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LOCALIZATION OF MULTIPLE NEUROTRANSMITTERS IN SURGICALLY DERIVED SPECIMENS OF HUMAN ATRIAL GANGLIA

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Abstract—Dysfunction of the intrinsic cardiac nervous system is implicated in the genesis of atrial and ventricular arrhythmias. While this system has been studied extensively in animal models, far less is known about the intrinsic cardiac nervous system of humans. This study was initiated to anatomically identify neurotransmitters associated with the right atrial ganglionated plexus (RAGP) of the human heart. Biopsies of epicardial fat containing a portion of the RAGP were collected from eight patients during cardiothoracic surgery and processed for immunofluorescent detection of specific neuronal markers. Colocalization of markers was evaluated by confocal microscopy. Most intrinsic cardiac neuronal somata displayed immunoreactivity for the cholinergic marker choline acetyltransferase and the nitrergic marker neuronal nitric oxide synthase. A subpopulation of intrinsic cardiac neurons also stained for noradrenergic markers. While most intrinsic cardiac neurons received cholinergic innervation evident as punctate immunostaining for the high affinity choline transporter, some lacked cholinergic inputs. Moreover, peptidergic, nitrergic, and noradrenergic nerves provided substantial innervation of intrinsic cardiac ganglia. These findings demonstrate that the human RAGP has a complex neurochemical anatomy, which includes the presence of a dual cholinergic/nitrergic phenotype for most of its neurons, the presence of noradrenergic markers in a subpopulation of neurons, and innervation by a host of neurochemically distinct nerves. The putative role of multiple neurotransmitters in controlling intrinsic cardiac neurons and mediating efferent signaling to the heart indicates the possibility of novel therapeutic targets for arrhythmia prevention.

Key words: cardiac ganglia, cholinergic, intrinsic cardiac nervous system, noradrenergic, neuronal nitric oxide synthase, neuropeptides.

Dynamic control of cardiac indices ultimately relies on efferent neuronal input to the heart from postganglionic neurons, some of which are located within the intrinsic cardiac ganglia (Ardell, 2004; Armour, 2004). While intrinsic cardiac ganglia were once viewed as simple efferent neuronal relay stations, detailed evaluation of this system in animal models has revealed its extensive structural and functional complexity (Ardell, 2004; Armour, 2004; Parsons, 2004). Intrinsic cardiac ganglia are widely distributed in fat on the surface of the heart, particularly in the atria (Yuan et al., 1994; Singh et al., 1996; Armour et al., 1997; Pauza et al., 2000; Ardell, 2004; Parsons, 2004). Interconnecting nerve bundles link these ganglia to form the extensive plexus representing the intrinsic cardiac nervous system.

Intrinsic cardiac ganglia contain neurons with several different functions (i.e., efferent, sensory, and interconnecting local circuit neurons) that control multiple cardiac indices (Ardell, 2004; Gray et al., 2004; Parsons, 2004). Animal studies indicate that the principal neurons of the ganglia receive and integrate inputs from several sources and exhibit a complex neurochemical phenotype, which goes beyond that of classical cholinergic neurons (Ardell, 2004; Armour, 2004; Parsons, 2004). The neuroanatomy of the human intrinsic cardiac nervous system, evaluated using postmortem hearts, includes numerous ganglionated plexuses associated with atrial and ventricular epicardial fat (Singh et al., 1996; Armour et al., 1997; Pauza et al., 2000). Each plexus contains multiple ganglia ranging in number from five to almost 200, and estimates for the total number of neurons in the human intrinsic cardiac nervous system range from 14,000 to over 40,000 (Armour et al., 1997; Pauza et al., 2000). The neurochemistry of intrinsic cardiac ganglia, evaluated via immunohistochemical studies of rats, guinea pigs, and mice, has uniformly demonstrated that a vast majority of their neurons label for cholinergic markers (Mawe et al., 1996; Richardson et al., 2003; Hoover et al., 2004; Mabe et al., 2006). Nevertheless, subpopulations of intrinsic cardiac neurons display immunoreactivity for other neurotransmitter markers such as neuropeptides, neuronal nitric oxide synthase (nNOS), and noradrenergic enzymes (Steele et al., 1994; Horackova et al., 1999; Richardson et al., 2003; Parsons, 2004; Parsons et al., 2006; Hoard et al., 2008). Furthermore, some intrinsic cardiac neurons receive inputs from sources in addition to their known innervation by preganglionic cholinergic neurons. These nonclassical inputs derive from peptidergic, nitrergic, and noradrenergic neurons (Steele et al., 1994; Horackova et al., 1999; Richardson et al., 2003; Parsons, 2004; Parsons et al., 2006; Hoard et al., 2008). As the neurochemical characteristics of intrinsic cardiac ganglia can vary between species and be-

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Abbreviations: BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; CHT, high affinity choline transporter; H&E, hematoxylin and eosin; IR, immunoreactive; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PBS, phosphate-buffered saline; RAGP, right atrial ganglionated plexus; SP, substance P; TH, tyrosine hydroxylase; VIP, vasoactive intestinal polypeptide; VMAT2, vesicular monoamine transporter type 2.

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cause the few studies that have examined the neurochemical anatomy of human cardiac ganglia have used postmortem samples with the potential for postmortem changes (Wharton et al., 1990; Singh et al., 1999; Weihe et al., 2005; Tan et al., 2006; Shuklin and Shvalev, 2006a,b), we set out to determine the neurochemical anatomy of ganglia obtained from living hearts. Furthermore, as only one of these reports specified the precise *in situ* location of the ganglia studied and since there is evidence for linkage of topography and function among intrinsic cardiac ganglia of animals and humans (Carlson et al., 1992; Quan et al., 2002; Ardell, 2004; Johnson et al., 2004; Gray et al., 2004), we collected tissue during open-heart surgery from a specific ganglionated plexus for immunohistochemical evaluation. Given the fundamental role of the intrinsic cardiac nervous system in human cardiac physiology and pathophysiology, learning the neurochemical complexities of this system is essential for understanding its potential as a novel target of pharmacotherapy.

