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MICROSCOPIC ANALYSIS OF SYMPATHETIC AND PARASYMPATHETIC DISTRIBUTION, TERMINAL MORPHOLOGY, AND INTERACTION IN WHOLE-MOUNT ATRIA OF C57BL/6 MICE

by

SCOTT WILLIAM HARDEN
B.S. Union University, 2007

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Molecular Biology and Microbiology in the Burnett School of Biomedical Sciences in the College of Medicine at the University of Central Florida Orlando, Florida

Spring Term
2009
ABSTRACT

The sympathetic (SNS) and parasympathetic (PSNS) branches of the autonomic nervous system (ANS) innervate the heart, exerting excitatory and inhibitory influences (respectively) over cardiac functions (heart rate, AV conduction velocity, and contractility). However, the distribution and structure of SNS and PSNS innervation has not yet been well studied. Detailed characterization of the distributional organization and structural morphology of the SNS and PSNS in normal states is essential to the study of pathological autonomic remodeling. The present study utilized double immunohistochemical labeling techniques to examine tyrosine hydroxylase (TH) immunoreactive (IR) SNS and vesicular acetylcholine transporter (VACHT) IR PSNS axons and terminal structures in whole-mount atria of C57BL/6 mice. We found that: (1) The atria contain a dense network of ANS axons. TH-IR, VACHT-IR, and dual cholinergic/dopaminergic TH+/VACHT-IR axons travel together in bundles on the epicardium before branching into differentiated terminal structures. (2) Parallel TH-IR and VACHT-IR axons often diverge from epicardial bundles and travel in parallel (less than 1μm apart) before forming terminal structures in the epicardium and myocardium. Such parallel SNS/PSNS axons interdigitize and have large alternating varicosities along their length adjacent to one other, suggesting possible antagonistic communication between both branches of the ANS at the prejunctional level. (3) Intrinsic cardiac ganglia (ICG) are targets for extrinsic sympathetic nerves which travel through ICG without forming large synaptic varicosities around cardiac principal neurons (PNs). (4) Small intensely
fluorescent (SIF) cells (presumably chemoreceptors and/or interneurons) exist near SNS bundles, inside ICG, and in the epicardium unaccompanied by ganglia and nerve bundles. (5) The subpopulation of TH+VACHT-IR PNs within ICG form loose terminals in the atria and do not project to other PNs. (6) Both TH-IR and VACHT-IR axons innervate atrial vasculature. (7) TH-IR axons innervate fat pads adjacent to the heart. (8) SNS/PSNS parallelism is not exclusive to the atria. Similar structures exist in the esophagus, right ventricle, and small intestine. This study provides a novel and overall view of the innervation and interaction of the SNS and PSNS in the atria. This will underlie a foundation for future physiological, pharmacological, and anatomical studies of SNS/PSNS innervation, interaction, and remodeling in pathological states (such as aging, intermittent hypoxia and diabetes).
# TABLE OF CONTENTS

LIST OF FIGURES........................................................................................................... ix
LIST OF TABLES.............................................................................................................. x
LIST OF ACRONYMS / ABBREVIATIONS....................................................................... xi

LITERATURE REVIEW ................................................................................................. 1
  Anatomy of a Neuron ................................................................................................. 1
  Functional Overview of the ANS ............................................................................. 2
  Anatomical Overview of the Cardiac ANS .............................................................. 3
    Parasympathetic Efferent Innervation of the Heart ............................................. 6
    Sympathetic Efferent Innervation of the Heart ................................................... 7
  Thoracic Ganglia and Cardiac Regulation .............................................................. 8
  The “Little Brain” on the Heart .............................................................................. 8
  Location of Integrative Processing ....................................................................... 11
  Previous Research Techniques ............................................................................. 12

INTRODUCTION ............................................................................................................. 16

MATERIALS AND METHODS...................................................................................... 17
  Animals ................................................................................................................... 17
  Tissue Preparation ................................................................................................. 17
  Immunohistochemistry ......................................................................................... 19
  Confocal Microscopy ............................................................................................. 21
  Colocalization Assessment ................................................................................... 21
  Utilization of Autofluorescence to Image Muscle and Vasculature .................... 22
  Image Modification ............................................................................................... 23
Montage Assembly .......................................................................................... 23

RESULTS ........................................................................................................... 25

Sympathetic Innervation of the Atria ................................................................. 25

Parasympathetic Innervation of the Atria ......................................................... 27

Sympathetic/Parasympathetic Interaction in the Atria ...................................... 28

Parallelism ....................................................................................................... 28

Innervation of the Epicardium vs. Myocardium ............................................ 29

Innervation of Vasculature ............................................................................ 30

Intrinsic Cardiac Ganglia .............................................................................. 31

SIF Cells .......................................................................................................... 32

Sympathetic/Parasympathetic Interaction in Other Tissues ............................. 33

Right Ventricle ............................................................................................... 33

Esophagus ....................................................................................................... 33

Small Intestine ............................................................................................... 34

DISCUSSION ..................................................................................................... 34

Methodology .................................................................................................... 34

Anatomical Overview ..................................................................................... 35

Intrinsic Cardiac Ganglia .............................................................................. 36

SIF Cells .......................................................................................................... 37

Sources of Myocardial Innervation ............................................................... 38

Sympathetic / Parasympathetic Parallelism ..................................................... 40

Theory of Sympathetic / Parasympathetic Antagonism .................................. 41

Developing Model of Autonomic Cardiac Regulation .................................... 43

Future Studies ................................................................................................. 44
LIST OF FIGURES

Figure 1: Anatomy of a Chemical Synapse ................................................. 49
Figure 2: Trends of ANS Innervation......................................................... 50
Figure 3: Mechanism of Cardiomyocyte Contraction .................................51
Figure 4: Anatomy of the Heart ................................................................. 51
Figure 5: Primary Nerves of the Thoracic Cavity ....................................... 52
Figure 6: Vagal Synapses............................................................................ 53
Figure 7: ICG on Atria ................................................................................ 53
Figure 8: Network of ICG ......................................................................... 54
Figure 9: SNS/PSNS Adjacent in Sectioned Tissue ..................................... 55
Figure 10: Guinea Pig Atrial Epicardium.................................................... 56
Figure 11: Layers of the Small Intestine ......................................................57
Figure 12: Sympathetic Innervation of the Atria (Montage) ...................... 58
Figure 13: TH, VACHT, and TH+VACHT IR Axons ................................. 59
Figure 14: TH-IR Density in the Auricles.................................................. 60
Figure 15: Innervation of the Myocardium .............................................. 61
Figure 16: Innervation at the Entrance of the Auricles ............................ 61
Figure 17: Fat Innervation by TH-IR Axons............................................. 62
Figure 18: Bundle Direction ................................................................. 63
Figure 19: Single Axon Parallelism............................................................ 64
Figure 20: TH/VACHT Colocalization ..................................................... 65
Figure 21: Epicardial Network ................................................................. 66
Figure 22: Vascular Innervation .............................................................. 67
Figure 23: Sympathetic Nerves Pass Through ICG ................................. 68
Figure 24: TH-IR ICGNs Innervated by the PSNS ................................. 69
Figure 25: Intrinsic Cardiac Ganglia in the Right Atrium ....................... 70
Figure 26: SIF Cells ............................................................................. 71
Figure 27: SIF Cells in Sympathetic Bundle........................................... 72
Figure 28: SNS/PSNS Interaction in the Right Ventricle ......................... 73
Figure 29: SNS/PSNS Interaction in the Esophagus............................... 73
Figure 30: SNS/PSNS Interaction in the Small Intestine ......................... 74
Figure 31: Speculative Mechanism of SNS/PSNS Antagonism............... 75
Figure 32: New View of Cardiac Autonomic Regulation ......................... 76
LIST OF TABLES

Table 1: Antibodies Used ............................................................................ 48
# LIST OF ACRONYMS / ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AF</td>
<td>Autofluorescence</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic Nervous System</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline Acetyltransferase</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>ICG</td>
<td>Intrinsic Cardiac Ganglia</td>
</tr>
<tr>
<td>ICGN</td>
<td>Intrinsic Cardiac Ganglionic Neurons</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemical / Immunohistochemistry</td>
</tr>
<tr>
<td>IR</td>
<td>Immunoreactivity</td>
</tr>
<tr>
<td>LCN</td>
<td>Local Circuit Neuron</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PN</td>
<td>Principal Neuron</td>
</tr>
<tr>
<td>PSNS</td>
<td>Parasympathetic Nervous System</td>
</tr>
<tr>
<td>SIF</td>
<td>Small Intensely Fluorescent</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic Nervous System</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>VACHT</td>
<td>Vesicular Acetylcholine Transporter</td>
</tr>
<tr>
<td>ZF</td>
<td>Zamboni’s Fixative</td>
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LITERATURE REVIEW

Few mammalian organs are as critical to immediate survival as the heart, temporary malfunction of which provokes life-threading pathological states and can even result in sudden death. Cardiovascular disease is the leading cause of mortality in the Western hemisphere (Kannel, 2002), claiming over 600,000 lives every year (killing one person every 34 seconds) in the United States alone (Hoyert et al., 2006). The interaction of the nervous system and the heart is critical to the maintenance of proper cardiac function, as even a healthy heart will stop performing adequately if it is not properly regulated (Chen and Tan, 2007). However, much is still unknown about the process by which the nervous system regulates cardiac output in response to body needs. Many of the current theories regarding the nature of the interactions throughout the cardiac neuroaxis are based upon indirect physiological evidence, and there are many questions about basic cardioneurological anatomy which remain unanswered. In order to advance the development of clinical treatments for common heart-related problems (such as cardiac arrhythmias, diabetic cardiac autonomic neuropathy, and sudden cardiac death), the regulatory interactions between the neurological system and the cardiovascular system must be further elucidated.

