The electrophysiological effects of nicotinic and electrical stimulation of intrinsic cardiac ganglia in the absence of extrinsic autonomic nerves in the rabbit heart

Emily Allen (PhD) [1,2]
John H Coote (DSc, FRSB, FBPhS) [1]
Blair D Grubb (BSc, PhD)[3]
Trevor FC Batten (PhD)[4]
Dainius H Pauza (PhD)[5]
G André Ng* (MBChB, PhD, FRCP (Glasg), FRCP, FESC, FEHRA) [1,2]
Kieran E Brack* (PhD) [1,2]

*G André Ng and Kieran E Brack contributed equally to this study.

1. Department of Cardiovascular Sciences, Cardiology Group, University of Leicester, Clinical Sciences Wing, Glenfield Hospital, Groby Road, Leicester, LE3 9QP.
2. NIHR Leicester Biomedical Research Centre, Glenfield Hospital, Groby Road, Leicester, LE3 9QP.
3. Institute of Life and Human Sciences, Life Sciences Building, Crown Street, Liverpool, L69 7ZB
4. AF3, Leeds Trinity University, Brownberrie Lane, Horsforth, Leeds, LS18 5HD.
5. Lithuanian University of Health Sciences, A. Mickeviciaus Street 9, Kaunas, LT 44307, Lithuania.

Corresponding Author:
Professor G André Ng
Professor of cardiac electrophysiology/Consultant cardiologist & electrophysiologist
Department of Cardiovascular Sciences, Cardiology Group, University of Leicester,
Glenfield Hospital,
Leicester, LE3 9QP, UK.
Tel: +44(0)1162502438
Fax: +44(0)1162875792
Email: gan1@leicester.ac.uk

Word Count: 7013
Short title: Stimulation of intrinsic cardiac ganglia
Subject codes: Electrophysiology, Autonomic Nervous System, Basic Science Research

ABSTRACT

Background

The intrinsic cardiac nervous system (ICNS) is a rich network of cardiac nerves that converge to form distinct ganglia and extend across the heart and is capable of influencing cardiac function.

Objective

To provide a picture of the neurotransmitter/neuromodulator profile of the rabbit ICNS and determine the action of spatially divergent ganglia on cardiac electrophysiology.

Methods

Nicotine injection or electrical stimulation was applied at discrete sites of the intrinsic cardiac nerve plexus in the Langendorff perfused rabbit heart. Functional responses to stimulation were measured including effects on sinus rate and atrioventricular conduction. Immunohistochemistry for choline acetyltransferase (ChAT), tyrosine hydroxylase (TH) and/or neuronal nitric oxide synthase (nNOS) was performed on whole-mount preparations.

Results

Stimulation within all ganglia produced either bradycardia, tachycardia or a biphasic brady-tachycardia. Electrical stimulation of the right atrial (RA) and right neuronal cluster (RNC) regions produced the greatest chronotropic responses (25 and 21% respectively). Significant prolongation of atrioventricular conduction (AVC) occurred during stimulation of all regions however, was predominant at the pulmonary vein-caudal vein region (PVCV). Neurons immunoreactive (IR) only for ChAT, or TH or nNOS were consistently located within the limits of the hilum and at the roots of the right cranial and right pulmonary veins. ChAT-IR neurons were most abundant (1946 ± 668 neurons). Neurons IR solely for nNOS were distributed within ganglia.

Conclusion

Stimulation of intrinsic ganglia, shown to be of phenotypic complexity but predominantly of cholinergic nature, indicates that clusters of neurons are capable of independent selective effects on cardiac electrophysiology, therefore providing a potential therapeutic target for the prevention and treatment of cardiac disease.

INTRODUCTION

In recent decades the presence of a rich network of intrinsic cardiac nerves that converge to form distinct ganglia and extend across the heart has been documented in several mammalian species such as dog, cat, pig, guinea pig, mouse as well as human [2]. Evidence suggests that activity within these ganglia may result in cardiac changes both locally and in other regions of the myocardium, independently of extrinsic autonomic nerves [2-5]. This has given rise to the notion that such an intrinsic cardiac nervous system (ICNS) acts as the hearts '*little brain*': capable of influencing cardiac function [6, 7] even in the absence of extrinsic autonomic input although this has not been directly tested.

The somata of intrinsic neurons occur within the epicardium [8] and spread widely over the walls of all chambers of the heart forming an interconnected "ganglionic plexus" (GP) [9-11]. Historically, it was presumed that the intrinsic cardiac ganglia were merely parasympathetic neuronal relay stations [2, 12] and would by assumption contain only cholinergic markers such as choline acetyltransferase (ChAT). However, a recent review by Wake and Brack [2] reviewed evidence showing these ganglia are neurophenotypically and neurochemically diverse [11, 13-15]. This would suggest that the ganglia are also functionally diverse. Knowledge of this functional diversity is important because remodelling of ganglia is a hallmark of cardiac diseases such as heart failure and atrial and ventricular rhythm disturbances [16, 17].

Our understanding of the cardiac regulatory functions of this dispersed epicardial GP is limited, presently depending either on electrophysiological studies of cells in isolated atria or on *in situ* heart studies in anaesthetised dogs [7] with intact extrinsic autonomic nerves. Therefore we considered that our Langendorff perfused rabbit heart [18, 19] could overcome these limitations and provide a valuable model to study the integrative action of the ICNS and its significance to cardiac performance in isolation of circulating and extraneuronal factors. Furthermore, little is known of the physiological actions of stimulating the ganglia in the rabbit heart, which is an important species of choice for studying normal and abnormal neurocardiac physiology.

Therefore the primary objective of the present study was to determine the influence of spatially divergent ganglia on cardiac electrophysiology, in the absence of extrinsic autonomic nerve influence. We tested the effects of nicotine and electrical stimulation, which have been the main methods used in previous studies in anaesthetized dogs [4, 20-22]. Unlike earlier studies, we compared the effects of applying stimuli to loci in four different regions whilst measuring effects on heart rate, perfusion pressure, left ventricular pressure, atrio-ventricular conduction and left ventricular monophasic action potential duration.

In addition to and with the aim of providing a more complete picture of the neurotransmitter/neuromodulator profile of the rabbit intrinsic cardiac network, immunofluorescent labelling for tyrosine hydroxylase (TH), neuronal nitric oxide synthase (nNOS) and ChAT was conducted using whole-mount atrial preparations.

METHODS

Ethical statement

All procedures were undertaken using Adult male New Zealand White rabbits (n=46, 1.5-3.4kg) in accordance with the UK Animals (Scientific Procedures) Act 1985, the Guide for the Care of Use of Laboratory Animals Published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and the European Union Directive on the protection of animals for scientific research (2010/63/EU). Local ethics approval was obtained from the University of Leicester animal welfare review board (AWERB) under the Home Office Project Licence PPL 70/8501.

Animal Preparation

Of the 46 animals utilised in this study, 28 were used to study the influence of spatially divergent ganglia on cardiac electrophysiology and a separate group of 18 used for immunohistochemical analysis. All animals were pre-medicated with ketamine (Ketaset, 10 mg/kg, Fort Dodge, UK), medetomidine hydrochloride (Sedator, 0.2 mg/kg, Dechra, UK) and butorphanol (Torbugesic, 0.05 mg/kg, Fort Dodge, UK) (via subcutaneous injection). Following stable sedation, animals were sacrificed with an overdose of pentobarbitone sodium (Sagatal, Rhone Merieux, UK; 111 mg/kg body weight, i.v.) containing heparin (1000 IU, Multiparin, UK) delivered via the marginal ear vein.

Isolation of the non-innervated heart preparation

Non-innervated hearts were isolated as previously described [23-25]. In brief, animals were pre-medicated and sacrificed with an overdose of pentobarbitone sodium (Sagatal, Rhone Merieux, UK; 111 mg/kg body weight, i.v.) containing heparin (1000 IU, Multiparin, UK. Hearts were rapidly excised, placed into ice cold Tyrode solution to reduce metabolic rate and retrogradely perfused through the ascending aorta in conditions of constant flow Langendorff mode (40 ml/min) using a Gilson Minipulse 3 peristaltic pump (Anachem, Luton, UK).

Langendorff perfusion

Hearts were perfused with Tyrode solution containing Na^+ 138.0; K^+ 4.0; Ca^{2+} 1.8; Mg^{2+} 1.0; HCO_3^- 24.0; $H_2PO_4^-$ 0.4; Cl^- 124.0; Glucose 11.0 (mM). The solution was maintained at a constant temperature of 37°C and pH of 7.4 by continuous bubbling with Carbogen [95% O_2/5% CO_2]. A 1mm ID, 2mm OD polypropylene catheter (Porlex, Kent, UK) was inserted through the left ventricular (LV) apex for Thebesian venous effluent drainage. Hearts were instrumented to record left ventricular pressure (LVP), coronary perfusion pressure (PP) and

ventricular monophasic action potentials (MAP) at the apex and base with MAP contact electrodes (73-0150, Harvard Apparatus, Kent, UK) using a custom made DC-coupled high input impedance differential amplifier (Joint Biomedical Workshop, University of Leicester, UK).

