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

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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

Nicotinic or electrical stimulation was applied at discrete sites of the intrinsic cardiac nerve plexus in the Langendorff perfused rabbit heart. Functional effects on sinus rate and atrioventricular conduction were measured. 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. Significant prolongation of atrioventricular conduction (AVC) 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, a rich network of intrinsic cardiac nerves that converge to form distinct ganglia and extend across the heart has been documented in mammalian species including 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, independent of extrinsic autonomic nerves [2-5]. This has given rise to the notion that such an intrinsic cardiac nervous system (ICNS) acts as the heart's '*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 and spread widely over the walls of all chambers of the heart forming an interconnected "ganglionic plexus"(GP)[8-10]. Historically, it was presumed that the intrinsic cardiac ganglia were merely parasympathetic neuronal relay stations[2,11], however, a recent review by Wake and Brack[2] showed evidence that these ganglia are neurophenotypically and neurochemically diverse[10,12,13]. This would suggest that the ganglia are also functionally diverse and could provide key knowledge in understanding the remodelling of ganglia in cardiac diseases such as heart failure and atrial/ventricular arrhythmias [14,15].

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 the Langendorff perfused rabbit heart[16] could overcome these limitations and provide a valuable model to study the integrative action of ICNS and its significance on cardiac performance in isolation of circulating and extraneuronal factors.

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 [4,17-19] and unlike earlier studies, we compared the effects of applying stimuli to loci in four different regions whilst measuring effects on cardiac electrophysiology. In addition and with the aim of providing a more complete picture of the neurotransmitter/neuromodulator profile of the rabbit intrinsic cardiac network, immunofluorescent labelling 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 PPL70/8501.

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 and following stable sedation, animals were sacrificed (see supplementary material).

Isolation of the non-innervated heart preparation

Non-innervated hearts were isolated as previously described[20,21]. In brief, animals were pre-medicated and sacrificed. Hearts were rapidly excised, placed into ice cold Tyrode solution and retrogradely perfused through the ascending aorta in conditions of constant flow Langendorff mode(40 ml/min)(see supplementary material).

Nicotinic stimulation of intrinsic cardiac ganglia

Stimulation of epicardial ganglia was performed at four regions(Fig.1) using the topographical map published previously[1]. These regions included 1) left neuronal complex(LNC), 2) right neuronal complex(RNC), 3) right atrial ganglia region(RAGP) and 4) between the middle pulmonary veins and the caudal vena cava (vena caudialis; ‘inferior vena cava’)(PVCV). Nicotine (0.1mg in 10µl saline) was directly injected into loci within LNC, RNC and PVCV and nicotine 0.1mg in 100µl saline[4] into loci within 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 stimulation, were recorded at stimulation frequencies

between 10 and 50Hz (stimulus strength 50% of cardiac pacing threshold), using a 0.1ms pulse[22].

Protocols and Pharmacological Agents

The effects of nicotine and electrical stimulation were determined both during sinus rhythm or constant cardiac pacing. To determine which types of autonomic receptors were involved in the cardiac responses, protocols were repeated in the presence of pharmacological blockers (see supplementary material).

Signal measurements and analysis

Functional responses were recorded with a PowerLab 16 channel system and digitised at 2kHz using Chart and Scope software (ADInstruments Ltd)(supplementary material).

Immunohistochemical analysis

In addition to studying the influence of spatially divergent ganglia on cardiac electrophysiology, a further 18 animals were used for immunohistochemical analysis. Immunofluorescent labelling for ChAT, TH and nNOS antibodies was performed on whole-mount atrial and ventricular preparations and subsequent microscopic and quantitative analysis completed (supplementary material).

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

I.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 - LNC in all 14 animals; RNC, RAGP and PVCV region tested in 13, 11 and 6 animals respectively.

Chronotropic and dromotropic responses following nicotine application

Following nicotine application, HR changes occurred in three distinct patterns: i) bradycardia alone, ii) tachycardia alone and iii) a biphasic response of bradycardia followed by tachycardia (Fig.2). No region contained sites which produced a single type of HR change. At PVCV and RAGP, bradycardia and biphasic HR responses prevailed.

Nicotine applied to RAGP produced the strongest bradycardia or tachycardia, whilst LNC produced the smallest HR decrease, and PVCV region the smallest HR increase (Fig.2).