Anatomy of a Neuron

The nervous system is composed of an intricate network of neurons which possess the ability to receive, process, and transmit signals through electrical and
chemical signals. Although many structural and functional variations exist differentiating various classes of neurons, the general nature of autonomic neurons is similar (see Figure 1 on page 49). Neurons of the ANS communicate via chemical messengers (neurotransmitters) stored within synaptic vesicles in their axons which are released in response to electrical impulses (action potentials) traveling down an axon. Once released from the signaling neuron, neurotransmitters bind to receptors on its target cell (usually other neurons or muscle cells). Action potentials are often initiated as a result of detected neurotransmitters released from another neuron. In this manner, signals can be relayed through a chain of neurons until they reach their destination. Different classes of neurons release different chemical neurotransmitters, allowing for multiple responses in the targeted cells depending on which neurons are active.

**Functional Overview of the ANS**

The autonomic nervous system (ANS) is the branch of the nervous system which regulates organ function to maintain homeostasis independent of conscious thought. With respect to the heart, the role of the ANS is to assess body needs through afferent (sensory) connections, and to respond appropriately by regulating cardiac indices through efferent (effecter) connections. For example, if blood pressure drops rapidly, the ANS detects the drop in pressure via afferent baroreceptors (pressure-sensitive neural structures) in the aortic arch (Cheng et al., 1997). In response, efferent axons stimulate the heart to increase heart rate in an attempt to compensate for the initial fall in blood pressure - a
reflexive response known as the baroreflex. Because it is virtually impossible to
directly examine the effectiveness of neurotransmitter communication in the
autonomic axons in the heart (especially in living organisms), most studies
involving autonomic / cardiovascular interaction are studied via indirect,
physiological measurements. For example, the sensitivity of the baroreflex is
often used as a tool of measurement to assess the effectiveness of the cardiac ANS
(La Rovere et al., 1997). Baroreflex sensitivity is a used as a risk factor to predict
cardiovascular problems, a severe decrease in which is often associated with an
sudden cardiac death (Stein, 2008) and other serious cardiac problems.

The ANS is composed of two subsystems: the sympathetic nervous system
(SNS) and the parasympathetic nervous system (PSNS). When the SNS becomes
strongly activated (typically in response to an increased demand for cardiac
output), heart function (heart rate, blood pressure, and contractile force) is
increased. The SNS is often referred to as the “fight or flight” branch of the ANS
because it is primarily active during times of stress. Alternatively, when the
PSNS becomes strongly activated (generally during periods of relaxation), cardiac
output is decreased. The PSNS is often referred to as the “rest and digest” branch
of the ANS because it is primarily active in the absence of stressors.

Anatomical Overview of the Cardiac ANS

Although the specific features of autonomic innervation vary in different
organs, there are several patterns the ANS usually follows when innervating
target organs (see Figure 2 on page 50). Autonomic signals are usually
transferred through peripheral ganglia (small clusters of neurons not directly associated with the brain or spinal cord) before reaching their targeted tissues. Autonomic neurons which project from the brain to these ganglia are termed “preganglionic”, and neurons which project from within these ganglia to their target tissues are termed “postganglionic”. Parasympathetic postganglionic neurons are usually located in ganglia very close to the target tissues that they innervate, whereas sympathetic postganglionic neurons are usually located in ganglia near the spinal cord. Although both branches of the ANS innervate the same target tissues, the SNS and PSNS utilize separate ganglia and nerves to innervate these targets, and are not traditionally believed to interact with one another directly. Instead, regulation of the balance of sympathetic and parasympathetic activity is traditionally believed to occur primarily in the central nervous system (brain and spinal cord).

The role of the cardiac ANS is to properly modulate cardiac output in response to circulatory needs. This is accomplished primarily by controlling cardiac indices such as inotropy (contractility, influencing stroke volume), chronotropy (heart rate), and dromotropy (electrical conduction velocity within the heart) which influence cardiac output. Unlike skeletal muscle cells which contract only when electrically stimulated by neurons, cardiomyocytes (the striated muscle cells of the heart) contract spontaneously and rhythmically. The ANS loosely innervates cardiomyocytes, releasing neurotransmitters which serve
to modulate the properties of their automatic contractions (rate, force, speed, etc.) rather than directly initiating them.

The mechanism by which cardiomyocytes beat intrinsically relies on several key proteins and enzymes (See Figure 3 on page 51). Cardiomyocyte contraction requires available intracellular cAMP (produced from ATP by adenyl cyclase) which promotes calcium (Ca$^{2+}$) release from the sarcoplasmic reticulum (the primary site of Ca$^{2+}$ storage in muscle cells). High intracellular Ca$^{2+}$ promotes cardiomyocyte contraction via the actin/myosin contraction system. Since the process of muscle contraction relies on the catalysis of cAMP from ATP by adenyl cyclase, controlling the rate of catalysis by adenyl cyclase effectively modulates the ability of the cardiomyocyte to contract. Adenyl cyclase can be inhibited or stimulated as a result of g-protein coupled receptors (GPCs) in the outer membrane of the cardiomyocyte sensing specific neurochemicals. GPCs can be coupled with stimulatory or inhibitory proteins (G$_s$ or G$_i$, respectively) which are released intracellularly when they are activated by the extracellular detection of specific molecules. When these receptors bind to their respective extracellular neurotransmitters, they release the protein they were bound to, thereby modifying the properties of cardiomyocyte contraction accordingly.

Although cardiomyocytes automatically contract at a similar rate which can be adjusted by chemical neurotransmitters, they do not all intrinsically contract simultaneously. To accomplish this, a sophisticated network of
electrically active tissues is strategically located throughout the atria and ventricles. Rhythmic electrical signals produced by the sinoatrial (SA) node in the right atrium (an overview of basic cardiac anatomy is provided in Figure 4 on page 51) initiate each heartbeat, promoting synchronous contraction of cardiomyocytes within the atria. The atrioventricular (AV) node (located between the atria and the ventricles) serves to decrease the rate of electrical conduction to the ventricles, instilling a brief delay between atrial and ventricular stimulation. Once past the AV node, electrical impulses travel through the interventricular septum via the His-Purkinje system, a network of highly conductive fibers responsible for transferring these signals throughout the ventricles. The result is strong atrial contraction, followed by a brief pause before a strong ventricular contraction. The brief delay between the onset of atrial and ventricular contractions allows the atria to fully empty their contents into the ventricles before they begin to contract.

Parasympathetic Efferent Innervation of the Heart

Parasympathetic autonomic projections to the heart originate from neurons in the brainstem located in the ventral lateral region of the nucleus ambiguous (Hopkins and Armour, 1984), the dorsal motor nucleus of the vagus, and in the intermediary zone between the two (Cheng et al., 1999). These neurons project axons from the brainstem which travel through the right and left vagus nerves in the neck and enter the heart (Figure 5 on page 52), forming tight synaptic connections (Figure 6 on page 53) with the parasympathetic

postganglionic neurons in the intrinsic cardiac ganglia (ICG) located on the surface of the atria (Figure 7 on page 53). Parasympathetic postganglionic principal neurons of the ICG (ICGNs) project to the heart, releasing acetylcholine (ACh) when stimulated. ACh is detected by M₂ receptors on cardiomyocytes. These inhibitory GCP receptors inhibit adenyl cyclase activity, preventing cAMP from being synthesized from ATP, ultimately suppressing the rate of cardiomyocyte contraction. Thus, PSNS activity inhibits cardiac function.

**Sympathetic Efferent Innervation of the Heart**

Sympathetic preganglionic neurons responsible for cardiac regulation are located in the spinal cord and project from T1-T5 to form synapses with postganglionic neurons in the sympathetic chain of ganglia (Figure 2 on page 50). These neurons then project to the heart through large sympathetic nerves, and are traditionally believed to innervate the cardiac muscle directly. Sympathetic postganglionic neurons release norepinephrine (NE) when stimulated. NE is detected primarily by β₁ receptors located on the cardiomyocytes. These receptors are stimulatory GCP receptors which promote adenyl cyclase activity when stimulated, increasing the synthesis of cAMP from ATP, ultimately leading to an increase rate of cardiomyocyte contraction. Thus, SNS activity increases cardiac function.
Thoracic Ganglia and Cardiac Regulation

Thoracic autonomic ganglia include the ganglia in the sympathetic chain of ganglia near the spinal cord as well as the ICG located directly on the heart. The role of the thoracic ganglia in cardiac regulation is a subject of recent debate. Traditionally these ganglia were considered to be relay stations which simply dispatch signals from the brain to the target tissues, acting as simple, local amplifiers. However, a new emerging view of cardiac regulation places increased importance on these thoracic ganglia, suggesting that they can perform complex integrative data processing functions (Armour, 2008).

