Cardioprotection evoked by remote ischaemic preconditioning is critically dependent on the activity of vagal preganglionic neurones

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Abstract

Aims. Innate mechanisms of inter-organ protection underlie the phenomenon of remote ischaemic preconditioning (RPc) in which episode(s) of ischaemia and reperfusion in tissues remote from the heart reduce myocardial ischaemia/reperfusion injury. The uncertainty surrounding the mechanism(s) underlying RPc centers on whether humoral factor(s) produced during ischaemia/reperfusion of remote tissue and released into the systemic circulation mediate RPc, or whether a neural signal is required. Whilst these two hypotheses may not be incompatible, one approach to clarify the potential role of a neural pathway requires targeted disruption or activation of discrete central nervous substrate(s).

Methods and Results. Using a rat model of myocardial ischaemia/reperfusion injury in combination with viral gene transfer, pharmaco- and optogenetics, we tested the hypothesis that RPc cardioprotection depends on the activity of vagal preganglionic neurones and consequently an intact parasympathetic drive. For cell-specific silencing or activation, neurones of the brainstem dorsal motor nucleus of the vagus nerve (DVMN) were targeted using viral vectors to express a Drosophila allatostatin receptor (AlstR) or light-sensitive fast channelrhodopsin variant (ChIEF), respectively. RPc cardioprotection, elicited by ischaemia/reperfusion of the limbs, was abolished when DVMN neurones transduced to express AlstR were silenced by selective ligand allatostatin or in conditions of systemic muscarinic receptor blockade with atropine. In the absence of remote ischaemia/reperfusion, optogenetic activation of DVMN neurones transduced to express ChIEF reduced infarct size, mimicking the effect of RPc.

Conclusions. These data indicate a crucial dependence of RPc cardioprotection against ischaemia/reperfusion injury upon the activity of a distinct population of vagal preganglionic neurones.
Introduction

Our body is capable of recruiting powerful innate mechanisms which are highly effective in protecting tissues and organ function against ischaemia/reperfusion injury. A landmark study by Murry et al. (1986)\(^1\) demonstrated that exposure of the myocardium to short non-lethal ischaemia-reperfusion episodes, renders it more tolerant to subsequent severe, potentially lethal, periods of ischaemia; i.e., the heart becomes "preconditioned". A similar level of protection can be achieved by remote ischaemic preconditioning (RPc) - a phenomenon in which episode(s) of ischaemia and reperfusion in tissues remote from the heart protects the myocardium against ischaemia/reperfusion injury. Although, promising results of recent trials in patients with acute myocardial infarction\(^2\) may facilitate the introduction of RPc procedure(s) into clinical practice, the mechanisms underlying RPc cardioprotection remain unclear.

The importance of the autonomic nervous system in mediating myocardial protection against ischaemia/reperfusion injury has been suggested by the demonstration that RPc is abolished under ganglionic blockade following systemic administration of hexamethonium\(^3\), which blocks transmission in both sympathetic and parasympathetic ganglia. Vagus nerve stimulation reduces myocardial injury\(^4-6\), while remote preconditioning cardioprotection was found to be abolished in conditions of bilateral vagotomy\(^7, 8\). These data suggest that an intact parasympathetic activity is important for RPc cardioprotection. However, the vagus is a mixed nerve containing both sensory and motor fibers. Therefore, to avoid confounding factors associated with complete surgical vagotony or electrical stimulation of the whole nerve, selective vagal “de-efferentation” or selective recruitment of vagal motor outflow require cell-specific targeting of vagal preganglionic neurones. In this study we used pharmaco- and optogenetic approaches to
test the hypothesis that the functional integrity of the central nervous parasympathetic circuitry is required for myocardial protection established by ischaemia/reperfusion stimulus applied to a remote tissue.

Methods

All the experiments were performed in accordance with the European Commission Directive 86/609/EEC (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes) and the UK Home Office (Scientific Procedures) Act (1986) with project approval from the Institutional Animal Care and Use Committee.