During ventricular pacing, nicotine applied to RAGP sites failed to elicit any change in VA conduction, whilst nicotine applied to PVCV produced a prolongation in 60% of cases, with no effect in the remaining 40%. A combination of VA prolongation and VA shortening was observed when stimulating LNC and RNC, but there was a larger proportion resulting in VA prolongation (65% *vs*. 35%) at RNC. Quantitatively, there was no difference in the VA shortening when stimulating LNC / RNC. There was however a greater degree of VA prolongation at RNC compared to others, with LNC and PVCV sites being equipotent (Fig.3).

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

Electrical stimulation of intrinsic cardiac ganglia was examined in 14 animals - RNC in all 14 animals; LNC, RAGP and PVCV in 8, 9 and 8 animals respectively.

Chronotropic responses to electrical stimulation

The effects of electrical stimulation (10-50Hz) were examined at 56 sites. Decreases in HR occurred on stimulation at sites in all regions tested. As with nicotine, electrical stimulation of sites in ganglia, could elicit three types of response. Tachycardia occurred at 5% of sites tested electrically (3/14), all at RA. The most pronounced HR decreases were produced at RNC and RA regions (Fig.4A) (-25.5±4.2% and -21.0±12.0% respectively). A single biphasic response was noted at 1 locus within the RNC (155 to 121 to 220bppm).

At frequencies ≥20Hz, there was a significant reduction in the average HR during stimulation within RNC and RA (Fig.4B). Small HR reductions were noted during stimulation within LNC and PVCV (Fig.4).

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 %change in LVP when each group was compared. There was also no significant change in monophasic action potential duration (MAPD).

Dromotropic changes during electrical stimulation

Atrioventricular conduction (AVC) during constant atrial pacing was measured from the pacing stimulus to the beginning of ventricular MAP. At 9 sites out of 54 tested, significant prolongations of AVC ranging from >20ms to atrioventricular block (AVB) were measured. Significant AV prolongation occurred during electrical stimulation of at least one locus within RA, RNC, and PVCV. Stimulation within LNC failed to elicit changes in AVC. The largest AVC changes occurred in stimulation within RA, the only region where electrical stimulation elicited atrioventricular block (AVB)(Fig.5).

III.Pharmacology of chronotropic responses to nicotinic and electrical stimulation

The effects of pharmacological blockade are summarised in Figure.6. All HR responses to electrical or nicotinic stimulation were abolished in the presence of hexamethonium. The bradycardic responses were blocked by atropine and tachycardic responses blocked by metoprolol. None of these pharmacological antagonists had effects on baseline values during electrical stimulation, especially evidenced by lack of changes during tests at LNC and PVCV (Fig.6).

Immunohistochemical characterisation and distribution of the rabbit ICNS

The location of intrinsic cardiac neuronal somata and ganglia positive for ChAT and/or TH and/or nNOS was reproducible from animal to animal in both atrial and ventricle preparations. Despite this, the precise anatomical location and size of individual ganglia varied between hearts. Neurons immunoreactive (IR) only for ChAT, or TH or nNOS were consistently located at the heart hilum, root of pulmonary veins and root of the right cranial vein (RCV), corresponding to neurons previously identified in these regions[1].

ChAT-IR neurons were significantly more abundant than all other phenotypes studied (1946±668 neuronal somata per heart)(Table.1). ChAT-IR somata formed large ganglia with a large proportion of neuronal somata of LNC and RNC being cholinergic. In comparison, markedly smaller numbers of neurons immunoreactive for TH or nNOS were identified (326±106 and 111±20 respectively)(Table.1). Neurons IR for TH or nNOS were often found dispersed throughout larger ganglia containing numerous cell phenotypes(Fig.7). Neurons which were solely nNOS positive were also identified forming smaller ganglia(Fig.7L) both atrially and on the ventricular epicardium. As well as singularly labelled cells(i.e. ChAT, TH *or*

nNOS only), numerous ganglia containing biphenotypic neurons were observed(Fig.7) which were consistent and dispersed throughout ganglia.

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 roots of pulmonary veins and root of RCV; extending between individual ganglia(Fig.7).

