Topographical Distribution and Morphology of Sympathetic Postganglionic Innervation and Chronic Intermittent Hypoxia (CIH) Induced Remodeling of the Whole Heart at Single Cell/Axon/Varicosity Scale

Ariege Bizanti
University of Central Florida

Part of the Cardiovascular Diseases Commons, and the Cardiovascular System Commons

Find similar works at: https://stars.library.ucf.edu/etd2020

STARS Citation
Bizanti, Ariege, "Topographical Distribution and Morphology of Sympathetic Postganglionic Innervation and Chronic Intermittent Hypoxia (CIH) Induced Remodeling of the Whole Heart at Single Cell/Axon/Varicosity Scale" (2023). Electronic Theses and Dissertations, 2020-. 1517.
https://stars.library.ucf.edu/etd2020/1517
TOPOGRAPHICAL DISTRIBUTION AND MORPHOLOGY OF SYMPATHETIC
POSTGANGLIONIC INNERVATION AND CHRONIC INTERMITTENT HYPOXIA (CIH)
INDUCED REMODELING OF THE WHOLE HEART AT SINGLE
CELL/AXON/VARICOSITY SCALE

by

ARIEGE BIZANTI
B.S., University of Central Florida 2016
M.S., University of Central Florida 2018
M.S., University of Central Florida 2021

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the of Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Spring Term
2023

Major Professor: Zixi (Jack) Cheng
ABSTRACT

The sympathetic nervous system is crucial for controlling multiple cardiac functions and its overactivity is associated with many cardiovascular diseases (CVD). Chronic intermittent hypoxia (CIH) is a current model for sleep apnea, which constitutes a major risk factor for CVD through sympathetic overactivity. However, a comprehensive neuroanatomical map of the sympathetic innervation of the heart is unavailable which impedes our understanding of the remodeling of this map in pathological conditions. First, we used a combination of state-of-the-art techniques, including flat-mount tissue processing, immunohistochemistry for tyrosine hydroxylase (TH, a sympathetic marker), confocal microscopy and Neurolucida 360 software to trace, digitize, and quantitatively map the topographical sympathetic innervation in the whole heart of mice. Then we integrated our tracing data onto a 3D heart scaffold. Second, we determined the remodeling of sympathetic innervation in CIH, by exposing mice to either room air or CIH for 8-10 weeks. We found that (1) 4–5 extrinsic TH-IR nerve bundles entered the right atrium from the superior vena cava and the left atrium from the left precaval vein. Although these bundles projected to different areas of the atria, their projection fields partially overlapped. (2) TH-IR axon and terminal density varied considerably between different sites of the heart with the greatest density of innervation near the sinoatrial node region (P < 0.05, n = 6). (3) TH-IR axons also innervated blood vessels and adipocytes. (4) In ventricles: TH-IR axons formed dense terminal networks in the epicardium, myocardium, and vasculature. (5) TH-IR axons were traced and integrated into 3D heart scaffolds. (6) CIH significantly increased TH-IR innervation and complexity in the heart. Collectively, this work provided detailed mapping of
catecholaminergic axons and terminal structures in the whole heart at single-cell/axon/varicosity scale in normal and CIH conditions. This work may provide a foundation for the functional study of sympathetic control of the heart and valuable neuromodulation strategies to treat CVD.
To my beloved ones
I would like to thank everyone who helped me to achieve my goals. First, I would like to extend my sincerest gratitude to my Ph.D. mentor and dissertation chair, Dr. Zixi (Jack) Cheng, who provided me with continuous and invaluable guidance and support throughout my journey. He was very encouraging and allowed me to grow academically and personally to achieve more as a scientist. Second, I would like to thank my committee members for their time, extreme patience, and for letting my annual meetings be an enjoyable moment with their brilliant comments and suggestions. I would also like to thank my lab partners for their consistent source of support, experimental aid, and advice. I must also extend my gratitude to our collaborators Dr. Gozal, Dr. Hoover, Dr. Peter Hunter group (especially Dr. Richard Christie), Dr. Tappan, Ms. Maci Heal, Dr. Shivkumar, and Dr. Tompkins for sharing their expertise, help, and open communication. I am thankful to the University of Central Florida, Burnett School of Biomedical Sciences, and College of Medicine, for giving me the opportunity to grow academically. I want to give special thanks to my wonderful family (my heroes and major support), partner, and friends for their continuous support, love, patience, and prayers that sustained me this far. I am forever in debt for their patience, support, and love. Thanks must also go to my cat “Prince”.

Finally, I would like to thank Allah, for letting me through all my difficulties and showering me with blessings and guidance day by day. “Alhamdulillah”.

ACKNOWLEDGMENTS
# TABLE OF CONTENTS

LIST OF FIGURES ............................................................................................................................................. xi

LIST OF TABLES .................................................................................................................................................. xiv

CHAPTER ONE: INTRODUCTION ..................................................................................................................... 1

  Sympathetic-heart connection ......................................................................................................................... 1

  History and anatomical origin ......................................................................................................................... 2

  SNS role in the heart physiological and pathophysiological state ............................................................... 3

  Remodeling of cardiac sympathetic innervation following chronic intermittent hypoxia ......................... 5

CHAPTER TWO: TOPOGRAPHICAL MAPPING OF CATECHOLAMINERGIC AXON INNERVATION IN
THE FLAT-MOUNTS OF THE MOUSE ATRIA: A QUANTITATIVE ANALYSIS ................................................. 8

  Introduction .................................................................................................................................................... 8

  Materials and Methods .................................................................................................................................. 11

    Animals and Ethical Statement .................................................................................................................... 11

    Tissue preparation ....................................................................................................................................... 12

    Immunohistochemistry (IHC) ..................................................................................................................... 13

    Fluoro-Gold (FG) counterstaining ............................................................................................................... 16

    Image acquisition ....................................................................................................................................... 16

    Density and size quantification .................................................................................................................. 17

  Results ......................................................................................................................................................... 20

    Topographical projections of TH-IR axons in the flat-mount of the whole left and right atria (connected):
    Neurolucida Tracing and Digitization ......................................................................................................... 20
TH-IR axon innervation of the right and left atrium: Density, Distribution and Morphology ................................................. 26

TH-IR neurons and SIF cells and TH-IR axons in ICG ................................................................. 34

TH-IR axon innervation of vasculature and fat cells ............................................................... 37

Discussion .......................................................................................................................... 40

Topographical distribution of TH-IR axon innervation in the flat-mount of the whole atria at single cell/axon/varicosity scale ................................................................. 41

TH-IR ICG neurons and TH-IR axons ................................................................. 43

TH-IR innervation of fat cells and vasculature .............................................................. 45

Functional implications ......................................................................................... 46

Limitations .............................................................................................................. 48

Summary and future directions .............................................................................. 49

CHAPTER THREE: CHRONIC INTERMITTENT HYPOXIA INDUCES REMODELING OF CATECHOLAMINERGIC AXON INNERVATION IN MOUSE ATRIA .................................................. 51

Introduction ............................................................................................................. 51

Methods .................................................................................................................. 52

Animals .................................................................................................................... 52

Tissue preparation .................................................................................................. 53

Immunohistochemistry (IHC) ................................................................................ 54

Density quantification ............................................................................................ 56

Axon Tracing and quantification ............................................................................. 57

Results .................................................................................................................... 58

An overview of TH-IR axon innervation in the flat-mount of whole right and left atrium in RA and CIH ....... 58

CIH increased regional density of TH-IR axons and terminals ........................................ 66
LIST OF FIGURES

Figure 2. 1: Pipeline for calculation of TH-IR axon density..............................................................19

Figure 2. 2: Distribution of TH-IR axons in the flat-mount of the whole atria (RA and LA connected). ..........21

Figure 2. 3: Comparison of TH-IR axon innervation across five animals..................................................22

Figure 2. 4: Neurolucida 360 tracing and digitizing of the TH-IR bundles and axons innervating the RA and LA (connected)...............................................................................................................................25

Figure 2. 5: TH-IR and PGP 9.5-IR neurons and axons in the ICG in the atria: Double-labeled. ..................27

Figure 2. 6: TH-IR axons in the LA of a representative mouse........................................................................28

Figure 2. 7: Regional density of TH-IR axon innervation in the RA..........................................................30

Figure 2. 8: TH-IR axons in the LA of a representative mouse.......................................................................32

Figure 2. 9: Regional density of TH-IR axon innervation in the LA.............................................................33

Figure 2. 10: ICG contained a subpopulation of TH-IR neurons...................................................................35

Figure 2. 11: TH-IR axons passed through ICG without apparent innervation................................................37

Figure 2. 12: Small intensely fluorescent (SIF) cells in the atria.................................................................37

Figure 2. 13: TH-IR axons innervated the blood vessels (B.V).................................................................38

Figure 2. 14: TH-IR axons innervated adipose tissue (white and brown fat cells).............................................40

Figure 2. 15: Summary figure describing the main findings of this study..................................................50
Figure 3.1: Distribution of TH-IR axon innervation in the whole right atrium flat-mount of representative mice. 61

Figure 3.2: Depth-encoded montages and Arivis vision 4D tracing of TH-IR axon innervation in the flat-mount of the right atrium. 62

Figure 3.3: Distribution of TH-IR axon innervation in the whole left atrium of RA and CIH. 64

Figure 3.4: Depth-encoded montages and heatmaps of TH-IR axon innervation in the flat-mount of the whole left atrium. 66

Figure 3.5: Regional density of TH-IR axons in the right atrium in RA and CIH. 67

Figure 3.6: Regional density of TH-IR axons in the left atrium in RA and CIH. 68

Figure 3.7: Neurolucida 360® reconstruction/tracing of TH-IR axons in the SAN following RA and CIH conditions. 72

Figure 3.8: TH-IR innervation in different tissue layers of the auricle following RA and CIH conditions. 74

Figure 3.9: Large TH-IR bundles, axons, and varicosities in the epicardium and myocardium layers. 76

Figure 3.10: TH-IR axons around ICG neurons change patterns following CIH. 78

Figure 3.11: TH-IR axons in adipocytes near the atrial tissue of RA and CIH. 79

Figure 3.12: Dense transmural innervation of TH-IR axons on the atrial vasculature. 80

Figure 4.1: Distribution of TH-IR axon innervation in the flat-mount of whole left ventricle in a representative mouse. 93

Figure 4.2: Distribution of TH-IR axon innervation in the flat-mount of whole right ventricle in a representative mouse. 94
Figure 4. 3: Distribution of TH-IR axon innervation in the flat-mount of whole interventricular septum in a representative mouse.................................................................95

Figure 4. 4. Distribution of TH-IR axons across the depth of ventricular cardiomyocyte............................96

Figure 4. 5. Extensive innervation of TH-IR axons and terminals in the inner layer of LV and RV.................98

Figure 4. 6. Regional distribution of TH-IR axons in the LV.................................................................99

Figure 4. 7. Regional distribution of TH-IR axons in the RV.................................................................100

Figure 4. 8. Regional distribution of TH-IR axons in the SP.................................................................100

Figure 4. 9. TH-IR axons around the blood vessels.............................................................................101

Figure 4. 10. Illustration for the integration of digitized axons in the flat-mount of the whole ventricles into a scaffold.................................................................103

Figure 4. 11. Regional changes of TH-IR axon innervation pattern in the left ventricle following CIH ..........105

Figure 4. 12: Distribution of TH-IR axon innervation in a flat-mount of whole left ventricle in RA and CI ..........106

Figure 4. 13: 3D Anatomical mapping of TH-IR reconstructed axons on the heart scaffold in RA and CIH.........107
LIST OF TABLES

Table 2.1: Antibodies used in this study .................................................................15

Table 3.1: Antibodies used in this study .................................................................55
CHAPTER ONE: INTRODUCTION

Sympathetic-heart connection

The autonomic nervous system (ANS) works unconsciously to regulate the cardiovascular response to physiologic and pathologic stimuli via the heart-brain interaction (1-3). The heart is controlled by the two branches of the ANS (sympathetic and parasympathetic). The sympathetic nervous system (SNS) was traditionally known for its response in stressful conditions as its reflex is referred to as the “fight or flight”. However, new emerging roles of cardiac sympathetic innervation have been recently established including the regulation of the size of the cardiomyocytes and providing a neurotrophic signal to the heart (4, 5). SNS uses norepinephrine as a neurotransmitter to increase the heart rate (chronotropic), conduction velocity (dromotropic) and cardiac muscle contraction (inotropic). On the other hand, the parasympathetic system release acetylcholine to reduce heart rate. The parasympathetic innervation of the heart is a current funded project in our lab and preliminary data was presented in the Society of Neuroscience 2022, but the focus of this dissertation will be mainly on the SNS.

Moreover, the topology of sympathetic neurons and their local communication with the heart which influence cardiac functions were characterized (4, 5). In those studies, it was shown that sympathetic neurons directly communicate with cardiomyocytes and the density of innervation correlates with the size of cardiomyocytes which emphasizes the need to determine the differential regional innervation of the heart. The innervation of the heart is not uniform and
regional variations in sympathetic nerve density in the heart correspond to distinct areas of influence over heart function that work in concert to efficiently regulate cardiac performance (6).

Cardiac sympathetic innervation density is altered in pathological conditions like myocardial infarction (MI) (7) and unbalanced innervation density has been shown to lead to lethal arrhythmia through ion channel modulation in the cardiomyocytes (8). For example, upregulation of the sympathetic efferent neural tone is observed in the failing heart leading to overactivity of the SNS and pathophysiological effects (9). Hence, SNS performance is critical to maintaining proper cardiac functions and establishing homeostasis (10). The essential role of the sympathetic system in cardiovascular function and its complexity necessitates the understanding of its topographical structure in the entire heart and how it is remodeled in disease. Therefore, this chapter will provide a background of the sympathetic innervation in the heart in normal and pathological conditions. The rest chapters will present our work of mapping catecholaminergic axon innervation in the whole heart.

**History and anatomical origin**

The anatomical pathway of the autonomic nervous system was first described by the great anatomists, Galen and Thomas Willis. In the 17th century, Thomas Willis provided the first recognizable images of the SNS. Later, in the 19th century, physiologist Bernard, Waller, and
Brown-Sequard, identified them as pressor nerves and showed that sympathetic nerves lead to a tonic vasoconstriction (11).

