*

Selective nNOS inhibition attenuated HR responses to VNS in WT, +EX and *nNOS*$^{+/-}$, +EX atria at 3 Hz (see Table 2), an effect reversed by excess L-arginine ($P < 0.05$, ANOVA with repeated measures). Furthermore, L-VNIO normalized the enhanced vagal responsiveness in WT, +EX atria with respect to *nNOS*$^{+/-}$, +EX atria, suggesting that the enhanced vagal responsiveness in WT *versus nNOS*$^{+/-}$ atria is nNOS dependent.

Immunolocalization of eGFP and nNOS following adenoviral gene transfer nNOS staining in cholinergic ganglia was increased in Ad.*nNOS*-treated atria compared with Ad.*eGFP*-treated atria. In addition, eGFP fluorescence was only evident in atria from Ad.*eGFP*-treated mice (see Fig. 4). nNOS localization in cholinergic ganglia, shown by co-localization of nNOS and ChAT, was also increased in atria from Ad.*nNOS*-treated mice.

Effect of gene transfer on total atrial nNOS protein levels

Western blot analysis showed that our percutaneous adenoviral gene transfer method was effective at increasing total eGFP or nNOS protein expression in atria (see Fig. 5, $P < 0.001$).

Effect of gene transfer on phenylephrine-induced bradycardia and vagal bradycardia

Ad.*nNOS* treatment increased phenylephrine-induced bradycardia in WT and *nNOS*$^{+/-}$ mice compared to Ad.*eGFP*. This effect was statistically significant in both *nNOS*$^{+/-}$, −EX and +EX mice, but only WT, −EX mice (see Fig. 6*A*). The absence of any effect of *nNOS* gene transfer in WT, +EX groups may be related to the fact this group demonstrated enhanced parasympathetic activity and *nNOS* expression compared with all other groups in response to exercise training.

The effect of *nNOS* gene transfer on vagal function *in vitro* mirrored that seen *in vivo* (see Fig. 6*B*). Vagal bradycardia was enhanced in all Ad.*nNOS* groups compared to Ad.*eGFP* except in the *nNOS*$^{+/-}$, −EX group which displayed high variance in responsiveness to vagal stimulation following *nNOS* gene transfer, most likely reflecting variability in the transfection efficiency in this group. In addition, the effects of *nNOS* gene transfer could be blocked by selective nNOS inhibition *in vitro* ($P < 0.05$, ANOVA with repeated measures, see Table 2).

Discussion

Regular aerobic exercise training induces numerous beneficial structural and regulatory adaptations in the cardiovascular system. Neurally mediated adjustments in the heart underlie an important part of this milieu of changes and lead to both sympathoinhibition and enhanced vagal activity (Buch *et al.* 2002). Although sympathoinhibition can largely be attributed to a reduction in circulating catecholamines, desensitization

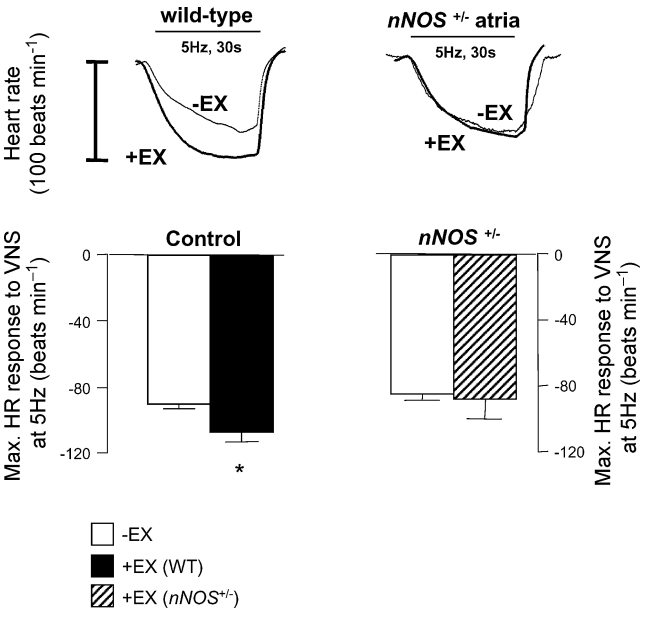

Figure 3. Heart rate responses to vagal stimulation *in vitro*
The decrease in heart rate associated with vagal nerve stimulation (5 Hz) in isolated atria *in vitro* was enhanced in trained (+EX) wild-type atria with respect to untrained (−EX; $^*P < 0.05$, unpaired *t* test). This effect was not evident in *nNOS*$^{+/-}$ atria.