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 RCV(Figs.7D,E,F). These neural bundles ran adjacent to large ganglia where the predominant neurochemical phenotype was cholinergic(Fig.7).

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[25], 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 RA, RNC, LNC and PVCV regions(Fig.1). These are regions within which stimulation has previously been shown to modify sino-atrial rate and atrio-ventricular conduction in anaesthetised dogs[3] or mice[22]. 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, we separately tested the action of nicotine, a postsynaptic receptor stimulant. At several loci at both RNC and LNC (Figs.2,3) both types of stimuli could evoke similar cardiac effects such as HR increase alone, HR decrease alone or a biphasic effect, as well as effects on 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[4,26]. 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[27], 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 RA region demonstrated their dominance, compared to left sided ganglia in HR control. This is consistent with previous functional studies in dogs[4] and 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)[28], 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 is unclear but 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 neurons at these loci were more isolated or in smaller functionally similar groups which concurs with the neuroanatomy.

An interesting observation was the significant AV prolongation evoked at PVCV (commonly around the root of the caudal vein or inferior vena cava). This was a region from which very small HR changes could be elicited which suggests that these ganglia play a dominant role in AV nodal innervation rather than SA nodal control, correlating with previous findings in dogs[4,29]. Taken together these data support the notion that the mammalian heart possesses its own nervous system whereby groups of neurons connect with spatially diverse intrinsic cardiac ganglia to influence myocardial activity and function [7,30].

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 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 activation. 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[13,31], 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[9,10,12,13,32,33]. 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.7L). 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 [23,34,35]. 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.

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Disclosures: None

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TABLE 1

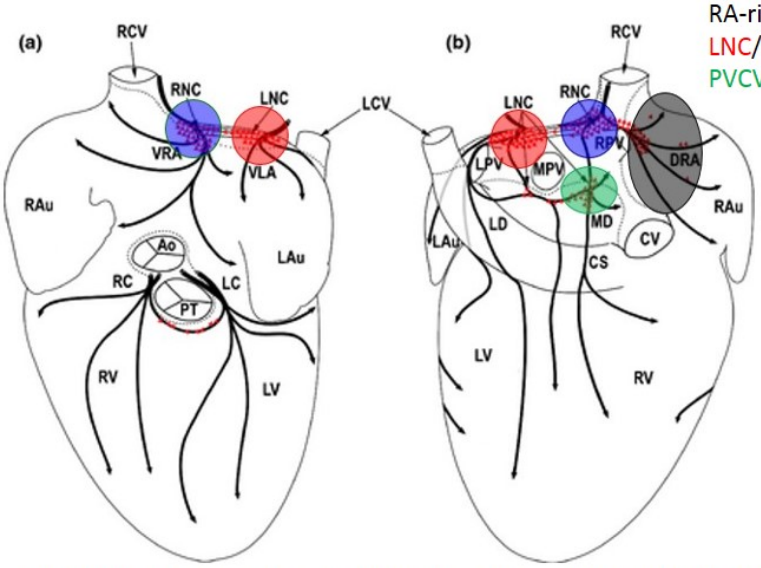
The mean number, range and size of immunohistochemically distinct intrinsic cardiac neuronal somata identified in whole-mount preparations of the rabbit.

	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

FIGURES

Figure 1.

