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Analysis of intrinsic cardiac neuron activity in relation to neurogenic atrial fibrillation and vagal stimulation

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RÉSUMÉ

La fibrillation auriculaire est le trouble du rythme le plus fréquent chez l'homme. Elle conduit souvent à de graves complications telles que l'insuffisance cardiaque et les accidents vasculaires cérébraux. Un mécanisme neurogène de la fibrillation auriculaire mis en évidence. L'induction de tachyarythmie par stimulation du nerf médiastinal a été proposée comme modèle pour étudier la fibrillation auriculaire neurogène. Dans cette thèse, nous avons étudié l'activité des neurones cardiaques intrinsèques et leurs interactions à l'intérieur des plexus ganglionnaires de l'oreillette droite dans un modèle canin de la fibrillation auriculaire neurogène. Ces activités ont été enregistrées par un réseau multicanal de microélectrodes empalé dans le plexus ganglionnaire de l'oreillette droite. L'enregistrement de l'activité neuronale a été effectué continument sur une période de près de 4 heures comprenant différentes interventions vasculaires (occlusion de l'aorte, de la veine cave inférieure, puis de l'artère coronaire descendante antérieure gauche), des stimuli mécaniques (toucher de l'oreillette ou du ventricule) et électriques (stimulation du nerf vague ou des ganglions stellaires) ainsi que des épisodes induits de fibrillation auriculaire. L'identification et la classification neuronale ont été effectuées en utilisant l'analyse en composantes principales et le partitionnement de données (cluster analysis) dans le logiciel Spike2. Une nouvelle méthode basée sur l'analyse en composante principale est proposée pour annuler l'activité auriculaire superposée sur le signal neuronal et ainsi augmenter la précision de l'identification de la réponse neuronale et de la classification. En se basant sur la réponse neuronale, nous avons défini des sous-types de neurones (afférent, efférent et les neurones des circuits locaux). Leur activité liée à différents facteurs de stress nous ont permis de fournir une description plus détaillée du système nerveux cardiaque intrinsèque. La majorité des neurones enregistrés ont réagi à des épisodes de fibrillation auriculaire en devenant plus actifs. Cette hyperactivité des neurones cardiaques intrinsèques suggère que le contrôle de cette activité pourrait aider à prévenir la fibrillation auriculaire neurogène. Puisque la stimulation à basse intensité du nerf vague affaiblit l'activité neuronale cardiaque intrinsèque (en particulier pour les neurones afférents et convergents des circuits locaux), nous avons examiné si cette

intervention pouvait être appliquée comme thérapie pour la fibrillation auriculaire. Nos résultats montrent que la stimulation du nerf vague droit a été en mesure d'atténuer la fibrillation auriculaire dans 12 des 16 cas malgré un effet pro-arythmique défavorable dans 1 des 16 cas. L'action protective a diminué au fil du temps et est devenue inefficace après ~ 40 minutes après 3 minutes de stimulation du nerf vague.

Mots-clés: arythmies auriculaires, le système nerveux cardiaque intrinsèque, les neurones de circuit local, la stimulation du nerf vague, interactivité neuronal stochastique

ABSTRACT

Atrial fibrillation is the most frequent sustained rhythm disorder in humans and often leads to severe complications such as heart failure and stroke. A neurogenic mechanism of atrial fibrillation has been hypothesized. Tachyarrhythmia induction by mediastinal nerve stimulation has been proposed as a model to study neurogenic atrial fibrillation. In this thesis, we studied the activity of intrinsic cardiac neurons and their interactions inside the right atrium ganglionated plexus in a canine model of neurogenic atrial fibrillation. These activities were recorded by a multichannel microelectrode array that was paled into the right atrium ganglionated plexus. The recording was done for up to 4 hours and it covered the neuronal activity during different interventions such as vascular (aorta occlusion, inferior vena cava occlusion, left anterior descending coronary artery occlusion), mechanical (touching atrium and ventricle) and electrical (stimulating of vagus nerve or stellate ganglion) stimuli as well as atrial fibrillation induction. Neuronal identification and classification were done using the principal component analysis and cluster on measurements analysis in Spike2 software. New method based on principal component analysis was proposed to cancel superimposed atrial activity on neuronal signal to increase the accuracy of the neuronal response identification and classification. Based on the neuronal response, we defined subtypes of neurons (afferent, efferent and local circuit neurons) and their related activity to different stressors which provided a more detailed description of the intrinsic cardiac nervous system. The majority of recorded neurons reacted to episodes of atrial fibrillation by becoming more active. This hyperactivity of intrinsic cardiac neurons during atrial fibrillation suggested that controlling that activity might help preventing neurogenic atrial fibrillation. Since low-level vagus nerve stimulation obtunds the intrinsic cardiac neuronal activity (especially for afferent and convergent local circuit neurons), we investigated whether this intervention could be applied as a therapy for atrial fibrillation. Our results showed that right vagus nerve stimulation was able to mitigate atrial fibrillation in 12 of 16 cases and showed an adverse pro-arrhythmic effect in 1 of 16 cases. The protective action however decreased over time and became ineffective after ~40 minutes for 3 minutes vagus nerve stimulation.

Keywords: Atrial arrhythmias, intrinsic cardiac nervous system, Local circuit neurons, vagus nerve stimulation, stochastic neuronal interactivity

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LIST OF ABBREVIATIONS

µm	:	Micrometer
AA	:	Atrial Activity
AF	:	Atrial Fibrillation
Aff.	:	Afferent
Ang	:	Angiotensin
Ao	:	Aortic
AOR	:	Descending Aortic Occlusion
APD	:	Atrial Premature Depolarization
ART	:	Autonomic Regulation Therapy
AT	:	Atrial Tachycardia
ATP	:	Adenosine Triphosphate
AV	:	Atrio-Ventricular
beat/min	:	Beat/Minute
BL	:	Baseline
$C.mol^{-1}$	:	Coulombs per mole
Ca^{2+}	:	Calcium
cAMP	:	Cyclic Adenosine Monophosphate
CAO	:	Coronary Artery Occlusion
Cl^-	:	Chloride
CO	:	Cardiac Output
CO_2	:	Carbon dioxide
CP	:	Conditional Probability
CSN	:	Carotid Sinus
CV	:	Cardiovascular
D/A	:	Digital to Analog
DC	:	Direct Current
DRG	:	Dorsal Root Ganglia
ECG	:	Electrocardiogram
Eff.	:	Efferent
Ext.	:	Exterior
FDA	:	Food and Drug Administration
FF	:	Firing Frequency
G.P	:	Ganglionated Plexus
Hz	:	Hertz
IC	:	Intrinsic Cardiac
ICN	:	Intrinsic Cardiac Neuron
ICNS	:	Intrinsic Cardiac Nervous System
Int.	:	Interior
IR	:	Isovolumetric relaxation
IV	:	Intravenous
IVC	:	Inferior Vena Cava
$J.K^{-1}.mol^{-1}$	:	Joules per Kelvin per mole
K	:	Kilo

K^+	:	Potassium
Kg	:	Kilogram
L.	:	Left
LAD	:	Left Anterior Descending
LCN	:	Local Circuit Neuron
LCV	:	Left Cervical Vagosympathteic complex
LMA	:	Linear Microarray
LSS	:	Left Stellate ganglion Stimulation
LV	:	Left Ventricle
LV dp/dt	:	first derivative (+ positive, - negative) of left ventricular pressure
LVP	:	Left Ventricular Pressure
LVSP	:	Left Ventricular Systolic Pressure
mA	:	Milliamp
mg/kg	:	Milligram per kilogram
mg/kg/hr	:	Milligram per kilogram per hour
mM	:	Milli mole
mm	:	Millimeter
mmHg	:	Millimeter of mercury
MNS	:	Mediastinal Nerve Stimulation
ms	:	Millisecond
mV	:	Milli Volts
MΩ	:	Megaohm
N/A	:	Not Applicable
Na^+	:	Sodium
NTS	:	Nucleus Tractus Solitaries
Occl	:	Occlusion
PA	:	Pulmonary Artery
Parasym	:	Parasympathetic
PCA	:	Principal Component Analysis
R.	:	Right
RAE	:	Right Atrial Electrogram
RAGP	:	Right Atrium Ganglionated Plexus
RCV	:	Right Cervical Vagosympathetic complex
RSS	:	Right Stellate ganglion Stimulation
RTX	:	Resiniferatoxin
RV	:	Right Ventricle
S	:	Second
SA	:	Sinoatrial
SCS	:	Dorsal column spinal cord
SD	:	Standard Deviation
sec	:	Second
SVC	:	Superior Vena Cava
SVR	:	Systemic Vascular Resistance
Sympath	:	Sympathetic
T1	:	Frist thoracic vertebra
T5	:	Fifth thoracic vertebra

TLV	:	Touch Left Ventricle
TRV	:	Touch Right Ventricle
VNS	:	Vagus Nerve Stimulation

I dedicate this thesis to my father Mozafar Salavatian and my sister Sara Salavatian for their lifetime support and to my wife Sara khosravi for her love. You are all the heart of my life and I hope this heart never stops.

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To my family, I hope you will be always beside me and I wish you to be always healthy and joyful.

CHAPTER I: INTRODUCTION

The heart of human is a pump made of muscles which circulates the blood in the body to bring oxygen to different organs and tissues and to collect carbon dioxide waste in systemic loop and bring the deoxygenated blood to the lungs to release carbon dioxide and collect oxygen from the lungs in pulmonary loop. The heart has 4 chambers: the right atrium, the left atrium, the right ventricle and the left ventricle. Each of these chambers has an important task to do.

In the systemic loop the oxygenated blood enters into the left atrium and into the left ventricle after atrial contraction and from there the blood will be sent to all organs and tissues by different vessels (arteries). In the pulmonary loop, the deoxygenated blood enters the right atrium and by contraction of right atrium it goes to the right ventricle and from there, the deoxygenated blood is pushed to the lungs to oxygenate the blood for the systemic loop. This important blood circulatory mechanism is vital for all organs and the functionality of this system is depending on a control system which regulates the heart. If any problem arises in this system, it can cause a delay in the delivery of the blood to vital organs and it may cause a major damage to these organs due to lack of oxygenated blood. Therefore the regular functioning of this blood circulatory system is very crucial to the human body.

There are different heart diseases which are related to the malfunctioning of one of the heart chambers. For example atrial fibrillation is caused by the malfunctioning of the atria. In Canada, in every 7 minutes one person dies from the heart disease or stroke [1], therefore studying the heart disease and trying to find potential cures is very important for public health. In this thesis, we study the atrial fibrillation disease. In particular we study in a dog model how neurally induced atrial fibrillation could be induced and what is the role of cardiac neurons in initiating and maintaining this disease and finally we try to propose a method to suppress or mitigate atrial fibrillation.

I.1. General physiology of the heart

I.1.1. Heart muscle

The function of heart muscles is to pump the blood to all the body throughout the whole life continuously and regularly without any rest. The atrial and ventricular muscles have a longer duration of contraction than skeletal muscles and more importantly there are some excitatory and conductive fibers in heart which spread the rhythmical electrochemical signals to all cells. This signal transmission is crucial for an efficient and regular contraction of a group of cardiac cells together. The timing and the sequence of contraction of each chamber of the heart is very important for the pumping function and blood circulation and is controlled by these electrochemical signals called action potentials.

I.1.2. Cardiac action potential

Potential difference is created across any membrane by having a high potential in one side and low potential in another side of the membrane. The potential difference across a biological cell membrane is created by different ions which carry electric charges. The sodium (Na^+) and potassium (K^+) ions play an important role in creating this potential difference. There are several more ions which contribute less to create the difference potential across the membrane like Ca^{2+} or Cl^-. Note that although Ca^{2+} is not contributing a lot in creating the potential difference, it acts a crucial role in cardiac muscles. Let us assume that we have a membrane which is only permeable to potassium ions and the concentration of potassium ions is higher inside the membrane than outside. Since there is a high concentration gradient towards outside the membrane, the potassium ions tend to diffuse out of the membrane. While the potassium ions diffuse from interior to exterior, they carry positive charges to the exterior of the membrane and it creates the difference in potential. This continues until the potential difference reaches a level which blocks the diffusion of more potassium ions. For the mammalian nerve fibers this level is -95 mV for potassium ion and +61 mV for sodium ion (interior potential- exterior potential). This level is called equilibrium potential or Nernst potential which is reached when the electrical gradient (which tends

to carry charges from higher potential to lower) and the chemical gradient (which tends to carry more ions from high ion concentration side to low ion concentration side) are the same in magnitude and opposite in direction. The Nernst or equilibrium potential for each ion is calculated based on the following formula:

$$V_{eq.} = \frac{RT}{zF} \ln \frac{[X]_o}{[X]_i} \qquad (1)$$

R is the universal gas constant which is equal to 8.314 $J.K^{-1}.mol^{-1}$ (Joules per Kelvin per mole). T is the temperature in Kelvin. Z is the valence of the ion (i.e. +1 for Na^+ and K^+, +2 for Ca^{2+} and -1 for Cl^-). F is the Faraday constant and is equal to 96,485 $C.mol^{-1}$ (Coulombs per mole). $[X]_{out}$ is the extracellular ion concentration and$[X]_{in}$ is the intracellular ion concentration. $[X]_{out}$ and$[X]_{in}$ have the same unit (mole or millimole).

Table 1 shows the Intracellular and extracellular concentrations and Nernst equilibrium potential value for three cardiac muscle related important ions Na^+, K^+ and Ca^{2+} [2].

Table1. Ion concentration of mammalian myocytes

Ion	intracellular concentration(mM)	extracellular concentration(mM)	equilibrium voltage(mV)
Sodium(Na^+)	5 — 34	140	89 — 38
Potassium(K^+)	104 — 180	5.4	-79 — -94
Chloride(Cl^-)	4.2	117	-89
Calcium(Ca^{2+})	0.1	3	45

When there are several ions involved and the membrane is permeable to different ions, the membrane potential could be calculated by the different equation called Goldman-Hodgkin-Katz equation. The following formula is a Goldman-Hodgkin-Katz equation when the membrane is permeable to Potassium, Sodium and calcium ions:

$$V_m = \frac{RT}{F} \ln\left(\frac{p_K[K^+]_o + p_{Na}[Na^+]_o + p_{Cl}[Cl^-]_i}{p_K[K^+]_i + p_{Na}[Na^+]_i + p_{Cl}[Cl^-]_o} \right) \quad (2)$$

The R, T, F, $[X]_{in}$ and $[X]_{out}$ are the same as equation 1 and P_X is the relative membrane permeability for ion X.

The resting potential for atrial myocyte is approximately -75 mV[3].

Heart control signals are transmitted by action potentials which are rapid changes in the membrane potential that spread rapidly along the cardiac cells membrane. The action potential starts from the resting potential and rises to a high positive membrane potential and then ends with the same negative resting potential. There are different ions and ion channels that are involved in this membrane potential change. There are different phases of cardiac action potential which are associated with the contribution of different ions. These phases are different for the pacemaker cardiac cells and non-pacemaker cardiac cells.

I.1.2.1. Non-pacemaker cardiac cells action potential

The atrial and ventricular cells are non-pacemaker cells in the heart. Purkinje cells are located inside the ventricle wall and they are able to conduct the action potentials rapidly and also cause the synchronized contraction of the ventricle. These non-pacemaker cells are not creating the initial impulse and they are mostly propagating the action potential which is created by a pacemaker cardiac cell. The action potential for these type of cells are shown in figure 1 [4].

Different phases for the non-pacemaker cells action potentials are:

Phase 0: When the voltage across the membrane of non-pacemaker cells reaches to a threshold voltage (about -70 mV) which could be triggered by an action potential in the adjacent cell, there would be a rapid depolarization which is caused by the rapid influx of Na^+. This transition is done through the fast Na^+ channel.

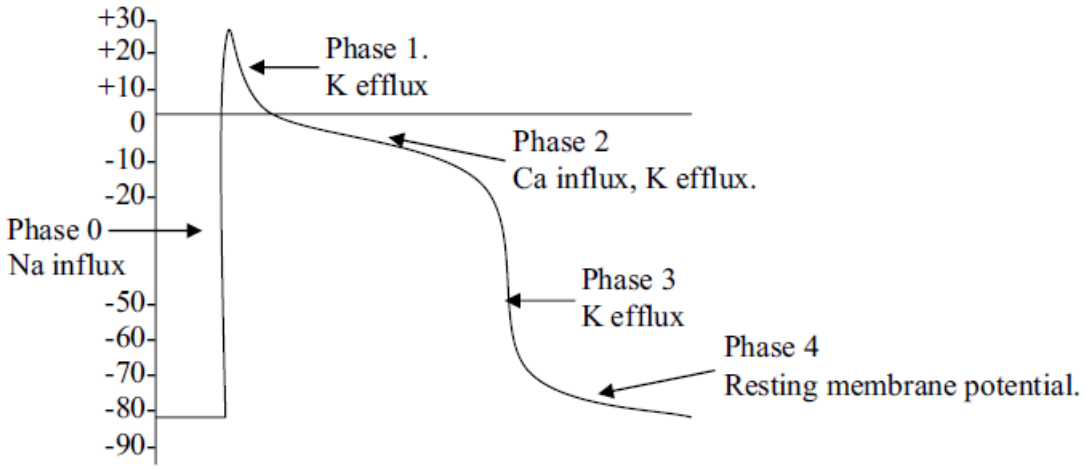

Figure 1 Non-Pacemaker cardiac cells action potential(reproduced from [4])

At the same time the potassium channels close and the efflux of the K^+ decreases. These two mechanisms causes the membrane potential to raise from the resting potential which is close to K^+ equilibrium potential to the potential which is close to Na^+ equilibrium potential.

Phase 1: This phase is the initial repolarization which is caused by the efflux of the K^+.

Phase 2: The plateau in phase 2 is caused by influx of Ca^{2+} which is due to specific long lasting calcium currents. This phase shows one of the big differences between cardiac action potential and nerve or skeletal muscle action potentials which is caused by the contribution of Ca^{2+} in changing the membrane potential.

Phase 3: In this phase the final repolarization occurs because of the efflux of the K^+.

Phase 4: In this phase the membrane is in the resting state. This phase is associated with the leave of K^+ ions from inside the cell which happens through the specific potassium channels. At this phase the sodium and calcium channels are closed.

I.1.2.2. Nodal cell action potential

The nodal action potential represents the impulse created by the pacemaker nodes like sinoatrial or atrioventricular nodes. This type of action potential is shown in the figure 2 [4].

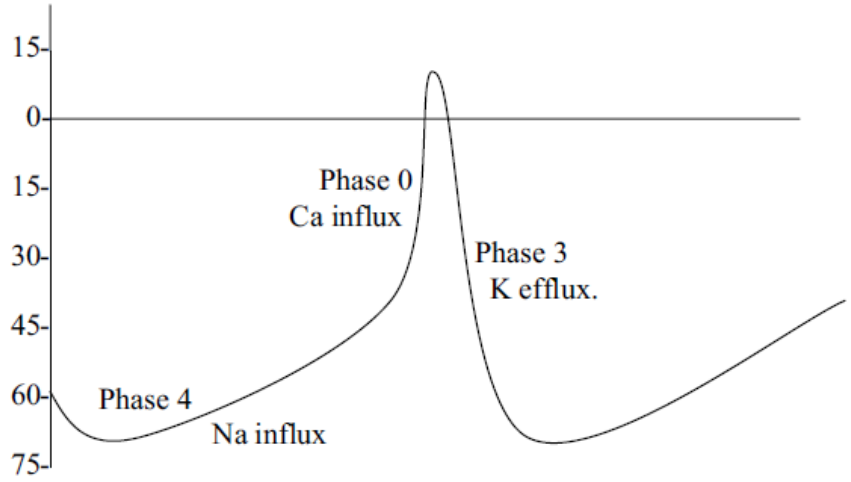

Figure 2 Nodal action potential (reproduced from [4])

The depolarization phase in the nodal cells is slower and is carried by the Ca^{2+} instead of Na^{+}. Actually in the nodal action potential the Na^{+} ions have a very small contribution to the action potential. As the depolarization with Ca^{2+} is slower than the depolarization with the Na+ due to the difference in their ion channels, the nodal action potential is called slow response action potential and the non-pacemaker action potentials are called fast response action potentials. Another important characteristic of the nodal cells membrane potential is that they have an unstable resting potential.

There are three different phases associated with the nodal action potential.

Phase 0: Depolarization is caused by the influx of the Ca^{2+} through specific calcium channels. Note that the depolarization in nodal cells is slower than the depolarization in non-pacemaker cells because of the different characteristics of the sodium and calcium channels.

Phase 3: In this phase the efflux of K^+ is repolarizing the cell and the calcium channels is inactivated.

Phase 4: Na^+ influx caused by the repolarisation of the preceding action potential raise the membrane potential.

I.1.2.3. Effective Refractory Period

Once an action potential is initiated in one cell, there is a period of time in which the action potential cannot be initiated from the same cell. This period of time is called effective refractory period or absolute refractory period. While a cell is in the refractory period it would not be excited by the action potential from the adjacent cell and therefore the action potential does not propagate through this cell. As the action potential propagation is one of the crucial points in the heart beating control system, the refractory period plays an important role in stopping the propagation of the action potential when it should not propagate to other cells.

I.1.2.4. Propagation of an action potential in cardiac cells

Once the action potential is created by a nodal cell, it propagates to other cardiac cells through gap junctions. The gap junctions are the junctions connecting adjacent cells in the heart which allows the spontaneous depolarization of the neighboring cells and propagation of the action potential.

I.1.3. Sinoatrial and atrioventricular nodes

The sinus node (also called sinoatrial or SA node) is a special part of cardiac muscle in the superior posterolateral wall of the right atrium. The muscle of the SA node is different from the other non-pacemaker cells in the heart. The SA node creates the first impulse which propagates through the atria and ventricles and is essential for heart contraction. This node is connected to the atrial muscles;

therefore the action potential created by the SA node can propagate to the right atrium and from the right atrium to the rest of the heart. The frequency of the impulses created by the SA node is very important, because it is directly related to the rate of heart contraction and variations in blood pressure. As we will discuss in future chapters, if there would be any problem with the SA node that it cannot create the impulse regularly the heart may not function properly and this could lead to different heart diseases. The contraction system in the heart starts from the atrium and then the blood is ejected to the ventricles. When the impulse from the SA node is generated and propagates throughout the atrial muscles, it causes the atrial muscle to contract and eject the blood to the ventricle. If the action potential propagated to the ventricles at the same time then atrial and ventricular contraction would be approximately simultaneous, which would causes incomplete blood ejection from the atria to the ventricle and some blood would remain in the atrium. To solve this problem, the heart has another important node called atrioventricular or AV node. The AV node is located in the posterior wall of the right atrium, immediately behind the tricuspid valve. The important role of the AV node is to delay (by approximately 0.13 s) [5] the propagation of the action potential coming from the atrium to the ventricles .

I.1.4. Blood circulation

A complete circulatory pathway of the heart is shown in figure 3.Oxygen poor-CO_2 rich blood goes to the right atrium through the venae cava vessels. The inferior vena cava is collecting the used blood from the lower part of the human body and superior vena cava is collecting the used blood from the upper part the body. When the SA node creates the action potential it propagates to the muscles in right atrium and causes these muscles to contract and eject the used blood to the right ventricle. When the action potential reaches the AV node the cardiac impulse is transmitted by the AV node after some delay and reaches the right ventricle muscle which contracts the right ventricle and the blood is pushed to the pulmonary arteries. Lung receives the used blood from the pulmonary arteries and the lung oxygenated the blood and removes the carbon dioxide from blood. The oxygenated blood goes to the left atrium. Left atrium contracts because of the action potential coming from the right atrium and it ejects the blood to the

left ventricle. The action potential coming from the AV node causes the left ventricle muscles to contract and the oxygenated blood will be pushed to the different organs of the body through the aorta. Like vena cava, aorta also has different branches to serve the body with the oxygenated blood. The ascending aorta is serving the upper part of the body and the descending aorta is providing blood to the lower part of the body.

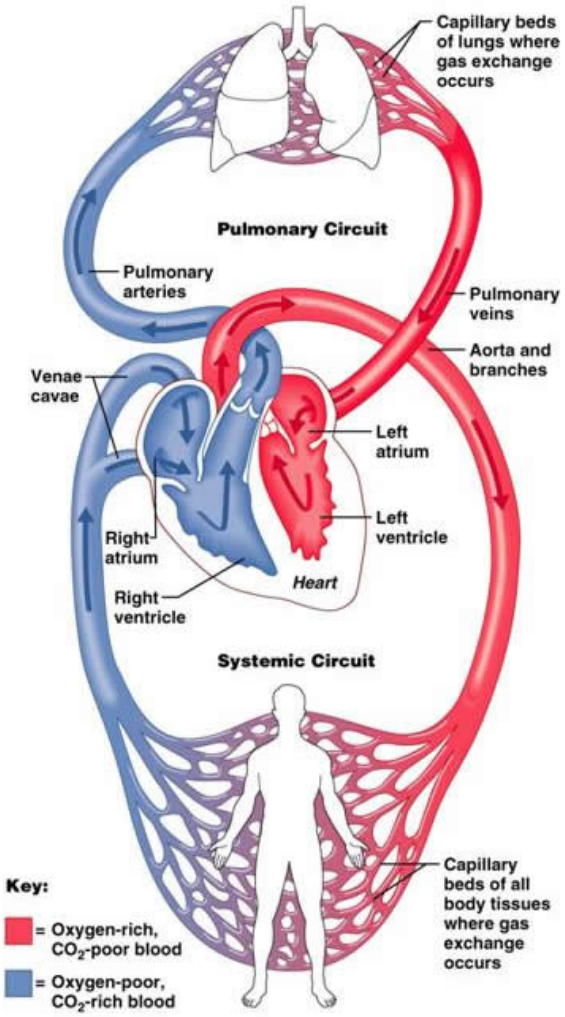

Figure 3 Circulatory pathway of the cardiac system[2]

I.2. Neurocardiology

The field of neurocardiology studies the interactions between the brain and the heart and it is composed of two sciences, neuroscience and cardiology. It is an emerging field which studies how the heart and the brain collaborate. It is known that the interaction among cardiac neurons is essential for the cardiac functionality and regulation. Recently the study of neurocardiological therapeutic methods has received greater attention because of the growing evidences that show the relation between heart diseases and autonomic nervous system.

I.2.1. Autonomic nervous system

The internal organs functions of the body are controlled involuntarily by the autonomic nervous system which is part of the peripheral nervous system. This nervous system controls the heart rate, blood pressure, respiratory rate and body temperature, all of which are vital signs of the body. The autonomic nervous system has two main divisions called sympathetic nervous system and parasympathetic nervous system. The following figure shows the branches of the sympathetic and parasympathetic nerves.

The sympathetic division of the autonomic nervous system is active during the “fight or flight” states which are in general stressful or emergency situations. When a person is in this kind of situations, the sympathetic nervous system may increase the heart rate and the blood pressure.

In contrast to sympathetic nervous system, parasympathetic nervous system is general active during the ordinary tasks of the human body. It can decrease the heart rate and blood pressure and slow down the respiratory rate. In most organs that have both sympathetic and parasympathetic nerves, both sympathetic and parasympathetic nervous systems are active, therefore the final effect of the autonomic nervous system on that organ is the accumulation of the effect of these two nervous systems. Note that typically when one is more active, the other nervous system is less active.

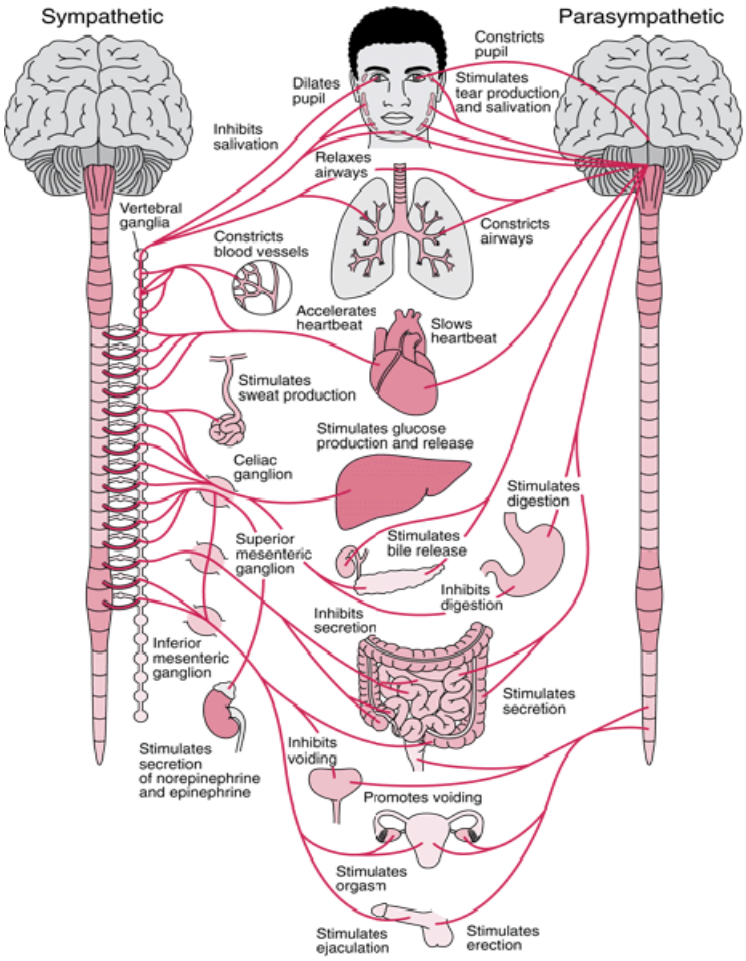

Figure 4 Sympathetic and parasympathetic nervous system

Two main neurotransmitters are used in the autonomic nervous system: acetylcholine and norepinephrine. Epinephrine neurotransmitter is also used by some nerves. Nerve fibers that use acetylcholine are called cholinergic and the nerve fibers that use norepinephrine or epinephrine are called adrenergic fibers. Norepinephrine and epinephrine are mostly used in the sympathetic nervous system and acetylcholine is mostly used in the parasympathetic nervous system.

The function of the nerves in the autonomic nervous system could be explained by their type. They could be afferent, efferent or local circuit neurons. The afferent neurons (sensory neurons) are the neurons which transmit the information carried by nerve impulses from different organs to the central nervous system which includes the brain and the spinal cord. On the other hand, efferent neurons (motor

neurons) transmit the information from central nervous system to different organs. Neurons that are only participating in a local processing of the information are called local circuit neurons or interneurons.

I.2.2. Autonomic innervation of the heart: Intrinsic and extrinsic cardiac nervous systems.

The function of the heart is controlled by two nervous systems: the intrinsic and the extrinsic cardiac nervous system. In the extrinsic nervous system, efferent parasympathetic nerves originate from the medulla in brainstem. These nerves are connected to the postganglionic neurons in different ganglionated plexi on the heart [6]. Sympathetic branch of the extrinsic cardiac nervous system originates from the interomediolateral nucleus of spinal cord and it is connected to the sympathetic postganglionic neurons of the intrathoracic and intrinsic cardiac ganglionated plexi [7]. The afferent neurons in the extrinsic cardiac nervous system transmit the local information from different cardiac regions and the major intrathoracic and cervical vessels to the central nervous system [8].

Recent studies show that three different types of neurons play important roles in the intrinsic cardiac nervous system: efferent, afferent and local circuit neurons [9-11]. Different ganglionated plexi distributed at different locations within the heart are part of the intrinsic cardiac nervous system. This includes epicardium, myocardium and endocardium [12]. The number, size and shape of the ganglionated plexi are different in different species [13]. The intrinsic cardiac ganglionated plexi are distributed over different atria and ventricle regions. Figures 5 and 6 show the locations of the ganglionated plexi on the surface of the atria and ventricles. Different aspects of the cardiac function are regulated by the neurons in the intrinsic cardiac nervous system. This includes regulation of the heart rate and blood flow [14] or modulation of intrathoracic and central cardiovascular-cardiac reflexes and coordination of parasympathetic and sympathetic efferent postganglionic neuronal input to the heart [15].

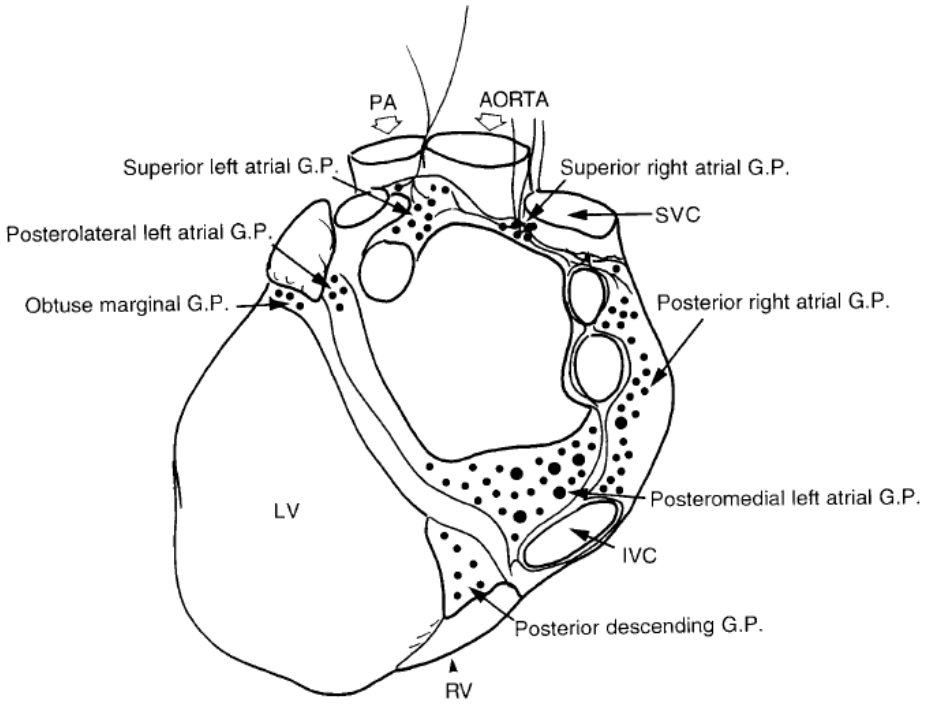

Figure 5 Drawing of a posterior view of the human heart and major vessels illustrating the locations of the posterior atrial and ventricular ganglionated plexi. Note the mediastinal nerves coursing adjacent to the aortic root and joining two superior atrial ganglionated plexi. Positions of the superior vena cava (SVC), inferior vena cava (IVC),right ventricle(RV), and left ventricle(LV) are shown(reproduced from [16]).

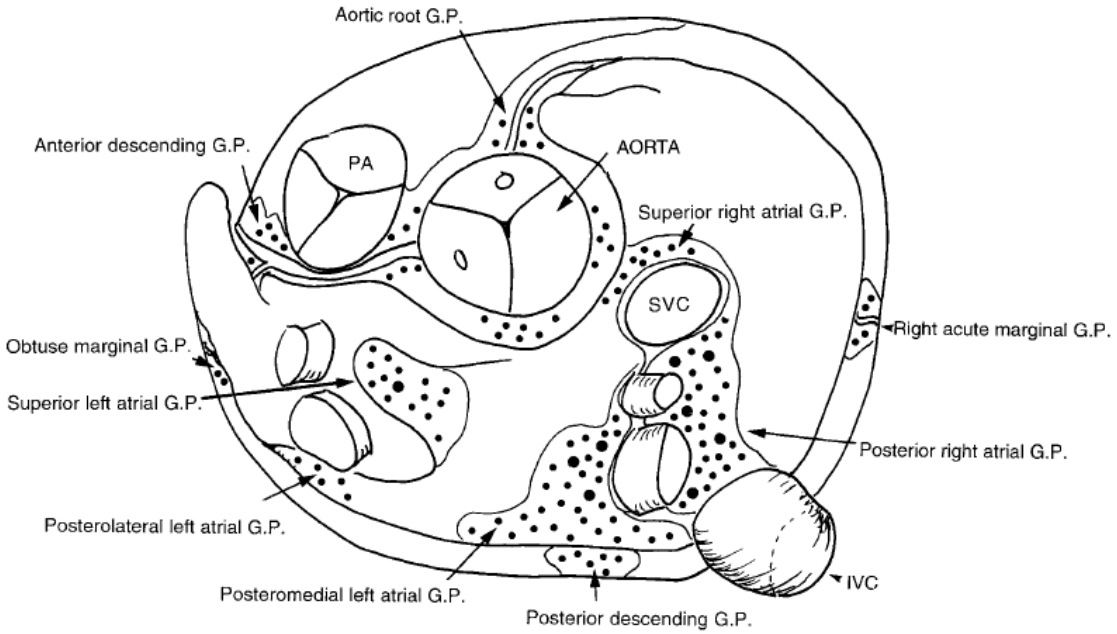

Figure 6 Drawing of a superior view of the human heart illustrating the distribution of ganglionated plexi on the surface of the atria and ventricles. For abbreviations, see figure5(reproduced from [16]).

Different studies suggested that intrinsic cardiac ganglionated plexus can affect the adjacent myocardial regions [17-19]. It was also proposed that the electrical and mechanical properties of the adjacent tissues and cardiac chambers can be influenced by the intrinsic cardiac ganglionated plexi [6]. For example, in a canine model study by Yuan et al [20] it was reported that cholinergic neurons of the right atrium ganglionated plexi decrease the discharge rage of sinoatrial node, depress the atrioventricular node conduction, and change ventricular contractility. Moreover the repolarization of ventricular muscle can also be influenced by intrinsic cardiac neurons [21].

In general both intrinsic and extrinsic cardiac nervous systems are influenced by central nervous system, baroreceptor reflex, chemoreceptor reflex and local neuronal activity by the intrinsic cardiac neurons [6].

I.2.3. Anatomy and function of intrinsic cardiac nervous system

Neural control of the heart includes extrinsic and intrinsic cardiac nervous systems. The intrinsic cardiac ganglia are the final pathway for autonomic modulation of regional cardiac function. This intrinsic cardiac nervous system is not just a relay for the extrinsic cardiac nervous system. It is able to mediate the regional cardiac reflexes and modulate the projection of extrinsic cardiac nervous system to the heart by acting as a local integrative neural network [22]. The intrinsic cardiac nervous system contains parasympathetic and sympathetic efferent neurons as well as afferent neurons. It also contains local circuit neurons which receive inputs from both efferent and afferent neurons. Inputs from cardiopulmonary afferent and extrapericardial autonomic ganglia can modulate the neurons in intrinsic cardiac nervous system[23]. These inputs are basically modulated by brainstem and spinal cord neurons associated with cardiovascular regulation [24-26]. Sinoatrial and atrioventricular nodes as well as regional contractile function are affected by vagi (cardiac efferent parasympathetic preganglionic neurons), stellate and middle cervical ganglia (cardiac efferent sympathetic neurons) [27, 28]. The intrinsic neural control of the heart is done by specific intracardiac convergence points where bilateral autonomic inputs merge together [29-32].

The anatomy of the human intrinsic cardiac nervous system was studied by Armour [16] in six human hearts. In this study, ganglionated plexi were identified in 10 different atrial and ventricular locations. In principle, intrinsic cardiac neurons in each ganglion modify the regional tissue, for example intrinsic cardiac neurons located on the atria primarily and not exclusively modify the atrial tissue (the same holds for the ventricles) [20].The locations of these ganglionated plexi are shown in Figs. 5-6. Different ganglionated plexi with various sizes and different number of neurons (from a few neurons to more than 200 neurons) were identified in each of these locations. The numbers of ganglia in different cardiac regions are shown in Table 2 based on the result from six human hearts.

Table 2. Numbers of ganglia in different cardiac regions grouped according to their estimated neuronal complement (n =6 human hearts) [16].

Ganglionic plexus	5–10 Neurons	11–50 Neurons	50–100 Neurons	100–200 Neurons	>200 Neurons	Total no. ganglia per heart
Atrial ganglionated plexuses						
Superior right atrial	19.2 ± 2.9	9.5 ± 2.8	2.2 ± 0.4	0.3 ± 0.1	0	31 ± 5
Superior left atrial	29.4 ± 5.9	19.7 ± 5.1	5.3 ± 1.9	2.2 ± 0.7	0.5 ± 0.2	56 ± 12
Posterior right atrial	90.1 ± 13.7	66.4 ± 7.6	22.8 ± 1.9	9.7 ± 0.7	4.7 ± 0.7	194 ± 22
Posteromedial left atrial	82.8 ± 13.5	56.4 ± 9.8	18.2 ± 4.1	4.5 ± 0.9	1.8 ± 0.6	161 ± 27
Posterolateral left atrial	8.2 ± 2.2	5.7 ± 1.1	1.7 ± 0.4	0.3 ± 0.1	0	16 ± 2
Total per heart						458 ± 43
Ventricular ganglionated plexuses						
Aortic root						
Right	12.2 ± 1.5	3.5 ± 0.7	0.3 ± 0.1	0	0	16 ± 2
Anterior	4.2 ± 1.2	1.2 ± 0.6	0	0	0	5.2 ± 1.8
Left	15.1 ± 2.3	5.5 ± 1.6	1.2 ± 0.5	0.4 ± 0.1	0	21.7 ± 4.0
Posterior	12.0 ± 1.0	5.5 ± 0.8	0.3 ± 0.1	0.2 ± 0.1	0	17.8 ± 2.0
Anterior descending	7.5 ± 1.2	3.7 ± 0.5	0.2 ± 0.1	0	0	11.2 ± 1.1
Posterior descending	4.1 ± 2.2	1.6 ± 0.6	0	0	0	5.2 ± 1.9
Right acute marginal	4.5 ± 0.8	1.5 ± 0.6	0	0	0	6.2 ± 2.8
Obtuse marginal	4.3 ± 1.6	1.0 ± 0.4	0	0	0	5.2 ± 2.0
Total per heart						88 ± 7

Using this table, it was reported that there are approximately over 14,000 neurons in each human heart [16]. The anatomical configuration and sizes of these ganglionated plexi were not exactly the same for different human hearts [16]. Since there were no identified ganglia in extensive regions of atrial or

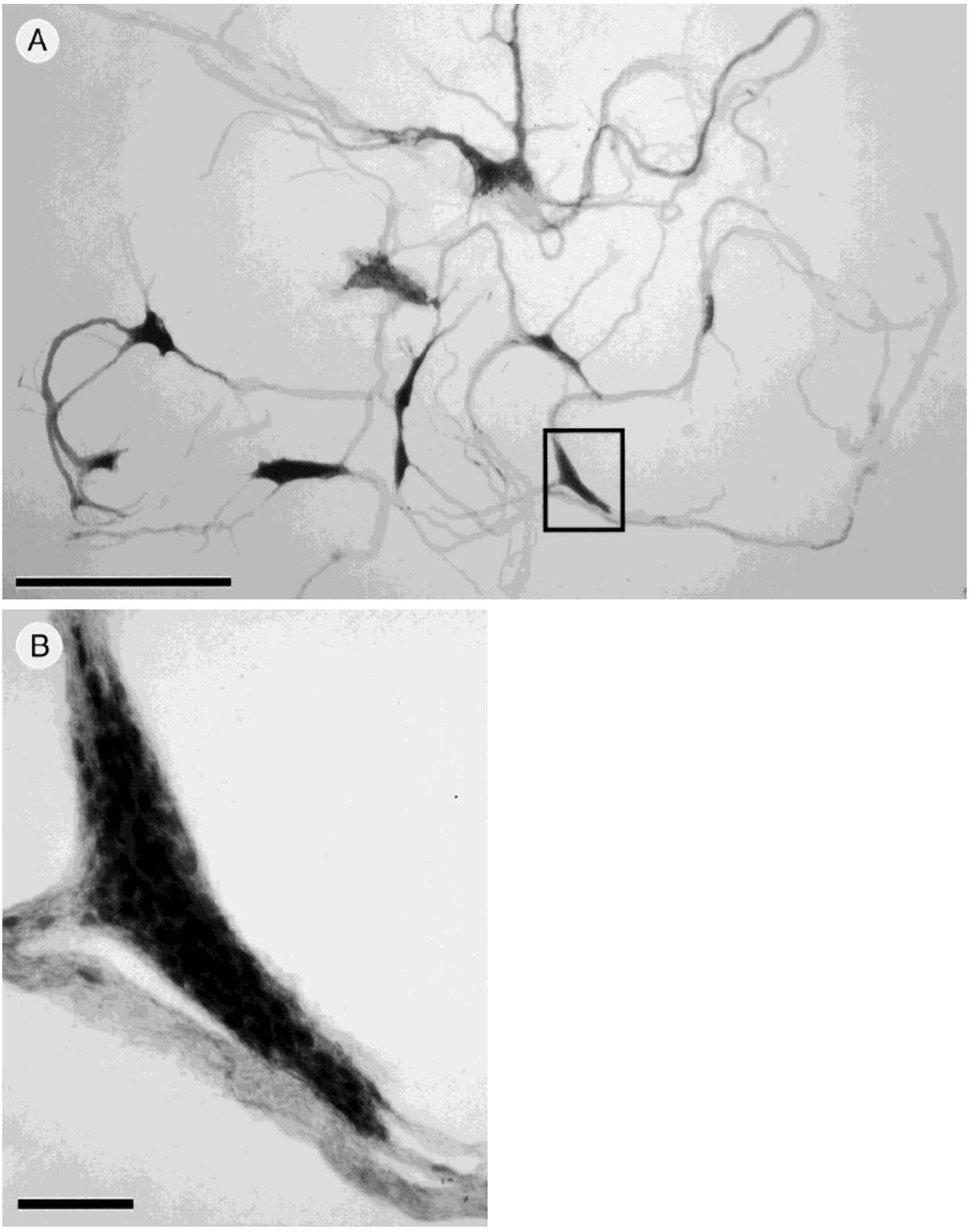

Figure 7 Light photomicrographs of human intrinsic cardiac nerves, ganglia, and neurons. A: Network of ganglia and nerves stained with methylene blue and dissected from the posteromedial left atrial ganglionated plexus. The ganglia appear as expansions along the length of a nerve, often at branch points (box). B: Enlargement of boxed area in A illustrating a ganglion composed of approximately 150–200 nerve cell bodies. Note the presence of individual neurons in adjacent nerves(reproduced from[16]).

ventricular fat, these intrinsic cardiac ganglionated plexi are loose regional neural networks which include the nerves that may form complete loop with diameter of less than 2mm to 1 cm while connecting two different ganglia within a ganglionated plexus (Fig.7A) [16].Various neuronal somata with long (39 ±10 μm) or short (34 ±7 μm) axes exist in intrinsic cardiac ganglionated plexi and some ganglia are estimated to have more than 200 neurons (Fig. 7) [16].