EXPERIMENTAL PROCEDURES

Collection of tissue samples

Cardiac fat pad biopsies derived from the right atrial ganglionated plexus (RAGP) were obtained with informed consent from eight patients undergoing cardiac surgery at the Hôpital du Sacré-Coeur de Montréal. Patients (six males and two females) ranged in age from 47 to 76 years. All had coronary artery disease and underwent coronary artery bypass grafting. Three patients also had aortic valve replacement. Five patients had diabetes and hypertension, and all patients were being treated with multiple medications, which included a β-blocker and angiotensin converting enzyme inhibitor. Samples to be resected were identified by the pale yellow appearance of the fatty tissue located just anterior of the right superior pulmonary vein, one-third overlapping the Waterston groove and the other two-thirds over the lateral right atrial free wall. An incision was first made in the epicardial layer near the junction of the fat pad with the atrial wall. Sharp dissection was then carried out to carefully separate the fat pad from the atrial myocardium without damaging underlying muscle. The fat pads were removed en bloc, held from their epicardial edge in order not to damage the center of the fat pad containing neural ganglia, and placed immediately in fixative solution.

Tissue processing for histology

Samples were stored in the fixative solution (4% paraformaldehyde and 0.2% picric acid in phosphate-buffered saline (PBS), pH 7.4) overnight at 4 °C and cryoprotected for 1 week at 4 °C in PBS containing 20% sucrose. Samples were then frozen with dry ice and shipped in dry ice to East Tennessee State University. There, tissues were sectioned using a Leica CM3050S cryostat (Leica Microsystems Inc., Bannockburn, IL, USA) and sections collected in identical sets on charged slides. Each set contained representative sections that spanned equivalent regions of the specimens. The cutting temperature was adjusted in the range from −23 to −38 °C in order to obtain uniform 16-μm sections. Two of the eight specimens required cutting at 30-μm thickness to obtain uniform sections. Each set of tissue sections was boxed separately, wrapped in aluminum foil, and stored at −20 °C until further processing.

Hematoxylin and eosin (H&E) staining and analysis

One set of sections from each patient sample was stained with H&E to locate the cardiac ganglia within the fat pad and to estimate the number of neurons per set. Stained sections were viewed and photographed with an Olympus BX41 microscope equipped with an Optronics MagnaFire SP charged-coupled device (CCD) camera (Olympus America Inc., Center Valley, PA, USA). Identified ganglia were counted and cataloged based on section number and patient. Neurons within each ganglion were then counted manually.

Immunohistochemistry

Once slide-mounted tissue sections were brought to room temperature, each was stained for specific neural markers using routine methods of fluorescence immunohistochemistry (Hoover et al., 2004; Mabe et al., 2006; Hoard et al., 2008). Slides were rinsed 4×10 min with 0.1 M PBS (pH 7.3), incubated for 20 min in PBS containing 0.4% Triton X-100 and 0.5% bovine serum albumin (BSA), and blocked for 2 h in PBS containing 1% BSA, 0.4% Triton X-100, and 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Sections were then double or triple labeled for 15–18 h using various combinations of primary antisera (Table 1). This was followed by washing 4×10 min with 0.1 M PBS and 20 min incubation in PBS containing 0.4% Triton X-100 and 0.5% BSA. Species-specific donkey secondary antibodies conjugated to Alexa Fluor 488, 555, or 647 (Molecular Probes, Eugene, OR, USA) were applied at a 1:200 dilution in PBS containing 0.4% Triton X-100 and 1% BSA, and sections were incubated for 2 h before final washing in PBS. Coverglasses were applied with Citifluor mounting medium (Ted Pella, Redding, CA, USA) and sealed with clear fingernail polish. The specificity of primary antibodies used in this study has been documented by the suppliers and in several published studies (Mawe et al., 1996; Ferguson et al., 2003; Hoard et al., 2008; Hoover et al., 2004, 2008; Parsons et al., 2006). Additionally, specific staining did not occur in negative control sections processed without the addition of the primary antibodies.

Image analysis and confocal microscopy

The labeled tissue sections were viewed and photographed with the Olympus BX41 Microscope and Optronics MagnaFire SP CCD camera. Regions of interest were selected for further evaluation

Table 1. Primary and secondary antibodies for immunohistochemistry

Antibody (abbreviation)	Host	Dilution	Supplier (Cat #)
Choline acetyltansferase (ChAT)	Goat	1:50	Millipore (AB144P)
High affinity choline transporter (CHT)	Mouse	1:1000	Millipore (MAB5514)
Neuronal nitric oxide synthase (nNOS)	Rabbit	1:500	Santa Cruz (sc648)
Tyrosine hydroxylase (TH)	Sheep	1:200	Millipore (AB1542)
Vesicular monoamine transporter 2 (VMAT2)	Rabbit	1:200	Millipore (AB1767)
Substance P (SP)	Rabbit	1:1000	Immunostar (20064)
Calcitonin gene-related peptide (CGRP)	Mouse	1:1000	Abcam (Ab10987)
Vasoactive intestinal polypeptide (VIP)	Rabbit	1:100	CURE Res Ctr, UCLA