Nicotinic stimulation of intrinsic cardiac ganglia

Stimulation of epicardial ganglia was performed at four sites (Fig. 1) using the topographical map previously published by Saburkina *et al.* [1]. These sites included 1) the left neuronal complex (LNC), 2) the right neuronal complex (RNC), 3) the right atrial ganglia region (RAGP) and 4) between the middle pulmonary veins and the caudal vena cava (vena caudialis; 'inferior vena cava') (PVCV). Nicotine (0.1 mg nicotine in 10 µl saline) was directly injected into loci within the LNC, RNC and PVCV and nicotine 0.1 mg in 100 µl saline [4] into loci within the RAGP to ensure a larger area of infiltration.

Electrical stimulation of intrinsic cardiac ganglia

Electrical stimulation was performed within the four regions (Fig. 1) using a custom-made bipolar silver electrode (0.5mm diameter, Advent research materials, Oxford, UK). Electrical stimulation was delivered using a single-channel constant voltage square-pulse stimulator (SD9, Grass Instruments, Astro-Med) connected via a constant current stimulator (DS7A, Digitimer Ltd, UK). Responses to electrical stimulation, applied using a 0.1ms pulse [26] in an attempt to limit the stimulus to neuronal somata, were recorded at stimulation frequencies between 10 and 50Hz at a stimulus strength 50% of the cardiac pacing threshold.

Protocols

The effect of nicotine and electrical stimulation at each GP site was determined during sinus rhythm; followed by application / stimulation during constant cardiac pacing. In order to account for the maximum anticipated increase in heart rate based on observations during preliminary investigations, hearts were paced at a 240ms cycle length at double the pacing threshold. This approach enabled the determination of chronotropic, dromotropic, inotropic and LV electrophysiological effects following ganglionic activation in the fewest protocols.

Pharmacological Agents

To determine which types of autonomic receptors were involved in the cardiac responses, protocols were repeated in the presence of the muscarinic (M2) receptor antagonist, atropine (0.1 µM; [19]), the β-adrenergic receptor blocker, metoprolol (1.8 µM; [19]) and the autonomic ganglia blocker, hexamethonium (0.5 mM; [27, 28]). Each agent was dissolved in a small

volume of Tyrode solution before being added to the Tyrode solution perfusing the heart, with measurements being made 5-10 minutes following exposure. Measurements were also made following a period of washout in order to confirm test effects were due to application of a pharmacological agent.

Signal measurements and analysis

Functional responses of the heart and MAPs were recorded with a PowerLab 16 channel system and digitised at 2kHz using Chart and Scope software (ADInstruments Ltd). During sinus rhythm, HR and LV pressure were measured along with contact MAP duration measured at 90% repolarisation ($MAPD_{90}$); calculated from an average of 10 cardiac cycles during baseline and subsequently during the steady state response. The PR Interval was calculated using AD instruments ECG measuring software. During constant atrial pacing, parameters were measured from averaged data taken from 30 cardiac cycles during steady state using AD instruments Scope software. The time from the cardiac pacing stimuli to the activation of atrial / ventricular electrical signal was used to determine atrio-ventricular delay. $MAPD_{90}$ was measured using custom-made NewMap analysis software (Dr F Burton, University of Glasgow).

Immunohistochemical analysis

Whole-mount preparations

Following euthanasia, the chest was opened by bilateral thoracotomy to expose the lungs and heart and the pericardium was cut to allow access to the heart. The caudal vein (vena caudalis; 'inferior vena cava') was identified and cannulated, therefore providing perfusate to the right side of the heart. A small incision was subsequently made at the superior edge of the left atrium and a cannula inserted and secured, thereby perfusing the left side of the heart. To prevent the continuation of blood flow from the body and head back into the heart, the right cranial vein (vena cranialis dextra; superior vena cava) and left cranial vein (vena cranialis sinistra) were clamped. Hearts were perfused with ice-cold phosphate-buffered saline (PBS; 0.01M, 4˚C, pH7.4) and pressure-inflated *in situ* [1]. Hearts were removed from the chest, placed into ice-cold PBS (pH 7.4, 4^{o}C) and the surrounding pericardium, mediastinal fat were removed and pulmonary vessels sectioned. Hearts were prefixed using 4% PFA (0.01M PB, pH7.4) for 30 minutes, followed by incubation in a hyaluronidase solution (PBS, 0.5mg/100ml) for 60 minutes.

Immunolabelling procedure

The walls of the atria and interatrial septum were separated from the ventricles along the atrioventricular groove and pinned flat into a custom made silicone based dissecting dish. In order to assess the distribution of ventricular neurons, the wall of the conus arteriosus and left ventricle was dissected and pinned to a separate dish, allowing for subsequent washes (3 x 10 minutes) in ice cold PB (0.01M, pH.4, 4^{o}C). To prevent non-specific protein binding, each whole mount preparation was incubated in a blocking buffer of 0.01M PB containing 10% normal horse serum (Vector Laboratories, UK) and 0.5% Triton X-100 (Sigma Aldrich, UK) for 30 minutes. Preparations were then incubated in a double primary antibody mixture (Table. 1) (antibodies were diluted in a solution of 0.01M PB containing 10% normal horse serum and 0.5% Triton X-100) for 48 hours at 4˚C in a humidity chamber [29].

After a washing step (3 x 10 minute washes in 0.01M PB), all sections were incubated in the combination of corresponding secondary antibodies (Table.1) for 2 hours in a dark humid chamber at room temperature. After a final wash step (3 x 10 minute washes in 0.01M PB) each section was mounted using Vectashield Hardset Mounting Medium (Vector Laboratories, UK), covered with a coverslip and sealed using clear nail varnish ready for microscopic analysis.

Microscopic examination and quantitative analysis

Immunohistochemically stained neural structures were visualised and imaged using an upright fluorescence microscope (Axioscope Imager.Z2, Carl Zeiss Ltd, UK). Images were captured using a digital camera (AxioCam HRc, Carl Zeiss Ltd, Cambridge, UK). Stereoscopic examination was achieved between x10-40 magnification. Overall topography was constructed offline and image examination and analysis was completed using ZEN software (Carl Zeiss Ltd, Cambridge, UK) and Image J 1.49v software. For counting and identifying, neuronal cell bodies and their areas were systematically scanned over the whole mount.

Chemicals

Unless stated, all chemicals were purchased from Sigma Aldrich, UK.

Statistical Analysis

Data analysis was performed using GraphPad Prism7 software. Statistical comparisons were made using Student's paired t-tests, one- or two-way ANOVA where appropriate with Bonferroni post-hoc test. Data are presented as mean ± SEM; $P<0.05$ was considered significant.

RESULTS

The effects of nicotinic stimulation of cardiac intrinsic ganglia

Nicotine was applied in a total of 14 hearts at a number of sites within the regions shown in Figure 1. Sites in the LNC located at the roots of the left and middle pulmonary veins were tested in all 14 animals. Other regions including the RNC, RAGP and PVCV region (located between the roots of the middle pulmonary vein and the caudal vein) were tested in 13, 11 and 6 animals respectively.

Chronotropic and dromotropic responses following nicotine application

Following nicotine application, the profile of heart rate changes occurred in three characteristic forms: i) bradycardia alone, ii) tachycardia alone and iii) a biphasic response of decreased heart rate (bradycardia) followed by an increase (tachycardia) (Fig.2). The incidence of nicotine induced heart rate changes are shown in the bar graph in figure 2. No region contained sites which produced a single type of heart rate change. However in the PVCV and RAGP regions, bradycardia sites and sites eliciting biphasic heart rate responses prevailed.

There were differences in the magnitude of the heart rate changes induced by nicotine applied at sites in each of the regions. Nicotine applied to the right atrial GP region produced the strongest bradycardia or tachycardia, whilst nicotine in the left neuronal complex produced the smallest decrease in heart rate, and in the PVCV region it produced the smallest increase in heart rate (Fig. 2).

During ventricular pacing, nicotine applied to sites in the right atrial GP failed to elicit any change in VA conduction, whilst nicotine applied to sites in the PVCV region produced a prolongation in VA conduction in 60% of cases, with no effect in the remaining 40%. A combination of VA prolongation and VA shortening was observed when stimulating sites in the LNC and RNC, but there was a larger proportion resulting in VA prolongation (65% *vs*. 40%) at sites in the right neuronal complex. Quantitatively, there was no difference in the VA shortening when stimulating the LNC / RNS sites. There was however a greater degree of VA prolongation by sites at the right neuronal complex compared to any other site, with the LNC and RVCV sites being equipotent (Fig. 3).

To pharmacologically characterise the chronotropic effect of ganglionic plexus stimulation, the application of nicotine was tested in the presence of classical autonomic blocking drugs

either with atropine, or metoprolol or hexamethonium in the perfusate. All bradycardias and tachycardias were blocked with atropine and metoprolol respectively, whilst hexamethonium completely blocked all chronotropic responses (Fig. 4)

The effects of electrical stimulation at sites in the cardiac intrinsic ganglionated plexus

Electrical stimulation of intrinsic cardiac ganglia was examined in 14 animals. The right neuronal cluster, located medial to the root of the RCV and dorsal to the root of the RPV, was examined in all 14 animals. Loci within the LNC, RAGP and PVCV regions were tested in fewer animals (8/14, 9/14 and 8/14 respectively).