There are different functions associated with each ganglia cluster. For example the neurons in the right atrial ganglionated plexus control the sinoatrial node [28, 30, 33-35], inferior venacava-inferior atrial ganglia neurons have an effect on the inferior atrial and atrioventricular conductile tissues [28, 30, 33, 34, 36]. The coordination of neural activity within intrathoracic autonomic ganglia and the central nervous system regionally control the cardiac function [37]. Different studies that used electrophysiological and neuropharmacological techniques showed that intrathoracic ganglia are not only simple relay stations for autonomic efferent neuronal control of the heart [37, 38].

I.2.4. Right atrium ganglionated plexus

The location of the right atrium ganglionated plexus (RAGP) is shown in Fig. 5 and Fig. 6. Generally the intrinsic neurons in RAGP modify atrial tissues and have been associated with neural control of the sinoatrial node [22]. There have been different studies on the activity of neurons in this ganglionated plexus. In these studies RAGP ablation was proposed as an antiarrhythmic/fibrillation therapy. For example, Moss et al. [39] reported that RAGP ablation may contribute to prevent the development of a tachycardia-dependent atrial fibrillation substrate or in another recent study [40] RAGP ablation was used as an anti-arrhythmic strategy. The neuronal activity of RAGP was also assessed during heart failure and it was reported that neuronal activity in RAGP was decreased by heart failure [41].

I.2.5. Intrathoracic autonomic neurons

Intrathoracic autonomic ganglia contain different population of neurons including afferent, efferent and local circuit neurons [37].

I.2.5.1. Afferent neurons: Cardiac afferent neurons

Cardiovascular information about the blood pressure, blood volume, blood gases and mechanical and chemical milieu of the heart is sent to the autonomic nervous system by cardiac afferent neurons [15].

The cardiopulmonary sensory inputs are provided by nodose and dorsal root ganglia to brain stem and spinal cord neurons [42]. There are more sensory inputs coming from baroreceptor and chemoreceptors located along the aortic arch, carotid arteries and carotid bodies which control the cardiac autonomic efferent neurons. The cardiac autonomic efferent neurons are also controlled by the afferent neural elements within the central nervous system [26].

I.2.5.1.1. Cardiac sensory neuronal transduction

Cardiac sensory neurites (nerve ending) are associated with somata located in nodose, dorsal root and intrathoracic ganglia [28, 43-45]. The somata for the cardiac afferent neurons which are located close to the target organ generate high frequency activity (phasic) and has a direct effect on the target organ efferent neurons [37] therefore there is a fast transmission of the information when these cardiac afferent neurons are active. The cardiac afferent neurons' somata which are not close to their sensory neurite like the cardiac afferent neurons in nodose or dorsal root ganglia affect the efferent neurons with a longer latency [46] which suggest that providing the cardiac information is depending on their sensory neurites and also their somata. These afferent neurons are functionally classified as fast responding and slow responding neurons[15].

I.2.5.1.2. Nodose ganglia afferent neurons

Nodose ganglion, also called inferior ganglion of vagus nerve, is a large sensory ganglion of vagus located in the height of the transverse process of atlas (Fig. 8).

Some cardiac afferent neurons have a sensory neurite on the heart and somata on the nodose ganglion somata. These sensory neurites respond mostly to chemical stimuli and rarely to the mechanical

stimuli [47-49]. As these cardiac afferent neurons receive inputs from cardiac receptors they contribute to the overall cardiovascular regulation but it is not easy to perceive their activity [50, 51].

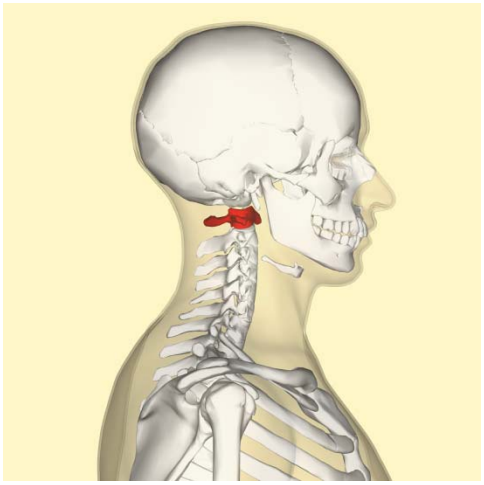

Figure 8 Location of Nodose ganglion in Atlas or the first cervical vertebra. Copyright:"BodyParts3D, © The Database Center for Life Science licensed under CC Attribution-Share Alike 2.1 Japan."

I.2.5.1.3. Dorsal root ganglia afferent neurons

Dorsal root ganglia (also known as posterior root ganglion) are located along the vertebral column by the spine in C6-T6 (Fig. 9) [42]. The sensory neurites of this ganglion respond to mechanical and chemical stimuli [52]. The cardiovascular regulation is sub-served by the sensory input of these cardiac afferent neurons [50, 51, 53].

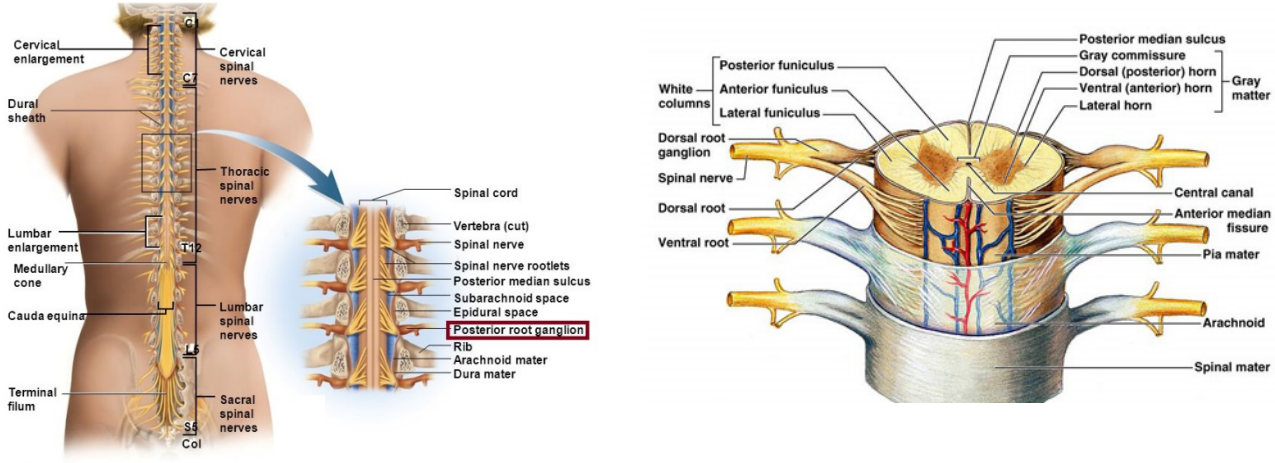

Figure 9 Dorsal root ganglion (posterior root ganglion)
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I.2.5.1.4. Intrathoracic afferent neurons

There exist afferent somata in intrathoracic autonomic ganglia [16, 54, 55]. The neurites of the intrathoracic afferent neurons are located in atrial, ventricular, major vascular and pulmonary tissues [37]. These neurons respond to both mechanical and chemical stimuli [56] and they affect intrathoracic efferent postganglionic outflows to the heart even after long-term decentralization of the intrathoracic ganglia [9, 57, 58]. The neural circuits receiving inputs from intrathoracic afferent neurons dynamically control the regional cardiac function in each cardiac cycle [37].

I.2.5.1.5. Arterial baroreceptors

Arteries are the blood vessels carrying the blood from the heart to different organs. It is important for the arterial blood pressure to be in a normal range (mean of 85-100 mmHg in adults). This normal pressure should be maintained for assurance of adequate of blood flows to different organs of the body. When arterial blood pressure is changing dramatically or it is not in the normal range the pressure sensors called baroreceptors react rapidly to this change via negative feedback systems. Carotid sinus and aortic sinus arterial baroreceptors are the most important arterial baroreceptors. Carotid sinus located at the bifurcation of external and internal carotids aortic arch is located on the arch of aorta on the top in ascending aorta (Fig.10).

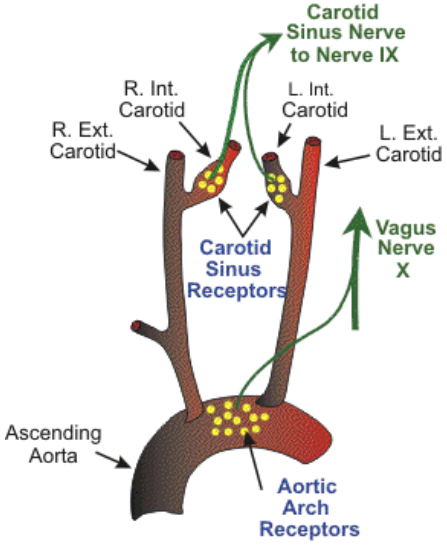

Figure 10 Location of arterial baroreceptors

These two baroreceptors are the most important because they control the blood flow of the arteries which serve blood to important organs (i.e. brain). Baroreceptors are sensitive to both rise and fall of the blood pressure. When the blood pressure rises in a vessel, the baroreceptors expand the wall of vessels and increase the firing frequency of receptor action potentials. When a sudden fall in blood pressure happens, baroreceptors decrease the stretch of the arterial walls and decrease the firing rate receptor firing frequency. For example an aorta occlusion leads to the dramatic change of pressure and activates the baroreceptors.

A branch of IX cranial nerve is connected to the carotid sinus baroreceptors and the X cranial nerve (vagus nerve) is connected to the aortic nerve and aortic arch baroreceptors. IX and X cranial nerves synapses are located in the nucleus tractus solitaries (NTS) in the medulla of the brainstem. The activities of sympathetic and parasympathetic neurons are modulated in nucleus tractus solitaries and it regulates the autonomic control of the heart and blood vessels. The carotid sinus and aortic arch baroreceptors respond (firing) is displayed in Fig. 11.

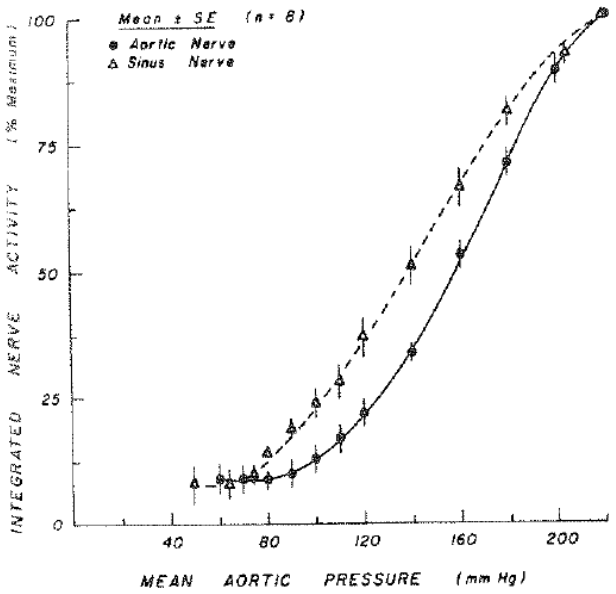

Figure 11 Relation of mean arterial pressure to integrated baroreceptor nerve activity obtained from the aortic (closed circles) and carotid sinus (open triangles) nerves. No change in afferent impulse activity occurred in the aortic nerve traffic is displaced to the right of that for the carotid sinus. For a given level of baroreceptor nerve activity a greater arterial pressure is required in the aortic receptor system.(Reproduced from "baroreceptor regulation of the heart" by S.Evans Downing)

The aortic arch receptor is less sensitive than the carotid sinus receptor at low pressure. Since maximum carotid sinus sensitivity (slope of the curve) occurs near the mean normal arterial blood pressure, if any changes (even small) in blood pressure occur and tends to put the blood pressure outside normal range, the carotid sinus will rapidly react to that and try to bring back the blood pressure to the normal range. The maximum carotid sinus sensitivity may change during exercise or hypertension. Also at a given mean arterial pressure, decreasing the pulse pressure (which is a difference between systolic and diastolic pressure) decreases the nerve activity of baroreceptor. This could become more important in the case where both pulse pressure and mean pressure decrease because baroreceptor responds to both low mean pressure and low pulse pressure and will decrease more the baroreceptor nerve activity.

When the arterial blood pressure becomes low, it can cause an irreversible damage to the organs therefore the baroreceptors are very sensitive to low pressure. Low blood pressure can happen because of blood loss or simply because of standing up suddenly. Decrease in the arterial blood pressure results in decreased firing rate of baroreceptor, increase in sympathetic response and decrease in parasympathetic response. In normal condition baroreceptors have a tonic inhibitory influence on sympathetic response and excitatory influence on parasympathetic response. So when the blood pressure drops, the firing of baroreceptor drops a lot and it results in disinhibition of the sympathetic activity. This causes vasoconstriction (increase in systemic vascular resistance (SVR)), tachycardia (increased heart rate) and positive inotropy (increase the force of heart contraction). These effects are intended to increase blood pressure towards the normal pressure.

The important point about the changes of baroreceptors activity is that this activity is temporary and baroreceptors adapt to constant changes in arterial pressure. Therefore if the low blood pressure continues, the baroreceptor activity return back to near normal activity. Hormonal or renal regulation of arterial pressure will have a long term effect on arterial blood pressure.

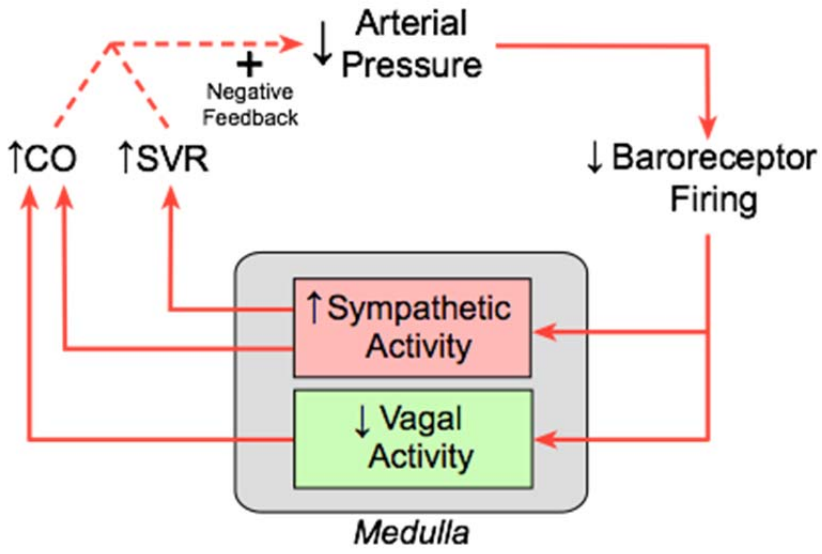

Figure 112 A sudden decreases in arterial pressure decreases baroreceptor firing, which activates sympathetic neurons and inactivates vagal neurons in the medulla. The resulting increases in cardiac output(CO) and SVR act as a negative feedback mechanism to attenuate the fall in arterial pressure.

I.2.5.2. Efferent neurons

I.2.5.2.1. Sympathetic efferent neurons

The sympathetic efferent preganglionic neurons which regulate heart rate have somata within the intermediolateral cell column of the spinal cord and their axon is projected from rami T1-T5 to synapses of postganglionic efferent neurons within intrathoracic intrinsic and extrinsic cardiac ganglia [37, 59]. When these sympathetic efferent neurons activate, they increase heart rate, heart contraction force and change impulse conduction's pattern and speed through the heart [27, 60, 61]. Sympathetic efferent postganglionic neurons' somata (for those that innervate the heart) are located in stellate ganglia [62], thoracic middle cervical, mediastinal and intrinsic cardiac ganglia [23, 47, 57, 59, 63, 64].

I.2.5.2.2. Parasympathetic efferent neurons

Cardiac parasympathetic efferent preganglionic neurons' somata are located in the brainstem and primarily within the nucleus ambiguous [37]. Some of these cardiac parasympathetic efferent

preganglionic neurons are located in the dorsal motor nucleus and regions in between [25, 65, 66]. The axons of these parasympathetic efferent preganglionic neurons are connected to the synapses of efferent postganglionic neurons within intrinsic cardiac ganglia via X cranial nerve (vagus nerve) [67, 68]. Unlike sympathetic efferent neurons, activation of parasympathetic efferent neurons decreases heart rate and heart contraction force and slows down the speed of the impulse conduction through the heart [22, 69, 70].

I.2.5.3. Local circuit neurons

There is a group of neurons within extracardiac and intrinsic cardiac intrathoracic autonomic ganglia that interconnect neurons and receive inputs from both afferent and efferent neurons; these neurons are called local circuit neurons [22, 23, 59]. Local circuit neurons are involved in the processing of cardiovascular afferent information to manage sympathetic and parasympathetic efferent outflows [56]. These neurons also contribute to generate the basal activity of cardiac neurons within peripheral autonomic ganglia [37]. This contribution becomes more important when intrathoracic ganglia are disconnected from the influence of central neurons [57].

The latest schematic for interactions that occur within intrathoracic autonomic neurons and among these neurons and central nervous system to control of cardiac function was proposed in 2015 [71](Fig. 13).

This schematic view indicates that beat to beat regulation of the cardiac function depends on the interaction of different neurons within different ganglions via nested feedback loops. Therefore intrathoracic extracardiac and intracardiac neurons controlling the cardiac function are affected by the influence of higher centers in the spinal cord and brain stem. The autonomic outflow that is coming from higher centers to the heart is influenced by afferent feedback from cardiac, intrathoracic vascular and pulmonary sensory neurites. Four functionally types of neurons have been identified within intrinsic cardiac nervous system: parasympathetic postganglionic efferent neurons [64, 72-74], adrenergic postganglionic efferent neurons [20, 75-78], local circuit neurons [47, 63, 64, 79], and afferent neurons

[47, 57, 63, 64]. The cholinergic and adrenergic efferent neurons located in intrathoracic autonomic ganglia project their axons to cardiac electrical and mechanical tissues [37]. Local circuit neurons connect neurons which are close to each other within a ganglion as well as neurons which are in two different clusters of intrathoracic ganglia [10, 79]. Mechanosensitive and chemosensitive inputs from cardiopulmonary regions are provided by cardiac afferent neurons which are playing an important role in the intrathoracic neuronal feedback system [57, 80]. Regional cardiac tone is influenced by the activity of neurons in peripheral autonomic ganglia and preganglionic efferent neurons in the brain stem and spinal cord [25, 27, 28]. The preganglionic efferent neurons are influenced by afferent feedback from peripheral cardiopulmonary afferent neurons [81, 82], and higher centers of central nervous system [26]. Additional details of Figure 13 related to mechanism of different receptors (i.e beta-1 and M2) are not discussed in this thesis. It was shown that subpopulations of intrinsic cardiac neurons get activated by stimulating the efferent neurons' projection to the heart (parasympathetic and/or sympathetic) [47, 63, 64]. With respect to control chronotropic function, direct vagal input to the sinoatrial node could be removed by disruption of right atrial ganglionated plexus [83] but sympathetic efferent neuronal effect and the inhibiting effect of vagal activation on sinus tachycardia are remained [83]. These remaining effects are suggested to depend on neuronal activity in other ganglia of intrinsic cardiac nervous system [37, 83, 84]. The neuronal activities within the intrinsic cardiac nervous system are mostly influenced by their common shared afferent inputs as well as interconnections mediated via local circuit neurons [37].

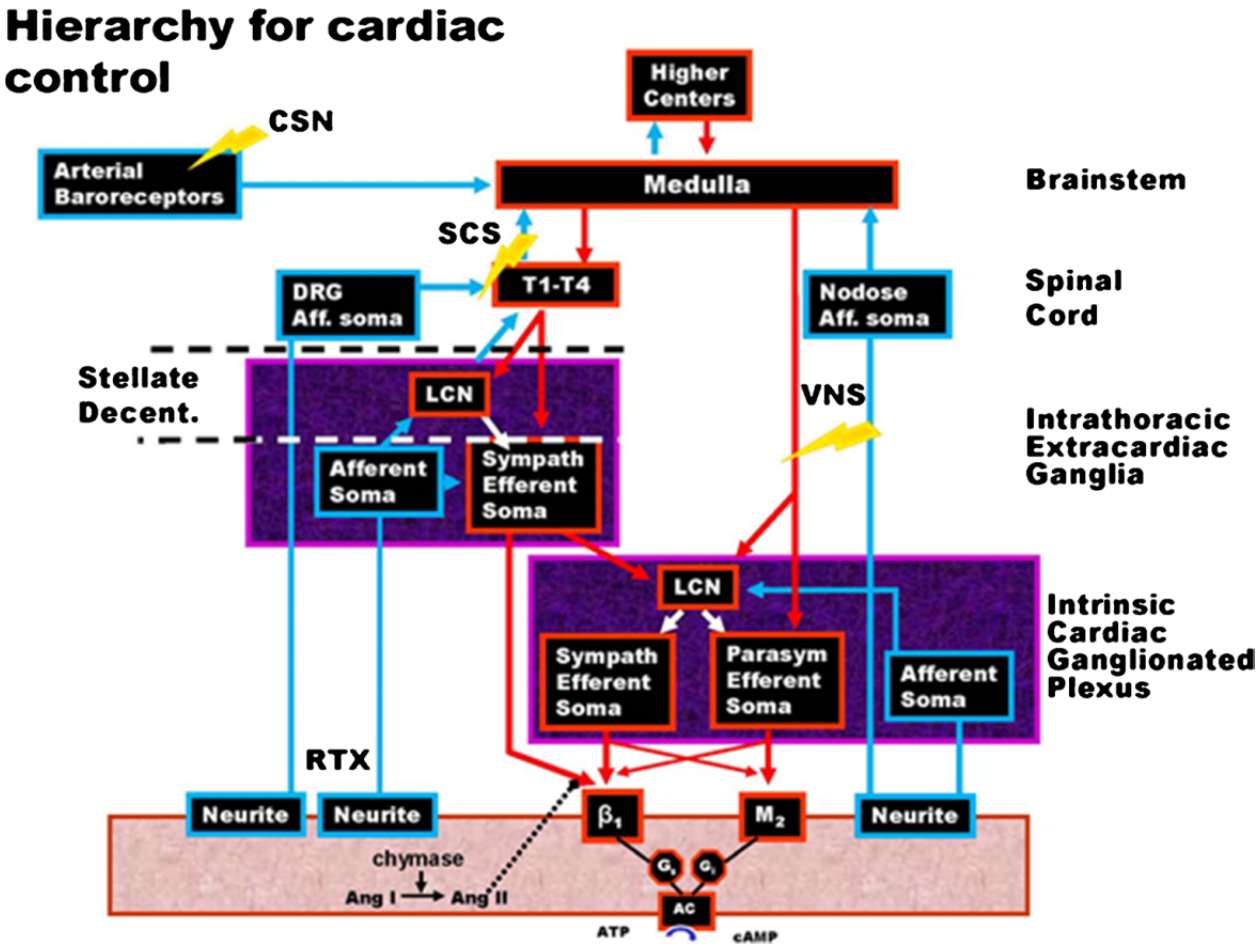

Figure 123 Schematic of proposed interactions that occur within and among intrathoracic autonomic neurons and between them and central neurons. Intrinsic cardiac ganglia possess afferent neurons, sympathetic (Sympath) and parasympathetic (Parasym) efferent neurons and interconnecting local circuit neurons (LCN). Extracardiac intrathoracic ganglia contain afferent neurons, local circuit neurons and sympathetic efferent neurons. Neurons in these intrinsic cardiac and extracardiac networks form separate and distinct nested feedback loops that act in concert with CNS feedback loops involving the spinal cord and medulla to coordinate regional cardiac function on a beat-to-beat basis. The complexity of the neuronal hierarchy for cardiac control. symbols: CSN: carotid sinus, SCS: dorsal column spinal cord, VNS: cervical vagus electrical stimulation ,RTX: resiniferatoxin ,DRG: dorsal root ganglia, Aff.: afferent, T1-T4 :first to fourth level of thoracic cord, Ang: angiotensin, β:beta adrenergic receptor, M: muscarinic receptor, Gs andGi: G proteins, AC: adenylate cyclase, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, Neurite: sensory endings embedded in the myocardium, Decent: decentralization (reproduced from figure 1 [71])

I.2.5.4. Extracellular recording of neuronal activity

The extracellular recording from neuronal activity is done using an electrode which is placed near a neuron. This recording can record the action potentials fired by a neuron but it cannot record subthreshold activity[85]. The extracellular recording is widely used for in vivo studies. There exist different recording electrodes in the market with different capabilities of recording. For example

multichannel linear microelectrode array can record from multiple channels; therefore it can cover more recording areas.

I.2.6. Electrophysiology of cardiac ganglia

I.2.6.1. Cardiac innervation intact

Many cardiac neuronal activities are related to the cardiac or respiratory cycles [47, 63, 64]. Figure 14 illustrates the percentage of active neurons within the ventral right atrial and ventricular ganglionated plexi. Bars denoted by "I" in Fig.14 represent the responses for the animals (n=20) with all innervation to the heart intact and bars denoted by "D" (decentralized) represent the responses for another group of animal (n=8) two weeks after interruption of all extrinsic input to the heart. Some neuronal activities are correlated to either cardiovascular or respiration and some other activities are not related to cardiac cycles or respiration. Among ventral right atrial ganglionated plexus neurons, 39% and 8% showed spontaneous activity that correlated with cardiac cycle and respiratory cycle respectively. For the ventral ventricular ganglionated plexus neurons 81% and 17% exhibited cardiac related and respiration related activity respectively [64]. It was also shown that vagus nerve stimulation or stellate ganglia stimulation activates a population of neurons in atrial and ventricular ganglia [47, 63, 64]. The recorded neuronal activity in these studies [47, 63, 64] suggest that although some intrinsic cardiac neurons could be directly affected by efferent sympathetic and parasympathetic input, most of them are not receiving a direct input from brainstem and intrathoracic ganglia. It is also suggested that there exist neuronal interconnection within the intrinsic cardiac nervous system and that these interconnections coordinate autonomic outflow at these ganglia sites [22]. In vitro studies on intracellular/extracellular recording of intrinsic cardiac neurons from rats [86-88], cats[89], pigs[90] and dogs [91-93] also showed that complex neural interactions occur within the intrinsic cardiac nervous system[22].

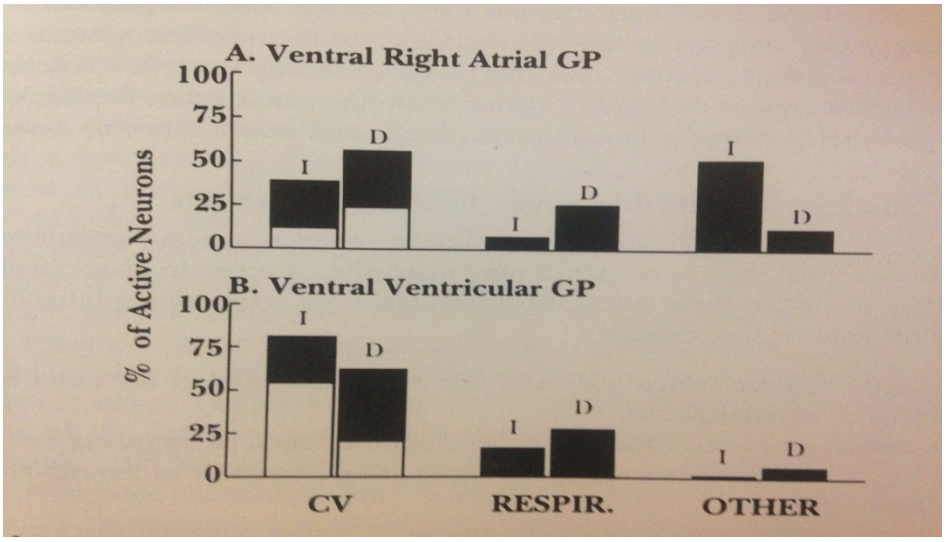

Figure 134 "percent of active neurons within the (A) ventral right atrial and (B) ventral ventricular ganglionated plexi, which display spontaneous activities correlated to cardiovascular(CV) or respiratory(respire.) events or exhibit irregular discharges unrelated to cardiorespiratory events (other). Responses are shown for animals(n=20) with all innervation to the heart intact(I) ,and for a second group of animals (n=8) 2 weeks following interruption of all extrinsic input to the heart(decentralization (D) of the intrinsic cardiac ganglionated plexus). For intracardiac neurons with cardiovascular related activities the stacked bars indicate the percentage of those neurons that were directly activated by discrete mechanical stimulation of epicardial ventricular and /or atrial sites "(Reproduced exactly from [22])

I.2.6.2. Decentralized cardiac nerve plexus

As cardiac neurons receive inputs from autonomic efferent neurons [10], decentralization of cardiac autonomic ganglia by acute transection of efferent projection reduces the spontaneous activities of cardiac neurons [47, 63, 64]. Despite decentralization of the cardiac ganglia, cardio/respiratory related residual activities by many neurons were reported [47, 63, 64]. More importantly, activities of some neurons are affected by afferent inputs from mechanoreceptors during decentralization [47, 63, 64]. The results shown in these studies [47, 63, 64] suggest that the coordination of intrinsic cardiac nerve activities with cardiorespiratory events are not absolutely dependent on the extracardiac feedback [22].They also suggest that some afferent dependent neuronal mechanisms within intrinsic cardiac neurons remain even after decentralization of cardiac ganglia and elimination of extra cardiac neural connection to the intrinsic cardiac neurons [22].

Decentralization of peripheral autonomic ganglia is associated with a high decrease in intraganglionic nerve activity [47, 63, 64, 80, 94] but this suppression in nerve activity lasts for some time after decentralization and after that it begins to recover toward control values [9, 94]; for example in the dog, two weeks after decentralization of intrinsic cardiac ganglia, the activity of atrial and ventricular neurons recovered to high levels of spontaneous activity [57]. After decentralization most spontaneous activities still remained correlated with either cardiac or respiratory cycles ("D" bars in Fig. 14) [57].

I.2.6.3. Intrathoracic nervous system and cardiac arrhythmias

Unlike the extracardiac autonomic ganglia which tend to amplify central nervous system and afferent feedback, intrinsic cardiac nervous system tries to limit cardiac excitability and acts as a low pass filter to minimize transient neuronal imbalances [37]. Mediation of local cardiac reflexes is also done by afferent feedback mechanisms within intrathoracic ganglia [37]. Moreover, efferent cardiac neurons control the heart via descending parasympathetic neuronal projections to the heart. The local circuit neurons also contribute with efferent cardiac neurons to mediate the cardiac function [83, 84, 95]. Ventral right atrial [28] and posterior atrial [84] ganglionated plexi have an important role in heart beat control. Inferior vena cava-inferior atrium ganglionated plexus is primarily controlling the AV conduction [28]. Imbalance control of cardiac electrical activity could happen when the end-effectors such as the sinoatrial node malfunction [96] or when neuronal parts are disrupted from the intrinsic nervous system as after ablation [84, 97, 98].This imbalance may be one of the important causes of cardiac arrhythmias [37]. For example it was indicated that stimulation of left-sided peripheral autonomic neurons induce dysrhythmias [99].

I.3. Atrial arrhythmias

I.3.1. Description of atrial fibrillation

Irregular heart rhythm is called arrhythmia. Arrhythmia could occur when the heart is beating much slower (bradycardia), much faster (tachycardia) or completely irregularly. The most common

arrhythmia is atrial fibrillation. During atrial fibrillation, disorganized propagation of action potentials in the atria causes the atria to fibrillate (irregular and fast contraction); therefore when atria are filled with blood, they are not able to efficiently pump the blood to ventricle and some blood remains in the atria. This may cause a lack of blood for different organs due to atrial pumping disability or the remaining blood in the atria may create a clot which increases the risk of stroke. There are nearly 250,000-350,000 patients with atrial fibrillation disease in Canada (more than 4 million in United States [100, 101]).It often leads to heart failure and stroke [102].The prevalence of atrial fibrillation increases with age [102, 103]. It results to approximately one-third of hospitalization for cardiac rhythm disorders [104] and it is estimated that 20% of all strokes are caused by atrial fibrillation [105].

I.3.2. Initiation and maintenance of atrial fibrillation

Atrial fibrillation is initiated when the action potential does not originate from the sinoatrial node and it is initiated by another part in the atria (mostly near pulmonary veins). When this electrical impulse is generated, it does not pass the same route as SA node action potential. As a result, the signal propagates throughout the atria in a disorganized way and it contracts different parts of atria in a disorganized manner which causes fibrillation. This signal may or may not pass through the AV node to the ventricles. The origin of atrial fibrillation could be neurogenic (the role of the intrinsic cardiac nervous system is critical in this case), myogenic (related to abnormalities or remodeling in the heart muscle), or due to a combination of both [106]. After the onset of focal atrial fibrillation, it is also possible to maintain it with very long duration by injecting carbachol into the fat pad at the base of right superior pulmonary vein [107]. The idea that the autonomic nervous system is involved in atrial arrhythmias dates from the 19th century [106, 108] and is now well established [109-116].

The occurrence of atrial fibrillation in patients is reported in both paroxysmal and chronic form. Remodeling of the underlying substrate is the main cause for the progress from the paroxysmal to the chronic form [117-119]. At the early stage of atrial fibrillation development, a focal origin located around the pulmonary veins was identified in many patients by several electrophysiological studies [120, 121].

Therefore, isolation of these pulmonary veins through catheter ablation has been a relatively successful strategy to terminate atrial fibrillation.

Clinical evidences show that the ganglionated plexus at the pulmonary vein-atrial junctions has a critical role in the initiation and maintenance of the focal form of atrial fibrillation [122]. It has also been proposed that the number of active ganglionated plexus can predict the atrial fibrillation recurrence after minimally invasive surgical atrial fibrillation ablation [123]. Therefore ganglionated plexus ablation was shown to be effective at preventing atrial fibrillation recurrence when combined with pulmonary vein isolation [122, 124, 125].

The implication of the autonomic nervous system in atrial fibrillation has also been established in a subgroup of patients in which atrial fibrillation episodes typically occurred overnight or after dinner and were preceded by bradycardia [115]. Patients with this vagally-mediated form of paroxysmal atrial fibrillation tended to be younger and had a normal P wave, and thus presumably a normal conduction substrate [126]. The exact role of the autonomic nervous system, however, remains unclear [127].

There are growing clinical evidences that the autonomic nervous system is involved in the initiation and recurrence of atrial fibrillation [128-132]. Experimental evidence of a neural origin of ectopic beats was provided by applying high-frequency stimulation pulmonary vein during atrial refractory period [133]. Autonomic nerve stimulation evoked ectopic beats, resulting in focal atrial fibrillation. This ectopic activity was eliminated by injecting a neuronal blocker [133].

Microelectrode recordings in excised pulmonary vein preparations showed action potentials with early after-depolarization and triggered activity following ganglionated plexus high-frequency stimulation [134]. Vagus nerve stimulation was also shown to create an arrhythmogenic substrate that promoted atrial fibrillation more strongly than chronic atrial rapid pacing for similar reduction in atrial effective refractory period [135].

These clinical observations have motivated the development of dog models to investigate the relation between atrial fibrillation and the autonomic nervous system. In these models, atrial

tachyarrhythmias could be induced by electrically stimulating the vagosympathetic trunks in the neck [136] or mediastinal nerves [137-139].

Recently, neurogenic effects have received growing attention, notably due to potential therapeutic targets for catheter ablation [140],drugs [141] and nerve stimulation [142, 143].

I.3.3. Induction of atrial fibrillation by stimulating mediastinal nerve

Atrial fibrillation can be induced neurally by stimulating small branches of mediastinal nerve of the thoracic vagosympathetic complex [144-146] or pulmonary vein related mediastinal nerves [147, 148]. Mediastinal nerves are the nerves in the mediastinum area showed in Figure 15.

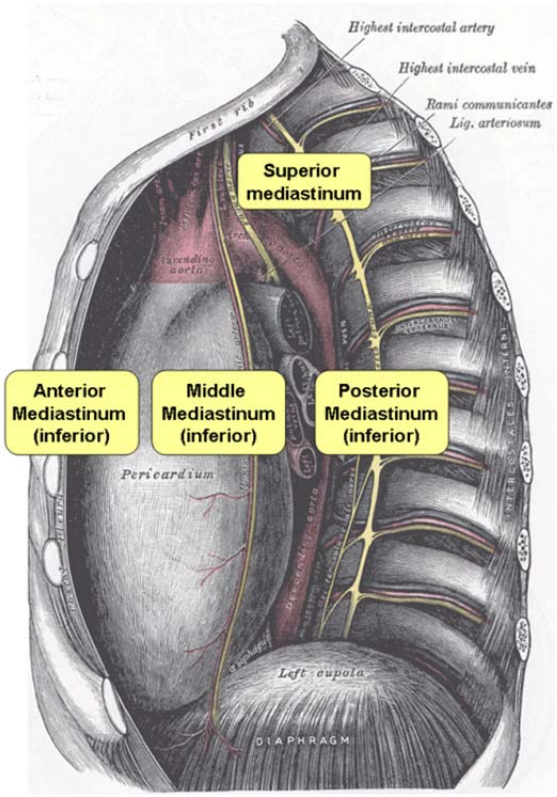

Figure 145 Mediastinum

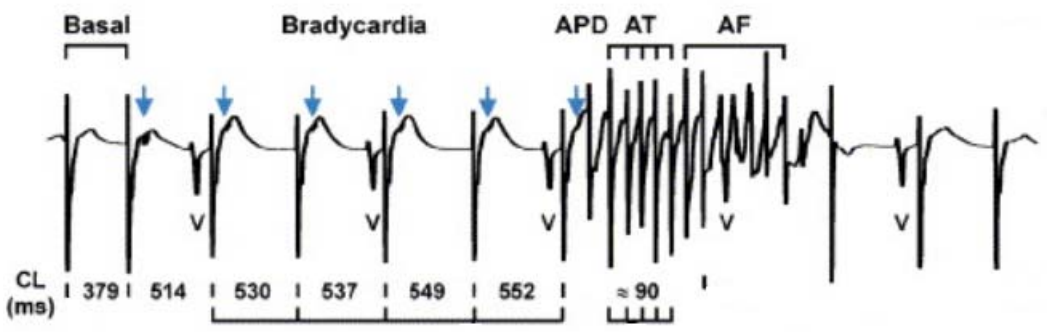

Figure 156 Arrhythmia caused by the stimulation of mediastinal nerve. "The unipolar electrogram (upper tracing) illustrates the bradycardic response (cycle length, CL, increased from 379 to 552 ms) followed by spontaneous atrial premature depolarization (APD), atrial tachycardia (AT) and paroxysmal atrial fibrillation (AF). Electrical stimuli (blue arrows) were applied to a right-sided, extra-pericardial mediastinal nerve. Deflections corresponding to dissociated ventricular depolarizations are identified (V). "(reproduced from [139])

Mediastinum contains different parasympathetic and sympathetic nerves. In this thesis we use "mediastinal nerve" term to point a small branch of vagus nerve. Applying electrical stimuli to the right sided mediastinal nerves could induce atrial fibrillation in canines [139]. These mediastinal nerves are identified by their accompanying vessels [145]. Figure 16 illustrates the effect of mediastinal nerve stimulation on the activity of atria.

I.3.4. Therapeutic approaches to atrial fibrillation

I.3.4.1. Pharmacotherapy

It was shown that the heart rhythm of 50% of atrial fibrillation patients that used antiarrhythmic drugs went back to the normal rhythm after one year [149]. Therefore using antiarrhythmic drugs is not an ultimate solution for treating atrial fibrillation and the patient who are not treated by these drugs, should try other atrial fibrillation treatments. The essential drug for the patient at risk of stroke is anticoagulant drugs [150]. The anticoagulant drugs help to prevent blood clotting. The pharmacotherapy is usually combined with other treatments like catheter ablation [17, 130].