The “Little Brain” on the Heart

Recent physiological evidence suggests the intrinsic cardiac nervous system is far more complex than previously assumed (Armour, 2008). It is known that the central nervous system exhibits control over the heart. However, the fastest rate of signal transduction from the heart to the brain and back has been estimated to be 125 to 300 ms (Armour, 1976). It has therefore been proposed that heart can rapidly detect perturbations (such as the development of single dysrhythmic heart beats) and respond to them rapidly (in 20 – 40 ms). Such short-latency reflexes are likely the result of local processing within the network of intrinsic cardiac ganglia located directly on the heart (Armour, 1976). Intermediate-latency reflexes (which respond in 100 – 200 ms) are believed to modulate cardiac indices over the period of a few cardiac cycles, and are likely the result of information processing in the thoracic ganglia.
Indeed, it has been reported that the extracardiac sympathetic ganglia contain many local circuit neurons (LCNs), suggesting that it is capable of some degree of information processing (Armour 1986; Smolen, 1988). Physiological studies (Bosnjak and Kampine, 1989) suggest these thoracic ganglia possess afferent (sensory) properties, further supporting the theory of complex information processing in ganglia outside of the central nervous system. The combination of proposed afferent, local circuit and efferent qualities in the network of thoracic ganglia, along with its apparent integrative processing abilities has led it to be referred to as the “little brain” on the heart (Armour, 2008).

Richardson et al. (2003) demonstrated in rats that large bundles of axons travel between ICG (Figure 8 on page 54). However, it is still unclear as to whether these interganglionic “neural highways” represent ganglion-to-ganglion local circuit connections, or if these bundles of axons (sympathetic postganglionic, parasympathetic preganglionic, and/or parasympathetic postganglionic) simply travel along the same paths, passing through the ICG rather than forming specific connections with ICGNs.

Injecting individual ganglia with neuronal tracers (fluorescent dyes which are absorbed by neurons and transported throughout their axons to aid in their morphological assessment) into ICG produces interesting results. Gray et al. (2004) injected fast-blue tracer into individual canine ICG and observed retrogradely-labeled fluorescent cell bodies in adjacent ganglia. Although it
appears result of this experiment provides evidence of local circuit connections between ICG, the same result could be explained by axons of the adjacent ICG passing through the tracer-injected ganglia, absorbing tracer from the middle of their axons, rather than synaptic terminals around neurons of other ICG.

Evidence in support of a complex function of ICG lies in the array of different phenotypical classes of ICGNs. Specific subpopulations of ICGNs contain phenotypical markers such as calbindin, nitric oxide synthase, and tyrosine hydroxylase (Richardson et al., 2003). The diverse collection of populations of ICGNs with distinctive neurochemical phenotypes within ICG can be interpreted as an indication of high complexity.

Tyrosine hydroxylase (TH, an enzyme which assists in the synthesis of NE and is considered a sympathetic efferent neuronal marker) is present in ~30% of all ICGNs (Hoard et al., 2008), suggesting that some ICGNs (traditionally regarded as purely PSNS postganglionic efferents) may serve roles similar to that of the SNS. However, despite the fact that many ICGNs contain TH, these ICGNs are reported to lack the vesicular monoamine transporter VMAT2 (Hoard et al., 2008). Since VMAT2 is responsible for the transport of dopamine into synaptic vesicles, the lack of it suggests that although some ICGNs possess TH and may synthesize NE, they are unable to load it into vesicles for synaptic release as a neurotransmitter. Currently, the role of TH-IR ICGNs remains a mystery.
Location of Integrative Processing

The degree of local information processing in the intrinsic cardiac nervous system is capable of ultimately depends upon interactions of the sympathetic and parasympathetic branches of the ANS. If the network of ICG were to possess complex integrative information processing functions, assessing centrifugal input from local cardiac afferent neurons and centripetal input from the central nervous system and independently determining the proper outcome, that network would have to control the activity of both branches of the ANS. For example, in order to rapidly correct the development of an arrhythmic heartbeat, the network of ICG would have to be able to rapidly override the tonic, long-latency signals transmitted from the brain through the SNS and PSNS to correct the problem. Therefore, if the heart is capable of these complex and rapid functions, the central nervous system is not likely to be the only location where the SNS and PSNS interact.

However, peripheral SNS and PSNS interaction is not usually addressed in the traditional view of autonomic regulation. It is generally believed that the SNS and PSNS innervate their target tissues independently, with the only interaction between the two branches being within the central components of the nervous system. The morphological structure and distribution of the SNS and PSNS components of the cardiac nervous system have not yet been well studied.
Previous Research Techniques

Most of what is known today about the intrinsic cardiac nervous system is theory based upon indirect, physiological evidence. Morphological evidence is often advantageous over physiological evidence when attempting to document morphological and distributional properties of the ANS because it allows direct visualization of the neurons of study, rather than basing conclusions upon indirect physiological data. However, techniques which can directly reveal the morphology and topography of various neural elements within the cardiac nervous system are usually limited to immunohistochemical (IHC) methods and tracer injections, both of which contain significant limitations when studying the cardiac nervous system. The atria of the heart are the primary focus of the study of cardiac regulation because they are the primary locations where sympathetic and parasympathetic bundles enter the heart, and because they contain the network of ICG. Although the ventricles of the heart provide a majority of the contractile force responsible for circulating blood throughout the body, the atria are the primary location of autonomic regulation. For these reasons, the atria are the primary location of study for autonomic regulation of cardiac function.

IHC is a process whereby individual proteins within tissues are labeled by fluorescent molecules. Performing IHC to label neuronal markers, such as enzymes responsible for the synthesis of NE or ACh, allow for the selective identification of sympathetic or parasympathetic efferent neurons and axons which are immunoactive (IR) for each, respectively. However, atrial tissue poses
several problems which limit IHC analysis. Most significantly, it is extremely thick and highly autofluorescent. To overcome these problems, atrial tissues are routinely sectioned utilizing a cryostat or microtome, producing several-micron-thick cross sections of the atria which can be processed by IHC to reveal labeled axons which appear as dots or small lines (as seen in Figure 9 on page 55).

Although sectioning tissue allows for the identification of neurons and axons, it provides an extremely limited amount of information regarding the morphology, distribution, interaction, or source of the identified structures. Sectioning atrial tissue prior to IHC destroys the large-scale network of autonomic axons, limiting the information that can be obtained, but is often necessary because performing IHC on whole-mount (non-sectioned) atrial tissue is extremely difficult due to a lack of antibody penetration and an extraordinarily high degree of autofluorescence. Leger et al. (1999) performed whole-mount IHC on peeled guinea pig atrial epicardium (Figure 10 on page 56), but the high degree of tissue autofluorescence, the fact that only the epicardium was observed (preventing autonomic/cardiomyocyte connection analysis), and the lack of detailed morphological examination severely limited the study.

In an attempt to elucidate the interaction between the SNS and PSNS in the atria of the heart, (Hoard et al., 2008) performed IHC on sectioned mouse atria and noted that SNS and PSNS axons occasionally ran adjacent to one another (see Figure 9 on page 55). This intriguing finding suggested the possibility that some interaction between the SNS and PSNS may occur at the
axonal level, but such interaction could not be further elucidated due to the limitations of studying sectioned atrial tissue. If a method to perform IHC analysis on unpeeled whole-mount atria were developed which overcame antibody penetration and autofluorescence limitations, it would serve as a significant contribution to the field cardiac neuroanatomy, allowing for the direct visualization of autonomic innervation of the heart. Such a method would provide invaluable information as to the large scale distribution of autonomic innervation in the atria, as well as the small-scale morphology and interactions of the axons and terminals within it.

An alternate method of direct, morphological assessment of cardiac autonomic innervation involves tracer injections. Tracers injected in various regions of the body are absorbed by neurons and can aid in identification of the processes which extend from them. For example, tracers injected into the brain were successfully used to label parasympathetic afferent terminal structures in the aortic arch (Cheng et al., 1997) as well as parasympathetic efferent connections to the neurons of the ICG as seen in Figure 6 on page 53 (Cheng et al., 1999).

Theoretically, one could inject tracer into a single ICG in an attempt to identify individual neurons projecting from one ICG and selectively innervating a neuron within another ICG (thus proving the theory that LCNs exist within and among ICG). However, tracer injections cannot differentiate between local connections and extrinsic connections which may simply pass through the tracer-
injected ganglion, producing positive, yet inconclusive results. In mice (an animal commonly used due to its ease of transgenic modification), accurate tracer injection of ICG is extremely difficult because the ganglia are exceptionally small and transparent (unlike the large, opaque ICG of cats, dogs, and larger mammals) and also because the heart is so small that injection anywhere within the pericardium would non-specifically label all neural fibers within the heart.

Because of the limitations opposing direct observation of the distribution and morphology of neurons in the heart, many basic questions such as “Do cardiac sympathetic and parasympathetic postganglionic neurons interact in the atria?” remain unanswered. If methods were developed which can overcome the challenges currently preventing whole-mount atrial IHC studies, it would allow the study large-scale autonomic innervation in the atria, providing a detailed depiction of normal cardiac autonomic innervation. This would open the door for future studies to elucidate the nature of pathological autonomic remodeling in disease states.
INTRODUCTION

The heart is innervated by the sympathetic (SNS) and parasympathetic (PSNS) branches of the autonomic nervous system (ANS). However, the distribution, morphology, and interaction of such innervation has not yet been well studied. Detailed characterization of the nature of cardiac autonomic innervation in normal states is critical to the assessment of pathological autonomic remodeling caused by cardiovascular disease. The goal of this study is to elucidate the distribution, morphology, and interaction of the SNS and PSNS branches of the ANS in the atria (the primary location of autonomic regulation on the heart) by performing multi-fluorophore immunohistochemical analysis on whole-mount mouse atria to simultaneously and specifically label sympathetic and parasympathetic efferent neurons. To accomplish this feat, new techniques were developed to overcome the previous hindrances limiting the study of whole-mount mouse atrial tissues (including poor antibody penetration and high autofluorescence). This study utilizes laser scanning confocal microscopy to demonstrate the large-scale distribution of the ANS in the atria, as well as detailed structures of individual terminals along the epicardium and within the myocardium. Additionally, the simultaneous labeling of SNS and PSNS efferent neurons in the heart provides new clues as to their distribution, neurite structure, and interaction. This study will serve as the substrate for future studies involving autonomic remodeling in pathological states.
MATERIALS AND METHODS

Animals

C57BL/6 mice (n=6, age 3 to 6 months) were used. All animals were housed in a room in which light/dark cycles were set to 12 h/12 h (6:00 AM to 6:00 PM light cycle) and provided food and water ad libitum. All procedures were approved by the University of Central Florida Animal Care and Use Committee and strictly followed the guidelines established by NIH. All experiments conformed to the University of Central Florida guidelines on the ethical use of animals. Efforts were made to minimize the number of animals used and their suffering.