Chronotropic responses to electrical stimulation

The effects of electrical stimulation at five frequencies 10Hz, 20Hz, 30Hz, 40Hz, and 50Hz, on heart rate and atrioventricular conduction were examined at 56 sites. Statistically significant heart rate increases were evoked at some sites in 2 regions, the RA and the RNC, out of the 4 regions explored when the frequency of stimulation was 20Hz or more (Fig. 5). Decreases in heart rate occurred on stimulation at some sites in all regions tested. As with nicotine, electrical stimulation of sites in ganglia, could elicit three types of response i) bradycardia alone, ii) tachycardia alone and iii) bradycardia followed by tachycardia. The increase in heart rate slowly developed reaching a peak around 5s post stimulation whereas the bradycardia responses occurred almost instantly following the initiation of stimulation. Sole tachycardia responses occurred at 5% of sites tested electrically (3/14 animals), all were at sites within the RA region around the root of the right pulmonary vein. The most pronounced decreases in heart were produced in response to stimulation of the hilum region of the RNC and the region of the RA (Fig. 5) with the average decreases in heart rate being -25.5 ± 4.2% and -21.0 ± 12.0% respectively. A single incidence of bradycardia followed by tachycardia was noted at 1 locus within the RNC (with a heart rate first decreasing from 155 to 121 and then increasing to 220bpm).

At frequencies of 20Hz or more, there was a significant reduction in the average heart rate during stimulation of loci within the RNC and RA regions (Fig. 5B). Small reductions in heart rate were noted during stimulation of loci within the LNC and PVCV (Figs. 5D,E).

Left ventricular pressure (LVP) and monophasic action potential duration (MAPD) changes

There was no statistically significant difference in mean LVP during electrical stimulation compared to baseline between sites stimulated in each of the 4 regions as well as no significant difference in the percentage change in LVP when each group was compared. Monophasic action potential duration (MAPD) was recorded during all experiments but no significant change in MAPD was evident even during significant heart changes.

Dromotropic changes during electrical stimulation

Atrioventricular conduction during constant atrial pacing was measured by the interval between the pacing stimulus and the ventricular monophasic action potential recorded from the left ventricle. At 9 sites out of 54 tested, significant prolongations of AVC ranging from more than 20ms to atrioventricular block (AVB) were measured. Significant AV prolongation occurred during electrical stimulation of at least one locus within RA, RNC, and PVCV out of the 4 regions examined. Stimulation at sites within the LNC failed to elicit significant changes in AV delay at any of the stimulated loci. The largest changes in AV conduction occurred following stimulation within the RA, the only region where electrical stimulation elicited atrioventricular block (AVB) (Fig. 6)

Pharmacology of chronotropic responses to electrical stimulation

The effects of pharmacological blockade are summarised in Figure 7. All heart rate responses to electrical stimulation were abolished in the presence of the cholinergic nicotinic receptor antagonist hexamethonium. The bradycardic response was blocked by the cholinergic muscarinic receptor blocker atropine and the tachycardic response was blocked by the beta adrenergic receptor antagonist metoprolol. None of these pharmacological antagonists had an effect on baseline values which is especially evidenced by the lack of any changes during tests in regions LNC and PVCV (Fig. 7).

Immunohistochemical characterisation and distribution of the rabbit ICNS

Both atrial and ventricle preparations were examined and results included in the following descriptions. The location of intrinsic cardiac neuronal somata and ganglia positive for ChAT and/or TH and/or nNOS was reproducible from animal to animal. Despite this, the precise anatomical location and size of individual ganglia varied between hearts for all antibodies tested. Neurons immunoreactive (IR) only for ChAT, or TH or nNOS were consistently located on the heart hilum, at the root of the pulmonary veins and at the root of the right cranial vein, corresponding to regions where neurons have previously been identified using the pan neuronal AChE stain by Saburkina *et al.* [1].

ChAT-IR neurons were significantly more abundant than all other phenotypes studied, with an average of 1946 ± 668 neuronal somata per heart (Table 2). ChAT-IR somata formed large ganglia with a large proportion of neuronal somata of the left and right neuronal clusters being cholinergic. In addition, a significant proportion of smaller ganglia contained cholinergic neurons (Fig.8). In comparison, markedly smaller numbers of neurons immunoreactive for TH or nNOS were identified (326 ± 106 and 111 ± 20 respectively) (Table.2). Neurons IR for TH or nNOS were often found dispersed throughout larger ganglia containing numerous cell phenotypes (Fig.8). Neurons which were solely nNOS positive were also identified forming smaller ganglia (Fig. 8L) both atrially and on the ventricular epicardium. As well as singularly labelled cells (i.e. ChAT only, TH only or nNOS only), numerous ganglia containing biphenotypic neurons were observed (Fig. 8). These biphenotypic neurons were consistently present and also found dispersed throughout ganglia. When comparing the morphological profile of neurons immunoreactive for ChAT, TH and nNOS, there was no significant difference in cellular dimensions including cell area and length (Table. 2).

In all hearts examined, intrinsic cardiac ganglia were regularly interconnected by thinner commissural nerves reactive for ChAT, TH and nNOS. Numerous thin commissural nerves were recognised epicardially connecting ganglia on the heart hilum, at the roots of the pulmonary veins and the root of the right cranial vein; extending between individual ganglia (Fig. 8).

Larger ChAT-IR and TH-IR nerve fibres passed epicardially over the venous portion of the heart hilum, extending to numerous ganglia. In all preparations examined for ChAT and TH immunoreactivity, larger bundles of nerve fibres were present with ChAT-IR axons extending in parallel to TH-IR axons. The majority of large neural bundles however contained primarily TH-IR axons including nerves accessing the heart on the medial side of the root of the right cranial vein (Figs. 8D, E and F). These neural bundles ran adjacent to large ganglia where the predominant neurochemical phenotype was cholinergic (Fig. 8).

DISCUSSION

This is the first study to investigate the functional effects of activating sites within ganglia of the ICNS in rabbit hearts. Furthermore unlike previous studies on dogs [30], the tests were performed on a Langendorff perfused heart preparation to avoid the influence of extrinsic autonomic nerves and circulatory factors. The results of this study also characterise the immunohistochemical profile of the rabbit atrial epicardial network as a whole for the first time.

Using pharmacological and electrical stimuli applied to discrete sites, the results indicate that these stimuli activated neurons and synapses that lie in the intrinsic neural plexus located within the right atrial, right neuronal cluster, left neuronal cluster and pulmonary vein-caudal vein regions (Fig. 1). These are regions within which electrical stimulation at discrete sites has previously been shown to modify sino-atrial rate and atrio-ventricular conduction in anaesthetised dogs [3] or mice [26]. The similarity of the present data in the isolated perfused rabbit heart would appear to strengthen the conclusions of the latter studies that the ICNS can independently influence cardiac functions. However, electrical current will activate afferent and efferent axons traversing the ganglia as well as nearby neuronal somata and dendrites. Therefore, in order to discriminate the elements affected at different sites, in addition we separately tested the action of nicotine, a postsynaptic receptor stimulant. Both nicotine and electrical stimulation was applied at discrete sites in different experiments, which accorded with the location of intrinsic cardiac ganglia as observed in our immunohistological study. At several loci in both the right and left neuronal complex (Fig. 2) both types of stimuli could evoke similar cardiac effects such as increases in heart rate alone, decreases in heart rate alone or a biphasic effect, as well as decreases in atrio-ventricular conduction. Therefore, it appears most likely that the effects of either stimulus on cardiac activity were caused by activating ICNS neurons acting as components of the efferent limb of the ICNS rather than extrinsic nerve terminals. Despite similar cardiac responses being elicited between individual hearts, known anatomical variability of the ganglionated nerve plexus [1] as well as the dispersed distribution of intrinsic ganglionic cells between different ganglia and neuronal cluster is hypothesised to be the reason for the highly variable nature of responses to stimulation [4].

By performing this study in the Langendorff perfused heart preparation, it is possible to interpret the functional effects of electrical and nicotinic stimulation in the absence of extrinsic autonomic inputs. The application of nicotine at discrete sites produced responses in line with those shown previously in anaesthetised canines [4]. Previous functional studies investigating the effects of nicotinic stimulation of intrinsic cardiac ganglia illustrated similar responses induced in both *in situ* intact preparations as well as in acutely decentralised preparations [4, 31]. By comparison, prior study utilising the auto-transplant model to investigate the effect of loss of the intrinsic-extrinsic cardiac autonomic interaction indicated that neurons found in acutely transplanted heart preparations were still capable of considerable cardiac augmentation, albeit with slightly reduced responses compared with those noted prior to transplantation [32], further reiterating the involvement of intrinsic cardiac ganglia in the contribution to modulation of cardiac function, independent of central neural inputs.