Physiological studies, first done by W.H. Gaskel and John Newport Langley of Cambridge, suggested that sympathetic outflow is from the spinal cord and the existence of two antagonistic involuntary systems. Langley developed the notion of antagonist between sympathetic and parasympathetic and demonstrated the preganglionic and postganglionic neurons in the sympathetic (12).

Pre-Sympathetic neurons arise from the rostral ventrolateral medulla (RVLM) in the brain which projects to synapse with preganglionic cholinergic sympathetic neurons of the heart in the intermediolateral cell column in the upper thoracic levels of the spinal cord. Then, these projection synapses in the cervicothoracic stellate ganglia and the noradrenergic postganglionic axons innervate the atria, and ventricles. The stellate ganglion is the primary ganglion of the sympathetic trunk which modulates cardiac function via the SNS (13).

**SNS role in the heart physiological and pathophysiological state**

SNS plays an important role in the regulation of cardiac functions. At times of stress or physical activity, the SNS is engaged, which results in an increase in heart rate, contractility, and cardiac output. Cardiac afferent neuron inputs govern ANS cardiac function, and these inputs come from neurons in the baroreceptors, volume receptors, chemoreceptors, and osmoreceptors (Park et al., 2020). The Brain activates the appropriate branch of the ANS to stimulate or
depress cardiac properties, or vascular features like resistance (arteries and arterioles) or capacitance (in veins and venules). The central components of the sympathetic outflow include the paraventricular nucleus (PVN), the nucleus of the solitary tract (NTS) and rostral ventrolateral (RVLM) regions of the medulla oblongata (14-17). These regions regulate basal activity, bursting patterns and affect the nerve discharge of sympathetic efferent outflow.

SNS postganglionic efferent neurons are adrenergic and their terminals release norepinephrine (NE) as their main neurotransmitter. NE is a catecholaminergic neurotransmitter made from L-tyrosine and the key enzyme of its conversion is called tyrosine hydroxylase (TH, a sympathetic marker). NE stimulates heart dromotropy and inotropy by activating adrenoceptors (β1, β2, and α1) to regulate cardiac function.

The mounting evidence associating the overactivity of SNS to pathological conditions drew a lot of attention to study its mechanisms which helped the translation of knowledge to the management and treatment of diseases. For example, knowledge of cardiac neural pathophysiology led to the development of beta-adrenergic blockers which helped treat severe hypertension (18, 19). Even though surgical sympathectomy was shown to be lifesaving in patients with hypertension. However, it is poorly tolerated, and other effective treatments are in need.

Recent ablations were shown to be successful in treating conditions like atrial flutter, but they also showed some recurrence. There is a missing link that is hindering the development of
effective treatment with minimal side effects. A better understanding of our detailed neuroanatomical structure could help. Inspired and supported by the NIH Common Fund Program, Stimulating Peripheral Activity to Relieve Conditions (SPARC) program and an NIH R15 grant, we mapped the connections of the peripheral nerves (sympathetic) and the heart, and reconstructed the whole axonal network in the heart using tools (Neurolucida 360, MBF Bioscience) developed by other SPARC-supported groups to accomplish this common goal. Our work can be used as an atlas for future studies that modulate electrical activity in nerves to improve organ function “bioelectronic medicine”.

Remodeling of cardiac sympathetic innervation following chronic intermittent hypoxia
Obstructive sleep apnea (OSA) is a common sleep-related breathing disease that affects 17% of women and 34% of men and it is a modifiable cardiovascular and neurological diseases and behavioral disorders (20). Patients with OSA have poor sleep quality that can result in serious complications, including memory deficit, hypertension, impaired baroreflex, depression, and cardiovascular consequences (21-24). Chronic intermittent hypoxia (CIH) is a widely used model of obstructive sleep apnea. CIH showed deleterious impacts on post-myocardial infarction remodeling and contractile dysfunction (25). The previous study used echocardiographic measurements of ejection fractions and demonstrated early occurrence of sustained sympathetic overactivity and hypoxia inducible factors following CIH exposure. As abnormal sympathetic activity leads to serious diseases like congestive heart failure, arrhythmias and sudden cardiac death (26), changes of the sympathetic innervation of the pacemaker of the
heart can ameliorate the heart rhythm stability and exacerbate arrhythmias. Catecholamines are involved in the facilitation of breathing, bronchodilation, cardiomyocyte contractility, conduction velocity which contribute to high blood pressure via the increase of cardiac output. Currently, it is known that CIH leads to sustained autonomic imbalance, but the exact mechanism of that imbalance is not well understood. There is an incomplete picture of how CIH induces brain-heart reorganization due to previous technical limitations, and this impedes the development of effective treatments. To understand the effect of CIH on multiple neural components and their connections within the brain-heart circuity, many studies have established the role of the central components in the initiation and progression of CVD following CIH (27-29). Anatomical changes in the cardiac sympathetic innervation pattern led to functional abnormalities that are apparent in many diseases. However, the remodeling of the cardiac peripheral neural network following CIH is still unclear. This hindered our knowledge of the role of TH-IR axon innervation in the atria in heart control in normal and pathological conditions like OSA. Therefore, untangling the TH-IR axon innervation of the heart and how it is remodeled in CIH is paramount to our understanding of its role. Using a combination of techniques including flat-mounts of whole tissue processing, immunohistochemistry, advanced imaging, Neurolucida 360© and vision 4D nerve tracing and digitization, and Scaffold Mapping tools enabled us to determine the cardiac-sympathetic 3D map in normal and CIH models. This will eventually help us in applying this knowledge to develop effective interventions for OSA.
and measure their efficacy by utilizing our map as a sympatho-cardiac atlas in normal and CIH conditions.
CHAPTER TWO: TOPOGRAPHICAL MAPPING OF CATECHOLAMINERGIC AXON INNERVATION IN THE FLAT-MOUNTS OF THE MOUSE ATRIA: A QUANTITATIVE ANALYSIS

Introduction

The sympathetic nervous system (SNS) plays a pivotal role in regulating cardiac functions including heart rate, contractility, and conduction velocity, which are essential for our survival (26, 30). Contrary to conventional belief, not only does the SNS play a role in the “fight or flight” integrated response, but it also regulates heart rate and contractility in both resting and non-resting conditions (31). In fact, new emerging roles of cardiac sympathetic innervation were revealed including the regulation of cardiomyocyte size and providing a neurotrophic signal to the heart (4). Furthermore, any disturbance of the SNS functions, including structural remodeling and overactivity, may promote progression of various cardiovascular diseases (32).

Although the functional roles of the SNS have been well established, a comprehensive organization map of the sympathetic postganglionic innervation of the atria remains insufficiently delineated. In addition, the regional density of the sympathetic innervation of the heart has yet to be quantified. There are numerous unanswered questions related to the detailed anatomy of the heart's sympathetic nervous system and how it is modified by disease states, such as atrial fibrillation, arrhythmia, and heart failure (33). For example, a complete understanding of the morphological and the quantitative assessment to explain the complexity of sympatho-cardiac communication and the differential regional distribution of the atrial nerve plexus remains to be elucidated (26, 31, 34).
Previous studies investigated the structure and function of sympathetic neurons and axons in different species (35-40) using sectioned heart preparations or focused only on specific regions of the atria, which disrupted the continuity of axons and terminals, preventing large scale morphological characterization of these structures. Great effort has been made to better characterize the intrinsic cardiac plexus in the whole-mount mouse heart, which increased our knowledge on the distribution of noradrenergic innervation of the mouse heart (41, 42).

Nevertheless, the complete fine details of TH-IR axon terminals and varicosities were not fully visualized in the whole-mount. Additionally, thick regions of the auricle and other structures were partially or completely removed. These structures include right cranial vein (RCV), left cranial vein (LCV), and caudal vein (CV) (43, 44) which we refer to in this study and our previous work as superior vena cava (SVC), left precaval vein (LPCV), and inferior vena cava (IVC) (40, 45, 46), respectively. Moreover, the topology of sympathetic neurons and their local communication with the heart, which influence cardiac functions were characterized (4, 5). In those studies, it was shown that sympathetic neurons directly communicate with cardiomyocytes in the ventricles and the density of innervation correlates with the size of cardiomyocytes, all of which emphasize the need to determine the differential regional innervation of the heart. Recently, researchers were able to generate two- and three-dimensional reconstructions of the sympathetic innervation of the myocardium. However, these studies provided imaging from only a few myocardial sections and a small segment of the heart (47). Alternatively, they revealed the big bundles without a clear visualization of the fine
axons and terminals or cardiac targets (48). Both studies used tyrosine hydroxylase (TH) as a sympathetic marker and showed that sympathetic nerves and intrinsic cardiac ganglia were mainly distributed in both atria of the heart, predominantly near the SAN, AVN and around the junction of left and right atria (49, 50).

Despite substantial advances in knowledge on the anatomy and physiology of cardiac nerves that contribute to therapeutic responses, there are still many gaps that need to be filled as neuromodulation treatments move away from pharmaceuticals and non-specific treatments to more guided and specific therapeutic targets for cardiovascular diseases. To facilitate these transitions, the architecture of cardiac sympathetic nerves needs to be carefully and precisely determined. More studies are needed to determine the structural organization of the sympathetic postganglionic innervation of whole-mount preparations of the heart atria and ventricles to improve understanding of sympathetic control of the heart.

Previously, we have determined the distribution and morphology of parasympathetic afferent and efferent axons in the atria in wild-type rat and mouse preparations (51-55) as well as in disease models (e.g., aging, sleep apnea, and diabetes) (27, 51, 56). Collectively, the present work provides a comprehensive topographical map of the catecholaminergic efferent axon distribution, density, and morphology of the atria at the single cell/axon/varicosity resolution. This anatomical map will provide a foundation for future functional studies of sympathetic control of the heart and its remodeling in pathological conditions.
Materials and Methods

Animals and Ethical Statement

All procedures were approved by the University of Central Florida Animal Care and Use Committee (HURON PROTO202000150) and strictly followed the guidelines established by the National Institutes of Health (NIH) and the ARRIVE 2.0 guidelines. This study was performed on healthy male C57Bl/6J mice (RRID: IMSR_JAX000664, The Jackson Laboratory, Bar Harbor, ME) (n = 20, age 2–3 months, weighing 20-30 g). Mice were housed in a plastic cage (n= 5/cage) with sawdust bedding (changed three times a week) in a room with controlled environmental conditions of humidity and temperature in which light/dark cycles were set to 12h/12h (6:00 AM to 6:00 PM light cycle) and provided food and water ad libitum. Mice were divided into 3 groups. Connected atria TH-IR axon innervation mapping group (n=5) were used to show topographical innervation and reconstruction of nerves. Quantification analysis of separate right and left atria group (n=6) were used to perform regional density analysis. Control group (n=5) were used to ensure there were not any nonspecific labeling and that labelled structures represent neuronal and axonal structures. This was performed by omitting the primary antibody (n=1) or omitting the secondary antibody (n=1) and labelling with PGP9.5 (2). Additional animals were used to counter-stain neurons with Fluorogold (n=4). All efforts were made to minimize the number of mice and their suffering.
Tissue preparation

Mice were deeply anesthetized with isoflurane (4%) induction in an anesthetic chamber. Absence of the hind paw pinch withdrawal reflex was used as an indicator of sufficient depth of anesthesia. Mice were injected with 0.2 mL heparin into the left ventricle followed by a cut to the inferior vena cava to drain the blood. After 2 minutes, a needle was inserted into the left ventricle and the mice were perfused with 0.9% saline at 38-40 °C for 5 minutes, followed by fixation with 4% paraformaldehyde. Hearts along with the lungs and trachea were removed from the chest and postfixed overnight in 4% paraformaldehyde at 4 °C. The heart was placed and pinned into a dissecting dish lined with Sylgard and containing PBS (0.1 M, pH = 7.4), and the specimen was further dissected using a Leica Stereo microscope as described previously (52, 53, 55, 57, 58). To reveal the intact network of sympathetic postganglionic atrial innervation, we removed the heart from the surrounding tissues (lungs, aortic arch and trachea). Then, the atria (both right and left atrium connected at the interatrial septum on the ventral side) were separated from the ventricles (n=5). The whole atria were processed as a montage of several hundred (~260) maximal projections of image stacks. To gain more insight into TH-IR axon innervation and regional density, the right and left atria (RA and LA) were separated. The auricles were cut along the boundary into two halves. The part of the auricle facing more exteriorly and connected to the big vessels is referred to in this study as the outer auricle and the other half is referred to as the inner auricle. Then, flat-mounts were scanned using the confocal microscope at higher magnification (40X oil lens). The separation of the atria
was necessary to avoid areas of overlap between RA and LA. Montages of the maximal projections of the right and left atria were prepared (n=6/group).