Tissue Preparation

All tissues utilized in this experiment were prepared identically. Mice were anesthetized with sodium pentobarbital injection (i.p., 100 μg/g). Surgical removal of the tissues of study began only when depth of anesthesia was sufficient to prevent suffering to the animal, determined by the absence of the hind paw pinch withdrawal reflex. A nearly circular transverse incision was made inferior to the plane of the diaphragm, exposing the abdominal cavity with the spinal cord and primary vasculature intact. The diaphragm was severed along its attachment to the ventral cavity wall, exposing the thoracic cavity. The thoracic cavity was further exposed by performing two longitudinal incisions from the ventral edge of the severed upper abdominal cavity to the superior region of the
thoracic cavity on each side of the internal thoracic arteries. The region of flesh
between these two incisions was clamped and folded upon itself, exposing the
heart and lungs. Heparin (1 ml) was injected directly into the left ventricle and
allowed to circulate for 2 minutes to prevent blood coagulation during the
following procedures. The inferior vena cava was then severed at the level of the
diaphragm and the heart was perfused through the left ventricle with warm
(40°C) PBS (1 M phosphate-buffered saline, pH=7.4) at a rate of approximately
40 ml/min, effectively draining the animal of blood. A strongly-beating heart
was always observed at the beginning of this perfusion. After 5 minutes
perfusion with cold (4°C) Zamboni’s fixative (ZF, 15% picric acid and 2%
paraformaldehyde in PBS, pH=7.4) began. After approximately 100mL of ZF had
been perfused through the animal, the heart and lungs, esophagus, and small
intestine were removed and further fixed in ZF for 24 hours at 4°C. During the
entire procedure, extreme care was taken to ensure that no part of the heart or
intestine became dry in an effort to minimize autofluorescence during later
examination.

Following adequate time in fixative, the heart and lungs were further
dissected. Briefly, the pulmonary veins were separated from the lungs with fine
tweezers and the lungs and trachea were discarded. Atria were separated from
the ventricles with fine scissors, and the aorta was removed from the atria. The
right and left atria were then separated, producing a right atrium with the
entrance of the inferior vena cava (IVC), superior vena cava (SVC), and left
precaval vein (LPCV) attached, and a left atrium with the entrance of the 4 pulmonary veins (PVs) attached. In some cases, the right and left atria were not separated, but rather the auricle (Au) of the left atrium (LAu) was removed, leaving the left atrium intact and attached to the right atrium. In some cases the right ventricle was isolated following its separation from the atria by cutting along the edge of the interventricular septum (IVS) at the attachment of the left ventricle. The esophagus and small intestine (opened by a longitudinal incision along the mesenteric attachment) were also isolated and saved.

Preparations of the outermost muscle layers of the small intestine (containing the circular and longitudinal muscle layers and the myenteric plexus as depicted in Figure 11 on page 57) were created by first removing the microvilli then separating the submucosa from the circular muscle with fine tweezers. The esophagus was prepared in a similar manner, with the outermost muscle layers saved for later analysis.

**Immunohistochemistry**

All immunohistochemical (IHC) procedures were performed on a shaker at room temperature, with each piece of tissue immersed in 0.5 ml of reaction liquid in its own chamber of a covered 24-well plate. Following tissue preparation, residual ZF was removed from tissues by 5 washes (10 min each) in PBS. Tissues were then immersed for 24-48 hr in a blocking reagent (2% bovine serum albumin, 10% of normal goat serum, 10% normal donkey serum, .08% Triton X-100, .08% NaNH$_3$ in .01 M PBS, pH=7.4) to reduce nonspecific binding
of the primary antibody and to increase antibody penetration. Primary antibodies (12 μl/ml each, see Table 1 on page 48 for details) were added to primary solution (2% bovine serum albumin, 4% of normal goat serum, 4% normal donkey serum, .08% Triton X-100, .05% NaNH₃ in .01 M PBS, pH=7.4) and administered for 48 hr. Unbound primary antibodies were removed from tissues by six 5 min washes in PBST (0.3% Triton X-100 in .01 M PBS, pH=7.4). Secondary antibodies (12μl/ml each, see Table 1 on page 48 for details) where then applied for 3 hr. Unbound secondary antibodies were removed from tissues by six 5 min washes in PBS.

Tissues were then mounted with their cranial surface (in the case of the atria) or distal surface (in the case of the small intestine, right ventricle, and esophagus) down onto positively-charged glass slides, crushed for 2 hours, and air-dried under a fume hood for 1 hour. Slides were dehydrated by immersing them for 2 minutes in each of 4 ascending concentrations of ethanol (75%, 95%, 100%, 100%), followed by two 10 min washes in 100% xylene. Slides were then coverslipped with DEPEX mounting medium (Electron Microscopy Sciences #13514) and allowed to dry overnight. Care was taken during every step of this procedure to minimize tissue exposure to light and to prevent tissue from becoming dry. Preparations were also created in which combinations of primary antibodies were omitted, serving as negative controls. In every case these preparations presented no labeling, confirming that nonspecific binding of secondary antibodies did not occur.
Confocal Microscopy

Slides were observed with a Leica SP5 laser scanning confocal microscope. In an effort to minimize autofluorescence (AF, the natural fluorescence in the blue-green emission spectrum due to the intrinsic properties of the atrial tissue) in the fluorophore emission region, many preparations contained secondary antibodies conjugated with far-red and/or infrared fluorophores which are indistinguishable or invisible to human eyes. However, these fluorophores were readily detected by the confocal microscope system, allowing clear imaging of thick tissue with minimal autofluorescent interference.

Due to the whole-mount nature of the auricles of the atria, the cranial epicardium was pressed flat against the slide, the proximal surface pressed (not quite as flat) against the glass cover slide, and the endocardium was highly distorted in 3D space so that in any given frame only a small region of it was observable, pressed strongly between bundles of myocardium, making it difficult to represent in a single optical section. For this reason, only confocal imaging of the cranial epicardium and the myocardium was performed, as presented in all representative images.

Colocalization Assessment

All confocal images were obtained sequentially (recording only one fluorescence channel at a time) to minimize fluorophore crossover. Scan-time negative control images were routinely obtained in tissues labeled with multiple fluorophores (by exciting in the region of one fluorophore and imaging the
emission wavelength of the other fluorophore) to ensure that significant crossover fluorescence did not occur. In cases where green (488 nm) and red (500 nm and above) fluorophores were compared, no crossover was ever observed. Due to the nature of the 594 fluorophore (with a wide emission spectra), a minimal amount of crossover occurred in which the 594 fluorophore was excited by and released light in the range of the 660 nm fluorophore. Due to the possibility of far-red/infrared crossover, conclusions about colocalization were only based upon images with green/red fluorophores. However, images displaying red/infrared fluorophores were excellent at revealing trends in TH/VACHT interaction due to the minimal amount of interfering AF normally experienced below 500 nm.

**Utilization of Autofluorescence to Image Muscle and Vasculature**

Autofluorescence is often considered a nuisance to fluorescence microscopy and has traditionally prevented high quality images from being obtained of IHC-labeled axons in whole mount atrial preparations (Figure 10 on page 56) and even in sectioned tissue only a few microns in thickness (Figure 9 on page 55). However, the use of far-red and infrared fluorophores allows AF to be utilized for a constructive purpose. By obtaining AF-only images in the green spectrum, the highly-AF heart and cardiac vasculature can be clearly imaged alongside IHC-labeled axons, producing a more complete picture which depicts IR features as well as the appearance of the surrounding tissue. In multicolor figures, TH is presented as green and VACHT is presented as magenta or blue. In
figures which utilize AF imaging to depict the texture of the tissue, TH is presented as green, VACHT is presented as blue, and the AF features (usually muscle cells, vasculature, or collagenous fibers) are presented as red.

**Image Modification**

Care was taken to minimize modification of confocal images. All confocal images were scanned with similar settings. The only adjustments made after scanning (with the exception of confocal montage images) involved modification of brightness and/or contrast of the entire image. Images were obtained with Leica LAS software. All modification, adjustments, cropping, scale bar additions, etc. was conducted utilizing NIH ImageJ software.

