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Review

Characterization of the intrinsic cardiac nervous system

Emily Wake, Kieran Brack Dr *

Department of Cardiovascular Sciences, Cardiology Group, Glenfield Hospital, Groby Road, University of Leicester, LE3 9QP, United Kingdom
Leicester NIHR Biomedical Research Unit in Cardiovascular Disease, Glenfield Hospital, Leicester LE3 9QP, United Kingdom

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ABSTRACT

Heart disease is the number one cause of mortality in the developed world and it is well recognised that neural mechanisms are important in pathology. As well as peripheral autonomic nerves, there is a rich intrinsic innervation of the heart that includes cardiac ganglia, collectively termed ganglionic plexuses (GP). Understanding the role that the intrinsic cardiac nervous system (ICNS) play in controlling cardiac function and how it interacts with information between central command centers and its integration with sensory information from the myocardium could prove crucial for prophylactic and corrective treatments of heart disease. This article in the timely and important special issue on central and peripheral nervous control of the heart in Autonomic Neuroscience; Basic and Clinical will focus on the anatomical and physiological characteristics that define the ICNS.

Contents

* Corresponding author at: Leicester NIHR Biomedical Research Unit in Cardiovascular Disease, Glenfield Hospital, Leicester LE3 9QP, United Kingdom.
E-mail address: keb18@le.ac.uk (K. Brack).

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The heart is supplied and controlled by centrally derived sympathetic and parasympathetic nerves. The classical augmenting influence of sympathetic inputs is accepted as dogma, but the inhibitory influence of the cervical vagus, in particular on ventricular function and electrophysiology is still passionately debated. Traditionally, autonomic control of the heart has focused on centrally derived extrinsic signals or the investigation of electrical stimulation of peripheral nerves. However, neurocardiac control is more complex owing to the existence of an extensive network of intrinsic cardiac neurons i.e. the intrinsic cardiac nervous system (ICNS) that has been collectively called the hearts '*little brain*' (Randall et al., 1996; Ardell, 2004). These neurons can be characterised broadly speaking by 1) their anatomical / topographical layout, 2) their chemical phenotype and 3) their functional influences on the heart. This review will characterise the ICNS in each of these components.

1. Anatomy of the intrinsic cardiac nervous system

Historically, methylene blue was used to visualize neurons from organs but it is often unstable following routine chemical fixation (Müller, 1990) so cannot reliably be used to characterize the ICNS of the heart. The most popular, cost effective, and reliable method used to identify nerves and their cell bodies was initially described by Karnovsky and Roots (1964) at various levels of the rat and guinea pig gastrointestinal tract, the rat ovary and guinea pig striated muscle i.e. the histochemical stain for acetylcholinesterase (AChE). This method allows a distinct and permanent signal that has permitted a more complete picture of the intrinsic cardiac nervous system to be developed from a wide range of mammalian species including mice, rat, guinea pig, rabbit, cat, dog, sheep, pig and humans.

AChE histology results in a dark brown stain and is considered a pan-neuronal marker so indiscriminately stains all cell bodies and nerve fibres. The ICNS comprises of collections of neuronal somata and connecting nerve fibres known as ganglionic plexuses (GPs). (See Fig. 1) Somata are typically between 15 and 30/20–45 μm (Rysevaite et al., 2011a; Leger et al., 1999) on the short and long axis respectively and occur as individual entities, gathered into ganglia containing anywhere between 2 and 1500 neurons, or grouped into 'clusters' with a number of smaller groups of cell bodies in close vicinity. The majority (≈90%) of somata reside on supra-ventricular tissues, lying flat on the epicardial surface but can also occur within fat pads outside the heart hilum. Ganglia are primarily found on the dorsal atrial surface, around the base of aorta / pulmonary artery, dorsal and ventral to the pulmonary veins and on the anterior ventricular surface (Table 1).

The number of cardiac ganglia is species dependent ranging from 19 in the mouse (Rysevaite et al., 2011a) to over 800 in humans (Pauza et al., 2000; Armour, 1997; Singh et al., 1996) (Table 2). In general, GPs in the hearts of smaller mammals such as mice and rats (Batulevicius et al., 2003; Rysevaite et al., 2011a) are comparatively similar to that of larger mammals including sheep (Saburkina et al., 2010) and pigs (Batulevicius et al., 2008). There is however a reduced density of innervation by cell bodies in smaller mammals compared to larger mammals, where cardiac neuronal innervation is more numerous and more liberally distributed across the hilum of the heart.

Ganglia from small mammals appear to be discretely located, but become progressively scattered and profusely distributed in larger mammals. Fields of ganglionated plexuses have been generalised into 5–7 regions (dependent on species), which importantly take into account the nervous supply and projections to different effector sites (Table 2). These regions are the right dorsal atrial (DRA), ventral right atrial (VRA), left dorsal (LD), ventral left atrial (VLA), middle dorsal (MD), right coronary (RC) and left coronary (LC) plexuses (Table 3). GPs in all of these regions have been demonstrated in the rabbit (Saburkina et al., 2014), dog (Yuan et al., 1994), sheep (Saburkina et al., 2010) and human (Pauza et al., 2000).

1.1. Extrinsic innervation into and cardiac innervation from ganglia

Evidence from the guinea pig (Batulevicius et al., 2005) and rabbit (Saburkina et al., 2014) suggests that ganglia are heterogeneously innervated by bilateral autonomic inputs but confirmation from neuronal tracing studies in experimental species is very much needed. In larger mammalian species such as sheep, pigs and dogs, extrinsic mediastinal nerves access the heart at multiple sites via arterial and venous routes. As is seen in many experimental mammalian species, extrinsic cardiac nerves access the heart arterially, around the roots of the pulmonary artery (PA) and aortic root (Ao) and at the venous portion of the heart hilum around the roots of the pulmonary veins (PVs) and superior vena cava (SVC) (Batulevicius et al., 2008; Saburkina et al., 2010; Pauza et al., 2002a,b, Richardson et al., 2003; Batulevicius et al., 2003).

Intrinsic cardiac nerves extend epicardially from GPs on the heart to innervate the atria, interatrial septum and the ventricles (Pauza et al., 2000; Saburkina et al., 2014). Two subplexus routes extend from the arterial region of the hilum between the pulmonary trunk (PT) and the aorta (Ao), to effector sites on the left and right ventricles: the left (LC) and right coronary (RC) subplexuses respectively. Another five subplexuses originate from the venous region on the heart hilum around the PVs. In general, 1) the dorsal right atrial subplexus originates from either the right caudal vein (RCV) or the superior vena cava branching out to supply the sinoatrial node (SAN) and the dorsal region of the right atrium. 2) The middle dorsal (MD) subplexus branching from amongst the pulmonary veins in the direction of the dorsal coronary groove with neuronal connections with 3) the left dorsal subplexus, terminating on dorsal left atrial and ventricular regions. Two ventral subplexuses also exist with the sparse nerves of 4) the ventral left atrial subplexus, beginning ventrally to the left PV and joining ganglia on the ventral left atrial region and finally, connecting the ventral right atrium to the ventro-medial region around the superior vena cava, 5) the right ventral subplexus. These defined routes noted in the rabbit are comparatively similar to those previously identified in several other mammalian species demonstrating a common trait for heterogeneous neurocardiac control by the ICNS. For a cross species illustration of the generalised GP layout and innervation route see Fig. 2.

1.2. Ventricular innervation

In contrast to the data regarding cardiac innervation of the atria, that of the ventricles still remains relatively unknown and more importantly widely underappreciated. Mammalian ventricles were historically believed to be devoid of ganglia and any innervation from the ICNS until Gagliardi et al. (1988) described ganglia present on human ventricular myocardium at locations ventral to the coronary groove and around the region of the conus arteriosus (CA). These findings have since been replicated in humans (Pauza et al., 2000), sheep (Saburkina et al., 2010), dog (Yuan et al., 1994), cat (Johnson et al., 2004) and rabbit (Pauziene et al., 2016; Saburkina et al., 2014). An illustration of ventricular ganglia in the rabbit is shown in Fig. 3. Unlike larger species where ventricular ganglia are clearly evident, smaller species appear to lack the presence of such ganglia. Ventricles are now known to be innervated by ganglia located adjacent to the aortic root and the root of the pulmonary trunk as well as at the cranial aspect of the ventral interventricular groove and a smaller ganglia at the region of the left atrioventricular sulcus (Armour, 1997; Pauza et al., 2000). In the rabbit, the pattern of innervation originates as described previously via the accessing mediastinal nerves entering arterially around the Ao and PT or venously around the roots of the PVs on the heart hilum. The numbers of ganglia present around the region of the CA varies dramatically between hearts, ranging from 11 to 220 (Pauziene et al., 2016).