Immunohistochemistry (IHC)

Tissue processing and immunolabeling were performed as described previously (58). Following dissection, the tissues were washed 6 x 5 min in 0.1 M PBS (pH = 7.4), then immersed for 48 hr in a blocking reagent (2% bovine serum albumin, 10% normal donkey serum, 2% Triton X-100, 0.08% NaN₃ in 0.1 M PBS, pH = 7.4) to reduce nonspecific binding of the primary antibody and to promote increased antibody penetration. Primary antibodies (1:100) were added to the primary solution (2% bovine serum albumin, 4% normal donkey serum, 0.5% Triton X-100, 0.08% NaN₃ in 0.1 M PBS, pH = 7.4) and incubated for 48 hr. Unbound primary antibodies were removed by 6x5 min tissue washes in PBST (0.5% Triton X-100 in 0.1 M PBS, pH=7.4). Secondary antibodies (1:50 in PBST) were then applied for 24 hr. Unbound secondary antibodies were removed by 6x5 min tissue washes in PBS. Negative control tests (in which primary antibodies were omitted) were also performed, and these preparations presented no labeling, confirming that nonspecific binding of secondary antibodies did not occur. Lastly, we verified the accuracy of our TH labeling by using PGP 9.5 (ubiquitin carboxyl-terminal hydrolase-1), a general neuronal marker that visualizes different populations and subtypes of nerves. A list of the antibodies used in this study is summarized in Table 2.1.
Flat-mounts were placed on a microscope slide with their dorsal side against the glass, 
coverslipped, crushed for 2 days with lead weights, and air-dried under a fume hood for 1 day. 
Slides were dehydrated by immersion for 2 minutes in each of 4 ascending concentrations of 
ethanol (75%, 95%, 100%, and 100%), followed by 2x10 min washes in xylene. Slides were then 
covered with coverslips and DEPEX mounting medium (Electron Microscopy Sciences #13514) 
and allowed to dry overnight.
Table 2.1: Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Host</th>
<th>Company</th>
<th>Catalog</th>
<th>RRID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-TH</td>
<td>1:100</td>
<td>Rabbit</td>
<td>Pel-Freeze</td>
<td>Cat# P40101-0</td>
<td>AB_461064</td>
</tr>
<tr>
<td>Anti-TH</td>
<td>1:100</td>
<td>Sheep</td>
<td>Millipore</td>
<td>AB1542</td>
<td>AB_90755</td>
</tr>
<tr>
<td>Anti-PGP9.5</td>
<td>1:100</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab108986</td>
<td>AB_10891773</td>
</tr>
<tr>
<td>Alexa Fluor 594</td>
<td>1:50</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A-11016</td>
<td>AB_2534083</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>1:50</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A21206</td>
<td>AB_2535792</td>
</tr>
</tbody>
</table>
Fluoro-Gold (FG) counterstaining

To counterstain, four additional animals were used. Fluoro-Gold (0.3 mL of 3 mg/mL per mouse; Fluorochrome, LLC, FG 50 mg) was injected (i.p.) to counter stain neurons in the peripheral ganglia. Mice were perfused 3-5 days after FG injection and the hearts were removed and dual labeled with TH.

Image acquisition

The Nikon 80i fluorescence microscope (Lens: 20X and 40X) was first used to survey the TH labeling in the whole flat-mounts of the atria. Then, a Leica TCS SP5 laser scanning confocal microscope (Lens: 20X and 40X oil) was used to acquire images and assemble image montages of whole connected atria, including left atrium and right atrium flat-mounts. An argon-krypton laser (488 nm) was used to image Alexa Fluor 594-labeled TH-IR axons and a HeNe laser was used to image background autofluorescence of the tissues. The connected atria were scanned using a 20X oil immersion objective lens (Z-step 1.5 μm), to produce approximately 400 confocal image stacks per montage. The confocal projection images of these stacks were used to assemble montages of whole atria flat-mounts using either Mosaic J or photoshop. To better visualize the topographical distribution and morphology of TH-IR innervation in the atria, the separate whole left atrium and right atrium and regions of interest were scanned at high magnification (40X oil immersion objective lens, Zoom X1 or X 1.5, Z-step 1.5 μm). The higher
magnification resulted in approximately 800 frames for each atrium. We were able to overcome the thickness of the flat-mount whole atria with our optimized tissue processing techniques and flattening of the tissue which allowed us to visualize fine details of TH-IR axon innervation. We also used a Zeiss M2 Imager microscope with an autostage (20X NA 0.8) to scan the samples which produced images with high quality that were comparable to the images obtained with confocal microscopy (20X objective lens). This approach will make future methodology less laborious and more efficient.

Tracing of TH-IR axons was performed using Neurolucida 360 (MBF Bioscience). Additionally, Neurolucida Explorer (MBF Bioscience), an analytical software built within Neurolucida 360, was used to perform morphometric analysis on traced axon reconstructions. Branched structure analysis was performed, and parameters (number of trees, nodes, terminals, total length and surface area) were selected for all connected atria tracings (n = 6).

**Density and size quantification**

To quantify the regional density of TH-IR fibers in the atria, we segregated images into specific regions of interest (ROIs): SAN, AVN, SVC, IVC, right outer and inner auricle, LA-PV junction, left PV, middle PV, right PV, left outer and inner auricle. The steps of density quantification were as follows *(Figure 2.1)*: 1) Subtracted the background with radius of 80 pixels to reduce noise and enhance contrast. 2) Applied particle removal to remove small debris. 3) Applied a binary threshold (Otsu method) to isolate immunoreactive structures. 4)
Quantified the signal above the threshold. 5) Averaged the signal of different ROIs windows using six counting frames. 6) Ran the Shapiro-Wilk normality test. Axontracer algorithm was used to trace and confirm axon quantification (59). Axon density was represented as total axon length per ROI. Total axon length in pixels was converted to µm using appropriate conversion factors. Statistical significance of the difference between the means was performed using one-way ANOVA and Tukey’s HSD (Honestly Significant Difference). Data are expressed as means +/- SEM. Significance is accepted at $P < 0.05$.

Heatmaps were created after applying a modified version of the freely available open-source automated software algorithm that trace and quantify axons (Axon tracer plugin, ImageJ)33. The percentage of TH-IR neurons was counted using all single optical sections of different ICG image stacks.
Figure 2. 1: Pipeline for calculation of TH-IR axon density.

Six frames within each region of interest (ROI) were selected, converted to grayscale, thresholded using Otsu algorithm, skeletonized and the signal pixels were measured in each ROI.
Results

Topographical projections of TH-IR axons in the flat-mount of the whole left and right atria (connected): Neurolucida Tracing and Digitization

Four major extrinsic TH-IR axon bundles entered the atria (short yellow arrows in figure 2), branched into the smaller bundles, and finally ramified into individual axons which covered the entire atria (Figure 2.2). Across animals, the number of large TH-IR bundles and their entry locations and innervation fields of the atria were quite consistent. In all atrial tissue preparations, most TH-IR bundles were identified consistently at the medial side of superior vena cava (SVC), entrance of the pulmonary veins (PVs) to the left atrium, and left precaval vein (LPCV) (Figure 2.3).
Figure 2. 2: Distribution of TH-IR axons in the flat-mount of the whole atria (RA and LA connected).

A montage of 260 maximal intensity projection images showed several large TH-IR bundles that entered the atria and bifurcated into small bundles that innervated the entire connected RA and LA. TH-IR neurons in the ICG were more densely distributed in the LA, junction of LA and RA, entrance of LA to PV. Intrinsic cardiac ganglia (asterisks), Fat (white arrows), junction of LA and RA (dotted line), entrance points (yellow arrows) LA: left atrium, RA: right atrium, PV: pulmonary vein, SVC: superior vena cava, LPCV: left precaval vein, SAN: sinoatrial node region, AVN: atrioventricular node region. Scale bar: 500 μm
Figure 2.3: Comparison of TH-IR axon innervation across five animals.
Large TH-IR bundles entered the atria mainly through the SVC (bundles 1, 2), LPCV (bundle 3) or the entrance of the PVs to LA (bundle 4). The bundles entering the RA mainly sent their projections to innervate the right atrium, whereas bundles entering the LPCV mainly extended their projections towards the LA. A certain degree of overlap of innervation could be seen in the junction of LA and RA, SA, and AVN. Note: some fibers could have been cut or folded during the dissection which contributed to the few differences across animals. 1’ indicates the additional bundle found in some animals near bundle. Scale bar: 500 μm

The tracing of TH-IR axons using the Neurolucida system highlighted the trajectory of major bundles quite well. These bundles innervated different regions with a certain degree of overlap (Figure 2.4a). TH-IR bundles projected their axons towards the atria via four main topographical pathways:

- Bundle 1 entered the atria at the medial side of the SVC and branched into smaller bundles that proceeded towards the SAN, conductive fibers, AVN, right PV and the lower part of the right auricle (Figure 2.4b).
- Bundle 2 formed a loop around the origin of the SVC (probably folded during dissection) and sent projections mainly to the upper part of the right auricle and junction of LA and RA (Figure 2.4c).
- Bundle 3 entered the atria at the IVCLPIVC and ramified into individual axons that projected towards the entire left auricle (Figure 2.4d)
- Bundle 4 entered the atria at the lower edge of the LPCV and projected towards the LA-PV junction, left and middle PVs and junction of LA and RA (Figure 2.4e).

Most animals showed a similar trend of TH-IR axon distribution. Some of the variations observed could be due to unintentional folding of bundles during dissection and interindividual
variation. To confirm TH-IR axons and neurons were accurately representing neural processes, pan-neuronal marker PGP 9.5 was used. All TH-IR axons and neurons were also PGP 9.5-IR (Figure 2.5), indicating that TH-IR fibers (Figure 2.5a-c) and neurons (Figure 2.5d-f) were indeed neural processes. Additionally, negative controls further confirmed the labeling specificity.
Figure 2. 4: Neurolucida 360 tracing and digitizing of the TH-IR bundles and axons innervating the RA and LA (connected).

a. Tracing of TH-IR bundles revealed ~4 major bundles entered the atria mainly at the SVC, LPCV or LA-PV junction then bifurcated into small bundles that eventually ramified into individual varicose axons. Two bundles extended the TH-IR axons towards the RA while the other two bundles mainly innervated the LA. These four bundles innervated distinct regions with a certain degree of overlap. b. TH-IR bundle that extended projections mainly towards the base of SVC, SAN, AVN, the lower part of the RAu. c. A TH-IR bundle that extended projections towards the upper part of the RAu, SAN, LA and RA junction, and the medial part of the LA. d. A TH-IR bundle that accessed the LA through the LPCV and mainly innervated the LPCV, the LAu, and the middle of the LA. e. TH-IR bundles entered the LA between LPCV and the left PV to mainly innervate the LPCV, the left and middle PVs, the middle part of the LA and the junction of LA and RA. Arrows indicate the beginning of the parent axon bundle. RA: right atrium,
TH-IR axon innervation of the right and left atrium: Density, Distribution and Morphology

The distribution of TH-IR axons in the whole right atrium was consistent in all animals. A couple of large TH-IR bundles entered the right atrium through the SVC and LPCV (Figure 2.6). These large bundles branched into smaller bundles that either passed through the intrinsic cardiac ganglia (ICG) or extended directly to other cardiac targets and ramified into individual axons. The overall density heatmap (Figure 2.6a) revealed that TH-IR axon innervation was significantly higher within the region of the SAN compared to other areas ($P < 0.05, n = 7$). The steps for the quantification of TH-IR axon density were delineated in Figure 1. TH-IR axon density at several regions of interest in the RA is shown in Figure 2.7b-g. The inner and outer walls of the auricles were separated due to their thickness. The density of TH-IR axon innervation in these regions was in the following order from high to low: SAN (687.3 μm/mm$^2$ ± 21.63), AVN (401.7 μm/mm$^2$ ± 51.03), inner auricle (303.1 μm/mm$^2$ ± 36.78) and outer auricle (243.4 μm/mm$^2$ ± 27.22), SVC (239.5 μm/mm$^2$ ± 33.09), IVC (113.6 μm/mm$^2$ ± 14.19) (Figure 2.7h).
Figure 2. 5: TH-IR and PGP 9.5-IR neurons and axons in the ICG in the atria: Double-labeled.

All TH-IR axons and neurons were also positive for PGP 9.5. A few TH-IR neurons were present. ICG: intrinsic cardiac ganglia, PGP 9.5: protein gene product 9.5. Arrows indicate the axons and neurons that were dual labeled with TH and PGP 9.5.
Figure 2. 6: TH-IR axons in the LA of a representative mouse.

The LA was separated from the RA of the same representative mouse in Figure 4. A montage of ~700 maximal projections in the LA showed that large TH-IR bundles entered the atrium at the LA-PV junction, traveled through cardiac ganglia, branched out to smaller axon bundles and extended individual axons that innervated the whole atrium with dense terminal varicosities. (Please zoom in 400x to view details as well as see the following figures). ICG (asterisks), fat (arrow), white dotted line (boundaries of cut to separate the sinner auricle), yellow dotted line (opening of atrium), LA-PV: left atrium-pulmonary vein junction, PV: pulmonary vein. Scale bar: 500 μm
Figure 2.7: Regional density of TH-IR axon innervation in the RA.

a. Heatmap of the regional density of TH-IR axon innervation in the RA (hot spots are indicated by red color). b-g. Representative images of the regions of interest to show the detailed TH-IR axon innervation in different regions. h. Quantification of TH-IR axons in the RA ROIs of a representative mouse. (Heatmaps were created by Ariege Bizanti with ImageJ 1.53t, https://imagej.net/software/fiji/). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. n = 6. Scale bars: 500 μm (in a); 50 μm (in b-g)

The distribution of TH-IR bundles and axons in the flat-mount of whole left atrium was determined (Figure 2.8). A couple of TH-IR bundles entered the left atrium through the LA-PV junction then bifurcated into smaller bundles. These bundles either extended towards the ICG or directly to other cardiac targets and eventually ramified into numerous axon terminals covering the entire left atrium. This montage clearly showed a holistic view of the sympathetic innervation of the left atrium at single axon/cell/varicosity scale. The overall heatmap of a representative mouse (Figure 2.8a) showed the highest density of TH-IR immunoreactivity in the regions of the left atrium within the LA-PV junctions and the roots of pulmonary veins. Regional density analysis of ROIs in the LA (Figure 2.9b-g) showed the density of TH-IR axon innervation as following from high to low: LA-PV junction (mean 348.2 μm/mm² ± 26), inner auricle (217 μm/mm² ± 19.17), outer auricle (197 μm/mm² ± 17.42), and pulmonary veins (left PV 179 μm/mm² ± 5.25, middle PV 165 μm/mm² ± 28.44, right PV 144.8 μm/mm² ± 11.85) (Figure 2.9h). There was a significantly higher density of TH-IR axons in the middle area of the left atrium represented as LA-PV junction than the auricle or pulmonary vein (P < 0.05, n = 6).

A comparison of the TH-IR axon density in the RA and LA showed the highest density of innervation was at the SAN. Of note, TH-IR bundles and ICG were excluded from the density
calculations and ROIs selected contained only TH-IR axons to avoid any bias in the quantitative analysis. In the LA, the junction of LA-PVs showed very dense innervation of TH-IR axons in most samples (Figure 2.9h). Interestingly, even though the density of TH-IR axons in the PVs were less than that at the LA-PV junction, the axons in the PVs were more continuous and had a more defined pattern. The bundles seen on LA are most likely branches of the large TH-IR bundles on the RA that were dislocated during the separation of RA and LA.
Figure 2.8: TH-IR axons in the LA of a representative mouse.