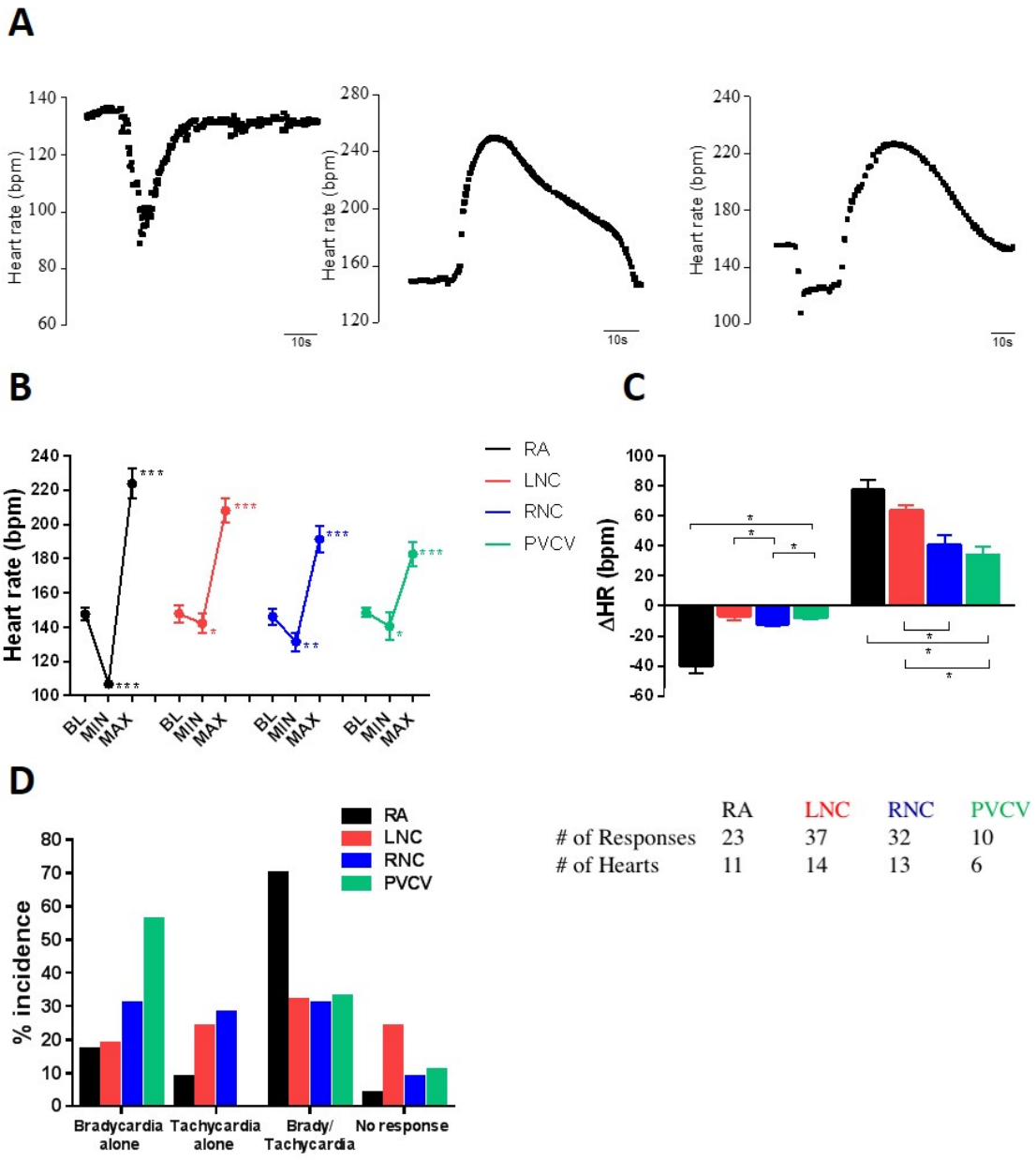
Stimulation sites within the encircled areas
RA-right atrial ganglia region
LNC/RNC–left/right neuronal complex
PVCV–pulmonary vein-caudal vein region



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.

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.

Figure 2.



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

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

Figure 3.