**Montage Assembly**

Confocal montages (large images assembled from many smaller images) were created from images which underwent considerable modification. First, stacks of confocal sections were obtained with a 10x lens. Background AF from each optical section was reduced by the iterative minimum ranking background subtraction algorithm distributed with NIH ImageJ. All stacks where then maximum-projected to yield a series of image tiles. MosaicJ (Thévenaz P and Unser M, 2007) was utilized to assist in the assembly of these tiles to create large montage images. Artifacts (air bubbles trapped in the mounting media) were manually removed from the resulting image, but only outside of the perimeter of the tissue. No selective modification of the montage image was ever performed.
over the area of the tissue. Due to the fact that the montage image was susceptible to non-uniform modification (in the background subtraction and automated montage assembly steps), such montages should only be assessed for their visual and qualitative properties.
RESULTS

Sympathetic Innervation of the Atria

Confocal montage assembly of whole-mount mouse atria IHC-labeled for TH-immunoreactivity (IR) allowed for the large-scale visualization of sympathetic innervation of the atria (Figure 12 on page 58). Two large bundles of smooth TH-IR axons (likely postganglionic sympathetic efferent axons from the sympathetic chain of ganglia) travel from each side of the heart to the sinoatrial (SA) and atrioventricular (AV) node, ultimately projecting to the muscle through a network of branching structures. In all cases, large projections from the atria to the entrance of the auricles were observed. These projections bifurcated multiple times along their length, forming branching tree-like structures as they traveled across the auricle.

The epicardium (identified by the presence of an autofluorescent network of collagenous fibers) was the primary layer in which large bundles (usually with more than 10 axons) were observed in the auricles (See panel A of Figure 13 on page 59). Single axon terminal structures were also observed in the epicardium. The myocardium contained mostly single TH-IR axons, some small bundles (usually with 10 or fewer axons), and no large bundles.

The auricles of the atria consist of many large, thick muscle bundles which provide a majority of the contractile force in the atria. TH-IR axons were observed forming dense networks of long varicose neurites in the auricles which
travel in small bundles along the epicardium and project to the myocardium. TH-IR axons were also observed innervating short regions of vasculature. Individual axons innervating vasculature were also observed to loosely innervate the cardiomyocytes. Fat cells near the atria were observed to be heavily innervated by TH-IR axons (see Figure 17 on page 62).

Although terminal structures were identified in the myocardium and on the epicardium, the large size of the terminal structures combined with the dense nature of the surrounding immunoreactive axons made individual axon visualization extremely difficult, if not impossible by IHC means. Refer to Figure 14 on page 60 for a representation. Although visual inspection of the confocal montage presented earlier (Figure 12 on page 58) demonstrates that TH-IR axons target the ICG, closer inspection reveals that TH-IR axons pass through ICG with forming large synaptic varicosities around the ICGNs within them (compare sympathetic innervation in Figure 26 on page 71 with vagal innervation in Figure 6 on page 53). Large bundles of smooth axons commonly travel through ICG, and often individual axons can be distinguished which travel through ICG, but clearly defined (vagal-like) large and tight TH-IR synaptic terminal endings around individual neurons in the ICG were never observed. Additionally, a subpopulation of approximately 30% of ICGNs were observed to be TH-IR. Small, intensely fluorescent (SIF) cells were also observed throughout the atria and will be further described below.
Parasympathetic Innervation of the Atria

Similar to the observed TH innervation, VACH-T-IR axons were present in dense bundles in the atria. These bundles projected to the auricles, bifurcating into smaller bundles and eventually formed individual terminal structures, observed in both the epicardium (see panel B of Figure 13 on page 59) and myocardium. Unlike the strong, smooth, and homogenous labeling observed in the TH-IR axons, VACH-T-IR axons were weakly labeled along the length of their axons, with stronger labeling in their varicosities. Similarly, ICGNs presented weak VACH-T labeling in their somata, with strongly-labeled punctate IR structures on their surface (likely the location of synaptic varicosities from pre-ganglionic neurons).

In the auricles of the atria a dense network of VACH-T-IR terminal structures innervated the epicardium and myocardium. Their terminal structures (single, lose, varicose neurites loosely innervating cardiomyocytes) were very similar in structure to TH-IR axons. Similarly, specific terminal structures were difficult to assess due to the extremely dense nature of VACH-T-IR axons in the atria. VACH-T-IR axons were observed innervating vasculature (with individual axons forming terminals along vessels and the myocardium). VACH-T-IR innervation of fat cells near the atria was extremely rare, and virtually all fat cells observed were innervated by TH-IR axons in the absence of VACH-T-IR axons.
Sympathetic/Parasympathetic Interaction in the Atria

Parallelism

IHC performed to simultaneously label TH-IR and VACHT-IR axons allowed for the visualization of the interactions of the SNS and PSNS in the atria of the heart (see panel C of Figure 13 on page 59 and Figure 15 on page 61). Individual TH-IR and VACHT-IR axons were commonly observed paralleling each other in the atria as axon bundles or as two parallel adjacent axons of opposing phenotypes. Larger bundles of parallel axons were observed at the entrance of the auricles than in the edges of the auricles (Figure 16 on page 61).

In addition to individual parallel axons, parallel TH-IR and VACHT-IR axons were commonly observed in large bundles consisting of many axons (Figure 18 on page 63) which traveled in a direction unassociated with cardiomyocyte structure. Smaller axons (usually parallel axons, one of each phenotype) often traveled in the direction of cardiomyocyte distribution, perpendicular to their striations. The only individual (non-parallel) axons observed were always varicose terminal structures (see panels A and B of Figure 19 on page 64). Such features were observed throughout the atria.

In every case, parallel axons consisted of at least one axon of each class (TH-IR or VACHT-IR). These parallel axons were always varicose in nature, with interdigitized varicosities alternating between TH-IR and VACHT-IR neurites along their length (panel C Figure 19 on page 64). Although a majority of the neurites in paralleling axons or bundles contained exclusively TH-IR or VACHT-IR.
IR, colocalization was also observed in some axons in atria (Figure 20 on page 65).

**Innervation of the Epicardium vs. Myocardium**

Although TH/VACHT parallelism was observed throughout the atria, different types of neural networks were observed in the epicardial and myocardial layers of the atrial auricles. These two layers were distinguished by the texture of the autofluorescent images, in which the collagenous epicardium (with clearly visible connective fibers as seen in Figure 21 on page 66) was distinctly different in appearance than the myocardium (identified by the presence of striated smooth muscle cells) as seen in panel A of Figure 15 on page 61.

The epicardium contains an organized network of bundled axons with individual neurites branching out from these bundles and forming flat, 2-dimensional terminal structures on the epicardium, allowing them to be easily imaged in a single confocal optical section (panel B in Figure 21 on page 66). TH-IR, VACHT-IR, and colocalized TH-VACHT-IR terminal structures were identified in the epicardium of the auricle of the right atrium. TH-IR, VACHT-IR, and colocalized TH-VACHT-IR terminal structures were also identified in the myocardium of the auricles (Figure 15 on page 49). However, due to the 3-dimensional and overlapping nature of these structures, morphological assessment of individual terminal structures proved to be difficult.

No significant differences were observed in the morphological nature of TH-IR and VACHT-IR axons in the atria. In both cases, axons innervated the
muscle loosely forming a dense network of varicose axons. Specific, tight, large terminal structures similar to intramuscular junctions (such as those seen in skeletal muscle innervation) were never observed. In all cases, axons traveled smoothly in bundles, parallel axons, or single axon terminal formations.

**Innervation of Vasculature**

In addition to the working muscle of the atria, autonomic axons also target the atrial vasculature. Short regions of vascular innervation were observed in all atrial tissues studied (Figure 22 on page 67). However, the dense nature of the surrounding tissue combined with the nature of the compressed tissue being studied often made its visualization a challenge. Clear images of vasculature are only available at the entrance of the veins to the atria where axonal innervation less dense. Locations of innervated vasculature were first identified by the structure of the fluorescently-labeled autonomic axons, and later confirmed by autofluorescence micrographs. Such structures were only definitively classified as vasculature upon the observation of smooth (collagenous) axons running parallel to the direction of the vessel combined with the lack of striated muscle tissue observed in the region.

TH-IR and VACHT-IR axons were commonly observed running alongside vasculature in bundles, often forming small varicose structures around the vasculature. Although colocalized TH-IR/VACHT-IR axons were not observed innervating the vasculature, this possibility has not been excluded as only several short regions of vasculature were successfully imaged in detail. TH-IR axons,
VACht-IR axons, and sometimes parallel TH-IR/VACht-IR axons were often distinguished which paralleled and innervated vasculature as well as projected to the muscle.

Intrinsic Cardiac Ganglia

ICG were always observed near the epicardium of the cranial surface of the atria. Within these ganglia were principal motoneurons (PNs), identified by their size and shape (large somata/nucleus ratio, with a maximum diameter between 20 and 40 μm). All ICG PNs were weakly VACht-IR, with strong, punctate VACht-IR dots around their somata (Figure 23 on page 68 and Figure 24 on page 69). Approximately 30% ICG PNs expressed a dual dopaminergic (TH-IR) / cholinergic (VACht-IR) phenotype. However, TH-IR intensity varied among PNs, making differentiation between weak TH-IR and non-TH-IR neurons difficult (see Figure 24 on page 69). The size of TH-IR PN somata appeared similar to non-TH-IR PNs. Punctate VACht-IR dots around PNs were always observed, regardless of phenotype.

Intrinsic cardiac ganglia of different sizes were also observed, but in most cases ganglia were near dense TH-IR nerve bundles from the sympathetic chain of ganglia (Figure 25 on page 70). Although punctate VACht-IR dots appeared around the somata of ICGNs, similar TH-IR punctate structures were never observed.
SIF Cells

Many small, intensely fluorescent (SIF) cells were observed in the atria (Figure 26 on page 71), distinguished from PNs by their small size (less than 15\(\mu m\) in diameter), intense TH-IR, and small somata/nucleus ratio. SIF cells were observed along preganglionic sympathetic bundles (Figure 27 on page 72), in, around, or near ICG (Figure 26 on page 71), and in the epicardium unaccompanied by large bundles or ganglia. SIF cells were never VACHt-IR. Additionally, SIF cells were not observed to be innervated by TH-IR or VACHt-IR axons, although their intense fluorescence in relation to other IR structures may have masked such innervation so this observation can not be considered definitive.