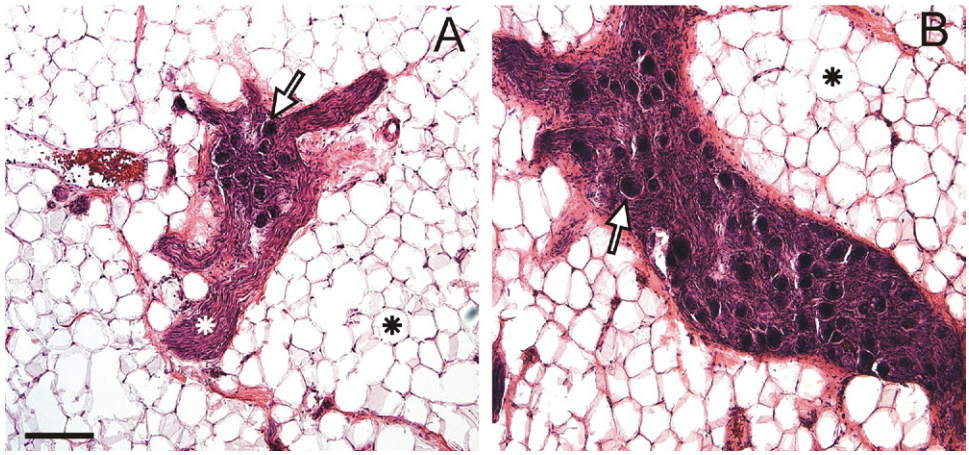

Fig. 1. Photomicrographs showing H&E stained ganglia and nerve bundles surrounded by adipose tissue (black asterisks). White arrows point to single neurons within the ganglia. White asterisk indicates nerve bundle. Scale bar=100 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

by confocal microscopy. Confocal images of cardiac ganglia were obtained with a Leica TCS SP2 confocal microscope system (Leica Microsystems Inc., Bannockburn, IL, USA). Sections were scanned sequentially to avoid crosstalk between fluorochromes. Maximum projection images derived from each series were exported into Corel Draw 11 and adjusted for brightness and contrast.

RESULTS

Characteristics of biopsy samples

Tissue samples were all of similar size, being approximately 10 mm thick and having a maximum length and width of 24±5 and 12±2 mm (mean±SD, n=8), respectively. All tissue samples contained multiple ganglia surrounded by fat, as identified by H&E staining (Fig. 1). Quantitative evaluation of H&E stained sections from all samples revealed 3±3 ganglia per section (mean±SD, n=328 sections). For this purpose, ganglia were defined as clusters of three or more neurons. Single and paired neurons were seen as well in many sections. The actual number of ganglia per section ranged from zero to 15, with a median of two ganglia per section. Within individual sections, the ganglia ranged in size from three neurons to a maximum of 106, with a median of eight neurons per ganglion (mean±SD of 13±12 neurons per ganglion, n=815 ganglia identified in all H&E sections). The larger ganglia could be followed through several sections. Ganglia commonly had one or more connecting nerve bundles (Fig. 1). The total number of neuronal somata per set of H&E stained sections ranged from a low of 446 to a high of almost 2000 neurons. No differences in morphology were observed between samples.

Localization of cholinergic markers

Antibodies to choline acetyltransferase (ChAT) and the high affinity choline transporter (CHT) were used to identify cholinergic structures. Synthesis of acetylcholine is catalyzed by ChAT; CHT is required for high affinity choline uptake by cholinergic nerve fibers. ChAT immunoreactivity was associated with many neuronal cell bodies and some processes surrounding these neuronal somata (Fig. 2). Double labeling showed punctate staining for CHT surrounding many ChAT-positive somata (Fig. 2). These findings support the classical view that intrinsic cardiac ganglia contain postganglionic cholinergic neurons, which receive preganglionic cholinergic input. However, there were clear examples in each biopsy where cardiac neuronal somata lacked cholinergic input based on the absence of CHT-positive varicosities around neuronal somata (Fig. 3).

Nitrergic and noradrenergic markers

A vast majority of examined neurons displayed intense immunoreactivity for nNOS (Fig. 4). Staining for nNOS was also prominent in axons within ganglia and in nerve bundles connecting to or bordering ganglia. In several cases, staining for nNOS was evident in the proximal axon of nNOS-positive somata and in varicose axons surrounding nNOS-positive neurons (Fig. 4).

The noradrenergic phenotype of human intrinsic cardiac neurons was evaluated using antibodies to tyrosine hydroxylase (TH) and the vesicular monoamine transporter type 2 (VMAT2). TH catalyzes the initial, rate-limiting step in the synthesis of catecholamines; VMAT2 facilitates the transport of norepinephrine into storage vesicles. A small population of neurons showed immunoreactivity for both TH and VMAT2 (Fig. 5). VMAT2 immunoreactivity was also prominent in many neurons that lacked staining for TH (Fig. 5). Quantitative evaluation of sections double labeled for these markers showed that 20.4±10.5% of VMAT2 positive neuronal somata (1280 neurons in sections from seven samples) also showed TH immunoreactivity. Additionally, some VMAT2 immunoreactive (IR) axons in the ganglia appeared to surround neuronal cell bodies (Fig. 5). TH-positive nerve fibers were far less common, but they also labeled for VMAT2.