The LA was separated from the RA of the same representative mouse in Figure 4. A montage of ~700 maximal projections in the LA showed that large TH-IR bundles entered the atrium at the LA-PV junction, traveled through cardiac ganglia, branched out to smaller axon bundles and extended individual axons that innervated the whole atrium with dense terminal varicosities. (Please zoom in 400x to view details as well as see the following figures). ICG (asterisks), fat (arrow), white dotted line (boundaries of cut to separate the sinner auricle), yellow dotted line (opening of atrium), LA-PV: left atrium-pulmonary vein junction, PV: pulmonary vein. Scale bar: 500 μm
Figure 2. Regional density of TH-IR axon innervation in the LA.

a. Heatmap of the regional density of TH-IR axon innervation in the LA (hot spots are indicated by red color). b-g. Representative images of the regions of interest to show the detailed TH-IR axon innervation in different regions. h. Quantification of TH-IR fibers in LA ROIs of a representative mouse. (Heatmaps were created by Ariege Bizanti with
In the whole atrial flat-mounts, several intrinsic cardiac ganglia were distributed in the epicardium. The majority of these ganglia were identified near the SAN region, AVN region, and interatrial groove in the connected atria (Figure 2.3). When separated, the left atrium had the majority of intrinsic cardiac ganglia in the middle area of the left atrium at the attachment points with the right atrium in the SA and AV regions and the entrance of the pulmonary veins (Figure 2.8). Some ganglia were also located in the right atrium around the SA region and the epicardial bundles on the LPCV (Figure 2.5). ICG were mostly located on the dorsal surface of the mice LA and TH-IR neurons comprised 18-30% of total ICG neurons in maximal intensity projections (Figure 2.10a-c) and optical sections (Figure 2.10a’-c’). TH-IR fibers were mostly observed passing through the individual ICG (Figure 2.11). Even though maximal projection images showed TH-IR axons near the ICG (Figure 2.11a), a more detailed evaluation of single optical sections (Figure 2.11a’) or partial projections of different ICG (Figure 2.11b-e) showed that no TH-IR axon terminals wrapped tightly around the individual ICG neurons. Additionally, small intensely fluorescent cells (SIF) cells were strongly TH-IR (Figure 2.12) and were observed in clusters of 3-8 cells, usually dispersed within ICG or near big TH-IR bundles. Optical sections of SIF cells in selected clusters (Figure 2.12a’, a’’) showed that they have a smaller diameter (<10 μm) compared to TH-IR neurons in the ICG (~20 μm).
Figure 2. ICG contained a subpopulation of TH-IR neurons.

a. Partial projection confocal image of FG-labeled neurons (yellow) in the ICG located on the dorsal surface of the LA. b. TH-IR neurons (red). c. Merged image revealed TH-IR neurons relative to FG-labeled neurons and TH-IR axons passing through the ICG neurons. a’-c’. Single optical sections of the representative images (a-c). FG: Fluoro-Gold, ICG: intrinsic cardiac ganglia. Scale bar: 50 μm
Figure 2. 11: TH-IR axons passed through ICG without apparent innervation.

a. Maximal projection image showed TH-IR axons mainly passing through a group of TH-IR neurons. a’. Single optical section of the corresponding image in a. b-e. Partial projection image of different ICG represented the pattern of TH-IR axons passing through rather than directly innervating the TH-IR neurons. Arrows indicate TH-IR axons passing by TH-IR neurons. Zoom 1X (a,a’), Zoom 2X (b-e). Scale bar: 20 μm

Figure 2. 12: Small intensely fluorescent (SIF) cells in the atria.

a. TH-IR SIF cells were grouped into clusters of 3-8 and expressed stronger TH-IR labeling than TH-IR neurons in the ICG. a’,a”. Two optical sections showed two separate SIF cell clusters in the dotted boxes in a indicating the intense labeling and small diameter (<10 μm) of SIF cells. Zoom 1.5X (a), Zoom 2X (a’,a”). Scale bar: 20 μm

TH-IR axon innervation of vasculature and fat cells

In addition to the major veins (SVA, IVA, PVs and LPCV) we identified clearly contoured blood vessels (arterioles) in the left and right atria with TH-IR axons running in parallel to the blood vessel walls or across them (Figure 2.13). In the montages, the blood vessels were much less apparent due to the overlays of multiple layers in the maximal projection masking the detailed vascular structures.
TH-IR axons also densely innervated the fat tissues at different layers of the atrial wall. White adipose tissue (WAT) and brown adipose tissue (BAT) were identified by their morphological characteristics using Brightfield (Figure 2.14a, b) or autofluorescence (Figure 2.14d, e). Figure 2.14c showed TH-IR axons innervated the fat cells in a cluster with numerous varicose terminals. Additionally, the optical sections of the same region showed that TH-IR axons specifically targeted individual adipocytes (Figure 2.14c'). TH-IR axon terminals were observed around the boundaries and in between WAT recognized by spherical cells with most of the volume occupied by cytoplasmic lipid droplets and peripherally located nucleus (Figure 2.13a', d). On the other hand, BAT was recognized by multiple vacuoles and darker shade and showed higher innervation by TH-IR axon terminals compared to WAT (Figure 14b', e).
Discussion

Here, we show that several TH-IR axon bundles (presumably sympathetic) entered the atria from the right and left sides, branched out into individual axons and projected to different fields of the atria with a certain degree of overlap. There was a clear lateralization with the right bundles projecting mainly to the right atrium, whereas the left bundles preferably projected to the left atrium. Asymmetry and regional differences in the cardiac sympathetic distribution were observed in many physiological studies in mice (60), pig (61), and humans (62). Our study provides anatomical evidence for differential regional distribution in mouse atria. TH-IR axon bundles were distributed in the epicardium, then bifurcated and formed a terminal network in the myocardium. Moreover, TH-IR axons were observed along/encircling small blood vessels and around WAT and BAT. Regional density analysis showed that the SAN had the highest TH-IR axon innervation. To our knowledge, this work, for the first time, provides a topographical map with quantitative assessment of the TH-IR axon innervation of the mouse whole atria at single cell/axon/varicosity scale.
Topographical distribution of TH-IR axon innervation in the flat-mount of the whole atria at single cell/axon/varicosity scale

**Innervation field of TH-IR axons**

Several studies have reported the distribution of catecholaminergic nerve fibers utilizing sectioned or whole mounts of partial atrial preparations (41, 42, 63, 64). The main limitation of such approaches is that the experimental approach damaged the intricate three-dimensional structures of axons and terminals in these tissues. Additionally, sections or partial flat mounts did not provide a comprehensive topographical map to assess the distribution and morphology of sympathetic postganglionic efferent axons and terminals across the entire atria. Recently, tissue clearing procedures have permitted an enhanced 3D view of the whole heart innervation (48). However, visibility of fine axons and terminals in the whole heart remained restricted with tissue clearance procedures. In addition, tissue clearance diminished the visibility of other cardiac targets such as ganglion cells, muscles, blood vessels, and adipocytes. In order to highlight the complex patterns of TH-IR axons and their terminal networks in atrial and targets, greater resolution imaging is required.

Our study has addressed these limitations by providing a comprehensive topographical map of the distribution, and morphology of TH-IR axons and terminals in the atria of mice using flat-mounts of the whole atria. Consistent with previous studies on mouse and other species (43, 65-67), we found a very dense TH-IR axon innervation in the atria. Additionally, the
entrance points of the major TH-IR bundles to the atria, which were determined in our study, are similar to those that were ascertained previously (40, 68, 69). Different from prior reports, our study provided a complete, comprehensive map of TH-IR axons in the atria at single cell/axon/varicosity scale. In the connected atria, we observed that several TH-IR axon bundles (4-5) entered the atria through the SVC and LPCV and bifurcated into smaller bundles that eventually ramified into individual axons forming different projection fields with a certain degree of overlap. Presumably, these bundles were mostly from the left and right sympathetic stellate ganglia. Previous studies using retrograde tracer and stellate ganglionectomy showed that the majority of sympathetic postganglionic innervation originates from the stellate ganglia (70, 71).

Our tracing of TH-IR axons showed clear lateralization as bundles from the right mainly projected towards the right atrium and SAN, while bundles from the left side showed preferential innervation of the left atrium. Our findings reveal detailed regional differences of TH-IR innervation in the entire atria, which enriches our knowledge regarding the differential sympathetic control over distinct regions.

Quantitative analysis of TH-IR regional density

Catecholaminergic axon innervation of the atria displays significant anatomical heterogeneity and several studies have attempted to assess the density of cardiac sympathetic nerves at different sites of the heart (60, 72). Although previous studies quantified the density
of TH-IR axons at specific sites, they only utilized sections or partial atrial preparations. Thus, a more complete quantitative analysis of TH-IR axon density in the whole heart has not been determined. In our study, we addressed the mentioned shortcomings and analyzed the distribution and density of TH-IR axons in the flat-mount of the whole RA and LA at a high resolution (40X oil lens). The density of TH-IR axons showed regional differences across the atrial wall. In the RA, TH-IR axons and terminals were the densest in the SAN region, followed by the AVN region and other regions, which is similar to what was found in other studies (73-75). In the LA, the density of TH-IR axons was the highest at the LA-PV junction which was pointed out to be an area richly innervated with sympathetic nerves (76). The auricles, one of the most prominent structural features of the right and left atrium, play an important role in pumping the blood within the heart with its capacity to expand during each heartbeat (77). The differential regional distribution of TH-IR axon innervation indicated by our density assessment gives insight to localized effects of catecholaminergic innervation of the atria. Our results could set the foundation for future physiological studies of anatomical remodeling in pathological conditions.

TH-IR ICG neurons and TH-IR axons

Traditionally, it was thought that all ICG neurons in guinea pigs and rats were exclusively cholinergic (40, 78). However, recent studies demonstrated that ICG neurons exhibit diverse neurochemical phenotypes (including TH, ChAT, nNOS, VIP, NPY) (79, 80) that extend beyond
the traditional concept of cholinergic neurons. A subpopulation of the ICG neurons were also
found to be TH-IR in mice which aligns with our findings (36, 81). Similar to previous studies, we
have observed the ICG being located primarily on the outer surface of the atria near the
entrance of the pulmonary veins to the LA and near the SAN and AVN (41, 52). Our work in
mice showed TH-IR neurons in the ICG with TH-IR axons going through the ganglia without
apparent innervation. This differs from what was found in guinea pigs and rats where some TH-
IR varicosities were seen around ICG neurons (40, 82). These previous findings may be
somewhat overestimated by their use of partial preparations that cannot be extrapolated to all
ICG neurons. In this study we aimed to assess TH-IR axons that cross through all ICG located on
the RA and LA. We found only a few TH-IR axons (if any) were in close contact with ICG neurons.
Higher magnification should be used in the future to ensure there is no underestimation of TH-
IR axons presence around ICG neurons. In support of this finding in mice, our recent study in
pigs showed that TH-IR axons traveled through the ICG without forming varicosities
surrounding the principal neurons (PNs) (83). The lack of TH-IR varicosities wrapping tightly
around TH-IR neurons in the ICG contrasts with what was observed in the gastrointestinal tract
where TH-IR varicosities tightly surround the PNs in the myenteric ganglia (84). Prior research
indicated that mice ICG are immunoreactive to dopamine-beta-hydroxylase (DBH) and
norepinephrine transporter (NET), but they lack vesicular monoamine transporter 2 (VMAT2)
(36). This is in contrast to the nerve fibers and stellate neurons which are positive for DBH, NET,
and VMAT2. The lack of VMAT2 renders the neurons in the mice ICG functionally non-
noradrenergic due to their inability to transport dopamine and norepinephrine into synaptic vesicles (85). However, there were limited studies on the function of TH-IR neurons in the ICG, and further studies are needed to explore the functions of TH-IR neurons in the ICG of different species.

**TH-IR innervation of fat cells and vasculature**

The sympathetic nervous system plays a crucial role in BAT thermogenesis and WAT lipolysis through its direct innervation of peripheral fat depots (86-89). Epicardial adipose tissue is an unusual visceral fat depot and has been shown to express its own specific transcriptomic signature (90). Epicardial fat was described as white adipose tissue with brown-fat-like features (91, 92). We noticed the presence of both types of adipose tissue at multiple locations with predominance of WAT on the atrial epicardium. Similar to our study, recent work that utilized iDISCO tissue clearance, confocal and light sheet microscopy showed a differential density of TH-IR axonal varicosities in BAT and WAT 67. Further functional studies to investigate the physiological effects of sympathetic innervation of both BAT and WAT in the atria would be highly valuable.

As expected, TH-IR axons were observed in close proximity (running parallel or wrapping around) the vasculature (93). Identification of the ultrastructure to confirm TH-IR axons formed contacts with the blood vessels using electron microscopy (54) or physiological studies will be needed. It has been demonstrated that the sympathetic nerves have a major influence on the
control of blood flow, blood pressure, and total vascular resistance via its innervation of small arteries (94). In particular, the sympathetic nervous system has an essential role in maintaining cardiovascular homeostasis and normal physiological activities, including vascular tone and blood pressure.

**Functional implications**

Although several studies have described the atrial sympathetic innervation, comprehensive studies that delineate the topographical TH-IR axon innervation of the whole atria and regional differences are currently lacking (62). Our tracing of the TH-IR axon innervation of the whole atria unraveled the complex axonal network and preferential innervation of distinct regions. The mapping data could be utilized to understand the sympathetic specific control of different regions of the atria and their autonomic responses. In our map, the bundles entering the right side of the atria provided the majority of the sympathetic innervation to the right auricle, right PV, SAN and conductive fibers while the left bundles provided the majority of the sympathetic innervation to the left auricle, interatrial groove (junction of LA and RA) and PVs. Regional and lateral differences in the function of the heart have been indicated previously via the functional studies (mainly in humans) of cardiac sympathetic innervation by the right and left stellate ganglia (SG) (62). SG block revealed that the right SG is largely responsible for increasing heart rate, slowing atrioventricular conduction, and primarily affects the right atrium as opposed to the left atrium. In contrast, the left SG has a lesser effect on heart rate and atrioventricular conduction and primarily affects the left atrium.
as opposed to the right atrium (95, 96). Modulating the sympathetic innervation of the atria is becoming an increasingly important therapeutic approach (32), for example, neuromodulation therapy by electrical stimulation or renal denervation has shown great success in treating diseases like atrial fibrillation via remodeling of stellate ganglion and reducing sympathetic output (97). Therefore, selective targeting of sympathetic innervation of either side of the heart can have different effects. Our topographical map of TH-IR axon innervation in the atria could be used as a cardiac sympathetic atlas to navigate more precise control of different heart regions.