High quality images of TH-IR SIF cells were not obtainable while simultaneously imaging TH-IR axons and PNs because the degree of SIF cell fluorescence intensity was far greater than any other TH-IR structures. Therefore, quality representations of SIF cell / TH-IR ICGN and axon interactions were created by imaging the same region of tissue twice using different laser intensity settings (as seen in panels A-D of Figure 26 on page 71). To produce single images in which both TH-IR axons and PNs are visible alongside SIF cells, merged images were created in which the high-powered laser image (showing TH-IR PNs and axons) was tinted blue, and the SIF cell image was added (in grayscale) to it (as seen in panel E of Figure 26 on page 71).
Sympathetic/Parasympathetic Interaction in Other Tissues

Because of the unique and fascinating nature of the parallel interactions between TH-IR and VAChT-IR axons in the auricles of the atria, several tissues of the same mice were processed via the same IHC protocol. The phenomenon of SNS/PSNS parallelism, it seems, is not exclusive to the atria.

Right Ventricle

A whole-mount right ventricle IHC-processed to label VAChT and TH displayed parallel features similar to those observed in the atria. (Figure 28 on page 73) However the extremely dense nature of whole-mount ventricles made confocal microscopy difficult, and no images obtained from the ventricle whole-mounts were of as high quality as those obtained from the atria. This could likely be improved by further optimization of the protocol to produce better results in the ventricles.

Esophagus

The outer layers of the esophagus IHC-processed to label VAChT and TH displayed parallel features similar to those observed in the atria (Figure 29 on page 73). In addition, esophageal tissues were observed to possess small, dense, oval-shaped terminal structures exclusively IR for VAChT in the muscle layers, likely parasympathetic intramuscular junctions (not shown).
The outer layers of the small intestine IHC-processed to label VACHT and TH displayed a unique interaction of SNS and PSNS axons. Due to the extremely thin nature of peeled small intestine (often less than 10 μm in thickness), IHC-treated whole-mounts produced strikingly clear results (Figure 30 on page 74). Postganglionic PSNS neurons in the myenteric plexus were a target of SNS axons, which traveled to (and heavily innervated) the neurons inside. Although the smooth, varicose structures of the TH-IR axons innervating the neurons of the myenteric plexus are not as tight as the preganglionic/postganglionic connections of the vagus nerve and the neurons of the ICG (refer to Figure 6 on page 53), it still appears far more extensive than that of the ICG where no dense TH innervation of PNs was ever observed (refer to Figure 23 on page 68). Similar to the atria, some of the axons innervating the muscle of the small intestine travel adjacent to axons of the opposite phenotype, but this is not observed to a lesser extent in the small intestine compared to the heart.

DISCUSSION

Methodology

This study demonstrates that the use of the described tissue processing methods, combining IHC techniques with infrared fluorophores and laser scanning confocal fluorescence microscopy, allows for the simultaneous study of SNS and PSNS cardiac innervation at both large (distributional-level) and the
small (axon and terminal structure-level) scales. The same method can be applied to study autonomic remodeling in pathological states, and serves as a significant contribution to the field of cardiac neuroanatomy. In addition, it can be adapted to facilitate the study of a wide selection of neurochemical markers in the atria, as well as other tissues in other organs.

**Anatomical Overview**

IHC performed on whole-mount mouse atria allowed for the examination of the general distribution of autonomic innervation (Figure 12 on page 58). Large SNS nerve bundles of TH-IR axons enter the heart, travel through one or more ICG, then travel in bifurcating bundles along epicardium of the atria before forming loose terminal structures in the epicardium or myocardium. With respect to the auricles of the atria, large bundles enter the auricles (Figure 16 on page 61), branching to form a net-like network of smaller bundles on the epicardial surface (Figure 21 on page 66). Terminal structures deep within the myocardium are formed by single axons which deviate from epicardial axon bundles, often paralleled at some point by an axon of the opposite autonomic phenotype. Similar SNS/PSNS parallelism was observed throughout the atria of every animal studied.

The general distribution of ANS innervation in the atria (bundle-rich epicardium with some terminals, and the terminal-rich myocardium) may be explained by a study conducted by Ieda and Fukada (2009) which investigated the molecules responsible for sympathetic innervation in the atria. It was
concluded that nerve growth factor (NGF, a potent neuron attractant) is expressed heavily in the myocardium, encouraging nerve sprouting and terminal formation in the myocardium from the sympathetic bundles transversing the epicardium. Such branching into the myocardium is balanced by Sema3a, a neuron repellent expressed specifically in the subendocardium. The epicardial-to-endocardial transmural gradient of NGF and Sema3a results in the unique structural qualities of SNS innervation in the auricles of the atria, and may also similarly affect axons of the PSNS as well. Whatever the case, due to the similarity observed in the structures of the SNS and PSNS axons in the heart, as well as the abundance of adjacent parallelism, it is very likely that both branches of the ANS are affected similarly by the same neural growth factors which encourage them to grow adjacently – a possibility which merits further investigation at the molecular level.

Intrinsic Cardiac Ganglia

Large bundles of TH-IR axons entering the heart (likely projections from the sympathetic chain of ganglia) were observed passing through ganglia (Figure 23 on page 68 and panel E of Figure 26 on page 71), but never forming large and tight vagal-like connections within ganglia as seen in PSNS vagal connections (as seen in Figure 6 on page 53). This suggests the sympathetic postganglionic projections from the sympathetic chain of ganglia do not directly communicate with the somata of parasympathetic postganglionic ICGNs.
Additionally, the lack of TH-IR synaptic varicosities around the somata of ICGNs suggest that no TH-IR ICGNs form local circuit connections (where ICGNs form synapses with the somata of ICGNs in the same or other ganglia). This finding is in contrast to the emerging “little brain” view of the intrinsic cardiac nervous system in which ICG are described as a highly interconnected plexus of neurons which are in constant communication. Although interactions may occur, tight synaptic formations around PN somata formed by local circuit neurons have yet to be definitively demonstrated. In this study, we concluded that TH-IR ICG PNs (a phenotypic subpopulation which comprises approximately 30% of all ICG PNs) do not form local circuit connections with neurons in the same or other ICG.

SIF Cells

The role of SIF cells is often debated. They are usually considered to serve roles as chemosensory paraneurons or local circuit interneurons (Heym and Williams, 1979), generally associated with the sympathetic branch of the ANS. However, previous studies have demonstrated that SIF cells in the heart are directly innervated by PSNS preganglionic axons (Cheng 1999). Previous IHC studies (Slavíková et al., 2003) have reported that cardiac SIF cells lack dopa-β-hydroxylase, suggesting that they cannot synthesize NE, and are most likely to release dopamine as their primary neurotransmitter.

Our study identified three classes of SIF cell formations in the atria (refer to panels A-D in Figure 26 on page 71), allowing speculation as to their functional...
role based upon their location. Extremely dense clusters of SIF cells were often observed associated with (inside or immediately beside) large TH-IR nerves from the sympathetic chain of ganglia (Figure 27 on page 72 and panels B and D of Figure 26 on page 71). These cells may serve chemosensory functions, modifying sympathetic activity in response to chemical changes. SIF cells were also observed associated with ICG (panels C and E of Figure 26). Because most ICG are innervated by TH-IR bundles which pass through them, it is difficult to differentiate between ganglion-associated SIF cells and SNS nerve bundle-associated SIF cells. Ganglionic SIF cells may serve as local circuit interneurons, although IHC/confocal analysis of their processes is difficult due to the extremely small size of their axons and the extreme intensity of their somata. Additionally, clusters of individual SIF cells were observed in regions of the atria far from sympathetic bundles or ganglia (panel A of Figure 26), the function which is difficult to interpret. Although numerous numbers and multiple classes of SIF cells were observed in the atria, IHC analysis and confocal microscopy only allow speculation as to their function. Ultrastructural electron microscopic studies should be performed to further analyze the structure of SIF cell synapses, internal vesicles, and other features in with the goal of definitively defining their function.

Sources of Myocardial Innervation

The source of neurites located in the atria can be determined by examining the neurochemical markers they possess. We observed three distinct classes of terminal phenotypes in the myocardium, all with similar morphology (Figure 20
on page 65). First, axons which were only IR for TH were presumed to originate from the sympathetic chain of ganglia, which release NE as their primary neurotransmitter and do not synthesize ACh. Secondly, we identified VACHT-IR axons innervating the myocardium. Because previous tracing studies labeling vagal efferent axons never displayed terminals in the muscle (Cheng et al., 1997), it is believed that the only VACHT-IR PSNS axons innervating the myocardium are postganglionic axons from the network of ICG located on the atria. However, a third class of terminal structures expressing both TH-IR and VACHT-IR was observed. Since the only cardiac autonomic neurons possessing both TH-IR and VACHT-IR are the ~30% of ICGNs described earlier, it can therefore be assumed that TH-IR ICGNs project to and form terminals within the myocardium and epicardium.

Previously it was hypothesized that TH-IR ICGNs served a role as local circuit interneurons due to their phenotypic similarity to SIF cells (Slavikova et al., 2003). In the present study, not only did we demonstrate that these axons innervate the working muscle of the atria, but we also reported the absence of large TH-IR synaptic varicosities within ICG, suggesting that these TH-IR ICGNs never function as LCNs.