and down-regulation of cardiac β-adrenergic receptors and increased central blood volume load on central cardiopulmonary baroreceptors (Shi *et al.* 1996), the mechanisms leading to enhanced vagal responsiveness are less well defined. Wheel-running activity in mice can significantly enhance the action of the parasympathetic nervous system on the heart (i.e. the decrease in beats per minute or increase in interbeat interval in milliseconds) by direct electrical stimulation of the vagus nerve *in vitro*, an effect that is blocked by NOS inhibition (Danson & Paterson, 2003). Given that this response is seen through vagal activation at the level of the vagal trunk suggests that an important response to training is peripheral neuronal modulation of cardiac excitability by NO. Here we provide direct evidence that this effect can be seen through central activation of the vagus, and that enhanced vagal responsiveness *in vivo* and *in vitro* brought about by physical training requires the presence of two intact alleles of *nNOS*. Furthermore, dysfunctional regulation of the *nNOS* gene in heterozygous mice can be overcome by *nNOS* gene transfer to intracardiac ganglia which restores vagal responsiveness and the autonomic phenotype, seen after exercise training.

Although the main limitation of our adenoviral atrial gene transfer technique over exercise training continues to be the lack of specificity in cell transfection and marginally more variable protein expression, our data are consistent with the responses seen following exercise training. In addition we show that *nNOS* gene transfer increases baroreflex-mediated parasympathetic bradycardia or pulse interval response in $nNOS^{+/-}$ mice. This appears to be mediated predominantly by increased NO bioavailability at intracardiac cholinergic ganglia, since bradycardia in response to VNS *in vitro* is enhanced in an NO-dependent manner. Furthermore, the margin of increase in vagal responsiveness resulting from *nNOS* gene transfer is greater in our *in vitro* preparations than *in vivo*. This is particularly striking in certain groups, e.g. in the WT, +EX group (see Fig. 6), suggesting that the central nervous system may, under certain circumstances, actually limit peripheral vagal facilitation by NO. This idea would be consistent with data showing NO-dependent inhibition of baroreflex vagal outflow in the nucleus tractus solitarii (Paton *et al.* 2001). Another possibility is that NO acts in a biphasic concentration-dependent manner in modulating cardiac parasympathetic signalling, and high levels of neuronal NO (from augmented nNOS activity resulting from the combined effects of training and gene transfer in WT mice) may begin to inhibit vagal control of heart rate.

It has been suggested that both eNOS (within endothelial cells and cardiomyocytes) and nNOS protein