Knowledge of cardiac sympathetic postganglionic innervation location and density may also help to elucidate the normal physiology and abnormal patterns in certain pathological conditions. Our quantitative analysis shed light onto the atrial regions that received the highest TH-IR axon innervation that could potentially indicate a more precise control in these areas. In the RA we found the highest innervation density of TH-IR axons in the SAN, which supports the fact that the sympathetic nervous system has a role in the fine tuning of heart rate. This could also indicate potential therapeutic targets as blockade of neuronal input with propranolol (beta blocker) leads to a decrease in heart rate (98-100). In the LA, the highest density of TH-IR axons was observed at the entrance of PV to the LA. The junction of the left atrium and pulmonary veins has been indicated to be a focal source which is responsible for the initiation of atrial fibrillation (101). Therefore, further functional studies of these great vein-atrial junction
regions, which were the densest with TH-IR axons in our quantitative analysis, are valuable to better understand the physiology and pathology of atrial fibrillation.

Considering that understanding how sympathetic neurons communicate to their cardiac targets is essential for understanding how the heart works (102), our results provide a basis for understanding the role that TH-IR axons specific innervation play in the control of the normal heart as well as in the diseased heart.

Limitations

A couple of limitations must be acknowledged:

1. Neurolucida 360 TH-IR axon tracing: Despite our effort to trace TH-IR axon bundles and their projection field, it was not feasible to trace the smallest branches and individual axons in the whole atria. Our continuous collaboration with MBF Bioscience in SPARC MAP-CORE to improve the customized settings for autotracing of our labeled axons should ensure more precise and faster tracing.

2. Density of single or double layers: Due to great differences in thickness of atria in different regions, some areas had to be separated into single layers to ensure fair comparison of the density. Moreover, our regional density analysis of TH-IR axon innervation in the axon was performed using 2D projection images that present the dense structures along the z-axis in a single bidimensional image. To gain a more accurate representation of the innervation considering the depth of the tissue, 3D representation
of the entire image stack of the atria should be reconstructed to quantify the density for each image stack.

**Summary and future directions**

We have determined the topographical innervation of TH-IR axons in the flat-mount of the whole atria at single cell/axon/varicosity scale. Several TH-IR axon bundles entered the atria through the SVC and LPCV, and these bundles had different projection fields. A clear lateralization preference was found: the right and left bundles preferably innervated the right and left atrium, respectively. In addition, the regional density analysis showed that TH-IR axon innervation in the RA was more abundant than in the LA. In the RA, the SAN, AVN region and internodal conducting fibers showed higher density than the other regions. LA-PV junction had the densest TH-IR axon innervation in the LA. Furthermore, TH-IR bundles and axons passed through the ICG with very limited innervation around ICG neurons, but densely innervated the blood vessels and fat cells. A schematic diagram that summarizes our main findings is shown in **Figure 15**. This work contributes to the cardiac-sympathetic brain connectome. However, anterograde tracer injections into the stellate ganglia to specifically map sympathetic postganglionic projections to the heart should be conducted in the future to address some limitations, including identifying the source of postganglionic TH-IR axons and characterizing terminal structures. In addition, our work provides an anatomical foundation for functional mapping of sympathetic control for the heart as well as evaluation of the remodeling of cardiac
sympathetic innervation in chronic disease models (hypertension, diabetes, sleep apnea, heart failure, aging).

Figure 2.15: Summary figure describing the main findings of this study.

Efferent postganglionic neurons send sympathetic postganglionic fibers to innervate the heart. TH, a commonly used catecholaminergic marker, labeled bundles of nerves, axons, and terminals innervating the atria. Additionally, TH-IR bundles were observed mainly passing through ICG and innervating fat cells and blood vessels. TH-IR neurons comprised about ~30% of ICG neurons. The presence of varicosities around TH-IR ICG neurons and whether there is direct innervation remains to be determined in the future (indicated by question mark). (Created by Ariege Bizanti with Biorender (2022), https://biorender.com/).
Introduction

Obstructive sleep apnea (OSA) is a chronic condition with a global prevalence of almost 1 billion people (103). Chronic intermittent hypoxia (CIH) plays a major role in the pathogenesis of OSA’s deleterious consequences including cardiovascular diseases (CVD) and neurocognitive dysfunction. In fact, previous research has established the existence of relatively strong and independent correlations between CIH and cardiovascular comorbidities, such as systemic hypertension, coronary artery disease, ischemic stroke, arrhythmia, and heart failure (104, 105). One contributing factor to OSA-related CVD is the autonomic dysregulation and the increased peripheral sympathetic discharges as indicated by shifts of the heart rate variability towards low frequency band (related to sympathetic modulation) (106, 107). Recently, it was demonstrated that OSA therapy improved autonomic function measures which may reduce the risk of cardiovascular disease through decreased sympathetic activity (108, 109). This further confirms the major role of the sympathetic nervous system in the pathogenesis of OSA and its associated CIH. Yet, standard OSA treatments have shown either controversial or poor outcomes, necessitating the identification of possible mechanisms and the development of more effective therapies (110) (Badran and Gozal, 2022).

Despite the broad and compelling evidence of CIH being an independent risk factor for cardiovascular diseases, the mechanism and effects of CIH on catecholaminergic axon
innervation in the heart is still not well described (111, 112). Although studies using tissue sections have enriched us with information regarding the distribution of tyrosine hydroxylase (TH, a marker for sympathetic axons) axon innervation in the normal and diseased heart, the remodeling of the topographical distribution and regional differences are still lacking. This renders the effect of CIH to cardiovascular morbidity and mortality unclear. Previously, we established that CIH induced the death of cardiac vagal motor neurons in the nucleus ambiguus of the brainstem which led to an overall reduction of the parasympathetic control of the heart rate (113, 114).

Our current aim is to study the effect of CIH on sympathetic axon innervation in the atria using TH as a marker. Recently, we have provided a map of TH-IR axon innervation in the normal heart (57). In this study, we determined CIH-induced structural remodeling of TH-IR axon innervation in the flat-mount of the whole left and right atrium at a cell/axon/varicosity scale. Our findings indicated that CIH significantly remodeled TH-IR axonal innervation in the atria.

Methods

Animals

All procedures were approved by the University of Central Florida’s Institutional Animal Care and Use Committee (IACUC) and strictly followed the guidelines established by the NIH. All experiments conformed to the University of Central Florida’s guidelines on the ethical use of animals. C57BL/6J male mice (n=7/group, 2 months) were housed in chambers with oxygen
levels controlled by an Oxcycl (BioSpherix Instruments) for intermittent hypoxia. The light and
dark cycle of the room was set to 12:12h (light 7:00 AM to 7:00 PM) at 21-22°C. Cycling changes
of the hypoxic conditions consisted of alternating 21% and 5.7% O2, every 6 minutes during the
10 hours light cycle and maintained at 21% for O2 for the rest of the circadian cycle for ~8
weeks. Ambient CO2 in the chambers was periodically monitored and maintained at 0.03% by
adjusting overall chamber ventilation. Humidity was measured and maintained at 40-50%. The
room air animals were exposed to the same conditions as CIH-exposed animals except that O2,
was maintained at 21% throughout the duration of exposure.

Tissue preparation

Animals were deeply anesthetized with isoflurane (4%) induction in an anesthetic chamber.
Absence of the hind-paw pinch withdrawal reflex was used as an indicator of a sufficient depth
of anesthesia. The chest cavity was opened with minimal incision and mice were perfused by
injecting 0.2 mL heparin to the left ventricle followed by a cut to inferior vena cava to drain
blood. After 2 minutes, a needle was inserted into the left ventricles and the mice were
perfused with 0.9% saline at 38-40 °C for 5 minutes, followed by fixation with 4 °C Zamboni’s
fixative (15% picric acid and 2% paraformaldehyde in PBS, pH=7.4). The heart, lung and trachea
were removed from the chest and postfixed overnight in 4% paraformaldehyde at 4 °C. The
heart was placed and pinned into a dissecting dish containing PBS (pH 7.4) and the specimen
was further dissected using the Leica Stereo microscope as described previously (53, 56, 115).
In brief, the lungs and trachea were removed with fine tweezers, the atria were separated from the ventricles and the aortic arch was removed from the atria. The right and left atria were separated along the interatrial septum producing the right atrium with the great vessels, including the inferior vena cava (IVC), superior vena cava (SVC), and left precaval vein (LPCV) attached, as well as the left atrium with the junctions of all pulmonary veins (PVs) attached. The sinoatrial node (SAN) region was identified based on its known anatomical location at the junction of the SVC and right atrium and further based on our previous staining with HCN4. The atrioventricular node (AVN) region was less clearly defined due to its deeper location and parts of it associated with the superior interventricular septum, thus we used the term “region” to refer to its the approximate location. Tissues were stored in 4 °C PBS until immunohistochemical analysis was initiated.

**Immunohistochemistry (IHC)**

Tissue processing and immunolabeling were performed as described previously (Li et al., 2014). Following dissection, the tissues were washed 6 x 5 min in 0.1 PBS (pH 7.4), then immersed for 48 hr/5 days in a blocking reagent (2% bovine serum albumin, 10% normal donkey serum, 2% Triton X-100, 0.008% NaN, in 0.1 M PBS, pH=7.4). To reduce nonspecific binding of the primary antibody and to promote increased antibody penetration. Primary antibodies (6 µl/ml) were added to primary solution (2% bovine serum albumin, 4% normal donkey serum, 0.08% Triton X-100, 0.008% NaN, in 0.1 M PBS, pH=7.4) and incubated administered for 48 hr/72 hr. Unbound
primary antibodies were removed from tissues by 6x5 min washes with PBST (0.53% Triton X-100 in 0.1 M PBS, pH=7.4). Secondary antibodies (12 μl/ml in PBST) were then applied for 2448 hr. Unbound secondary antibodies were removed from tissues by 6x5 min washes in PBS. Negative control tests (in which primary antibodies were omitted) were also performed, and in every case these preparations presented no labeling, confirming that nonspecific binding of secondary antibodies did not occur. Antibodies used in this study are listed in (Table 3.1).

Whole-mounts were placed on a microscope slide with their dorsal side against the glass, coverslipped, crushed for 2 days with lead weights, and air-dried under a fume hood for 1 day. Slides were dehydrated by immersion for 2 minutes in each of 4 ascending concentrations of ethanol (75%, 95%, 100%, and 100%), followed by 2x10 min washes in 100% xylene. Slides were then covered with coverslips and DEPEX mounting medium (Electron Microscopy Sciences #13514) and allowed to dry overnight.

Table 3.1: Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Host</th>
<th>Company</th>
<th>Catalog</th>
<th>RRID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-TH</td>
<td>1:10012 μL/mL</td>
<td>Rabbit</td>
<td>Pel-Freeze</td>
<td>Cat# P40101- 0</td>
<td>AB_461064</td>
</tr>
<tr>
<td>Antibody</td>
<td>Dilution</td>
<td>Species</td>
<td>Vendor</td>
<td>Catalogue Number</td>
<td>Accession Number</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------</td>
<td>------------</td>
<td>----------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Anti-TH</td>
<td>1:10012 μL/mL</td>
<td>Sheep</td>
<td>Millipore</td>
<td>AB1542</td>
<td>AB_90755</td>
</tr>
<tr>
<td>Anti- PGP9.5</td>
<td>1:10012 μL/mL</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab108986</td>
<td>AB_10891773</td>
</tr>
<tr>
<td>Alexa Fluor 594 anti-sheep##</td>
<td>1:5024 μL/mL</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A-11016</td>
<td>AB_2534083</td>
</tr>
<tr>
<td>Alexa Fluor 488##</td>
<td>1:50 24 μL/mL</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A21206</td>
<td>AB_2535792</td>
</tr>
</tbody>
</table>

**Density quantification**

To quantify the regional density of TH-IR fibers in the atria, we segregated images into specific regions of interest (ROIs): SA node, SVC, IVC, right outer and inner auricle, LA-PV junction, left PV, middle PV, right PV, left outer and inner auricle. The steps of the density quantification were as follows: 1) subtracted the background with radius of 80 pixels to reduce noise and enhance contrast. 2) applied particle removal to remove small debris. 3) Applied a binary threshold (Otsu method) to isolate immunoreactive structures and then images were skeletonized. 4) Quantified the signal above the threshold. 5) averaged the signal of different
ROIs windows using six counting frames. 6) Divided the number of signal pixels by ROI area. 7) ran the Shapiro-Wilk normality test. Statistical significance of the difference between the means was performed using one-way ANOVA and Tukey’s HSD (Honesty Significant Difference). Data are expressed as mean density % +/- SEM. Significance as accepted at P<0.05.

Heatmaps of the signal intensity were created after applying a modified version of the freely available open-source automated software algorithm that trace and quantify axons (Axon tracer plugin, ImageJ)(59).

Axon Tracing and quantification

**Neurolucida 360 tracing:** We used Neurolucida 360 software (https://www.mbfbioscience.com/neurolucida360, RRID:SCR_016788) to digitize and trace axonal structures. Additionally, small areas 3d z stack were selected to perform a semiautomated tracing by choosing the settings for automatic tracing followed by manual adjustment to ensure the tracing reflects the original nerves and avoid false positive and false negative tracings. Another software by MBF Biosciences, namely Neurolucida Explorer® (https://www.mbfbioscience.com/neurolucida-explorer, RRID:SCR_017348) was used in combination to perform morphometric analysis including (complexity : number of bifurcation points per unit of area) and varicosity distance.
**Arvis Vision4D** was utilized to autotrace large size deconvolution data of the whole right and left atrium through customized pipeline. The analysis pipeline was used for quantification of the 3D image data complete tracing. Features selected to measure included Axons total length, average tortuosity, and total bifurcation points.