In smaller animals, such as rats and mice, ventricular nerve supply originates entirely from the atria, with the ventral surface of the ventricles being principally supplied by the subplexus route originating from the right ventral atrial region (Batulevicius et al., 2003). This is in stark

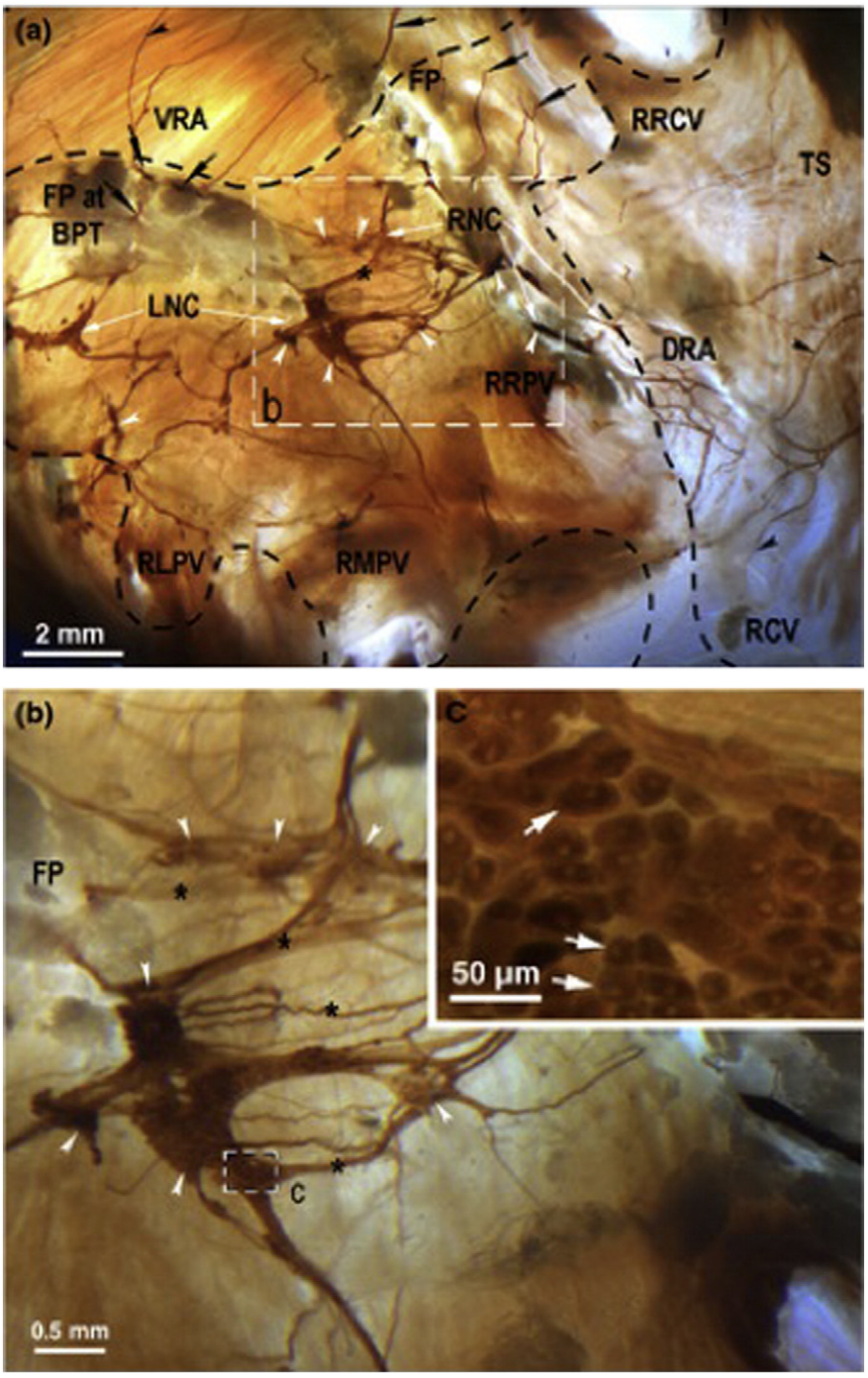

Fig. 1. Macrophotographs to illustrate the location and the structure of the nerve plexus of the heart hilum (NPHH) in the rabbit heart stained histochemically for acetylcholinesterase. The boxed area in (a) outlines the area on the rabbit heart that is enlarged in (b). The boxed area in (b) outlines the area on the rabbit right neuronal cluster (RNC) that is enlarged as the inset (c). Black arrows indicate the access routes of extrinsic cardiac nerves into the heart through the venous part of the heart hilum. White arrowheads indicate ganglia within the neuronal clusters. Black arrowheads point to subplexal nerves extending to the innervation regions, while the white solid arrows in the inset (c) point to intrinsic cardiac neurons within the RNC. Asterisks indicate commissural nerves that connect the neuronal clusters within the venous part of the heart hilum. Dashed line demarcates the heart hilum. DRA, dorsal right atrial subplexus; FP, fat pad under interatrial septum; FP at BPT, fat pad at bifurcation of pulmonary trunk; LNC, left neuronal cluster; RCV, root of caudal vein (inferior caval vein); RLPV, root of left pulmonary vein; RMPV, root of middle pulmonary vein; RNC, right neuronal cluster; RRCV, root of right cranial vein (superior caval vein); RRPV, root of right pulmonary vein; TS, terminal groove; VRA, ventral right atrial subplexus. Used with permissions from Saburkina et al., 2014.

contrast to larger species, where the left and right coronary subplexuses originating from the ganglia at the PT/Ao are responsible for the majority of ventricular innervation. These data further reiterate the increasing level of neuronal complexity with alternative models. The fact that subplexus neuronal projections branch onto the ventricles, emphasises the role of the ICNS in ventricular control and robust anatomical evidence from a number of species of sub-atrial ganglia suggest a pivitol physiological role in ventriclular control.

In all species, ganglia are linked via small interconnecting commissural nerves. The presence of such nerves further demonstrates the notion that ganglia and even individual neurons within ganglia are capable of communicating with each other in order to provide autonomic

Table 1
Cross species variability of GP location. Key; AV, atrioventricular; CA, conus arteriosus; CS, coronary sinus; CV, caudal vein; DA, dorso-atrial; GP, ganglionic plexus; HH, heart hilum; IAS, interatrial septum; IVC-ILA, inferior vena cava-inferior left atrial; LA, left atrium; LAV, left azygos vein; LCV, left caudal vein; LPV, left pulmonary vein; LV, left ventricle; MPV, middle pulmonary vein; OM, obtuse marginal; PA, posterior atrial; PL, posterior left; Post RA, posterior right atrium; PVs, pulmonary veins; RA, right atrium; RAM, right acute marginal; RCV, right caudal vein; RPV, right pulmonary vein; RV, right ventricle; RV FP, right ventricle fat pad; SA, superior atrial; Sup, superior; SVC, superior vena cava.

	Cluster/region	Ganglia position		Cluster/region	Ganglia position
Mouse (Pauza et al., 2013)	Right atrium	Ventral to RPV, Medial to RCV, Ventral surface of RA	Rat (Batulevicius et al., 2004)	Right atrium	Medial to sinus of RCV, Superior interatral groove, Ventral to CV
	Left atrium	Ventral to LPV, Between MPV and CV, Ventral LA		Left atrium	Cranial surface of LA, Dorsal wall of the LCV, Medial and ventral to LPV, Ventral to RPV & MPV
Guinea Pig (Batulevicius et al., 2005)	Right atrium	Right region of HH, Dorsal RA region, Ventral RA region			
	Left atrium	Left region of HH		Non-hilum	Medial surface of the CS
		Dorsal and ventral LA region	Rabbit (Saburkina et al., 2014)	Right atrium	Medial to RCV
	Right ventricle	Dorsal RV			Ventral region of RA
					Cranial aspect of IAS
	Left ventricle	Dorsal LV		Left atrium	Ventral to LPV & MPV, Dorsal to MPV, Between MPV and caudal vein
	Non-hilum	Surface of LCV, Para-aorta, Intra-atrial & Intra-ventricular septum		Non-hilum	Conus arteriosus, Intra-atrial septum
Cat (Johnson et al., 2004)	Right atrium	Fat pad between SVC / aorta 'PA GP'	Sheep (Saburkina et al., 2010)	CS / AVN	Conorary sinus
		Fat pad RPV- SVC junction 'SA GP'			Dorsal inferior RA
	Left atrium	IVC / inferior LA 'AV GP'		SAN region	Root of Cranial caval vein
	Atrial-septum	Intra-atrial septum 'IAS GP'			Superior surface of RA
	RV	CA – 'RV FP'		Distal to SAN	Dorsal superior RA
	Left ventricle	Cranio LV margin fat pad 'CV GP'			Ventral superior RA
		Epicardial fat of LV 'LV2 GP'		Left azygous vein	Cardiac portion of LAV
	Ventricular-septum	Mid-level LV / RV septum		Ventral atrial	Ventral superior LA
					Ventral inferior LA and RA
Pig (Batulevicius et al., 2008)	Left atrium	Dorsal and ventral LA, Ventral LA			Inferior surface of RA
		Left heart hilum			
	Right atrium	Dorsal and ventral LA, Right heart hilum		Hilum	Hilum
				Aorta / PT	Pre and post aorta & CA
	Para/Aortic	Para/Aortic		Ventricular	Ventral LV & RV
Dog (Yuan et al., 1994)	Right atrium	Ventro-dorsal RA fat pad 'RA GP'			
	Left atrium	Cranial-ventral LA fat pad	Human (Pauza et al., 2000)	Posterior-superior RA	Around SVC & RA 'Sup RA'
		Intermediate-ventral fat pad LA			Interatrial groove ' Post RA'
					LA/PVs 'Sup LA'
		Caudal-ventral fat pad LA			
		Dorsal atrial fat pad (DA-GP)		Posterior-superior LA	Posteriormedial surface of LA 'PM LA'
		IVC-inferior atrial fat pad (IVC-ILA GP)			Atrial side of the AV groove 'PL LA'
	Ventricular	Right marginal CA fat pad		Intra-atrial septum	Posterior RA/Posteromedial LA 'IAS GP'
		Left marginal CA fat pad		Ventricular	Aortic root
		Cranial ventricular fat pad (para-aortic)			Anterior & Posterior descending CA
					Origin of R acute marginal CA 'RAM GP'
					Origin of left obtuse CA "OM GP'

feedback and ensure the heart as a whole is physiologically functioning to full capacity.