Animal preparation

Adult male Sprague-Dawley rats (280-340 g) were anaesthetised with pentobarbitone sodium (induction 60 mg kg\(^{-1}\) i.p.; maintenance 10 mg kg\(^{-1}\) h\(^{-1}\) i.v.). Adequate anaesthesia was ensured by monitoring heart rate, arterial blood pressure and the absence of a withdrawal response to a paw pinch. Animals were placed supine and a right carotid artery and left jugular vein cannulated for measurement of arterial blood pressure and administration of anaesthetic, respectively. The trachea was cannulated and animals were ventilated with room air using a positive pressure ventilator (Harvard Rodent Ventilator). A tidal volume of ~8-10 ml kg\(^{-1}\) and a ventilator frequency similar to the normal respiratory frequency (~60 strokes min\(^{-1}\)) were used. Partial pressures of O\(_2\) and CO\(_2\) as well as pH of the arterial blood were measured every hour. The rate and volume of mechanical ventilation were adjusted to maintain blood gases within the physiological range. Standard
lead II ECG was recorded throughout the experiment. Body temperature was maintained with a servo-controlled heating pad at 37.0±0.2°C.

*Induction of RPC*

To establish RPC cardioprotection the protocol described by Shahid et al (2008) was used. Blood supply to the limbs was interrupted for 15 min by placing vessel clamps on both femoral arteries at the proximal level ~1 cm below the inguinal ligament. Sham-RPC procedure involved dissection of both femoral arteries without occlusion.

*Myocardial ischaemia/reperfusion*

The heart was exposed via a left thoracotomy. A 5-0 monofilament polypropylene suture was passed around the left anterior descending (LAD) coronary artery to induce a temporary occlusion. The animals were subjected to 30 min of LAD coronary artery ligation, followed by 120 min of reperfusion. Successful coronary artery occlusion was confirmed by elevation of the ST segment in the ECG and an immediate 15–30 mmHg fall in BP.

*Measurements of infarct size*

At the end of the reperfusion period, the LAD artery was re-occluded and 1 ml of 1.5% Evans Blue dye was injected into the jugular vein for assessment of the area at risk. The animal was then given an anaesthetic overdose (pentobarbital 250 mg kg⁻¹ i.v.), the heart was excised, left ventricle was isolated, frozen and sectioned into 5-6 transverse slices from the apex to the base. The slices were weighed and photographed. The area at risk
was demarcated by the absence of Evans Blue staining. The slices were then incubated with 1% 2,3,5-triphenyltetrasodium chloride (TTC) in Tris buffer (pH 7.4) for 15 min at 37°C, fixed in 4% formalin for 24 h, and photographed again. Viable myocardium is stained red by TTC, whereas necrotic myocardium appears pale yellow. The area at risk and the necrotic area were determined by computerized planimetry, normalized to the weight of each slice, with degree of necrosis (i.e. infarct size) expressed as the percentage of area at risk.

Targeting vagal preganglionic neurones with viral vectors

Cholinergic vagal preganglionic neurones of the dorsal motor nucleus of the vagus nerve (DVMN) express the transcriptional factor Phox2 and were targeted using an artificial Phox2-activated promoter – PRSx8\(^{10}\) – incorporated into lenti- and adenoviral vectors\(^{11}\). Expression driven by PRSx8 is dependent on the activity of Phox2 and, therefore, expression in DVMN neurones is not surprising, given the putative role of Phox2 in the development of these neurones\(^{12}\).

The lentiviral construct used to express the \(G_{i}\)-protein-coupled *Drosophila* allatostatin receptor (AlstR) in DVMN neurones has been described previously\(^{13, 14}\). In brief, the plasmid pTYF-PRSx8-AlstR-IRES2-eGFP was used to generate the lentiviral vector (LV). The LV system used was HIV-1-derived and pseudotyped with the VSV-G envelope\(^{15}\). Titres of PRSx8-AlstR-eGFP-LV and the control vector (PRSx8-eGFP-LV) were between 1 \(x 10^9\) to 1 \(x 10^{10}\) transducing units ml\(^{-1}\). Viral concentration and titration were carried out as described in detail previously\(^{15}\).
To control the activity of DVMN neurones with high temporal resolution we generated a new adenoviral vector (AV) where a mutant of channelrhodopsin (ChR) – ChIEF - is fused with a red fluorescent protein – the tandem dimer Tomato (tdTomato) protein. The ChIEFtdTomato clone was kindly provided by Dr JY Lin (University of California). ChIEF is a chimeric ChR variant constructed from the N-terminal part of the ChR1 and the C-terminal part of ChR2 and also incorporates an isoleucine 170 to valine mutation. ChIEF combines the reduced inactivation characteristics of ChR1 with more favourable cation permeability properties conferred by ChR2. The I170V mutation further improves the channel closure kinetics. Therefore, ChIEF allows greater temporal control of neuronal activation by light pulses. Its spectral properties are similar to those of ChR2 but it shows more efficient membrane expression and trafficking in the mammalian cells.