Other studies have reported that newborn rats exhibit a higher fraction of TH-IR ICG PNs than adults, implying that they may provide intrinsic sympathetic-type stimulation during early life while the SNS is still developing, switching to the parasympathetic phenotype once the SNS becomes fully
developed (Slavíková et al., 2003). If this is the case, the observed TH contained within some ICGNs exists as a remnant of development and no longer serves a functional role in the synthesis of catecholamines as neurotransmitters. This theory is supported by the IHC evidence presented herein, demonstrating that TH-IR ICG PNs express the full adrenergic (VAChT-IR) phenotype and innervate the myocardium similarly to non-TH-IR ICG PNs.

**Sympathetic / Parasympathetic Parallelism**

An important finding of this study is the discovery that SNS/PSNS parallelism is extremely common in the atria, whereby individual (non-paralleled) axons are only found forming terminal structures. The suggestion that TH-IR and VAChT-IR axons parallel each other in the atria was previously noted by Hoard et al. (2008) in sectioned tissue (Figure 9 on page 55). However, due to the nature of sectioned tissue studies, limited information was available as to the frequency and terminal morphology of these adjacent axons. As a result, the overwhelmingly high frequency of parallelism in the atria was previously under-reported, and the occurrence of dual-labeled VACH+TH-IR axons and terminals innervating cardiomyocytes was overlooked. The IHC-labeling of both neurochemical markers in whole-mount mouse atria provides a much more complete picture of the interaction between these two branches of the ANS, allowing the visualization of their general distribution and structure.
Theory of Sympathetic / Parasympathetic Antagonism

Unique structural features are often indicative of distinctive functional qualities. Although the structural nature of sympathetic/parasympathetic parallelism in the atria is easy to observe utilizing IHC methods, the functional ramifications of this unique structural phenomenon remain elusive. Physiological or morphological electromicroscopic studies may eventually determine the functional role of sympathetic/parasympathetic parallelism. Until then, one can only speculate as to its purpose based upon the structural features it demonstrates. The close proximity of adjacent paralleling sympathetic and parasympathetic axons suggests the possibility of communication between them. Due to their incredibly close proximity to one another, their method of interaction could be via prejunctional chemical communication.

One possible method of communication between parallel sympathetic / parasympathetic axons is neurotransmitter-based, whereby each axon has receptors sensitive to the neurotransmitters released by the other axon. It has already been established that SNS varicosities contain synaptic regulatory elements which serve to suppress nerve activity to in response to NE detected by $\alpha_2$ receptors, resulting in a local negative feedback loop (Gilsbach et al., 2009). Prejunctional sympathetic varicosities in the heart are also known to possess $M_2$ receptors (Trandelenburg et al., 2005), allowing their activity to be suppressed by ACh released from parasympathetic nerve terminals. In this way, the PSNS (the
network ICGNs) has the potential to directly suppress SNS activity prejunctionally.

Whether sympathetic suppression of parasympathetic activity is also possible merits investigation. Postganglionic PSNS axons in the heart are not believed to possess NE receptors of any type. However, physiological evidence from Roquebert et al. (1991) suggests that parasympathetic axons in the heart possess prejunctional inhibitory dopamine (D₂) receptors which inhibit parasympathetic activity (and the release of ACh) in response to epinephrine (dopamine), a precursor of NE. Traditionally, sympathetic postganglionic axons are believed to release NE. However, if synaptic vesicles contain a combination of NE and dopamine (due to incomplete conversion of dopamine to NE by dopa-β-hydroxylase), they would be able to stimulate muscle contraction (via NE release) while simultaneously inhibiting parasympathetic activity (via DA release). This would allow sympathetic suppression of parasympathetic activity, representing a new theoretical mechanism of direct, prejunctional SNS/PSNS antagonism (Figure 31 on page 75).

Functionally, such a method of neurotransmitter-based SNS/PSNS antagonism could allow the ICG to modulate tonic inputs from the sympathetic chain ganglia, effectively controlling SNS input to the heart. In such a configuration, the ICG would serve as the primary integrative center for cardiac autonomic control. Conversely, sympathetic ganglia may possess the ability to override the postganglionic PSNS control exhibited by the ICG via the speculated
SNS/PSNS antagonism mechanism. In this model, the sympathetic chain of ganglia would serve as the primary integrative center for the heart, modulating the signals from the network of ICG. Alternatively, it is possible that both branches of the ANS work together evenly throughout most of the active cycle, with no branch chronically suppressing the other to dominate control over cardiac function, but rather each taking over (and inhibiting the opposite branch) only when dramatic modulation of cardiac output is necessary in response to physiological extremes such as excessive stress (SNS inhibition of PSNS) or deep sleep (PSNS inhibition of SNS).

**Developing Model of Autonomic Cardiac Regulation**

The results presented in this study provide new clues as to the methods by which ANS regulates cardiac output, adding the prejunctional level to the list of sites of sympathetic / parasympathetic interaction and possible communication. Data from this report provide support both for and against the “little brain” theory of advanced cardiac regulation via local information processing in the network of ICG. On one hand, the lack of dual-labeled VACHT+TH-IR synaptic terminals forming connections with neurons in ICG and the presence of such axons forming terminal structures in the myocardium suggests that TH-IR IGCNs (~30% of all ICGNs) never form local circuit connections, but rather project only to the muscle, providing evidence against a complex and interconnected network of neurons within the ICG. On the other hand, the overwhelming majority of traveling autonomic axons in the atria exhibited
adjacent sympathetic / parasympathetic parallelism with alternating varicose structures suggesting the possibility of complex prejunctional communication between these two systems. If such a communication system were to function antagonistically, the ICGNs would have the ability to modulate the activity of neurons from the sympathetic thoracic ganglia, and visa versa, increasing the level of functional complexity of the intrinsic cardiac nervous system.

**Future Studies**

This study utilized IHC techniques to provide high quality images of anatomical features of both branches of the ANS and the unique interactions between their neurites. However, future studies using different techniques should be conducted to further elucidate the connections within the cardiac nervous system. First, elucidating the role of the ICG in cardiac regulation (including the presence and nature of local circuit connections) is of paramount importance to the development of methods of treatment for severe cardiac arrhythmia and other heart-related health problems. Studies involving fluorescent tracer injection directly into the somata of individual ICGNs to label their connections are already underway to provide information about the network of ICG and its ability to process information. Additionally, methods of quantification of autonomic indices (such as three-dimensional axon density) should be perfected so that pathological changes to the ANS in disease states (such as diabetes and chronic intermittent hypoxia) can be quantified, possibly
providing insight into the mechanisms of heart disease and cardiac autonomic neuropathy.
CONCLUSION

The immunohistochemical studies performed on whole-mount mouse atria described herein provide much information as to the distribution, function, and interaction of both branches of the ANS in the heart. This study demonstrated that large sympathetic bundles from the sympathetic chain of ganglia project to ICG, but pass through them rather than forming large synaptic varicosities with the ICGNs contained within them. This study also demonstrated that sympathetic (TH-IR) and parasympathetic (VACHt-IR) postganglionic neurons are often parallel adjacent to one another in the atria, with alternating varicosities between paralleling TH-IR and VACHt-IR axons, suggestive of communication between them at the prejunctional level. Anatomical details of SNS and PSNS innervation and interaction in the epicardium, myocardium, fat pads, and vasculature of the heart were reported (Figure 32 on page 76), as well as in portions of tissue from the esophagus and small intestine. This study not only contributes much information to the field of cardiac neuroanatomy, but underlies the foundation for future studies investigating pathological cardiac autonomic remodeling in disease states.
APPENDIX: TABLES AND FIGURES
Table 1: Antibodies Used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Host</th>
<th>Company</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-TH†</td>
<td>12 μl/ml</td>
<td>Rabbit</td>
<td>Pel-Freez</td>
<td>n/a</td>
</tr>
<tr>
<td>Anti-VAcT†</td>
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<td>Guinea Pig</td>
<td>Millipore</td>
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<tr>
<td>Anti-Rabbit††</td>
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<td>Donkey</td>
<td>Invitrogen</td>
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</tr>
<tr>
<td>Anti-Rabbit††</td>
<td>24 μl/ml</td>
<td>Goat</td>
<td>Invitrogen</td>
<td>488 nm</td>
</tr>
<tr>
<td>Anti- Guinea Pig††</td>
<td>24 μl/ml</td>
<td>Goat</td>
<td>Invitrogen</td>
<td>660 nm*</td>
</tr>
</tbody>
</table>

†primary  
‡‡secondary  
*infrared fluorophores are invisible to human eyes
Figure 1: Anatomy of a Chemical Synapse
Most neurons of the autonomic nervous system (ANS) communicate via chemical synapses. The neuron transmitting the message (top right) produces an electrical charge which travels down the length of an axon (a long branch of a neuron) and causes the release of neurotransmitters from within synaptic vesicles. Receptors sensitive to the released neurotransmitters located on the target cells allow the detection of the chemical message. The connection between two neurons is called a synapse.
Figure 2: Trends of ANS Innervation
Autonomic signals from the central nervous system generally pass through peripheral ganglia before innervating their targeted tissues. Colors depict neuron classifications: blue, sympathetic preganglionic; pink, sympathetic postganglionic; red, parasympathetic preganglionic; green, parasympathetic postganglionic
**Figure 3: Mechanism of Cardiomyocyte Contraction**

Neurotransmitters released by the ANS (such as NE or ACh) promote the release of stimulatory or inhibitory molecules (respectively) which modulate the rate of catalysis of cAMP from ATP by AC. Since muscle contraction is ultimately the product of AC efficiency, NE and ACh can effectively modify muscle contraction properties.