2. Neurochemical phenotype

Historically, it was thought that intrinsic ganglia were simple relay stations for parasympathetic inputs and by assumption would only contain cholinergic markers e.g. choline acetyltransferase (ChAT); the enzyme responsible for the synthesis of acetylcholine (ACh). This would therefore imply that all ganglionic somata would be parasympathetic post-ganglionic efferents, however, over the years it has been shown using immunohisotchemical methods that a variety of neurochemicals are present (Table 4). Ganglia show immunoreactivity (IR) to neuromodulators and neurotransmitters including, but not exclusively to, ChAT; vasoactive intestinal peptide (VIP, known to be co-released alongside ACh); tyrosine hydroxylase (TH, responsible for the production of the sympathetic nerve neurotransmitter noradrenaline [NA]); neuropeptide Y (NPY, known to be co-released alongside NA); neuronal nitric oxide synthase (nNOS, involved in the production of NO of parasympathetic, sympathetic nerves and non-adrenergic non-cholinergic (NANC) nerves); synaptophysin (a marker of presynaptic fibres); substance P (sub P) and calcitonin gene related peptide (CGRP). Cardiac ganglia are therefore much more than a vagal relay station but are highly complex structures including efferent pre- and post-ganglionic parasympathetic neurons and presumed putative post-ganglionic sympathetic neurons, all of which all lie in close proximity to sensory nerves. This is perhaps not surprising, since neuromodulation of various aspects of cardiac control are already known to be dependent on some of the neurotransmitters and neuromodulators mentioned above.

Table 2
Estimated number of intrinsic cardiac neurones and ganglia across species.

Species	Average number of intrinsic neurons per heart	Average number of intrinsic ganglia per heart
Mice	1082 ± 160	19 ± 3
Rat	6576 ± 317	Not documented
Guinea Pig	2321 ± 215	262 ± 28
Rabbit	2200 ± 262	Unknown
Dog	Estimated 80,000	Approx. 2000
Sheep	Estimated 17,000	769 ± 52
Pig	Approximately 12,000	362 ± 52
Human	43,000–94,000	836 ± 76

2.1. Choline acetyltransferase (ChAT)

Whilst a number of neurotransmitters and neuromodulators have been found in the intrinsic network, it is generally appreciated that across species, cholinergic cell bodies predominate (i.e. AChE-IR; ChAT-IR). More recent studies have focused on innervation of supraventricular myocardium in and around the SAN and atrioventricular

Table 3
Origin and supply of intrinsic ganglia cross species. Key - Ao, Ascending aorta; AVN, atrioventricular node; IVS, interventricular septum; LA, left atrium; LAa, left atrial appendage; LAV, left azygos vein; LCT; LCV, left cranial vein; LIPV, left inferior pulmonary vein; LPV, left pulmonary vein; LV, left ventricle; LVC, ; LVN, ; PA, pulmonary artery; PT, pulmonary trunk; RA, right atrium; RCT; RCV, right cranial vein; RIPV, right inferior pulmonary vein; RSPV, right superior pulmonary vein; RV, right ventricle; RVN, ; SAN, sinoatrial node; SVC, superior vena cava; T, thoracic vertebrae.

Nerve subplexus		Mouse (Pauza et al., 2013)	Rat (Batulevicius et al., 2004)	G Pig (Batulevicius et al., 2005)	Rabbit (Saburkina et al., 2014)
Right dorsal atrial (DRA)	Origin	*Venous; RCV*, RCT, RVN	*Venous; RCV*	*Venous; RCV*	*Venous; RCV*
	Supply	Dorsal RA, SAN	Dorsal RA, SAN	Dorsal RA, SAN	Dorsal RA, SAN
Ventral RA (VRA / RV)	Origin	*Venous; RCV*, RCT, RVN	*Venous; RCV*	*Venous RCV*	*Venous; RCV*
	Supply	Ventral RA, Posterior L/R V	Ventral RA	Ventral RA, AV groove	Ventral RA/LA
Left Dorsal (LD)	Origin	*Venous; LCV*, LCT, LVC	*Venous; LCV*	*Venous; L / RCV*	*Venous; LCV*
	Supply	Posterior LV /RV	Posterior and lateral L/R V	Posterior & Lateral L/R V, Lateral RA	Posterior & lateral LV
Ventral LA (VLA)	Origin	*Venous; LCV*, LCT, LVN	*Venous; LCV*	*Venous; LCV*	*Venous;* RCV
	Supply	Ventral LA	Ventral LA	Ventral LA, Lateral LV	Ventral LA
Middle dorsal (MD)	Origin				*Venous*; RCV
	Supply				Posterior RV, AVN
Right coronary (RC)	Origin		*Arterial*; Ao/PA	*Arterial*; Ao/PA	*Arterial*; Ao/PA
	Supply		Anterior & Lateral RV	Anterior & Lateral RV	Anterior and lateral RV
Left coronary (LC)	Origin	*Arterial; Ao/PA*	*Arterial*; Ao/PA	*Arterial*; Ao/PA	*Arterial*; *Ao*/PA
	Supply	Anterior LV	Anterior LV, Lateral LV, IVS	Anterior and Lateral LV, IVS	Anterior and lateral LV
Nerve subplexus		Dog (Yuan et al., 1994)	Pig (Batulevicius et al., 2008)	Sheep (Saburkina et al., 2010)	Human (Pauza et al., 2000)
Right dorsal atrial (DRA)	Origin	*Venous; SVC*,	*Venous;* dorsal SVC	*Venous; RCV*, RCT, T2-T3, RVN	*Venous; SVC - RIPV*
	Supply	Dorsal RA, SAN, appendage	Dorsal RA, SAN	Dorsal RA, SAN	Dorsal RA, SAN
Ventral RA (VRA / RV)	Origin	*Venous; medial SVC*,	*Venous*; *Mentromedial SVC*	*Venous; RCV*, RCT, T2-T3, RVN	*Venous; medial SVC*
	Supply	Ventral RA	Ventral L/R A, Anterior L/R V	Anterior LV /RV, Ventral LA / RA	Ventral L/R A
Left Dorsal (LD)	Origin	*Venous; LAV*,	*Venous*; *Dorsomedial LAV*	*Venous; LAV*, LCT, T4-T6, LVN	*Venous; LIPV*
	Supply	Lateral LV, Posterior LV / RV. LA	Dorsal LA, Posterior LV / IVS	Lateral LV, Posterior LV / RV	Ventral LA, LAa, Posterior LV
Ventral LA (VLA)	Origin	*Venous; medial SVC*,	*Venous; Ventral LPV*	*Venous; LAV*, LCT, T4-T6, LVN	*Venous; RSPV*
	Supply	Ventral LA	Ventral LA, Anterior RV	Ventral LA	Ventral LA
Middle dorsal (MD)	Origin	*Venous; RCV*,	*Venous; pulmonary veins*	*Venous; RCV*, RCT, T2 / T3, RVN	*Venous; RSPV – LIPV*
	Supply	AVN, Dorsal RA, cranial atria	Dorsal LA, Posterior RV	Posterior RV	Posterior & lateral RV, dorsal LA
Right coronary (RC)	Origin	*Arterial; Ao/PT*		*Arterial; Ao/PT*, Right T4-T5, L/RVN	*Arterial; Ao/PT*
	Supply	Anterior, lateral & posterior RV		Anterior & Lateral RV	Anterior, lateral & posterior LV
Left coronary (LC)	Origin	*Arterial; Ao/PT*,		*Arterial; Ao/PV*, Right T4-T5, L/RVN	*Arterial; Ao/PT*
	Supply	Anterior & Lateral LV		Anterior & Lateral LV	Anterior, lateral & posterior LV

(AVN) nodes with the most detailed and convincing images produced by Pauza's group (Richardson et al., 2003; Rysevaite et al., 2011b). This is not surprising considering our understanding of vagal control in these regions. In general, between 60 and 100% of cardiac ganglia are ChAT-IR (Horackova et al., 2000; Hoard et al., 2007; Rysevaite et al., 2011b; Richardson et al., 2003). Even with the most abundant neurotransmitter in the network, it is evident that there is a high level of neurochemical variability in the ICNS. Recently, robust evidence has been provided illustrating the presence of ChAT-IR in cardiac ganglia that are known to innervate ventricular myocardium in the rabbit heart (Pauziene et al., 2016). Thereby providing the anatomical evidence to support our long-standing belief that the cardiac vagus has a prominent role in physiological control of the ventricles (Coote, 2013).

2.2. Tyrosine hydroxylase (TH)

There are numerous cell types within the ICNS including principal neurons, which are defined as those that are directly innervated by peripheral autonomic neurons and have long axons capable of transmitting information over relatively longer distances, inter- and intraganglionic nerves and small intensely fluorescent cells (SIF cells), (Rysevaite et al., 2011b). SIF cells were historically found to be TH-IR and are generally located within larger ganglia, grouped into small clusters or dispersed on the walls of the atria and ventricles (Rysevaite et al., 2011b). Currently, SIF cells have an unknown function in the heart. In addition, it is becoming increasingly apparent that TH is also present within larger diameter cell bodies of ganglia (Horackova et al., 2000). The presence of catacholamines in regular GP neurons could suggest GP involvement in the functional effects from sympathetic activation, as vagal stimulation does not generally elicit an adrenergic sensitive physiological response.