Electrical and nicotinic stimulation of ganglia within the right atrial region demonstrated their dominance, compared to the left located ganglia in the control of heart rate. This is consistent with previous functional studies in dogs [4] and is congruent with anatomical data from several species and with the present study in rabbits. It accords with anatomical studies in rabbits [1] which reported right atrial ganglia located along nerves extending epicardially from the right neuronal cluster, along with minute epicardial ganglia coursing towards the region of the sinus node. In comparison, stimulation of ganglia at the limits of the heart heart hilum had little or no effect on haemodynamic parameters or MAPD. It has previously been comprehensively demonstrated that myocardial innervation of the rabbit ventricles occurs mainly via the epicardial nerve plexus (ENP) [33], however the results shown here draw attention to the likely possibility that the effects of the ENP on the ventricular myocardium are highly localised and further investigation into the complete involvement of the ENP on ventricular electrophysiology is warranted.

Notable differences between electrical and nicotinic stimulation were evident in this study. The electrical current effects in the left neuronal cluster were absent or weak. The reason for this are unclear but it may have been a consequence of the very short pulse duration of 0.1ms and could be understood to suggest that either the current strength was insufficient or that there were few neuronal somata in these loci. Unfortunately, a direct comparison between both methods of stimulation at the same site could not be made due to technical difficulties in placing electrodes or micropipettes at the same locations. However, the results serve to emphasise the complexity of the ICNS and the individual capabilities of specific ganglia.

The incidence of biphasic effects is unsurprising since stimuli were applied to ganglia in which networks of connected neurons are present as can be seen on whole mount preparations. Spread of chemical or electrical current from a micropipette or a bipolar electrode would be difficult to restrict in such an environment. Nonetheless the fact that on many occasions sole bradycardia or sole tachycardia was observed, suggests that at these loci neurons were more isolated or in smaller functionally similar groups which concurs with the neuroanatomy.

There was a greater incidence of bradycardiac effects, which were mainly associated with right neuronal complexes. This accords with the predominance of immunohistochemically identified cholinergic neurons we located in these regions. Whilst some of these neurons are likely to be parasympathetic postganglionic cells, the anatomical evidence also indicated there were many cholinergic neurons with fine commissural inter-ganglion connections at these locations. Therefore, it would be surprising if they had not also been stimulated. We acknowledge that with micropipette application of solutions or electrical stimulation it is difficult to limit the area stimulated but the rapid onset of a response indicated that neurons or fibres close to the tip of the micropipette or the bipolar silver wire electrode were likely responsible for the effects.

In comparison to the effects of stimulation of intracardiac ganglia on cardiac chronotropy, one noteworthy effect was the influence of ganglia within the PVCV region on atrioventricular conduction with significant AV prolongation evoked by activation of sites within this region (commonly around the root of the caudal vein or inferior vena cava) being evident in the rabbit. This was a region from which very small heart rate changes could be elicited. This suggests that these ganglia play a dominant role in AV nodal innervation and not SA nodal control, correlating with and emphasising previous findings in dogs [4, 34].

Therefore these data obtained in an isolated heart preparation expand previous more limited results from anaesthetised dogs [3, 4, 34]. Thus the rabbit accords with the hypothesis that the mammalian heart possesses its own nervous system whereby groups of neurons connect with spatially diverse intrinsic cardiac ganglia to influence myocyte activity and function [7, 8].

The present study also tested the synaptic interaction and junctional receptor transmission involved in the cardiac responses. We showed that the chemical or electrical effects we observed depended on cholinergic nicotinic ganglion transmission and that bradycardiac effects were mediated by cholinergic muscarinic receptors whilst tachycardic effects were

mediated via postganglionic beta adrenoreceptor sites. These observations are unsurprising but are the first of their kind. Unlike in previous studies, where the focus has been on either the quantitative or morphometric characterisation [1] of neuronal somata related to distinct regions of the heart [15, 35], the study investigates and characterises the atrial epicardial network in the rabbit as a whole. The immunohistochemical study of whole mount preparations of atria and ventricles demonstrated a wide phenotypic complexity of the intrinsic cardiac nerve plexus indicating that the cholinergic and adrenergic connections provide only a brief glimpse of the functional neurochemistry of the ICNS [10, 11, 13, 15, 29, 36]. In this context and of particular significance was the clear demonstration of neurons immunoreactive solely for nNOS with nerve fibres connecting with neurons in linking ganglia (Fig. 8L). Exploration of the physiology of these nNOS neurons requires further study but there is an important correlation of the present data to our earlier studies in isolated innervated rabbit heart preparations, showing the protective effects of cervical vagus nerve stimulation against ventricular fibrillation to be solely dependent on nitrergic postganglionic fibres [27, 37-39]. The results shown here are the first to provide anatomical support for the likely involvement of these neurons in the neurocardiac effects.

CONCLUSION

Over recent decades it has become increasingly evident that the ICNS is a key network involved in the cardiac neuronal hierarchy. The results of this study reveal the previously uncharacterised neurochemical phenotype of the atrial epicardial network as well as drawing attention to a significant capability of clusters of neurons in independently and selectively modulating cardiac electrophysiology. This study therefore provides an important foundation for further exploration into the involvement and potential therapeutic target of the ICNS in cardiac disease.

Funding acknowledgements

Work included in this manuscript has been supported by British Heart Foundation Programme Grant (RG/17/3/32774). EA and KEB were both supported on a British Heart Foundation Intermediate Basic Science Fellowship (FS 12/2/29300). DHP was supported on a grant from the Research Council of Lithuania (MIP-13037).

Disclosures: None

REFERENCES

1. Saburkina, I., L. Gukauskiene, K. Rysevaite, K.E. Brack, A.G. Pauza, N. Pauziene, and D.H. Pauza, *Morphological pattern of intrinsic nerve plexus distributed on the rabbit heart and interatrial septum.* J Anat, 2014. **224**(5): p. 583-93.
2. Wake, E. and K. Brack, *Characterization of the intrinsic cardiac nervous system* Auton Neurosci, 2016. **199**: p. 3-16.
3. Butler, C.K., F.M. Smith, R. Cardinal, D.A. Murphy, D.A. Hopkins, and J.A. Armour, *Cardiac responses to electrical stimulation of discrete loci in canine atrial and ventricular ganglionated plexi.* Am J Physiol 1990. **259**(5): p. H1365-H1373.
4. Cardinal, R., P. Page, M. Vermeulen, J.L. Ardell, and J.A. Armour, *Spatially divergent cardiac responses to nicotinic stimulation of ganglionated plexus neurons in the canine heart.* Auton Neurosci, 2009. **145**(1-2): p. 55-62.
5. Armour, J.A., M.H. Huang, and F.M. Smith, *Peptidergic modulation of in situ canine intrinsic cardiac neurons.* Peptides, 1993. **14**(2): p. 191-202.
6. Brack, K.E., *The heart's 'little brain' controlling cardiac function in the rabbit* Exp Physiol, 2015. **100**(4): p. 348-353.
7. Armour, J.A., *The little brain on the heart* Cleveland Clinic Journal of Medicine 2007. **74**: p. S48-51.
8. Randall, W.C., R.D. Wurster, D.C. Randall, and S.X. Xi-Moy, *From cardioaccelerator and inhibitory nerves to a heart brain: an evolution of concepts.*, in *Nervous Control of the Heart* S. J.T. and S.F. Vainer, Editors. 1996, Hardwood Academic Publishers: Amsterdam. p. 173-200.
9. Pauza, D.H., V. Skripka, and N. Pauziene, *Morphology of the intrinsic cardiac nervous system in the dog: a whole-mount study employing histochemical staining with acetylcholinesterase.* Cells Tissues Organs, 2002. **172**(4): p. 297-320.
10. Pauza, D.H., I. Saburkina, K. Rysevaite, H. Inokaitis, M. Jokubauskas, J. Jalife, and N. Pauziene, *Neuroanatomy of the murine cardiac conduction system: a combined stereomicroscopic and fluorescence immunohistochemical study.* Auton Neurosci, 2013. **176**(1-2): p. 32-47.
11. Richardson, R.J., I. Grkovic, and C.R. Anderson, *Immunohistochemical analysis of intracardiac ganglia of the rat heart.* Cell Tissue Res, 2003. **314**(3): p. 337-50.
12. Thompson, G.W., K. Collier, J.L. Ardell, G. Kember, and J.A. Armour, *Functional interdependence of neurons in a single canine intrinsic cardiac ganglionated plexus.* J Physiol, 2000. **528**(Pt 3): p. 561-71.
13. Rysevaite, K., I. Saburkina, N. Pauziene, R. Vaitkevicius, S.F. Noujaim, J. Jalife, and D.H. Pauza, *Immunohistochemical characterization of the intrinsic cardiac neural plexus in whole-mount mouse heart preparations.* Heart Rhythm, 2011. **8**(5): p. 731-8.
14. Horackova, M., J. Slavikova, and Z. Byczko, *Postnatal development of the rat intrinsic cardiac nervous system: a confocal laser scanning microscopy study in whole-mount atria.* Tissue Cell, 2000. **32**(5): p. 377-88.
15. Hoover, D.B., E.R. Isaacs, F. Jacques, J.L. Hoard, P. Page, and J.A. Armour, *Localization of multiple neurotransmitters in surgically derived specimens of human atrial ganglia.* Neuroscience, 2009. **164**(3): p. 1170-9.
16. Brack, K.E., J. Winter, and G.A. Ng, *Mechanisms underlying the autonomic modulation of ventricular fibrillation initiation--tentative prophylactic properties of vagus nerve stimulation on malignant arrhythmias in heart failure.* Heart Fail Rev, 2013. **18**(4): p. 389-408.
17. Ng, G.A., *Neuro-cardiac interaction in malignant ventricular arrhythmia and sudden cardiac death.* Auton Neurosci, 2016. **199**: p. 66-79.