I.3.4.2. Catheter ablation

In this treatment flexible thin wires (catheters) are inserted in to the blood vessel and they are guided to the specific regions of the heart that needs to be removed. Usually radiofrequency energy is transmitted via these catheters to the heart tissue where is causing the problem (i.e. arrhythmias) to destroy these parts. Catheter ablation showed a very good success rate in terminating heart rhythm disorders but it was not very successful in treating atrial fibrillation [151]. The success rates of surgical approaches to treat atrial fibrillation vary among different patients. In the study by Cappato et al [152], it was reported that catheter ablation was effective in ≈70% of patients. This percentage increased to ≈80% in patients when the catheter ablation was combined with antiarrhythmic pharmacological therapy [152] .Therapeutic success rates of such therapy was 87, 81 and 63 % at the end of 1, 3 and 5 years, respectively [153]. Moreover, catheter ablation exhibits complications [152] such as the left atrial stiffness syndrome [154], micro-embolic episodes [155], as well as risk of symptomatic or silent cerebral ischemia detected via magnetic resonance imaging [156]. The disadvantages of catheter ablation have encouraged the development of new non-pharmacologic therapies for atrial fibrillation treatment. New procedures have been designed to improve success rate by targeting complex fractionated atrial electrograms [157] or the ganglionated plexi [140]. Evidences of ganglionated plexus involvement in pulmonary vein ectopy [158, 159] supported the idea of ablating right and/or left ganglionated plexus to treat paroxysmal atrial fibrillation [160-164], and sometimes persistent atrial fibrillation [165] and neurocardiogenic syncope [122, 166, 167]. A recent retrospective meta-analysis of 342 patients demonstrated the clinical relevance of ganglionated plexus in atrial fibrillation [168]. Other new therapies like vagus nerve stimulation [169-171] or spinal cord stimulation [172-175] have been evaluated for the treatment of atrial fibrillation. Antiarrhythmic effect of vagus nerve stimulation was demonstrated and it was shown that it has minimal adverse effects [133, 143, 171]. Low level vagus nerve stimulation therapy is able to suppress atrial fibrillation induced by cholinergic neuronal activation in ambulatory canines, by suppression of stellate ganglion hyperactivity [170]. It has been hypothesized

that decreasing the activity of intrinsic cardiac local circuit neurons by vagus nerve stimulation might be the reason of antiarrhythmic effect [169, 176].

I.4. Vagus nerve stimulation

I.4.1. Description of vagus nerve stimulation

The vagus nerve is the tenth cranial nerve (it is also called cranial nerve X, pneumogastric nerve). The position of vagus nerve is shown in figure 17. The vagus nerve is connected to many organs in the body (Figure 18) and it mostly controls the parasympathetic innervation of these organs. The main substance released by vagus nerve as a neurotransmitter is acetylcholine.

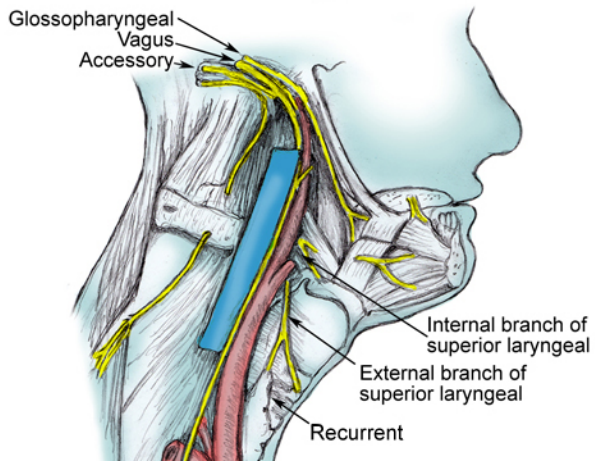

Figure 167 Location of Vagus nerve (reproduced from [177])

In vagus nerve stimulation, the vagal nerve is stimulated by a light electrical signal which increases the parasympathetic tone. Vagus nerve stimulation is a FDA-approved therapy for refractory epilepsy and depression [133, 134, 178, 179]. Since it affects cardiac electrophysiology [133], vagus nerve stimulation has the potential to become a therapy for cardiac arrhythmias as well. Treatment of heart failure appears to be a promising application for vagus nerve stimulation [133, 180-184]. Recently the advances in technology made it possible to develop the implantable device that can test the vagus

nerve stimulation therapy on heart failure in animal model [182, 185]. CardioFit system (BioControl Medical, Yehud, Israel; Figure 19) is a device that implements the vagus nerve stimulation.

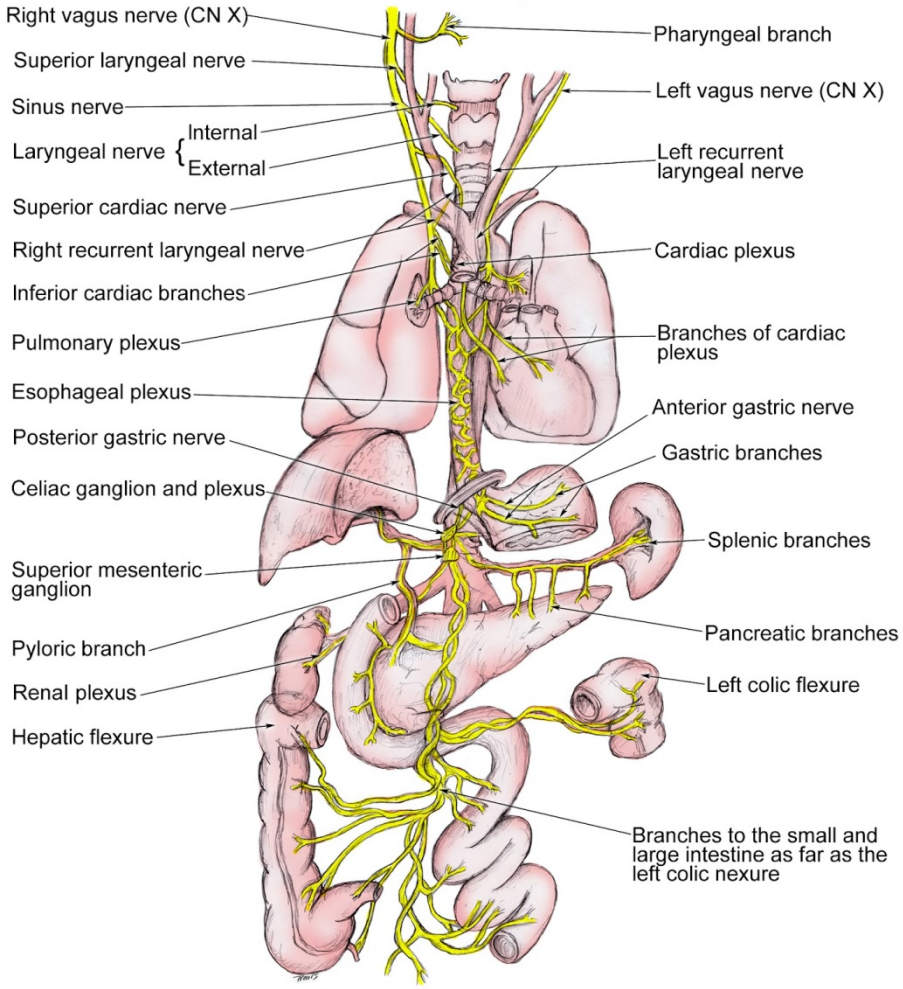

Figure 18 Connection of Vagus nerve branches to some body organs (reproduced from [177])

This system generates a small electrical current by an implantable pulse generator and delivers the pulses to the vagus nerve by the first lead placed surgically around the right cervical vagus nerve [185]. The heart rate detection and electrocardiogram sensing are done by another lead placed in the right ventricle and used to stop vagus nerve stimulator when heart rate drops below a predefined level [69].

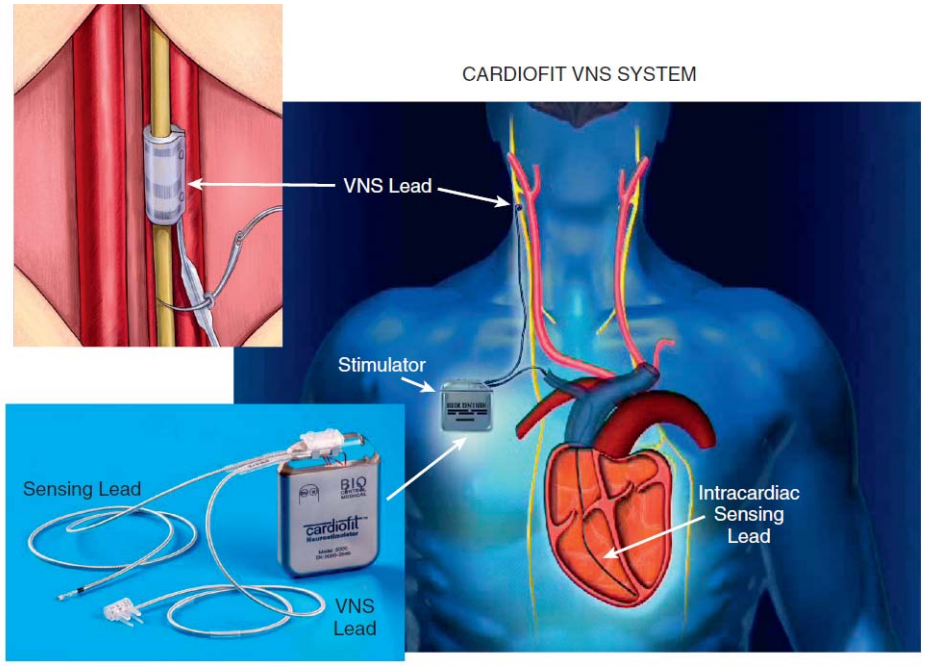

Figure 19 Right, Depiction of an implanted CardioFit Vagus Nerve Stimulation (VNS) device showing the position of the VNS lead on the right vagus nerve, the intracardiac pacing lead in the right ventricular apex, and the implantable CardioFit neurostimulator in the right subclavicular region. Left top, Depiction of the positioning of the CardioFit stimulation lead around the right vagus nerve. Left bottom, Photograph of the Cardiot VNS implantable neurostimulator, sensing lead, and VNS lead.Courtesy BioControl Medical, Ltd., Yehud, Israel.(reproduced from [69])

I.4.2. Vagus nerve stimulation therapy

Although intensive vagus nerve stimulation has been used to trigger and maintain atrial fibrillation, low level vagal nerve stimulation is able to mitigate atrial fibrillation. Different studies showed the anti-arrhythmogenic effect of vagus nerve stimulation [133]. Zhang et al. [186] reported the non-arrhythmogenic effect of therapeutic chronic vagus nerve stimulation in conscious animals, and concluded that moderately intense vagus nerve stimulation can be used to deliver therapeutic benefits without arrhythmogenic risk. It has also been proposed that low level vagus nerve stimulation may prevent episodic atrial fibrillation caused by rapid pulmonary vein and non-pulmonary vein firing [142] or caused by rapid atrial pacing [171], significantly decrease acetylcholine-induced atrial fibrillation duration [187] and even suppress atrial fibrillation [171, 188]. Since atrial fibrillation can be triggered by

vagus nerve stimulation in dogs (for a specific range of pacing parameters), there was a concern that atrial fibrillation episodes might occur as a side effect of chronic vagus nerve stimulation. This does not seem to be the case in epilepsy patients [180, 189, 190]. In a study that used low level vagus nerve stimulation, it was claimed that low level vagus nerve stimulation can suppress atrial fibrillation induced by strong cholinergic stimulation and it was hypothesized that the inhibition of the intrinsic cardiac nervous system by low level vagus nerve stimulation may be responsible for this anti- arrhythmogenic effect [171, 188, 191]. It was also reported that continuous low level vagus nerve stimulation reduces paroxysmal atrial tachyarrhythmias in ambulatory canines [170] and suppresses atrial fibrillation inducibility [169, 192]. Inhibition of atrial fibrillation inducibility could also be done by low level transcutaneous electrical stimulation [191].

I.5. Conceptual framework

Hypothesis 1 : The intrinsic cardiac nervous system acts as a local processor (not only as a relay between the brain and the heart), using multiple nested feedback control loops to modulate cardiac function[56]. Mediastinal nerve stimulation induces imbalance of neuronal activity and enhances arrhythmogenicity.

Atrial fibrillation is the most frequent persistent rhythm disorder in humans (nearly 250,000 patients in Canada) and often leads to heart failure and stroke[193]. The most frequent clinical conditions that are associated with atrial fibrillation are: “ischemic heart disease, diabetes, hypertension, cardiomyopathy, valvular heart disease and heart failure”[194]. Despite decades of investigations, many questions related to the principal mechanisms of the initiation and maintenance of atrial fibrillation remain open [195].

Atrial fibrillation could be induced neurally by stimulating mediastinal nerves [137-139] and one of the approaches to terminate atrial fibrillation is to isolate pulmonary veins(due to the origin of ectopic beat) through catheter ablation which showed success rates in the range 70-85%, often after a second

intervention [140, 196-198]. As intrinsic cardiac ganglionated plexi are involved in the pulmonary vein ectopy [158, 159], new approaches target the ganglionated plexi to treat paroxysmal atrial fibrillation [161-163, 199, 200]and persistent atrial fibrillation [165]. Hence ganglionated plexi ablation and pulmonary vein isolation are promising treatments for preventing atrial fibrillation [122, 124, 201]. While ablation remains the main focus[202, 203], ganglionated plexi may also be targeted by pharmacological intervention[204] but the exact role of ganglionated plexi remains unknown[127].Therefore a better understanding of a possible link between ganglionated plexi and atrial fibrillation would benefit to the development [129] and validation of therapies.

Among the intrinsic cardiac ganglia the right atrium ganglionated plexus is the most easily accessible plexus during open chest surgery because of its location on the superior surface of the right atrium. The right atrium ganglionated plexi receives inputs from mediastinal nerves and from the vagal nerve and it exerts predominant parasympathetic regulation of sinus node [205].The activity generated by neurons located in right atrium ganglionated plexus can be recorded in situ by means of a multichannel microelectrode.

In this thesis, we recorded the activity of intrinsic cardiac neurons in right atrium ganglionated plexus in a dog model and studied their activity during atrial fibrillation which was induced by mediastinal nerve stimulation. Experiments were done at Quillen College of Medicine, East Tennessee State University, Johnson City, TN.

During my internship which led to PhD. studies at Hôpital du Sacré-Coeur de Montréal under the supervision of Dr. Jacquemet, I was introduced to Dr.Beaumont who is specialized in neuroscience and neurocardiology and who was a professor at Hôpital du Sacré-Coeur de Montréal. When I started my PhD. the canine experiments had been done by him and our collaborators Dr.Ardell and Dr.Armour in Tennessee. Dr.Ardell and Dr.Armour are leaders and influential in neurocardiology field who worked more than 40 years in neurocardiology related projects. As these recordings were new recordings, there

was a need to improve the tools to get the data in a reasonable time and explore the data analysis methods that could be applied. The tools improvement and engineering parts were done at Hôpital du Sacré-Coeur de Montréal by Dr.Jacquemet, Dr.Vinet and me. Dr.Jacquemet and Dr.Vinet are specialized in cardiac electrophysiology, signal processing, statistics and data analysis methods. Therefore we had a good team in Tennessee who were working on the experiments and another team in Montreal who were working on the analysis of data which were transferred from Tennessee to Montreal. During the first year of my PhD. studies, Dr.Beaumont was the person who kept the collaboration efficient by contacting Dr.Ardell and Dr.Armour and visiting their lab in Tennessee. When Dr.Beaumont moved from Montreal to Tennessee, the Montreal team (Dr.Jacqumet,Dr.Vinet and me) kept the efficient contact with Dr.Beaumont and we had discussion through telephone or conference call. Although working with collaborators in another country is not very easy, our collaboration went well.

Hypothesis 2: Low level vagus nerve stimulation mitigates the atrial fibrillation by attenuating the activity of intrinsic cardiac local circuit neurons.

Recent treatment such as low level vagus nerve stimulation [171, 188] were also proposed to mitigate atrial fibrillation due to its effect on cardiac electrophysiology [133, 206]. Although there are some hypothesis about the suppression of intrinsic cardiac neuronal activity during vagal stimulation [169, 176], the effect of vagal stimulation on different types of intrinsic cardiac neurons (afferent, efferent, local circuit neurons) is not investigated and remains unclear.

In this thesis, we tried to understand a link between vagal nerve stimulation, atrial fibrillation and intrinsic cardiac neuronal activity and to investigate if vagus nerve stimulation could be applied as a therapy for atrial fibrillation.

Chapter 2 will discuss the activity of intrinsic cardiac neurons in right atrium ganglionated plexus in relation to different interventions. The interventions include mechanical, vascular, and electrical stimuli as well as induction of atrial fibrillation. This chapter will show how the neuronal recording, identification and classification processes were done and it will show the methods that were used to evaluate the data. It will also give some insights about the responses of each neuronal subtype to different stimuli.

Chapter3 will discuss a new method which enhances the neuronal identification process by cancelling the atrial activity from neuronal recorded signals. This chapter will explain the basis of the method and the results of applying the method.

Chapter 4 will discuss and study the intrinsic cardiac neuronal activity in relation to atrial fibrillation and vagus nerve stimulation. Moreover it will evaluate the efficacy of vagus nerve stimulation on mitigation of atrial fibrillation.

CHAPTER II: NETWORK INTERACTIONS WITHIN THE CANINE INTRINSIC CARDIAC NERVOUS SYSTEM: IMPLICATIONS FOR REFLEX CONTROL OF REGIONAL CARDIAC FUNCTION

Contribution of student:

The candidate did the neuronal identification and classification process, developed tools to analyze data and analyzed data to obtain the results. The candidate also contributed to writing the manuscript and creating figures.

Network interactions within the canine intrinsic cardiac nervous system: Implications for reflex control of regional cardiac function

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Running title: Network interactions within the intrinsic cardiac nervous system

TOC category: Cardiovascular

Key words: intrinsic cardiac nervous system; Linear micro-array electrode recording; local circuit neurons; parasympathetic; sympathetic; cardiac afferents; myocardial ischemia; atrial fibrillation

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Key Point Summary

- Control of regional cardiac function, as mediated by the intrinsic cardiac (IC) nervous system, is dependent upon its cardiac afferent neuronal inputs, changes in its central neuronal drive and interactions mediated within via local circuit neurons.
- The majority of such local circuit neurons receive indirect central (sympathetic and parasympathetic) inputs, lesser proportions transducing the cardiac milieu.
- 50% of IC neurons exhibit cardiac cycle related periodicity that is primarily related to direct cardiac mechano-sensory afferent inputs and, secondarily, to indirect central autonomic efferent inputs.
- In response to mediastinal nerve stimulation, most IC neurons became excessively activated in the induction of atrial arrhythmias such that their stochastic interactivity precedes and persists throughout neuronally induced atrial fibrillation.
- Modulation of such stochastic IC local circuit neuronal recruitment may represent a novel target for the treatment of select cardiac disease, including atrial arrhythmias.

Abstract

Objectives: To determine how aggregates of intrinsic cardiac (IC) neurons transduce the cardiovascular milieu versus responding to changes in central neuronal drive. To determine IC network interactions subsequent to induced neural imbalances in the genesis of atrial fibrillation (AF). *Methods:* Activity from multiple IC neurons in the right atrial ganglionated plexus was recorded in 8 anesthetized canines using a 16-channel linear microelectrode array. Induced changes in IC neuronal activity were evaluated in response to: (1) focal cardiac mechanical distortion; (2) electrical activation of cervical vagi or stellate ganglia; (3) occlusion of the inferior vena cava or thoracic aorta; (4) transient ventricular ischemia and (5) neurally induced AF. *Results:* Low level activity (ranging from 0 to 2.7 Hz) generated by 92 neurons was identified in basal states, activities that displayed functional interconnectivity. The majority (56%) of IC neurons so identified received indirect central inputs (vagus alone: 25%; stellate ganglion alone: 27%; both: 48%). 50% transduced the cardiac milieu responding to multimodal stressors applied to the great vessels or heart. 50% of IC neurons exhibited cardiac cycle periodicity, with activity occurring primarily in late diastole into isovolumetric contraction. Cardiac related activity in IC neurons was primarily related to direct cardiac mechano-sensory inputs and indirect autonomic efferent inputs. In response to mediastinal nerve stimulation, most IC neurons became excessively activated; such network behavior preceded and persisted throughout AF. *Conclusion:* Stochastic interactions occur among IC local circuit neuronal populations in the control of regional cardiac function. Modulation of IC local circuit neuronal recruitment may represent a novel approach for treatment of cardiac disease, including atrial arrhythmias.

Abbreviations

AF, atrial fibrillation; Ao, aortic; CAO, coronary artery occlusion; CV, cardiovascular; IC, intrinsic cardiac; ICNS, intrinsic cardiac nervous system; IVC, inferior vena cava; LAD, left anterior descending coronary artery; LCV, left cervical vagosympathetic complex; LSS, left stellate ganglion stimulation; LCN, local circuit neuron; LMA, linear microarray; LV, left ventricle; LV dp/dt, first derivative (+ positive, - negative) of left ventricular pressure; LVP, left ventricular pressure; LVSP, Left ventricular systolic pressure; Occl, occlusion; RAGP, right atrial ganglionated plexus; RCV, right cervical vagosympathetic complex; RSS, right stellate ganglion stimulation; RV, right ventricle.

II.1. Introduction

It has been proposed that the intrinsic cardiac nervous system (ICNS) acts as the final coordinator of regional cardiac indices, doing so under the modulating influence of higher centers of the cardiac nervous system including intrathoracic, spinal and brainstem mediated reflexes (Armour & Janes, 1988;Armour & Hopkins, 1990;Ardell, 2004;Gray *et al.*, 2004b;Zucker & Gilmore, 1991). Coordination occurring within the ICNS is dependent on three factors: convergence of afferent neuronal inputs (mechano-sensitive, chemo-sensitive, ischemic sensitive), central efferent neuronal inputs (both sympathetic and parasympathetic) and interconnections mediated via local circuit neurons (Ardell *et al.*, 1991;Armour *et al.*, 1998;Herring & Paterson, 2009;Taylor *et al.*, 1999;McAllen *et al.*, 2011;Gray *et al.*, 2004a). The latter neuronal population likely sub serves complex reflex processing within the ICNS (Armour, 2008). Neuronal imbalances within any of these elements can exert deleterious effects on cardiac function, including arrhythmia induction (e.g. AF; (Gibbons *et al.*, 2012;Shen *et al.*, 2011;Scherlag *et al.*, 2006) and progression into congestive heart failure (Dell'Italia & Ardell, 2004;Zucker *et al.*, 2012;Liu *et al.*, 2012).

In order to characterize the ability of different neuronal populations within the ICNS to transduce altered cardiac status, the correlative interactions exhibited among specific IC neuronal populations within the heart need to be assessed with respect to whether they receive common shared cardiovascular sensory inputs or not (Armour & Kember, 2004;Kember *et al.*, 2001;Thompson *et al.*, 2000) as well as how they are impacted by central neuronal inputs (Ardell, 2004;McAllen *et al.*, 2011;Andresen *et al.*, 2004;Herring & Paterson, 2009). This information would form the basis for determining: (1) how they differentially transduce afferent inputs from different cardiac regions and the major thoracic vasculature (Thompson *et al.*, 2000;Waldmann *et al.*, 2006); (2) how individual neurons distributed throughout an intrinsic cardiac ganglionated plexus interact under the direct versus indirect control of central cholinergic preganglionic (medullary) or adrenergic (spinal cord/stellate/middle cervical ganglia) efferent neuronal inputs (Gagliardi *et al.*, 1988;Armour *et al.*, 2002;Armour & Hopkins, 1990); and (3) how their sensory

activity and interactive behavior are affected by transient regional ventricular ischemia (Armour *et al.*, 2002;Huang *et al.*, 1993;Armour *et al.*, 1998). These questions have implications for atrial and ventricular arrhythmia induction which might arise as a consequence of abnormal interactions among the various populations of intrinsic cardiac neurons (Cardinal et al, 2009), an issue yet to be defined.

The present study addresses these questions in the anesthetized canine preparation. With the emergence of linear microarray electrode technology, it is now feasible to evaluate *in situ* activities within and between different aggregates of IC neurons over relatively long periods of time, including in response to complex afferent and efferent stressors. Data so derived indicate that the majority of neurons in the ICNS are local circuit neurons that simultaneously transduce inputs from cardiac and major intrathoracic vascular receptors, as well as direct or indirect inputs from central (spinal cord and medullary) neurons. Surprisingly few IC neurons proved to be under the direct (monosynaptic) influence of medullary or spinal cord efferent preganglionic neurons. The interactive behavior displayed among most of this ICNS network was of a primarily stochastic nature. Imbalance of intrinsic cardiac control in the induction of atrial arrhythmia indicates that targeting excessive, stochastic interactions among intrinsic cardiac local circuit neurons may be relevant to understanding how to mitigate such pathology therapeutically.

II.2. Methods

II.2.1. Ethical approval

Eight mongrel dogs (either sex), weighing 18-27 kg, were used in this study. All experiments were performed in accordance with the guidelines for animal experimentation described in the "Guiding Principles for Research Involving Animals and Human Beings" (Am.Physiol.Society, 2002). The Institutional Animal Care and Use Committee of East Tennessee State University approved these experiments.

II.2.2. Animal preparation

Animals were pre-medicated with sodium thiopental (15 mg/kg, i.v), intubated and anesthetized using 2% isoflurane. The left femoral vein was catheterized to allow fluid replacement as well as the administration of anesthetic and pharmacological agents. Left ventricular chamber pressure was measured via a 5-Fr Mikro-Tip pressure transducer catheter (Millar Instruments, Houston, TX) inserted into the chamber via the left femoral artery. The right femoral artery was catheterized to monitor aortic pressure using another Mikro-Tip transducer. Heart rate was monitored via ECG lead II. Depth of anesthesia was assessed by monitoring corneal reflexes, jaw tone and alterations in cardiovascular indices. Following completion of the surgery, anesthesia was changed to α-chloralose (75 mg/kg i.v. bolus), with continuous infusion (16 mg/kg/hr) adjusted as required throughout the duration of each study. Body temperature was monitored rectally and maintained steady via a circulating water heating pad (T/Pump, Gaymar Industries Inc., Orchard Park, NY). Respiration was controlled using an artificial ventilator (at 12-16 cycles/min) supplied with oxygen. Acid-base status was evaluated hourly (Irma TruePoint blood gas analyzer, International Technidyne Corp., Edison NJ); tidal volume was adjusted and bicarbonate infused as necessary to maintain blood gas homeostasis.

II.2.3. Neuronal activity recording

Following a transthoracic thoracotomy (T4), a pericardial cradle was formed. The activity generated by neurons located in the right atrial ganglionated plexus (RAGP) was recorded *in situ* by means of a multichannel linear microelectrode array (MicroProbes Inc., Guithersberg, MD). This microelectrode linear array, consisting of 16 platinum/iridium electrodes (25 μm diameter electrode with an exposed tip of 2 mm; impedance 0.3-0.5 MΩ at 1 kHz), was embedded in the right atrial fat that contained the RAGP such that its tip was placed adjacent to right atrial musculature.

The probe was attached to a flexible lead, allowing the probe to be semi-floating. The density of tissue in the ventral right atrial fat helped to maintain position stability over prolonged periods of time (6-8 hrs of recording). The connecting wires of the multichannel electrode, along with ground and reference

wires, were attached to a 16-channel microelectrode amplifier with headstage preamplifier (A-M systems, Inc., model 3600; Carlsborg, WA). For each channel, filters were set to 300-3K Hz and gain to 5K. A hook electrode was sewn to the atrial myocardium close to the RAGP to provide a reference right atrial electrogram. This atrial electrogram was utilized for determination of atrial rate, duration and characterization of atrial arrhythmias, including atrial fibrillation, and for identification of the timing of atrial electrical artifacts contained within the neural recording data. The 16 microelectrode array signals, along with recorded cardiovascular indices (ECG, right atrium electrogram and hemodynamic data), were digitized via a Cambridge Electronics Design (model 1401) data acquisition system for off-line analysis. The sampling frequency for neuronal data was 5.26 kHz; it was six time lower (0.877 kHz) for all other signals.

II.2.4. Cardiac and vascular mechanical stimuli

In order to determine whether identified right atrial neuronal populations transduce mechanosensory inputs from select cardiac tissues, the right ventricular conus and the left ventricular lateral wall were sequentially touched gently by a finger during 10 s intervals with at least 2 min baseline data obtained between stimulus applications. A length of saline soaked umbilical tape was placed around the base of the inferior vena cava and another one around the descending thoracic aorta. Silk ligatures were placed around the left anterior descending coronary artery about 1 cm from its origin. This enabled us to repeatedly occlude the inferior vena cava (for 20 seconds), the descending aorta (for 20 seconds) and the left anterior descending coronary (for 1 minute) while recording evoked changes in IC neuronal activities. At least 5 min separated each of these stressors, thereby allowing for return to basal activities.

II.2.5. Extracardiac efferent neuronal inputs

The left and right cervical vagosympathetic complexes and stellate ganglia were exposed. Bipolar electrodes were placed around each of them. Thereafter, these neural structures were stimulated individually via a Grass Model S88 Stimulator (Grass Co., Warwick, RI). To established threshold for

vagal efferent activation, the stimulus frequency was initially set to 20 Hz, pulse width to 2 ms and voltage increased until heart rate decreased by 10%. To establish threshold for sympathetic efferent activation, frequency was set to 4 Hz, pulse width to 2 ms, with threshold defined as the voltage necessary to increase heart rate or LV dP/dt by 10%. During the course of each experiment, these efferent neural stimuli (RCV, LCV, RSS or LSS) were periodically delivered for 1 min at 1 Hz, 2 ms pulse width, with stimulus voltages being 3x threshold. This was done to identify potential direct versus indirect inputs to IC neurons.

II.2.6. Mediastinal nerve stimulation

The right-sided mediastinal nerves that coursed over the ventral and ventro-lateral surfaces of the intrapericardial aspects of the superior vena cava were identified visually. In order to consistently elicit brief episodes of atrial arrhythmias, one or more of these mediastinal nerve sites were stimulated repeatedly via a bipolar electrode (inter-electrode distance 1.5 mm), as done previously (Armour *et al.*, 2005;Richer *et al.*, 2008). Each active site was marked with India ink for identification during subsequent stimulations. The stimulator was controlled externally by the Cambridge Electronics Design data acquisition system running Spike 2 software, with the macro for D/A output triggered by on-line atrial wave-front detection. Trains of five electrical stimuli (1-2 mA, 1 ms duration, and 5 ms pulse interval) were delivered for up to 20s to each selected mediastinal site during the refractory period of each atrial beat. Contact between the bipolar electrode and the tissue was discontinued immediately after the onset of the atrial tachyarrhythmia.

II.2.7. Data analysis: Signal processing of recorded multi-unit IC neuronal activity

Recorded neuronal activity generated by individual neuronal somata located throughout the RAGP was recorded, as depicted above. Recorded neuronal signals were contaminated by the electrical activity arising from the adjacent atrial myocardium located below the RAGP. Electrical artifacts induced during electrical stimulation of autonomic neural structures or mediastinal nerves could also be identified in the recorded signals. Artifact removal was thus necessitated, using the software Spike2 program

(Cambridge Electronic Design, Cambridge, England). Four channels with neuronal activity were selected by visual inspection from all 16 channels of information. Simultaneously occurring activity displaying similar waveforms in each of 3 adjacent channels was interpreted as being artifactual as, for instance representative of electrical activity generated by the adjacent atrial tissue. As such, these artifacts were identified using the template matching functionalities of Spike2. The right atrial electrogram channel and the stimulator signal served to validate such artifact identification. In this manner, artifact waveforms were eliminated from all 16 channels. Figure 1 illustrates such an analysis process. These blanking intervals represented only 3% of the signal durations during sinus rhythm and up to 18% of signal duration during AF.

Following artifact removal, the activity generated by individual IC neuronal somata could be characterized by their specific amplitudes and waveforms derived from each of the 16 recorded LMA channels. These signals were processed by analyzing the activities recorded from pairs of adjacent electrodes (stereotrode). An action potential was considered to arise from a single IC neuronal somata and/or dendrites when two (but not more) adjacent electrodes displayed similar waveforms that occurred simultaneously in two adjacent electrodes. Action potentials derived from a single electrode were consistent with the waveforms observed from adjacent (but not distant) electrodes, this association remaining unchanging over time. Automatic waveform classification (i.e. identifying all the action potential corresponding to the same IC neuron) were performed by template matching (template length: 5-6 ms, ~30 samples) and validated by principal component analysis. Further manual validation was performed by visual inspection of the templates such that artifact-related templates could be eliminated. Two similar templates (as established by principal component analysis) were merged when evidence arose (e.g. complementary intermittent firing) that the two firing time series so identified corresponded to a single neuron. Using that procedure, consistent waveforms derived from individual somata could be identified *in situ*, as has been done previously using a single unipolar electrode (Gagliardi *et al.*, 1988;Ardell *et al.*, 1991;Ardell *et al.*, 2009). Figure 1 illustrates this process to identify the activities generated by two separate IC neurons, as recorded concurrently from adjacent channels of the LMA

electrode. Using these techniques and criteria, action potentials generated by individual somata and/or dendrites (not axons of passage) could be identified for up to 8 hours (Ardell *et al.*, 1991;Ardell *et al.*, 2009). At the completion of neural recording, animals were terminated under deep anesthesia (50mg/kg alpha choloralose) using DC current induced ventricular fibrillation.

II.2.8. Data Analysis: Monitoring individual neuron activity

Neuronal activity was compared in different time windows (before versus during an intervention such as touch or autonomic efferent nerve stimulation) by calculating the evolution of average neuronal activity rate. The time window before an intervention (baseline) was assessed for 1 minute time periods. The time window during an intervention covered the actual duration of that intervention. The significance level of the observed differences in firing rate was assessed using a statistical test developed for cortical neurons and based on the Skellam distribution (Shin *et al.*, 2010) (see Appendix for a detailed description of this analysis). The resulting p-value was a function of the duration of the two time windows that were compared as well as the number of action potential identified in each time window. Two levels of significance were employed: $0.01<p<0.05$ (moderate change) and $p<0.01$ (strong change). Figure 2 illustrates such an analysis for baseline activity intervals of 60 sec contrasted with stressor-evoked intervals of 60 sec (panel A) or 5 sec (panel B). Using this statistical approach, changes in action potential generation rates recorded before and during each intervention (e.g., afferent activation, stimulation of efferent inputs to IC network) were quantitatively evaluated for all identified IC neurons for each stressor.

II.2.9. Data Analysis: Conditional Probability

This type of analysis quantifies whether a neuron that responded to one stressor also responds to another stressor. For that purpose, a neuron was said to respond to a stressor when a significant change in its activity rate ($p<0.05$; either an increase or a decrease) was observed before and during each intervention (see above and Figure 2). The potential for a functional relationship between stressors X and Y was quantified within neurons identified in each dog as a conditional probability that a neuron that

responded to stressor Y also responded to a stressor X. The conditional probability (Prob: response to Y | response to X) was estimated as the number of neurons that responded to both X and Y, divided by the number of neurons that responded to X.

II.2.10. Data Analysis: Chi Square

Chi square analysis was used to compare potential inter-relations between response characteristics of IC activity (e.g. basal activity with or without cardiac related periodicity) and corresponding activity effects evoked by activation of specific afferent and efferent pathways.

II.3. Results

II.3.1. Spontaneous activity in physiological states

In the 8 anesthetized animals investigated, the activities generated by a total of 92 neuronal somata and/or dendrites (referred to as IC neurons hereafter) located at different depths (superficial, intermediate and deep) within the RAGP were identified using the LMA electrode (average: 11.5 neurons/dog). Spontaneous activity generated by each was assessed by pooling the data from the 1-min time interval baselines obtained before each of the ten interventions. Spontaneous spiking activity varied considerably among neurons. As shown in Figure 3, baseline firing frequency ranged from 0 to 2.7 Hz, 68% being < 0.1 Hz and 8% being > 0.4 Hz. Although 12% of these neurons were never active during the 1-min intervals right before the interventions commenced, all of these became active at some time during or after one or more of the interventions.

Within these control intervals, some neurons (43 of 92) generated spikes that clustered around specific phases of the cardiac cycle. Figure 4 illustrates this relationship such that neuron 1 was preferentially active during the left ventricular ejection phase and another one (neuron 2) was preferentially active during left ventricular isovolumetric contraction. Other identified neurons (e.g. Fig 4, neuron 3) generated activity patterns that did not relate to a specific phase of the cardiac cycle. Using spike-triggered averaging, it is possible to demonstrate the temporal correlation between some IC neurons

thereby indicating interdependent relationships within IC networks. Figure 5 demonstrates such a relationship for two IC neurons that generated activities with cardiac related periodicity. For instance, the activity generated by neuron 1 was, on average, followed within a few milliseconds by activity generated by neuron 2. Note that this interdependent relationship was dynamic in nature such that they displayed inter-spike intervals that varied over time in the temporal relationship of their activities to each other (minimum 8 ms; peak of the histogram at 12 ms, see Fig. 5C) and that furthermore the activities displayed by these two neurons was not always coupled during every cardiac cycle.

II.3.2. Cardiovascular mechanoreceptor activation evoked IC responses

Within identified populations of IC neurons of the RAGP, 22% responded significantly to touching the ventricular epicardial surface in one or more region (RV conus, RV sinus or ventral LV). A typical neuronal response to touch is shown on Fig. 6. In this example, during touch, the activity generated by two neurons became inhibited, while others were activated. Note that some other ones (e.g. neuron 8) were relatively unaffected by this gentle epicardial touch. The significance of changes induced in the activity rates of each of the identified neuron in each of the dogs during each of the stressors studied is summarized in Fig. 7 and Table 1. Note that even within a given animal, a common stressor (e.g. touch of RV or LV) can evoke differential neuronal effects, even from closely adjacent IC neurons. For instance, of IC neurons modulated by ventricular touch, activity generated by 76% (16 of 21) increased while that of 24% (5 of 21) decreased (Table 1).

The activities generated by 41% (38 of 92) of identified IC neurons changed when the IVC or the descending thoracic aorta was occluded briefly; of these 39% were affected by both stressors (15 of 38). As indicated in Table 1, IC activity was either increased (n=37) or decreased (n=16) among these differing neuronal populations in response to one stressor, even within a given animal (Figure 7). In the case of ventricular touch and occlusion of the great vessels, IC neurons generating lower level basal activity tended to be activated by these stressors while the activities of those with higher levels of basal

activity tended to be suppressed by these same stressors (Table 1). Overall, 50% of recorded IC neurons (46 of 92) were modified by ventricular touch or occlusion of the great vessels.

II.3.3. Evoked IC response to stimulation of central inputs

Previous studies using a single unipolar electrode found that few RAGP neurons received direct inputs from medullary (parasympathetic efferent preganglionic) neurons or stellate ganglia neurons, as determined by fixed latency evoked responses between RAGP neuronal activity and individual electrical stimuli applied at low frequencies to a stellate ganglion or a cervical vagus nerve (Gagliardi *et al.*, 1988). Based upon these fixed latency criteria, none of the 92 RAGP neurons evaluated in this study received direct central neuronal inputs. On the other hand, the activity of 42% (39/92) of identified IC neurons was modified in response to 1 Hz stimulation of either stellate ganglion, indicating indirect input activation of such neurons with non-fixed latencies (Fig. 7 and Table 1). Of these IC neurons, 10 were influenced by RSS alone, 12 by LSS alone and 17 other were affected by both of these central neuronal inputs. The predominant effect of stellate stimulation on IC activity was excitation (Table 1). Likewise, when low frequency electrical stimuli (1 Hz) were applied to either cervical vagus, the IC activity from 41% (38/92) of identified neurons was modified - doing so after non-fixed latencies (Fig. 7 and Table 1). Of these IC neurons, 17 were impacted by RCV alone, 7 by LCV alone and 14 by both vagi. The predominant effect of vagal stimuli on affected IC neurons was excitatory in nature (Table 1). At 1 Hz stimulation frequencies, induced changes in LVSP or LV -dp/dt were less than 5% of baseline in response to either cervical vagal or stellate stimulation indicating minimal evoked changes in cardiac inotropic function. While induced chronotropic responses to 1 Hz VNS were less than 5% difference from baseline ($p=.94$), LSS increased heart rate on average from 96.6±11.9 beats/min to 103.2±10.1 beats/min ($p=.30$) and RSS from 101.1± 12.6 beat/min baseline to 120.2±0.3 beats/min ($p<0.01$).

Some IC neurons receive convergent inputs from both efferent limbs of the autonomic nervous system. Fifty-six percent (52/92) of identified IC neurons responded to some combination of cervical vagal nerve (RCV, LCV) or stellate ganglia (RSS, LSS) nerve stimulation. Of these 52 IC responders, 5

were activated by all 4 inputs, 12 were activated by 3 of 4 inputs, 17 responded to 2 inputs and 18 responded solely to 1 input. Overall, 48% (25/52) have evoked response to both vagal and sympathetic inputs, 25% (13/52) respond sole to vagal (RCV or LCV) stimulation and the remained 27% (14/52) respond solely to central sympathetic (RSS or LSS) efferent neuronal inputs.

II.3.4. Cardiac related IC periodicity: relationship to afferent and efferent inputs

Figure 8 shows activity histograms obtained for all IC neurons that generated at least 100 action potentials during baseline recording periods. Of the 49 neurons so identified, 43 generated activity that clustered around specific phases of the cardiac cycle. The majority of that cardiac periodicity was evident during diastole (14 neurons) or isovolumetric contraction (22 neurons), with lesser aggregate activity being evident in the ejection phase (10 neurons) or during isovolumetric contraction (7 neurons). 23% of IC neurons with cardiac related periodicity exhibited dual activity peaks in relationship to LV pressure during the cardiac cycle.

Figures 7 and 9 demonstrate the differential effects of afferent and efferent inputs on IC activity from neurons that displayed cardiac related periodicity versus those that did not. Following Chi-square analysis of the neuronal data, it is evident that those IC neurons that generated cardiac related activities were preferentially modified by mechano-sensitive inputs (RV touch, LV touch, Aortic occlusion or IVC occlusion) as well as by activation of each of the primary central efferent neuronal inputs to the IC network, both sympathetic (stellate stimulation) and parasympathetic (vagal stimulation) in origin. In contrast, transient occlusion of the LAD evoked similar changes in the activity of neurons, whether they did or did not display cardiac related periodicity during basal states.

II.3.5. LV ischemia and evoked IC responses

Myocardial ischemia induced by transient LAD occlusion triggered a response in 32% (26/82) of identified IC neurons. Transient myocardial ischemia increased activity in 14 of these neurons and depressed that of 12 neurons (Table 1). Such activity changes were manifest during ischemia and during early reperfusion. Figure 10 (top panel) summarizes the corresponding sensitivity to activation by cardiac

mechano-sensitive inputs (RV touch, LV touch, Aortic occlusion and IVC occlusion) in IC neurons that responded to LAD occlusion compared to IC neurons that were ischemia insensitive. Chi-square analysis indicated that ventricular ischemic sensitive IC neurons also displayed increased responsiveness to cardiac afferent inputs. In contrast, responsiveness to either vagal or stellate stimulation was similar between ischemic sensitive versus insensitive IC neurons (Figure 7 and Chi-square analysis with P greater than 0.05).

II.3.6. Atrial arrhythmia induction

Short episodes of atrial arrhythmia (duration 5 to 20 s) were initiated repeatedly when brief bursts of electrical current were delivered to selected right sided mediastinal nerves during the atrial refractory period. Arrhythmia induction via MNS was the stressor, by itself, that affected the largest number of identified neurons (Table 1: 48 of 92 neurons). Of those IC neurons affected by mediastinal nerve stimulation, 88% (42 of 48) increased their activity and 12% (6 of 48) exhibited reduced activity during AF (Table 1 and Figure 7). Chi-square analysis also indicated that the sensitivity of IC neurons that responded to mediastinal nerve stimulation was reflective of an increased responsiveness to cardiac afferent inputs (Figure 10, bottom panel). In contrast, IC responsiveness to either vagal or stellate ganglion inputs was similar when comparing MNS sensitive versus MNS insensitive neurons (Figure 7 and Chi-square analysis with P greater than 0.05).