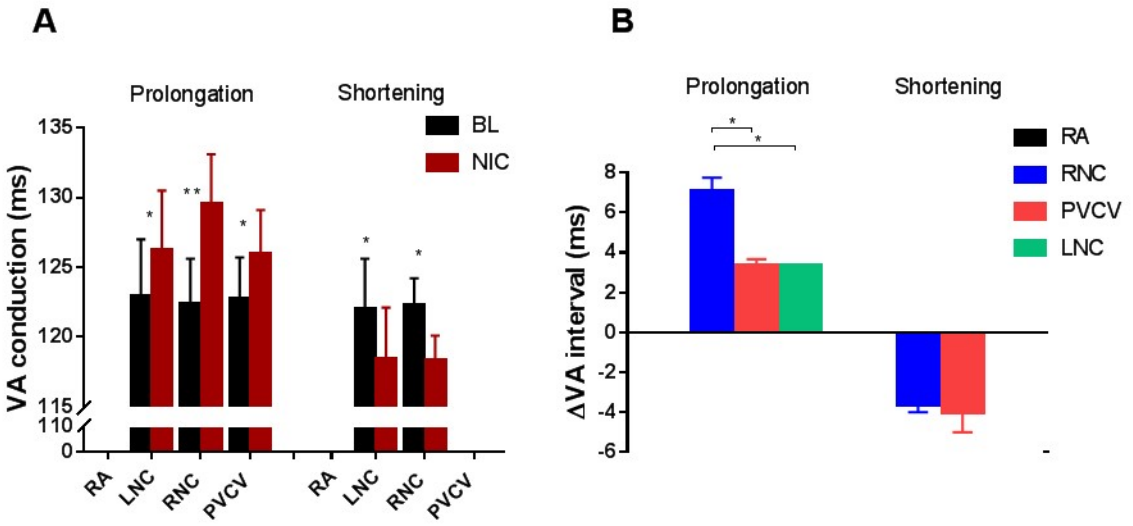


Figure 3. HR independent changes in atrioventricular conduction during constant right ventricular pacing.
A) Mean data representing the interval from ventricular pacing to atrial electrogram activation at baseline(BL) and following nicotine(nic) application. B) Mean data representing the change in VA interval calculated from (A). *P<0.05,**P<0.01 vs BL.

Figure 4.