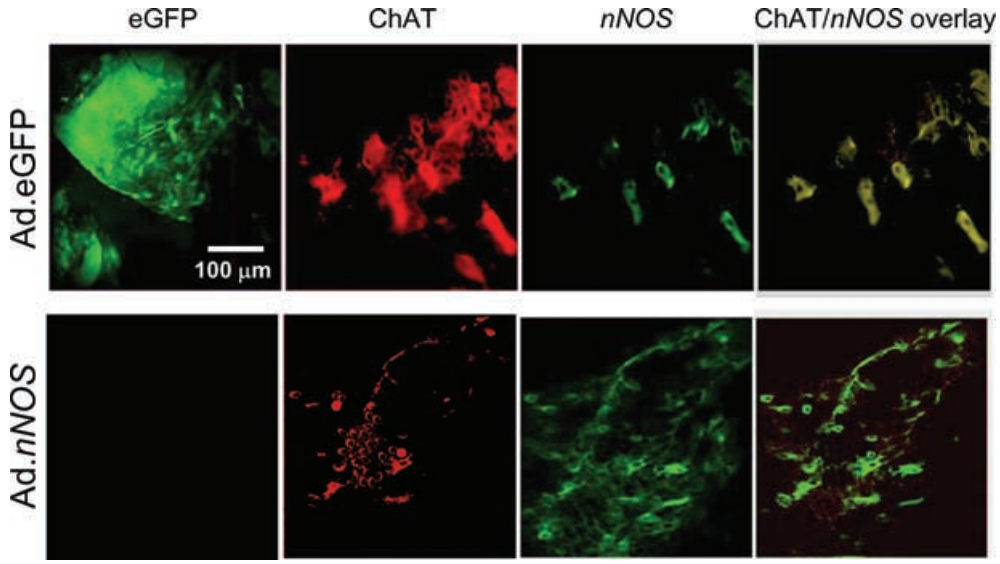

Figure 4. Immunohistochemical localization of nNOS in cholinergic ganglia
Confocal micrograph illustrates enhanced green fluorescence (far left, green panels) immunoreactivity against choline acetyltransferase (ChAT, middle, red panels) and nNOS (far right, green panels) in atria from $nNOS^{+/-}$, +EX mice pretreated with adenoviruses containing either *eGFP* (top row) or *nNOS* genes (bottom row). The panels show the presence of enhanced green fluorescence located with ChAT-positive cholinergic ganglia in an Ad.*eGFP* specimen with some neurones coexpressing nNOS. In the Ad.*nNOS* specimen, enhanced green fluorescence is not present, and coexpression of nNOS in cholinergic neurones is increased with respect to the Ad.*eGFP* group.

(from intracardiac neurones) may play important roles in establishing the peripheral neuronal response to exercise as the expression of both isoforms increases following training (Sessa *et al.* 1994; Mohan *et al.* 2000). However, the putatively selective nNOS inhibitor L-VNIO can normalize the enhanced vagal response *in vitro* suggesting that NO bioavailability at the level of ganglia is important in bringing about the vagal response to training (Danson & Paterson, 2003). In addition, although *eNOS* gene knockout results in mild bradycardia *in vivo* (Kojda *et al.* 1999), these effects are thought to result entirely from hypertension and baroreflex cardioinhibition since this effect has not been consistently demonstrated in normotensive animals or in isolated hearts. Furthermore, non-selective NOS inhibition has no effect on the heart rate response to carbachol (Sears *et al.* 1998), suggesting that there is little or no action of NO on heart rate at sinoatrial node myocytes. When *eNOS* is overexpressed in cardiomyocytes (40–90 times normal rate) – albeit without any direct evidence of changes in expression in sinoatrial node myocytes – there is also no effect on heart rate (Brunner *et al.* 2001). Conversely, increases in nNOS mRNA or protein in WT relative to $nNOS^{+/-}$ mice were associated with significant increases in vagal bradycardia *in vivo* and *in vitro*. Although we cannot conclusively rule out that nNOS expression may have been influenced over the course of the *in vitro* protocol, relative changes in protein and mRNA levels from this preparation were similar to those reported from freshly isolated tissue. We believe that the major source of this nNOS is from cholinergic ganglia, and evidence suggests that NO derived from nNOS can modulate neurotransmission at the pre-postganglionic synapse (Markos *et al.* 2002), and pre-synaptically at the postganglionic neuroeffector junction (Mohan *et al.* 2004). In contrast, there is no compelling evidence that nNOS is expressed at significant levels in sinoatrial node myocytes; however, it is now widely recognized that nNOS derived from ventricular myocytes may be involved the regulation of contractility (Ashley *et al.* 2002), highlighting the site-specific action of this isoform.

Although evidence for the shared functionality of eNOS and nNOS in the regulation of heart rate may be weak, there may be important similarities in the factors responsible for triggering an increase in gene expression during exercise. Despite extensive knowledge about the promoters of *eNOS* expression during exercise, those for the *nNOS* gene are not as well documented. Unlike *eNOS* which responds to vascular shear during exercise (Ziegler *et al.* 1998), direct evidence for mechanical stimuli in the regulation of *nNOS* during exercise has not been established. The global perfusion with chemical stimuli such as H_2O_2 regulates *eNOS* in cultured endothelial cells (Drummond *et al.* 2000). Likewise, indirect evidence suggests that *nNOS* responds to the increased oxidative stress experienced during myocardial infarction (Takimoto *et al.* 2002) and in hypertension (Piech *et al.* 2003). This implicates a role for free radical production in the regulation of *nNOS* which takes place during exercise (Ji *et al.* 1998). In addition, there is evidence that the reduction in plasma angiotensin