II.3.7. Interdependence of neuronal function in response to different stressors

Figure 11A summarizes, in matrix format the relationships of the various identified IC neurons when comparing their responses to all 10 stressors tested. It was found that all ten of these conditional probabilities generated specific neuronal relationships. As such, their corresponding ($CP \geq 0.6$) functional interconnectivities can be represented as a network (c.f., Fig. 11B). These relations so displayed do not necessarily imply a mechanistic link between neurons initiated by these stressors. Rather, these relationships appear to reflect the concordant behavior among RAGP neuronal populations induced by pairs of independent stressors. Note that conditional probability links responses initiated among afferent

stressors (e.g. IVC and aorta occlusion) contrast to those initiated by differential central neuronal efferent neuronal inputs to the IC network (RCV, LCV, RSS and LSS). Furthermore, these data indicate a convergence point of the IC neuronal population that transduces both afferent and efferent inputs – a local circuit neuronal population engaged by MNS in the induction of atrial arrhythmias.

II.4. Discussion

Neural control of the heart depends upon the dynamic interplay between a series of nested feedback loops involving peripheral and central aspects of the cardiac nervous system (Armour, 2008;Zucker & Gilmore, 1991). The intrinsic cardiac nervous system (ICN) represents the most distal of its control loops and, as such, functions as the final common pathway for cardiac control (Ardell *et al.*, 1991;Ardell, 2004;Armour, 2008). The data derived from the experiments reported herein demonstrates the primary inherent characteristics of the major neuronal subpopulations within the ICNS, including the fact that the integrative characteristics of its different local circuit neuronal populations differ in the transduction of specific cardiac afferent or central efferent derived neuronal inputs.

The majority of intrinsic cardiac (IC) neurons that were functionally identified in this study exhibited low levels of spontaneous activity in control states, 68% exhibiting basal activity less than 0.1 Hz. The data derived from this study further demonstrates the state dependence of the majority of these intrinsic cardiac neurons that predicated their response characteristics to select cardiovascular stressors (Kember *et al.*, 2001;Waldmann *et al.*, 2006). For example, the baseline activity displayed by many IC neurons appeared to determine how such neurons responded to specific afferent versus efferent neuronal inputs to the ICN. Those IC neurons exhibiting low basal firing levels in control states were for the most part activated by cardiovascular stressors, while those exhibiting higher basal firing rates tended to be suppressed by cardiovascular stressors.

Fundamental to our understanding of control of regional cardiac indices, the various subsets of IC neurons identified in this study demonstrated interdependent neuronal behavior, even during basal states. Previous work from our laboratory has suggested that such neuronal activity interdependence is indicative

of common shared cardiopulmonary afferent inputs to the ICN, ones that primarily rely upon functional interconnectivity mediated via i) local network interactions and ii) divergent descending projections from higher centers to the distributive IC networks (Waldmann *et al.*, 2006;Armour *et al.*, 1998;Thompson *et al.*, 2000;Randall *et al.*, 2003). As such, we have proposed that this overlapping control system allows for effective local reflex modulation of regional cardiac electrical and mechanical indices while, at the same time, providing a peripheral substrate for higher centers to impact cardiac function (Armour, 2008;Ardell, 2004).

The intrinsic cardiac nervous system is a distributed neural network capable of independent and interdependent reflex processing of cardiac sensory and central neuronal inputs (Ardell *et al.*, 1991;Thompson *et al.*, 2000;Waldmann *et al.*, 2006). While efferent postganglionic neuronal outputs to the target organ arising from the various aggregates of intrinsic cardiac ganglia exert preferential control over different regions of the heart, many exert modulating effects over divergent cardiac regions because of ICNS interconnectivity (e.g. RAGP neurons evaluated herein are primarily associated with control of atrial function) (Ardell & Randall, 1986;Yuan *et al.*, 1994;Gray *et al.*, 2004a). Thus, changes can occur in the function of neurons located in multiple sites throughout the intrinsic cardiac nervous system in response to activation of select populations throughout the intrinsic cardiac ganglionated plexus (Cardinal *et al.*, 2009;Yuan *et al.*, 1993). This distribution/cascade of IC control ultimately results in a combined capacity to influence tissues throughout the atria and ventricles.

The intrinsic cardiac nervous system is composed of a heterogeneous population of neurons (Gagliardi *et al.*, 1988;Adams & Cuevas, 2004;Parsons, 2004;McAllen *et al.*, 2011). Prior neurophysiological studies using unipolar recording electrodes demonstrated that its neurons can be functionally sub-divided into afferent, local circuit and efferent neurons, the latter involving both sympathetic and the expected parasympathetic post-ganglionic neurons (Armour, 2008;Ardell, 2004). Prior anatomical and immunohistochemistry approaches have supported this stratification in both humans and various animal models (Yuan *et al.*, 1994;Armour *et al.*, 1997;Parsons, 2004;Hoover *et al.*, 2009). Utilizing classical neurophysiological-based definitions, afferent neurons would be defined as those transducing a

circumscribed cardiac receptor field with preferential modality sensitivity (Armour & Kember, 2004;Kember *et al.*, 2001;Brown, 1979;Zucker & Gilmore, 1991). Classically, autonomic efferent post-ganglionic neurons would be defined as those somata that can be activated mono-synaptically following application of electrical stimuli to their pre-ganglionic neuronal inputs (c.f., the vagi or stellate ganglia) (Langley, 1921). The population of local circuit neurons (LCN's) would be the remainder of the neurons of the ICNS that receive i) secondary inputs from the IC afferent/efferent subpopulations depicted above, in addition to ii) interconnecting inputs derived from other LCN's located in the same or other ganglia.

It has been proposed that the primary functions of the IC LCN's are to: i) process and coordinate dynamic afferent and efferent derived inputs which, in turn, ii) modulate efferent postganglionic neuronal outputs to various cardiac regions (Armour, 2008;Ardell, 2004). Given these assumptions and, as summarized in Figure 11, we now propose that LCN populations should be subdivided into three basic sub-classes: i) secondary afferent LCN's, ii) secondary efferent LCN's and iii) the convergent LCN's. Each of these sub-populations exhibit preferential response characteristics to various stressors. For instance, secondary afferent LCNs transduced multiple and divergent cardiopulmonary mechanical and chemical stressors in a secondary fashion. In like manner, the secondary efferent LCN's received multiple indirect (not monosynaptic), but consistent, inputs from central sympathetic and/or parasympathetic efferent neuronal sources. Our data further indicates that many of these secondary afferent and efferent LCN's interconnect with other populations - the convergent LCN's. The convergent LCN's can be best represented by their collective responsiveness to excessive nerve input imbalances – as induced in this study by mediastinal nerve stimulation.

II.4.1. Central neuronal command

Central and peripheral aspects of the cardiac nervous system act synergistically to regulate regional cardiac function (Ardell, 2004;Armour, 2008;Herring & Paterson, 2009;Armour *et al.*, 1998;McAllen *et al.*, 2011). Data derived from this study indicate that over half (56%) of IC neurons received indirect and frequently convergent central efferent neuronal inputs via the vagi and/or stellate

ganglia. In accord with that finding, there was a substantial convergence of right and left-sided autonomic efferent inputs onto identified IC neurons. For instance, of the 42% of the population that responded indirectly to sympathetic efferent neuronal inputs, 56% were modulated by unilateral inputs and 44% by inputs from both stellate ganglia. Correspondingly, of the 41% of IC neurons that responded to vagal nerve stimulation, 63% responded to unilateral inputs and 37% to bilateral inputs. The predominant effect of these central neuronal inputs to ICN was excitatory in nature. Of those IC neurons responding to electrical stimulation of these preganglionic inputs, 25% responded solely to stellate stimulation and 25% responded solely to vagal activation; these neurons are defined as secondary efferent LCN's since they did not respond with fixed latencies in response to preganglionic efferent stimulation. The remaining 50% of IC neurons identified by autonomic stimulation responded to inputs from both central neuronal sources: at least one stellate ganglion and one cervical vagus; these neurons are defined as convergent LCN's. This latter population may sub serve, in part, the role of sympathetic/parasympathetic interactive control of regional cardiac function - as occurs at ICN sites separate from the end-effectors (Herring & Paterson, 2009;McGuirt *et al.*, 1997;Furukawa *et al.*, 1996;Randall *et al.*, 1998).

II.4.2. Sensory neural inputs

For a given CV afferent stressor or combination of stressors, the evoked change in neuronal activity of a given responsive IC neuron was found to be reproducible. Few of these afferent could be classified as primary afferent neurons, ones that responded to one modality arising from a restricted receptive field (Armour & Kember, 2004;Brown, 1979). Thus, the majority of the neurons so identified can be more appropriately classified as secondary afferent LCN's.

Secondary afferent LCN's transduce multimodal inputs from the great thoracic vessels and/or different regions of the heart. In fact, in some cases mechano-sensitive afferent inputs arising from these different cardiopulmonary regions evoked directionally differential responses in various IC neurons, even when studying the evoked activities generated by adjacent IC neurons. About 50% of the IC neurons that exhibited cardiac cycle related periodicity displayed activity primarily during late diastole into

isovolumetric contraction. Furthermore, those IC neurons that displayed cardiac cycle related periodicity were found to preferentially transduce not only mechano-sensitive inputs but also descending central neuronal efferent inputs. This observation demonstrates the diverse nature of the processing capabilities of individual IC neurons. It also suggest an ability of the network to respond appropriately to multiple afferent inputs arising from different cardiac regions or from major vessel adjacent to the heart. All of which indicates that the afferent neuronal transduction capabilities of the ICN may account for the fact that many of its neurons involved in the synchrony of regional cardiac contractile function display beat-to beat activity patterns reflective of regional cardiodynamics (Armour, 2008;Armour *et al.*, 1998).

It has been proposed that common shared CV afferent neuronal inputs can sub serve a primary role in the interactive behavior among neuronal somata in i) the ICN and ii) extracardiac intrathoracic autonomic ganglia (including the mediastinal, middle cervical and stellate ganglia) in the determination of local network interactions (Kember *et al.*, 2001;Armour & Kember, 2004;Waldmann *et al.*, 2006;Armour *et al.*, 1998). As such, these can be impacted by cardiovascular stressors, including: 1) asymmetric activation of extracardiac neuronal inputs into and between IC nerve networks in the induction of atrial arrhythmogenesis (Armour *et al.*, 2005;Richer *et al.*, 2008); and 2) ischemic-induced excessive activation of ventricular afferent neurons inducing heterogeneous and excessive activation of local circuit neurons (Huang *et al.*, 1993;Waldmann *et al.*, 2006;Armour *et al.*, 1998). Such derangements of cardiac control networks can contribute to the potential for sudden cardiac death (Schwartz *et al.*, 1992;Vanoli *et al.*, 2008;Billman, 2006). Moreover, disruptions and remodeling of network interconnections within the cardiac nervous system, including its ICNS elements (Hardwick *et al.*, 2012;Bibevski & Dunlap, 2011), are associated with chronic ischemic heart disease or congestive heart failure and likely contribute to the evolution of these pathologies (Chen *et al.*, 2001;Zucker *et al.*, 2012;Bibevski & Dunlap, 2011;Arora *et al.*, 2003;Nguyen *et al.*, 2012;Lopshire *et al.*, 2009;Shinohara *et al.*, 2012).

II.4.3. Atrial arrhythmia induction

Heterogeneous activation of the cardiac nervous system can exert destabilizing influences on cardiac electrical indices (Armour *et al.*, 2005;Scherlag *et al.*, 2006;Billman, 2006). In response to mediastinal nerve stimulation, most IC neurons became excessively activated – including many previously inactive ones; such alteration in network behavior preceded and persisted throughout such induced AF (Gibbons *et al.*, 2012). We have previous shown that suppression of IC function via targeted neuromodulation therapy mitigates this MSN induced AF potential (Gibbons *et al.*, 2012).

In the current study we found that most IC neurons that responded to excessive MSN inputs likewise demonstrated preferential and extensive inputs from cardiopulmonary afferents. While some MSN-sensitive IC neurons received inputs from both the vagi and stellate ganglia, their response characteristics to central efferent autonomic inputs was not predictive of their potential contribution to local neuronal imbalance leading to AF induction. Based upon their integrated response to imposed afferent and efferent stressor, the data derived herein suggest that IC neurons that responded to MNS with enhanced activity were most likely made up of the convergent LCN population. Future studies should focus on which sub-populations of IC neurons are targeted by neuromodulation based therapies, either electrical or pharmacological (Armour *et al.*, 2005;Lopshire *et al.*, 2009;Gibbons *et al.*, 2012), for effective control of cardiac arrhythmias.

II.4.4. Myocardial ischemia

Transient myocardial ischemia impacts cardiomyocytes as well as neurons in multiple levels of the cardiac nervous system that regulate them (Waldmann *et al.*, 2006;Ardell *et al.*, 2009;Southerland *et al.*, 2007;Southerland *et al.*, 2012). The resultant local metabolic factors, coupled with reflex evoked changes in neurotransmitter release, are principal factors in augmenting the arrhythmogenic substrate, including the potential for sudden cardiac death, and subsequent apoptosis of affected myocytes within the ischemic zone (Cohen & Downey, 2011;Southerland *et al.*, 2012;Billman, 2006;Crow *et al.*, 2004).

As determined previously and confirmed herein, myocardial ischemia impacts IC neural network interactions, doing so primarily via cardiac afferent neuronal activation (Huang *et al.*, 1993;Armour *et al.*, 2002;Waldmann *et al.*, 2006). Activation of ischemic-sensitive afferent neurons can impact various elements within the ICN (Armour *et al.*, 2002;Foreman *et al.*, 2000), even when they are disconnected from higher centers of the cardiac neuronal hierarchy (Huang *et al.*, 1993). Data derived in this study demonstrates that myocardial ischemia transducing IC neurons which are preferentially influenced by cardiac afferent activation exhibit no selectivity with respect to central efferent neuronal inputs when compared to populations of non-ischemic sensitive IC neurons. These data apparently reflect the differential processing capabilities of the ICNS convergent local circuit neuronal population. Recent studies have demonstrated that neuromodulation therapy has the potential to modulate arrhythmogenic and apoptotic potentials to ischemic stressors (Southerland *et al.*, 2007;Southerland *et al.*, 2012;Ardell *et al.*, 2009;Lopshire *et al.*, 2009). Future studies should consider whether selective sub-populations of peripheral autonomic (intrathoracic extracardiac and intrinsic cardiac) neurons can be targeted therapeutically to provide cardioprotection in the presence of stressors.

II.5. Conclusion and significance

The data derived from these studies indicate that the intrinsic cardiac nervous system is composed of heterogeneous populations of neurons, as identified functionally herein and in the past by anatomical means (Armour *et al.*, 1997;Hoover *et al.*, 2009;Parsons, 2004;Yuan *et al.*, 1994), that act synergistically with one another and with neurons in intrathoracic extracardiac ganglia, the spinal cord, the brainstem and higher centers in the control of regional cardiac function (Brown, 1979;Zucker & Gilmore, 1991;Armour, 2008;McAllen *et al.*, 2011;Southerland *et al.*, 2012). Such an arrangement subtends in both normal and stressed states. The relative contribution of central versus peripheral aspects of the cardiac nervous system varies (Lopshire *et al.*, 2009;Southerland *et al.*, 2007;Southerland *et al.*, 2012;Zhang *et al.*, 2009), according to the stressor applied (e.g., regional cardiac mechanical vs ischemic perturbations) and the adapations so engendered in the neurohumoral control systems (Armour *et al.*, 1998;Zucker *et al.*,

2012;Mill *et al.*, 2011;Hardwick *et al.*, 2012). As such, neurons located in each level of the cardiac neuronal hierarchy (intrinsic cardiac, intrathoracic extracardiac and central) interact in an ongoing dynamic fashion to insure adequate cardiac output that meets the demands imposed by transient alterations in bodily functions (Armour, 2008;Zucker *et al.*, 2012).

This study makes evident the fact that the major population of intrinsic cardiac neurons involved in cardiac regulation is local circuit in nature. Thus for efferent control, while a limited population of identified IC neurons received direct centrally derived preganglionic inputs, a sizable population of intrinsic cardiac neurons transduced indirect inputs from one or more central neuronal source (medulla vs spinal cord neurons). As such the population of LCNs that receive preferential, but not direct, central neuronal inputs we have defined as secondary efferent LCN's. This subpopulation of IC neurons may subserve a major role in coordinating autonomic interactions on the target organ itself. Importantly, this population is likely involved in arrhythmia formation when its stochastic interactions become activated excessively.

A number of LCN's received preferential and divergent cardiopulmonary afferent inputs (Fig. 11). While some of the IC neurons that responded to touch or great vessel occlusions could be primary afferents, the majority exhibited wide-field distributions and responded to multi-modal inputs; these we define as secondary afferent LCN's. As such, this may be the population that becomes excessively activated in the transduction of ventricular ischemia. In addition, it appears that the networks interactions that occur among the various LCN populations distributed throughout the ICN do so primarily via the convergent LCN population.

Under basal conditions the low level, stochastic interactivity that occurs among the different ICN populations apparently acts to coordinate regional cardiac function. Yet, asymmetric changes in afferent or efferent inputs to that population can evoke disorganized responses with resultant imbalances in efferent distributions to tissues throughout the heart (Armour *et al.*, 2005;Scherlag *et al.*, 2005;Issa *et al.*, 2005). Such a state can readily lead to cardiac arrhythmia induction (Scherlag *et al.*, 2006;Billman, 2006;Armour, 2008). By inference, stabilization of IC/LCN stochastic interactivity may represent a novel

approach for the suppression of atrial arrhythmia formation (Gibbons *et al.*, 2012). Centrally mediated coordination of disparate IC neuronal networks likewise may find applications in the therapeutic management of compromised contractile function, such as occurs during the evolution of CHF with its attendant abnormal neurohumoral engagement (Zucker *et al.*, 2012;Lopshire *et al.*, 2009;Liu *et al.*, 2012)**.** Future studies should consider the contribution of differential remodeling of select neuronal populations within the cardiac neuronal hierarchy in response to evolution of cardiac pathology in order to exploit potential neural targets to mitigate the adverse consequences of abnormal cardiac neuronal hierarchy function that attends cardiac disease.

Author contributions

J.L.A., J.A.A., E.B. and E.M.S. designed and performed the experiments. V.J. and A.V. developed the signal processing methodology. S.S. implemented the signal processing tools. J.L.A., S.S. and E.B. analyzed the data. All authors contributed to writing the paper and approved the manuscript.

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Reference List

Adams DJ & Cuevas J (2004). Electrophysiological properties of intrinsic cardiac neurons. In *Basic and Clinical Neurocardiology*, eds. Armour JA & Ardell JL, pp. 1-60. Oxford Univ. Press, NY.

Am.Physiol.Society (2002). Guiding principles for research involving animals and human beings. *Am J Physiol Regulatory Integrative Comp Physiol* **283**, R281-R283.

Andresen MC, Kunze DL, & Mendelowitz D (2004). Central nervous system regulation of the heart. In *Basic and Clinical Neurocardiology*, eds. Armour JA & Ardell JL, pp. 187-219. Oxford Univ. Press, NY.

Ardell JL (2004). Intrathoracic neuronal regulation of cardiac function. In *Basic and Clinical Neurocardiology*, eds. Armour JA & Ardell JL, pp. 118-152. Oxford University Press, New York.

Ardell JL, Butler CK, Smith FM, Hopkins DA, & Armour JA (1991). Activity of in vivo atrial and ventricular neurons in chronic decentralized canine hearts. *Am J Physiol* **260**, H713-H721.

Ardell JL, Cardinal R, Vermeulen M, & Armour JA (2009). Dorsal spinal cord stimulation obtunds the capacity of intrathoracic extracardiac neurons to transduce myocardial ischemia. *Am J Physiol Heart Circ Physiol* **297**, R470-R477.

Ardell JL & Randall WC (1986). Selective vagal innervation of sinoatrial and atrioventricular nodes in canine heart. *Am J Physiol* **251**, H764-H773.

Armour JA (2008). Potential clincial relevance of the 'little brain' on the mammalian heart. *Exp Physiol* **93.2**, 165-176.

Armour JA, Collier K, Kember G, & Ardell JL (1998). Differential selectivity of cardiac neurons in separate intrathoracic autonomic ganglia. *Am J Physiol* **274**, R939-R949.

Armour JA & Hopkins DA (1990). Activity of in vivo ventricular neurons. *Am J Physiol* **258**, H326-H336.

Armour JA & Janes RD (1988). Neuronal activity recorded extracellularly from *in situ* mediastinal ganglia. *Can J Physiol Pharmacol* **66**, 119-127.

Armour JA & Kember G (2004). Cardiac Sensory Neurons. In *Basic and Clinical Neurocardiology*, eds. Armour JA & Ardell JL, pp. 79-117. Oxford Univ. Press, NY.

Armour JA, Linderoth B, Arora RC, DeJongste MJL, Ardell JL, Kingma JG, Hill M, & Foreman RD (2002). Long-term modulation of the intrinsic cardiac nervous system by spinal cord neurons in normal and ischemic hearts. *Autonomic Neuroscience: Basic and Clinical* **95**, 71-79.

Armour JA, Murphy DA, Yuan BX, MacDonald S, & Hopkins DA (1997). Gross and microscopic anatomy of the human intrinsic cardiac nervous system. *The Anatomical Record* **247**, 289-298.

Armour JA, Richer LP, Pagé PL, Vinet A, Kus T, Vermeulen M, Nadeau R, & Cardinal R (2005). Origin and pharmacological response of atrial tachyarrhythmias induced by activation of mediastinal nerves in canines. *Autonomic Neuroscience: Basic and Clinical* **118**, 68-78.

Arora RC, Cardinal R, Smith FM, Ardell JL, Dell'Italia LJ, & Armour JA (2003). Intrinsic cardiac nervous system in tachycardia induced heart failure. *Am J Physiol Regulatory Integrative Comp Physiol* **285**, R1212-R1223.

Bibevski S & Dunlap ME (2011). Evidence for impaired vagus nerve activity in heart failure. *Heart Fail Rev* **16**, 129-135.

Billman GE (2006). A comprehensive review and analysis of 25 years of data from an in vivo canine model of sudden cardiac death: Implications for future anti-arrhythmic drug development. *Pharmacology & Therapeutics* **111**, 808-835.

Brown AM (1979). Cardiac reflexes. In *Handbook of Physiology, The Cardiovascular System, Section 2, Vol.1, The Heart*, eds. Berne RM, Sperelakis N, & Geiger SR, pp. 677-689. American Physiological Society (Williams and Wilkins), Bethesda.

Cardinal R, Pagé P, Ardell JL, & Armour JA (2009). Spatially divergent cardiac responses to nicotinic stimulation of ganglionated plexus neurons in the canine heart. *Autonomic Neuroscience: Basic and Clinical* **145**, 55-62.

Chen P-S, Chen LS, Cao J-M, Sharifi B, Karagueuzian HS, & Fishbein MC (2001). Sympathetic nerve sprouting, electrical remodeling and the mechanisms of sudden cardiac death. *Cardiov Res* **50**, 409-416.

Cohen MV & Downey JM (2011). Ischemic postconditioning: from receptor to end-effector. *Antioxid Redox Signal* **14**, 821-831.

Crow MT, Mani K, Nam YJ, & Kitsis RN (2004). The mitochondrial death pathway and cardiac myocyte apoptosis. *Circ Res* **95**, 957-970.

Dell'Italia LJ & Ardell JL (2004). Sympathetic nervous system in the evolution of heart failure. In *Basic and Clinical Neurocardiology*, eds. Armour JA & Ardell JL, pp. 340-367. Oxford University Press, New York.

Foreman RD, Linderoth B, DeJongste MJL, Ardell JL, & Armour JA (2000). Central and peripheral mechanisms evoked by spinal cord stimulation (SCS) for angina pectoris. In *Management of Acute and Chronic Pain*, eds. Krames E & Reig E, pp. 597-604.

Furukawa Y, Hoyano Y, & Chiba S (1996). Parasympathetic inhibition of sympathetic effects on sinus rate in anesthetized dogs. *Am J Physiol Heart Circ Physiol* **271**, H44-H50.

Gagliardi M, Randall WC, Bieger D, Wurster RD, Hopkins DA, & Armour JA (1988). Activity of in vivo canine cardiac plexus neurons. *Am J Physiol* **255**, H789-H800.

Gibbons DD, Southerland EM, Hoover DB, Beaumont E, Armour JA, & Ardell JL (2012). Neuromodulation targets intrinsic cardiac neurons to attenuate neuronally mediated atrial arrhythmias. *Am J Physiol Regul Integr Comp Physiol* **302**, R357-R364.

Gray AL, Johnson CI, Ardell JL, & Massari VJ (2004a). Parasympathetic control of the heart. A novel interganglionic intrinsic cardiac circuit mediates neural control of the heart. *J Applied Physiol* **96**, 2273-2278.

Gray AL, Johnson TA, Lauenstein JM, Newton GE, Ardell JL, & Massari VJ (2004b). Parasympathetic control of the heart III. Neuropeptide Y-immunoreactive nerve terminals synapse on three populations of negative chronotropic vagal preganglionic neurons. *J Applied Physiol* **96**, 2279-2287.

Hardwick JC, Southerland EM, Girasole AE, Ryan SE, Negrotto S, & Ardell JL (2012). Remodeling of intrinsic cardiac neurons: effects of beta-adrenergic receptor blockade in guinea pig models of chronic heart disease. *Am J Physiol Regul Integr Comp Physiol* **303**, R950-R958.

Herring N & Paterson DJ (2009). Neuromodulators of peripheral cardiac sympatho-vagal balance. *Exp Physiol* **94**, 46-53.

Hoover DB, Isaacs ER, Jacques F, Hoard JL, Pagé P, & Armour JA (2009). Localization of multiple neurotransmitters in surgically derived specimens of human atrial ganglia. *Neurosci* **164**, 1170-1179.

Huang MH, Ardell JL, Hanna BD, Wolf SG, & Armour JA (1993). Effects of transient coronary artery occlusion on canine intrinsic cardiac neuronal activity. *Integ Physiol Behav Sci* **28**, 5-21.

Issa ZF, Zhou X, Ujhelyi MR, Rosenberger J, Bhakta D, Groh WJ, Miller J.M., & Zipes DP (2005). Thoracic spinal cord stimulation reduces the risk of ischemic ventricular arrhythmias in a post-infarction heart failure canine model. *Circ* **111**, 3217-3220.

Kember G, Fenton GA, Armour JA, & Kalyaniwalla N (2001). Competition model for aperiodic stochastic resonance in a Fitzhugh-Nagumo model of cardiac sensory neurons. *Phys Rev E Stat Nonlin Soft Matter Phys* **63**, 1-6.

Langley G (1921). *The Autonomic Nervous System* Cambridge University Press, Cambridge, UK.

Liu Y, Yue WS, Liao SY, Zhang Y, Au KW, Shuto C, Hata C, Park E, Chen P, Siu CW, & Tse HF (2012). Thoracic spinal cord stimulation improves cardiac contractile function and myocardial oxygen consumption in a porcine model of ischemic heart failure. *J Cardiovasc Electrophysiol* **23**, 534-540.

Lopshire JC, Zhou X, Dusa C, Ueyama T, Rosenberger J, Courtney N, Ujhelyi M, Mullen T, Das M, & Zipes DP (2009). Spinal cord stimulation improves ventricular function and reduces ventricular arrhythmias in a canine postinfarction heart failure model. *Circulation* **120**, 286-294.

McAllen RM, Salo LM, Paton JFR, & Pickering AE (2011). Processing of central and reflex vagal drives by rat cardiac ganglion neurones: an intracellular analysis. *J Physiol* **589.23**, 5801-5818.

McGuirt AS, Schmacht DC, & Ardell JL (1997). Autonomic interactions for control of atrial rate are maintained after SA nodal parasympathectomy. *Am J Physiol* **272**, H2525-H2533.

Mill JG, Stefanon I, & Baldo MP (2011). Remodeling in the ischemic heart: the stepwise progression for heart failure. *Braz J Med Biol Res* **44**, 890-898.

Nguyen BL, Li H, Fishbein MC, Lin SF, Gaudio C, Chen PS, & Chen LS (2012). Acute myocardial infarction induces bilateral stellate ganglia neural remodeling in rabbits. *Cardiovasc Pathol* **21**, 143-148.

Parsons RL (2004). Mammalian cardiac ganglia as local integration centers: histochemical and electrophysiological evidence. In *Neural Mechanisms of Cardiovascular Regulation*, eds. Dun NJ, Machado BH, & Pilowsky PM, pp. 335-356. Kluwer Academic Publishers, Boston.

Randall DC, Brown DR, Li SG, Olmstead ME, Kilgore JM, Sprinkle AG, Randall WC, & Ardell JL (1998). Ablation of posterior atrial ganglionated plexus potentiates sympathetic tachycardia to behavioral stress. *Am J Physiol* **275**, R779-R787.

Randall DC, Brown DR, McGuirt AS, Thompson G, Armour JA, & Ardell JL (2003). Interactions within the intrinsic cardiac nervous system contribute to chronotropic regulation. *Am J Physiol Regulatory Integrative Comp Physiol* **285**, R1066-R1075.

Richer LP, Vinet A, Kus T, Cardinal R, Ardell JL, & Armour JA (2008). □□Adrenoceptor blockade modifies neurally induced atrial arrhythmias. *Am J Physiol Regulatory Integrative Comp Physiol* **295**, R1175-R1180.

Scherlag BJ, Nakagawa JH, Jackman WM, Yamanashi WS, Patterson E, Po S, & Lazzara R (2005). Electrical stimulation to identify neural elements on the heart: their role in atrial fibrillation. *J Interv Card Electrophysiol* **13**, 37-42.

Scherlag BJ, Patterson E, & Po SS (2006). The neual basis of atrial fibrillation. *J Electrophysiology* **39**, S180-S183.

Schwartz PJ, La Rovere MT, & Vanoli E (1992). Autonomic nervous system and sudden cardiac death. Experimental basis and clinical observations for post-myocardial infarction risk stratification. *Circulation* **85**, I77-I91.

Shen MJ, Choi E-K, Tan AY, Han S, Shinohara T, Maruyama M, Chen LS, Shen C, Hwang C, Lin S-F, & Chen P-S (2011). Patterns of baseline autonomic nerve activity and development of pacing-induced atrial fibrillation. *Heart Rhythm* **8**, 583-589.

Shin HC, Aggarwal V, Acharya S, Schieber MH, & Thakor NV (2010). Neural decoding of finger movements using Skellam-based maximum-likelihood decoding. *IEEE Trans Biomed Eng* **57**, 754-760.

Shinohara T, Shen MJ, Han S, Maruyama M, Park HW, Fishbein MC, Shen C, Chen PS, & Lin SF (2012). Heart failure decreases nerve activity in the right atrial ganglionated plexus. *J Cardiovasc Electrophysiol* **23**, 404-412.

Skellam JG (1946). The frequency distribution of the difference between two Poisson variates belonging to different populations. *J R Stat Soc Ser A* **109(Pt 3)**, 296.

Southerland EM, Milhorn D, Foreman RD, Linderoth B, DeJongste MJL, Armour JA, Subramanian V, Singh M, Singh K, & Ardell JL (2007). Preemptive, but not reactive, spinal cord stimulation mitigates transient ischemia-induced infarction via cardiac adrenergic neurons. *Am J Physiol Heart Circ Physiol* **292**, H311-H317.

Southerland EM, Gibbons DD, Smith SB, Sipe A, Williams CA, Beaumont E, Armour JA, Foreman RD, & Ardell JL (2012). Activated cranial cervical cord neurons affect left ventricular infarct size and the potential for sudden cardiac death. *Auton Neurosci* **169**, 34-42.

Strackee J & Deneir van der Gan JJ (1962). The frequency distribution of the difference between two Poisson variates. *Statistica Neerlandica* **16**, 17-23.

Taylor EW, Jordan D, & Coote JH (1999). Central control of the cardiovascular and respiratory systems and their interactions in vertebrates. *Physiol Rev* **79**, 855-915.

Thompson GW, Collier K, Ardell JL, Kember G, & Armour JA (2000). Functional interdependence of neurons in a single canine intrinsic cardiac ganglionated plexus. *J Physiol* **528**, 561-571.

Vanoli E, Adamson PB, Foreman RD, & Schwartz PJ (2008). Prediction of unexpected sudden death among healthy dogs by a novel marker of autonomic neural activity. *Heart Rhythm* **5**, 300-305.

Waldmann M, Thompson GW, Kember G, Ardell JL, & Armour JA (2006). Stochastic behavior of atrial and ventricular intrinsic cardiac neurons. *J Applied Physiol* **101**, 1-7.

Yuan BX, Ardell JL, Hopkins DA, & Armour JA (1993). Differential cardiac responses induced by nicotine sensitive canine atrial and ventricular neurons. *Cardiov Res* **27**, 760-769.

Yuan BX, Ardell JL, Hopkins DA, Losier AM, & Armour JA (1994). Gross and microscopic anatomy of the canine intrinsic cardiac nervous system. *The Anatomical Record* **239**, 75-87.

Zhang Y, Popovic ZB, Bibevski S, Fakhry I, Sica DA, Van Wagoner DR, & Mazgalev TN (2009). Chronic vagus nerve stimulation improves autonomic control and attenuates systemic inflammation and heart failure progression in a canine high-rate pacing model. *Circ Heart Fail* **2**, 692-699.

Zucker IH & Gilmore JP. Reflex Control of the Circulation. 1991. Boca Raton, CRC Press.

Zucker IH, Patel KP, & Schultz HD (2012). Neurohumoral stimulation. *Heart Fail Clinics* **8**, 87-99.

Appendix 1: Significance of changes in firing rate

Changes identified in neuronal activity were compared at different time windows by calculating the average firing rate over time. The significance level of the observed differences in firing rate was assessed using a statistical test recently utilized in a study of primary motor cortex neurons (Shin *et al.*, 2010). The resulting p-value is a function of four parameters: the duration of the two time windows and the number of firings in each time window.

The null hypothesis is that the two firing rates are equal. For this analysis, it is assumed that the number of action potentials identified follows a Poisson distribution in each time window and that the difference in the activities follows a Skellam distribution (Skellam, 1946;Strackee & Deneir van der Gan, 1962). Parameters can then be estimated using the maximum likelihood approach. From the Skellam cumulative distribution function, the probability that the difference in number of firings is larger than the observed value provides the desired p-value (unilateral test). The test was implemented in Matlab and adapted from the R package "skellam" by Jerry W. Lewis. Two significance levels were used: 1% and 5%. Figure 2 shows the firing rate-dependent thresholds of significant increase and decrease of firing rate. Note that when the duration of the time windows are different the regions are asymmetric (Fig. 2B).

The advantages of this method are its simplicity, the robustness of its parameter estimation and its applicability in the case of low firing rates, including when the firing rate is zero. Its limitations are the assumptions of stationarity and Poisson-distributed firings. In contrast with cortical neurons, neurons in the right atrium ganglionated plexus tend to fire at low frequency unless a special event (e.g. atrial fibrillation) occurs. As a result, the limited number of firings in the time windows prevents robust estimation of a larger number of parameters.

Figure legends

Table 1. IC neuronal activity (mean ± SD) at baseline and in response to indicated stressors (see abbreviations). Responses are subdivided based upon evoked increases in activity (top panels) and decreased activity (bottom panels). P values derived based on analysis detailed in appendix and Fig. 2. Based on that analysis, responses are subdivided based upon evoked responses with $p<0.01$ (left panels) and $p<0.05$ (right panels).

Figure 1. Methodology for the identification of individual IC neurons. Traces indicate: (*a*) Left ventricular chamber pressure (LVP); (b) electrocardiogram (ECG); (*c*) right atrial electrogram (RAE); (*d*) event channel created from identified electrical/mechanical artifacts; (*e, g*) raw signal recordings of two channels from the multichannel linear microarray electrode; (*f, h*) neuronal recordings from channels (*e*) and (*g*) after artifact removal based on the event channel (*d*); and (*i, j*) two final neuronal waveforms extracted from a stereotrode built from channels (*f*) and (*h*) using principal component analysis. These final waveforms (*i, j*) represent basal activity from two separate IC neurons located within the right atrial ganglionated plexus; such activity can be evaluated continuously and concurrently for hours and in response to imposed stressors.

Figure 2. Quantitative assessment of significance (P-values) when comparing the firing rate in two intervals: baseline to stress-evoked response. P-values are computed using the Skellam test and displayed as a function of the average firing rate in the first and in the second interval. The dark red and light red colors mean that the firing rate is strongly ($p<0.01$) or moderately decreasing ($0.01<p<0.05$) respectively. The dark green and light green colors mean that the firing rate is strongly ($p<0.01$) or moderately increasing ($0.01<p<0.05$) respectively. (A) Both intervals last 60 sec (baseline interval 1 and response during stressor interval 2). (B) The first interval (baseline) has a duration of 60 sec and the second (response during stressor) has a duration of 5 sec.

Figure 3. Histogram of baseline frequencies of all identified IC neurons.

Figure 4. Subpopulations of IC neurons demonstrate cardiac-related neuronal activity. (A) LV pressure and representative examples of neurons that are primarily active during left ventricular ejection (neuron 1), during isovolumetric contraction phase for LV (neuron 2) or with activity independent of LVP (neuron 3). (B) For each of these neurons, the probability density of firing as a function of the position within the LVP cycle (expressed as a phase between 0 and 2π) is indicated along with the average LVP profile.

Figure 5. Long-term interdependent activity of two IC cardiovascular-related neurons. (A) ECG and concurrent spontaneous activity of two IC neurons. (B) Zoom of panel A over 4 cardiac cycles. (C) Spike triggered (neuron 1 to neuron 2) histogram of spontaneous activity for these two IC neurons recorded over 2 hours. Note maintained temporal relationship, but with some variation in such interdependent activity.

Figure 6. Left ventricular touch differentially modifies IC activity. The spiking activities concurrently recorded from 8 selected IC neurons in a single animal are shown. Vertical dotted lines indicate onset and offset of touch. Note that subpopulations of IC neurons shown diminished activity during touch (e.g. neurons 2 and 3), some are activated by touch (e.g. neuron 7) and some are unaffected (e.g. neuron 8).

Figure 7. Varied responses displayed by each neuron studied in response to differing sensory or central efferent neuronal stressors. Each horizontal column represents how each identified RA neuron in all 8 dogs responded to each of the stressors applied (horizontal row above). Each column is associated with a specific stressor: afferent activation (touch of right [RV] or left [LV] ventricle;

occlusion of inferior vena cava [IVC] or descending aorta; myocardial ischemia evoked by transient occlusion of left anterior descending coronary artery), activation of efferent inputs to the RAGP via electrical stimulation of the right (RCV) or left (LCV) cervical vagus or stellate ganglia (right, RSS; left, LSS), or global activation of the IC network evoked by electrical stimulation of mediastinal nerves (MNS) at levels sufficient to evoke atrial fibrillation (AF). The number (n) of neurons so identified in each animal is indicated to left. The significance of each change ranged from greatest ($p<0.01$) to moderate ($0.01<p<0.05$) to insignificant (N/A) (grey bars means that an intervention was not performed). Right hand column characterized whether neurons CV related activity occurred during diastole (D), isovolumetric contraction (C), LV ejection (E) or isovolumetric relaxation (R).

Figure 8. IC neurons with cardiac-related activity are preferentially active during diastole to isovolumetric contraction phases. Activity histograms for all identified IC neurons that generated at least 100 spikes at baseline (49 of 92). Shaded area indicates time of LV contractile phase. The activities of these IC neurons are sorted according to entropy of distribution. Classification of firing patterns, relative to cardiac cycle, indicated above each neuron based upon bin (or immediately adjacent bin) counts that exceed 30% of total activity. Distribution of firing was: 14 neurons active in diastole (D), 22 neurons active during involumetic contraction (IC), 10 neurons active during ejection phase (E), 7 units active during isovolumetric relaxation (IR). 10 neurons showed dual peaks. Six neurons that exhibit adequate basal activity failed to demonstrate cardiac-related periodicity in firing.

Figure 9. **IC neurons displaying cardiac related activity are modified differentially by afferent and efferent stressors.** Proportion (% responders) of IC neurons with basal cardiac (cardiac periodicity) vs non-cardiac cycle (no cardiac periodicity) related periodicities whose activity was modified by afferent neuronal inputs (top left panel: RV touch, LV touch, transient occlusion of IVC or descending Aorta), efferent neuronal inputs (top right panel, stellate ganglia; bottom left panel, cervical

vagi) or transient occlusion of the LAD (bottom right panel). Chi-square P values are indicated for each subclass of stressor.

Figure 10. Afferent sensitivity to mechanical stressors predicts IC responsiveness to ischemic or MNS stressors. Proportion of IC neurons modified by afferent stressors (RV touch, LV touch, transient occlusion of IVC or descending aorta) divided according to their sensitivity to transient LAD CAO (top panel) vs mediastinal nerve stimulation (bottom panel). Chi-square P values are indicated for each subclass of stressor.

Figure 11. Interdependent activity among IC neurons in response to transient afferent or efferent stressors. Panel A indicates the conditional probability that one neuron responding to one stressor (X axis) responded to another stressor (Y axis). Aor: aorta occlusion; other acronyms as in Fig. 7. Gray-scale indicates level of probability of each occurrence (0 to 1 in 0.2 increments). Panel B graphically indicates the pattern of interdependent interactions between applied stressors. Arrow thickness is proportional to the strength of conditional probability, whose value is also indicated next to each arrow. Only links with conditional probabilities ≥ 0.6 are displayed. Mediastinal nerve stimulation (MNS) is a preeminent stressor, evoking changes in 52% of recorded IC neurons (48 of 92). Interdependent interactions among the IC neurons in response to stressors fall into two principal categories, efferent dependent and afferent dependent; stressor evoked activity in both subpopulations of IC neurons likewise being predictive of activation in response to MNS. Seventeen % of recorded IC neurons (16 of 92) were not significantly affected by any of the stressors applied.