18. Brack, K.E., J.H. Coote, and G.A. Ng, *Interaction between direct sympathetic and vagus nerve stimulation on heart rate in the isolated rabbit heart.* Exp Physiol, 2004. **89**(1): p. 128-39.
19. Ng, G.A., K.E. Brack, and J.H. Coote, *Effects of direct sympathetic and vagus nerve stimulation on the physiology of the whole heart - a novel model of isolated Langendorff perfused rabbit heart with intact dual autonomic innervation. .* Exp Physiol, 2001. **86**(3): p. 319-29.
20. Huang, M.H., J.L. Ardell, B.D. Hanna, S.G. Wolf, and J.A. Armour, *Effects of transient coronary artery occlusion on canine intrinsic cardiac neuronal activity.* Integr Physiol Behav Sci, 1993. **28**(1): p. 5-21.
21. Yuan, B.X., J.L. Ardell, D.A. Hopkins, A.M. Losier, and J.A. Armour, *Gross and microscopic anatomy of the canine intrinsic cardiac nervous system.* Anat Rec 1994. **239**(1): p. 75-87.
22. Zarzoso, M., K. Rysevaite, M.L. Milstein, C.J. Calvo, A.C. Kean, F. Atienza, D.H. Pauza, J. Jalife, and S.F. Noujaim, *Nerves projecting from the intrinsic cardiac ganglia of the pulmonary veins modulate sinoatrial node pacemaker function.* Cardiovasc Res, 2013. **99**(3): p. 566-75.
23. Brack, K.E., R. Narang, J. Winter, and G.A. Ng, *The mechanical uncoupler blebbistatin is associated with significant electrophysiological effects in the isolated rabbit heart.* Exp Physiol, 2013. **98**(5): p. 1009-27.
24. Patel, V.H., K.E. Brack, J.H. Coote, and G.A. Ng, *A novel method of measuring nitric-oxide-dependent fluorescence using 4,5-diaminofluorescein (DAF-2) in the isolated Langendorff-perfused rabbit heart.* Pflugers Arch, 2008. **456**(3): p. 635-45.
25. Winter, J., K.E. Brack, J.H. Coote, and G.A. Ng, *Cardiac contractility modulation increases action potential duration dispersion and decreases ventricular fibrillation threshold via β1-adrenoceptor activation in the crystalloid perfused normal rabbit heart.* Int J Cardiol, 2014. **172**(1): p. 144-54.
26. He, B., Z. Lu, W. He, L. Wu, B. Cui, X. Hu, L. Yu, C. Huang, and H. Jiang, *Effects of ganglionated plexi ablation on ventricular electrophysiological properties in normal hearts and after acute myocardial ischemia.* Int J Cardiol, 2013. **168**(1): p. 86-93.
27. Brack, K.E., J.H. Coote, and G.A. Ng, *Vagus nerve stimulation protects against ventricular fibrillation independent of muscarinic receptor activation.* Cardiovasc Res, 2011. **91**(3): p. 437-46.
28. Winter, J., A.S. Tanko, K.E. Brack, J.H. Coote, and G.A. Ng, *Differential cardiac responses to unilateral sympathetic nerve stimulation in the isolated innervated rabbit heart.* Auton Neurosci, 2012. **166**(1-2): p. 4-14.
29. Pauza, D.H., K. Rysevaite-Kyguoliene, J. Vismantaite, K.E. Brack, H. Inokaitis, A.G. Pauza, V. Rimasauskaite-Petraitiene, J.I. Pauzaite, and N. Pauziene, *A combined acetylcholinesterase and immunohistochemical method for precise anatomical analysis of intrinsic cardiac neural structures.* Ann Anat, 2014. **196**(6): p. 430-40.
30. Armour, J.A., *Potential clinical relevance of the 'little brain' on the mammalian heart.* Exp Physiol, 2008. **93**(2): p. 165-76.
31. Murphy, D.A., S. O'Blenes, B.D. Hanna, and J.A. Armour, *Functional capacity of nicotine-sensitive canine intrinsic cardiac neurons to modify the heart. .* Am J Physiol 1994. **266**(4): p. R1127-35.
32. Murphy, D.A., S. O'Blenes, B.D. Hanna, and J.A. Armour, *Capacity of intrinsic cardiac neurons to modify the acutely autotransplanted mammalian heart.* J Heart Lung Transplant 1994. **13**(5): p. 847-56.
33. Pauziene, N., P. Alaburda, K. Rysevaite-Kyguoliene, A.G. Pauza, H. Inokaitis, A. Masaityte, G. Rudokaite, I. Saburkina, J. Plisiene, and D.H. Pauza, *Innervation of the rabbit cardiac ventricles.* J Anat, 2016. **228**(1): p. 26-46.
34. Hou, Y., B.J. Scherlag, J. Lin, J. Zhou, J. Song, Y. Zhang, E. Patterson, R. Lazzara, W.M. Jackman, and S.S. Po, *Interactive atrial neural network: Determining the connections between ganglionated plexi.* Heart Rhythm, 2007. **4**(1): p. 56-63.

35. Inokaitis, H., N. Pauziene, K. Rysevaite-Kyguoliene, and D.H. Pauza, *Innervation of sinoatrial nodal cells in the rabbit.* Ann Anat, 2016. **205**: p. 113-21.
36. Parsons, R.L., S.A. Locknar, B.A. Young, J.L. Hoard, and D.B. Hoover, *Presence and co-localization of vasoactive intestinal polypeptide with neuronal nitric oxide synthase in cells and nerve fibers within guinea pig intrinsic cardiac ganglia and cardiac tissue.* Cell Tissue Res, 2006. **323**(2): p. 197-209.
37. Brack, K.E., V.H. Patel, J.H. Coote, and G.A. Ng, *Nitric oxide mediates the vagal protective effect on ventricular fibrillation via effects on action potential duration restitution in the rabbit heart.* J Physiol, 2007. **583**(Pt 2): p. 695-704.
38. Brack, K.E. and G.A. Ng, *Autonomic control of cardiac arrhythmia* in *Cardiac Arrhthmias: from Basic Mechanisms to State-of-the-Art Management* A.S. Kibod, et al., Editors. 2014, Springer-Verlag: London. p. 43-60.
39. Coote, J.H., *Myths and realities of the cardiac vagus.* J Physiol, 2013. **591**(Pt 17): p. 4073-85.

TABLES

Primary Antibody	Host Species	Dilution	Catalogue Number and Supplier
ChAT	Goat	1:100	AB144P Merck Millipore
TH	Mouse	1:2000	22941 Immunostar
nNOS	Mouse	1:300	SC-5302 Santa Cruz
nNOS	Sheep	1:1000	Gift from Professor P. Emson, University of Cambridge
Secondary Antibody			
Anti-goat (AF594)	Donkey	1:300	Jackson Immunoresearch
Anti-goat (FITC)	Donkey	1:100	Jackson Immunoresearch
Anti-mouse (AF594)	Donkey	1:300	Jackson Immunoresearch
Anti-mouse (FITC)	Donkey	1:100	Jackson Immunoresearch

Table 1. Primary and secondary antisera used within this study.

	ChAT n = 9	TH n = 10	nNOS n = 9	ChAT/TH n = 4	ChAT/nNOS n=3	TH/nNOS n=5
Average number of somata per heart	1946 ± 668	326 ± 106	111 ± 20	616 ± 161	203 ± 58	112 ± 36
Average number of somata per heart (range)	1014 - 3240	35 - 854	54 - 193	340 - 899	107 - 308	22 – 211
Area of neurons (μm^2)	557 ± 31	507 ± 35	478 ± 32	515 ± 30	600 ± 48	519 ± 45
Short axis (μm)	25.0 ± 0.9	22.6 ± 1.1	23.1 ± 1.3	23.2 ± 0.4	25.9 ± 1.7	22.6 ± 1.1
Long axis (μm)	32.2 ± 1.4	29.4 ± 1.6	29.6 ± 1.7	31.0 ± 1.0	32.2 ± 2.7	29.6 ± 1.6

Table 2. The mean number, range and size of immunohistochemically distinct intrinsic cardiac neuronal somata identified in whole-mount preparations of the rabbit. No significant differences were noted in cellular dimensions including cell area and length between all groups.