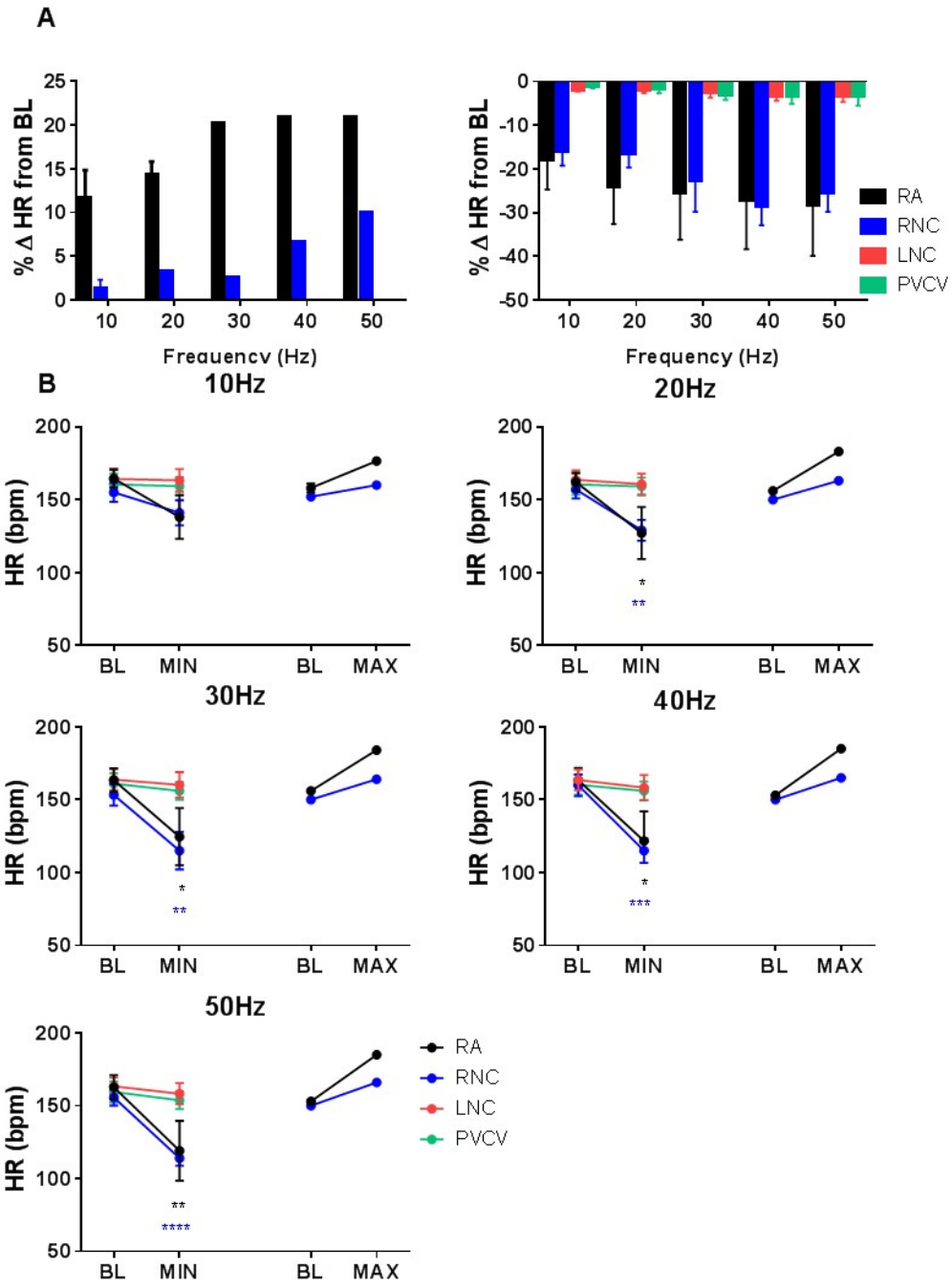


Figure 4. HR responses induced by electrical stimulation at sites in RA, RNC, LNC, and PVCV. Comparison of the percentage change in HR during electrical stimulation at different regions (A). Comparison of the maximum reductions and maximum increases in HR compared to baseline(BL) during electrical stimulation 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 HR.

Figure 5.