In this study ChIEFtdTomato was engineered into an AV under the control of an enhanced PRSx8 promoter. AVs were produced as described previously.

Viral gene transfer

Rats were anaesthetised (ketamine [60 mg kg\(^{-1}\); i.m.] and medetomidine [250 µg kg\(^{-1}\); i.m.]) and placed in a stereotaxic frame. Adequate surgical anaesthesia was ensured by the absence of a withdrawal response to a paw pinch. DVMN neurones were targeted with two microinjections per side (0.25 µl each, 0.05 µl min\(^{-1}\)) of a viral suspension containing PRSx8-AlstR-eGFP-LV, PRSx8-eGFP-LV, PRSx8-ChIEFtdTomato-AV or PRSx8-DsRed-AV (control for optogenetic experiments; this vector drives the expression of DsRed in the DVMN neurones). The PRSx8 promoter is also active in a population of noradrenergic neurones (A2 cell group) of the nucleus of the solitary tract located dorsal to the DVMN. To avoid transduction of A2 neurones, injections were placed immediately ventral to the
DVMN using the following coordinates from *calamus scriptorius* (1) 0.5 mm rostral, 0.6 mm lateral, 0.8 mm ventral and (2) 1.0 mm rostral, 0.8 mm lateral, 0.6 mm ventral. In our preliminary validation experiments conducted in 6 animals, along with widespread expression of the transgene in the DVMN we only found occasional (2-5 per brainstem) neurones expressing ChIEFtdTomato and stained positive for the noradrenergic marker DBH. Similarly, in contrast to strong AlstR expression in almost the entire population of DVMN neurones, the majority of dorsally located noradrenergic neurones were not transduced (Fig 1A,B). Thus, precise stereotaxic targeting of the viral vector ensured specific transduction of DVMN neurones. Anaesthesia was reversed with atipemazole (1 mg kg\(^{-1}\)) and no complications were observed after the surgery.

In vitro electrophysiology - verification of transgene functionality

Rapid inhibitory and readily reversible effects of allatostatin were previously reported to be selective for AlstR-expressing neurones\(^{22}\). Binding of allatostatin peptide to AlstR\(^{23}\) should lead to opening of inwardly rectifying K\(^+\) channels, neuronal hyperpolarisation and cessation of action potential firing\(^{22,24}\). Naturally, AlstRs are only expressed in insects and allatostatin itself does not interfere with endogenous receptor complexes in mammals\(^{22,23}\). Conversely, activation of ChIEFtdTomato using blue light should lead to immediate neuronal depolarization and action potential firing. Visually guided patch-clamp recordings in the brainstem slice preparation were used to verify efficacy of these approaches in silencing and activating DVMN neurones, respectively.

Seven to ten days after the delivery of PRSx8-AlstR-eGFP-LV or PRSx8-ChIEFtdTomato-AV into the DVMN, rats were sacrificed with halothane overdose and 200 µm coronal brainstem slices were cut and maintained as described previously\(^{25}\). Patch pipettes (3-6
MΩ) were pulled from thin-walled borosilicate capillary glass (Clark Electromedical Instruments, Pangbourne, UK). Electrodes were filled with (in mM) 120 K-gluconate, 5 HEPES, 5 BAPTA, 1 NaCl, 1 MgCl₂, 1 CaCl₂, 2 K₂ATP (pH 7.2). Recordings were carried out under an epifluorescence microscope (Zeiss Axioskop 2 FS, Zeiss, Germany) in artificial cerebrospinal fluid (aCSF, containing in mM; 118 NaCl, 3 KCl, 25 NaHCO₃, 1 MgCl₂, 2 CaCl₂, and 10 glucose) saturated with 95% O₂/5% CO₂ (pH 7.4) at 28-32°C. The recording chamber (volume 2 ml) was perfused with aCSF at a rate of 4-5 ml min⁻¹. Recordings were performed in cell attached configuration and whole-cell mode using an EPC-9 amplifier and Pulse/Pulsefit software (Heka Elektronik, Lambrecht, Germany). Currents or membrane potentials were filtered at 1 kHz and digitized at 3 kHz.