Currently, the overall proportion of TH-IR neurons in experimental models as well as the presence of TH in principal cells is under debate with numbers varying greatly between studies (Richardson et al., 2003; Hoover et al., 2009; Rysevaite et al., 2011b). Some studies report a complete lack of principle cells with a catecholaminergic phenotype (Richardson et al., 2003) whilst others indicate catecholaminergic ganglionic neurons of larger diameter (20–40 μm) (Horackova et al., 2000; Slavikova et al., 2003). One interesting feature regarding the presence of ChAT and TH cells, is that there are a number of reports of populations of biphenotypic neurons e.g. neurons showing both ChAT-IR and TH-IR, which represent between 10 and 20% of all neurons (Rysevaite et al., 2011b; Zarzoso et al., 2013). Why some neurons would have the capability to produce and release both neurotransmitters is interesting and requires further investigation.

In contrast to the situation in the atria where cholinergic somata and nerve fibres dominate, the situation in the cardiac ventricles is reversed. Here, there is a dominance of adrenergic nerve fibres within the left and right coronary subplexuses that innervate the ventricles (Pauziene et al., 2016) as well around the blood vessels (Pauza et al., 2013).

2.3. Neuronal nitric oxide synthase (nNOS)

One of the most interesting neurotransmitters from our groups' perspective (Brack et al., 2007, 2011; Brack and Ng, 2014; Coote, 2013) is for the enzyme nNOS, which is responsible for the production of nitric oxide (NO). nNOS has been found in GPs and in cardiac nerves in mice (Maifrino et al., 2006), rat (Klimaschewski et al., 1992), rabbit (Brack, 2015), guinea pig (Klimaschewski et al., 1992), and human (Hoover et al., 2009). The proportion of nNOS-IR cell bodies ranges from 7 to 67% (Maifrino et al., 2006) and is commonly recognised to be co-localised with ChAT (Richardson et al., 2003; Hoover et al., 2009; Herring et al., 2002)). This data is in accord with functional studies where it has been shown that nNOS acts as a co-transmitter either postsynaptically or presynaptically to modulate vagal effects on ventricular electrophysiology and the inducibility of ventricular fibrillation (Brack et al., 2007; Herring et al., 2002). As well as the axons from specific nNOS–IR neurons projecting to and from specific ganglia, fibres from neurons are

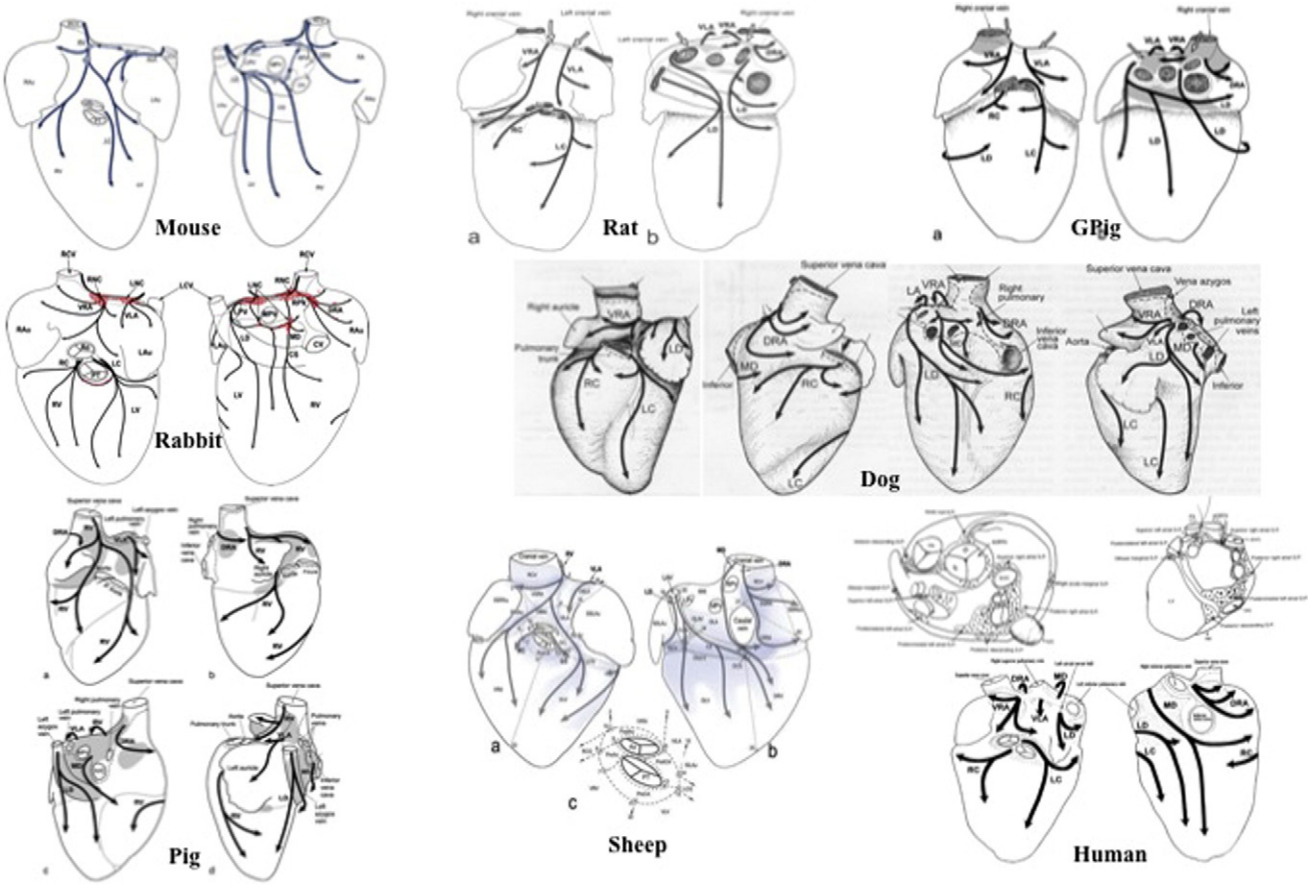

Fig. 2. Generalised GP layout and innervations route across species. Used with permissions; mouse (Rysevaite et al., 2011a,b); rat (Batulevicius et al., 2003); guinea pig (Batulevicius et al., 2005); rabbit (Saburkina et al., 2014); pig (Batulevicius et al., 2008); dog (Pauza et al., 2002a,b); sheep (Saburkina et al., 2010); human (Pauza et al., 2000).

also distributed within ganglia and acting as interneurons and suggesting the possibility of crosstalk between neurons within ganglia (Hoover et al., 2009). More importantly, recent anatomical evidence, again from the Pauza group has demonstrated the co-localisation of nNOS and ChAT in the rabbit cardiac ventricle (Pauziene et al., 2016) (Fig. 4). In the later article, there was an equal nNOS/ChAT density in the epicardiam and endocardium of the left ventricle, with a basal > apical distribution. Interestingly, there was ×8 more nNOS nerve fibres in the mid-myocardium over ChAT, suggesting a preferential and dominant nitregic innervation in this region. In addition, this article also reiterates the existence of ChAT/TH biphenotypical neuronal somata and TH-IR SIF cells.

2.4. Other neurotransmitters/neuromodulators

2.4.1. VIP

There is increasing evidence for the presence of a diverse range of neuropeptides within the ICNS, raising questions to their functional roles. Recently, markers for vasoactive intestinal peptide (VIP), which is known to be co-released alongside acetylcholine (Kuncová et al.,

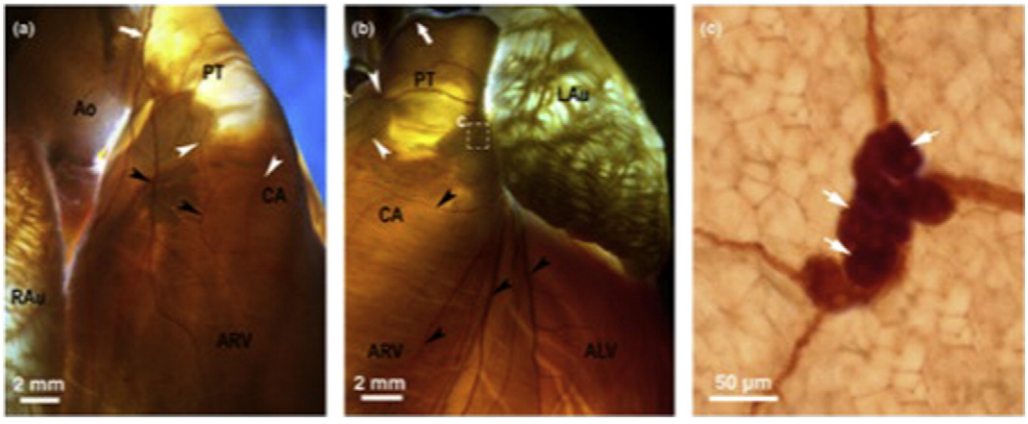

Fig. 3. Ventricular cardiac ganglia in the rabbit. Macrophotographs of the right (a) and the left (b) coronary subplexuses (RC and LC) in the rabbit heart stained histochemically for acetylcholinesterase. White arrows indicate the access of extrinsic cardiac nerves into the heart through the arterial part of the heart hilum. The boxed area 'c' in the panel (b) is enlarged as the panel (c). Black arrowheads point to some subplexal nerves extending to the innervation regions, the white ones point to some small epicardial ganglia on the conus arteriosus (CA) and the root of pulmonary trunk (PT). ALV, anterior wall of the left ventricle; Ao, root of the aorta; ARV, anterior wall of the right ventricle; LAu, left auricle; RAu, right auricle. Used with permissions from Saburkina et al., 2014.