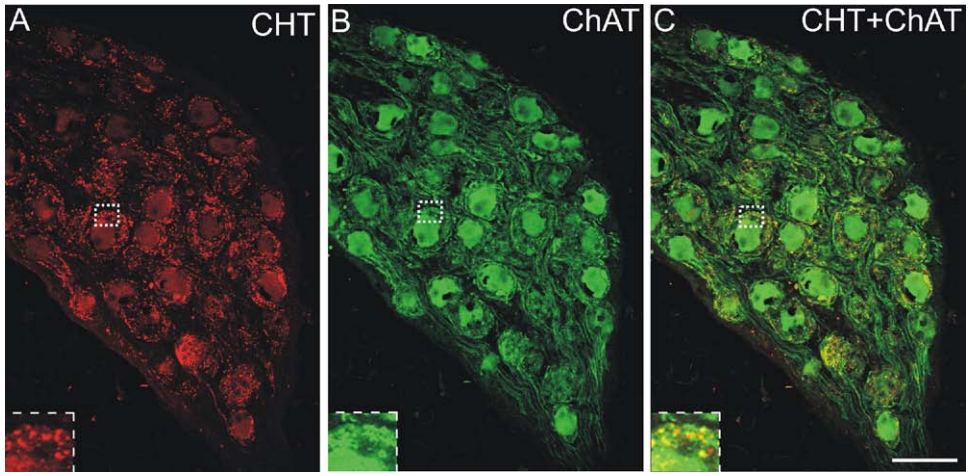

Fig. 2. Intrinsic cardiac neurons show the cholinergic phenotype and receive cholinergic input. (A–C) Confocal images of a ganglion that was double labeled to show CHT (A) and ChAT (B). Staining for CHT (A, red) was prominent in varicose nerve fibers around intrinsic cardiac neurons and faint or absent in the neuronal cell bodies. ChAT immunoreactivity (B, green) was prominent in neuronal cell bodies and generally less intense in surrounding nerve processes. (C) Colocalization of CHT and ChAT was evident from the yellow color of some cell bodies and nerve processes in the overlay image (CHT+ChAT). Inserts at lower left show boxed regions at higher magnification. All panels contain maximum projection images compiled from confocal scans that spanned 8 μm. Scale bar=100 μm in A–C. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Localization of neuropeptides

The neuropeptides vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), and substance P (SP) were localized to nerve fibers in cardiac ganglia derived from all patients. These fibers frequently formed a plexus surrounding several neurons, but neuronal somata lacked staining for these neuropeptides. SP was always colocalized with CGRP, but many CGRP-positive nerve fibers lacked SP (Fig. 6). VIP occurred in separate nerve fibers since it was not colocalized with CGRP (Fig. 7). Likewise, neither SP nor VIP was colocalized with CHT (Fig. 7), indicating that these neuropeptides were associated with non-cholinergic nerves.

DISCUSSION

Results of this study provide clear evidence that the human RAGP has a complex neurochemical anatomy, which rivals that demonstrated previously for the intrinsic cardiac

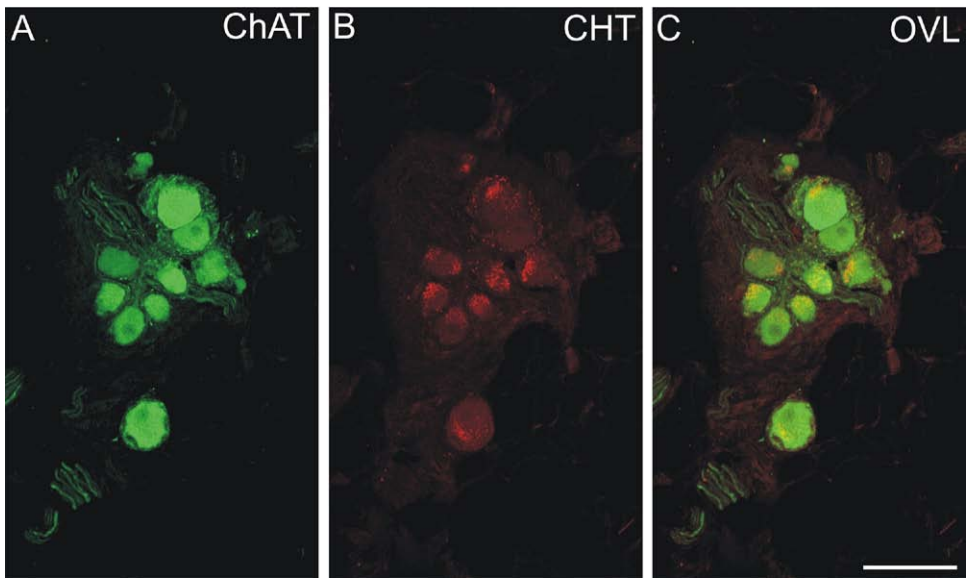

Fig. 3. Some ChAT-immunolabeled neurons are not surrounded by CHT-positive varicosities. (A–C) Confocal images of a section that was double labeled to show ChAT (A, green), and CHT (B, red). (A) All neurons demonstrate ChAT-immunoreactivity. (B) In this ganglion, very few CHT-labeled varicosities were observed. (C) The overlay image (OVL) shows cholinergic, ChAT-positive cell bodies, most of which are not surrounded by CHT varicosities. All panels contain maximum projection images compiled from confocal scans that spanned 8 μm. Scale bar=100 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