DVMN neurones expressing AlstR were identified by eGFP fluorescence. To test neuronal responses to activation of AlstR, allatostatin (0.5-1 µM; Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH₂, Phoenix Pharmaceuticals, USA) was added to the perfusate. For recordings from the DVMN neurones expressing ChIEFtdTomato, cells were identified by red (tdTomato) fluorescence. A light fibre connected to a 445 nm laser (Omicon, Germany) was submerged into the recording chamber and pointed towards the slice. Duration and intensity of light pulses were controlled by laser software and triggered from the EPC-9 amplifier. Control recordings verified that neither allatostatin (n=5) nor 445 nm light (n=4) had an effect on electrical activity in untransduced DVMN neurones.

Immunohistochemistry - verification of transgene expression

At the end of the in vivo experiments, the rats transduced to express AlstR/eGFP or ChIEFtdTomato in the DVMN neurones were perfused through the ascending aorta with 0.9% saline solution followed by 4% phosphate-buffered (0.1M, pH 7.4) paraformaldehyde.
After 12 hours of post-fixation and subsequent cryoprotection (30% sucrose), 30 µm-thick coronal sections were collected along the rostro-caudal extent of the medulla oblongata. Sections were processed for immunohistochemical detection of choline acetyl-transferase (ChAT) or tyrosine hydroxylase (TH). Tissue was incubated in goat anti-ChAT (1:500, Chemicon) or sheep anti-TH (1:250, Abcam) followed by donkey anti-goat Alexa Fluor568 or biotinylated rabbit anti-sheep (1:500) amplified by AMCA avidin DCS (1:250, Vector laboratories). Identification of AlstR-expressing neurones was enhanced by eGFP immunostaining as described previously\textsuperscript{13}. All brainstem sections from this group, including those stained for ChAT and TH, were subsequently incubated in chicken anti-GFP antibody (1:250, Avés) for 48 hours followed by goat anti-chicken Alexa Fluor488 (1:1000, Molecular Probes).

**Experimental protocols**

1. **RPc in conditions of DVMN silencing.** A small occipital craniotomy was performed and a miniature polyethylene catheter was slid around the caudal surface of the cerebellum and secured in place with dental impression material with the catheter tip placed in the *cisterna magna*. All rats transduced with either PRSx8-AlstR-eGFP-LV or PRSx8-eGFP-LV received slow continuous infusion of allatostatin (100 µM in aCSF; 4 µl h\textsuperscript{-1}) starting 15 min prior to RPc or sham-RPc procedures and lasting till the end of the myocardial ischaemia/reperfusion period.

2. **Optogenetic conditioning of the heart.** Occipital craniotomy was performed and cerebellum was partially removed to expose the dorsal part of the medulla oblongata overlaying dorsal vagal complex, including the DVMN. DVMN neurones were stimulated using blue light (445 nm, 10 ms pulses, 10 Hz) delivered via an optrode placed against the
brainstem surface. Light stimulation commenced 25 min prior to myocardial ischaemia (to follow the timeline of R Pc protocol) and lasted 10 min into the reperfusion period. Atropine was administered (initial bolus dose 2 mg kg\(^{-1}\) i.v. 15 min prior to light onset; followed by infusion at a rate of 1 mg kg\(^{-1}\) h\(^{-1}\) i.v.) to separate groups of animals in order to determine the effect of systemic muscarinic receptor blockade on cardioprotection elicited by optogenetic stimulation of the DVMN neurones.

3. R Pc in conditions of systemic muscarinic receptor blockade with atropine. Systemic muscarinic receptor blockade was achieved by intravenous infusion of atropine methyl nitrate 15 min prior to R Pc or sham-R Pc procedures (initial bolus dose 2 mg kg\(^{-1}\) i.v.; followed by infusion at a rate of 1 mg kg\(^{-1}\) h\(^{-1}\) i.v.).

Statistical Analysis

Data are reported as mean ± s.e.m. Data were compared by Kruskal–Wallis ANOVA by ranks or Student’s t test, as appropriate. Values of \(P<0.05\) were considered to be significant.