Table 4
Neurochemical profile of the intrinsic cardiac nervous system across species. Key - AVN, atrioventricular node; ChAT, choline acetyltransferase; GP, ganglionic plexus; IAS, interatrial septum; LA, left atrium; LNC, left neuronal cluster; NADPH, nicotinamide adenine dinucleotide phosphate; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; NPY, neuropeptide Y; PGP9.5, protein gene product 9.5; PV, pulmonary vein; RA, right atrium; RAGP, right atrial ganglionic plexus; RCV, right cranial vein; RNC, right neuronal cluster; RPVs, right pulmonary veins; SAN, sinoatrial node; sLPV, superior left pulmonary vein; sRPV, superior right pulmonary vein; Sub P, substance P; TH, tyrosine hydroxylase; VC, ; VIP, vasoactive intestinal peptide.

Chemical	Species	Region	GP	Quantity	Additional information	Reference
ChAT	Mouse	LNC	Dorsal to PV	83% of all GP cells	Size; 20–14 μm	Rysevaite et al. (2011a,b)
		RNC	Medial RCV			
	Rat	LNC	Ventral to sLPV	100% of cell bodies	Localised with NPY; localised with NPY/NOS	Richardson et al. (2003)
			Ventral to sRPV	100% of cell bodies		
			Medial to RPVs	100% of cell bodies		
		LNC	Close to IAS	Around 80% of GP cells		Kuncová et al. (2003)
		RNC	SAN	Unknown	Often localised with VIP	
	GPig	RNC	IAS	'Majority' of cell bodies	In small (10–40 μm) & large diameter cells (50–60 μm)	Horackova et al. (1999)
			'Adjacent to VC'		Most cell bodies localised with NPY, some with VIP. Small portion localised with Sub P positive cell bodies.	
		Atria	Left Atria	94% All regions; 92%	Size; 27–21 μm, monopolar in nature	Leger et al. (1999)
			Right atria	88%		
			Septum	94%		
	Human	Atria	RAGP	'Many cell bodies'	Some cell bodies surrounded with marker of pre-ganglionic cholinergic fibres. Some localised with nNOS.	Hoover et al. (2009)
VIP	Rat	LNC	Sup to PVs	Large # of GP cells	Fibres often visualised	Kuncová et al. (2003)
		RNC	SAN	Small # of GP cells, sized 2–5 cells	Few solely labelled, majority localised with ChAT	
	GPig	RNC	IAS next to VC	Cell bodies found	Often associated with ChAT	Horackova et al. (1999)
		Atria	Unknown	2.7% of all GP cells	Fibres present in 69% of ganglia, also seen in interganglionic tracts. Localised with nNOS and ChAT	Parsons et al. (2006)
	Human	Atria	RAGP	Fibres only surrounding cells	Not localised with any neurotransmitter studied	Hoover et al. (2009)
nNOS	Mouse	LNC	Dorsal to PVs	67% of population '10% of heart'	NADPH staining, unipolar, bipolar and multipolar cell bodies found	Maifrino et al. (2006)
			Ventral to PVs	27% of population		
		RNC	Inter-atrial groove	7% of population		
	Rat	RA	Not known	Cell bodies found	Not localised with TH	Klimaschewski et al. (1992)
	GPig	RA	Atria	Cell bodies found	Fibres in SAN (and AVN) region	Klimaschewski et al. (1992)
		LA	PV	Cell bodies found	Surrounded by Sub P fibres	
	Human	Atria	RAGP	'Vast majority of cell bodies'	Some somata solely labelled and localised with ChAT. Prominent in fibres	Hoover et al. (2009)
TH	Mouse	ND	'Ubiquitous'	3% of all GP cells	22 by 15 μm in size Co-localised with ChAT (14% of all GP cells)	Rysevaite et al. (2011a,b)
	Rat	LNC	PV	Only fibres present	Often around ChAT cell bodies	Richardson et al. (2003)
	GPig	RNC	IAS next to VC	Cell bodies found	Often localised with NPY	Horackova et al. (1999)
		Atria	Left Atria	Cell bodies; 6% All regions; 8%	Size of cell bodies; 7–10 μm. Mono- and bi-polar cells	Leger et al. (1999)
			Right atria	Cell bodies; 12%		
			Septum	Cell bodies; 6%	80% in TH positive cells are in small ganglia (1–8 cells) No cells were localised with the general neuronal marker PGP 9.5	
	Human	Atria	RAGP	'Small proportion present'	Few fibres found	Hoover et al. (2009)
NPY	Rat	LNC	PV	100%	Few localised with NOS	Richardson et al. (2003)
	GPig	RNC	IAS adjacent to VC'	'majority' of cell bodies	In small (10–40 μm) diameter cells, Mostly localised with ChAT. Small portion localised with Sub P positive cell bodies.	Horackova et al. (1999)
CGRP	Mouse	RNC	Adjacent to hilum	Fibres present	Varicose fibres in contact and en passent with GP cells in accessing nerves, intrinsic bundles and inter-ganglionic tracts to PV roots	Rysevaite et al. (2011a,b)
	Rat	LNC	PV	Fibres present	Often localised with Sup P	Richardson et al. (2003)
	Human	Atria	RAGP	Fibres only surrounding cells	Non-cholinergic and always localised with Sup P	Hoover et al. (2009)
Sub P	Mouse	RNC	Adjacent to hilum	Fibres only	Varicose fibres in contact and en passent with GP cells in accessing nerves, intrinsic bundles and inter-ganglionic tracts to PV roots	Rysevaite et al. (2011a,b)
	Rat	LNC	PV	Fibres Only	Often localised with CGRP	Richardson et al. (2003)
	GPig	RA	Atria	Fibres only	Surrounding NOS GP cells	Klimaschewski et al. (1992)
		RNC	IAS adjacent to VC	Cell bodies and fibres	Some associated with ChAT positive cell bodies	Horackova et al. (1999)
	Human	Atria	RAGP	Fibres only surrounding cells	Non-cholinergic and always localised with CGRP	Hoover et al. (2009)

2003) have been identified in the ICNS and has been used to identify fibres and cell bodies in the cardiac hilum in several mammalian species (Steele et al., 1994; Parsons et al., 2006). VIP-IR somata are either absent within the ICNS or present in only small numbers (see Table 4). On the other hand, as many as 100% of the nerve fibres within ganglia can be immunoreactive for VIP (Parsons et al., 2006; Hoover et al., 2009; Steele et al., 1996). The origin of these fibres is unclear but it has been suggested that such fibres originate from extrinsic sources i.e. the central nervous system (Li et al., 2014). Physiological data from the rabbit, suggest that these VIP-IR fibres and cell bodies may not be involved in vagal control of ventricular electrophysiology (Brack et al., 2011), but that does not rule out a role in other actions, such as a control over