[this figure will be re-created]

**Figure 4: Anatomy of the Heart**
Figure 5: Primary Nerves of the Thoracic Cavity
Schematic diagram of the primary nerves in the human thoracic cavity. Note the location of the vagus nerve in relation to the sympathetic chain of ganglia (superior cervical ganglia, middle cervical sympathetic ganglia, stellate ganglia, and the rest of the sympathetic ganglia on the thoracic sympathetic trunk). Unlike humans, mice are not believed to have a cardiac plexus at the base of the aortic arch, but rather a plexus of ganglia located directly on the epicardial surface of the atria.
Figure 6: Vagal Synapses
Tracer-labeled preganglionic vagal projections to the ICG form dense, tight synaptic varicosities around individual ICGNs (arrows). Image adapted from Ai et al. (2007)

Figure 7: ICG on Atria
Location of intrinsic cardiac ganglia (circular and triangular markings) on the surface of the atria. IVC, inferior vena cava; SVC, superior vena cava; LP-CV, left precaval vein; RX, right vagus nerve; LX, left vagus nerve; RA, right atrium; LA, left atrium; a, auricle. Image adapted from Cheng et al. (1999).
Figure 8: Network of ICG

Network of PSNS ICG in peeled rat epicardium labeled via IHC (inverted fluorescence micrograph). Although it appears that various ICG are interconnected, it remains to be clarified whether these axons represent axons which form synapses with, or simply pass through, other ICG.

Image adapted from Richardson et al., 2003
Figure 9: SNS/PSNS Adjacent in Sectioned Tissue
Adjacent TH-IR and VACChT-IR axons in sectioned mouse atrial myocardium. Note the background intensity due to myocardial autofluorescence even in tissues only several microns in thickness. Image adapted from Hoard et al. (2008).
Figure 10: Guinea Pig Atrial Epicardium

Sympathetic (TH-IR) network in the peeled epicardium of guinea pig atria. Note the intense autofluorescence remaining in the epicardium. The removed myocardium (containing cardiomyocytes) are similarly fluorescent. LAA, left atrium auricle; RAA, right atrium auricle; SVC, superior vena cava; IVC, inferior vena cava; PV, pulmonary vein. Image adapted from Leger et al. (1999).
Figure 11: Layers of the Small Intestine
Figured obtained from (somewhere) depicting the layers of the small intestine. In preparations examined via IHC in this study, the circular muscle was separated from the submucosa with fine forceps, and the outermost layers examined.
Image adapted from Furness and Costa (1980).
Figure 12: Sympathetic Innervation of the Atria (Montage)
TH-IR innervation in unpeeled whole-mount mouse atria. RA, right atrium; Rau, right auricle; LA, left atrium; LAu, left auricle (removed); LPCV, left precaval vein; IVC, inferior vena cava; SVC, superior vena cava; AV, atrioventricular node; SA, sinoatrial node. A-F are represented in Figure 25 on page 70. G is represented in Figure 23 on page 68 and Figure 26 on page 71.
Figure 13: TH, VACHT, and TH+VACHT IR Axons
TH-IR (A, green) and VACHT-IR (B, blue) innervation of the atrial myocardium and their interaction (C). Red fibers represent autofluorescent collagen of the epicardium.
Figure 14: TH-IR Density in the Auricles
The region of the right atrium enclosed by the square in (A) was scanned at high resolution and maximum-projected (B), demonstrating the extremely dense network of TH-IR innervation of the atrium. VAChT-IR innervation is of similar density (projection not shown).
Figure 15: Innervation of the Myocardium
All types 3 classes of axons innervate the muscular myocardium, but in a rich 3D network. This single optical section demonstrates the interaction of TH-IR (green) and VACHT-IR axons (blue) with the thick, dense network of cardiomyocytes (red).

Figure 16: Innervation at the Entrance of the Auricles
Unlike the edges of the auricles in which single paralleling axons were common, the entrance of the auricles consists mostly of large parallel axon bundles.
Figure 17: Fat Innervation by TH-IR Axons
TH-IR axons (green) were commonly observed innervating fat pads around the heart, in the absence of VACHT-IR axons.
**Figure 18: Bundle Direction**

Bundled parallel axons are often observed traveling in random directions, whereas the individual axon terminals and single parallel axons are usually observed travelling in the direction of the muscle. Green=TH, Blue=VAChT, Red=Autofluorescence
Figure 19: Single Axon Parallelism
Sympathetic (TH-IR, green) and parasympathetic (VACHT-IR, blue) axons are often observed paralleling each other in the atrial myocardium. Individual (non-parallel) axons are only observed forming terminal structures (A and B). Adjacent parallel axons are often interdigitized, with varicosities alternating along the length of the parallel axons (C), suggestive of some form of prejunctional communication between the two.
Figure 20: TH/VACHT Colocalization
TH-IR (left arrow, green), VACHT-IR (right arrow, deep blue), and colocalized TH+VACHT-IR (center arrow, light blue) axons are all observed in the atria.
Figure 21: Epicardial Network

A 2d network of parallel axons in bundles (B1), parallel axons (B2), single terminals (B3), and colocalized axons (B4) are also visible in the epicardium, identified by fibrous collagen (A). Green=TH, Deep Blue=VAChT, Light Blue=TH+VAChT colocalization, Red=Autofluorescence (A only)
Figure 22: Vascular Innervation
TH-IR (green) and VACht-IR (blue) axons were both observed innervating atrial vasculature. The same axons observed paralleling vasculature were observed innervating the muscle as well. Vessels were identified by the collagenous network which envelops them, as well as the lack of cardiomyocytes in their vicinity in the autofluorescent channel (red).
Figure 23: Sympathetic Nerves Pass Through ICG
Although most large bundles of sympathetic (TH-IR) axons target ICG, they always pass through them without forming large terminal structures around the ICGNs within them. Note the small, punctate dots around ICGNs, representative of sites of preganglionic synapse formation from the vagus nerve. Green=TH, Blue=VAChT, Red=Autofluorescence. This region is indicated as marker F in Figure 12 on page 58.
Figure 24: TH-IR ICGNs Innervated by the PSNS
All ICGNs are VACbT-IR (magenta). Approximately 30% of ICGNs are also TH-IR (green). Many VACbT-IR synaptic varicosities are visible as punctate dots around the somata of ICGNs, likely the result of vagal innervation. TH-IR ICGNs were innervated similarly to non-TH-IR ICGNs. Similar TH-IR synaptic varicosities around ICGNs were never observed.
Figure 25: Intrinsic Cardiac Ganglia in the Right Atrium

Six ganglia from the atrial preparation presented in Figure 12 on page 58 presented as confocal maximum projections. A and B represent the SA and AV node, respectively. Locations of each ganglion are represented by letters A-F in Figure 12 on page 58.
Figure 26: SIF Cells

SIF cells were commonly observed along sympathetic nerve bundles, alone in the epicardium, or nested among principal neurons in ICG. To emphasize SIF cells, standard images were taken to image surrounding TH-IR (A-D), with the same regions re-imaged at lower laser power (A’-D’). The ganglion in E is indicated as marker F in Figure 12 on page 58, and represents two merged images (standard IR intensity, blue; low laser power, white)
Figure 27: SIF Cells in Sympathetic Bundle
Many SIF cells were observed along bundles of TH-IR axons from the sympathetic chain ganglia.
Figure 28: SNS/PSNS Interaction in the Right Ventricle
Parallel SNS/PSNS axons were demonstrated in the epicardial layer of the right ventricle. VACht, magenta; TH, green.

Figure 29: SNS/PSNS Interaction in the Esophagus
Single parallel SNS/PSNS axons (A) and bundles of parallel axons (B) are present in the outer muscle layers of the esophagus. VACht, magenta; TH, green.
Figure 30: SNS/PSNS Interaction in the Small Intestine
Dual immunohistochemical labeling of the PSNS (VACHT, magenta) and SNS (TH, green) in the myenteric plexus of the small intestine. Note the presence of “tight” synaptic varicosities of both PSNS and SNS axons within the ganglia and contrast their morphology observed in images of the ICG from similarly treated tissue. On the right side of this image a remnant of the circular muscle is visible, with vertically-running TH-IR and VACHT-IR axons occasionally traveling adjacently.
Theoretical mechanism of SNS/PSNS antagonism. In this model, ACh released by PSNS neurons inhibits SNS action potentials via the suppressive M2 receptor. DA released by SNS neurons inhibits PSNS action potentials via the suppressive D2 receptor. Although the mechanism involving PSNS suppression of SNS activity is already established, the reverse has not yet been proven.
Data presented in this study provide a new view of cardiac autonomic innervation which contributes much to the under-studied field of cardiac autonomic innervation. In this new view, sympathetic nerves project to the ganglia, passing through them (without forming large synaptic connections with ICGNs) to target the muscle of the atria. Axons (from PSNS and SNS sources) travel together in large bundles which bifurcate along the epicardium. Individual, parallel SNS/PSNS axons with interdigitized varicosities diverge from such bundles and project to the epicardium and myocardium, only presenting single, non-paralleled formations as large and small (respectively) terminal structures. Both branches of the ANS were observed innervating vasculature, and SNS axons were observed innervating fat cells in fat pads adjacent to the heart.
REFERENCES


Hoard JL, Hoover DB, Mabe AM, Blakely RD, Feng N, Paolocci N. 2008. Cholinergic neurons of mouse intrinsic cardiac ganglia contain noradrenergic enzymes, norepinephrine transporters, and the
neurotrophin receptors tropomyosin-related kinase A and p75. *Neuroscience* 156: 129-142.