Results

A lentiviral vector bearing the PRSx8 promoter was highly efficient in driving the expression of AlstR/eGFP in DVMN neurones (Fig 1A,B). First, patch-clamp recordings in brainstem slices were used to confirm that activation of AlstR inhibits the activity of transduced neurones by opening inwardly rectifying K\(^+\) channels, hyperpolarisation and cessation of action potential generation\(^{22}\). DVMN neurones expressing AlstR/eGFP recorded in cell-attached configuration had a mean firing rate of 1.7±0.4 Hz (n=6).
Application of allatostatin reversibly abolished action potential firing within 2 min in 5 cells (Fig 1C) and decreased firing rate by 55% in one remaining cell. In whole-cell current clamp recording configuration, allatostatin induced a 5.3±0.8 mV hyperpolarisation and cessation of firing, caused by activation of an inwardly rectifying K⁺ conductance, as revealed in voltage clamp recordings (n=4) (Fig 1D). Thus, targeted stereotaxic delivery of the viral vector (avoiding transduction of dorsally located catecholaminergic neurones, Fig 1B), the specificity of the PRSx8 promoter and the effective silencing of transduced AlstR-expressing neurones by allatostatin, allowed accurate assessment of the functional role of DVMN vagal preganglionic neurones with high temporal resolution.

The efficacy of RPc cardioprotection was then assessed while DVMN neurones were inhibited by allatostatin (100 µM) continuously infused (4 µl h⁻¹) into the cisterna magna (Fig 2A). RPc induced by ischaemia/reperfusion of the limbs reduced myocardial ischaemia/reperfusion injury as evident from a significant (P<0.01) reduction in infarct size (Fig 2B). Selective DVMN silencing abolished RPc cardioprotection (P=0.004) while having no effect on infarct size in animals not subjected to RPc (Fig 2B). There were no differences in the areas at risk between groups of animals recruited into the experimental groups. There were also no differences in mean arterial blood pressure or heart rate between the groups of animals either before, or during myocardial ischaemia and reperfusion (Table 1). Thus, RPc cardioprotection appears to be crucially dependent on the activity of DVMN neurones.

By extension, activation of DVMN neurones in the absence of remote ischaemia/reperfusion should result in cardioprotection. To test this hypothesis we used optogenetics to increase DVMN neuronal activity with high resolution and specificity. DVMN neurones transduced to express ChIEFtdTomato (Fig 3A,B) displayed precisely-
timed depolarizations and action potential firing in response to flashes of blue (445 nm) light (Fig 3A). Voltage clamp analysis revealed that light stimulation induces an inward current with half maximal activation by a 5 ms light pulse (Fig 3C). Consequently, optogenetic stimulation of DVMN in vivo was performed with 10 ms pulse duration at a frequency of 10 Hz (a stimulation regime not associated with changes in heart rate or blood pressure; Table 1). Optogenetic stimulation of DVMN markedly reduced infarct size (P=0.008; Fig 3D), indicating that increased activity of this specific neuronal population is sufficient to limit ischaemia/reperfusion myocardial injury, effectively mimicking the effect of RPc. In conditions of systemic administration of atropine optogenetic stimulation of the DVMN neurones failed to establish cardioprotection (Fig 3D).

This observation prompted the experiment to determine whether a muscarinic receptor mechanism mediates RPc cardioprotection. RPc conferred no cardioprotection in conditions of systemic muscarinic receptor blockade with atropine methyl nitrate (Fig 4) which has a limited ability to cross the blood-brain barrier. Atropine had no effect on heart rate in animals subjected to RPc procedure (Table 2), consistent with the data showing that in rats anesthetized with pentobarbital chronotropic vagal tone is significantly reduced26.