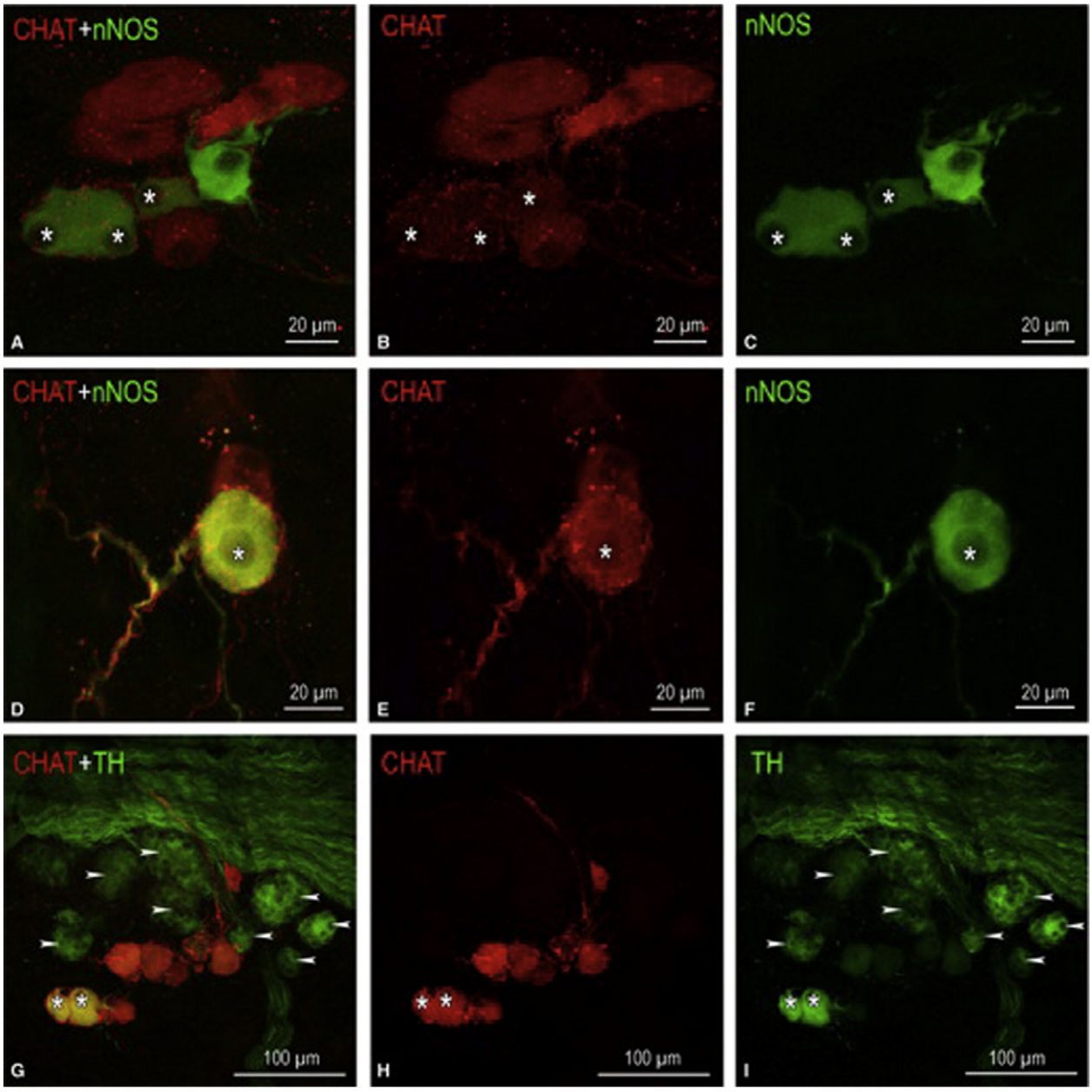

Fig. 4. Ventricular nNOS innervation. Neuronal somata (NS) distributed on the conus arteriosus and the root of the pulmonary trunk of rabbit ventricles. (A–F) Small epicardial ganglia found on the arterial cone that contain NS positive for choline acetyltransferase (ChAT; in red), for neuronal nitric oxide synthase (nNOS; in green), and biphenotypic somata (labelled by asterisks), i.e. simultaneously positive for ChAT and nNOS. In (A) and (C), note the comparatively small size of NS positive for nNOS; (D–F) ganglion of two neurons, one of which is ChAT-positive and another biphenotypic with positivity for nNOS (*). (G–I) A little ganglion located nearby a thick nerve that accessed cardiac ventricles throughout the arterial part of the heart hilum. NS positive for ChAT are shown in red, for tyrosine hydroxylase (TH) in green, and biphenotypic somata, i.e. simultaneously positive for ChAT and TH, are labelled with asterisks. Numerous SIF cells strongly positive for TH (labelled arrowheads) and clustered into tiny groups are located in between NS and a nerve containing plentiful NFs positive for TH. Used with permission from Pauziene et al., 2016.

the vasculature (Henning and Sawmiler, 2001) or the sinoatrial node (Hogan and Markos, 2006).

2.4.2. NPY

Neuropeptide Y is known to be co-released with NA and to be involved in sympathetic-parasympathetic control on heart rate (Herring et al., 2008). The presence of NPY in the ICNS is therefore not surprising and is found in the left neuronal complex (LNC) of the rat (Richardson et al., 2003) and the right neuronal complex (RNC) of the guinea pig (Horackova et al., 1999). Interestingly, these studies have shown that NYP can be localised with nNOS and also with ChAT in up to 100% of cell bodies that were found (Richardson et al., 2003). Despite this data, some of this information illustrating the extent of their existence must be taken with some caution because the main limitation that prevents a proper interpretation of some of these neurotransmitters/neuromodulators is that studies often only study a small proportion of the entire network. Nevertheless, their presence cannot be disputed, but it is vital that investigation into neuronal populations is systematically undertaken.

2.4.3. Afferent markers

Afferent nerves detect changes in the chemical milieu and physical state of the myocardium and are a vital part of the autonomic system that allows the coordination of beat to beat function and the response to cardiac disease, such as reductions oxygen levels or restrictions in myocardial blood flow. Historically, CGRP and SP would be the key

targets to identify sensory nerve fibres and it is therefore not surprising that both neurotransmitters have been found in a number of species. CGRP nerve fibres (see Table 3) are most abundant adjacent to the heart hilum, where large axons enter the atria, bifurcating multiple times to produce fine fibres that branch over the entire atrial region and around the roots of the PVs (Rysevaite et al., 2011b). This pattern of innervation is similar to that seen with SP although in a lesser abundance (Rysevaite et al., 2011b). Studies show that the majority of nerves showing SP-IR are also co-localised with CGRP. Interestingly, SP fibres have been found to surround nNOS-IR and ChAT-IR cell bodies (Klimaschewski et al., 1992) suggesting a putative role for afferent led reactive control of nitrergic and cholinergic nerves. A unique characteristic of these neurotransmitters is that they are always found in varicosities of nerve fibres and are recently confirmed in the rabbit ventricle (Pauziene et al., 2016). Generally speaking, CGRP-IR or SP-IR cell bodies are not present in the ICNS and accords with the likelihood that they are afferents with cell bodies in the nodose ganglia or the paravertebral sympathetic chains, with one exception where SP-IR somata were identified in the ICNS of the Guinea Pig (Horackova et al., 1999).

3. Physiology of the ICNS

Despite the longstanding appreciation that the autonomic nervous system modulates cardiac dynamics and electrophysiology, data regarding the involvement of the ICNS remains limited and only a handful of studies have investigated what each component of the GP network is responsible for modulating. Traditionally, it was considered that the GP network was a simple relay station for nerve activity that was coming from the cervical vagus nerve. However, the aforementioned anatomical and neurochemical evidence, provides structural evidence that this is not the case and more recent physiological data by Beaumont et al. (2013) confirms that theory.

3.1. Direct actions from GP activation

Neuronal activity is modified *in situ* mechanically and/or via locally applied neuroactive chemicals including nicotine, histamine, α and β-adrenergic agonists, angiotensin, bradykinin, NO, NPY, CGRP, substance P (Armour et al., 1993; Table 5A) as well as following the activation of sensory nerves (Thompson et al., 2000). Evidence suggests that interactions within the ICNS and activation of a single plexus can result in local and/or remote cardiac changes (Table 5B) producing often-overlapping effects in cardiac performance and atrial/ventricular electrophysiology (Table 5C) that are dependent on location. Reports of small populations of biphenotypic GP neurons (Rysevaite et al., 2011b) are in accord with the electrophysiology of isolated intrinsic cardiac neurons responsive to the application of noradrenaline in vitro (Xu and Adams, 1993) and implies that the role of an adrenergic input to cholinergic neurons needs further investigation.

Using both electrical and chemical stimulation of ganglia and neurons, possible intra-ganglionic interactions have been identified. Chemical stimulation of specific GPs through an injection of nicotine to simulate cholinergic activation, elicits 3 typical responses: 1) bradycardia alone, 2) tachycardia alone and 3) a biphasic response of bradycardia followed by tachycardia (Cardinal et al., 2009). This functional data suggests the presence of both catecholaminergic and adrenergic neurons within the same loci to produce such a biphenotypic response that corresponds with anatomical and neurochemical data discussed earlier.

Previous beliefs surfaced around the idea that individual GPs were solely responsible for the innervation of a specific adjacent region of the heart, e.g. the right atrial GP to innervate and affect the SAN and the inferior vena cava-inferior left atrial GP regulating the atrioventricular node (Lazzara et al., 1973). It is now well documented that GPs innervate adjacent regions but can also alter cardiac outputs further afield, via intra- and interganglionic communication (Hou et al., 2007; Liao et al., 2015). The co-ordination of neuronal inputs and outputs within the ICNS depends upon the nature of the afferent nerve supply, the efferent neuronal inputs received from the central / peripheral sympathetic and parasympathetic nerves and the intrinsic connections within the ICNS via local circuit neurons.

3.2. Interaction with peripheral autonomic nerves

An important question regarding GP physiology is their role during and interaction with peripheral autonomic nerve activity. Intracellular recordings made from postganglionic vagal neurons in the right atrium (McAllen et al., 2011) have shown that GPs can determine the level of postganglionic output. However, questions remain as to the influences of other GPs on other parasympathetic inputs, as well the influence on extrinsic postganglionic sympathetic nerves. Studies in the *in vitro* innervated rabbit heart consolidate knowledge that there is a high degree of lateralisation in the effects of sympathetic (Winter et al., 2012) and vagus nerve stimulation (Ng et al., 2001) on cardiodynamics, suggesting that the heterogeneous effects could also reflect selective GP involvement and with some data to support this. Using the ganglionic blocker hexamethonium, studies suggest that ganglia in the right atrial, inferior vena caval/ventral left atrium and cranial medial GPs selectively modulate vagal control of heart rate, atrio-ventricular conduction and left ventricular inotropy respectively (Gray et al., 2004; Gatti et al., 1995; Dickerson et al., 1998).

Concerning adrenergic control, dogma teaches that cardiotropic sympathetic ganglia reside in bilateral paravertebral chains with postganglionic left and right-sided nerves projecting via the dorsal nerve plexus to differentially innervate the left and right ventricle (Momose et al., 2001). More importantly, the often-overlooked discovery of TH containing somata in most intrinsic ganglia should challenge this dogma and possibly redefine the nature of cardiac sympathetic innervation. Some neurons exist between cardiac ganglia and are termed local circuit neurons communicating information between and within individual ganglia (Armour, 1986). The presence of such linkages significantly increases the capability to process sensory and motor information and influence cardiodynamics. A recent study by Beaumont et al. (2013) demonstrated that interneurons involved in the transduction of afferent signals into mechanical efferent effects, when excessively active, are partly responsible for the induction of atrial fibrillation (AF) and can be altered when the vagus nerve is stimulated.

Electrical stimulation of such ganglia alters ventricular indices supporting a functional role of the ICNS (Thompson et al., 2000). Understanding ICNS anatomy has allowed functional studies to explore the roles of specific ganglia.