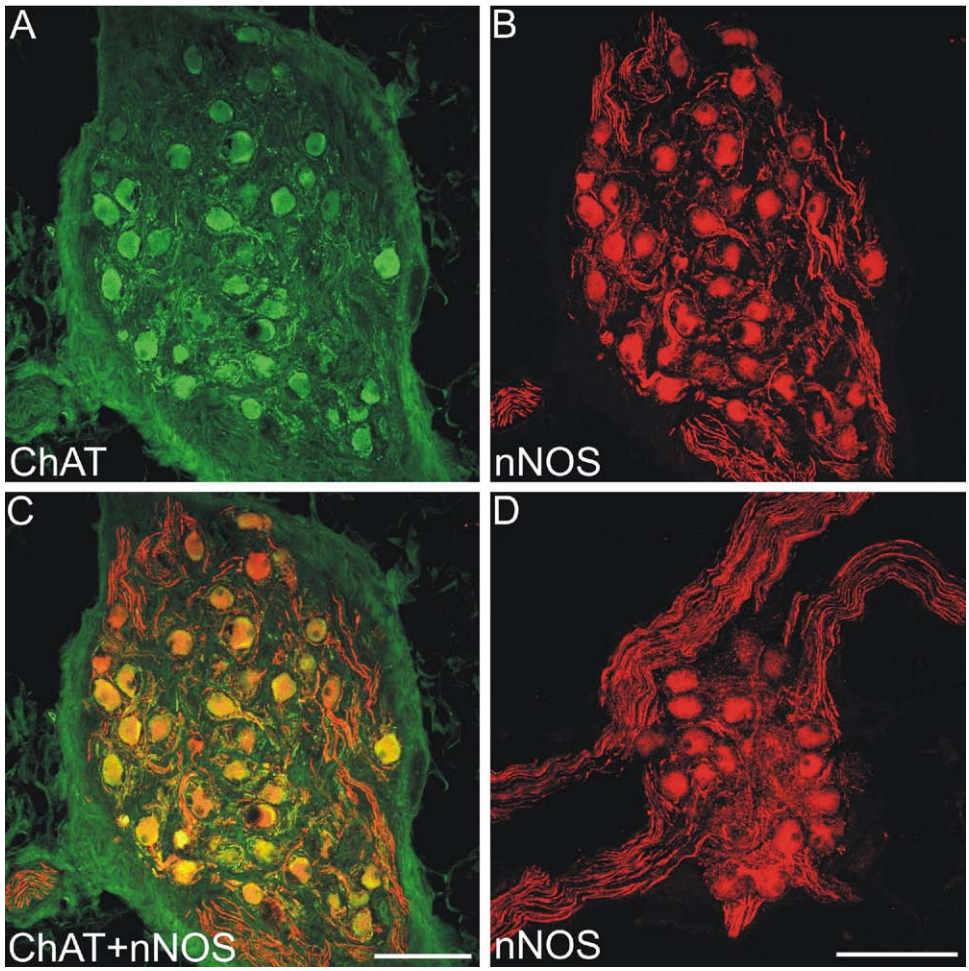

Fig. 4. The nitrergic marker nNOS was localized to most cholinergic neurons of the ICG and to non-cholinergic nerve fibers within the ganglia and nerve bundles. (A–C) Confocal images of a section that was double labeled to show ChAT (A) and nNOS (B). (A) ChAT (green) was localized to a subpopulation of neuronal cell bodies in this ganglion and to nerve fibers within the ganglion. (B) Intense staining for nNOS (red) occurred in most neuronal cell bodies, in varicose and non-varicose nerve fibers within the ganglion, and in numerous fibers in nerve bundles. (C) Colocalization (yellow) of ChAT and nNOS was limited to neuronal cell bodies and their proximal axons. (D) nNOS immunolabeled fiber bundles entering/leaving a different ganglion. All panels contain maximum projection images compiled from confocal scans that spanned 8 μm. Scale bar=150 μm in A–D. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

nervous system of experimental animals. This complexity goes well beyond the classic view of intrinsic cardiac ganglia as cholinergic efferent neuronal relay stations that merely transmit vagal impulses to the heart. The use of acute biopsy samples in this study permitted optimum tissue preparation for fluorescence immunohistochemistry. Evaluation of these samples revealed that most cardiac parasympathetic postganglionic neurons also contain nNOS, which confers the nitrergic phenotype. Subpopulations of these neurons also contain TH and VMAT2, which suggests a capacity for the synthesis, storage, and release of catecholamines. Lastly, our studies demonstrate that neurons of the human RAGP receive a plethora of non-cholinergic inputs that include nerve fibers staining for VIP, CGRP, SP, nNOS, and noradrenergic markers. These findings provide strong, neuroanatomical support for an integrative function of the human intrinsic cardiac nervous system.

This study focused specifically on the RAGP, which is one of several regional ganglionated plexuses and is very accessible during cardiothoracic surgery. Previous functional studies of canine and human RAGP established that this ganglionated plexus has a major role in the regulation of heart rate and may serve as an integration center for inputs from other cardiac ganglionated plexuses (Ardell, 1994; Pagé et al., 1999; Hou et al., 2007; Cardinal et al., 2009). Accordingly, our findings are especially relevant to the regulation of chronotropic function. Nevertheless, there is clear evidence that the RAGP can also affect a broader region of right atrial myocardium beyond the sinus node (Pagé et al., 1999) and, in canine hearts also affects the posterior right ventricular wall (Cardinal et al., 2009).

Most of the identified intrinsic cardiac neurons contained the cholinergic marker ChAT, as reported previously for postmortem ganglia (Singh et al., 1999). The additional observation that CHT–IR varicosities encircle many of