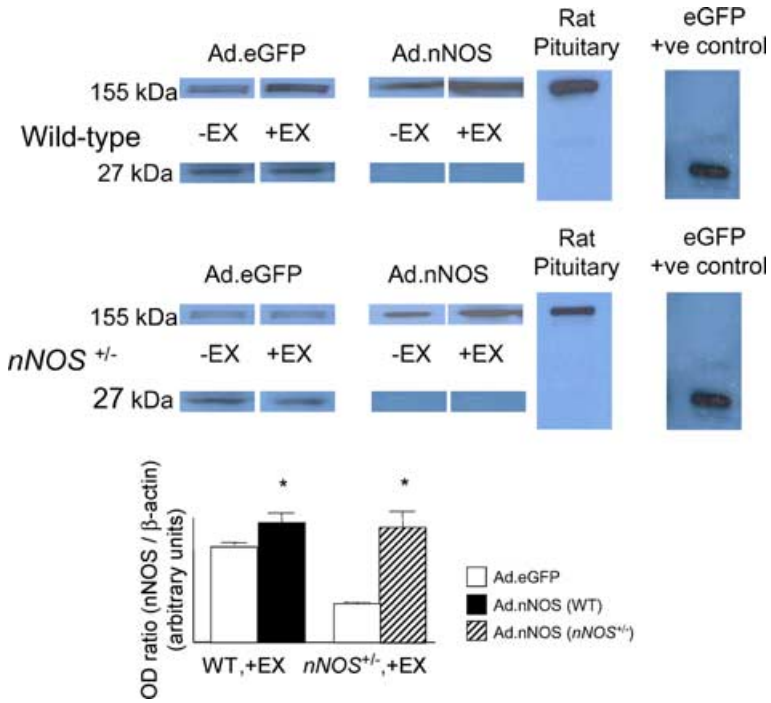

Figure 5. nNOS and eGFP protein levels in adenovirus-transfected atria
Western Blot analysis showed expression of nNOS (155 kDa) and eGFP (27 kDa) in wild-type, −EX and +EX; and $nNOS^{+/-}$, −EX and +EX atria treated with Ad.*nNOS* or Ad.*eGFP* adenovirus. Ad.*eGFP* treatment was successful in causing atria to express eGFP protein. Ad.*nNOS* significantly increased atrial expression of nNOS protein in all groups (*$P < 0.001$, unpaired *t* test).

II associated with exercise training (Kiyonaga *et al.* 1985) may be coupled to the NO-dependent actions on the autonomic nervous system (Zucker *et al.* 2001). Increases in angiotensin II result in both hypertension and interruption of the trafficking of eNOS in vascular myocytes (Gerzanich *et al.* 2003) indicating a strong regulatory link between these two signalling pathways.

The resemblance in the dysfunctional regulation of *eNOS* and *nNOS* in the absence of one allele also suggests that there may be involvement of similar allele silencing and recruitment mechanisms during rest and exercise. The heterozygous *eNOS* knockout mouse shows unperturbed regulation of blood pressure (Kurihara *et al.* 1998) and vascular reactivity compared to its wild-type control and exercise capacities also appear to be identical during the first 3 weeks of training (Kojda *et al.* 2001). However, the physiological responses to exercise training brought about by augmented NO production appear ablated in both $eNOS^{+/-}$ and $nNOS^{+/-}$ mice. In a similar study, Kojda *et al.* (2001) elegantly described the ablated response to exercise in aorta and coronary vessels from the $eNOS^{+/-}$ mouse compared to its wild-type control, despite there being no difference in either vascular reactivity to ACh or basal eNOS expression before exercise. Although underscoring the importance of cardiac NO in preserving homeostasis during stress, this suggests that the functional role played by one *NOS* allele whilst the animal remains relatively unstressed may be minimal or redundant.