FIGURES

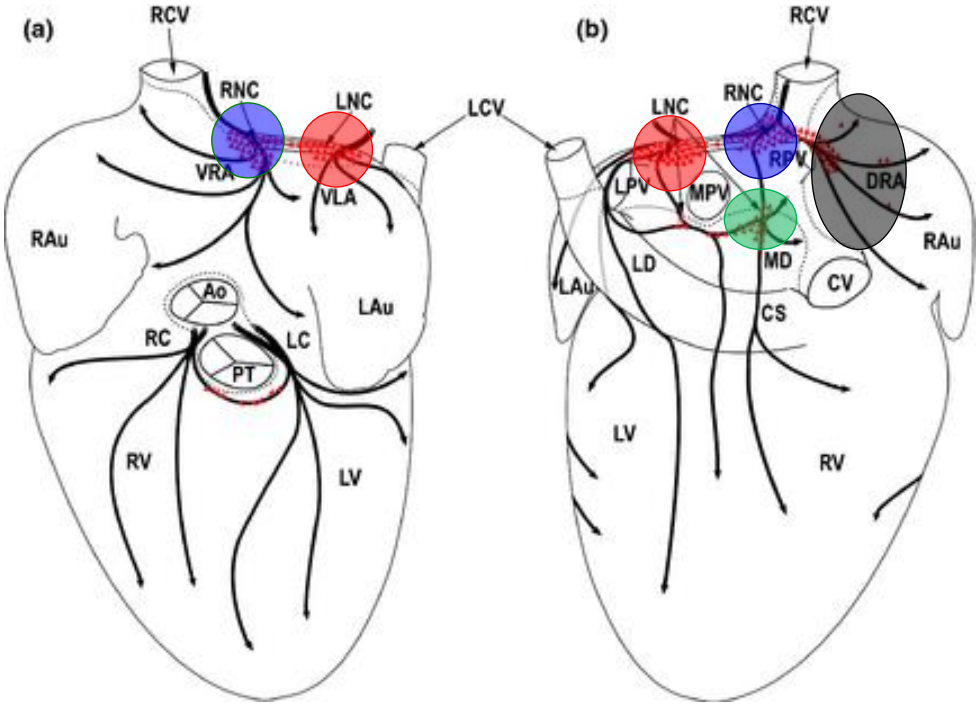
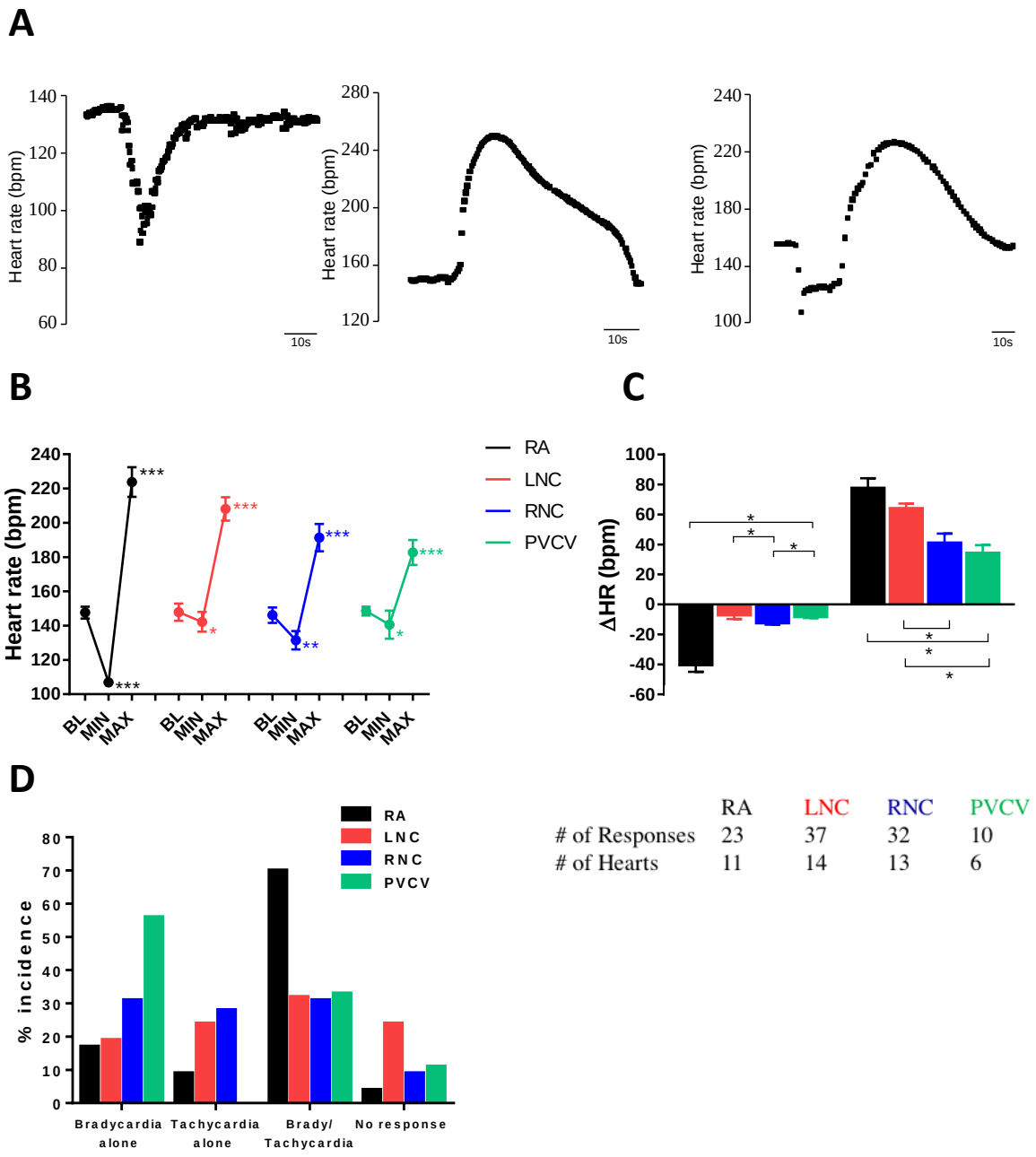


Figure 1: Anterior (a) and posterior (b) view of the heart indicating sites of ganglionic stimulation in the present study. Image modified from [1]. Red triangles indicate the location of neuronal clusters and epicardial ganglia. Abbreviations: Ao, aorta; CS, coronary sinus; CV, caudal vein; DRA, dorsal right atrial subplexus; LAu, left auricle; LC, left coronary subplexus; LCV, left cranial vein; LD, left dorsal subplexus; LNC, left neuronal cluster; LPV, left pulmonary vein; LV, left ventricle; MD, middle dorsal subplexus; MPV, middle pulmonary vein; PT; pulmonary trunk; RAu, right auricle; RC, right coronary subplexus; RCV, right cranial vein; RNC, right neuronal cluster; RPV, right pulmonary vein; RV, right ventricle; VLA, ventral left atrial subplexus; VRA, ventral right atrial subplexus.
Stimulation sites within the encircled areas
RA – right atrial ganglia region
LNC / RNC – left / right neuronal complex
PVCV – pulmonary vein-caudal vein region



	RA	LNC	RNC	PVCV
# of Responses	23	37	32	10
# of Hearts	11	14	13	6

Figure 2: Quantification of nicotine induced heart rate responses.
A. Characteristic heart rate responses by the epicardial application of nicotine to individual cardiac regions known to contain ganglia. **B**. Heart rate at baseline (BL) and during the bradycardia (Min) and tachycardia (Max) phase of the response. **C**. The change in heart rate of each phase. * P<0.05, ** P<0.01, *** P<0.001 *vs.* corresponding baseline [BL]. **D**. Incidence of each heart rate response according to response type and region. **Table:** Number of responses (nicotine applications) and hearts studied.

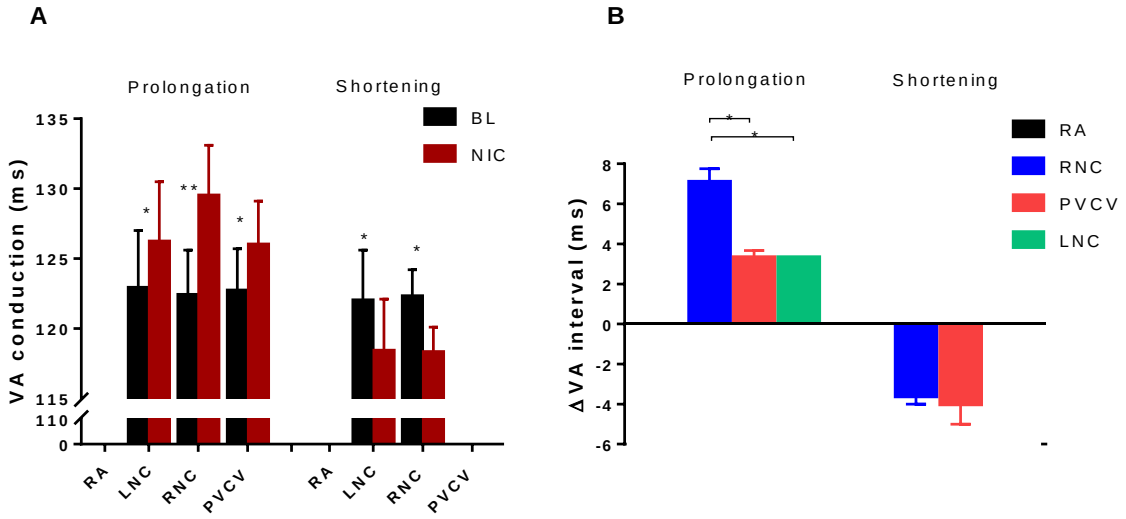


Figure 3. HR independent changes in atrioventricular conduction during constant right ventricular pacing at 250bpm

A) Mean data representing the interval from ventricular pacing to atrial electrogram activation at baseline (BL) and following nicotine (nic) application. B) Mean data representing the change in VA interval calculated from (A). *P<0.05, **P<0.01 vs BL.