Table 1: Evoked changes in IC activity in response to afferent and efferent stressors.

increased FF (p<0.01)

Stressor	Baseline	Stress	p value	n
RV	0.01 ± 0.01	0.42 ± 0.19	p = 0.03	6
LV	0.25 ± 0.02	0.91 ± 0.19	p = 0.50	2
Ao Occl	0.05 ± 0.08	0.98 ± 1.03	p = 0.00	18
IVC Occl	0.12 ± 0.27	0.59 ± 0.87	p = 0.00	12
RCV	0.06 ± 0.10	0.73 ± 0.56	p = 0.00	17
LCV	0.13 ± 0.24	0.57 ± 0.58	p = 0.00	12
RSS	0.14 ± 0.13	0.71 ± 0.45	p = 0.00	10
LSS	0.16 ± 0.17	0.66 ± 0.37	p = 0.00	14
MNS	0.18 ± 0.90	1.43 ± 1.90	p = 0.00	35
LAD CAO	0.18 ± 0.12	0.78 ± 0.75	p = 0.03	6

increased FF (p<0.05)

Baseline	Stress	p value	n
0.03 ± 0.05	0.44 ± 0.28	p = 0.00	12
0.15 ± 0.12	0.64 ± 0.33	p = 0.12	4
0.04 ± 0.08	0.89 ± 1.01	p = 0.00	20
0.09 ± 0.23	0.46 ± 0.75	p = 0.00	17
0.05 ± 0.09	0.57 ± 0.56	p = 0.00	23
0.11 ± 0.22	0.51 ± 0.56	p = 0.00	14
0.15 ± 0.30	0.48 ± 0.50	p = 0.00	23
0.19 ± 0.20	0.60 ± 0.36	p = 0.00	21
0.15 ± 0.82	1.24 ± 1.79	p = 0.00	42
0.26 ± 0.23	0.66 ± 0.56	p = 0.00	14

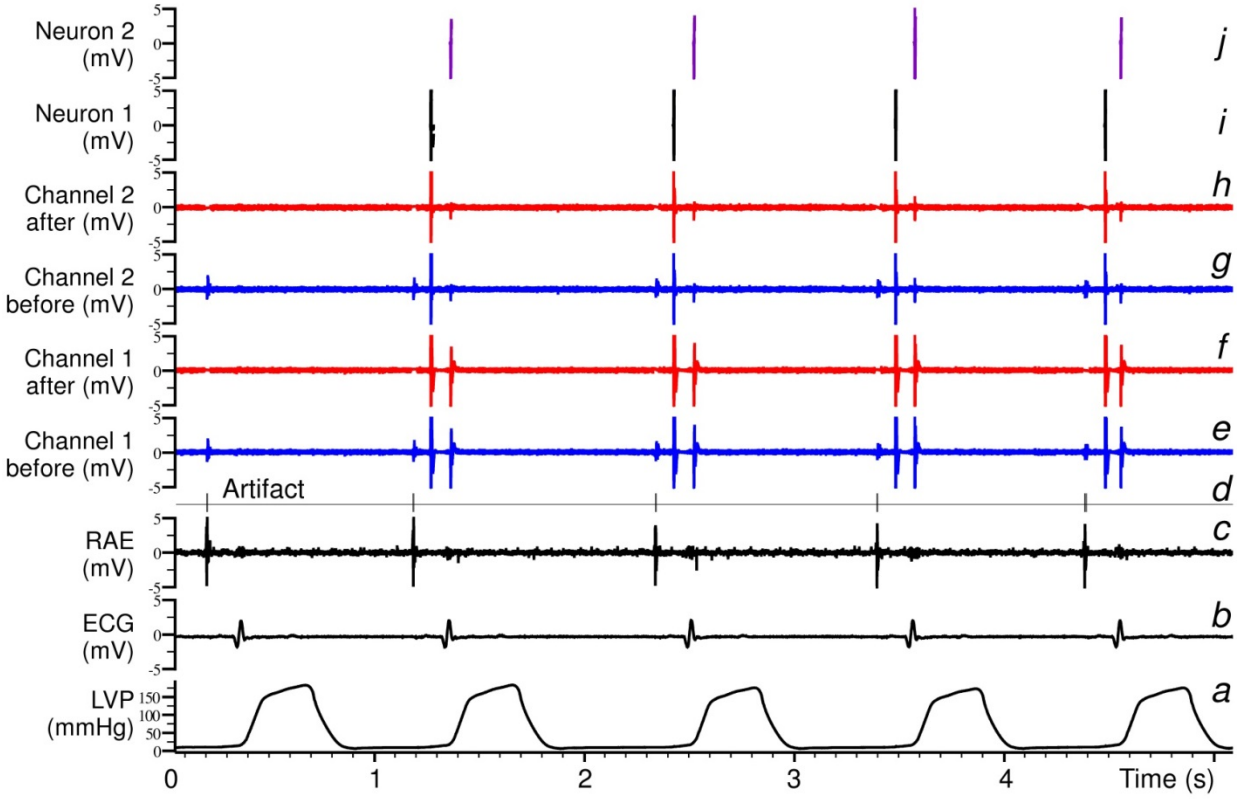
decreased FF (p<0.01)

RV	4.03 ± 0.00	0.12 ± 0.00	p = 1.00	1
LV	0.70 ± 0.52	0.26 ± 0.04	p = 0.50	2
Ao Occl	1.54 ± 0.93	0.51 ± 0.63	p = 0.03	6
IVC Occl	1.19 ± 1.39	0.12 ± 0.15	p = 0.06	5
RCV	0.74 ± 0.53	0.15 ± 0.19	p = 0.02	7
LCV	0.51 ± 0.40	0.12 ± 0.16	p=0.03	6
RSS	2.00 ± 2.44	1.29 ± 2.17	p = 0.25	3
LSS	1.54 ± 1.43	0.48 ± 0.55	p = .12	4
MNS	0.79 ± 0.21	0.08 ± 0.10	p = .25	3
LAD CAO	0.64 ± 0.54	0.17 ± 0.29	p = 0.01	8

decreased FF (p<0.05)

2.67 ± 1.92	0.12 ± 0.00	p = 0.50	2
0.67 ± 0.37	0.22 ± 0.08	p = 0.25	3
1.33 ± 0.89	0.44 ± 0.57	p = 0.01	8
0.79 ± 1.18	0.08 ± 0.13	p = 0.01	8
0.67 ± 0.53	0.14 ± 0.18	p = 0.01	8
0.53 ± 0.37	0.15 ± 0.18	p = 0.02	7
1.53 ± 2.20	0.97 ± 1.88	p = 0.12	4
1.00 ± 1.13	0.37 ± 0.43	p = 0.01	8
0.51 ± 0.35	0.07 ± 0.09	p = 0.03	6
0.48 ± 0.49	0.13 ± 0.24	p = 0.00	12

Figure 1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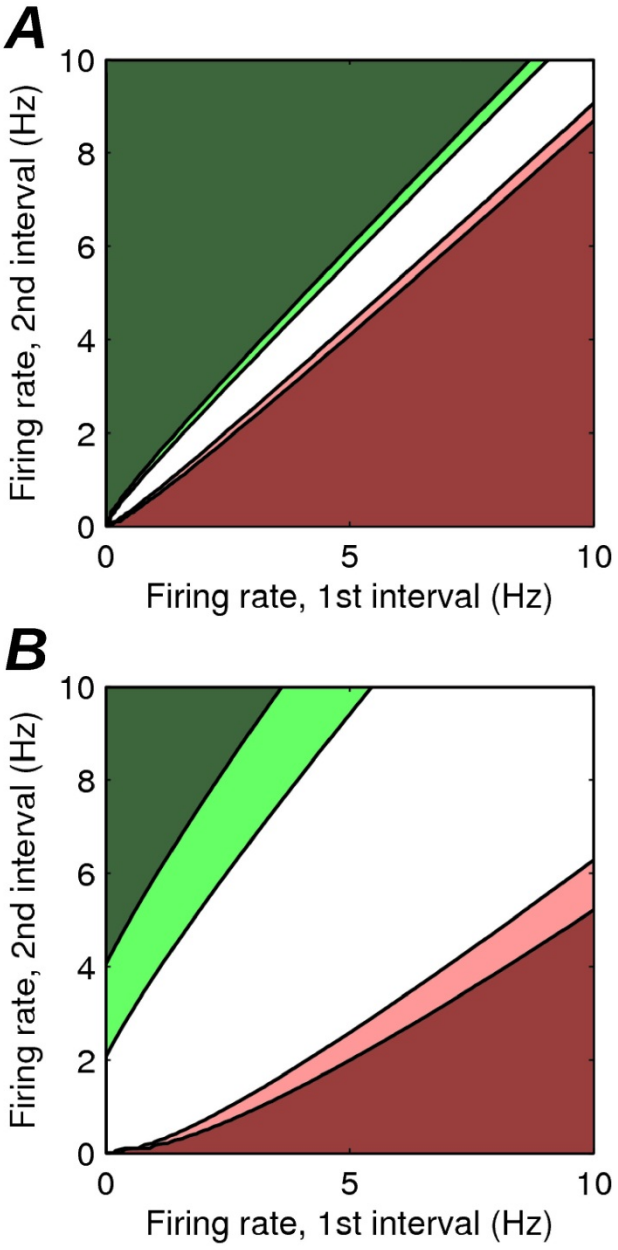
Time (s)

Figure 2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Firing rate, 1st interval (Hz)

Figure 3

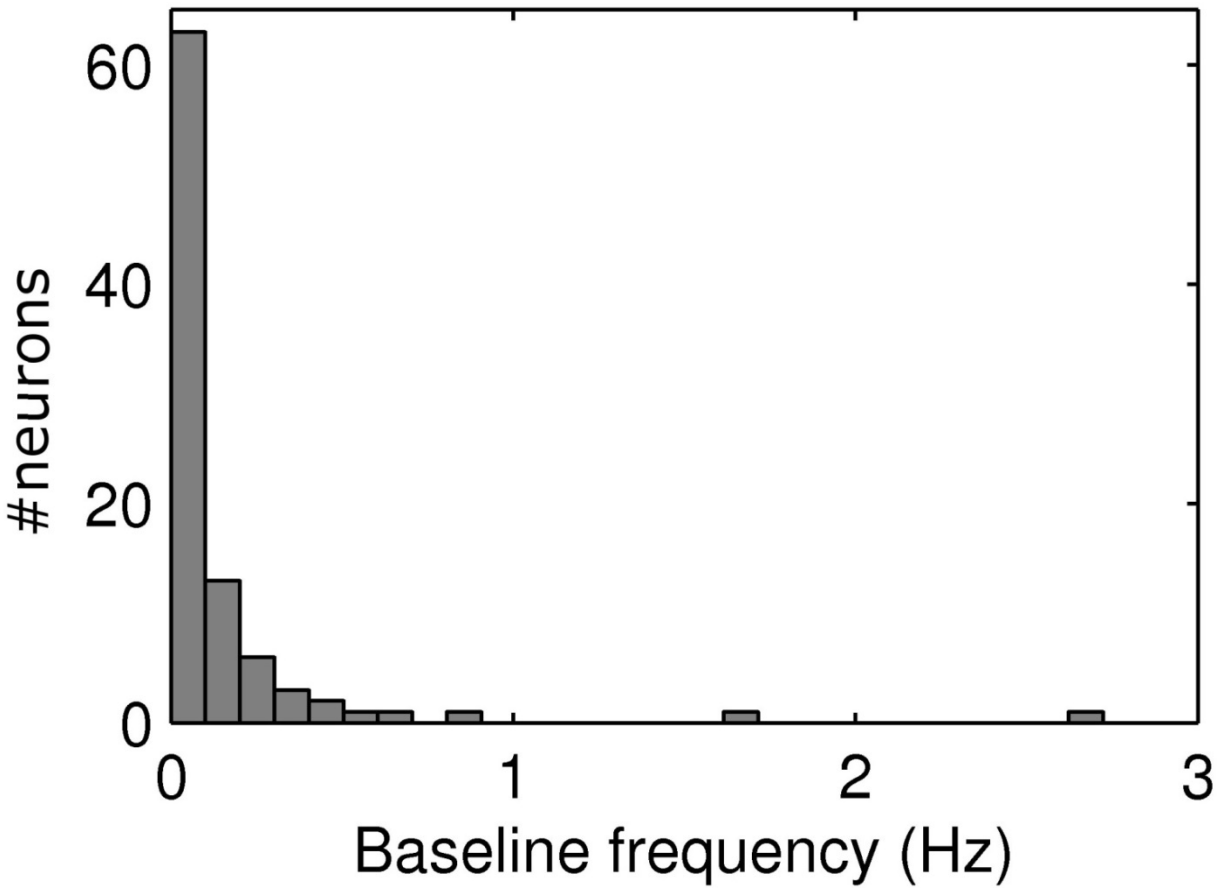
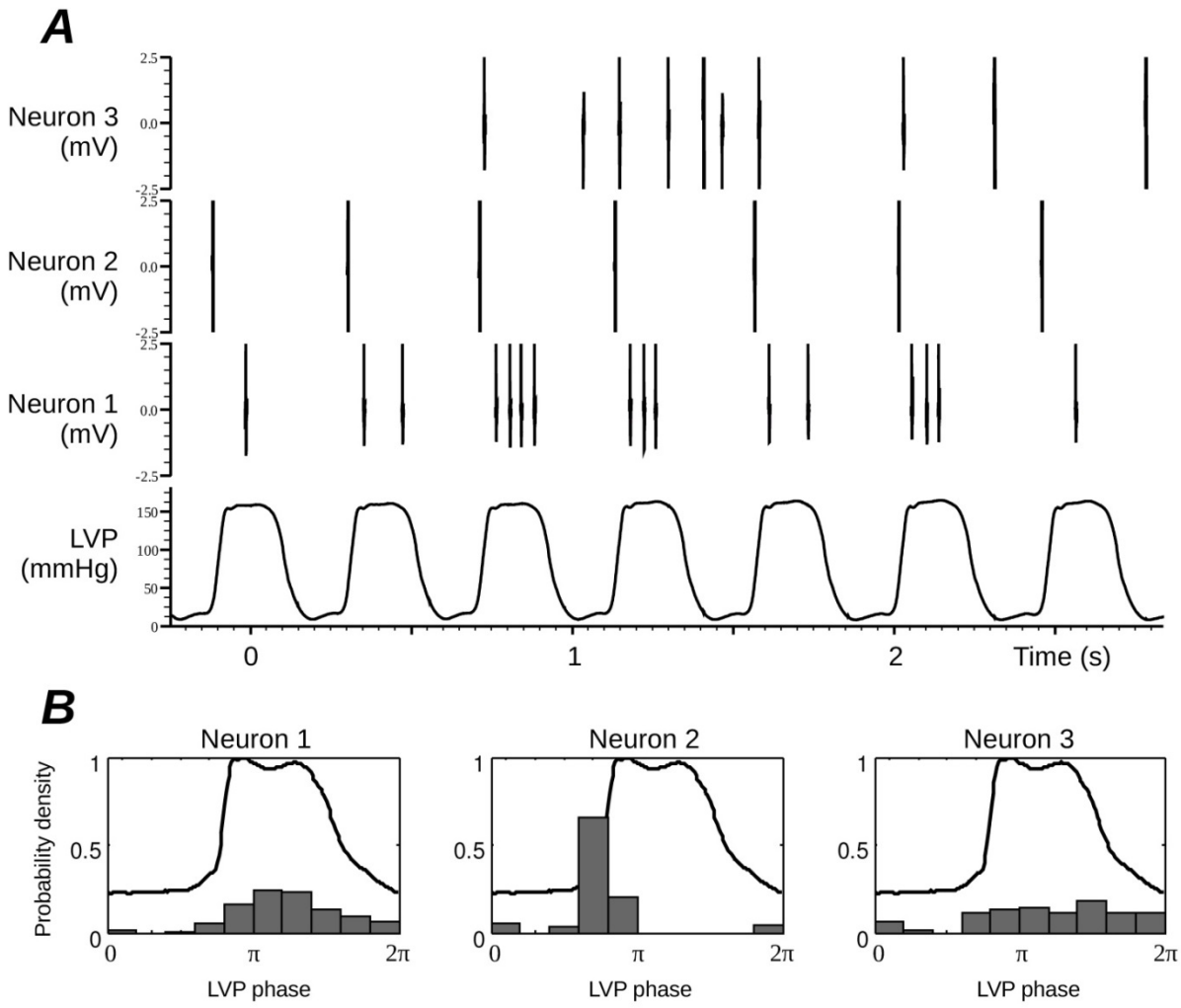


Figure 4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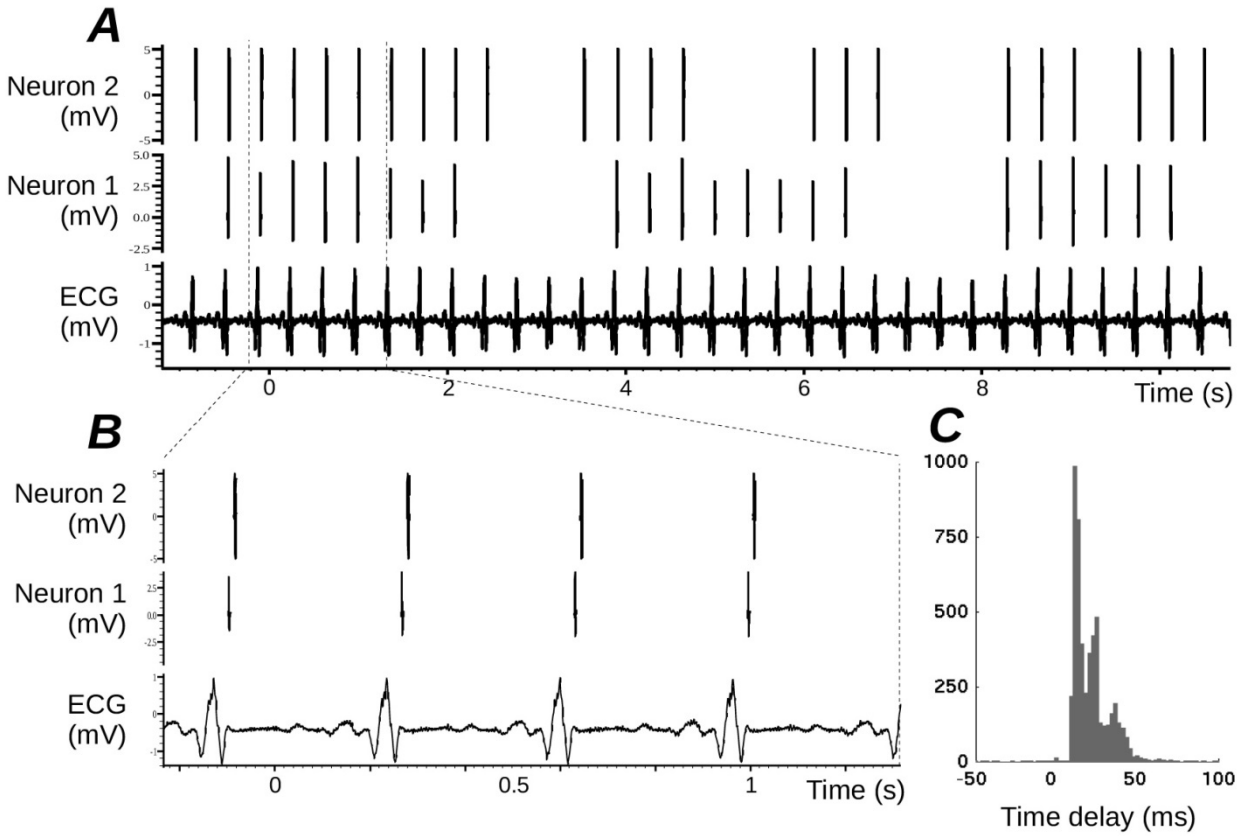
LVP phase

Figure 5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Neuron 1
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ECG
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Figure 6

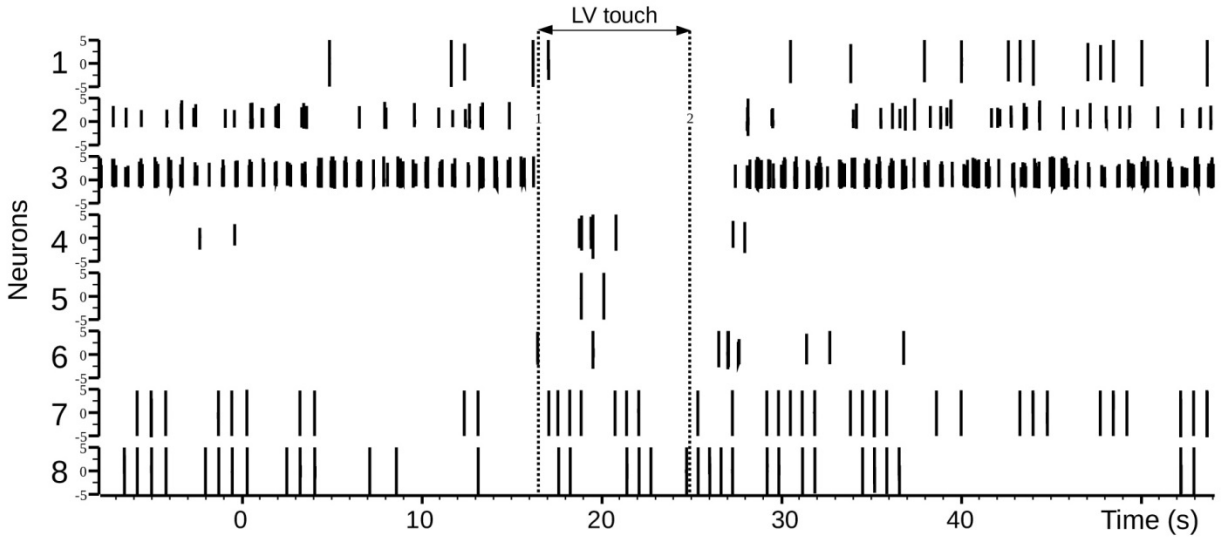


Figure 7

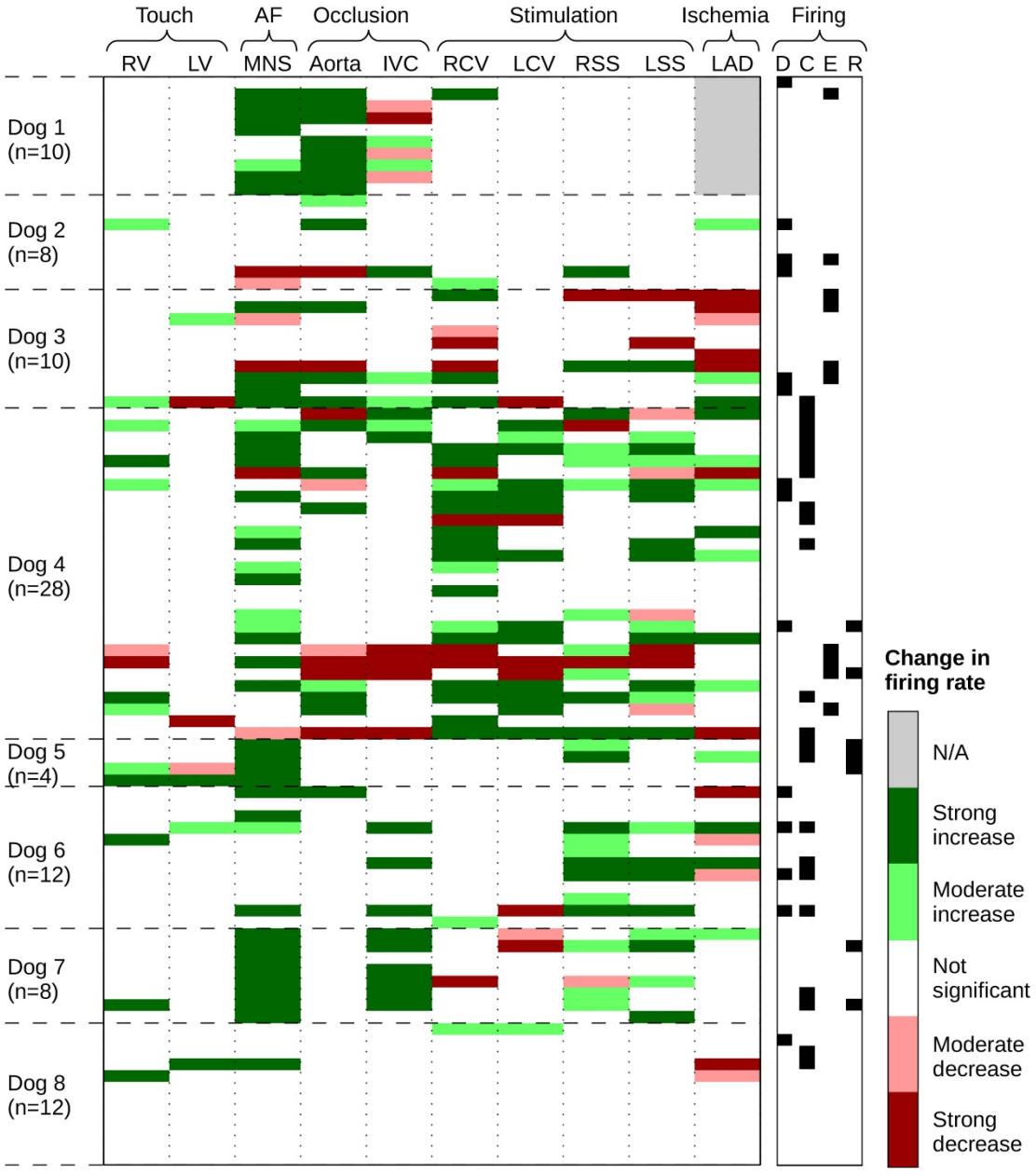

Touch
AF
Occlusion
Stimulation
Ischemia
Firing
RV
LV
MNS
Aorta
IVC
RCV
LCV
RSS
LSS
LAD
D
C
E
R
Dog 1 (n=10)
Dog 2 (n=8)
Dog 3 (n=10)
Dog 4 (n=28)
Dog 5 (n=4)
Dog 6 (n=12)
Dog 7 (n=8)
Dog 8 (n=12)
Change in firing rate
N/A
Strong increase
Moderate increase
Not significant
Moderate decrease
Strong decrease

Figure 8.

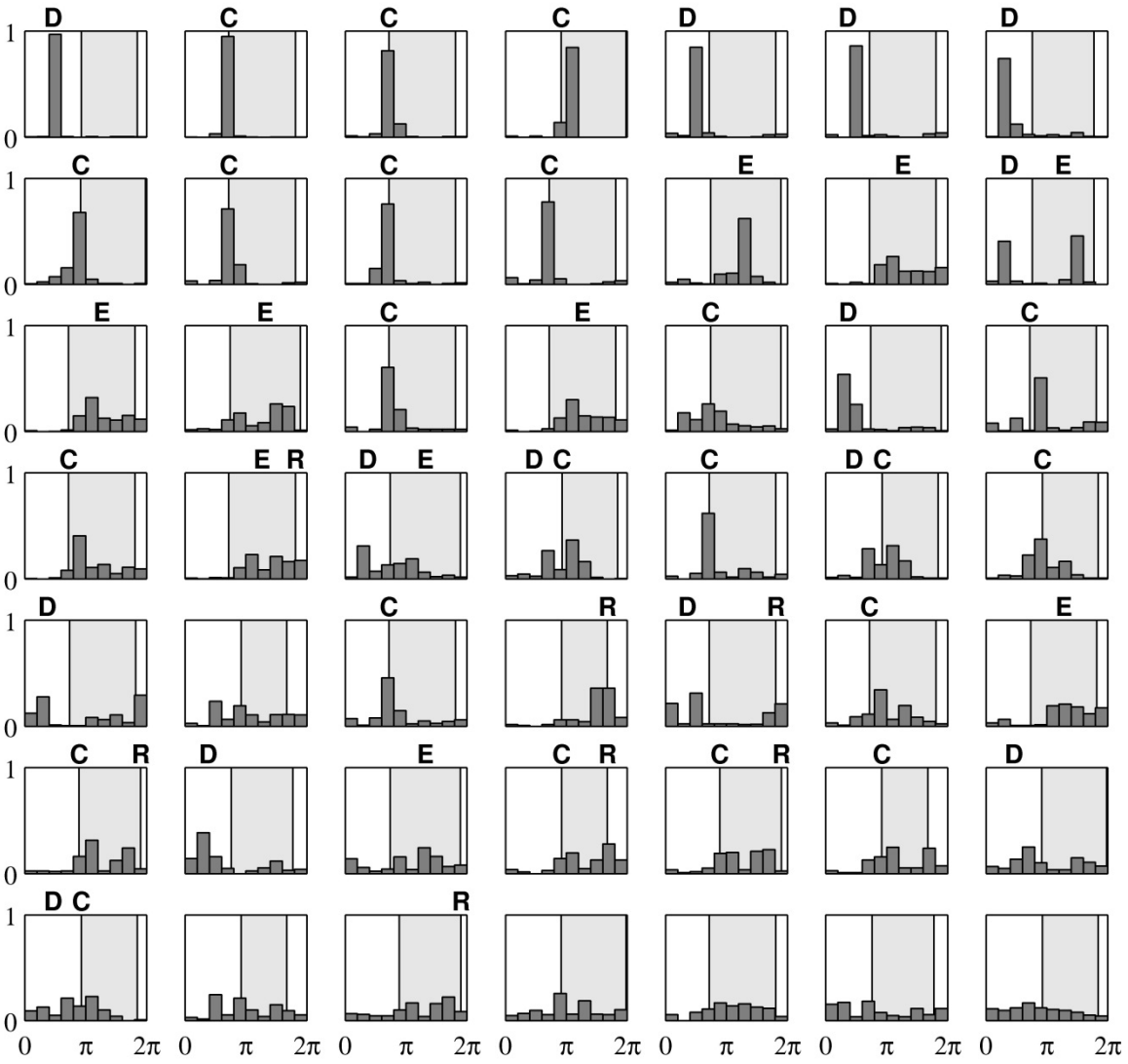

D C C C D D D
C C C C E E D E
E E C E C D C
C E R D E D C E R C D C
D C R E C R C R C D
C R D E C R C R C D
D C R
1
0
0 π 2π

Figure 9

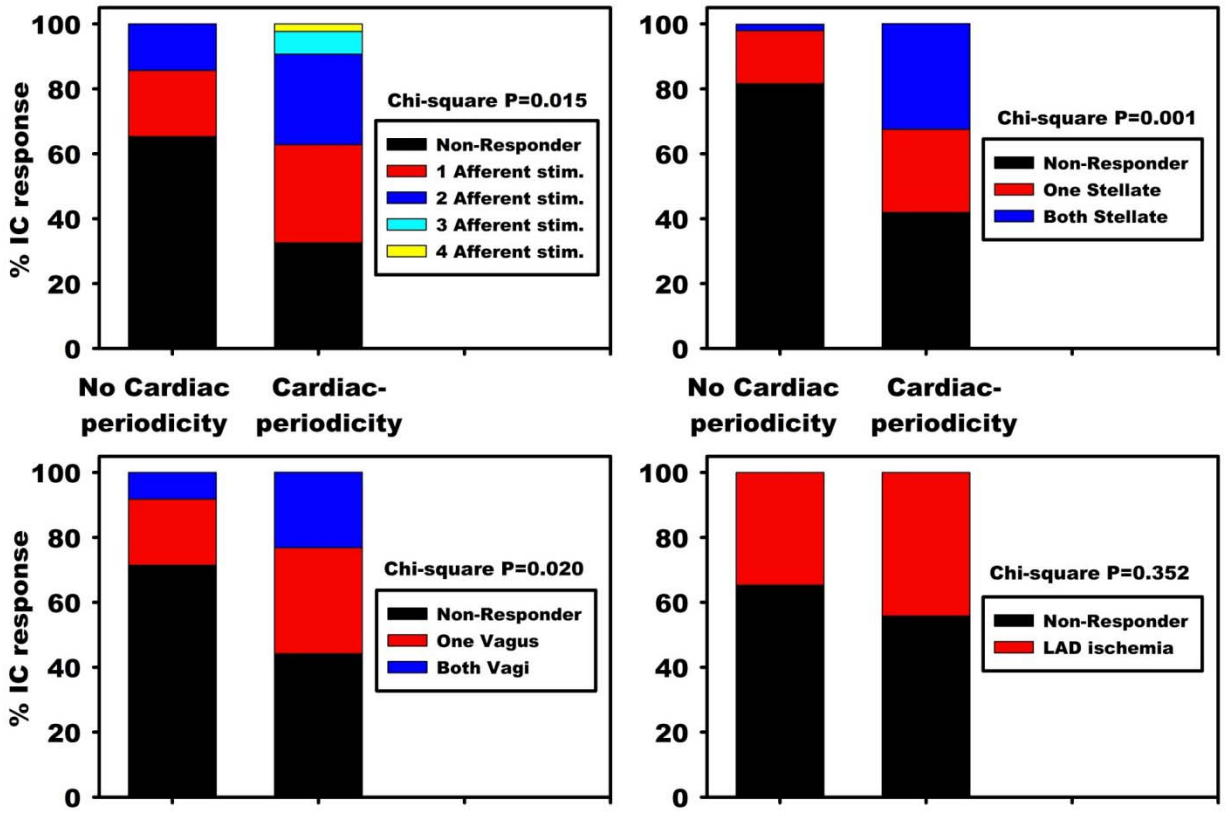

% IC response
100
80
60
40
20
0
Chi-square P=0.015
Non-Responder
1 Afferent stim.
2 Afferent stim.
3 Afferent stim.
4 Afferent stim.
No Cardiac periodicity
Cardiac-periodicity
Chi-square P=0.001
Non-Responder
One Stellate
Both Stellate
No Cardiac periodicity
Cardiac-periodicity
% IC response
Chi-square P=0.020
Non-Responder
One Vagus
Both Vagi
Chi-square P=0.352
Non-Responder
LAD ischemia

Figure 10

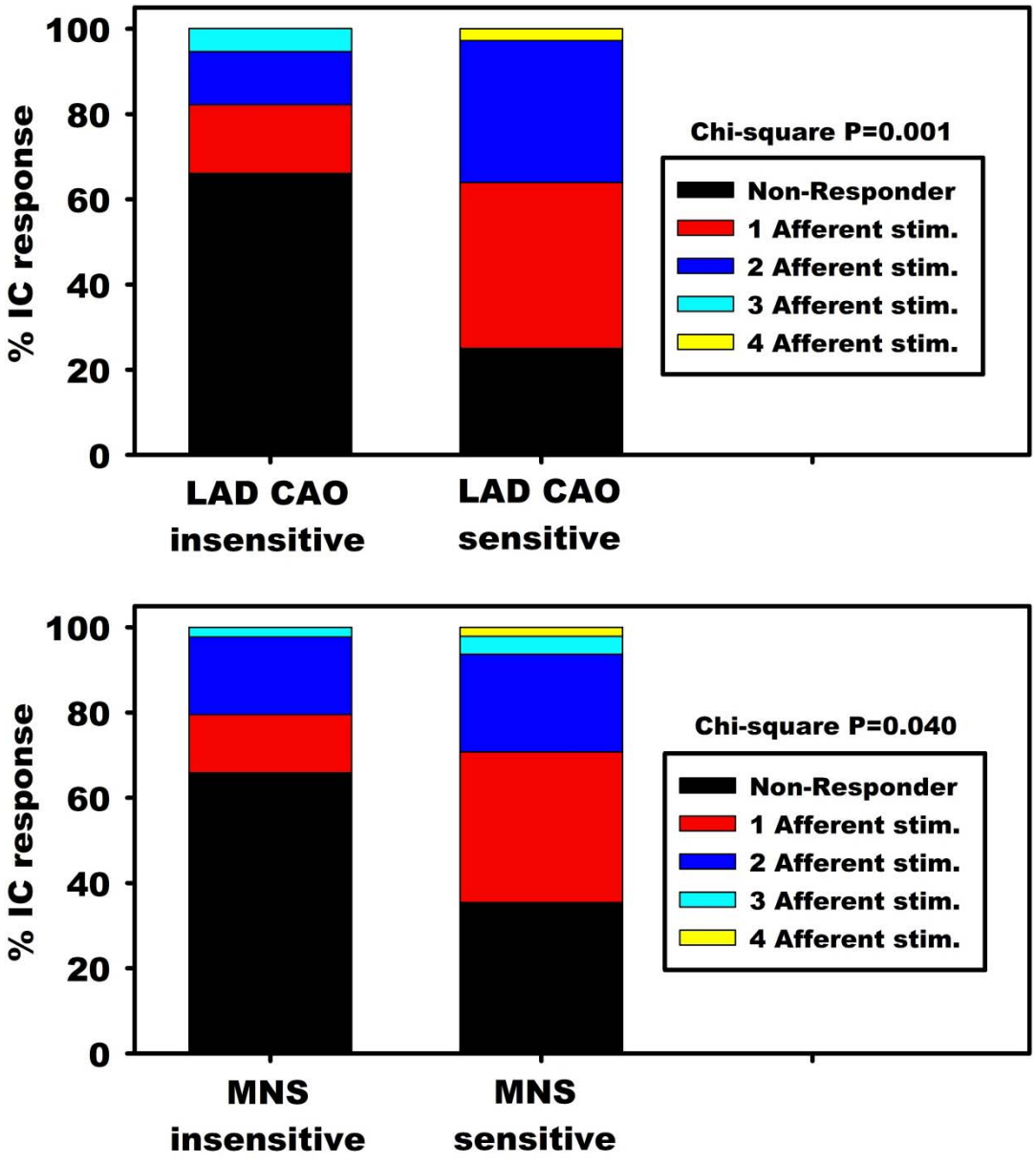
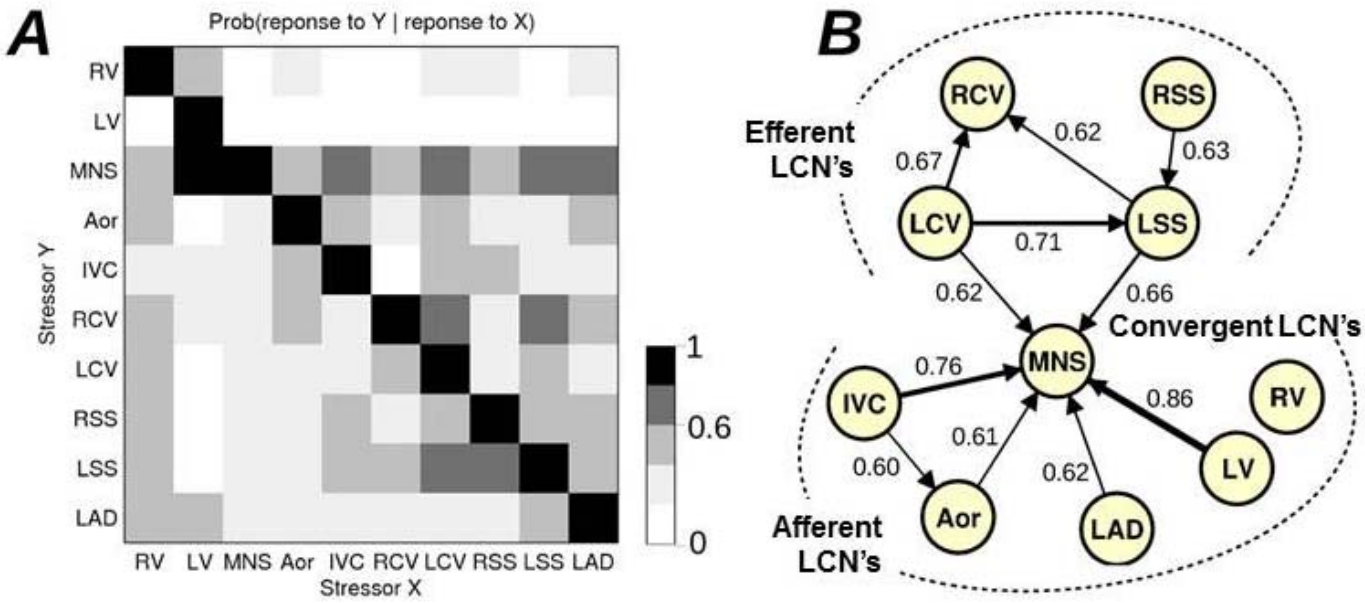

100
80
60
40
20
0
% IC response
Chi-square P=0.001
Non-Responder
1 Afferent stim.
2 Afferent stim.
3 Afferent stim.
4 Afferent stim.
LAD CAO insensitive
LAD CAO sensitive
100
80
60
40
20
0
% IC response
Chi-square P=0.040
Non-Responder
1 Afferent stim.
2 Afferent stim.
3 Afferent stim.
4 Afferent stim.
MNS insensitive
MNS sensitive

Figure 11


A
Prob(reponse to Y | reponse to X)
Stressor Y
RV
LV
MNS
Aor
IVC
RCV
LCV
RSS
LSS
LAD
Stressor X
1
0.6
0
B
Efferent LCN's
RCV
RSS
LCV
LSS
0.67
0.62
0.63
0.71
0.62
0.66
Convergent LCN's
MNS
0.76
IVC
0.86
RV
0.61
0.60
0.62
LV
Aor
LAD
Afferent LCN's

CHAPTER III: RECORDING AND IDENTIFICATION OF CARDIAC NEURON ACTIVITY IN THE RIGHT ATRIUM GANGLIONATED PLEXUS

Contribution of student:

The candidate did the neuronal identification and classification process, developed methodology and analyzed data to obtain the results. The candidate also wrote the manuscript and prepared the figures.

Recording and Identification of Cardiac Neuron Activity in the Right Atrium Ganglionated Plexus

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Abstract

Recent multichannel electrode array technology has enabled the simultaneous recording of multiple cardiac neurons located in ganglia on a beating heart. These new bioelectric signals are contaminated by the electrical activity of the atrial muscle just underneath. These atrial waveforms may mask relevant neuronal activity. In this paper, we evaluate the application of a principal component analysis technique to suppress atrial activity (AA) and reveal hidden neuronal activity. Neuronal signals were recorded in situ using a 16-channel electrode in an open-chest, anesthetized dog in sinus rhythm. Validation of AA cancellation was performed by comparing neuron spike waveforms extracted from within AA with those found in AA-free time intervals. Results showed that consistent neuronal waveforms can be identified within AA in order to improve the detection of neuron firings.

III.1. Introduction

The heart receives sympathetic and parasympathetic innervation through the intrinsic cardiac nervous system [1]. Cardiac neurons notably contribute to the control and the regulation of heart rate and contraction. Afferent and efferent terminations as well as sympathetic efferent post-ganglionic neurons are known to be located in patches of fatty tissue on the atrial surface [2]. These ganglionated plexi have been hypothesized to contain local circuit neurons acting as local processor of information (the so-called "little brain in the heart" [3]) to coordinate regional cardiac function. There are growing evidences that an imbalance in the electrical activity of cardiac neurons is involved in the initiation and maintenance of atrial arrhythmias [4,5]. Advances in neurocardiology raised the need for reliable in situ monitoring of the electrical activity of the cardiac neurons in the ganglionated plexi.

Electrophysiological recordings in ganglionated plexi are performed by inserting an electrode in the nervous tissue, enabling the measurement of extracellular potentials (spikes) generated by neuronal action potential [1]. In the atria, this task is complicated by two problems. First, the electrode is placed directly on a beating heart and moves with it. Second, the signals generated by cardiac neurons may be masked by the superimposed atrial activity (AA). The first issue is typically addressed by means of a probe tethered by a flexible lead. Proposed solutions to the second problem have been so far limited to blanking the signals during AA [1,6], thus ignoring possible neuronal activity in these intervals. This paper presents a first attempt to extract information about cardiac neurons during local atrial depolarization, which will be crucial for future studies during atrial fibrillation.

This AA cancellation problem is similar to the subtraction of ventricular activity in the ECG during an atrial arrhythmia [7,8]. By analogy, considerable gain in AA removal performance is expected from the use of multiple simultaneous signals. While multichannel electrodes are commonly used in the brain, recordings in intrinsic cardiac ganglia have been so far essentially limited to one or a few electrodes [1]. We are using linear multielectrode arrays, in which electrodes are far enough from each other to record different neurons, but sufficiently close so that AA manifestation (atrial waveforms) should remain

similar in all of them. Principal component analysis (PCA) appears to be a natural tool in this situation. In this paper, we follow this approach and evaluate its applicability to multichannel cardiac neuron recordings in dogs.

III.2. Methods

III.2.1. Experimental recordings

Mongrel dogs underwent bilateral open chest surgery. The activity generated by neurons located in the right atrium ganglionated plexus (RAGP) was recorded for 25 minutes by means of a multichannel microelectrode array (Linear Microelectrode Array, MicroProbes Inc., Guithersberg, MD) *in situ* in baseline conditions under anesthesia and controlled respiration. This microelectrode array, consisting of 16 platinum/iridium electrodes (25 μm diameter electrode with an exposed tip of 2 mm; impedance 0.3-0.5 MΩ at 1 kHz), was embedded in the right atrial fat that contained the RAGP such that its tip was placed adjacent to right atrial myocardium. In addition, an electrode was sewn to the atrial myocardium close to the RAGP to provide a reference atrial electrogram and assist AA identification. When low amplitude neuronal activity was observed by visual inspection in a channel during the setup phase of the experiment, the gain of that channel was manually adjusted. The gain therefore varied across the channels.

The signals were digitized at a sampling frequency of 5.6 kHz via a Cambridge Electronics Design (model 1401) data acquisition system.