Discussion

The present study reveals a crucial dependence of interorgan (limb-heart) protection against ischaemia/reperfusion injury upon the activity of a distinct population of vagal preganglionic neurones residing in the DVMN. It also demonstrates for the first time that activation of a specific central nervous substrate can effectively protect the heart against lethal ischaemia/reperfusion injury.
Here we tested the hypothesis that cardioprotection induced by ischaemia/reperfusion of the remote tissue requires functional integrity of the central nervous parasympathetic circuitry and, by extension, an intact parasympathetic activity. This hypothesis was based on the following earlier findings. First, RPc is abolished by systemic hexamethonium administration, which blocks transmission in both sympathetic and parasympathetic ganglia. Second, electrical stimulation of the vagus nerve is cardioprotective, limits myocardial ischaemia/reperfusion injury, and reduces the number of severe arrhythmias and overall lethality. Our recent observations also demonstrated that RPc cardioprotection is abolished by bilateral cervical vagotomy. However, whilst eliminating vagal efferent activity, cervical vagotomy also interrupts transmission of sensory information from the heart (and other internal organs) to the CNS. To avoid confounding factors associated with complete surgical vagotomy, here we performed a selective (albeit partial) vagal ‘de-efferentation’ by cell-specific targeting and silencing of vagal preganglionic neurones in the DVMN which contribute to tonic C-fibre-mediated innervation of the heart. In support of our hypothesis, we found that RPc fails to establish cardioprotection when DVMN neurones are silenced.

In this study all the experiments were conducted in rats anaesthetised with pentobarbital which is known to suppress chronotropic vagal tone, hence the heart rate in animals subjected to RPc was not affected by either silencing or optogenetic activation of the DVMN neurones or in conditions of systemic muscarinic receptor blockade with atropine. This is consistent with the data demonstrating that chronotropic control of the heart is provided predominantly by a population of vagal preganglionic neurones located in the nucleus ambiguus. Collectively these data indicate that while an intact vagal supply is essential for RPc cardioprotection, its beneficial effect is independent of heart rate...
modulation as demonstrated by prior studies involving electrical stimulation of the vagus nerve\textsuperscript{5}. In contrast to an earlier view still conveyed by the majority of the physiology textbooks, there is strong evidence which demonstrates functional vagal innervation of the ventricles (see for example\textsuperscript{29}). Here we demonstrate that a muscarinic receptor mechanism mediates RPc cardioprotection and confirm the existence of a significant cholinergic supply to the right and left ventricles of the heart in a rat strain used in this study (Sprague-Dawley) (\textbf{Supplementary Figure}). These results suggest that increased efferent vagal outflow reduces myocardial injury through the potent cardioprotective effects of acetylcholine (which is as potent as adenosine in eliciting cardioprotection) reported previously\textsuperscript{30-33}.

The mechanism of RPc-induced cardioprotection was suggested to involve humoral factor(s) produced during ischemia/reperfusion of the remote tissue and released into the systemic circulation\textsuperscript{34-38}, or a neural component\textsuperscript{3,37,39-42}, or both\textsuperscript{37,43}. Several studies clearly demonstrated that RPc cardioprotection requires intact sensory innervation of the peripheral ischaemic tissue\textsuperscript{37,39,43}. Based on those data together with the results of our studies we propose the existence of a ‘remote preconditioning reflex’\textsuperscript{7} which involves sensory input from the remote ischaemic organ/tissue and protects the heart via recruitment of a distinct population of vagal preganglionic neurones in the DVMN. Indeed, this study demonstrates that the key component of inter-organ remote protection against ischaemia/reperfusion injury is neural, since DVMN neurones are essential for RPc, while their activation (even in the absence of any humoral factor(s) released from the ischaemic limb) is sufficient to establish cardioprotection. These results do not exclude the involvement of humoral factor(s)\textsuperscript{37,43}, but demonstrate that in order to establish cardioprotection an enigmatic ‘humoral pathway’ of RPc requires functional integrity of DVMN neurones. Several disparate experimental models indicate that direct myocardial
preconditioning cardioprotection does not require intact connections between the heart and the central nervous system. Therefore, the parasympathetic pathway of myocardial protection described in this report may only be recruited under certain conditions, for example when RPc stimulus is applied to the tissue located away from the heart.

Ongoing trials of the efficacy of vagus nerve stimulation in chronic heart failure patients are expected to confirm the results of the preliminary report showing significant improvements in quality of life and left ventricular function. Intermittent arm ischaemia in humans was recently reported to increase parasympathetic tone. The results of the present study obtained with optogenetic stimulation of DVMN suggest that timely application of the vagus nerve stimulation or other procedures/treatments which reflexly increase vagal activity may be highly effective in protecting the heart against acute lethal ischaemia/reperfusion injury and decreasing morbidity and mortality of patients with ischaemic heart disease.