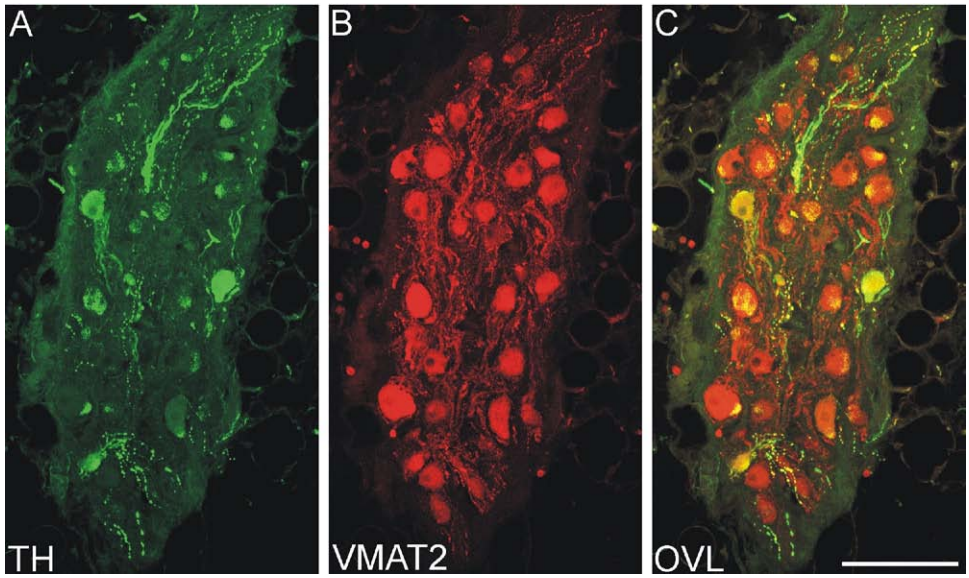

Fig. 5. Most intrinsic cardiac neurons stained for VMAT2 but only a subpopulation of these neurons was also TH-positive. (A–C) Confocal images of a section that was double labeled to show TH (A, green) and VMAT2 (B, red). (A) Only a few neurons and nerve fibers show TH immunoreactivity. (B) Prominent staining for VMAT2 occurred in most neurons and many nerves fibers. VMAT2-positive nerve varicosities are evident around several neurons. (C) OVL of TH and VMAT2 images shows that much of the TH is colocalized (yellow) with VMAT2. All panels contain maximum projection images compiled from confocal scans that spanned 10 μm. Scale bar=150 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

these neurons most likely reflects preganglionic cholinergic input to a major subpopulation of human intrinsic cardiac neurons since CHT is a highly specific cholinergic marker. Nevertheless, a number of intrinsic cardiac neurons lacked cholinergic varicosities. Whether such a lack of cholinergic inputs is normal or reflects a pathological state remains to be established. However, recent work has demonstrated that age-related loss of vagal efferent neuronal inputs to rat intrinsic cardiac neurons may occur (Ai et al., 2007). In fact, reduction of preganglionic vagal nerves could be one mechanism leading to impaired vagal control of heart rate in cardiovascular disease. The absence or very low abundance of CHT in ganglion cell bodies is consistent with our previous observations for guinea-pig cardiac parasympathetic neurons (Hoover et al., 2004) and probably reflects the export of CHT to cholinergic nerve fibers. This conclusion is supported by the observation that CHT accumulates in guinea-pig intrinsic cardiac neurons after treatment with colchicine, which blocks axoplasmic transport (Hoover et al., 2004). While it is most likely that CHT-positive varicosities surrounding intrinsic cardiac neurons have a vagal origin, we cannot eliminate the possibility that some cholinergic inputs derive from cholinergic neurons in other intrinsic cardiac ganglia. Such interganglionic connections have been identified in physiological studies of canine heart (Randall et al., 2003; Gray et al., 2004; Hou et al., 2007) but the neurotransmitter(s) utilized by these neurons remain unknown.

A vast majority of intrinsic cardiac neurons in the biopsy samples displayed prominent staining for the nitrergic marker nNOS. The further localization of nNOS to axons emanating from some intrinsic cardiac neurons and to many axons within nerve bundles suggests that nitric oxide (NO) probably functions as a co-transmitter in postganglionic parasympathetic nerves. Previous studies identified nNOS–IR neurons in the intrinsic cardiac ganglia of mice, rats, and guinea pigs (Klimaschewski et al., 1992; Calupca et al., 2000, 2001; Tanaka et al., 2001; Choate et al., 2001; Richardson et al., 2003). These were far less abundant than we observed in the human RAGP. In contrast, many neurons of canine, monkey, and human intrinsic cardiac ganglia stain for the surrogate nitrergic marker, NADPH diaphorase, along with acetylcholinesterase (Yoshida and Toda, 1996). Two recent reports have noted the presence of many nNOS–IR neurons in post-mortem samples of human atrial ganglia (Shuklin and Shvalev, 2006a,b) but the abundance of nNOS-positive neurons, judged to be about 50% in these studies, was less than we found in surgical samples of the RAGP where virtually all neurons stained for nNOS. This discrepancy could be due to differences in sampling location or their preservation status. Collectively, these data support the presence of a dual cholinergic/nitrergic phenotype for human intrinsic cardiac neurons.

NO can have an autocrine influence to enhance the release of acetylcholine from cardiac cholinergic nerves (Conlon and Kidd, 1999; Herring et al., 2000; Herring and Paterson, 2001; Choate et al., 2001). Additionally, NO can act directly on cardiac myocytes to stimulate guanylyl cyclase. Such postjunctional actions could be quite important clinically since recent work has demonstrated that NO of neuronal origin is released within the left ventricular wall during vagal stimulation (Brack et al., 2009) and mediates a decrease in susceptibility to ventricular fibrillation in the isolated rabbit heart (Brack et al., 2007). Also, decreased vagal tone is a common finding in many cardiovascular