III.2.2. AA waveforms detection

The 17 signals (16 neuronal channels + electrogram) were analyzed offline using the Spike2 software (Cambridge Electronics Design). The signals of the two distal microelectrodes of the array were discarded because of low signal-to-noise ratio, leaving 14 neuronal channels. Figure 1 shows three different channels as well as the right atrium electrogram. Neuronal activity is clearly visible on the first

two channels, while it remains very low on the third one. When the local cardiac tissue depolarizes, it generates a waveform not only on the electrogram, but also simultaneously on all other data channels (see Fig. 1).

Events (both neuronal responses and AA since they have similar amplitude) were detected using a threshold-based method provided in Spike2. Because of the inter-electrode distance, the activity of a single neuron can usually not be seen from more than two adjacent channels. To discriminate between neuronal response and AA, we therefore assumed that any event present simultaneously in three or more channels was AA. To facilitate the procedure, AA detection was performed on a subset of four channels (called four-trode) with low neuronal activity (selected by visual inspection). The right atrial electrogram served to ensure that AA detected in neuronal signals was indeed caused by an atrial activation. Note that atrial electrogram waveforms were approximately but not exactly aligned with the AA in neuronal channels since the myocardial electrode and the RAGP electrode were distant by a few millimeters.

III.2.3. Spike sorting outside AA

Spike sorting consists in grouping neuronal waveforms into clusters based on their shape [9]. Each waveform of a group presumably corresponds to the firing of the same neuron. As a first step, a 26-ms window was blanked in all channels around each detected AA waveform. Automatic spike sorting techniques available in Spike2 were applied to extract neuron spike trains. The standard approach for neuronal activity identification stops here. The next two paragraphs describe an attempt at finding neuronal spikes within the blanked intervals.

III.2.4. AA cancellation

Signals were then exported from Spike2 to Matlab for further analysis. Each AA window was processed separately. An example of signal waveforms in an AA window is shown on the left panel of Fig. 2. After mean subtraction, the signals were normalized by their standard deviation to compensate for channel-specific gain (see e.g. signals 4, 5, 7 and 9 in Fig. 2).

For each atrial beat, the 14 signals in the corresponding AA window were represented as a 146-

by-14 data matrix (windows were 146 samples long). AA cancellation was performed based on principal component analysis (PCA) of that matrix [207]. In all but rare cases, the first principal component (an example is shown at the bottom of Fig. 2) captured at least 70-90% of the variance. This confirmed that AA shape was very similar in all channels. The signals were then reconstructed after suppression of the first principal component. Removing the second principal component did not further improve the identification of neuronal activity in the residual, presumably due to its orthogonality constraint. The right panel of Fig. 2 shows examples of residuals after AA cancellation. One of them (channel 2) seems to contain a neuronal activity.

III.2.5. Spike identification within AA

To establish that spikes observed within AA after cancellation (and detected by thresholding) were real neuronal responses, two criteria were used. First, when simultaneous spikes occurred in more than two channels, they were considered as cancellation artifact. Otherwise, the spike waveform was compared to the templates of neuronal waveforms identified outside AA using Spike2. The maximum cross-correlation served as a quantitative measure to select the best candidate and associate the spike with a previously identified neuron.

III.3. Results

In the 25 min studied, 1600 AA waveforms were detected (64 per min). These corresponded to the segments that were blanked in the standard approach. Some AA corresponding to atrial activations were not detected. They had significantly lower amplitude (e.g. premature beats) than neuronal response and were not blanked in the standard approach because they did not prevent the identification of neuronal response. Decreasing the threshold to detect them would increase false positive detection of neuronal response.

Outside AA, a total of 18 neuronal waveforms were identified in the 14 channels, presumably corresponding to the activity of 18 different neurons. Their firing rates ranged from 0.06 to 2.09 Hz in

baseline conditions, in agreement with previous recordings in the RAPG [1]. Table 1 lists the number of neuronal spikes outside AA for the 10 neurons having the highest firing rates.

AA cancellation provides a tool for identifying additional neuronal spikes. The channel 2 of Fig. 2 has a spike in the residual after cancellation. Its amplitude is in the range of the amplitudes of spikes identified outside AA. To further confirm that this spike is a neuronal response, its waveform was compared to similar spike waveforms outside AA. Figure 3 gives a few examples of spikes with similar shapes found inside and outside AA. For a variety of morphologies, waveforms were consistent inside and outside AA. As a results, it was possible to associate each spike inside AA with a cluster of waveforms (i.e., with a neuron) outside AA. Table 1 summarizes for the neurons with highest firing rates the number of spikes inside and outside AA. Spikes inside AA contributed to about 1 to 2% of the total identified spikes, while the cumulated length of all AA represented 2.8% of total signal duration.

III.4. Discussion

This paper demonstrates the value of multichannel neuronal recording for investigating the intrinsic cardiac nervous system. First, the detection of simultaneous spikes in multiple channels facilitates the identification of AA. This is of particular importance since amplitude and shape alone are sometimes not sufficient to reliably classify waveforms as AA. Second, a simple PCA algorithm enabled us to reveal neuronal activity masked by AA. Combined with powerful spike sorting techniques in Spike2, this type of multichannel analysis opens new perspectives in neurocardiology by permitting to study neuron population dynamics and network interactions in atrial ganglionated plexi in relation to cardiovascular, chemical, mechanical or neuronal inputs/outputs [11].

When AA waveforms are blanked, neuron firing rates are underestimated. Our results suggest that the systematic error in firing rate is of the order of 1.5% during sinus rhythm, which is small as compared to changes in firing rates resulting from external input (e.g. increased blood pressure). When the activity of a neuron is cardiovascular-related (i.e., when it fires at specific phases within the cardiac

cycle), there may be a physiological reason for the presence or absence of firing during the AA. This may explain why the average firing rate in the AA is slightly lower than expected (1.5% of the spikes in 2.8% of signal duration). Another reason could be misdetection of lower amplitude waveforms due to cancellation artifacts.

The problem of removing AA becomes more critical during episodes of atrial arrhythmias. In these very relevant conditions, atrial rate increases markedly so that the cumulated duration of AA may represent up to 15-20% of signal duration, resulting in a more severe underestimation of firing rates. Future work will evaluate the applicability of our approach to these signals.

III.5. Conclusion

The application of multichannel microelectrode arrays to neurocardiology created new signal processing challenges. A combination of PCA and template matching enabled us to get more insight into RAGP neuronal activity hidden in AA. These tools and their future developments will form the basis for deeper investigations of neuronal activity in relation to the occurrence of atrial arrhythmias.

Acknowledgements

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References

[1] Armour JA, Ardell JL. Basic and Clinical Neurocardiology. New York: Oxford University Press, 2004.

[2] Levy MN, Martin PJ. The cardiovascular system. In: Berne RM, Sperelakins N, Geiger SR, editors. Handbook of physiology, vol. 1, 1979.

[3] Armour JA. Potential clinical relevance of the 'little brain' on the mammalian heart, Exp Physiol 2008;93:165-76.

[4] Olshansky B. Interrelationships between the autonomic nervous system and atrial fibrillation. Prog Cardiovasc Dis 2005;48:57-78.

[5] Scherlag BJ, Patterson E, Po SS. The neural basis of atrial fibrillation. J Electrocardiol 2006;39:S180-3.

[6] Thompson GW, Collier K, Ardell JL, Kember G, Armour JA. Functional interdependence of neurons in a single canine intrinsic cardiac ganglionated plexus. J Physiol 2000;528:561-71.

[7] Lemay M, Vesin JM, van Oosterom A, Jacquemet V, Kappenberger L. Cancellation of ventricular activity in the ECG: evaluation of novel and existing methods. IEEE Trans Biomed Eng 2007;54:542-6.

[8] Stridh M, Sornmo L. Spatiotemporal QRST cancellation techniques for analysis of atrial fibrillation. IEEE Trans Biomed Eng 2001;48:105-11.

[9] Lewicki MS. A review of methods for spike sorting: the detection and classification of neural action potentials. Network 1998;9:R53-78.

[10] D. Morrison. Multivariate statistical methods. New York: McGraw-Hill, 1976.

[11] Armour JA, Collier K, Kember G, Ardell JL. Differential selectivity of cardiac neurons in separate intrathoracic autonomic ganglia. Am J Physiol 1998;274:R939-49.

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Figure legend

Table 1. Number of neuron spikes inside and outside atrial activity (AA).

Figure 1. Electrical activity recorded in the right atrial ganglionated plexus (only 3 channels are displayed) and right atrial electrogram representing myocardial activity. Note that some events (AA) are aligned in all channels.

Figure 2. First column: signals (14 channels) containing an atrial activity (AA) waveform. Second column: the same signals after AA cancellation. The bottom trace shows the principal component.

Figure 3. (A) Examples of neuronal responses within an AA waveform after cancellation. (B) Best match with a neuronal response found outside AA.

Table 1. Number of neuron spikes inside and outside atrial activity (AA).

neuron	#spikes outside AA	#spikes within AA	fraction inside
1	3129	35	1.1%
2	1888	23	1.2%
3	1641	24	1.4%
4	1333	17	1.3%
5	1251	11	0.9%
6	1023	14	1.4%
7	897	7	0.8%
8	687	20	2.8%
9	584	25	4.1%
10	549	7	1.3%
others	1850	38	2.0%
total	14832	221	1.5%

Figure 1.

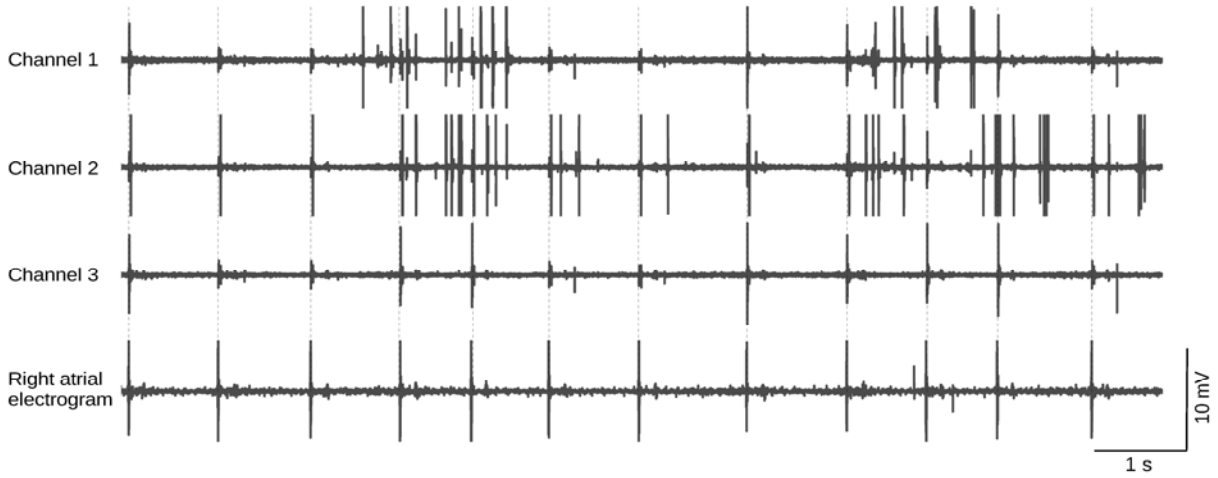

Channel 1
Channel 2
Channel 3
Right atrial electrogram
10 mV
1 s

Figure 2.

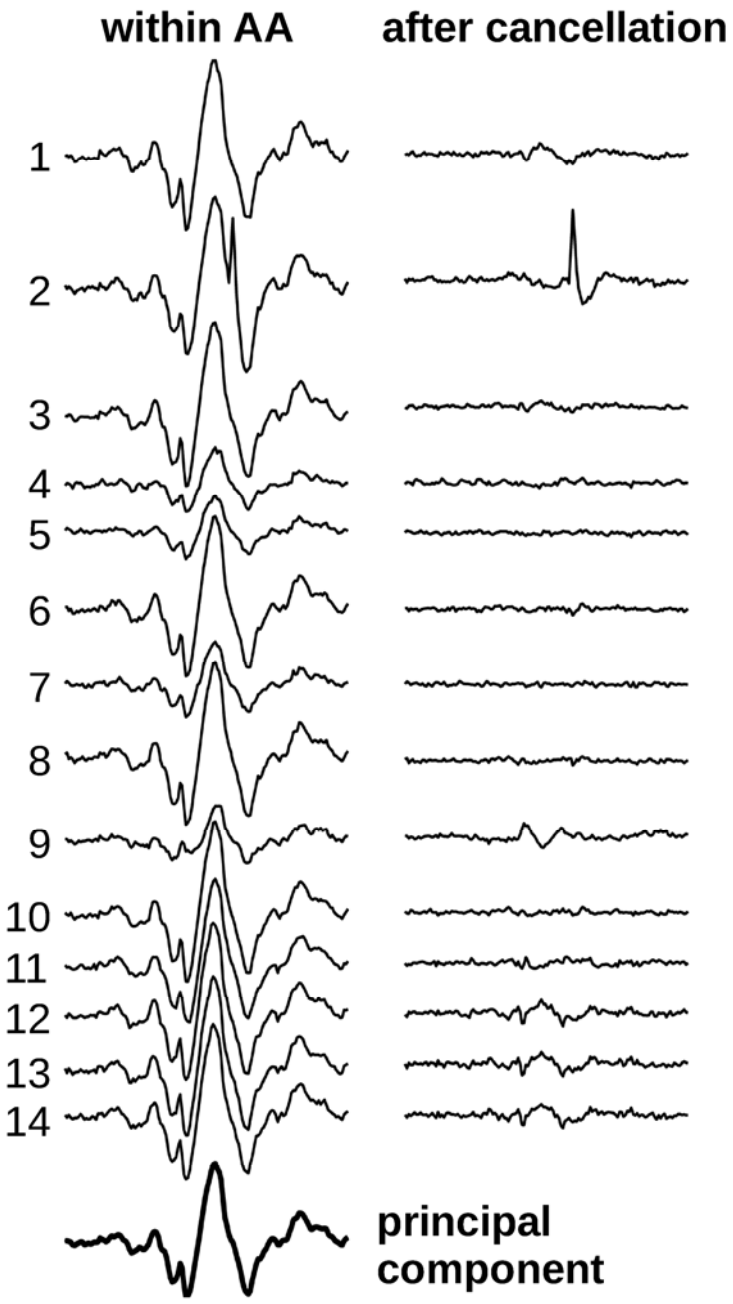
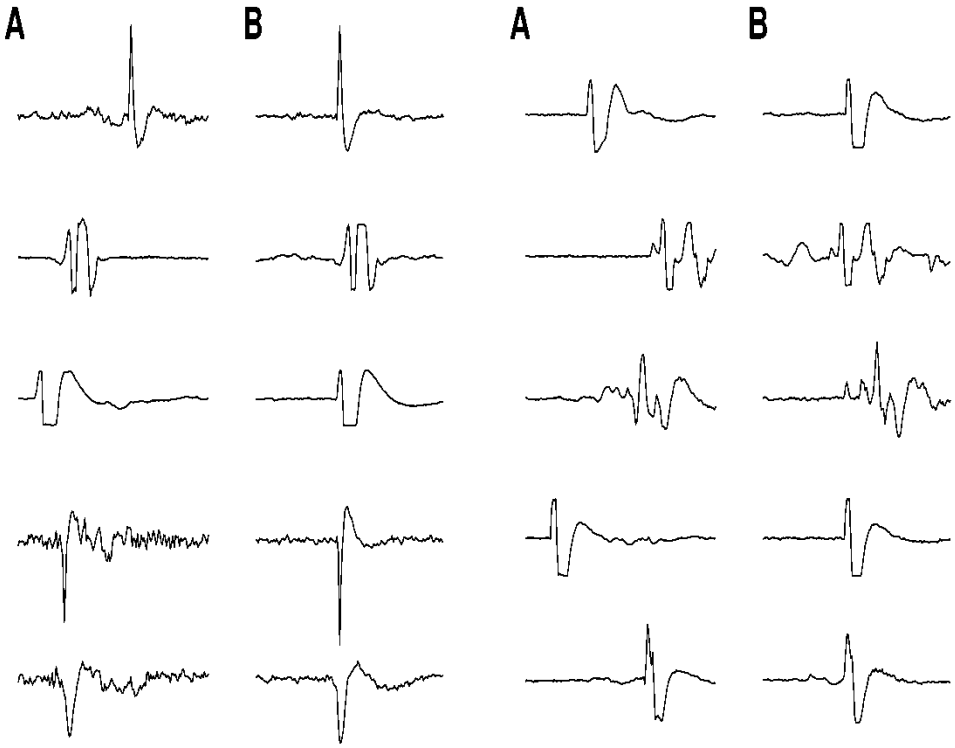

within AA
after cancellation
1
2
3
4
5
6
7
8
9
10
11
12
13
14
principal component

Figure 3.



CHAPTER IV: VAGAL STIMULATION TARGETS SELECT POPULATIONS OF INTRINSIC CARDIAC NEURONS TO CONTROL NEURALLY-INDUCED ATRIAL FIBRILLATION

Contribution of student:

The candidate did the neuronal identification and classification process, developed tools to analyze data and analyzed data to obtain the results. The candidate also contributed to writing the manuscript and creating figures.

Vagal stimulation targets select populations of intrinsic cardiac neurons to control neurally-induced atrial fibrillation

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UCLA Health System

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Los Angeles, CA 90095

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Running title: Vagal suppression of neurally induced atrial fibrillation

Key words: atrial arrhythmias; intrinsic cardiac nervous system; local circuit neurons; memory; stochastic neuronal interactivity; vagus

Abbreviations: AF,atrial fibrillation; AOR, descending aortic occlusion; ART, Autonomic Regulation Therapy; CV, cardiovascular; ECG, electrocardiogram; IVC, inferior vena cava; IC, intrinsic cardiac; ICNS, intrinsic cardiac nervous system; LCN, local circuit neuron; LCV, left cervical vagosympathetic complex; LMA, linear microarray; LSS, left stellate ganglion stimulation; LV, left ventricle; LVP, left ventricular pressure; MNS, mediastinal nerve stimulation; RAE, right atrial electrogram; RAGP, right atrial ganglionated plexus; RCV, right cervical vagosympathetic complex; RSS, right stellate ganglion stimulation; RV, right ventricle; TLV, touch right ventricle (epicardial); SCS, Spinal cord stimulation; TRV, touch left ventricle (epicardial); VNS, Vagus nerve stimulation.

Abstract:

Background: Mediastinal nerve stimulation (MNS) reproducibly evokes atrial fibrillation (AF), an effect dependent upon excessive activation of intrinsic cardiac (IC) neurons. This study evaluated whether pre-emptive vagus nerve stimulation (VNS) alters mediastinal nerve (MNS)-induced changes within the IC neural network to alter susceptibility to AF. *Methods:* Activity of IC neurons in the right atrial ganglionated plexus was directly evaluated in anesthetized canines (n=8) using a linear microelectrode array in response to: 1) epicardial touch or great vessel occlusion vs (2) stellate or vagal stimulation. The capacity of right-sided MNS to modify IC activity in the induction of AF was determined prior to and after pre-emptive right (RCV) vs left-sided (LCV) VNS (15 Hz, 500μsec; 1.2x bradycardia threshold; 3 min). *Results:* IC activity (baseline: 0.11±0.29Hz) increased during MNS-induced AF (0.51±1.30Hz; $p<0.001$). Convergent LCN's, defined as IC neurons responding to afferent and efferent inputs, were preferentially activated. Pre-emptive RCV reduced MNS-induced changes in convergent LCN's activity (by 70%), while mitigating the potential for MNS-induce AF (by 75%). Pre-emptive LCV reduced LCN activity by 60% while mitigating the AF potential by 40%. Increases in IC neuronal synchrony elicited during neurally-induced AF were also mitigated by pre-emptive VNS. Such anti-arrhythmic effects persisted post-VNS for, on average, 26 min. *Conclusions:* VNS preferentially targets convergent LCNs and their interactive coherence to mitigate AF resulting from IC neural imbalance. The anti-arrhythmic properties imposed by VNS exhibit memory.

IV.1. Introduction

Atrial fibrillation (AF) affects more than three million people a year in the United States, prevalence that has been projected to reach 5.6 - 12.1 million by 2050 (23, 37). Despite such prevalence, the underlying mechanisms of AF are not fully understood. Current treatments consist of pharmacological therapies that have been combined with localized atrial catheter-based or surgical ablation (17, 48). Although many surgical approaches to AF have been attempted, their success rates vary among patients. Cappato et al (14) reported that catheter ablation was effective in ≈70% of patients, increasing to ≈80% in patients when combined with antiarrhythmic pharmacological therapy. Weerasooriya et al (57) reported the therapeutic success rates of such therapy as 87, 81 and 63 % at the end of 1, 3 and 5 years, respectively. Moreover, ablation procedures are associated with complications such as the left atrial stiffness syndrome (22), micro-embolic episodes (45), and a risk of symptomatic or silent cerebral ischemia (20). Such drawbacks have increased the focus on understanding the mechanisms of AF and the development of novel non-pharmacologic therapeutic options for its management. Active neuromodulation based therapies for AF represent a new approach to such management. These include vagal nerve stimulation (VNS) (33, 47, 49) and spinal cord stimulation (SCS) (21, 52, 56); which target various aspects of the cardiac neuronal hierarchy.

The cardiac neuronal hierarchy is made up of neuronal somata located in the insular cortex, brain stem, spinal cord, intrathoracic sympathetic ganglia and the intrinsic cardiac nervous system (ICNS) (2, 6, 66). It has been proposed that the ICNS acts as the final coordinator of regional cardiac indices and that it is under the influence of intrathoracic, spinal cord and brainstem reflexes (6). Modulation of cardiac function by the ICNS is coordinated by afferent (mechanosensitive, chemosensitive and ischemia sensitive) inputs acting reflexly on both peripheral and central aspects of the cardiac nervous system (6, 8, 65) . These afferent and efferent projections interact via local circuit neurons (LCNs) at these locations to modulate both sympathetic and parasympathetic

efferent postganglionic projections to all regions of the heart (3, 18, 26, 35). It has been postulated that LCNs support complex reflex processing within the ICNS (6). It has been further demonstrated that neuronal imbalances within the ICNS can exert deleterious effects on cardiac function, including arrhythmia induction (7, 9, 44, 46).

Vagal stimulation modulates cardiac electrical function (58) and has the potential to either increase or decrease the propensity to arrhythmias (17). Higher intensity stimulations tend to increase atrial fibrillation inducibility (61, 63); lower intensity vagal stimulation can stabilize atrial electrical function (16, 53). Moreover, recent studies have demonstrated the anti-arrhythmic effects of vagal nerve stimulation (VNS) can be delivered with minimal adverse effects (49, 61, 62). It has also been reported that low level VNS therapy can suppress AF induced by cholinergic neuronal activation in ambulatory canines along with suppression of stellate ganglion hyperactivity (47). It has been hypothesized that obtunding intrinsic cardiac LCN transduction might be a mechanistic basis of such benefit (21).

In order to understand the efficacy of VNS therapy on atrial arrhythmia suppression, we studied the effects of right versus left-sided vagus nerve stimulation on various subgroups of intrinsic cardiac (IC) neurons in the canine right atrial ganglionated plexuses (RAGP) (60). This population of neurons is primarily associated with autonomic control of the sinoatrial node (5, 36). With direct IC neuronal recordings, using linear microarray electrode technology, it is possible to evaluate and sub-classify the activities generated by multiple populations of IC neurons *in situ* based on their responses to different cardiovascular stressors (11). Disruptions in ICNS network function can be evaluated with respect to its varied neuronal subtypes (afferent, efferent or local circuit) and, correspondingly, used to delineate neural targets for potential anti-arrhythmic therapies.

The model of atrial fibrillation utilized in this study is focal mediastinal nerve stimulation (9). This model provides a reproducible stress against which anti-arrhythmic therapies can be evaluated and optimized (4, 9, 21, 43). Previous work has demonstrated that such electrical stimuli

activate neural projections that directly innervate a discrete sub-population of intrinsic cardiac neurons (21), giving rise to overall ICNS network hyper-excitability (21), with resultant disparate efferent outflows to atrial tissues causing local heterogeneities in atrial function and induction of transient atrial fibrillation (9, 43). The present study demonstrates the fundamental contributions of LCN neurons within the ICNS in modulating the atrial arrhythmogenic substrate and their pivotal role as a target for neuromodulation of AF.

IV.2. Methods

IV.2.1. Animal preparation

All experiments were performed in accordance with the guidelines for animal experimentation described in the Guide for the Care and Use of Laboratory Animals, Eighth Edition, National Academy Press, Washington DC, 2010. The Institutional Animal Care and Use Committee of the East Tennessee State University approved these experiments. Eight mongrel dogs of either sex, weighing 18.6-26.9 kg, entered this study. Animals were sedated with propofol (3-8 mg/kg, intravenous (i.v.)), followed by endotracheal intubation and mechanical ventilation. General anesthesia was maintained with isoflurane (1-2%, inhalation). Following completion of surgery, anesthesia was changed to α-chloralose (50 mg/kg i.v. bolus), with continuous infusion (8-12 mg/kg/hr i.v.) adjusted to effect throughout the duration of each study. Throughout, the depth of anesthesia was assessed by monitoring corneal reflexes, jaw tone, and hemodynamic indices. Body temperature was maintained via a circulating water heating pad (Gaymar T/Pump, Gaymar Industries Inc., Orchard Park, NY). At the completion of the experiments, animals were humanely euthanized under deep anesthesia and by inducing ventricular fibrillation via direct current stimulation.

IV.2.2. Hemodynamic recording

The left femoral artery was catheterized to record arterial blood pressure (Ao BP). The left femoral vein was catheterized to allow for fluid replacement, as well as anesthetic and pharmacological

agent delivery. The right femoral artery was catheterized in order to monitor left ventricular chamber pressure (LVP) via placement into that chamber of a Mikro-Tip Pressure Transducer Catheter (Millar Instruments, Houston, TX). Heart rate was monitored via a Lead II electrocardiogram (ECG). Pressures (Ao BP, LVP) and ECG were input to a Cambridge Electronics Design Cambridge Electronics Design (model 1401) data acquisition system for continuous monitoring of hemodynamic stability.

IV.2.3. Vagal stimulation (VNS)

Following a midline incision in the ventral neck, the right and left cervical vagi were exposed and bipolar stimulation electrodes (PerrenialFlex, Model 304, Cyberonics, Inc.) placed around each. Each lead was connected individually to a Grass S88 stimulator via separate PSIU6 constant current isolation units. Bradycardia thresholds for each nerve stimulated were identified using 20 Hz, 500μs pulse width stimuli, as determined by progressive increases in current intensity until 10% bradycardia was evoked. For right-sided VNS this current was found to be, on average, 1.75 mA; for left-sided VNS it was 2.25 mA. VNS was applied to each vagus for 3 min periods (15 Hz; 500μs pulse width) at a current intensity that was 1.2x bradycardia threshold.

IV.2.4. Mediastinal nerve stimulation (MNS)

Following thoracotomy, an incision was made in the pericardial sac and a pericardial cradle formed. A bipolar electrode was affixed to the right atrium, 1 cm dorsal to the SA node, to record an atrial electrogram. Right-sided mediastinal nerves were identified visually on the ventral and ventro-lateral surface of the intrapericardial aspects of the superior vena cava. This nerves are aggregate of sympathetic and parasympathetic efferent projections as well as inter-ganglionic projections arising from local circuit neurons contained within the ICNS (9, 21, 24, 55) . Each nerve was stimulated individually using detailed published techniques (9, 21). Briefly, trains of five electrical stimuli (0.3-1.2 mA, 1 ms duration, 5 ms pulse interval) were delivered during individual atrial refractory periods to identified mediastinal sites for up to 20 seconds, as triggered by the reference atrial electrogram to avoid direct atrial capture. Electrical stimuli were delivered via a roving bipolar probe electrode (1.5 mm spacing)

connected to a constant current generator (PSIU6, Grass Instruments, Quincy, MA) affixed to a Grass S88 stimulator (Grass Instruments, Quincy, MA). The stimulator was externally controlled by a script running on the CED powerlab and triggered by atrial wave front detections. Active nerve sites were identified by the immediate induction of atrial tachyarrhythmias (including atrial fibrillation) when first exposed to focal electrical stimuli. Each active mediastinal nerve site so identified was marked with India ink for repeated stimulation. By these means, 2-4 active nerve sites were identified in each animal. Contact between the bipolar electrodes and tissue was discontinued immediately after the onset of the atrial tachyarrhythmia in order to limit their durations.

IV.2.5. Neuronal Recording

The activity generated by right atrial neurons *in situ* was identified using a multichannel linear microelectrode array (LMA; MicroProbes Inc., Gaithersburg, MD) that consisted of 16 platinum/iridium electrodes (25 μm diameter electrode with an exposed tip of 2 mm; impedance 0.3-0.5 MΩ at 1 kHz). The LMA electrode was embedded in the right atrial fat that contained the RAGP, as described previously (11). This probe was attached to a flexible lead, the density of right atrial fat helping to maintain position stability over prolonged periods of time (up to 8 hours of recording). The connecting wires of the multichannel electrode, along with ground and reference wires, were attached to a 16-channel microelectrode amplifier with a headstage preamplifier (A-M systems, Inc., model 3600; Carlsborg, WA). For each channel, filters were set to 300-3K Hz and gain to 5K. Another electrode was sewn to the atrial myocardium close to the RAGP to provide a reference right atrial electrogram (RAE) which was utilized to determine atrial rate, duration and characterization of atrial arrhythmias, along with a timing index for subsequent identification of atrial electrical artifacts in IC neural recording data. The 16 microelectrode array signals, along with recorded cardiovascular indices (ECG, right atrium electrogram and hemodynamic data), were digitized via a Cambridge Electronics Design (model 1401) data acquisition system for off-line analysis. The sampling frequency for neuronal data was 5.26 kHz; it was six time lower (0.877 kHz) for all other recorded signals.

IV.2.6. Identification of neuronal activity

The activity generated by individual neuronal somata located within the RAGP was recorded. Identification of the activity generated by individual neurons via the 16 channel electrodes was performed off line using Spike2 software program (Cambridge Electronic Design) in two steps: (1) artifact identification and blanking; (2) spike detection, waveform classification and validation with principal component analysis (11). Artifacts were identified related to electrical activity generated by the atrial myocardium beneath the RAGP as well as by MNS stimulation. Using the techniques depicted above, each artifact could be identified and eliminated to permit identification of action potentials generated by individual somata and/or dendrites (not axons of passage) for up to 8h periods (11, 19, 54).

IV.2.7. Monitoring IC neuron activity

The activity generated by individual IC neurons was identified in different time windows (c.f., before *versus* during each intervention depicted below). The time window before an intervention (baseline) was defined as the 1 min time interval preceding each intervention. The time window during an intervention covered the entire duration of that intervention. The significance level of the observed differences in firing rates was assessed using a statistical test based on the Skellam distribution developed for cortical neurons (50), as adapted for IC neurons (11). Using this statistical approach, the significance of changes in firing rates before and during each intervention was computed for all identified IC neurons during all interventions. This permitted matrix summarization of all responses that identified IC neurons underwent during each intervention. Change in neuronal activity so engendered was considered moderate when $p<0.05$ and strong when $p<0.01$.

IV.2.8. Determination of neuron subtypes

We classified the behavior of identified neurons according to their activity characteristics in response to the following interventions: (1) touching the ventral LV or RV (conus vs sinus); (2) 20 sec descending aorta occlusion; (3) 20 sec inferior vena cava occlusion; (4) stimulation (1 Hz for 1 min) of RCV vs LCV; and (5) stimulation (1 Hz for 1 min) of right vs left stellate ganglia. By these means, each

neuron was classified according to how it responded to each of those interventions and change in firing rate ($p<0.05$). When a neuron responded solely to one or more afferent stressors (interventions 1-3 above), it was classified as an afferent LCN. Efferent LCNs were identified as those responding indirectly (variable latency) to one or more efferent (vagal vs sympathetic motor) inputs. IC neurons that respond with a fixed latency to efferent inputs were classified as efferent IC neurons (6). IC neurons that responded indirectly to both afferent and efferent stressor were classified as convergent LCN (11). Identified neurons that did not respond to any stressor were classified as exhibiting unknown function.

IV.2.9. AF characteristics

Atrial electrograms were recorded from the ventral right atrial free wall and referenced to a Wilson Central terminal. From the atrial electrograms, the following response characteristics were determined during the atrial tachyarrhythmia: latency (defined as the interval from the first applied stimulus to tachyarrhythmia initiation); duration of the AF (defined as time from onset to self-termination of AF); and dominant frequency of atrial activity during induced AF episode. When AF was not initiated by MNS, AF duration was by definition set to zero. The duration of AF episodes occurring before and after VNS were compared by reference to the duration of each, as obtained from one or more AF episodes induced before and after the VNS protocols. The effects of VNS therapy was separated into 4 categories, using MNS as the constant defined stressor: i) AF prevention (AF initiation failed); ii) AF mitigation (AF duration reduced by 20% or more); iii) AF prolongation (AF duration increased by at least 20%) and iv) as having no effect. Results were considered to be not significant (no effect) when occurring up to the 20% range.

IV.2.10. Time dependence of VNS effect

Kaplan-Meier survival analysis was performed to estimate how long the effect of VNS lasted as the number (up to 7) and timing of successive AF initiation attempts (at 5 to 10 min intervals, if needed) varied. When a mediastinal nerve stimulus evoked an AF episode as long as the reference (control state) episode, sequential MNS trials were terminated. Accordingly, VNS efficacy at time *t* was defined as the

percentage of experiments for which the latest unsuccessful AF attempt (if any) occurred after time t. A second survival curve was also created based on the percentage of experiments in which the latest mitigated AF episode (if any) occurred after time *t* in order to determine how long neuronal effectiveness lasted.

IV.2.11. Neuronal synchrony

In each animal, a synchrony index (SI) was calculated (34) in order to characterize the possibility of synchrony of activity generated among different populations of IC neurons identified during: i) baseline states as compared to ii) during episodes of neurally-induced atrial arrhythmias. The potential of VNS to alter IC synchrony was likewise assessed. Since there was a limitation of analysis imposed by the limited number of action potentials identified during the 3 min of VNS, synchrony analysis was not performed in such instances. The synchrony of activities displayed by different populations of identified neurons, as defined by Agmon (1), was analyzed by assessing the activity generated by pairs of identified neurons in each animal. In order to calculate such a synchrony index (SI), one neuron was defined as the reference and the other as the target. Different SI values were obtained that depended on which neuron was considered reference, thus making the SI a non-symmetric measure. As such, calculation of this SI index required the identification of coincidences of activities among neurons when reference and target neurons both generate activity within a time window of selected duration τ. Our previous study defined the optimal value for τ with respect to intrinsic cardiac neurons to be 40 ms (34). The number of coincidences was obtained by counting reference spikes during which coincidence occurred. Given that some coincidences may be random in nature, the coincidence count was also estimated in surrogate data obtained by applying a random jitter to the reference spikes in each time window of duration 4τ (1). To obtain normalized SI values, the mean coincidence count in surrogate data was subtracted from the actual coincidence count identified. Thereafter, the resultant was divided by the number of reference spikes. Surrogate data also served to calculate a *p*-value in order to assess statistical significance of these data. When the number of neuron pairs demonstrating statistically significant synchrony was identified ($p <$

0.01 and SI > 0.01), a chi-square test was performed to assign statistical significance to changes in the number of synchronized pairs for each neuronal subtype combination studied (51).

IV.3. Results

IV.3.1. Functional response characteristics of identified right atrial neurons

A total of 89 neurons were identified in the 8 subjects studied (11.1±3.5 neurons per subject). Figure 1C shows the patterns of IC neural responses from one representative animal (n = 10 neurons) as ascribed to discrete afferent or efferent inputs. Based on these responses, IC neurons were sub-classified as afferent, efferent or convergent LCN's. The distribution of such grouping in this representative animal was reflected across all 8 animals (Fig. 1D). Of the 89 functionally identified right atrial neurons (those that generated spontaneous activity), 65 neurons were characterized as being i) afferent local circuit neurons (n = 15; 17%), ii) efferent local circuit neurons (n= 20; 22%) or iii) convergent local circuit neurons (n = 30; 34%) by their response to defined afferent and efferent stressors. The rest (n=24; 27%) did not respond to any of these imposed stressors; as such, their function was labeled as being unknown (Fig. 1D, right panel).

IV.3.2. Effects of right-sided mediastinal nerve stimulation on right atrial neurons and atrial electrophysiological stability

Figures 1A and 2A illustrate representative responses from individual animals in which a brief period of MNS stimulation elicited transient periods of atrial arrhythmia (atrial tachycardia/atrial fibrillation; AT/AF). Note that bradycardia usually precedes the onset of AT/AF (Figure 1A) and that there is an increase in IC activity during MNS and that activity persisted during progression of atrial arrhythmia (Figure 2A). In 5 of 9 neurons, activity persisted briefly even after spontaneous conversion back to sinus rhythm . Across all animals and in response to MNS, IC activity increased preferentially in response to MNS among the convergent LCN IC population (0.13±0.3 to 0.88±1.73 Hz, $p<0.001$), with less effects occurring in afferent LCNs (0.07±0.3 to 0.14±0.43 Hz, $p<0.032$) and no change occurring in the efferent LCN population (0.11±0.3 to 0.21±0.74 Hz, $p<0.24$). Average neuronal activity across all

recorded IC neurons was 0.11±0.29 Hz at baseline, increasing to 0.51±1.30 Hz ($p<.001$) during the MNS induced atrial tachyarrhythmia.

Figures 1B and 3 summarize the impact of VNS on the AF inducibility to right-sided MNS. The latency for AF induction at baseline was 2.68±2.32 seconds, this latency increased to 3.32± 2.82 seconds after RCV and decreased to 2.25±3.19 seconds after applying LCV. These changes were not significant. AF responses to pre-emptive VNS stratified into 4 subgroups, 32% were prevented (10 of 31 trials, see Fig 1B for example), 26% mitigated (8 of 31 trials), 23% were not impacted (7/31 trials) and 19% prolonged (6 of 31 trials) (Fig. 3A). AF prolongations were most evident with left-sided VNS evaluated against right-sided mediastinal nerve stimulation. When AF was induced by MNS, dominant frequency was similar with and without pre-emptive VNS. Dominant frequency (sham VNS) was not predictive of anti-arrhythmic effects for subsequent VNS.

IV.3.3. Effects of ipsilateral vagal nerve stimulation on IC activity and the potential for neurally induced atrial arrhythmias

Right-sided VNS impacted right atrial neural function in response to MNS (Figs 2B and 4A). It mitigated the potential for neurally-induced AF by 75% (Fig 4B). Prior to VNS, MNS increased the activity among both afferent and convergent LCN sub-populations (Fig 4A, black lines). Following pre-emptive right-sided VNS, basal activity was differentially decreased among efferent LCNs (0.16±0.4 vs 0.06±0.19 Hz; $p<0.01$). Post-VNS, MNS-induced excitation of convergent LCNs was blunted (0.91±1.73 vs 0.26±0.73 Hz; $p<0.002$), being totally eliminated among afferent LCN populations (Fig 4A).

IV.3.4. Effects of contralateral vagal nerve stimulation on IC activity and the potential for neurally induced atrial arrhythmias

In contrast to ipsilateral VNS, left-sided vagal stimulation exerted no significant change in basal IC neuronal activity (Fig 5A). However, as with right-sided VNS, LCV differentially mitigated the MNS-induced increase in convergent LCN activity (0.84±1.74 vs 0.34±0.49 Hz; $p=0.057$). The potential for

MNS-induced AF was mitigated in 40% by LCV VNS and without effect in 27% of cases. Pre-emptive left-sided VNS enhanced AF induced from 33% of right-sided MNS sites evaluated (Figure 5B).

IV.3.5. IC network characteristics: neuronal synchrony

The MNS-induced increases in IC activity are reflective of common shared input and/or interdependent local network interactions mediated by LCNs (6). Figure 6 illustrates this potential by evaluating synchrony among the various pairs of IC neurons identified within the RAGP during baseline conditions, as well as during MNS induced changes i) prior to (top panel) and following pre-emptive right-sided VNS (bottom panel). In the sham (unstimulated) VNS state, note that while there was minimal coherence of activity among the various sub-populations of IC neurons identified, in response to MNS there was a preferential increase in IC synchrony among convergent LCNs, as well as between convergent and efferent LCN sub-populations. Following right-sided VNS, while there was a differential increase in synchrony during baseline states among convergent LCNs (Fig 6, bottom panel); any MNS-induced change in IC synchrony was extinguished.

IV.3.6. IC network characteristics: memory

The efficacy of VNS therapy in terms of shortening/preventing MNS-induced arrhythmias (post-VNS) was assessed via Kaplan-Meier survival analysis (Fig. 7). Following right-sided VNS, anti-arrhythmic effects against repeated MNS-induced arrhythmias was attenuated for 20 min after VNS therapy (top panel); it was extinguished by ~40 min post VNS (Fig. 7A; fitting exponential function resulted in a time constant of 26±2 min [95% confidence interval]). While the overall anti-arrhythmic efficacy of contralateral VNS was reduced (Fig 7, bottom panel), the time constants derived from RCV vs LCV responses were not significantly different (logrank test). For corresponding MNS-induced changes in IC activity, the pre-VNS induced change in convergent activity (0.11 to 1.57 Hz, $p=0.023$) was suppressed immediately after VNS (0.04 to 0.38 Hz, $p=0.17$), and recovered ~30 min post VNS (0.07 to 1.28 Hz, $p=0.016$). Following recovery, characteristics of MNS-induced AF (latency, duration and dominant frequency) were similar to sham VNS control (data not shown).

IV.4. Discussion

The major findings of this study are: 1) VNS can attenuate AF through its effects of specific cardiac neuronal populations, the convergent LCN's; 2) Convergent LCN's are central to neurally-induced AF induction and are the primary neural targets for anti-arrhythmic effect of VNS; 3) Disruptive neural inputs to the ICNS differentially alter coherence of activity among IC neurons and pre-emptive VNS modifies such effects; 4) ipsilateral VNS imparts a greater impact on IC neural function and the ability to stabilize the ICNS against neural imbalance; and 5) The anti-arrhythmic effects imparted by VNS has memory.

IV.4.1. ICN modulation of cardiac function

The ICNS is composed of heterogeneously dispersed populations of neurons, with stratification into distinct classes of neurons (6, 11). These IC neurons can be functionally stratified by their *in situ* behavior based on their responses to different stressors according to whether they belong to either afferent, efferent or convergent LCN sub-types (6, 11). The convergent LCNs are responsible for primary reflex integration within the ICNS (6), coordinating atrial and ventricular tissues via its efferent outputs. With respect to central autonomic efferent preganglionic axons, they project directly onto intrinsic cardiac efferent post-ganglionic (intrinsic cardiac parasympathetic and sympathetic) neurons as well as convergent LCNs (11, 36, 39). These IC network interactions are critical to mediating sympathetic/parasympathetic cardiomotor outflow (36, 38).

IV.4.2. ICN processing and atrial arrhythmias

Asymmetric neural inputs to the IC network increase the potential for AT/AF (9, 17). Stochastic processing within that network underlies the instability that can occur within the ICNS to initiate arrhythmias (30, 31). The resultant "hyper-stochasticity" displayed among its convergent LCNs in response to MNS appears to be fundamental to any enhancement of an arrhythmia potential (21). Our study shows, any such enhancement of activity among IC LCNs can be associated with increases in their coherence to effect local efferent neuronal outflows (24, 36). Such coherence or lack thereof, is ultimately

dependent on intra-ganglionic interconnections (27, 54). Our data indicates that IC network interactions can be targeted therapeutically to modify atrial arrhythmia induction.