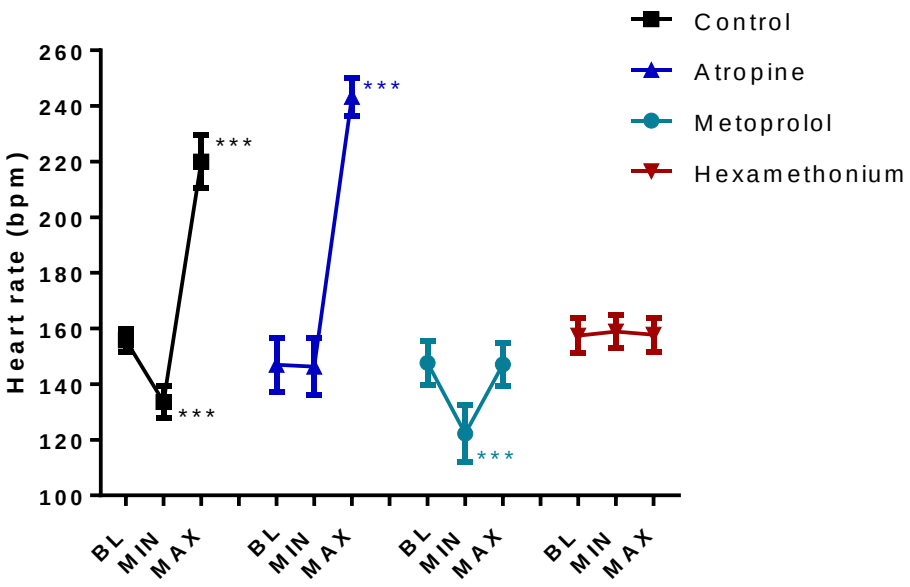


Figure 4. Pharmacological autonomic blockade of nicotine-HR response
Nicotine heart rate response during control (black), and in the presence of the muscarinic (cholinergic) receptor blocker Atropine (0.1 uM, blue, Ng et al, 2001), beta-adrenoreceptor blocker Metoprolol (1.8 uM, green, Ng *et al*, 2001) and the ganglionic blocker hexamthonium (0.5 mM, red, Hex, Brack et al, 2011). *** P<0.001 vs baseline [BL] heart rate.

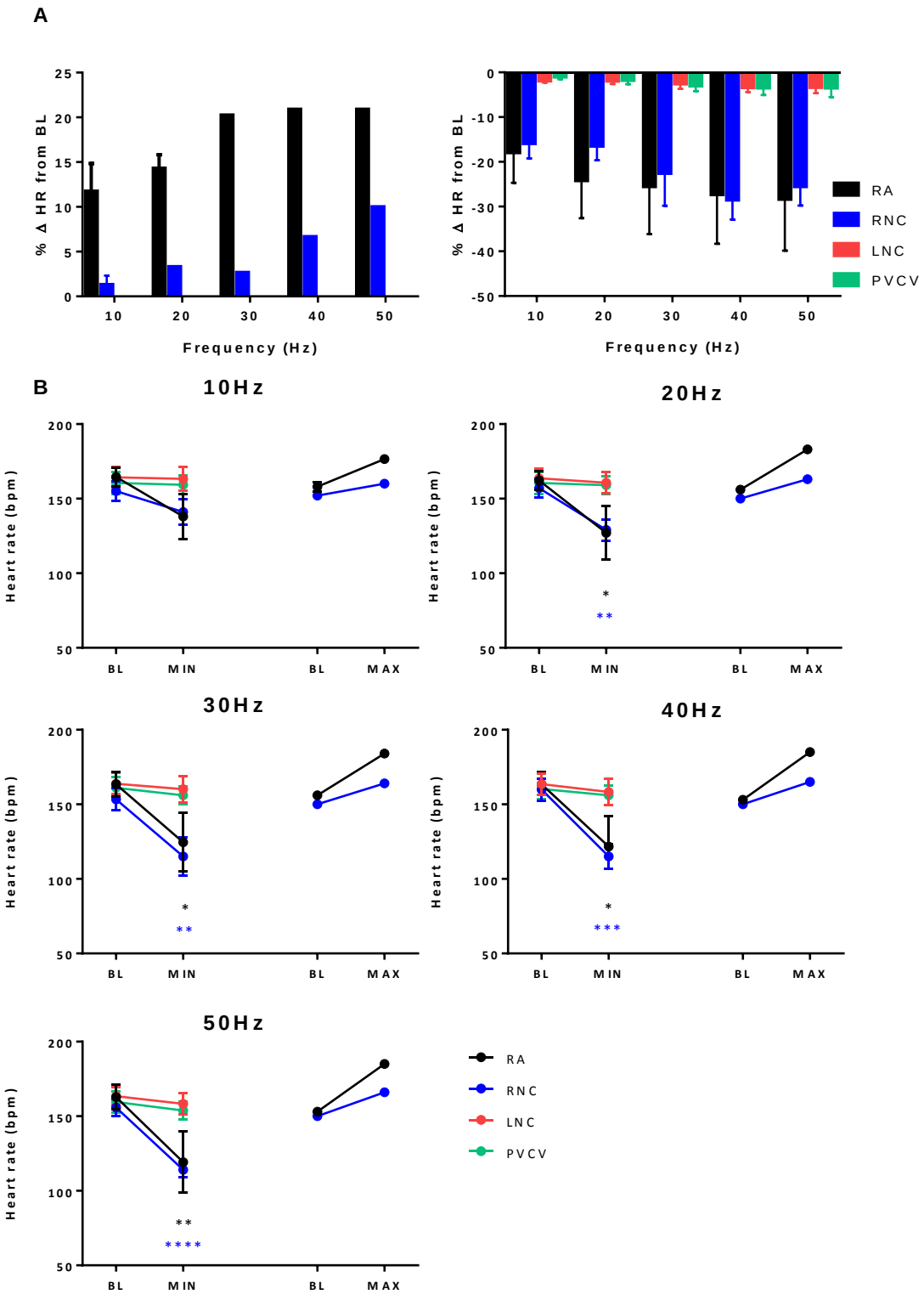


Figure 5. Heart rate responses induced by electrical stimulation at sites in RA, RNC, LNC, and PVCV. Comparison of the percentage change in HR during electrical stimulation at different regions (A). Comparison of the maximum reductions and maximum increases in heart rate compared to baseline (BL) during electrical stimulation at different frequencies (B) 10Hz, 20Hz, 30Hz, 40Hz and 50Hz at sites in the regions indicated RA (black) RNC (blue), LNC (red) and PVCV (green). Data represents mean ± SEM. *p <0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 *vs* BL heart rate.