Whilst the role of the right atrial and inferior vena cava-inferior left atrial ganglionic plexuses in chronotropy and AVN conduction has been confirmed by multiple studies of the same group (Ardell and Randall, 1986; Cardinal et al., 2009; Randall et al., 2003), it is now evident that intrinsic cardiac neurons from all GPs at the atrial level of the heart are involved in some sort of cardiac regulation. Selective ablation of specific ganglia within the ICNS leads to a disruption in neuronal modulation of the heart (Randall et al., 2003; Randall et al., 1992). Under normal circumstances, stress leads to a biphasic increase in heart rate (Randall et al., 1998) with an initial rapid acceleration followed by a prolonged further increase. Original hypotheses led to the idea that such a heart rate response would be diminished or even abolished when the right atrial GP were ablated. This however is not the case, with ablation of this GP only blunting the initial acceleration phase and is ineffective at impacting on the prolonged phase (Randall et al., 1998). Multiple intrinsic ganglia therefore innervate the same region i.e. the RAGP and the PAGP innervating the sinoatrial node. Functional interactions demonstrated anatomically (Gray et al., 2004) show the complex level of interaction not only between functional cardiac regions but also between individual ganglia, therefore suggesting a role for individual ganglia in the fine tuning of cardiac regulation.

Table 5
Functional effects from GP activation.

A. Chemical stimulation

	Species	GP	HR	AVC	Inotropy (all in SR)	Additional information	Ref
Nicotine	Mouse	LPV	↓-↑	–	–	Change in pacemaker site	Zarzoso et al. (2013)
	Dog	RA	↓	–	↓ L & R atria	Absent after decentralisation, atropine sensitive	Yuan et al. (1993)
			↑	↑	↑ L & R atria	Middle and caudal region of GP, propranalol sensitive	Yuan et al. (1993), Cardinal et al. (2009)
			↓-↑				
		LA	↓	↑	↓ L & R atria	Ventral loci, atropine sensitive	Yuan et al. (1993), Cardinal et al. (2009)
			↓-↑				
		DA	↓		↓ L & R atria *often in 1 atrium	Loci sensitive (loci not documented)	Yuan et al. (1993)
			↑	↑	↑ RA only,↑ RV conus & LV-wall	Loci sensitive (loci not documented)	Yuan et al. (1993), Cardinal et al. (2009)
			↓-↑				
		CM	↓	↑	↓ L & R atria	Present after decentralisation, atropine sensitive	Yuan et al. (1993)
			↑	–	↑ L & R atria & ventricle	Present after decentralisation, propranolol sensitive	Yuan et al. (1993) Cardinal et al. (2009)
			↓-↑				
		IVC-ILA	↑	↑		Minimal HR effects, significant site for AV prolongation	Yuan et al. (1993), Cardinal et al. (2009)
			↓-↑				
Histamine	Dog	RA	↑	–	↑ Left atrial & ventricle	↑ Neuronal activity	Armour (1996)
Pheynlephrine	Dog	RA	↑	–	↑ RV	↑ & ↓Neuronal activity in separate neurons	Armour (1997)
Clonidine	Dog	RA	↑	–	↑ LV	↑ Neuronal activity	Armour (1997)
Prenaterol	Dog	RA	↑	–	↑ LV & RV	↑ Neuronal activity	Armour (1997)
Endothelin	Dog	RA	↑	–	↑ LV & RV	↑ Neuronal activity	Armour (1996)
Glutamate	Dog	RA	↑	–	↑ LV	↑ & ↓Neuronal activity in separate neurons	Huang et al. (1993)
Aspartate	Dog	RA	↓ or ↑	–	↑ LV	↑ & ↓Neuronal activity in separate neurons	Huang et al. (1993)
GABA	Dog	RA	↑	–	↑ LV	↑ & ↓Neuronal activity in separate neurons	Huang et al. (1993)

B. Electrical stimulation

Species	GP	HR	AVC	Inotropy	Additional information	Ref
Mouse	LPV	↓-↑	–	–	Changed pacemaker, atropine sensitive bradycardia, propranolol sensitive tachycardia All responses blocked with hexamthonium	Zarzoso et al. (2013)
Dog	RA	↓	–	↓ L & R Atria	Atropine sensitive (replaced by ↑ in HR)	Butler et al. (1990)
	LA	↓	–	↓ L & R Atria		Butler et al. (1990)

C. Electrophysiological effects

Species	Loci	Left atria	Right atria	Additional information	Ref
Dog	RA	Small depression in free wall	Large free wall elevation	Unipolar ECG/atropine uncovered sympathetic like response	Cardinal et al. (2009), Page et al. (2006)
		Negative polarity changes	Positive polarity changes		
	RV	Small free wall elevation	Large free wall elevation	Unipolar ECG, associated with changes in HR	Cardinal et al. (2009), Page et al. (2006)
		Positive polarity changes	Large free wall depression		

		Left ventricle		Right ventricle		Additional information	Ref
Dog	RA	ST elevation	↓ARI	ST elevation	↓QRST (free wall) ↑ QRST (Posterior)	Unipolar ECG	Cardinal et al. (2009)
		↑APD, ↓RT slope	↑ ERP	↑APD, ↓RT slope	↑ ERP	Monophasic action potentials	He et al., 2013
	LA	↑APD, ↓RT slope	↑ ERP	↑APD, ↓RT slope	↑ ERP	Monophasic action potentials	He et al. (2013)

Functional heterogeneity between the left and right sympathetic paravertebral chains is well documented and quantification in the comparison between the outputs of both supplies confirms functional and electrophysiological differences in response to nerve stimulation and denervation (Winter et al., 2012). Nerves arising from the left sympathetic chain influence left ventricular contractility and electrical conduction via the atrioventricular node to a greater degree than the right, whilst the nerves arising from the right hand side have a more significant effect on sinus rate via the sinoatrial node (Winter et al., 2012). It is now a possibility that the nature of such cardiac innervation could be redefined by the presence of sympathetic neurons and nerve fibres within the ICNS and that the heterogeneity evident could be due to these ganglia.

3.3. Characteristic of the ICNS in disease states

Cardiovascular disease is a significant clinical, social and economic burden with the World Health Organization reporting in 2012 that cardiovascular disease kills more people than any other disease. With respect to heart disease, it has long been recognised that neural mechanisms contribute to pathology including atrial and ventricular arrhythmia (Brack and Ng, 2014), myocardial ischemia and heart failure (Brack et al., 2012) and renews attention onto neurocardiology particularly as some of this dysregulation is likely to result from altered intrinsic-extrinsic interaction and ICNS activity. Clinical studies demonstrate that dysfunction of the intrinsic cardiac nervous system is associated with cardiac diseases, including atrial (AF) and ventricular fibrillation (VF) (Scherlag and Po, 2006; He et al., 2013), so understanding the gross anatomy and function of the human ICNS is of increasing importance.

3.4. Irregular heart rhythms

3.4.1. Atrial arrhythmia

AF is a complex multifactorial phenomenon and the most common arrhythmia seen in clinical practice. A key contributory factor in AF is ectopic firing around the pulmonary veins (PVs), which are richly innervated by autonomic nerves and where numerous ganglia are located. Coupled with our knowledge that activation of intrinsic inputs heterogeneously affects atrial electrophysiology (Table 5), it is logical to see why intrinsic ganglia are involved. Experimentally, spectral analysis of the dominant frequency of AF can be used to identify hot spots of abnormal electrical substrate. Interestingly, these hot spots are around intrinsic ganglia (Chang et al., 2014), which help explain why GP stimulation

in this region can induce, whilst ablation can prevent AF (Lu et al., 2009). Interestingly, neuronal activity in non-pulmonary vein regions is also significantly increased during AF (Beaumont et al., 2013). Whether this hyperactivity involves cholinergic, adrenergic or non-adrenergic non-cholinergic systems remains to be confirmed and the extent of involvement of other GPs, in particular ventricular ones is unknown. AF is the most common cardiac arrhythmia and recent developments in treatments have revolved around ablation of the ICNS (Choi and Chen, 2015).

The regions around the roots of the pulmonary veins (PVs) that are known to contain intrinsic cardiac ganglia in humans and experimental mammalian species (Zarzoso et al., 2013; Saburkina et al., 2010) are strongly involved in the ectopic and rapid atrial firing seen with AF. The application of a series of premature stimuli to simulate ectopic firing at the PV-atrial level at the right superior PV (RSPV) was simultaneously accompanied by activation of an adjacent GPs and induction of AF (Scherlag et al., 2005). AF was more readily inducible at the roots of the PVs compared to elsewhere on the epicardium (Schauerte et al., 2001). Human PVs are supplied by 3 epicardial subplexuses; the dorsal right atrial, middle dorsal and left dorsal subplexuses, with an estimated 2000 neurons residing at the base of each PV (Vaitkevicius et al., 2009). Histochemical characterisation shows a direct neural connection linking PV ganglia to the sinoatrial node (SAN), a finding that would concur with clinical data suggesting that stimulation or ablation of such ganglia could trigger bradycardia, asystole and increased AF susceptibility (Kurotobi et al., 2015). PV isolation is a therapy used to target and prevent focal triggers around the PV leading to AF through electrical ablation. This approach is used to target intrinsic cardiac neuronal connections and improve AF outcome. Such selective ablation decreases firing around the PV and prolongs the effective refractory period (Chang et al., 2012) reducing the level of focal discharge and incidence of AF.

Despite ablation therapy commonly being used to prevent recurrent AF, such therapy doesn't take into account the level of plasticity the nerves and ganglia within the ICNS are capable of or the possibility of denervation of the ventricles. This is of clinical concern, as ablation of autonomic input in these regions has been known to lead to an increase in susceptibility of the ventricles to triggered arrhythmia (Osman et al., 2010). Following AF, significant remodelling of the neurons within the ICNS occurs (Yanmei et al., 2015). Changes including increased levels of adrenergic / sympathetic neurons in atrial ganglionic plexuses accompanied by an increased density of ACh positively labelled neurons (notably in the ventral left atrial region) could alter the effects of ablation therapy and render it inadequate in controlling the initiation of AF.