IV.4.3. VNS and ICN network function

VNS therapy not only impacts individual IC activity, but also coherence displayed among its neurons (29). Data presented herein shows VNS, through effects on IC network interactions, blunts IC responsiveness to excessive neural inputs. In fact, its convergent LCNs appear to be central to the stabilizing influence of the VNS therapy both in terms of neural activation and resulting cardiac electrophysiological stability. Previous studies have demonstrated that aggregates of the IC ganglionic plexus neurons exert preferential spheres of influence on cardiac tissues, manifested by their direct and indirect projections to cardiomyocytes (5, 59, 60). With respect to the RAGP, although it exerts preferential control of sinoatrial nodal pacemaker activity, it also influences distant atrial tissues along with ventricular electrical and contractile indices (5, 59).

Medullary derived parasympathetic efferent preganglionic projections likewise have spheres of influence, reflecting both their projections onto specific subpopulations of intrinsic cardiac ganglia and their targeting of convergent LCNs (6, 25, 35, 41). Our data shows that ipsilateral VNS exerts substantially greater anti-arrhythmic effects when targeting right atrial neuronal networks than contralateral preganglionic projections to such ganglia (Fig. 7). Presumably, this reflects insufficient preganglionic efferent coverage to respective (contralateral vs ipsilateral) aggregates of IC neurons (38, 40, 41). This anatomical-functional heterogeneity likely underlies any increased AF potential that right-sided ICNS neural imbalance elicits with left-sided VNS therapy. Regardless of VNS site of delivery, its anti-arrhythmic effects exhibit memory. In this study, 3 min of VNS conferred protection for up to 26 minutes.

IV.4.4. Perspectives and significance

Ablation and surgical approaches for AF, while in many cases exerting adequate short-term management (~80% success rate), show substantial failure rates over time (~60% success rate at 5 years) (14, 57). In fact, failures are not unexpected given the potential of IC networks to adapt and reorganize over time in the face of pathology (10, 32). In addition, there are at least five neuronal aggregates of atrial intrinsic cardiac ganglionated plexuses that modulate atrial function, each with preferential distributions/spheres of influence (15, 59). For example, the RAGP primarily modulates sinoatrial nodal function (5), the posterior atrial ganglionic plexus coordinates sympathetic and parasympathetic interactions for control of chronotropic function (36, 38), and the inferior vena cava-inferior atrial ganglionated plexus modulates inferior atrial electrical functions with effects extending into the atrioventricular node (5). This is in addition to potential contributions mediated by the dorsal atrial ganglionated plexus (59, 60) and its utility as a potential target in conjunction with ablation of the pulmonary vein complex for treatment of AF (28, 48).

A major advantage of electrical neuromodulation therapy, especially when applied at more rostral sites in the neuraxis, is that single point therapy can modulate a wide range of disparate ganglia within the ICNS (6, 41, 42, 62). VNS therapy represents an effective strategy to modulate global ICNS network function/stability. In addition the therapy is readily reversible, has a rapid therapeutic onset and, as the data in this paper shows; it has memory (effects that outlast application).

Mechanistic understanding of autonomic regulator therapy is essential for its effective application. Cervical VNS therapy influences both ascending and descending axonal projections to impact neuronal processing at different (central to target organ) levels of the cardiac neuraxis (12, 13). By understanding mechanistically the underlying pathology and its impact on the neural hierarchy for cardiac control, neuromodulation therapy can be applied to effectively manage the disease process and preserve end-organ function (13, 64).

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References

1. **Agmon A.** A Novel, Jitter-Based Method for Detecting and Measuring Spike Synchrony and Quantifying Temporal Firing Precision. *Neural Syst Circuits* 2: 5, 2012.

2. **Andresen MC, Kunze DL, and Mendelowitz D.** Central Nervous System Regulation of the Heart. In: *Basic and Clincial Neurocardiology*, edited by Armour JA and Ardell JL. New York: Oxford University Press, 2004, p. 187-219.

3. **Ardell JL.** Intrathoracic Neuronal Regulation of Cardiac Function In: *Basic and Clinical Neurocardiology*, edited by Armour JA and Ardell JL. New York: Oxford University Press, 2004, p. 118-152.

4. **Ardell JL, Cardinal R, Beaumont E, Vermeulen M, Smith FM, and Andrew Armour J.** Chronic Spinal Cord Stimulation Modifies Intrinsic Cardiac Synaptic Efficacy in the Suppression of Atrial Fibrillation. *Auton Neurosci* 186: 38-44, 2014.

5. **Ardell JL and Randall WC.** Selective Vagal Innervation of Sinoatrial and Atrioventricular Nodes in Canine Heart. *Am J Physiol* 251: H764-773, 1986.

6. **Armour JA.** Potential Clinical Relevance of the 'Little Brain' on the Mammalian Heart. *Exp Physiol* 93: 165-176, 2008.

7. **Armour JA, Hageman GR, and Randall WC.** Arrhythmias Induced by Local Cardiac Nerve Stimulation. *Am J Physiol* 223: 1068-1075, 1972.

8. **Armour JA and Kember G.** Cardiac Sensory Neurons. In: *Basic and Clinical Neurocardiology*, edited by Armour JA and Ardell JL. New York: Oxford University Press, 2004, p. 79-117.

9. **Armour JA, Richer LP, Page P, Vinet A, Kus T, Vermeulen M, Nadeau R, and Cardinal R.** Origin and Pharmacological Response of Atrial Tachyarrhythmias Induced by Activation of Mediastinal Nerves in Canines. *Auton Neurosci* 118: 68-78, 2005.

10. **Arora RC, Cardinal R, Smith FM, Ardell JL, Dell'Italia LJ, and Armour JA.** Intrinsic Cardiac Nervous System in Tachycardia Induced Heart Failure. *Am J Physiol Regul Integr Comp Physiol* 285: R1212-1223, 2003.

11. **Beaumont E, Salavatian S, Southerland EM, Vinet A, Jacquemet V, Armour JA, and Ardell JL.** Network Interactions within the Canine Intrinsic Cardiac Nervous System: Implications for Reflex Control of Regional Cardiac Function. *J Physiol* 591: 4515-4533, 2013.

12. **Bonaz B, Picq C, Sinniger V, Mayol JF, and Clarencon D.** Vagus Nerve Stimulation: From Epilepsy to the Cholinergic Anti-Inflammatory Pathway. *Neurogastroenterol Motil* 25: 208-221, 2013.

13. **Buckley U, Shivkumar K, and Ardell JL.** Autonomic Regulation Therapy in Heart Failure. *Curr Heart Fail Rep* 12: 284-293, 2015.

14. **Cappato R, Calkins H, Chen SA, Davies W, Iesaka Y, Kalman J, Kim YH, Klein G, Natale A, Packer D, Skanes A, Ambrogi F, and Biganzoli E.** Updated Worldwide Survey on the Methods, Efficacy, and Safety of Catheter Ablation for Human Atrial Fibrillation. *Circ Arrhythm Electrophysiol* 3: 32-38, 2010.

15. **Cardinal R, Page P, Vermeulen M, Ardell JL, and Armour JA.** Spatially Divergent Cardiac Responses to Nicotinic Stimulation of Ganglionated Plexus Neurons in the Canine Heart. *Auton Neurosci* 145: 55-62, 2009.

16. **Chen M, Yu L, Zhou X, Liu Q, Jiang H, and Zhou S.** Low-Level Vagus Nerve Stimulation: An Important Therapeutic Option for Atrial Fibrillation Treatment Via Modulating Cardiac Autonomic Tone. *Int J Cardiol* 199: 437-438, 2015.

17. **Chen PS, Chen LS, Fishbein MC, Lin SF, and Nattel S.** Role of the Autonomic Nervous System in Atrial Fibrillation: Pathophysiology and Therapy. *Circ Res* 114: 1500-1515, 2014.

18. **Fukuda K, Kanazawa H, Aizawa Y, Ardell JL, and Shivkumar K.** Cardiac Innervation and Sudden Cardiac Death. *Circ Res* 116: 2005-2019, 2015.

19. **Gagliardi M, Randall WC, Bieger D, Wurster RD, Hopkins DA, and Armour JA.** Activity of in Vivo Canine Cardiac Plexus Neurons. *Am J Physiol* 255: H789-800, 1988.

20. **Gaita F, Caponi D, Pianelli M, Scaglione M, Toso E, Cesarani F, Boffano C, Gandini G, Valentini MC, De Ponti R, Halimi F, and Leclercq JF.** Radiofrequency Catheter Ablation of Atrial Fibrillation: A Cause of Silent Thromboembolism? Magnetic Resonance Imaging Assessment of Cerebral

Thromboembolism in Patients Undergoing Ablation of Atrial Fibrillation. *Circulation* 122: 1667-1673, 2010.

21. **Gibbons DD, Southerland EM, Hoover DB, Beaumont E, Armour JA, and Ardell JL.** Neuromodulation Targets Intrinsic Cardiac Neurons to Attenuate Neuronally Mediated Atrial Arrhythmias. *Am J Physiol Regul Integr Comp Physiol* 302: R357-364, 2012.

22. **Gibson DN, Di Biase L, Mohanty P, Patel JD, Bai R, Sanchez J, Burkhardt JD, Heywood JT, Johnson AD, Rubenson DS, Horton R, Gallinghouse GJ, Beheiry S, Curtis GP, Cohen DN, Lee MY, Smith MR, Gopinath D, Lewis WR, and Natale A.** Stiff Left Atrial Syndrome after Catheter Ablation for Atrial Fibrillation: Clinical Characterization, Prevalence, and Predictors. *Heart Rhythm* 8: 1364-1371, 2011.

23. **Go AS, Hylek EM, Phillips KA, Chang Y, Henault LE, Selby JV, and Singer DE.** Prevalence of Diagnosed Atrial Fibrillation in Adults: National Implications for Rhythm Management and Stroke Prevention: The Anticoagulation and Risk Factors in Atrial Fibrillation (Atria) Study. *JAMA* 285: 2370-2375, 2001.

24. **Gray AL, Johnson TA, Ardell JL, and Massari VJ.** Parasympathetic Control of the Heart. Ii. A Novel Interganglionic Intrinsic Cardiac Circuit Mediates Neural Control of Heart Rate. *J Appl Physiol (1985)* 96: 2273-2278, 2004.

25. **Gray AL, Johnson TA, Lauenstein JM, Newton SS, Ardell JL, and Massari VJ.** Parasympathetic Control of the Heart. Iii. Neuropeptide Y-Immunoreactive Nerve Terminals Synapse on Three Populations of Negative Chronotropic Vagal Preganglionic Neurons. *J Appl Physiol (1985)* 96: 2279-2287, 2004.

26. **Herring N and Paterson DJ.** Neuromodulators of Peripheral Cardiac Sympatho-Vagal Balance. *Exp Physiol* 94: 46-53, 2009.

27. **Hoover DB, Isaacs ER, Jacques F, Hoard JL, Page P, and Armour JA.** Localization of Multiple Neurotransmitters in Surgically Derived Specimens of Human Atrial Ganglia. *Neuroscience* 164: 1170-1179, 2009.

28.**Katritsis DG, Giazitzoglou E, Zografos T, Pokushalov E, Po SS, and Camm AJ.** Rapid Pulmonary Vein Isolation Combined with Autonomic Ganglia Modification: A Randomized Study. *Heart Rhythm* 8: 672-678, 2011.

29.**Kember G, Ardell JL, Armour JA, and Zamir M.** Vagal Nerve Stimulation Therapy: What Is Being Stimulated? *PLoS One* 9: e114498, 2014.

30.**Kember G, Armour JA, and Zamir M.** Dynamic Neural Networking as a Basis for Plasticity in the Control of Heart Rate. *J Theor Biol* 317: 39-46, 2013.

31.**Kember G, Armour JA, and Zamir M.** Neural Control Hierarchy of the Heart Has Not Evolved to Deal with Myocardial Ischemia. *Physiol Genomics* 45: 638-644, 2013.

32.**Leiria TL, Glavinovic T, Armour JA, Cardinal R, de Lima GG, and Kus T.** Longterm Effects of Cardiac Mediastinal Nerve Cryoablation on Neural Inducibility of Atrial Fibrillation in Canines. *Auton Neurosci* 161: 68-74, 2011.

33.**Lin Y, Bian N, Li H, Chen J, Xing H, Li H, Huang D, Lan X, Gong B, Zhou L, Liu R, Guan M, Zhang D, Du G, Huang Z, Chen X, Zhang T, Feng J, Wu S, Wang L, Zhang A, and Li Z.** Effects of Low-Level Autonomic Stimulation on Prevention of Atrial Fibrillation Induced by Acute Electrical Remodeling. *ScientificWorldJournal* 2013: 781084, 2013.

34.**Longpre JP, Salavatian S, Beaumont E, Armour JA, Ardell JL, and Jacquemet V.** Measure of Synchrony in the Activity of Intrinsic Cardiac Neurons. *Physiol Meas* 35: 549-566, 2014.

35.**McAllen RM, Salo LM, Paton JF, and Pickering AE.** Processing of Central and Reflex Vagal Drives by Rat Cardiac Ganglion Neurones: An Intracellular Analysis. *J Physiol* 589: 5801-5818, 2011.

36.**McGuirt AS, Schmacht DC, and Ardell JL.** Autonomic Interactions for Control of Atrial Rate Are Maintained after Sa Nodal Parasympathectomy. *Am J Physiol* 272: H2525-2533, 1997.

37.**Naccarelli GV, Varker H, Lin J, and Schulman KL.** Increasing Prevalence of Atrial Fibrillation and Flutter in the United States. *Am J Cardiol* 104: 1534-1539, 2009.

38.**Randall DC, Brown DR, Li SG, Olmstead ME, Kilgore JM, Sprinkle AG, Randall WC, and Ardell JL.** Ablation of Posterior Atrial Ganglionated Plexus Potentiates Sympathetic Tachycardia to Behavioral Stress. *Am J Physiol* 275: R779-787, 1998.

39.**Randall DC, Brown DR, McGuirt AS, Thompson GW, Armour JA, and Ardell JL.** Interactions within the Intrinsic Cardiac Nervous System Contribute to Chronotropic Regulation. *Am J Physiol Regul Integr Comp Physiol* 285: R1066-1075, 2003.

40.**Randall WC and Ardell JL.** Selective Parasympathectomy of Automatic and Conductile Tissues of the Canine Heart. *Am J Physiol* 248: H61-68, 1985.

41.**Randall WC, Ardell JL, O'Toole MF, and Wurster RD.** Differential Autonomic Control of San and Avn Regions of the Canine Heart: Structure and Function. *Prog Clin Biol Res* 275: 15-31, 1988.

42.**Randall WC, Milosavljevic M, Wurster RD, Geis GS, and Ardell JL.** Selective Vagal Innervation of the Heart. *Ann Clin Lab Sci* 16: 198-208, 1986.

43.**Richer LP, Vinet A, Kus T, Cardinal R, Ardell JL, and Armour JA.** Alpha-Adrenoceptor Blockade Modifies Neurally Induced Atrial Arrhythmias. *Am J Physiol Regul Integr Comp Physiol* 295: R1175-1180, 2008.

44.**Scherlag BJ, Nakagawa H, Jackman WM, Lazzara R, and Po SS.** Non-Pharmacological, Non-Ablative Approaches for the Treatment of Atrial Fibrillation: Experimental Evidence and Potential Clinical Implications. *J Cardiovasc Transl Res* 4: 35-41, 2011.

45.**Schwarz N, Kuniss M, Nedelmann M, Kaps M, Bachmann G, Neumann T, Pitschner HF, and Gerriets T.** Neuropsychological Decline after Catheter Ablation of Atrial Fibrillation. *Heart Rhythm* 7: 1761-1767, 2010.

46.**Shen MJ, Choi EK, Tan AY, Lin SF, Fishbein MC, Chen LS, and Chen PS.** Neural Mechanisms of Atrial Arrhythmias. *Nat Rev Cardiol* 9: 30-39, 2012.

47.**Shen MJ, Shinohara T, Park HW, Frick K, Ice DS, Choi EK, Han S, Maruyama M, Sharma R, Shen C, Fishbein MC, Chen LS, Lopshire JC, Zipes DP, Lin SF, and Chen PS.** Continuous Low-

Level Vagus Nerve Stimulation Reduces Stellate Ganglion Nerve Activity and Paroxysmal Atrial Tachyarrhythmias in Ambulatory Canines. *Circulation* 123: 2204-2212, 2011.

48. **Shen MJ and Zipes DP.** Role of the Autonomic Nervous System in Modulating Cardiac Arrhythmias. *Circ Res* 114: 1004-1021, 2014.

49. **Sheng X, Scherlag BJ, Yu L, Li S, Ali R, Zhang Y, Fu G, Nakagawa H, Jackman WM, Lazzara R, and Po SS.** Prevention and Reversal of Atrial Fibrillation Inducibility and Autonomic Remodeling by Low-Level Vagosympathetic Nerve Stimulation. *J Am Coll Cardiol* 57: 563-571, 2011.

50. **Shin HC, Aggarwal V, Acharya S, Schieber MH, and Thakor NV.** Neural Decoding of Finger Movements Using Skellam-Based Maximum-Likelihood Decoding. *IEEE Trans Biomed Eng* 57: 754-760, 2010.

51. **Snedocor G and Cochrane W.** *Statistical Methods*: Iowa State University Press, 1980.

52. **Southerland EM, Gibbons DD, Smith SB, Sipe A, Williams CA, Beaumont E, Armour JA, Foreman RD, and Ardell JL.** Activated Cranial Cervical Cord Neurons Affect Left Ventricular Infarct Size and the Potential for Sudden Cardiac Death. *Auton Neurosci* 169: 34-42, 2012.

53. **Stavrakis S, Humphrey MB, Scherlag BJ, Hu Y, Jackman WM, Nakagawa H, Lockwood D, Lazzara R, and Po SS.** Low-Level Transcutaneous Electrical Vagus Nerve Stimulation Suppresses Atrial Fibrillation. *J Am Coll Cardiol* 65: 867-875, 2015.

54. **Thompson GW, Collier K, Ardell JL, Kember G, and Armour JA.** Functional Interdependence of Neurons in a Single Canine Intrinsic Cardiac Ganglionated Plexus. *J Physiol* 528: 561-571, 2000.

55. **Waldmann M, Thompson GW, Kember GC, Ardell JL, and Armour JA.** Stochastic Behavior of Atrial and Ventricular Intrinsic Cardiac Neurons. *J Appl Physiol (1985)* 101: 413-419, 2006.

56. **Wang S, Zhou X, Huang B, Wang Z, Liao K, Saren G, Lu Z, Chen M, Yu L, and Jiang H.** Spinal Cord Stimulation Protects against Ventricular Arrhythmias by Suppressing Left Stellate Ganglion Neural Activity in an Acute Myocardial Infarction Canine Model. *Heart Rhythm* 12: 1628-1635, 2015.

57. **Weerasooriya R, Khairy P, Litalien J, Macle L, Hocini M, Sacher F, Lellouche N, Knecht S, Wright M, Nault I, Miyazaki S, Scavee C, Clementy J, Haissaguerre M, and Jais P.** Catheter

Ablation for Atrial Fibrillation: Are Results Maintained at 5 Years of Follow-Up? *J Am Coll Cardiol* 57: 160-166, 2011.

58. **Yamakawa K, So EL, Rajendran PS, Hoang JD, Makkar N, Mahajan A, Shivkumar K, and Vaseghi M.** Electrophysiological Effects of Right and Left Vagal Nerve Stimulation on the Ventricular Myocardium. *Am J Physiol Heart Circ Physiol* 307: H722-731, 2014.

59. **Yuan BX, Ardell JL, Hopkins DA, and Armour JA.** Differential Cardiac Responses Induced by Nicotine Sensitive Canine Atrial and Ventricular Neurones. *Cardiovasc Res* 27: 760-769, 1993.

60. **Yuan BX, Ardell JL, Hopkins DA, Losier AM, and Armour JA.** Gross and Microscopic Anatomy of the Canine Intrinsic Cardiac Nervous System. *Anat Rec* 239: 75-87, 1994.

61. **Zhang Y, Ilsar I, Sabbah HN, Ben David T, and Mazgalev TN.** Relationship between Right Cervical Vagus Nerve Stimulation and Atrial Fibrillation Inducibility: Therapeutic Intensities Do Not Increase Arrhythmogenesis. *Heart Rhythm* 6: 244-250, 2009.

62. **Zhang Y and Mazgalev TN.** Arrhythmias and Vagus Nerve Stimulation. *Heart Fail Rev* 16: 147-161, 2011.

63. **Zhang Y, Scherlag BJ, Lu Z, Niu GD, Yamanashi WS, Hogan C, Fields J, Ghias M, Lazzara R, Jackman WM, and Po S.** Comparison of Atrial Fibrillation Inducibility by Electrical Stimulation of Either the Extrinsic or the Intrinsic Autonomic Nervous Systems. *J Interv Card Electrophysiol* 24: 5-10, 2009.

64. **Zipes DP.** Antiarrhythmic Therapy in 2014: Contemporary Approaches to Treating Arrhythmias. *Nat Rev Cardiol* 12: 68-69, 2015.

65. **Zucker IH and Gilmore JP.** *Reflex Control of the Circulation*. Boca Raton: CRC Press, 1991.

66. **Zucker IH, Patel KP, and Schultz HD.** Neurohumoral Stimulation. *Heart Fail Clin* 8: 87-99, 2012.

Figure legends

Figure 1. *VNS effects on MNS-induced AF and functional classification of IC neurons.* Atrial electrical activity recorded from a unipolar electrode on the ventral right atrial free wall. Bursts of electrical stimuli were applied to a caudal right-sided mediastinal nerve during the atrial refractory period (arrows above) before (A) and after (B) preemptive right-sided VNS. Note the prolongation of atrial cycle length (CL) that transitioned to atrial tachyarrhythmia before, but not after VNS. IC neurons are classified based on their functional responses to application of afferent [(touch of right (TRV) or left (TLV) ventricle; occlusion of descending aorta (AOR) or inferior vena cava (IVC)] vs efferent [(right (RCV) or left (LCV) cervical vagus or stellate ganglia (right, RSS; left, LSS) electrical stimulation] interventions. Panel C represents the distribution of response derived from 10 IC neurons in one representative animal. Panel D sub-classifies identified IC neurons based on their functional responses to defined inputs for the representative animal (left pie chart) vs the entire population of 89 IC neurons identified in all 8 animals (right pie chart). Afferent-related IC neurons responded differentially to at least one of the following stressors: TRV, TLV, AOR or IVC. Efferent-related IC neurons responded to cervical vagal and/or stellate stimulation. Convergent IC neurons were modulated by sub-sets of afferent and efferent inputs. Approximately 1/5 of IC neurons evaluated had basal discharge that was unaffected by any of the afferent or efferent stressors tested and, as such, are defined as unknown.

Figure 2. *Representative responses to mediastinal nerve stimulation (A) prior to and (B) following RCV VNS.* A right atrial electrogram (RAE) is shown with concomitant activities generated by 9 IC neurons. Panel A shows the control state where AF was induced right-sided

MNS. Panel B shows response when the same MNS site was stimulated 1 min following 3 min of pre-emptive of right-sided VNS. Solid arrows delimit time of the MSN nerve stimulations. Dashed line (panel A) indicates duration of AF induced by MNS. As shown in panel B, following RCV, MNS failed to induce AF, even when applied for longer periods of time (20 s) than before therapy.

Figure 3. *Characteristics of MNS-induced AF pre- and post- VNS.* Panel A summarizes induces changes in AF duration. Panel B summarizes dominant frequency of AF. Responses are sub-grouped into those where VNS prevented, mitigated, had no effect or prolonged atrial fibrillation. * $p<0.02$ from sham VNS.

Figure 4. A. Neural response of IC neurons to MNS in the absence (solid line) vs immediately following pre-emptive right-sided (ipsilateral) therapy (Dashed line: RCV VNS). IC neurons were sub-classified as convergent, afferent or efferent LCN's as defined by protocol outlined in Figure 1. In control states, right-sided MNS stimulations induced AF in 100% of cases reflecting the emergent and heterogeneous activation of IC neurons within the RAGP intrinsic cardiac ganglia. Convergent LCNs were the predominant population of IC's activated by MNS and the primary target for neuromodulation/suppression by pre-emptive RCV therapy. B. Impact of ipsilateral VNS therapy on the atrial arrhythmogenic potential to MNS. Effects of RCV VNS were classified according to whether it prevented, blunted or enhanced AF or exerted no effects on MNS-induced AF. * $p<0.05$ from baseline; # $p<0.05$ from control.

Figure 5. A. Neural response of IC neurons to MNS in the absence (solid line) vs immediately following pre-emptive of left-sided (contralateral) therapy (Dashed line: LCV

VNS). IC neurons were sub-classified as convergent, afferent or efferent LCN's as defined by protocol outlined in Figure 1. Convergent LCNs were the predominant population of IC's activated by MNS and the primary target for neuromodulation/suppression by pre-emptive LCV therapy. B. Impact of contralateral VNS therapy on the atrial arrhythmogenic potential to MNS. Effects of LCV VNS were classified according to whether it prevented, blunted or enhanced atrial fibrillation or exerted no effects on MNS-induced AF. While LCV VNS mitigated the AF potential for 40% of MNS sites tested, in contradistinction to RCV VNS, it enhanced that potential in 1/3 of MNS sites tested. * $p<0.05$ from baseline.

Figure 6. *MNS-induced changes in IC network synchrony*. The synchronized activities generated by identified pairs of IC neurons (SI>0.01 and $p<0.01$) were determined and the classified post-hoc according to the following subtypes: [A] afferent LCNs; [E] efferent LCNs; and [C] convergent LCNs. Panels show the degree of synchrony between the 6 combinations of IC subclass pairings elicited during: i) baseline states (black bars) and ii) in during MNS-induced arrhythmias (grey bars). The top panel shows these relationships in untreated (sham VNS) states as well as during neurally induced AF. Note that MNS-induced a differential increase in synchrony between efferent to convergent IC pairs (E:C) as well as the convergent to convergent pairings (C:C). The bottom panel illustrates the effects of pre-emptive RCV VNS on these same IC neurons. While pre-emptive RCV differentially increased synchrony between convergent LCNs at baseline, it eliminated the increase in synchrony across all 6 IC subclass pairings during MNS. * $p< 0.02$ from baseline; # $p<0.01$ sham to RCV VNS state.

Figure 7. *VNS induced neural memory and its anti-arrhythmic effects***.** (A) Evolution of the effects of right-sided VNS therapy on the capacity of MNS to induce AF (% efficacy), as a function of time post-therapy. Light gray curve represent the percentage of cases (Kaplan-Meier survival curve) in which AF duration was shortened or prevented; dark curve indicates when pre-emptive RCV prevented MNS-induced AF. (B) Similar data derived with respect to AF potential when left-sided (LCV) therapy was applied pre-emptively.

Figure.1

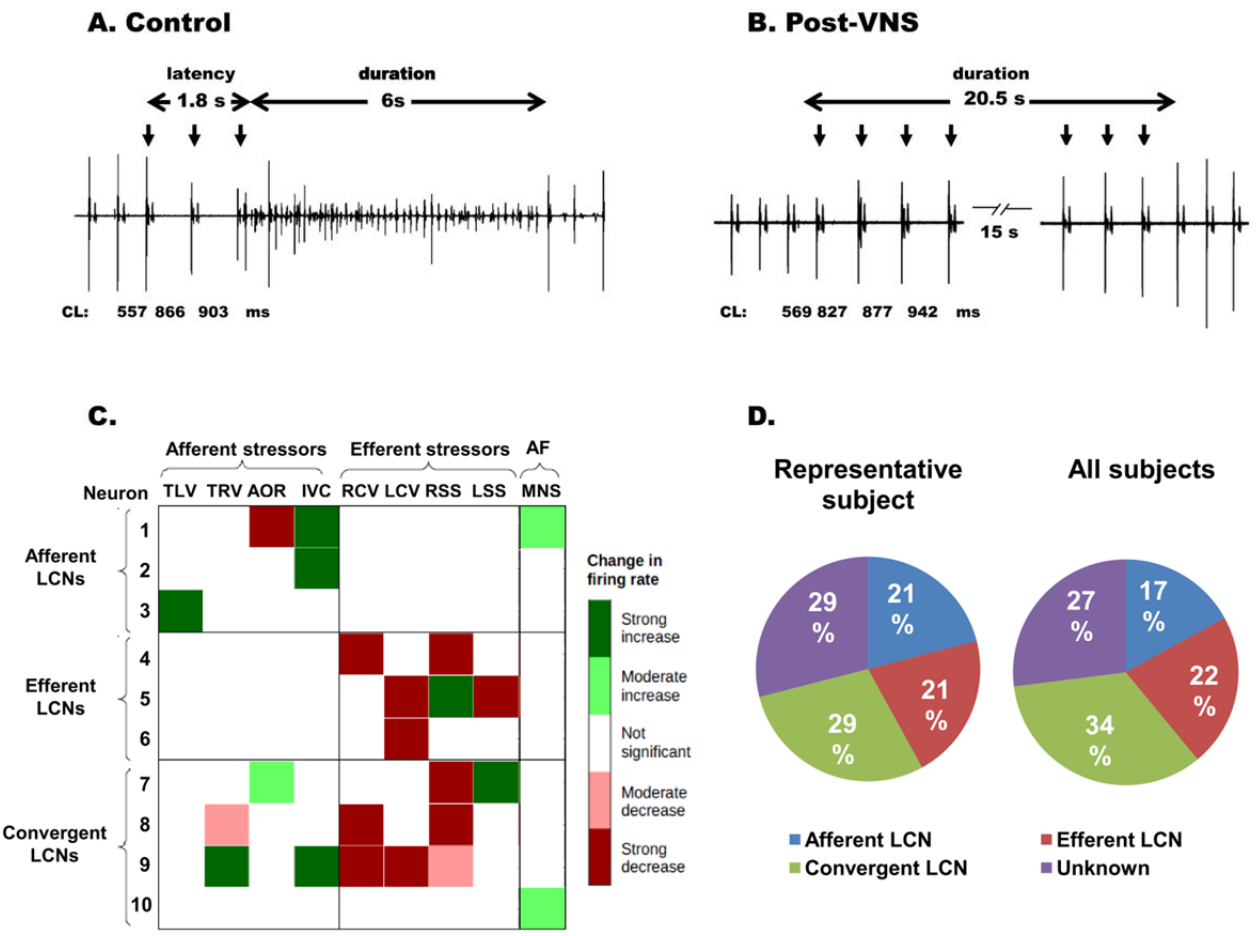

A. Control
latency
1.8 s
duration
6s
CL: 557 866 903 ms
B. Post-VNS
duration
20.5 s
15 s
CL: 569 827 877 942 ms
C.
Afferent stressors
Efferent stressors
AF
Neuron
TLV TRV AOR IVC RCV LCV RSS LSS MNS
Afferent LCNs
Efferent LCNs
Convergent LCNs
1
2
3
4
5
6
7
8
9
10
Change in firing rate
Strong increase
Moderate increase
Not significant
Moderate decrease
Strong decrease
D.
Representative subject
All subjects
29 %
21 %
21 %
29 %
27 %
17 %
22 %
34 %
Afferent LCN
Efferent LCN
Convergent LCN
Unknown

Figure 2.

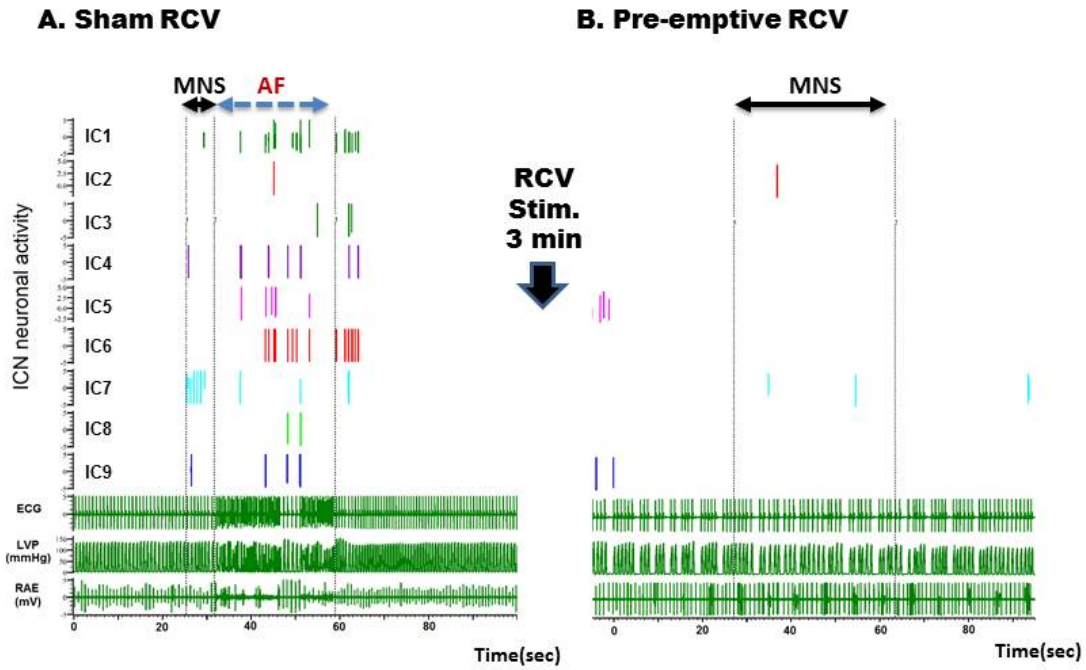

A. Sham RCV
B. Pre-emptive RCV
MNS
AF
MNS
RCV
Stim.
3 min
IC1
IC2
IC3
IC4
IC5
IC6
IC7
IC8
IC9
ICN neuronal activity
ECG
LVP
(mmHg)
RAE
(mV)
0
20
40
60
80
Time(sec)
0
20
40
60
80
Time(sec)

Figure 3.

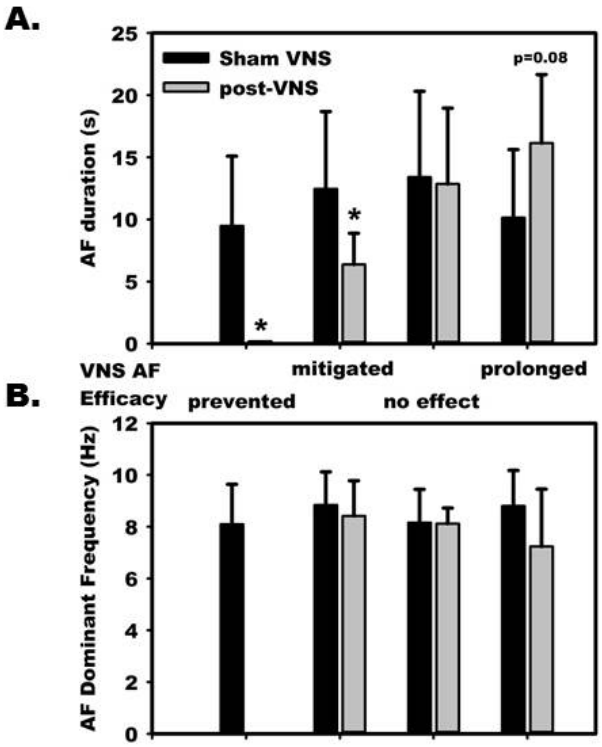

A.
Sham VNS
post-VNS
p=0.08
AF duration (s)
25
20
15
10
5
0
*
*
VNS AF Efficacy
prevented
mitigated
no effect
prolonged
B.
AF Dominant Frequency (Hz)
12
10
8
6
4
2
0

Figure 4.

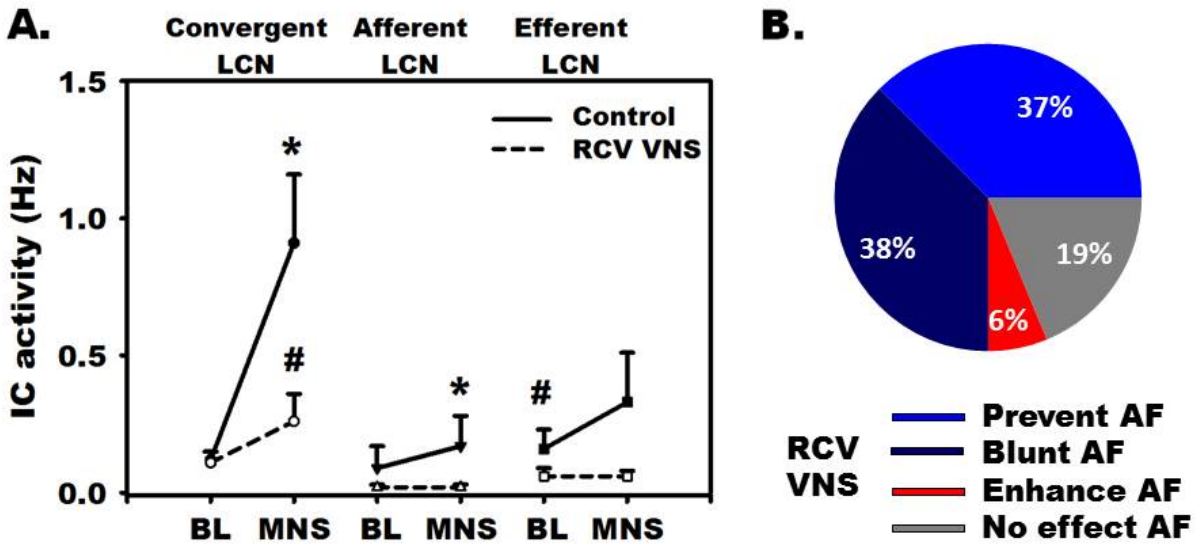

A.
Convergent LCN
Afferent LCN
Efferent LCN
Control
RCV VNS
IC activity (Hz)
1.5
1.0
0.5
0.0
BL MNS BL MNS BL MNS
B.
37%
38%
19%
6%
RCV VNS
Prevent AF
Blunt AF
Enhance AF
No effect AF

Figure 5.

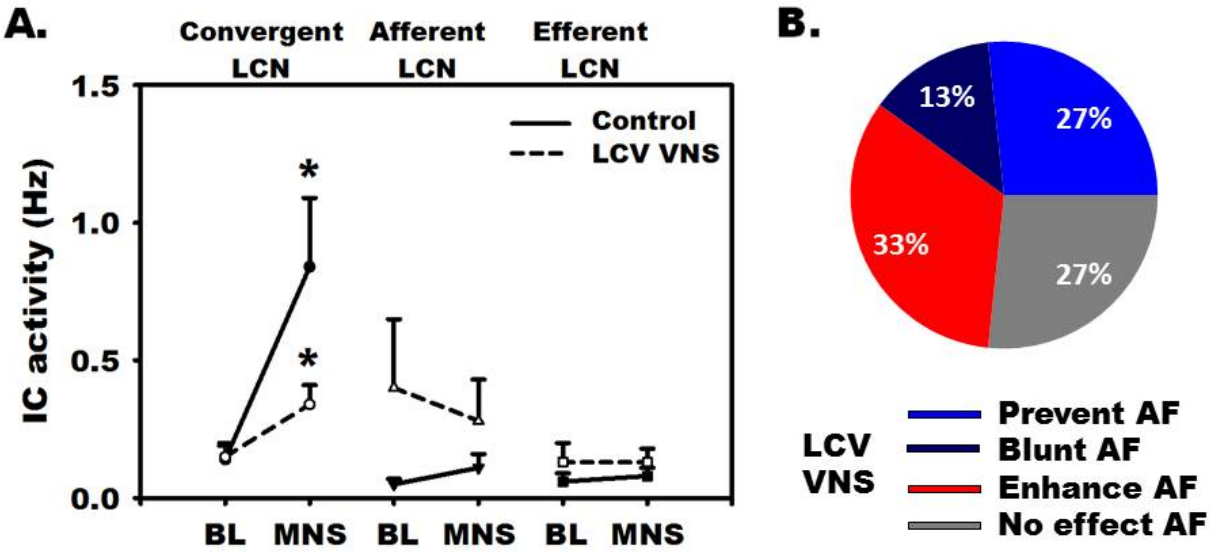

A.
Convergent LCN
Afferent LCN
Efferent LCN
Control
LCV VNS
IC activity (Hz)
1.5
1.0
0.5
0.0
*
*
BL MNS BL MNS BL MNS
B.
13%
27%
33%
27%
LCV VNS
Prevent AF
Blunt AF
Enhance AF
No effect AF

Figure 6.

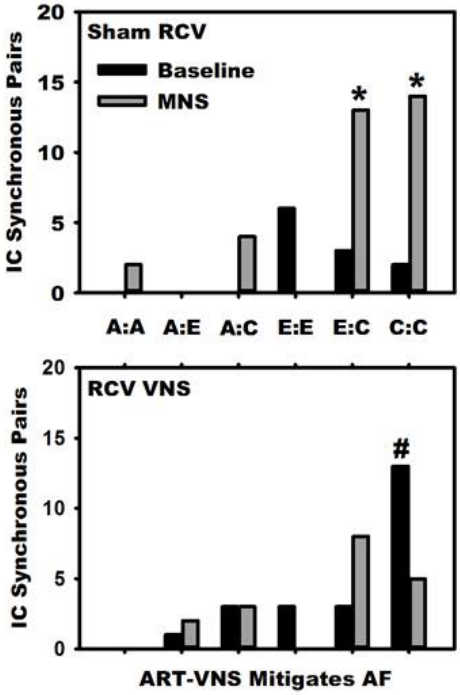
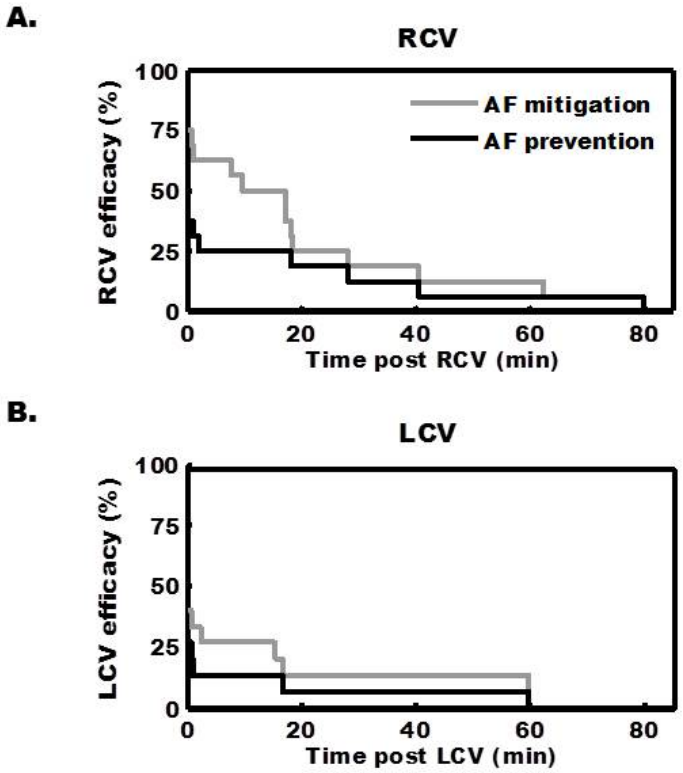

Sham RCV
Baseline
MNS
IC Synchronous Pairs
20
15
10
5
0
*
*
A:A
A:E
A:C
E:E
E:C
C:C
RCV VNS
IC Synchronous Pairs
#
ART-VNS Mitigates AF

Figure 7.