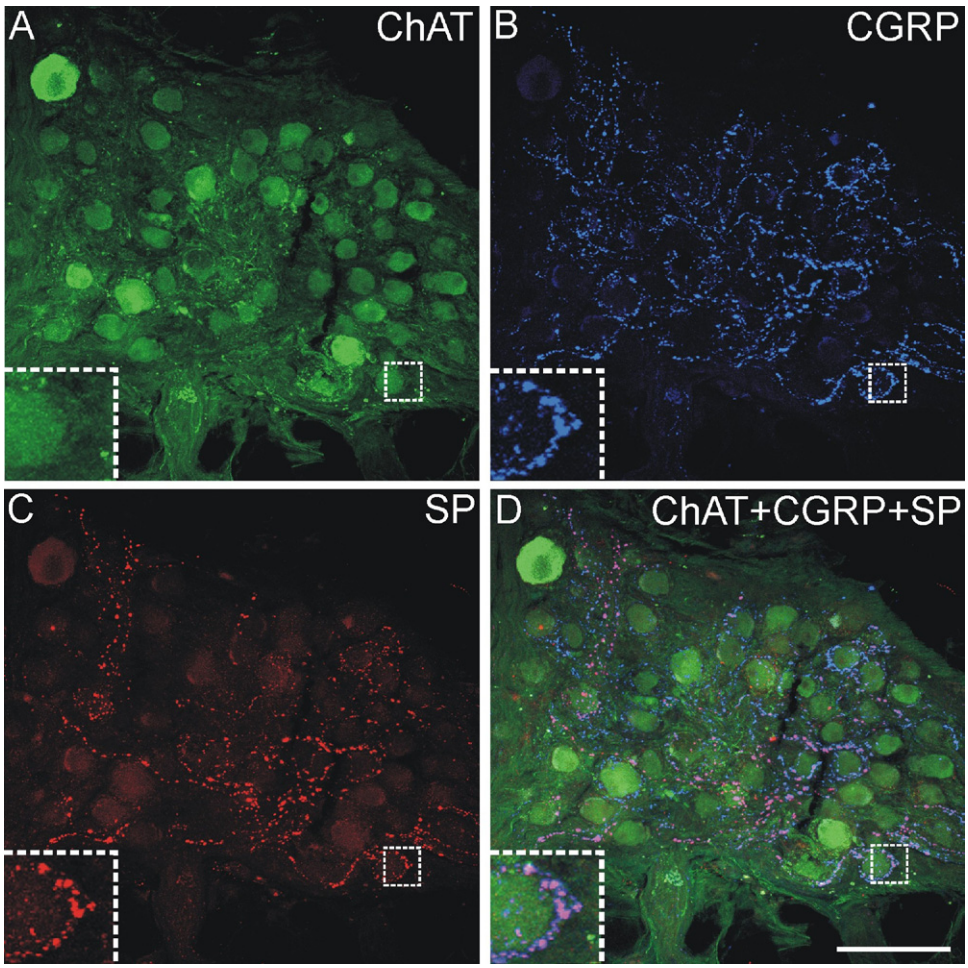

Fig. 6. CGRP and SP are colocalized in varicose nerve fibers that surround ChAT-immunoreactive cholinergic neurons. (A–D) Confocal images of a section that was triple labeled to show ChAT (A, green), CGRP (B, blue), and SP (C, red). (A) ChAT was localized to neurons and nerve fibers. (B, C) CGRP (B) and SP (C) occurred only in varicose nerve fibers that often surrounded intrinsic cardiac neurons. (D) OVL of images A–C shows that SP was predominantly colocalized with CGRP (lavender). Some varicose nerve fibers contained CGRP alone (blue). Neither CGRP nor SP occurred in ChAT-positive cholinergic nerve fibers. All panels contain maximum projection images compiled from confocal scans that spanned 8 μm. Scale bar=150 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

diseases and is recognized as an indicator of poor prognosis (Thayer and Lane, 2007). The dual cholinergic/nitrergic phenotype of human intrinsic cardiac neurons suggests that deficits of NO as well as acetylcholine might contribute to adverse outcomes in patients with impaired vagal tone.

An association of noradrenergic markers with some RAGP neurons is supported by previous evidence indicating that many human and subhuman primate intrinsic cardiac neurons exhibit a dual cholinergic/noradrenergic phenotype (Weihe et al., 2005). In fact, norepinephrine immunostaining has been reported for many human intrinsic cardiac neurons (Singh et al., 1999). Our results differ somewhat in as much as a broader distribution of VMAT2 was found compared to TH. That some intrinsic cardiac neurons have such a dual phenotype may correspond to adrenergic efferent neurons identified in functional studies of canine intrinsic cardiac ganglia (Ardell, 2004; Armour, 2004). It has been speculated that these dual phenotype neurons might be able to switch rapidly between neurotransmitters, depending on their neurotrophic factor environment (Weihe et al., 2005). Support for this scenario comes from studies of sympathetic neurons and cardiomyocytes in co-culture (Yang et al., 2002). The target of these "dual phenotype" neurons in human cardiac ganglia and their functional significance remain to be determined.

Many neuronal markers besides those representing the cholinergic phenotype were identified in the neuropil of ganglia within the RAGP. Intraganglionic nerve fibers that stained for TH and VMAT2 might derive from sympathetic efferent neurons, as shown previously in experimental animals (Parsons, 2004). That human intrinsic cardiac ganglia receive abundant inputs from peptidergic neurons confirms previous findings obtained employing postmortem samples (Wharton et al., 1990). In fact, many principle ganglionic neurons were surrounded by varicose nerve terminals that displayed immunoreactivity for VIP, SP, and CGRP. None of these neuropeptides were colocalized with CHT, suggesting that these were non-cholinergic nerve fibers. Furthermore, VIP occurred in a separate population