Studies using the homozygous *NOS* gene knockout mouse have proved useful in delineating the roles of separate isoforms in the heart, particularly with reference to the microdomain-specific mechanism of actions of NOS in cardiomyocytes (Barouch *et al.* 2002). However, the effects of *NOS* knockout on cardiac homeostasis associated with age or stress in these animals may be ultimately more interesting. The absence of dramatic differences observed between *NOS* homozygous – and even more commonly heterozygous – gene knockouts and wild-type mice may also be because the expression of *NOS* is low in certain tissues before stress (Kurihara *et al.* 1998), or that the mutant models commonly develop compensatory mechanisms which mask the effects of gene ablation (Huang *et al.* 2001).

In this respect, *NOS* gene polymorphisms may have very little effect in the young, unstressed heart, but cardiac autonomic signalling may be regulated very differently by physiological stresses such as exercise or by ageing. At present, *eNOS* polymorphisms identified in humans have been implicated in the impairment of endothelium-dependent vasodilatation in coronary artery disease (CAD) patients and may have an adverse effect on training-induced improvement in endothelial function (Erbs *et al.* 2003). Despite extensive investigation into *eNOS* polymorphisms with often striking results, research into polymorphisms of *nNOS* and the consequences of being a carrier of these has received relatively little attention. Recent investigations have found associations between hypertropic pyloric stenosis (Saur *et al.* 2004), depression (Yu *et al.* 2003), Parkinson's disease (Levecque *et al.* 2003) and acute chest syndrome in sickle cell patients (Sullivan *et al.* 2001). Since we know that nNOS plays a central role in cardiac parasympathetic neural modulation, it is conceivable that polymorphisms of the *nNOS* gene are associated with reduced parasympathetic activity. Impaired vagal function predisposes mice to arrhythmia (Cogliati *et al.* 2002) and is strongly correlated with mortality in humans (Cole *et al.* 1999). It remains to be established whether *nNOS* gene mutations and

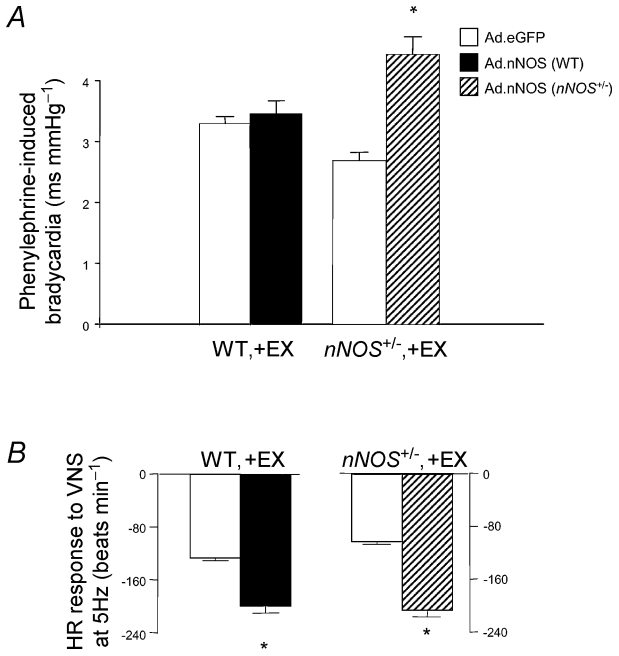

Figure 6. Effects of *nNOS* gene transfer on *in vivo* phenylephrine-induced bradycardia and *in vitro* vagal-induced bradycardia

A, phenylephrine-induced bradycardia, expressed as change in interpulse interval (ms) per change in mean arterial blood pressure (mmHg), in response to pressor challenge was increased in anaesthetized $nNOS^{+/-}$, +EX mice treated with Ad.*nNOS* compared to treatment with Ad.*eGFP* adenovirus (*$P < 0.05$, unpaired *t* test). Ad.*nNOS* treatment failed to have a significant effect on phenylephrine-induced bradycardia in WT, +EX mice, although Ad.*nNOS* increased bradycardia in WT, −EX mice by 64% compared to Ad.*eGFP* ($P < 0.05$, unpaired *t* test; data not shown). *B*, bradycardia to vagal nerve stimulation (5 Hz) in isolated atria *in vitro* was enhanced in $nNOS^{+/-}$, +EX mice treated with Ad.*nNOS* compared to treatment with Ad.*eGFP* adenovirus (*$P < 0.01$, unpaired *t* test).

polymorphisms interfere with *nNOS* regulation during exercise, since this could affect cardiac rehabilitation programmes that employ training.

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