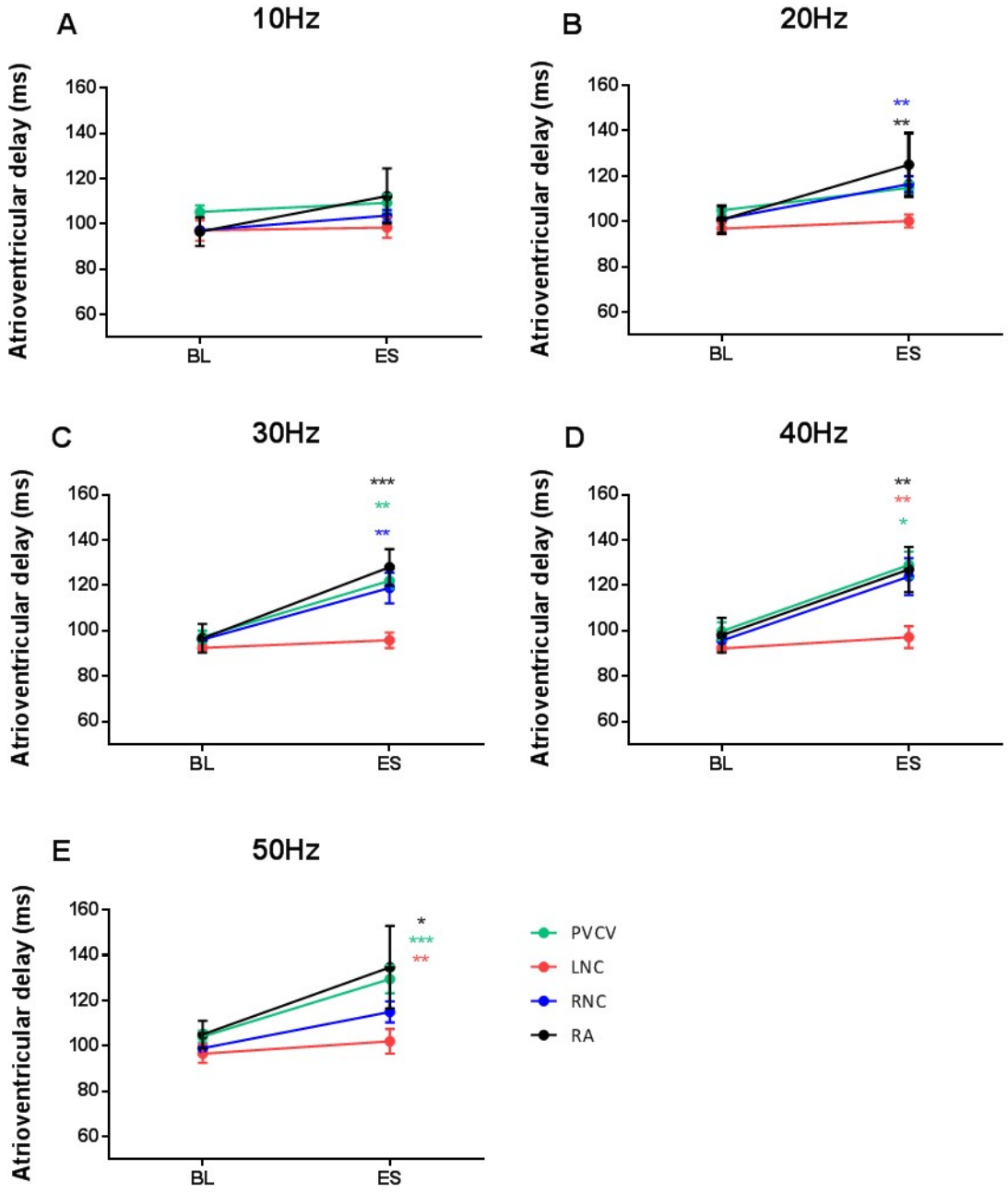


Figure 5. Electrical stimulation induced effects on atrioventricular conduction. Mean data representing the average change in AV delay during electrical stimulation of sites within intrinsic cardiac ganglia PVCV(n=9), LNC(n=7), RNC(n=27), RA(n=4) at different frequencies, A)10Hz, B)20Hz, C)30Hz, D)40Hz and E)50Hz. Data represents mean±SEM. BL=baseline, ES=electrical stimulation. *p<0.05,**p<0.01,***p<0.001.

Figure 6.

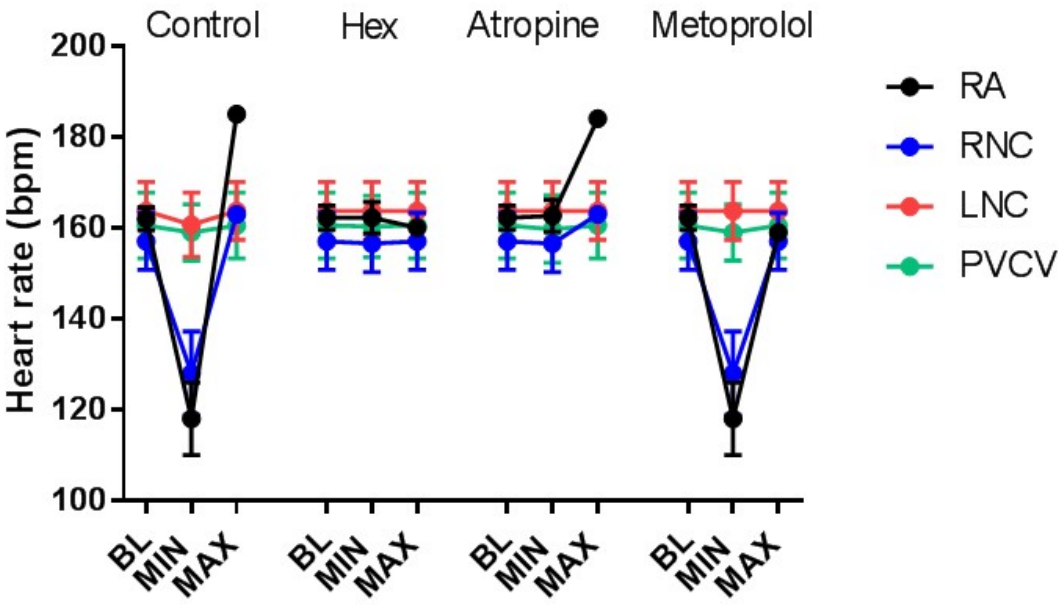


Figure 6. Pharmacological autonomic blockade of HR responses to electrical stimulation. HR response during control and in the presence of the atropine(0.1uM,[16]), metoprolol(1.8uM[16]) and hexamethonium(0.5mM[23]). Electrical stimulation applied at either the left neuronal cluster(LNC), pulmonary vein-caudal vein(PVCV), right atrial(RA) and right neuronal cluster(RNC). Data represents mean±SEM. *p<0.001 vs baseline[BL] HR.

Figure 7.