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Conflict of interest: None
References


Figure Legends

Figure 1. Genetic targeting and silencing of vagal preganglionic neurones in the dorsal motor nucleus of the vagus nerve (DVMN)

(A) Confocal image of the coronal section of the rat brainstem targeted to express allatostatin receptor (AlstR) in the DVMN. Figure illustrates a representative example of distribution of choline acetyltransferase (ChAT)-positive (i.e. cholinergic) (red) DVMN neurones transduced to express AlstR/eGFP (green). Colocalization appears yellow. Bregma level -14 mm. XII – hypoglossal motor nucleus (ChAT-positive, but not expressing eGFP). CC – central canal; (B) A representative example of distribution of AlstR/eGFP-transduced DVMN neurons in relation to the location of A2 noradrenergic cells identified by tyrosine hydroxylase (TH) immunohistochemistry (red). Along with strong expression of AlstR/eGFP in the DVMN only occasional noradrenergic neurons were found to be transduced. Colocalization appears yellow (arrows). Bregma level -13.8 mm. (C) Representative cell-attached recording from an AlstR/eGFP-positive DVMN neurone illustrating its rapid and reversible silencing in response to allatostatin (0.5 µM); (D) Current-voltage relationship (IV) of allatostatin-induced current. Transduced DVMN neurone was voltage-clamped to -30 mV and hyperpolarizing voltage ramps to -130 mV (700 ms duration) were applied before and during allatostatin application. The displayed IV was obtained by subtracting the whole-cell IV obtained under control conditions, from that obtained in the presence of allatostatin.

Figure 2. Remote preconditioning (RPc) cardioprotection critically depends on the activity of DVMN vagal preganglionic neurones

(A) Illustration of the experimental protocols. RPc was induced by 15 min occlusion of both femoral arteries, followed by 10 min reperfusion. Sham-RPc procedure involved dissection
of both femoral arteries without occlusion. Arrow indicates the start of allatostatin infusion into the *cisterna magna*. eGFP – animals transduced to express eGFP in the DVMN. AlstR – animals transduced to express AlstR/eGFP in the DVMN. MI – myocardial ischaemia. (B) Silencing DVMN neurones abolishes RPc cardioprotection. Infarct size is presented as percentage of area at risk. Individual data and means±s.e.m. are shown. **P<0.01 (Kruskal–Wallis ANOVA by ranks).

**Figure 3. Optogenetic stimulation of DVMN neurones mimics RPc cardioprotection.**

(A) Representative whole-cell current-clamp recording from CHIEFtdTomato-expressing DVMN neurone illustrating depolarization and action potential firing in response to blue light (20 ms pulses). Four consecutive traces are overlaid. Action potential was elicited in response to 15 out of 16 pulses; (B) DVMN neurones expressing CHIEFtdTomato. Scale bar=30 µm (C) Mean data from voltage-clamp recordings (n=6) at a holding potential of -50 mV demonstrating the relationship between duration of the light stimulus and the normalized amplitude of inward current elicited by opening CHIEF channel; (D) Optogenetic stimulation of DVMN neurones reduces myocardial ischaemia/reperfusion injury via a muscarinic receptor-mediated mechanism. Control animals were transduced to express DsRed in the DVMN. Infarct size is presented as percentage of area at risk. Individual data and means±s.e.m. are shown. **P<0.01 (Kruskal–Wallis ANOVA by ranks).

**Figure 4. Muscarinic receptor mechanisms mediate RPc cardioprotection**

RPc was induced by 15 min occlusion of both femoral arteries, followed by 10 min reperfusion. Sham-RPc procedure involved dissection of both femoral arteries without occlusion. Atropine infusion commenced 15 min prior to RPc or sham-RPc. RPc conferred
no cardioprotection in conditions of systemic muscarinic receptor blockade. Infarct size is presented as percentage of area at risk. Individual data and means±s.e.m. are shown.