**Results**

An overview of TH-IR axon innervation in the flat-mount of whole right and left atrium in RA and CIH

The montages of the whole right and left atrium allowed us to delineate the common features across all animals in both groups. The salient differences and specific features were more distinct in the detailed analysis of specific regions as demonstrated later in this paper.

*In the right atrium:*

In both RA and CIH right atrium, large TH-IR axon bundles entered through the superior vena cava (SVC) or left pre-caval vein (LPCV), branched out into smaller bundles, and eventually ramified into individual axons, and their terminals covered the entire atrium ([Figure 3.1 a,b](#)). The entrance points of large TH-IR axon bundles (yellow arrows) in RA and CIH flat-mount right atrium were relatively similar. However, the pattern of distribution was different between the two groups. ICG ganglia (yellow asterisks) in the right atrium were either near SAN or on LPCV close to the point of connection to the left atrium. Colored maps of TH-IR axon intensity...
showed the highest intensity areas around the SAN region, conductive fibers, AVN region and the medial side of the right auricle (Figure 3.1 a’,b’). To preserve the z-information and resolve details in the depth of the atrium, depth-coded layer projections showed that CIH increased the innervation of TH-IR axons in the range of (~5-35 um) compared to RA condition (Figure 3.2 a,c). Even though, both RA and CIH tissues were treated similarly (in terms of staining, flattening, and weight applied), the thickness of the whole right atrium cardiac muscle was increased following CIH in all animals (7/group). We developed a customized pipeline in Arivis Vision4D software which allowed auto-tracing of TH-IR axons from the z-stack volume of the entire right atrium and advanced analysis. Z-projection of the 3D tracing allowed the visualization of the distribution of TH-IR axon innervation in the whole atria as well as the regional heterogeneity in both RA and CIH (figure 3.2 b,d). Additionally, the reconstruction of the axons provided a combined pipeline for image processing and robust quantification analysis of the entire TH-IR axon innervation in the right atrium with a broad range of parameters (including total length, average tortuosity and branching points). Tortuosity (the twistedness of curvilinear structure) was increased following CIH (2.36 +/- 0.1) compared to RA (1.76 +/- 0.04).
Figure 3. 1: Distribution of TH-IR axon innervation in the whole right atrium flat-mount of representative mice.

Two montages of a few hundred maximal projection images demonstrate the catecholaminergic (TH-IR) axon innervation in RA (a) and CIH (b) conditions. Large TH-IR bundles enter the right atrium from the SVC and LPCV and bifurcate into smaller bundles and eventually into individual axons covering the entire atrium. Colored-maps of TH-IR axon intensity showed the regions with the highest intensity in the SAN region, conductive fibers, AVN region and medial side of the auricle in RA (a') and CIH (b')
Figure 3.2: Depth-encoded montages and Arivis vision 4D tracing of TH-IR axon innervation in the flat-mount of the right atrium.

Depth is displayed as yellow-red (outermost) and green (middle and inner) layers. Z-stack range in RA was ~0-70 (a) while in CIH ~ (0-84 micrometer) in CIH (c). Pipeline created in Vision 4D that included background subtraction, enhancement, seeds and tubularity filters resulted in complete tracing of the entire right atrium in RA (b) and CIH (d).
In the left atrium

In the flat-mount of the whole left atrium, large TH-IR bundles entered the left atrium from the middle region (yellow arrows, Figure 3 a,b) and bifurcated into smaller bundles and then single axons covering the entire left atrium. Colored maps of the signal intensity showed that the regions with the highest TH-IR axon density were located mainly through the middle area of the left atrium between the PVs and auricle and in the middle of the left auricle (Figure 3.3 a’,b’). Similar to the right atrium, the large TH-IR bundles were present on the epicardium layer and CIH increased the thickness of the atrial tissue (Figure 3.4 a,b). Tracing of the whole atrium using Arivis Vision 4D allowed the visualization of the detailed distribution of TH-IR bundles and their projection field in the entire atrium as well as the highly convoluted form of TH-IR axonal network in the middle area of the atrium (Figure 3.4 b,c). Furthermore, quantification of the complete tracing was possible utilizing the predefined workflow extracting the data from the autotracing of the entire atrium. Despite the common features of the TH-IR axon distribution in the montages of both atria in RA and CIH, zooming in the details can reveal the difference of innervation between groups.
Figure 3.3: Distribution of TH-IR axon innervation in the whole left atrium of RA and CIH.

Large TH-IR bundles (yellow arrows) entered the left atrium in the middle area near the LA-PV junction, bifurcated into smaller bundles and eventually into individual axons covering the entire atrium. Colored-map of TH-IR axon intensity showed the highest intensity regions around the LA-PV junction in RA and CIH (a', b').
**Figure 3.4:** Depth-encoded montages and heatmaps of TH-IR axon innervation in the flat-mount of the whole left atrium.

Depth is displayed as green-yellow (outermost) and green (middle and inner) layers. Z-stack range in the RA was 0-64 (a) while in CIH (0-87 micrometer) (c). Heatmap of TH-IR axons showing the overall intensity distribution and the hot spot of the highest regions. Pipeline created in Vision 4D that included background subtraction, enhancement, seeds and tubularity filters resulted in complete tracing of the entire right atrium in RA (b) and CIH (d).

CIH increased regional density of TH-IR axons and terminals

To visualize the distinct regional distribution in RA and CIH conditions, few regions of interests of the right atrium were quantified (**Figure 3.5 a-l**). CIH led to an increase of TH-IR axons in the following regions: (SAN regions, AVN regions, right auricle, LPCV, SVC, and IVC) with the highest density observed in the SAN region (**Figure 3.5m**). A closer look in those regions of interest shows a change in the pattern of innervation with more branching points, and random directionality. Examining regions of interest in the left atrium (**Figure 3.6 a-j**), indicated that CIH increased density of TH-IR axons in the flat-mount of the left atrium and showed a more complex network of TH-IR axon innervation in the CIH condition that was indicated by more branching points, tortuosity and random directionality. The increase of TH-IR axon density following CIH exposure was observed in LA-PV junction, left auricle, R.PV, and L.PV (**Figure 3.6k**).
Figure 3.5: Regional density of TH-IR axons in the right atrium in RA and CIH.

Representative images of regions of interest (ROIs) in the flat-mount of right atrium showed detailed TH-IR axon innervation in different regions (a-l). Quantification of TH-IR fibers in ROIs of a representative mouse (m). *P<0.05. n = 7. Scale bars: 50 μm.
Figure 3. 6: Regional density of TH-IR axons in the left atrium in RA and CIH.

Representative images of the regions of interest (ROIs) showed detailed TH-IR axon innervation in different regions of the left atrium (a-j). Quantification of TH-IR fibers in ROIs of a representative mouse (k). *P<0.05, **P<0.01. n = 7. Scale bars: 50 μm
CIH induced branching of TH-IR axons that traversed more layers of the atria

Considering the important role of SAN as a pacemaker and the region in which CIH increased density of TH-IR axon innervation mostly, we selected the SAN and reconstructed the axonal network in that region specifically. In both RA and CIH, the SAN was heavily innervated (Figure 3.7a, b). However, following CIH, bifurcation points per area increased and random directionality of TH-IR axon innervation was more noticeable (Figure 3.7b). Neurolucida 360 3D semi-automated tracing of a small area (350 um x 350 um) of the SAN showed substantial change in the innervation pattern following CIH indicated by less uniform directionality and more inter-axonal connections (Figure 3.7 c,d).

Furthermore, changes of the TH-IR axons were also observed at the different layers of the auricle (Figure 8). For example, near the epicardium TH-IR smaller bundles formed more horizontal connections with other axons and ran in less uniform direction in CIH (Figure 3.8d) compared to RA (Figure 3.8a). The non preferential directionality was also observed in the myocardium following CIH (Figure 3.8b, b'). In the deeper layers of the auricle tissue, more pronounced tortuosity and inter-axonal connections were induced by CIH (Figure 3.8d, d'). 3D tracing with Neurolucida 360 and orthogonal projection showed more distinct changes across the z layer. Traced TH-IR axonal structure spanned ~40 um in RA and ~60 um following CIH as seen in the XZ orthogonal projection (Figure 3.8 g,h)
Overall, TH-IR axons were more organized in RA while following CIH the innervation pattern became more disorganized. Tortuosity of large TH-IR bundles on the epicardium (Figure 3.9 a,a’,b,b’) and single axons (Figure 3.9c,d) and varicosities (Figure 3.9e,f) in the myocardium were more pronounced following CIH. TH-IR varicosities were seen in innervating cardiomyocytes in RA and CIH (arrowheads in Figure 3.9e,f).
Figure 3. 7: Neurolucida 360® reconstruction/tracing of TH-IR axons in the SAN following RA and CIH conditions.

Tracing of TH-IR axons in the SAN displays a more organized pattern of axonal innervation following RA conditions (a) in comparison to a less uniform and tortuous/complex pattern of innervation following CIH conditions (b). 3D axon tracing in a ROI of SAN displays an organized pattern of directionality following RA conditions (c) in comparison to a more non-uniform directionality following CIH conditions (d). Scale bar: 100 µm
Figure 3. 8: TH-IR innervation in different tissue layers of the auricle following RA and CIH conditions.

TH-IR axons displayed a uniform directional pattern in the epicardial layer following RA conditions (a) in comparison to a less uniform pattern following CIH conditions (d). TH-IR axons displayed a preferential directional pattern in the myocardial layer following RA conditions (b) in comparison to a non-preferential pattern following CIH conditions (e). TH-IR displayed a uniform directional pattern in the endocardial layer following RA conditions (c) in comparison to a non-uniform pattern following CIH conditions (f). Neurolucida 360® orthogonal projections of a ROI in the auricle displaying a more uniform directionality and an axonal span around 40 µm following RA conditions (g) compared to a more random directionality and an increased axonal span around 60 µm following CIH conditions (h).
Figure 3. 9: Large TH-IR bundles, axons, and varicosities in the epicardium and myocardium layers.

(a,b) large TH-IR bundles appear more tortuous in the CIH epicardial layer (b,b’) in comparison to RA (a,a’). In the myocardium, TH-IR axons were more uniformly distributed and running in a relatively straight path in RA (c) compared to CIH where TH-IR axons did not show preferential directionality and more tortuosity (d). TH-IR varicosities (white arrowheads) could be seen in the cardiomocytes in RA (e) and CIH (f). Scale bar 20.

TH-IR axons traveled through ICG

ICG were located mainly on the dorsal surface of the left atrium. ICG varied in size and 2-3 large ganglia were consistently found across all animals. Generally, in both large (Figure 3.10a,b) and medium-small ganglia (Figure 3.10c,d) ICG neurons were more aggregated following CIH compared to RA. Similar to our recent published work (Bizanti et al., 2023; Zhang et al., 2023), TH-IR axons were mainly observed to travel through TH-IR neurons in the ganglia rather than tightly encircling individual neurons like vagal efferent axons. Interestingly, we observed distinct irregular patterns of TH-IR axon terminals in ICG following CIH. For example, finger-like projections of TH-IR axons (arrows in figure 3.10 e, f) and large TH-IR varicosities were observed on FG-labeled neurons. Additionally, in contrast to RA, we found some axons encircling individual neurons of ICG (arrow in figure 3.10 g,h) following CIH.
**Figure 3. 10**: TH-IR axons around ICG neurons change patterns following CIH.

Neurons in large-size ICG (a,b) and medium/small size ICG (c,d) were more aggregated following CIH. Irregular patterns of TH-IR axons formed finger-like projections around ICG neurons (e,f) and encircled individual neurons (g,h) following CIH.

**RA vs CIH TH-IR axon innervation of fat and vasculature**

Utilizing autofluorescence to visualize adipocytes, we were able to identify both white adipose tissue (WAT) (characterized by one large droplet) and brown adipose tissue (BAT, characterized by multiple vacuoles) near the atrial tissue. TH-IR axons were densely distributed near both BAT (Figure 3.11 a,b) and WAT (Figure 3.11 c,d). Single axons were seen traveling around individual white adipocytes (arrowheads in Figure 3.11f) or around the circumference of brown adipocytes (dotted box in Figure 3.11 e,f). TH-IR axon innervation of the atria increased following CIH (Figure 3.11 g). TH-IR axons also wrapped around and ran along the boundaries of blood vessels near the atrial tissue in RA (Figure 3.12 a,a') and CIH (Figure 3.12 c,c'). Dense transmural innervation of TH-IR axons was also seen on vasculature in the atrial cardiac muscle (Figure 12 b,b',d,d'). TH-IR en passant varicosities on the smooth muscles of the major veins attached to the atria (SVC, IVC, L.PV, M.PV, R.PV) (Figure 3.12 f-o) were traced and auto-detected by Neurolucida 360 (Figure 3.12 f'-o') and had shorter distance between TH-IR varicosities across the axon following CIH compared to RA (Figure 3.12 e).
Figure 3. TH-IR axons in adipocytes near the atrial tissue of RA and CIH.

TH-IR axons were in BAT of RA (a-c) and CIH (d-f) with more TH-IR axons observed around BAT of CIH. TH-IR axons were mostly observed around WAT (a'-c') of RA, and (d'-f') of hypoxia. Scale bar 50 um. TH-IR axons were found around the circumference of individual adipocytes in RA (e) and CIH (f) around both BAT (dotted box) and WAT (white arrowheads) and TH-IR axons were more abundant around adipocytes following CIH (g).
Figure 3.12: Dense transmural innervation of TH-IR axons on the atrial vasculature.