3.4.2. Ventricular arrhythmia

VF is the leading cause of sudden cardiac death (SCD) where there is no effective prophylactic treatment. Clinical studies strongly support an association between lethal ventricular arrhythmia-linked mortality and autonomic tone and along with numerous animal studies, the evidence points towards the notion that adrenergic activity is deleterious whilst electrical stimulation of the cervical vagus nerve is anti-fibrillatory (Ng et al., 2007). However, information pertaining to which part of the ICNS are involved are only just beginning to become known.

Studies in the dual *in vitro* innervated rabbit heart (Ng et al., 2001) suggest that two separate electrophysiological cardiac mechanisms mediate this protection; 1) a muscarinic effect on heart rate and ventricular effective refractory period (Brack et al., 2011) and 2) a reduction in electrical restitution slope that is independent to the actions of acetylcholine and vasoactive intestinal peptide, but mediated by NO (Brack et al., 2007). These data suggest, that a select population of post-ganglionic nitrergic-nerves may exert this protection. Interestingly, this functional data is in accord with the recent data from the Pauza group highlighting a subgroup of nitrergic nerves in the mid-myocardium of the rabbit left ventricle (Pauziene et al., 2016). In the rabbit, the sub-group of nerves demonstrated in the aforementioned study are more than likely to originate from intrinsic ganglia on the conus arterious (Fig. 3), but studies from other animals may suggest alternative origins i.e. the left superior and anterior right atrial GP. Individual or simultaneous stimulation of these ganglia increases ventricular action potential duration whilst reducing the electrical restitution slope (He et al., 2013). More importantly, this was associated with a lower incidence of electrical alternans, in accord with our studies, suggesting a reduced propensity for normal hearts to ventricular arrhythmia.

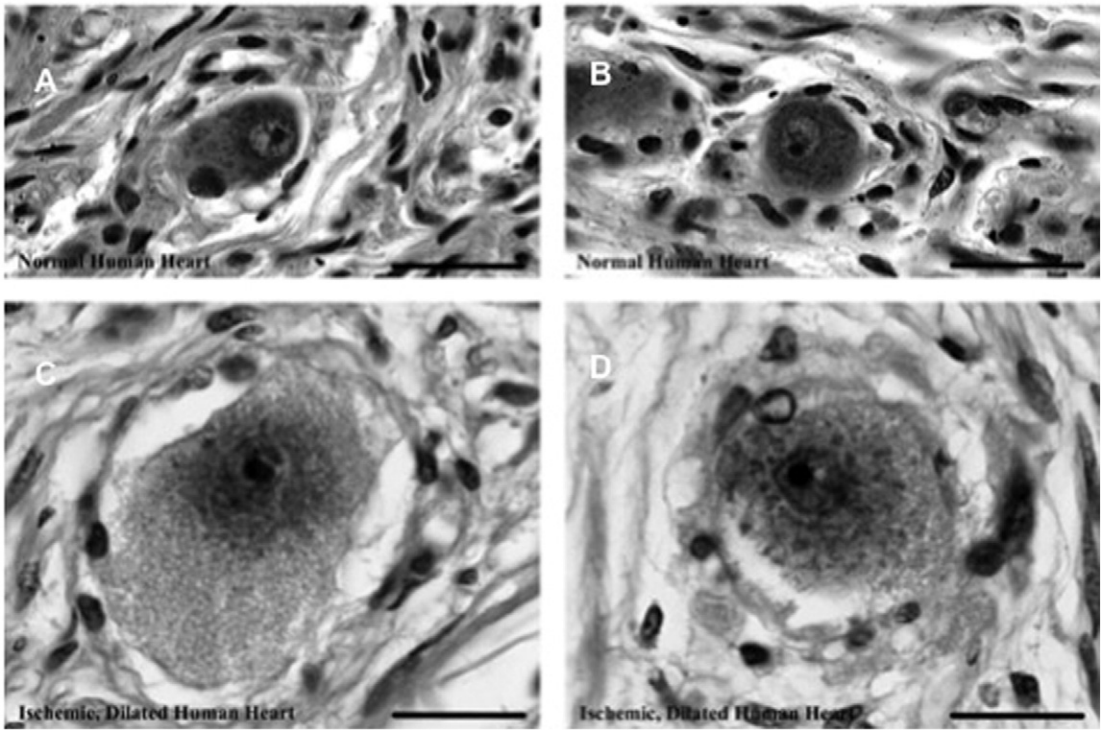

Fig. 5. Neuronal hypertrophy following heart failure. Micrographs showing examples of representative neurons taken from ganglia in human hearts. A and B show neurons of normal hearts and C and D are taken from a heart where ischemic heart failure was evident. Used with permission from Singh et al., 2013.

Furthermore, chemical stimulation of the right atrial GP neurons also induces ventricular arrhythmia (Huang et al., 1994). Clearly, the role of the ICNS in ventricular arrhythmia is not understood and further research is warranted.

3.5. Myocardial ischemia (MI) and heart failure (HF)

3.5.1. MI

Most cardiac nerves are perivascular making them susceptible to ischemic insults, whilst sensory nerves tend to innervate more the apical regions of the heart (Pauziene et al., 2016) thereby setting an environment for neuronal remodelling. Whilst coronary atherloscleriosis is the main underlying cause, some ischemic events are triggered by the autonomic nervous system e.g. emotional stress and central ischaemic strokes that lead to cardiac sympatho-vagal imbalance (Openheimer, 2006). This is observed as an increase in cardiac sympathetic activity and reduced vagal tone (Kochiadakis et al., 2000), although interaction between the ICNS and extrinsic vagal or sympathetic nerves in the setting of MI has not been fully realised. The overall effect of ischemia and the ICNS will ultimately be dependent upon the location of the cell damage and modified sensory signals. It is known however; that vagal-bradycardia (Du et al., 1998) and release of acetylcholine in non-ischaemic ventricular regions (Kawada et al., 2002) is attenuated following MI; providing clear evidence that regional ischemia affects both proximal and distal non-ischaemic sites. This implies that signals originating within and/or around ischemic regions mediate this response; however which intrinsic GPs and the chemical triggers are involved are not fully understood.

Occlusion of the blood supply to the coronary arteries leading to an ischemic insult of the myocardial tissue, results in alterations of neurohumoral control including changes in cardiac neuronal hierarchy and altered functional capability of GP neurons (Huang et al., 1993). It is now evident that not only does neuronal remodelling occur at the intrathoracic and central neural levels, but also within intrinsic cardiac neurons (Hopkins et al., 2000). The reorganisation and remodelling of the ICNS appears to be most dynamic within the first 7 days following an MI and is accompanied by increased adrenergic sensitivity and increased neuronal nitric oxide synthase (nNOS) expression within parasympathetic postganglionic intrinsic cardiac neurons (Hardwick et al., 2014). Neuronal remodelling occurs primarily within regions of non-infarcted myocardium, presumably enabling the ICNS to cope with the damage caused and allowing cardiac function to be maintained. It can be hypothesised that this increase in nNOS expression has evolved in order to provide a protective purpose, reducing the initial increase in centrally derived sympathetic drive and increasing the capabilities of the parasympathetic neuronal inputs (Hardwick et al., 2014).

Following compromised regional coronary artery blood supply to intrinsic cardiac neurons, pathological and degenerative changes to ganglia occur providing an anatomical basis for altered ganglionic control. Populations of neurons demonstrate abnormal appearance compared to those seen in control patients, with neurons becoming enlarged (Hopkins et al., 2000, Rajendran et al., 2015), evidence of degenerative changes to dendrites and axons and often indication of an increased presence of cytoplasmic inclusions, a resemblance of results commonly seen in patients with neuronal degeneration disorders (Rajendran et al., 2015).

3.5.2. HF

Deranged peripheral autonomic nerve activity is a hallmark of HF. During early stage HF there is little or no substantial alterations in ICNS function (Arora et al., 2003). The ICNS appears to demonstrate an ability to retain full functional capacity, with functionally intact connections between the intrinsic and extrinsic cardiac neuronal components remaining (Arora et al., 2003). In contrast, late stage HF secondary to coronary artery disease and ischemia causes cardiac and neuronal hypertrophy (Fig. 5), with neurons markedly oedematous and significantly less excitable (Singh et al., 2013). The hypertrophy caused by HF leads to an increased possibility of neurons failing to reach their excitability threshold and is potentially a cause of parasympathetic withdrawal (Singh et al., 2013). These changes would undoubtedly affect neuronal cell communication: detrimentally affecting ganglionic physiology and could, in part, explain the attenuation in GP neurotransmission following MI-induced HF (Bibevski and Dunlap, 2004). Thereby highlighting a key mechanism that could be involved in disease related reduced vagal tone. The most important question is, how this can be counteracted.

4. Concluding remarks

Recently there has been increasing recognition of the ICNS and the functional capabilities of GPs. However, the full extent to which this intrinsic system modifies cardiac performance and how things are changed in cardiac disease remain unclear. A more complete picture of the neurotransmitter/neuromodulator profile throughout the network is very much needed along with information pertaining to anatomical and functional connectivity from the periphery and between plexuses and the extent to which the ICNS can affect regional actions of extrinsic autonomic nerves. Understanding how GPs are involved in cardiac control could be crucial to reverse pathological autonomic imbalance.

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