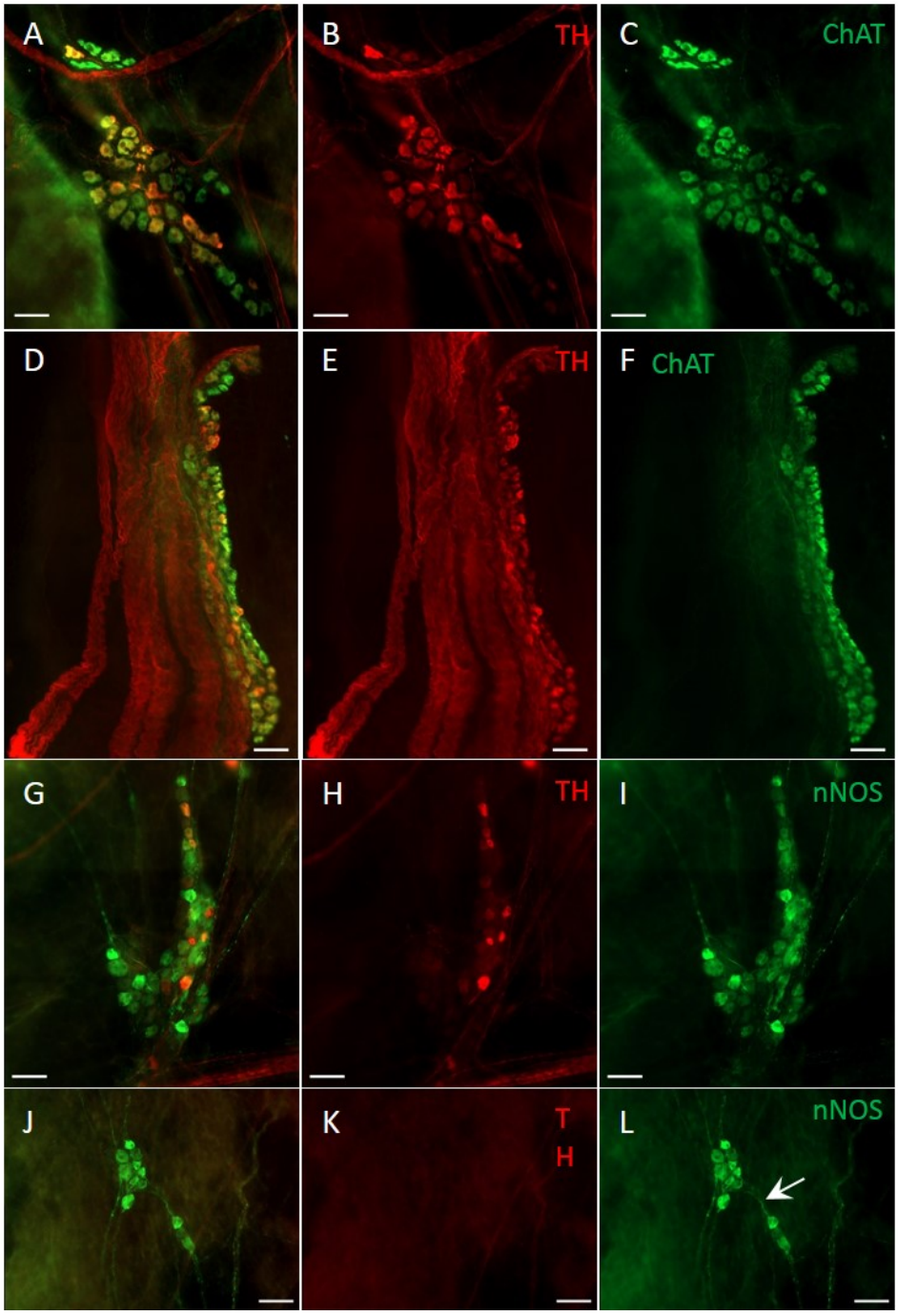


Figure 7. Microphotographs illustrating the predominance of cholinergic(ChAT-IR) neurons within ganglia. **A-C:** Representation of a ganglia containing both ChAT and TH-IR neurons located within the RNC of the rabbit ICNS. **D–F:** Microphotographs illustrating nerves accessing the heart on the medial side of the root of RCV, 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 RCV. **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. Note the thin nNOS-IR nerve fibres (white arrow) connecting two small neighbouring ganglia. Scale bars represent 100µm.