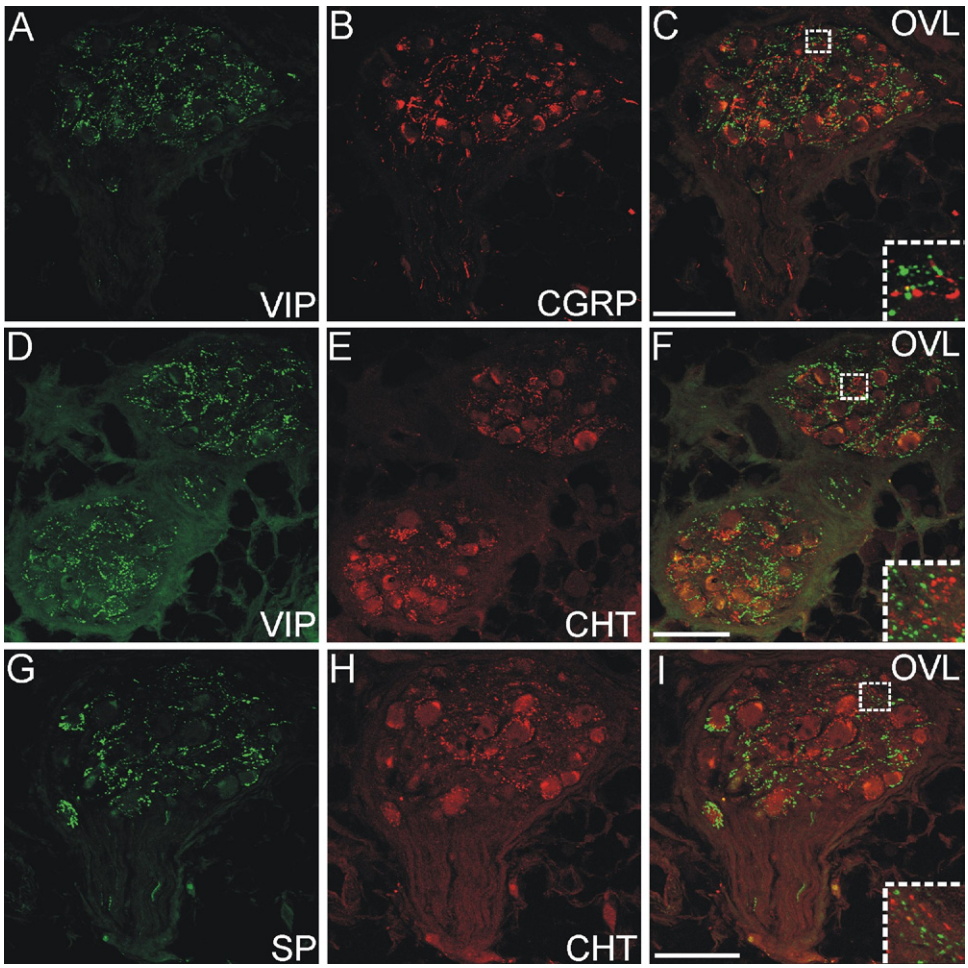

Fig. 7. VIP is localized to a separate populate of varicose nerve fibers that surround many intrinsic cardiac neurons. (A–C) Confocal images of a section that was double labeled to show VIP (A, green) and CGRP (B, red). (A) VIP occurred in varicose nerve fibers but not in neuronal cell bodies. (B) CGRP was localized to varicose nerve fibers in the same ganglion. (C) OVL shows that VIP and CGRP were not colocalized. (D–F) Confocal images of a section that was double labeled to show VIP (A, green) and CHT (B, red). (F) OVL shows that VIP and CHT were not colocalized, so VIP fibers were not cholinergic. (G–I) Confocal images of a section that was double labeled to show SP (A, green) and CHT (B, red). (I) OVL shows that SP and CHT were not colocalized, so SP fibers were not cholinergic. All panels contain maximum projection images compiled from confocal scans that spanned 10 μm. Scale bars=150 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

of nerve fibers compared to SP and CGRP. Based on evidence from previous studies of guinea-pig atrial ganglia and transplanted human hearts (Wharton et al., 1990; Parsons, 2004; Parsons et al., 2006), it is most likely that these peptidergic nerves originated from sensory neurons located outside of the heart. This conclusion is supported by the absence of neuropeptide immunoreactivity in neuronal somata identified in this study. However, we cannot exclude the possibility that some peptidergic innervation might derive from neurons located in other intrinsic cardiac ganglionated plexuses. It has been reported that VIP nerves in guinea-pig heart, including those innervating the intrinsic cardiac ganglia, appear to originate from vagal sensory ganglia (Parsons et al., 2006). VIP is known to augment the response of rat intrinsic cardiac neurons to acetylcholine (Cuevas and Adams, 1996). In addition, there is abundant evidence that CGRP and SP are colocalized in afferent nerve endings in the heart and cardiac ganglia (Parsons, 2004; Hoover et al., 2008). Cardiac afferent neurons that possess SP/CGRP may not only mediate pain but also efferent actions within the heart (Franco-Cereceda and Liska, 2000). SP in particular is noted for its ability to activate intrinsic cardiac neurons (Parsons, 2004). In this regard, SP/CGRP nerves that innervate intrinsic cardiac neurons could represent collateral branches of nociceptive neurons that mediate axon reflex responses triggered by myocardial ischemia (Hoover et al., 2000).

Limitations

Our data were obtained from patients who underwent surgery for cardiac valve replacement or coronary artery bypass. Accordingly, the findings may be influenced by the presence of chronic cardiovascular disease. As noted previously, the lack of normal control data precludes any definitive statement regarding the lack of cholinergic inner-

vation of some intrinsic cardiac neurons. Nonetheless, the neurochemical complexity identified herein is strikingly similar to that identified in previous studies of normal animal models. This similarity suggests that the rich neurochemical environment observed in this study reflects normal operating conditions rather than disease-related changes. Lastly, despite the similarity of neuronal types throughout the intrinsic cardiac nervous system, our observations were limited to the RAGP and therefore do not shed light on the neurochemical status elsewhere within the intrinsic cardiac nervous system.

Clinical significance

The human intrinsic cardiac nervous system displays an amazing degree of complexity of its neurochemical composition. Such complexity could function as a two-edged sword, on the one hand enabling robust, highly regulated control of regional cardiac indices while on the other providing numerous opportunities for dysfunction through alterations in their array of neurotransmitters and receptors. As such, disruption of processing within the system might occur as a consequence of adverse neuronal remodeling in cardiac disease or as a side effect of pharmacological therapy. As we gain more insight into the intrinsic cardiac nervous system and its capacity to coordinate regional cardiac indices, it may become possible to exploit its complexities to therapeutic advantage.

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