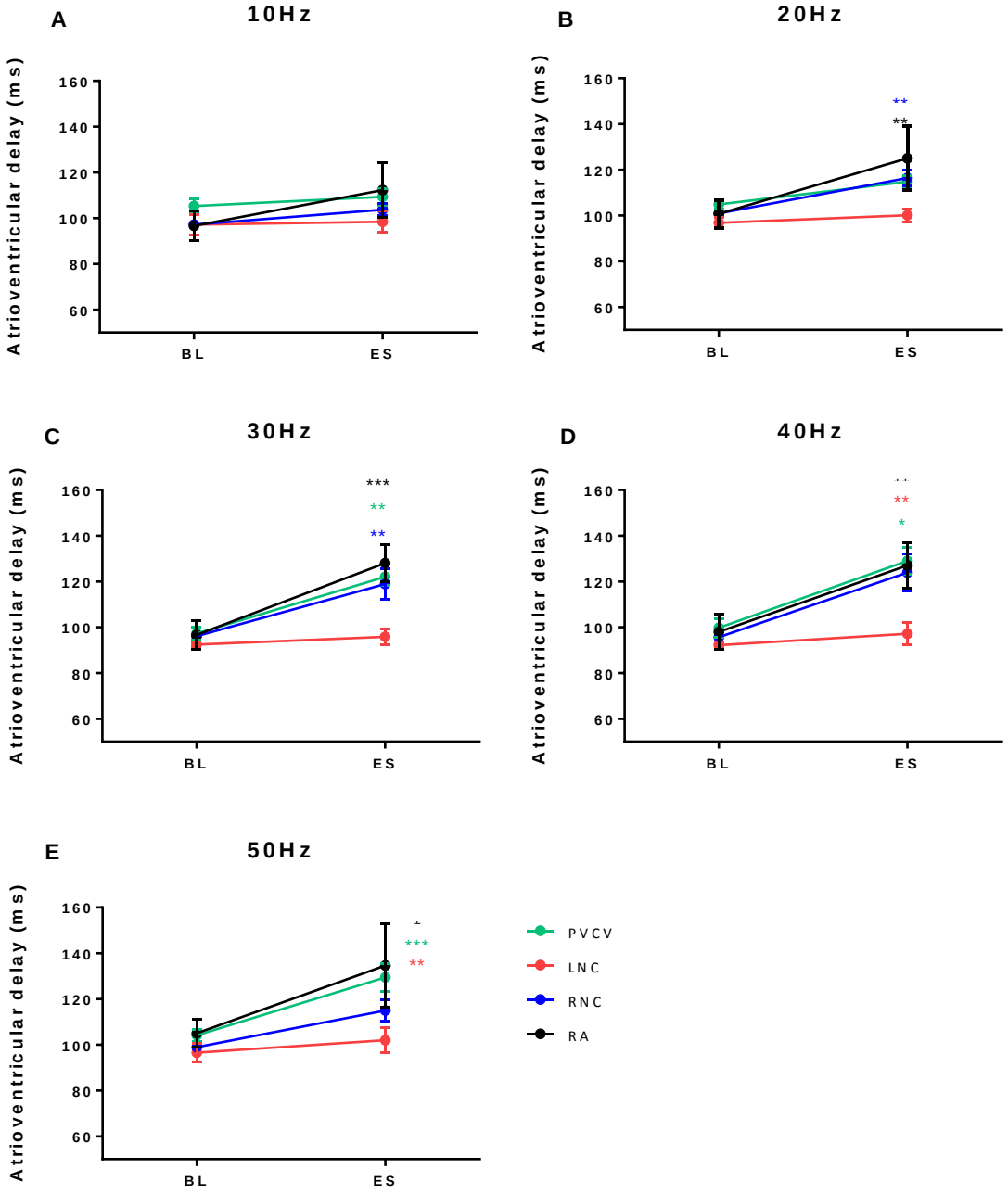


Figure 6. Electrical stimulation induced effects on atrioventricular conduction. Mean data representing the average change in AV delay during electrical stimulation of sites within intrinsic cardiac ganglia PVCV (n=9), LNC (n=7), RNC (n=27), RA (n=4) at different frequencies, A) 10Hz, B) 20Hz, C) 30Hz, D) 40Hz and E) 50Hz. Data represents mean ± SEM. BL=baseline, ES = electrical stimulation. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

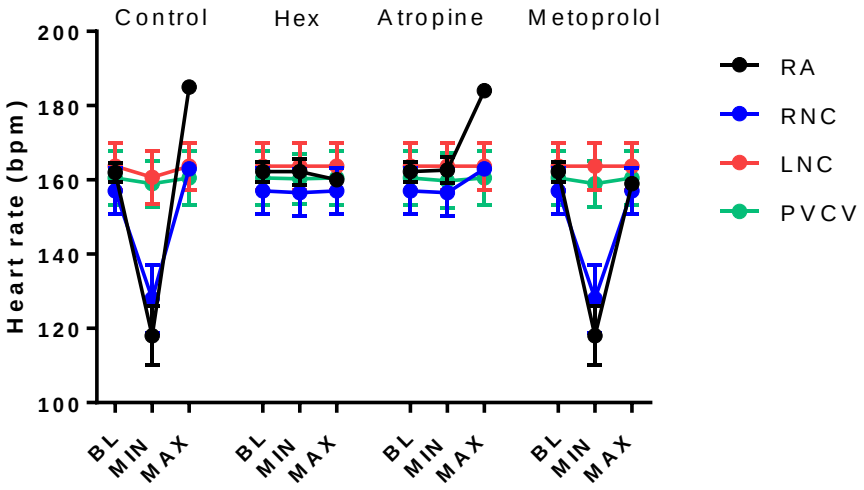


Figure 7. Pharmacological autonomic blockade of heart rate responses to electrical stimulation. Heart rate response during control and in the presence of the atropine (0.1 uM,[19]), metoprolol (1.8 uM, [19]) and hexamethonium (0.5 mM, [27]). Electrical stimulation applied at either the left neuronal cluster (LNC), pulmonary vein- caudal vein (PVCV), right atrial (RA) and right neuronal cluster (RNC). Data represents mean ± SEM. * $p < 0.001$ vs baseline [BL] heart rate.

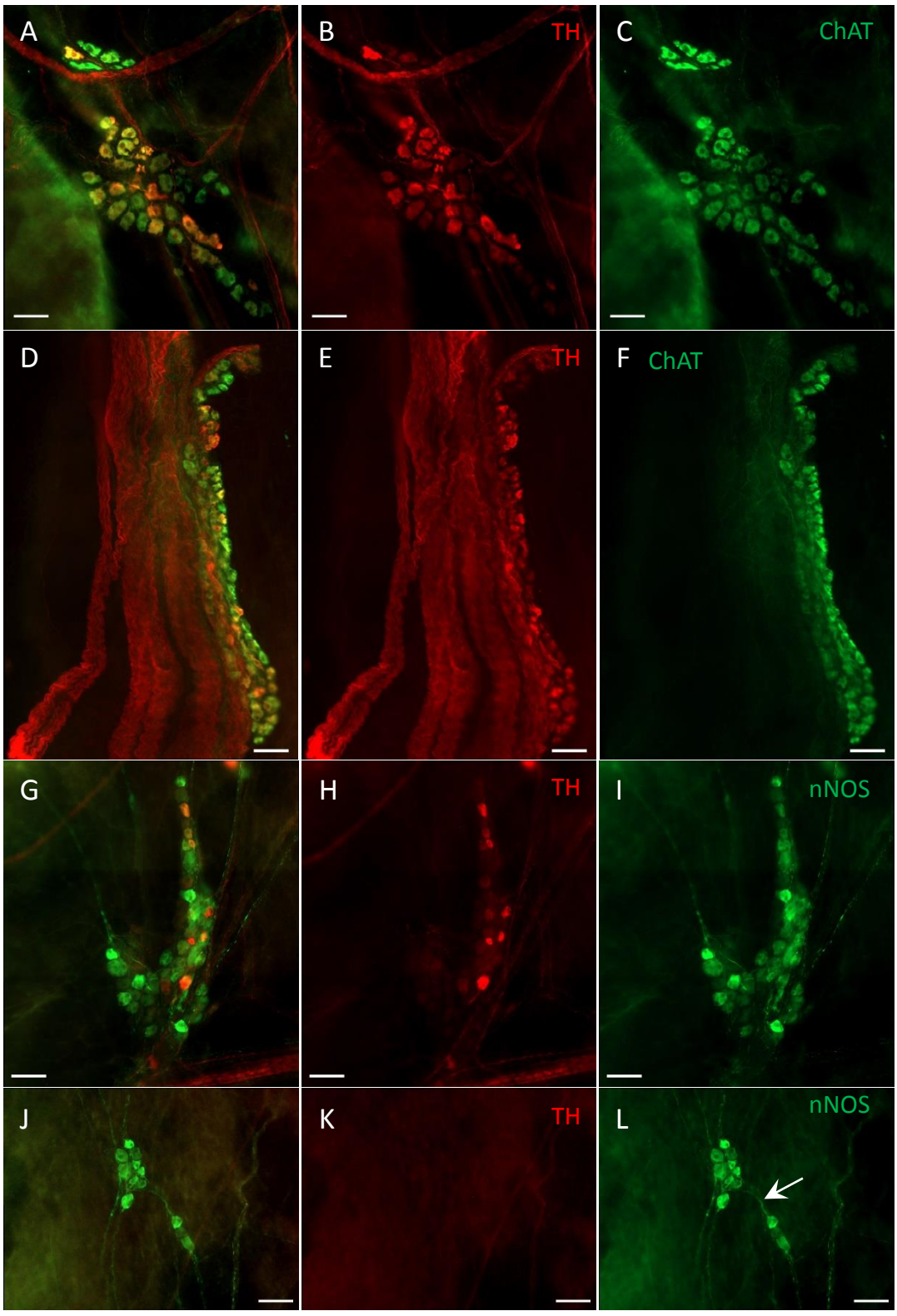


Figure 8. Microphotographs illustrating the predominance of cholinergic (ChAT-IR) neurons within ganglia. **A-C:** Representation of a ganglia containing both ChAT and TH-IR neurons located within the right neuronal cluster of the rabbit ICNS. **D – F:** Microphotographs illustrating nerves accessing the heart on the medial side of the root of right cranial vein, where TH-IR nerve fibres predominate. Microphotographs illustrating the presence of both TH-IR and nNOS-IR neurons (**G-I**) on the root of the right cranial vein. **J-L:** An illustration of a smaller ganglion located in close proximity to the ganglia shown in images G-H and

containing solely nNOS positive neurons. TH or adrenergic neuronal structures are shown in red with nNOS positive structures evident in green. Note the thin nNOS-IR nerve fibres (white arrow) connecting two small neighbouring ganglia. Scale bars represent 100µm.