**P<0.01 (Kruskal–Wallis ANOVA by ranks).
Table 1

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<td>MAP</td>
<td>115 ± 9</td>
<td>104 ± 10</td>
<td>99 ± 8</td>
<td>91 ± 7</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>401 ± 9</td>
<td>421 ± 13</td>
<td>397 ± 12</td>
<td>384 ± 12</td>
</tr>
<tr>
<td>allatostatin + RPc (n=8)</td>
<td>MAP</td>
<td>117 ± 9</td>
<td>103 ± 7</td>
<td>102 ± 5</td>
<td>101 ± 5</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>413 ± 15</td>
<td>430 ± 9</td>
<td>405 ± 7</td>
<td>407 ± 7</td>
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<td><strong>Optogenetic preconditioning experiment</strong></td>
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<tr>
<td>DsRed - DVMN blue light (n=7)</td>
<td>MAP</td>
<td>92 ± 9</td>
<td>82 ± 7</td>
<td>78 ± 3</td>
<td>77 ± 5</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>401 ± 12</td>
<td>403 ± 8</td>
<td>384 ± 5</td>
<td>380 ± 7</td>
</tr>
<tr>
<td>CHIEFtdTomato - DVMN blue light (n=6)</td>
<td>MAP</td>
<td>99 ± 6</td>
<td>79 ± 4</td>
<td>78 ± 4</td>
<td>80 ± 5</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>420 ± 6</td>
<td>415 ± 8</td>
<td>402 ± 7</td>
<td>397 ± 9</td>
</tr>
<tr>
<td>DsRed - DVMN blue light + atropine (n=7)</td>
<td>MAP</td>
<td>106 ± 11</td>
<td>85 ± 5</td>
<td>90 ± 4</td>
<td>90 ± 7</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>391 ± 17</td>
<td>392 ± 11</td>
<td>393 ± 10</td>
<td>410 ± 12</td>
</tr>
<tr>
<td>CHIEFtdTomato - DVMN blue light + atropine (n=8)</td>
<td>MAP</td>
<td>99 ± 10</td>
<td>76 ± 6</td>
<td>84 ± 5</td>
<td>79 ± 4</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>379 ± 14</td>
<td>392 ± 15</td>
<td>389 ± 11</td>
<td>380 ± 20</td>
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</tbody>
</table>

Abbreviations: AlstR – allatostatin receptor; DVMN - dorsal motor nucleus of the vagus nerve; eGFP – enhanced green fluorescent protein; HR – heart rate (min⁻¹); MAP – mean arterial blood pressure (mmHg); RPc – remote preconditioning.
Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-ischaemia</th>
<th>End ischaemia</th>
<th>Reperfusion (min)</th>
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<tr>
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<td>30</td>
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</table>

**Experiment with systemic muscarinic receptor blockade**

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP (mmHg)</th>
<th>HR (min⁻¹)</th>
<th>MAP (mmHg)</th>
<th>HR (min⁻¹)</th>
<th>MAP (mmHg)</th>
<th>HR (min⁻¹)</th>
<th>MAP (mmHg)</th>
<th>HR (min⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>sham-RPc (n=10)</td>
<td>98 ± 9</td>
<td>416 ± 12</td>
<td>98 ± 7</td>
<td>421 ± 12</td>
<td>100 ± 7</td>
<td>423 ± 9</td>
<td>103 ± 6</td>
<td>419 ± 11</td>
</tr>
<tr>
<td>RPc (n=12)</td>
<td>103 ± 4</td>
<td>427 ± 9</td>
<td>95 ± 5</td>
<td>431 ± 7</td>
<td>97 ± 5</td>
<td>437 ± 9</td>
<td>94 ± 4</td>
<td>436 ± 7</td>
</tr>
<tr>
<td>atropine + sham-RPc (n=5)</td>
<td>105 ± 10</td>
<td>412 ± 12</td>
<td>92 ± 5</td>
<td>414 ± 18</td>
<td>104 ± 2</td>
<td>410 ± 10</td>
<td>89 ± 10</td>
<td>424 ± 11</td>
</tr>
<tr>
<td>atropine + RPc (n=7)</td>
<td>107 ± 7</td>
<td>439 ± 22</td>
<td>95 ± 7</td>
<td>437 ± 15</td>
<td>103 ± 3</td>
<td>430 ± 11</td>
<td>103 ± 3</td>
<td>424 ± 13</td>
</tr>
</tbody>
</table>

Abbreviations: HR – heart rate (min⁻¹); MAP – mean arterial blood pressure (mmHg); RPc – remote preconditioning.