In the blood vessels near the atria, TH-IR axons were found wrapping around the B.V. of both RA (a) and CIH (c) and innervating the wall as seen in the optical section in RA (a’) and CIH (c’). TH-IR axons also traveled along the vasculature wall in the atrial muscle (b,d) with more interaxonal connection following CIH as seen by the Neurolucida 360 tracing (b’,d’). High-resolution images of ROIs on the major atrial veins muscle (f-n) showed TH-IR en passant varicosities which were also detected using Neurolucida 360 tracing (f’-n’). Following CIH, the distance between TH-IR varicosities decreased significantly (e).
Discussion

In this study, we unraveled the effect of CIH on the remodeling of TH-IR axon innervation in the flat-mount of the whole right and left atria. Compared to RA mice, CIH increased the density of TH-IR axon innervation at different regions including (SAN region, AVN region, auricles, and major veins attached to the atria). Additionally, TH-IR axons showed more complexity and disorganization following CIH indicated by increased branching, tortuosity, and terminal endings. These changes were observed at the epicardium and myocardium. CIH also caused an increase in TH-IR axon innervation of adipocytes and reduced the distance between varicosities on atrial major veins.

1. CIH remodeled TH-IR axon innervation in the atria

CIH alters the structure and function of multiple neural components within the brain-heart circuitry implying CIH-induced changes at multiple sites which can involve different mechanisms. There is mounting evidence supporting the association of overactivity of the sympathetic nervous system and OSA related consequences. Neurons that reside in the central components including the rostral ventrolateral medulla (RVLM), hypothalamus, and the nucleus of the solitary tract (NTS) regulate the basal sympathetic tone and crucial determinant of the control of blood pressure (116). Other studies have established the role of the central components in the initiation and progression of CVD following CIH (27-29). This study is an extension of these previous studies to determine the remodeling of TH-IR axonal network in the whole atria following CIH and improve our understanding of the peripheral neural component
role during CIH. OSA has been consistently associated with high levels of sympathetic drive that contribute to CVD consequences, and atrial muscle structural remodeling plays a key role in CIH-related CVD effects (117). Similarly, we found that CIH induced hyperinnervation and regional heterogeneity of TH-IR axons in the atria. Considering that the role of sympathetic and catecholaminergic activity in the pathogenesis of cardiovascular morbidity in individuals with OSA (118), our observation of the preferential innervation of atrial catecholaminergic axons in RA and CIH gave insight into the sympathetic regional control of the atria. Following CIH exposure, we found many alteration in innervation pattern and significant increase of TH-IR axon density around SAN region, AVN region, LA-PV junction, major veins attached to the atria which suggest that CIH can induce region-specific modulation of catecholamine response as indicated previously (119). Furthermore, Nerve tortuosity appears to be a promising indicator for nerve abnormalities, and may have potential applications in the diagnosis, management, and monitoring of nerve disorders. Tortuosity was mainly used as a morphological parameter associating corneal nerve tortuosity to ocular and systemic disease (120-122). Here, we used this parameter to investigate the curvature of TH-IR axons following CIH and this may potentially be another measurement to facilitate determining of the distinct changes associated with CIH. Additionally, the reduced distance between TH-IR varicosities may indicate the formation of new synaptic structures following CIH. Previous ultrastructural studies provided evidence of close contact between the adrenergic varicosities and cardiac muscle or sinus venosus muscle (123, 124).
1.1 Remodeling of TH-IR axons in the SAN region

We found denser and more complex innervation of TH-IR axons in the SAN region which may explain the functional alteration associated with CIH induced CVD. Hyperactivity of SNS elicit tachyarrhythmias (9) and abnormal activity of the sympathetic nervous system and catecholamine release are major determinants of hypertension development in patients with OSA (125). OSA has been associated with heart rhythm disturbance and the significant increase in SAN region innervation and disorganization that was demonstrated by our results may disturb the pacemaker control of the heart rhythm and exacerbate arrhythmias. Similar to our study, a prior study showed that CIH induces atrial fibrillation and atrial remodeling in sections of the atria along with hyperinnervation of TH-IR axons (117). However, we provided a more detailed regional differential distribution and changes in the innervation pattern of TH-IR axons following CIH.

1.2 Remodeling of TH-IR axons in the auricles

Our results also indicated hyperinnervation of TH-IR axons in other areas beside the SAN which may contribute to other functional implications. For example, the increase of TH-IR axon innervation in the auricle may have an effect of CIH deleterious impacts on post-myocardial infarction remodeling and contractile dysfunction (25). In our previous work (126), CIH significantly remodeled the structure of vagal cardiac axons and terminals in the atria. Similarly,
we observed disarray of TH-IR axons in the auricles which was amplified through the different z-stacks of the auricle muscles.

1.3 Remodeling of TH-IR axons in the pulmonary veins

The alteration in PVs innervation we observed may also indicate an important functional effect as it was reported that PVs and their junction with the left atrium represent critical areas for the initiation of atrial fibrillation (101). The autonomic innervation of PVs has been suggested to play an important mechanistic role in the initiation of AF (127). In fact, radiofrequency ablation of the atria eliminated sustained AF, but the distribution of catecholaminergic nerves in the atria were not delineated which hindered the explanation of the exact effect of radiofrequency ablation. Here, we found TH-IR axons structural changes in PVs which may give important insights in the CIH-related Atrial fibrillation mechanism.

2. TH-IR ICG neurons

Consistent with our previous studies (Zhang et al., 2023;(57)), we observed that TH-IR bundles and axons primarily traveled through the ICG without encircling individual ICG principal neurons (PNs) in both RA and CIH.

Following CIH exposure, we observed a clustering of ICG neurons as they were more crowded compared to RA. This observation is consistent with ICG remodeling in Goto-Kakizaki GK and STZ-induced diabetic rats and mice (128-130) which imply an association of ICG remodeling and
pathological conditions. Whether the structural remodeling of TH-IR ICG in CIH rats leads to functional cardiac deficits remains to be elucidated.

3. TH-IR axon innervation of adipocytes

CIH has variable effects on adipose tissue that exhibit location dependency (131). Here, we focused on the epicardial fat located near the atria. Epicardial fat has been identified as a putative risk factor for cardiovascular diseases as it appears to promote the activation of the cardiac autonomic nervous system along with the finding of a strong association of increased epicardial fat thickness in OSA among both obese and non-obese patients (132, 133). Our findings showed TH-IR axons hyperinnervation of the adipocytes following CIH suggesting that these structural changes may correlate with some of the functional alterations previously suggested by others. In earlier studies, the regulatory effect of CIH on the production of adipokines production was primarily dependent on activation of adipocyte adrenergic receptors and treatment with β-adrenergic receptor antagonist metoprolol reversed the metabolic remodeling induced by OSA (134, 135). However, although these studies showed an association of sympathetic innervation of the adipose tissue and OSA-related cardio-metabolic effects, a direct causal relationship is still not clear. Since epicardial fat can adversely influence the ANS of the heart via derangement of the extrinsic sympathetic system (133), the hyperinnervation innervation of TH-IR axons in the adipose tissue that we observed may play a role in this
mechanism. More functional studies are needed to investigate the metabolic effects of TH-IR increased innervation of epicardial adipose tissue following CIH.

4. TH-IR axon innervation of vasculature (Blood vessels)

The emergence of hypertension following CIH is mediated by neurogenic mechanisms involving increased sympathetic tonic activity (136). Additionally, CIH activation of the sympathetic nervous system promotes endothelial dysfunction and vascular inflammation contributing to the development of hypertension (137, 138). We observed dense innervation of TH-IR axons in the major blood vessels attached to the atria that increased following CIH along with structural changes in the pattern of innervation. Of note, CIH also induces substantial vasoconstriction and hypertrophy of the pulmonary veins. (Gao and Raj, 2005). Our observation of shorter distance between varicosities may give initial insights into the potential mechanism(s) of the increased sympathetic innervation of pulmonary veins that leads to the vasoconstriction following CIH. Similarly, we showed that the SVC and IVC had more innervation and shorter distance between TH-IR varicosities. The remodeling of TH-IR axons and en passant varicosities innervating the vasculature of the atria may provide opportunities for developing sympathetic modulation therapeutic strategies that reverse the CIH-related vascular dysfunction. For example, thoracic vena cava constrictions were shown to be associated with sympathetic efferent pathways, but the exact potential mechanism of this association was not clear (139).
Focusing on the structural changes of the sympathetic efferent to regain the normal
innervation distribution and reduction of the disorganization can potentially improve the
neuromodulation to specific targets such as blood vessels. However, assessments that include
noradrenaline spillover rate, direct catecholamine measurements, and heart rate variability
that are associated with the structural changes observed herein would help better delineate
the pathophysiology of OSA-related vascular dysfunction.

Limitations
The present study employed TH-IR axons as a sympathetic marker. Nevertheless, it is
imperative to conduct anterograde tracer injection into the stellate ganglia to validate that
these fibers are, in fact, sympathetic postganglionic fibers and to pinpoint their precise origin.
Furthermore, as the ventricles hold considerable clinical significance, an evaluation of the
sympathetic innervation within the ventricles in RA and CIH would provide valuable insight into
OSA-related CVDs and facilitate the translation of this knowledge into CVD management.
Although the application of this technique to the ventricles poses a challenge, we have recently
developed a strategy for addressing this concern. Therefore, utilizing our established protocol
can overcome the technical difficulty in staining the flat-mount whole ventricles and determine
the complete cardiac TH-IR innervation.
Summary

We have determined the CIH-induced structural changes to local cardiac circuitry, specifically the TH-IR axon innervation of the flat-mount of whole atria at single cell/axon/varicosity scale. Here, we showed that CIH induces hyperinnervation of TH-IR axons, regional heterogeneity, as well as increased branching and decreased distance between varicosities in the right and left atrium. Previously, it has been shown that CIH increased the central sympathetic output. Our study provides evidence that CIH increased sympathetic innervation of the heart including SAN region, AVN region and major blood vessels attached to the atria. This further indicates that CIH enhances sympathetic control of the heart, which may lead to increased heart rate and contractility and ultimately hypertension. This study provides more clues of another neural component within the brain-heart circuitry that CIH alters. Our morphometric analysis of TH-IR axons in RA and CIH indicated preferential innervation density and distinct patterns at different regions and layers of the atria. Our findings imply CIH effects of the highly specific control of the sympathetic innervation in the atria that can give insight into more complex modes of CVD adjustments in OSA. Along with our previous findings that CIH reduces parasympathetic control of the heart (27, 51, 126, 140, 141), our work supports the hypothesis that CIH induces chronic autonomic imbalance. Our anatomical techniques and strategies can be interpolated to study other pathological conditions that also induce autonomic imbalance.
CHAPTER FOUR: TOPOGRAPHICAL MAPPING OF CATECHOLAMINERGIC AXON INNERVATION IN THE FLAT-MOUNTS OF THE MOUSE VENTRICLES AND INTERVENTRICULAR SEPTUM IN NORMAL AND DISEASE MODELS

Introduction

The sympathetic innervation of the ventricles is a critical mediator of cardiac contractility, and arrhythmogenesis (142). The ventricles of the heart are richly innervated by sympathetic nerves, which originate from the sympathetic ganglia located along the thoracic and cervical spinal cord (143). These nerves project to the ventricles through the cardiac plexus and release norepinephrine (NE) in response to various stimuli, including stress and exercise. The distribution of sympathetic innervation in the ventricles is not uniform, with the apex and base of the heart exhibiting different patterns of innervation (144), and most of the innervation is located primarily in the subepicardium (6). The sympathetic nervous system (SNS) regulates ventricular function via its effect on the β-adrenergic receptors, which are widely distributed throughout the heart. The activation of these receptors results in the release of the second messenger cyclic adenosine monophosphate (cAMP), which modulates intracellular calcium handling, ultimately leading to increased contractility (2). Furthermore, SNS hyperactivity is known to play a critical role in the pathogenesis of various cardiac pathologies, including heart failure and myocardial infarction. In fact, sympathetic hyperactivity is a hallmark of these conditions and leads to deleterious effects on ventricular function. The chronic activation of the SNS results in sustained β-adrenergic receptor signaling, leading to myocardial remodeling and
increased risk of arrhythmias (145). Considering the pivotal role of SNS in the ventricular function and the established lateralization and remodeling of ventricle innervation in disease, a detailed neuroanatomical map of the ventricle sympathetic innervation is essential. Therefore, in our study we will determine the distribution of TH-IR axon innervation in the flat-mount of whole ventricles and interventricular septum.

Methodology

Animal

All animal handling and procedures strictly followed the guidelines established by the NIH which were approved by the University of Central Florida’s Institutional Animal Care and Use Committee (IACUC) and. All of our experiments conformed to the University of Central Florida’s guidelines on the ethical use of animals.

Chronic intermittent hypoxia and tissue processing

C57BL/6J male mice (n=7/group, 2 months) were housed in chambers with oxygen levels controlled by an Oxycl (BioSpherix Instruments) for intermittent hypoxia. The light and dark cycle of the room was set to 12:12h (light 7:00 AM to 7:00 PM) at 21-22℃. Cycling changes of the hypoxic conditions consisted of alternating 21% and 5.7% O2, every 6 minutes during the 10 hours light cycle and maintained at 21% for O2 for the rest of the circadian cycle for ~8 weeks. Ambient CO2 in the chambers was periodically monitored and maintained at 0.03% by adjusting
overall chamber ventilation. Humidity was measured and maintained at 40-50%. The room air animals were housed in the same room as CIH-exposed animals except O, was maintained at 21% throughout the duration of exposure.

**Tissue preparations**

We followed our established protocols (53, 56, 115) as well as the optimized version to stain the whole ventricles (57). In brief, animals were anesthetized deeply and perfused through the left ventricle. The hearts were removed and the ventricles were separated from the atria to produce the left ventricle (LV), right ventricle (RV) and interventricular septum (IVS). The flat-mounts of the whole ventricles and interventricular septum were immunohistochemistry stained with TH, then imaged using Confocal Microscopy or Zeiss Imager and stitched to create a montage of the entire tissue.

Furthermore, we determined the remodeling of the TH-IR axon innervation in the ventricles following CIH. Utilizing the same methodology we used in chapter 3, we analyzed the alterations in the TH-IR axon innervation patterns at different regions of the ventricles after CIH exposure.

**Anatomical mapping into a 3D scaffold model**

Using the MBF Neurolucida system 360, Zeiss Arivis Vision 4D, and the NIH SPARC mapping tool (by Auckland University), we integrated the anatomical maps into a scaffold model. After all TH-
IR axons in the flat-mount of the ventricles are traced and digitized, fiducial annotations in the scaffold and the tracings were created to be used as anchor points. By matching the fiducial annotation, the geometry fitter aligned the scaffold to the flat-mount left ventricle data. This allowed the scaffolding to conform to the flat-contours. Finally, the material coordinates of the traced innervation were obtained for data embedding based on their placements in the fitted scaffold.

Results

Topographical organization of TH-IR axons in the flat-mounts of whole ventricles and interventricular septum

We determined the structural organization of the TH-IR axons innervation in the flat-mounts of whole ventricles. Our findings showed that large TH-IR bundles entered the left ventricle (LV) (Figure 4.1), right ventricle (RV) (Figure 4.2), and interventricular septum (IVS) (Figure 4.3) from the base. These bundles traveled down towards the apex while branching into smaller bundles and eventually ramifying into individual axons covering the entire ventricles. We traced the large TH-IR individual bundles to represent their projection fields.
Figure 4.1: Distribution of TH-IR axon innervation in the flat-mount of whole left ventricle in a representative mouse.
Figure 4.2: Distribution of TH-IR axon innervation in the flat-mount of whole right ventricle in a representative mouse.
Figure 4.3: Distribution of TH-IR axon innervation in the flat-mount of whole interventricular septum in a representative mouse.
At closer examination, the TH-IR axons exhibited distinct patterns at different regions of the ventricles. Examining the pattern of innervation at different layers revealed that the large TH-IR bundles are present on the outer layer in the LV,RV and near the outer layer in the IVS (Figure 4.4). We were able to visualize TH-IR axons through the different depths of the tissue in both ventricles and IVS.

Additionally, TH-IR varicosities and terminals were visualized in the inner layer of both ventricles (Figure 4.5). In the LV, large TH-IR axon bundles were predominantly present around the base region which bifurcated into smaller bundles traveling through the middle region and change in the directionality of the axons were more salient at the apex region (Figure 4.6). In
the RV, large TH-IR bundles bifurcated multiple times to produce many smaller bundles in the middle region and dense innervation of TH-IR axons were found at different regions of the ventricle (Figure 4.7). TH-IR axons were more disorganized in the IVS as it showed multidirectional axons all over the IVS (Figure 4.8).
Figure 4.5: Extensive innervation of TH-IR axons and terminals in the inner layer of LV and RV
Figure 4. Regional distribution of TH-IR axons in the LV
TH-IR axons in the vasculature of the ventricles

Blood vessels (BV) with different diameters were present in the ventricles. Large BV were heavily innervated with TH-IR axons which ran along the wall of the vessel or wrapped around it (Figure 4.9). The vasculature in the ventricular myocardium were highly innervated and TH-IR axon innervation decreased in smaller blood vessels.
Figure 4.9. TH-IR axons around the blood vessels
Anatomical mapping on the 3D organ Scaffold

For the first time, we created an integrated tracing of TH-IR axon innervation in the left ventricle onto a 3D heart scaffold. In collaboration with Dr. Hunter group from Auckland bioengineering university, we utilized their mapping tool to integrate our tracing data onto a geometrically fitted heart scaffold.

We illustrated the integration of digitized axons in the flat mount of the whole left ventricles into a scaffold (Figure 4.10). Four main steps were used to create the final output: 1) Imaging: flattened whole left ventricle labeled with TH was imaged using Zeiss imager and several hundred tiles were stitched together to produce a montage. 2) Segmentation: All axons in the ventricles flat-mount were traced and digitized using Neurolucida and Arivis Vision4D. 3) Fiducial annotation and Scaffold fitting: Geometrical fitting of the tracing data to a scaffold using fiducial alignments in our flat-mount tissue and the flattened scaffold. 4) Scaffold mapping: all digitized axons are mapped onto the scaffold using calculated geometric coordinate relative to the fitted scaffold.
Figure 4. 10. Illustration for the integration of digitized axons in the flat-mount of the whole ventricles into a scaffold.
Remodeling of TH-IR axon innervation in the left ventricle following CIH

We noticed an increase in the density in the base, middle and apex in CIH compared to RA (Figure 4.5, 4.6). Additionally, we traced and digitized TH-IR axons in the ventricles of RA and CIH conditions using Vision 4D. Then, we utilized the mapping tool to integrate the traced axons onto a 3D heart scaffold (Figure 4.7). This allowed the visualization of the precise spatial distribution of sympathetic innervation on the heart scaffold.
Figure 4.11. Regional changes of TH-IR axon innervation pattern in the left ventricle following CIH
Figure 4.12: Distribution of TH-IR axon innervation in a flat-mount of whole left ventricle in RA and CI.
Figure 4.13: 3D Anatomical mapping of TH-IR reconstructed axons on the heart scaffold in RA and CIH
Discussion

Previously, we have demonstrated the topographical distribution of catecholaminergic axon innervation in the flat-mount of the whole atria, right ventricle, interventricular septum and partial left ventricle. We optimized our protocols to label the TH-IR axon innervation in the flat-mount of whole ventricles (right and left) and interventricular septum to provide a map of the sympathetic-cardiac connectome. Additionally, we determined the remodeling of the catecholaminergic axon innervation in the ventricles following CIH which indicated an increase in density of innervation and change of patterns. Our tracing data was mapped onto a 3D heart scaffold. This work provides an anatomical foundation for the future study of the heart-brain connectome in normal and disease conditions.

Topographical organization of TH-IR axon innervation in the flat-mount of whole ventricles in normal conditions

Previous work by Pauza group has demonstrated the TH-IR nerve fibers in the ventricles, but our study enhanced our knowledge of the fine details of those TH-IR individual axons and terminals that were not delineated previously (38, 39). Similar to our findings, it was reported that TH-IR axons are distributed unevenly throughout the different regions of the ventricles (39). Previous findings in the pig and rabbit, we observed axons alongside the blood vessels in the myocardium (39). Epicardium has significantly thicker and more numerous nerves going from the base of the ventricles to the apex than the endocardium.
Moreover, physiological studies established the influence of cardiac sympathetic innervation in normal and chronically infarcted myocardium (146). These studies suggested regional control of cardiac function by sympathetic nerves. Here, we provided anatomical evidence of the preferential innervation of cardiac sympathetic innervation which corresponds to their functional role.

Remodeling of catecholaminergic axon innervation in the flat-mounts of ventricles following CIH

CIH shows deleterious impacts on post-myocardial infarction remodeling and contractile dysfunction (25). The previous study used echocardiographic measurements of ejection fractions and demonstrated early occurrence of sustained sympathetic overactivity and hypoxia inducible factors following CIH exposure. As abnormal sympathetic activity leads to serious diseases like congestive heart failure, arrhythmias and sudden cardiac death (26, 147), changes of the sympathetic innervation of the pacemaker of the heart can ameliorate the heart rhythm stability and exacerbate arrhythmias. Summary

In this study, we determined the topographical distribution and morphology of TH-IR axon innervation in the flat-mount of whole ventricles. Moreover, we provided a novel 3D anatomical map of the sympathetic innervation onto 3D heart Scaffold. Finally, we showed that CIH induce remodeling of TH-IR axon innervation in the ventricles which may contribute to sympathetic overactivity in CVD.
Functional implications

Abnormal autonomic functions are attributed to CVD in people with OSA (148). Specifically, elevated sympathetic activity imposes a major risk factor for CVD in OSA and reduction of its activity could potentially reduce risks in those patients (108). Previously, we have demonstrated that CIH induced significant remodeling of vagal afferent innervation in the aortic arch and vagal efferent innervation of the cardiac ganglia which are associated with functional changes (126, 149). Here, we showed the structural remodeling of TH-IR axon innervation in the ventricles as indicated by increased density. Our study provides a new insight of the structural changes of TH-IR axons following CIH as it shows the entire sympathetic axonal network remodeling as well as detailed alterations of the catecholaminergic axons in the ventricles. Increased sympathetic innervation of the ventricles implies an increase in the sympathetic control of the heart following CIH. In particular, the increase of sympathetic drive to the cardiomyocytes will increase the contractility of the ventricular muscles. Our novel 3D anatomical map of the reconstructed sympathetic innervation on the ventricles provides new targets for future functional studies on CIH-induced autonomic imbalance along with its medical interventions. This will eventually help us in applying this knowledge to develop effective interventions sympathetic modulation to relieve OSA-related CVDs and measure their efficacy by utilizing our map as a sympatho-cardiac atlas in normal and CIH conditions.
Summary

In this study, we determined the topographical distribution and morphology of TH-IR axon innervation in the flat-mount of whole ventricles. Utilizing our optimized methodology, we were able to visualize TH-IR axons and terminals through all layers of the flat-mount ventricles and interventricular septum. Different patterns of innervation were exhibited at different regions (base, middle, and apex). Vasculature on the ventricular muscle was also heavily innervated with TH-IR axons. Moreover, we provided a novel 3D anatomical map of the sympathetic innervation onto 3D heart Scaffold. Finally, we showed that CIH induced remodeling of TH-IR axon innervation in the ventricles which may contribute to sympathetic overactivity in CVD. These structural observations provide an anatomical foundation for future functional and pathological studies in the heart.
CHAPTER FIVE: DISCUSSION

Summary

Using state-of-the-art anatomical techniques, we provided a comprehensive topographical map of the catecholaminergic efferent axon morphology, innervation, and distribution in flat-mounts of the whole heart (atria, ventricles and interventricular septum) at single cell/axon/varicosity scale.

In the atria, a few large TH-immunoreactive (IR) axon bundles entered both left and right atria, branched into small bundles, and then single axons that eventually formed very dense terminal networks in the epicardium, myocardium, and inlet regions of great vessels to the left and right atria. Varicose TH-IR axons formed close contact with cardiomyocytes, vessels, and adipocytes. Multiple intrinsic cardiac ganglia (ICG) were identified in the epicardium of both atria, and a subpopulation of the neurons in the ICG were TH-IR. Most TH-IR axons in bundles traveled through ICG before forming dense varicose terminal networks in cardiomyocytes. In the left and right ventricles and interventricular septum, TH-IR axons formed dense terminal networks in the epicardium, myocardium, and vasculature.

In the ventricles, few large TH-IR axon bundles entered both ventricles and interventricular septum from the base and traveled toward the apex. These bundles branched into smaller axon bundles and eventually ramifying into individual axons covering the entire ventricles. Regional
heterogeneity of TH-IR axon innervation was observed as indicated by change of density, TH-IR axon bundler diameter, and different patterns. Blood vessels with well-defined contour within the ventricles were present.

**Following CIH**, catecholaminergic axon innervation remodeling was apparent in both atria and ventricles. Specifically, an increase in the complexity as indicated by more branching points per unit area was apparent in CIH compared to RA. Moreover, hyperinnervation of TH-IR axons was observed at different regions including the SAN region, AVN region, the working cardiomyocytes of atria and ventricles, auricles, and the major veins attached to the atria. Hypertrophy of the atrial tissues and increase of innervation in the z-dimension after CIH exposure was demonstrated utilizing the Neurolucida 360 and Arivis Vision 4D techniques.

**3D Scaffold modeling**: Utilizing tools developed by our collaborators in the Auckland Bioengineering Institute, TH-IR axon innervation was traced, and reconstructed data was integrated onto 3D scaffold to provide more precise spatial distribution of the sympathetic-cardiac map in normal and CIH conditions.

**Implications**

This work contributes to the cardiac-sympathetic brain connectome and provides an anatomical foundation for functional mapping of sympathetic control for the heart as well as evaluation of the remodeling of cardiac sympathetic innervation in chronic disease models. We provided unprecedented resolution of the distribution of TH-IR axons in the whole heart (atria,
ventricles, interventricular septum) at a single cell/axon/varicosity scale. This mapping could be utilized to understand the sympathetic specific control of different regions of the heart and their autonomic responses. Additionally, this work determined the remodeling of structural organization of sympathetic postganglionic innervation of the whole-mount preparations of the heart following CIH. This could potentially facilitate the development of novel and effective therapeutic treatments for OSA by utilizing the map a cardiac sympathetic atlas to navigate more precise control of different cardiac regions.
LIST OF REFERENCES


35. Swissa M, Zhou S, Tan AY, Fishbein MC, Chen P-S, Chen LS. Atrial sympathetic and parasympathetic nerve sprouting and hyperinnervation induced by subthreshold electrical


106. Heart rate variability: standards of measurement, physiological interpretation and clinical use. 
   Task Force of the European Society of Cardiology and the North American Society of Pacing and 

107. Floras JS, Ponikowski P. The sympathetic/parasympathetic imbalance in heart failure with 

108. Dissanayake HU, Bin YS, Sutherland K, Ucak S, de Chazal P, Cistulli PA. The effect of obstructive 
   sleep apnea therapy on cardiovascular autonomic function: a systematic review and meta-
   analysis. Sleep. 2022;45(12).

   nervous system as a biomarker of well-ageing in the prevention of stroke. Front Aging Neurosci. 
   2022;14:969352.

110. Badran M, Gozal D. PAI-1: A Major Player in the Vascular Dysfunction in Obstructive Sleep 

    consequences of intermittent hypoxia: a matter of dose? A systematic review and meta-
    analysis in rodents. Eur Respir Rev. 2022;31(164).

112. Sánchez-de-la-Torre M, Sánchez-de-la-Torre A, Bertran S, Abad J, Duran-Cantolla J, Cabriada V, 
    et al. Effect of obstructive sleep apnoea and its treatment with continuous positive airway 
    pressure on the prevalence of cardiovascular events in patients with acute coronary syndrome 


dystrophic remodeling within the intrinsic cardiac nervous system of the streptozotocin-

130. Lin M, Ai J, Harden SW, Huang C, Li L, Wurster RD, et al. Impairment of baroreflex control of
heart rate and structural changes of cardiac ganglia in conscious streptozotocin (STZ)-induced

during Sleep Causes Browning of Interscapular Adipose Tissue Accompanied by Local Insulin

adipose tissue is associated with coronary artery disease and major adverse cardiovascular

133. Gaborit B, Sengenes C, Ancel P, Jacquier A, Dutour A. Role of Epicardial Adipose Tissue in Health

Epicardial Adipose Tissue in a Canine Model of Chronic Obstructive Sleep Apnea. J Am Heart

apnea-induced atrial fibrillation by inhibiting structural, sympathetic nervous and metabolic