A.
RCV
RCV efficacy (%)
100
75
50
25
0
0
20
40
60
80
Time post RCV (min)
AF mitigation
AF prevention
B.
LCV
LCV efficacy (%)
100
75
50
25
0
0
20
40
60
80
Time post LCV (min)

CHAPTER V: DISCUSSION

V.1. Technological challenges

V.1.1. Recording, identification and classification of intrinsic cardiac neurons activity

Recording the activity of intrinsic cardiac neurons in vivo has its own difficulties. As stated in the methodology part of chapter 2, while the heart is beating, the electrode is paled into the intrinsic cardiac ganglion and it should stay at the same location (while moving with heart) to avoid losing individual neuronal activity throughout whole experiment. Actually this is not the case in recording brain's neuronal activity in which the electrode is implanted and it is fixed during the experiment. Finding the appropriate ganglion with active neurons is also an important point in recording neuronal activities. Using new technologies, it is now possible to record and display the neuronal signal in real-time, therefore the place of electrode could be changed in case no neuronal activity is showed on the monitor; this could happen when the electrode is not close enough to neuronal somata in extracellular recordings. Using multichannel microelectrode array helped us to cover more recording areas and detect superimposed atrial activity on neuronal signal. Unfortunately these electrodes could not be used for many times, and they had to be replaced when the signal to noise ratio on the neuronal signal was very low. Using a new electrode helps to have a better clean recording. Normally the recorded activities on the first and last channels are not very useful, because they have very low signal to noise ratio. Generally the recording process could be improved by using a new electrode to increase signal to noise ratio and finding a spot where we can record from more neurons.

Identification and classification of cardiac activities were done in Spike2 software. This part of the analysis is very critical because all the rest of analyses which are related to neuronal activity are based on this identification and classification. The identification was done by using a threshold which is close to the noise level. Using a high threshold for detecting neuronal activity leads to detection of more obvious neuronal activity and it could be done very fast. However, using a low threshold leads to detection of

small action potentials (which are presumably generated by a neuronal soma not very close to the electrode), therefore it catches more neuronal activity. The big disadvantages of using low threshold is that it detects many artifacts as neuronal activity, and it is very time consuming to only extract neuronal activity from detected activities. Therefore there is always a trade-off between using a low or high threshold for neuronal activity detection. In this project the threshold was chosen to be close to the noise level, so there would be maximal neuronal activity detection and manageable number of artifact/noise detection. Classification part was done using principal component analysis, and other measurement tools with Spike2 which helped to classify the neuronal activity. The identification and classification part could be improved by applying principal component analysis and other new measurement tools combined by visual inspection[208]. This could be very time consuming in case the signal to noise ratio is low, the detection threshold is low and high accuracy is required. To accelerate the identification and classification process, one could only extract the part of signals that are going to be used for the further analysis and apply identification and classification process only on these extracted parts. In case we have different recording files for the same experiment, it is very important to merge all files and then apply the analysis; otherwise combining neurons from different files could be very complicated and time consuming.

Using all these tools and analysis made it possible to have the activity of individual neurons throughout 4 hours of recording, which was a big improvement to the previous single channel recording [176].

V.1.2. Atrial activity detection and cancelation

As discussed, identification of the neuronal activity is one of the most important parts in the analysis. Since we are recording from RAGP and it is very close to the atria, the superimposed electrical activity of atria makes the identification process difficult and time consuming; therefore we need to remove this superimposed atrial activity. By using multichannel microelectrode array, it was possible to detect the atrial activity part by simultaneous activity on all channels of electrode which is not the case in

the neuronal activity (a neuronal activity appears on maximum 2 channels simultaneously). As explained in chapter 3, our first method for removing these atrial activities was to blank the part of signal in which atrial activity was detected. Although blanking method is very fast, it does not allow the maximal detection of neuronal activity. In this method the neuronal activity that are superimposed with atrial activity are removed. This problem becomes more critical when some neurons are cardio-related neurons which fires close to atrial activity's time (chapter III, figure1) or during atrial fibrillation when the heart rate (i.e atrial activity) is increasing. In these cases many neuronal activities will be removed and the rest of analysis on the activities of these neurons will not be reliable (many activities were removed by blanking). In the new method that was discussed in chapter 3, we used principal component analysis to cancel the superimposed activity of atria instead of blanking. The results (chapter III, figure 2) showed that using the new method, we do not lose any activity that is superimposed by atrial activity which improves the identification analysis. In our case, the superimposed atrial activities were simultaneous on all our neuronal channels but if they were not simultaneous, the signals should be aligned first by using the cross correlation function. In a very noisy signal, our atrial activity cancellation method performs better if a low pass filter is applied before applying the method.

V.1.3. Neuronal response to different mechanical and electrical stimuli

The firing rate of majority of intrinsic cardiac neurons in our study was low in the baseline (Chapter 2, figure3). This is not the case in the recording from the neurons in the brain. For that reason, in our study, in most cases, it is difficult to do statistics on the activity of intrinsic cardiac neurons. The neuronal responses to different mechanical stressors and electrical stimuli were shown in chapter 2,figure 7. To calculate the response of neurons, it was assumed that the activity of neurons follow the Poisson distribution due to irregular firing time of neurons. The firing rate of each neuron was compared before and during intervention. As this is not a symmetric comparison because of different duration of compared segments, a correction was applied (see chapter 2). The neuronal responses to different stressors were investigated to understand the effect of these stressors on intrinsic cardiac neurons in RAGP. Moreover

these responses were used to identify the neurons' subtype (afferent, efferent or local circuit neurons). This identification of neurons' could be improved by repeating different stressors and increase the strength of each stressor when applicable (for example severe occlusion while applying aorta occlusion to affect baroreceptors more). However it could be some experimental constrains that prevents the application of more severe stressor. For example very severe aorta occlusion may have a very long lasting effect on the heart and it could be very time consuming to wait for the heart to go back to the baseline state to continue the experiment. Moreover there is a risk that heart does not return to the normal state after applying severe stressor.

V.1.4. Induction of atrial fibrillation and analysis of neuronal activity during atrial fibrillation

In our project we used the canine model and we induced atrial fibrillation by stimulating mediastinal nerve. This induction is related to the stimuli current in such a way that if the stimulation's current is higher, the atrial fibrillation will last longer and if it is not high enough, it is not possible to induce atrial fibrillation. Therefore selecting proper current amplitude for the stimulation is crucial and it should be used throughout the experiment. Another important parameter to induce atrial fibrillation is the location of mediastinal nerve that is being stimulated. In our study we tried different locations (sites) and after finding the best sites, we marked them and we used the same locations for all induction of atrial fibrillation in that canine. Normally after 3-4 pulses of the stimulation, the heart goes to the atrial fibrillation state (chapter IV, figure1) and the atrial fibrillation terminates by itself (this could last from couple of seconds to couple of minutes). As during atrial fibrillation the atria do not contract completely, it is not easy to detect the atrial rate during atrial fibrillation from right atrium electrogram. In a case where the atrioventricular node blockage happens, it is possible to detect it by looking at right atrium electrogram and ECG for the same beat. One of the big challenges for the neuronal activity analysis during atrial fibrillation is the short duration of atrial fibrillation. This could be an issue for the firing rate analysis and also synchrony analysis. For the firing rate analysis, knowing the low firing rate of intrinsic

cardiac neurons, comparing two neurons statistically which had 1 or 2 spikes during 5 seconds of atrial fibrillation does not give good insight. This could be more problematic for the synchrony analysis where we need a minimum amount of time to calculate synchrony index[209]. This issue could be addressed by using average firing rate over different trials of atrial fibrillation and merge different atrial fibrillation episodes for firing rate and synchrony analysis respectively. The result in chapter 2 shows the hyperactivity of intrinsic cardiac neurons during atrial fibrillation [210] therefore in chapter 4 we studied this neuronal activity and we tried to find a way to calm down this hyper activity.

V.1.5. Stimulating vagus nerve: effect on atrial fibrillation and intrinsic cardiac neuronal activity

In chapter 4, it was shown that low level vagus nerve stimulation is able to mitigate atrial fibrillation in a canine model. In this study the right vagus nerve stimulation showed better anti-arrhythmogenic effect and in contrast left vagus nerve stimulation showed some arrhythmogenic effect. This could be explained by knowing that atrial fibrillation was induced by stimulating the right sided mediastinal nerve which creates imbalance in the right sided neuronal network. Since right sided vagus nerve projects more axons extensively to the right atrium ganglionated plexus than left vagus nerve, it could be more effective in inducing network stability throughout this specific complex. This is a very important result for the vagus nerve stimulation therapy because if the vagus nerve stimulation is applied to the wrong side, it may have arrhythmognic effect. Vagus nerve stimulation memory was approximately 26 min for a 3 min vagus nerve stimulation. We hypothetize that if the vagus nerve stimulation could be applied for a longer period of time, the network will be more stabilized and as a result the anti arrhythmogenic effect will last longer .

In this thesis we proposed that the hyperactivity of convergent local circuit neurons could be a reason that the atrial fibrillation was induced or maintained. This hypothesis was based on the fact that when vagal stimulation showed the anti-arrhythmogenic effect, the firing rate of all convergent local

circuit neurons that had significant ($p<0.01$) change, decreased. This could show that the vagal stimulation is targeting the convergent local circuit neurons by attenuating their activity and as a result the atrial fibrillation could be mitigated because of the lower activity of convergent local circuit neurons caused by vagus nerve stimulation.

For many heart rhythm disorders catheter ablation of intrinsic cardiac ganglionated plexus has shown to be a reliable treatment. The similarity between ablation of ganglionated plexus and vagus nerve stimulation is they try to decrease the activity of intrinsic cardiac neurons. The disadvantage of the ganglionated plexus ablations beside the clinical complication is that all the neurons in the ganglionated plexus are removed and all their functionalities either good or bad are removed. However in vagus nerve stimulation, the treatment is less invasive and is more specific to attenuate the activity of convergent local circuit neurons which proposed to be the main cause of the maintenance of the atrial fibrillation.

V.2. Future work

As the neurocardiology field is a growing domain ,it brings new challenges to the research.

Recording part is one of the main parts of electrophysiology projects. Many works were done to enhance the recording techniques and data acquisition systems but still there is a need for better recording with higher signal to noise ratio and the electrodes which could capture more neuronal activities. Recording the activity of more neurons would be very beneficial for the neurocardiology projects, because in most cases the electrodes can only catch a few neurons. Ideally the neuronal activity's identification and classification is better to be done in real-time or with a small delay. But we are far from performing real time identification and classification. This is due to the algorithms and techniques which are used for these types of analysis and also some manual works that still need to be done to complete this process. This process could be improved by developing new identification and classification techniques. Understanding the "little brain in the heart" is advancing but there is still a lot to understand to create a model for this neural network.

In our study, vagus nerve stimulation showed to be a potential therapy for atrial fibrillation (chapter IV, figure 4). The effect of vagus nerve stimulation depends on different parameters of stimulation like frequency and amplitude. As high level vagus nerve stimulation leads to atrial fibrillation and low level vagus nerve stimulation leads to mitigation of atrial fibrillation, it is very important to find the best parameters for the vagal stimulation. This becomes very important in clinical trials. Another task that could be done is to apply vagus nerve stimulation for a longer period of time and evaluate the efficacy of this therapy.

REFERENCES

1. Turcotte, M., *Women and Health*, S. Canada, Editor. 2011.
2. Iaizzo, P.A., *Handbook of cardiac anatomy, physiology, and devices*. 2009: Springer Science & Business Media.
3. Opie, L.H., *Pacemakers, conduction system, and electrocardiogram.* Heart physiology from cell to circulation, 1998: p. 115-145.
4. Shah, S., et al., *The heart: Anatomy, physiology and exercise physiology*, in *Integrating Cardiology for Nuclear Medicine Physicians*. 2009, Springer. p. 3-22.
5. Hall, J.E., *Rhythmical Excitation of the Heart*, in *Guyton and Hall textbook of medical physiology*

2010, Elsevier Health Sciences. p. 119.
6. Armour, J.A., *Potential clinical relevance of the 'little brain' on the mammalian heart.* Exp Physiol, 2008. **93**(2): p. 165-76.
7. Armour, J.A., *Myocardial ischaemia and the cardiac nervous system.* Cardiovasc Res, 1999. **41**(1): p. 41-54.
8. Armour, J.A., *Cardiac neuronal hierarchy in health and disease.* Am J Physiol Regul Integr Comp Physiol, 2004. **287**(2): p. R262-71.
9. Armour, J., *Neuronal activity recorded extracellularly in chronically decentralized in situ canine middle cervical ganglia.* Canadian journal of physiology and pharmacology, 1986. **64**(7): p. 1038-1046.
10. ARMOUR, J.A., *Intrinsic cardiac neurons.* Journal of Cardiovascular Electrophysiology, 1991. **2**(4): p. 331-341.
11. Waldmann, M., et al., *Stochastic behavior of atrial and ventricular intrinsic cardiac neurons.* Journal of Applied Physiology, 2006. **101**(2): p. 413-419.
12. Marron, K., et al., *Distribution, morphology, and neurochemistry of endocardial and epicardial nerve terminal arborizations in the human heart.* Circulation, 1995. **92**(8): p. 2343-51.
13. Pauza, D.H., et al., *Comparative quantitative study of the intrinsic cardiac ganglia and neurons in the rat, guinea pig, dog and human as revealed by histochemical staining for acetylcholinesterase.* Ann Anat, 2002. **184**(2): p. 125-36.
14. Tsuboi, M., et al., *Inotropic, chronotropic, and dromotropic effects mediated via parasympathetic ganglia in the dog heart.* Am J Physiol Heart Circ Physiol, 2000. **279**(3): p. H1201-7.
15. Armour, J.A. and G.C. Kember, *Cardiac sensory neurons.* Basic and clinical neurocardiology, 2004: p. 79-117.
16. Armour, J.A., et al., *Gross and microscopic anatomy of the human intrinsic cardiac nervous system.* Anat Rec, 1997. **247**(2): p. 289-98.
17. Chen, P.S., et al., *Role of the autonomic nervous system in atrial fibrillation: pathophysiology and therapy.* Circ Res, 2014. **114**(9): p. 1500-15.
18. Gatti, P.J., et al., *The physiological and anatomical demonstration of functionally selective parasympathetic ganglia located in discrete fat pads on the feline myocardium.* J Auton Nerv Syst, 1995. **51**(3): p. 255-9.
19. Gatti, P.J., T.A. Johnson, and V.J. Massari, *Can neurons in the nucleus ambiguus selectively regulate cardiac rate and atrio-ventricular conduction?* J Auton Nerv Syst, 1996. **57**(1-2): p. 123-7.
20. Yuan, B.X., et al., *Differential cardiac responses induced by nicotine sensitive canine atrial and ventricular neurones.* Cardiovasc Res, 1993. **27**(5): p. 760-9.
21. Cardinal, R., et al., *Spatially divergent cardiac responses to nicotinic stimulation of ganglionated plexus neurons in the canine heart.* Auton Neurosci, 2009. **145**(1-2): p. 55-62.
22. Ardell, J.L., *Structure and function of mammalian intrinsic cardiac neurons.* Neurocardiology, 1994. **95**.

23. Armour, J.A., *Peripheral autonomic neuronal interactions in cardiac regulation.* Neurocardiology, 1994: p. 219-244.
24. Foreman, R., *Spinal cord neuronal regulation of the cardiovascular system*, in *Neurocardiology*. 1994, Oxford Univ. Press. p. 245-276.
25. Hopkins, D. and H. Ellenberger, *Cardiorespiratory neurons in the medulla oblongata: input and output relationships*, in *Neurocardiology*. 1994, Oxford University Press New York. p. 277-307.
26. Oppenheimer, S. and D. Hopkins, *Suprabulbar neuronal regulation of the heart.* Neurocardiology, 1994: p. 309-341.
27. Ardell, J.L., et al., *Differential sympathetic regulation of automatic, conductile, and contractile tissue in dog heart.* Am J Physiol, 1988. **255**(5 Pt 2): p. H1050-9.
28. Ardell, J.L. and W.C. Randall, *Selective vagal innervation of sinoatrial and atrioventricular nodes in canine heart.* Am J Physiol, 1986. **251**(4 Pt 2): p. H764-73.
29. Pardini, B.J., et al., *Location, distribution and projections of intracardiac ganglion cells in the rat.* J Auton Nerv Syst, 1987. **20**(2): p. 91-101.
30. Randall, W. and J. Ardell, *Functional anatomy of the cardiac efferent innervation.* Neurocardiology. New York: Futura Publishing, 1988: p. 3-24.
31. Roberts, L., *The sinoatrial ring bundle: A cardiac neural communication system?* American journal of anatomy, 1991. **191**(3): p. 250-260.
32. Roberts, L., G. Slocum, and D. Riley, *Morphological study of the innervation pattern of the rabbit sinoatrial node.* American journal of anatomy, 1989. **185**(1): p. 74-88.
33. Furukawa, Y., et al., *Cardiac electrical responses to vagal stimulation of fibers to discrete cardiac regions.* Am J Physiol, 1990. **258**(4 Pt 2): p. H1112-8.
34. Furukawa, Y., et al., *Chronotropic and dromotropic responses to stimulation of intracardiac sympathetic nerves to sinoatrial or atrioventricular nodal region in anesthetized dogs.* Circ Res, 1990. **66**(5): p. 1391-9.
35. Randall, W.C., et al., *Vagal postganglionic innervation of the canine sinoatrial node.* J Auton Nerv Syst, 1987. **20**(1): p. 13-23.
36. Randall, W.C., et al., *Parasympathetic ganglia innervating the canine atrioventricular nodal region.* J Auton Nerv Syst, 1986. **16**(4): p. 311-23.
37. Ardell, J.L., *Intrathoracic neuronal regulation of cardiac function.* Basic and clinical neurocardiology, 2004: p. 118-152.
38. Ardell, J.L., *Neurohumoral control of cardiac function.* Heart Physiology and Pathophysiology, 2001: p. 45-59.
39. Moss, E., et al., *Biatrial neuroablation attenuates atrial remodeling and vulnerability to atrial fibrillation in canine chronic rapid atrial pacing.* Auton Neurosci, 2015. **189**: p. 43-9.
40. Juan, S., et al., *Effect of Cardiac Autonomic Nerve Plexus on Atrial Vulnerability.* 2014.
41. Shinohara, T., et al., *Heart failure decreases nerve activity in the right atrial ganglionated plexus.* J Cardiovasc Electrophysiol, 2012. **23**(4): p. 404-12.
42. Hopkins, D.A. and J.A. Armour, *Ganglionic distribution of afferent neurons innervating the canine heart and cardiopulmonary nerves.* J Auton Nerv Syst, 1989. **26**(3): p. 213-22.
43. Malliani, A., *Cardiovascular sympathetic afferent fibers*, in *Reviews of Physiology, Biochemistry and Pharmacology, Volume 94*. 1982, Springer. p. 11-74.
44. Thorén, P., *Role of cardiac vagal C-fibers in cardiovascular control*, in *Reviews of Physiology, Biochemistry and Pharmacology, Volume 86*. 1979, Springer. p. 1-94.
45. WHITE, J.C., *Cardiac Pain Anatomic Pathways and Physiologic Mechanisms.* Circulation, 1957. **16**(4): p. 644-655.
46. Armour, J.A., *Physiological behavior of thoracic cardiovascular receptors.* Am J Physiol, 1973. **225**(1): p. 177-85.
47. Armour, J.A. and D.A. Hopkins, *Activity of canine in situ left atrial ganglion neurons.* Am J Physiol, 1990. **259**(4 Pt 2): p. H1207-15.

48. Thompson, G.W., M. Horackova, and J.A. Armour, *Chemotransduction properties of nodose ganglion cardiac afferent neurons in guinea pigs.* Am J Physiol Regul Integr Comp Physiol, 2000. **279**(2): p. R433-9.
49. Thoren, P.N., *Characteristics of left ventricular receptors with nonmedullated vagal afferents in cats.* Circ Res, 1977. **40**(4): p. 415-21.
50. Foreman, R.D., *Mechanisms of cardiac pain.* Annu Rev Physiol, 1999. **61**: p. 143-67.
51. Foreman, R.D., K.M. Garrett, and R.W. Blair, *Mechanisms of cardiac pain.* Compr Physiol, 2015. **5**(2): p. 929-60.
52. Huang, M.H., et al., *Polysensory response characteristics of dorsal root ganglion neurones that may serve sensory functions during myocardial ischaemia.* Cardiovasc Res, 1996. **32**(3): p. 503-15.
53. Sylven, C., *Angina pectoris. Clinical characteristics, neurophysiological and molecular mechanisms.* Pain, 1989. **36**(2): p. 145-67.
54. Cheng, Z., et al., *Vagal afferent innervation of the atria of the rat heart reconstructed with confocal microscopy.* J Comp Neurol, 1997. **381**(1): p. 1-17.
55. Yuan, B.X., et al., *Gross and microscopic anatomy of the canine intrinsic cardiac nervous system.* Anat Rec, 1994. **239**(1): p. 75-87.
56. Armour, J.A., et al., *Differential selectivity of cardiac neurons in separate intrathoracic autonomic ganglia.* Am J Physiol, 1998. **274**(4 Pt 2): p. R939-49.
57. Ardell, J.L., et al., *Activity of in vivo atrial and ventricular neurons in chronically decentralized canine hearts.* Am J Physiol, 1991. **260**(3 Pt 2): p. H713-21.
58. Armour, J.A., *Synaptic transmission in the chronically decentralized middle cervical and stellate ganglia of the dog.* Can J Physiol Pharmacol, 1983. **61**(10): p. 1149-55.
59. Armour, J., *Anatomy and function of the intrathoracic neurons regulating the mammalian heart.* Reflex control of the circulation, 1991: p. 1-37.
60. Randall, W.C., *Efferent sympathetic innervation of the heart.* Neurocardiology, 1994: p. 77-94.
61. Randall, W.C., et al., *Regional cardiac distribution of the sympathetic nerves.* Fed Proc, 1972. **31**(4): p. 1199-208.
62. Kuntz, A., *THE AUTONOMIC NERVOUS SYSTEM.* The American Journal of the Medical Sciences, 1935. **189**(1): p. 124.
63. Armour, J.A. and D.A. Hopkins, *Activity of in vivo canine ventricular neurons.* Am J Physiol, 1990. **258**(2 Pt 2): p. H326-36.
64. Gagliardi, M., et al., *Activity of in vivo canine cardiac plexus neurons.* Am J Physiol, 1988. **255**(4 Pt 2): p. H789-800.
65. Massari, V.J., T.A. Johnson, and P.J. Gatti, *Cardiotopic organization of the nucleus ambiguus? An anatomical and physiological analysis of neurons regulating atrioventricular conduction.* Brain Res, 1995. **679**(2): p. 227-40.
66. Plecha, D.M., et al., *Localization of vagal preganglionic somata controlling sinoatrial and atrioventricular nodes.* Am J Physiol, 1988. **255**(5 Pt 2): p. R703-8.
67. Blinder, K.J., et al., *Ultrastructural circuitry of cardiorespiratory reflexes: there is a monosynaptic path between the nucleus of the solitary tract and vagal preganglionic motoneurons controlling atrioventricular conduction in the cat.* Brain Res, 1998. **785**(1): p. 143-57.
68. Blinder, K.J., T.A. Johnson, and V. John Massari, *Negative inotropic vagal preganglionic neurons in the nucleus ambiguus of the cat: neuroanatomical comparison with negative chronotropic neurons utilizing dual retrograde tracers.* Brain Res, 1998. **804**(2): p. 325-30.
69. Schwartz, P., S. Priori, and C. Napolitano, *Cardiac Electrophysiology: From Cell to Beside*. 2000, Philadelphia: WB Saunders Co.
70. Levy, M. and M. Warner, *Parasympathetic effects on cardiac function.* Neurocardiology, 1994: p. 53-76.

71. Buckley, U., K. Shivkumar, and J.L. Ardell, *Autonomic Regulation Therapy in Heart Failure.* Curr Heart Fail Rep, 2015.
72. Bluemel, K.M., et al., *Parasympathetic postganglionic pathways to the sinoatrial node.* Am J Physiol, 1990. **259**(5 Pt 2): p. H1504-10.
73. Allen, T.G. and G. Burnstock, *Intracellular studies of the electrophysiological properties of cultured intracardiac neurones of the guinea-pig.* J Physiol, 1987. **388**: p. 349-66.
74. Fee, J.D., et al., *Selective ganglionic blockade of vagal inputs to sinoatrial and/or atrioventricular regions.* J Pharmacol Exp Ther, 1987. **242**(3): p. 1006-12.
75. Armour, J.A., M.H. Huang, and F.M. Smith, *Peptidergic modulation of in situ canine intrinsic cardiac neurons.* Peptides, 1993. **14**(2): p. 191-202.
76. Moravec, M., A. Courtalon, and J. Moravec, *Intrinsic neurosecretory neurons of the rat heart atrioventricular junction: possibility of local neuromuscular feed back loops.* J Mol Cell Cardiol, 1986. **18**(4): p. 357-67.
77. Moravec, M. and J. Moravec, *Intrinsic innervation of the atrioventricular junction of the rat heart.* Am J Anat, 1984. **171**(3): p. 307-19.
78. Moravec, M. and J. Moravec, *Adrenergic neurons and short proprioceptive feedback loops involved in the integration of cardiac function in the rat.* Cell Tissue Res, 1989. **258**(2): p. 381-5.
79. Xi, X., W.C. Randall, and R.D. Wurster, *Morphology of intracellularly labeled canine intracardiac ganglion cells.* J Comp Neurol, 1991. **314**(2): p. 396-402.
80. Huang, M.H., et al., *Effects of transient coronary artery occlusion on canine intrinsic cardiac neuronal activity.* Integr Physiol Behav Sci, 1993. **28**(1): p. 5-21.
81. Armour, J.A., *Activity of in situ middle cervical ganglion neurons in dogs, using extracellular recording techniques.* Can J Physiol Pharmacol, 1985. **63**(6): p. 704-16.
82. Armour, J.A., *Activity of in situ stellate ganglion neurons of dogs recorded extracellularly.* Can J Physiol Pharmacol, 1986. **64**(2): p. 101-11.
83. McGuirt, A.S., D.C. Schmacht, and J.L. Ardell, *Autonomic interactions for control of atrial rate are maintained after SA nodal parasympathectomy.* Am J Physiol, 1997. **272**(6 Pt 2): p. H2525-33.
84. Randall, D.C., et al., *Ablation of posterior atrial ganglionated plexus potentiates sympathetic tachycardia to behavioral stress.* Am J Physiol, 1998. **275**(3 Pt 2): p. R779-87.
85. Dayan, P. and L.F. Abbott, *Theoretical neuroscience*. 2001: Cambridge, MA: MIT Press.
86. Seabrook, G.R., L.A. Fieber, and D.J. Adams, *Neurotransmission in neonatal rat cardiac ganglion in situ.* Am J Physiol, 1990. **259**(4 Pt 2): p. H997-1005.
87. Selyanko, A.A., *Membrane properties and firing characteristics of rat cardiac neurones in vitro.* J Auton Nerv Syst, 1992. **39**(3): p. 181-9.
88. Selyanko, A.A. and V.I. Skok, *Synaptic transmission in rat cardiac neurones.* J Auton Nerv Syst, 1992. **39**(3): p. 191-9.
89. Nozdrachev, A.D. and A.G. Pogorelov, *Extracellular recording of neuronal activity of the cat heart ganglia.* Journal of the autonomic nervous system, 1982. **6**(1): p. 73-81.
90. Smith, F.M., D.A. Hopkins, and J.A. Armour, *Electrophysiological properties of in vitro intrinsic cardiac neurons in the pig (Sus scrofa).* Brain Res Bull, 1992. **28**(5): p. 715-25.
91. Xi, X., W.C. Randall, and R.D. Wurster, *Intracellular recording of spontaneous activity of canine intracardiac ganglion cells.* Neurosci Lett, 1991. **128**(1): p. 129-32.
92. Xi, X.H., et al., *Intracellular recordings from canine intracardiac ganglion cells.* J Auton Nerv Syst, 1991. **32**(2): p. 177-82.
93. Xi-Moy, S.X., W.C. Randall, and R.D. Wurster, *Nicotinic and muscarinic synaptic transmission in canine intracardiac ganglion cells innervating the sinoatrial node.* J Auton Nerv Syst, 1993. **42**(3): p. 201-13.
94. Ardell, J.L., S.M. Barman, and G.L. Gebber, *Sympathetic nerve discharge in chronic spinal cat.* Am J Physiol, 1982. **243**(3): p. H463-70.

95. Thompson, G.W., et al., *Functional interdependence of neurons in a single canine intrinsic cardiac ganglionated plexus.* J Physiol, 2000. **528**(Pt 3): p. 561-71.
96. Mangrum, J.M. and J.P. DiMarco, *The evaluation and management of bradycardia.* N Engl J Med, 2000. **342**(10): p. 703-9.
97. O'Toole, M.F., J.L. Ardell, and W.C. Randall, *Functional interdependence of discrete vagal projections to SA and AV nodes.* Am J Physiol, 1986. **251**(2 Pt 2): p. H398-404.
98. Randall, D.C., et al., *Heart rate control in awake dog after selective SA-nodal parasympathectomy.* Am J Physiol, 1992. **262**(4 Pt 2): p. H1128-35.
99. Schwartz, P.J., M.T. La Rovere, and E. Vanoli, *Autonomic nervous system and sudden cardiac death. Experimental basis and clinical observations for post-myocardial infarction risk stratification.* Circulation, 1992. **85**(1 Suppl): p. I77-91.
100. Kannel, W.B. and E.J. Benjamin, *Status of the epidemiology of atrial fibrillation.* Med Clin North Am, 2008. **92**(1): p. 17-40, ix.
101. Genovesi, S., et al., *Prevalence of atrial fibrillation and associated factors in a population of long-term hemodialysis patients.* Am J Kidney Dis, 2005. **46**(5): p. 897-902.
102. Go, A.S., et al., *Prevalence of diagnosed atrial fibrillation in adults: national implications for rhythm management and stroke prevention: the AnTicoagulation and Risk Factors in Atrial Fibrillation (ATRIA) Study.* JAMA, 2001. **285**(18): p. 2370-5.
103. Stewart, S., et al., *Population prevalence, incidence, and predictors of atrial fibrillation in the Renfrew/Paisley study.* Heart, 2001. **86**(5): p. 516-21.
104. European Heart Rhythm, A., et al., *ACC/AHA/ESC 2006 guidelines for the management of patients with atrial fibrillation--executive summary: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and the European Society of Cardiology Committee for Practice Guidelines (Writing Committee to Revise the 2001 Guidelines for the Management of Patients With Atrial Fibrillation).* J Am Coll Cardiol, 2006. **48**(4): p. 854-906.
105. European Heart Rhythm, A., et al., *Guidelines for the management of atrial fibrillation: the Task Force for the Management of Atrial Fibrillation of the European Society of Cardiology (ESC).* Europace, 2010. **12**(10): p. 1360-420.
106. Efimov, I.R. and V.V. Fedorov, *Chessboard of atrial fibrillation: reentry or focus? Single or multiple source(s)? Neurogenic or myogenic?* Am J Physiol Heart Circ Physiol, 2005. **289**(3): p. H977-9.
107. GENG, N., Y. SUN, and S. PO, *Relationship between right atrium ganglionated plexus and atrial fibrillation: A new canine model for sustained focal atrial fibrillation.* Chinese Journal of Interventional Cardiology, 2006. **3**: p. 013.
108. Hoffa, M. and C. Ludwig, *Einige neue versuche uber herzbewegung.* Zeitschrift Rationelle Medizin, 1850. **9**: p. 107-144.
109. Wickramasinghe, S.R. and V.V. Patel, *Local innervation and atrial fibrillation.* Circulation, 2013. **128**(14): p. 1566-75.
110. Linz, D., et al., *Atrial autonomic innervation: a target for interventional antiarrhythmic therapy?* J Am Coll Cardiol, 2014. **63**(3): p. 215-24.
111. Nattel, S., *Age, gender, and supraventricular arrhythmias: roles of ion channels, connexins, and tissue architecture?* Heart Rhythm, 2004. **1**(4): p. 397-8.
112. Chang, C.M., et al., *Nerve sprouting and sympathetic hyperinnervation in a canine model of atrial fibrillation produced by prolonged right atrial pacing.* Circulation, 2001. **103**(1): p. 22-5.
113. Liu, L. and S. Nattel, *Differing sympathetic and vagal effects on atrial fibrillation in dogs: role of refractoriness heterogeneity.* Am J Physiol, 1997. **273**(2 Pt 2): p. H805-16.
114. Coumel, P., et al., *[The atrial arrhythmia syndrome of vagal origin].* Arch Mal Coeur Vaiss, 1978. **71**(6): p. 645-56.
115. Coumel, P., *Autonomic influences in atrial tachyarrhythmias.* J Cardiovasc Electrophysiol, 1996. **7**(10): p. 999-1007.

116. Zipes, D.P., M.J. Mihalick, and G.T. Robbins, *Effects of selective vagal and stellate ganglion stimulation of atrial refractoriness.* Cardiovasc Res, 1974. **8**(5): p. 647-55.
117. Wijffels, M.C., et al., *Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats.* Circulation, 1995. **92**(7): p. 1954-68.
118. Nattel, S. and D. Li, *Ionic remodeling in the heart: pathophysiological significance and new therapeutic opportunities for atrial fibrillation.* Circ Res, 2000. **87**(6): p. 440-7.
119. Stiles, M.K., et al., *Paroxysmal lone atrial fibrillation is associated with an abnormal atrial substrate: characterizing the "second factor".* Journal of the American College of Cardiology, 2009. **53**(14): p. 1182-1191.
120. Haissaguerre, M., et al., *Electrophysiological breakthroughs from the left atrium to the pulmonary veins.* Circulation, 2000. **102**(20): p. 2463-5.
121. Haissaguerre, M., et al., *Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins.* N Engl J Med, 1998. **339**(10): p. 659-66.
122. He, B., et al., *The intrinsic autonomic nervous system in atrial fibrillation: a review.* ISRN Cardiol, 2012. **2012**: p. 490674.
123. Zheng, S., et al., *Active ganglionated plexi is a predictor of atrial fibrillation recurrence after minimally invasive surgical ablation.* J Card Surg, 2014. **29**(2): p. 279-85.
124. Yamashiro, K., et al., *Ganglionated plexi ablation plus antral pulmonary vein isolation for paroximal atrial fibrillation: clinical outcome after a single procedure at one-year follow-up.* European Heart Journal, 2013. **34**(suppl 1): p. P494.
125. Scherlag, B.J., et al., *The Autonomic Nervous System and Atrial Fibril-lation: The Roles of Pulmonary Vein Isolation and Ganglionated Plexi Ablation.* JAFIB: Journal of Atrial Fibrillation, 2009. **1**(8).
126. Nemirovsky, D., R. Hutter, and J.A. Gomes, *The Electrical Substrate of Vagal Atrial Fibrillation as Assessed by the Signal-Averaged Electrocardiogram of the P Wave.* Pacing and clinical electrophysiology, 2008. **31**(3): p. 308-313.
127. van den Berg, M.P., et al., *Role of the autonomic nervous system in vagal atrial fibrillation.* Heart, 2003. **89**(3): p. 333-5.
128. Zheng, Z., Sun, Zhang, Duan and Hou, *The Role of Autonomic Neural System in Atrial Fibrillation* in *Cardiovascular Disease II*, i.P. Ltd., Editor. 2014.
129. Brack, K.E. and G.A. Ng, *Autonomic Control of Cardiac Arrhythmia*, in *Cardiac Arrhythmias*. 2014, Springer. p. 43-60.
130. Shen, M.J. and D.P. Zipes, *Role of the autonomic nervous system in modulating cardiac arrhythmias.* Circulation research, 2014. **114**(6): p. 1004-1021.
131. Olshansky, B., *Interrelationships between the autonomic nervous system and atrial fibrillation.* Progress in cardiovascular diseases, 2005. **48**(1): p. 57-78.
132. Armour, J.A. and J.L. Ardell, *Basic and clinical neurocardiology*. 2004: Oxford University Press, USA.
133. Zhang, Y. and T.N. Mazgalev, *Arrhythmias and vagus nerve stimulation.* Heart Fail Rev, 2011. **16**(2): p. 147-61.
134. Schachter, S.C. and C.B. Saper, *Vagus nerve stimulation.* Epilepsia, 1998. **39**(7): p. 677-86.
135. Katsouras, G., et al., *Differences in atrial fibrillation properties under vagal nerve stimulation versus atrial tachycardia remodeling.* Heart Rhythm, 2009. **6**(10): p. 1465-72.
136. Sharifov, O.F., et al., *Spatial distribution and frequency dependence of arrhythmogenic vagal effects in canine atria.* J Cardiovasc Electrophysiol, 2000. **11**(9): p. 1029-42.
137. Cardinal, R., et al., *Atrial tachyarrhythmias and repolarization changes induced by discrete activation of dorsal mediastinal cardiac nerves in canines.* Circ Arrhythm Electrophysiol, 2010. **3**(5): p. 511-20.
138. Page, P., et al., *Differential effects of cervical vagosympathetic and mediastinal nerve activation on atrial arrhythmia formation in dogs.* Auton Neurosci, 2006. **128**(1-2): p. 9-18.

139. Armour, J.A., et al., *Origin and pharmacological response of atrial tachyarrhythmias induced by activation of mediastinal nerves in canines.* Auton Neurosci, 2005. **118**(1-2): p. 68-78.
140. Scherlag, B.J., E. Patterson, and S.S. Po, *The neural basis of atrial fibrillation.* J Electrocardiol, 2006. **39**(4 Suppl): p. S180-3.
141. Jones, D.L., J.M. Tuomi, and P. Chidiac, *Role of Cholinergic Innervation and RGS2 in Atrial Arrhythmia.* Front Physiol, 2012. **3**: p. 239.
142. Li, S., et al., *Low-level vagosympathetic stimulation: a paradox and potential new modality for the treatment of focal atrial fibrillation.* Circ Arrhythm Electrophysiol, 2009. **2**(6): p. 645-51.
143. Zhang, Y., et al., *Relationship between right cervical vagus nerve stimulation and atrial fibrillation inducibility: therapeutic intensities do not increase arrhythmogenesis.* Heart Rhythm, 2009. **6**(2): p. 244-50.
144. Hageman, G.R., et al., *Cardiac dysrhythmias induced by autonomic nerve stimulation.* Am J Cardiol, 1973. **32**(6): p. 823-30.
145. Armour, J.A., W.C. Randall, and S. Sinha, *Localized myocardial responses to stimulation of small cardiac branches of the vagus.* Am J Physiol, 1975. **228**(1): p. 141-8.
146. Armour, J.A., G.R. Hageman, and W.C. Randall, *Arrhythmias induced by local cardiac nerve stimulation.* Am J Physiol, 1972. **223**(5): p. 1068-75.
147. Scherlag, B.J., et al., *Endovascular stimulation within the left pulmonary artery to induce slowing of heart rate and paroxysmal atrial fibrillation.* Cardiovascular research, 2002. **54**(2): p. 470-475.
148. Schauerte, P., et al., *Focal atrial fibrillation: experimental evidence for a pathophysiologic role of the autonomic nervous system.* J Cardiovasc Electrophysiol, 2001. **12**(5): p. 592-9.
149. Wyse, D.G., et al., *A comparison of rate control and rhythm control in patients with atrial fibrillation.* N Engl J Med, 2002. **347**(23): p. 1825-33.
150. Singer, D.E., et al., *Antithrombotic therapy in atrial fibrillation: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy.* Chest, 2004. **126**(3 Suppl): p. 429S-456S.
151. Male, S. and B.J. Scherlag, *Role of neural modulation in the pathophysiology of atrial fibrillation.* Indian J Med Res, 2014. **139**(4): p. 512-22.
152. Cappato, R., et al., *Updated worldwide survey on the methods, efficacy, and safety of catheter ablation for human atrial fibrillation.* Circ Arrhythm Electrophysiol, 2010. **3**(1): p. 32-8.
153. Weerasooriya, R., et al., *Catheter ablation for atrial fibrillation: are results maintained at 5 years of follow-up?* J Am Coll Cardiol, 2011. **57**(2): p. 160-6.
154. Gibson, D.N., et al., *Stiff left atrial syndrome after catheter ablation for atrial fibrillation: clinical characterization, prevalence, and predictors.* Heart Rhythm, 2011. **8**(9): p. 1364-71.
155. Schwarz, N., et al., *Neuropsychological decline after catheter ablation of atrial fibrillation.* Heart Rhythm, 2010. **7**(12): p. 1761-7.
156. Gaita, F., et al., *Radiofrequency catheter ablation of atrial fibrillation: a cause of silent thromboembolism? Magnetic resonance imaging assessment of cerebral thromboembolism in patients undergoing ablation of atrial fibrillation.* Circulation, 2010. **122**(17): p. 1667-73.
157. Nademanee, K., et al., *A new approach for catheter ablation of atrial fibrillation: mapping of the electrophysiologic substrate.* J Am Coll Cardiol, 2004. **43**(11): p. 2044-53.
158. Lu, Z., et al., *Autonomic mechanism for initiation of rapid firing from atria and pulmonary veins: evidence by ablation of ganglionated plexi.* Cardiovasc Res, 2009. **84**(2): p. 245-52.
159. Lim, P.B., et al., *Intrinsic cardiac autonomic stimulation induces pulmonary vein ectopy and triggers atrial fibrillation in humans.* J Cardiovasc Electrophysiol, 2011. **22**(6): p. 638-46.
160. Chang, H.-Y., et al., *Relationship between intrinsic cardiac autonomic ganglionated plexi and the atrial fibrillation nest.* Circ J, 2014. **78**: p. 922-928.
161. Pokushalov, E., et al., *Left atrial ablation at the anatomic areas of ganglionated plexi for paroxysmal atrial fibrillation.* Pacing Clin Electrophysiol, 2010. **33**(10): p. 1231-8.
162. Pokushalov, E., et al., *Ganglionated plexi ablation directed by high-frequency stimulation and complex fractionated atrial electrograms for paroxysmal atrial fibrillation.* Pacing Clin Electrophysiol, 2012. **35**(7): p. 776-84.

163. Cui, B., et al., *Acute effects of ganglionated plexi ablation on sinoatrial nodal and atrioventricular nodal functions.* Auton Neurosci, 2011. **161**(1-2): p. 87-94.
164. Calo, L., et al., *Catheter ablation of right atrial ganglionated plexi in patients with vagal paroxysmal atrial fibrillation.* Circ Arrhythm Electrophysiol, 2012. **5**(1): p. 22-31.
165. Pokushalov, E., et al., *Ganglionated plexi ablation for longstanding persistent atrial fibrillation.* Europace, 2010. **12**(3): p. 342-6.
166. Yao, Y., et al., *Endocardial autonomic denervation of the left atrium to treat vasovagal syncope: an early experience in humans.* Circ Arrhythm Electrophysiol, 2012. **5**(2): p. 279-86.
167. Rebecchi, M., et al., *Ganglionated plexi ablation in right atrium to treat cardioinhibitory neurocardiogenic syncope.* J Interv Card Electrophysiol, 2012. **34**(3): p. 231-5.
168. Zhou, Q., Y. Hou, and S. Yang, *A meta-analysis of the comparative efficacy of ablation for atrial fibrillation with and without ablation of the ganglionated plexi.* Pacing Clin Electrophysiol, 2011. **34**(12): p. 1687-94.
169. Lin, Y., et al., *Effects of low-level autonomic stimulation on prevention of atrial fibrillation induced by acute electrical remodeling.* ScientificWorldJournal, 2013. **2013**: p. 781084.
170. Shen, M.J., et al., *Continuous low-level vagus nerve stimulation reduces stellate ganglion nerve activity and paroxysmal atrial tachyarrhythmias in ambulatory canines.* Circulation, 2011. **123**(20): p. 2204-12.
171. Sheng, X., et al., *Prevention and reversal of atrial fibrillation inducibility and autonomic remodeling by low-level vagosympathetic nerve stimulation.* J Am Coll Cardiol, 2011. **57**(5): p. 563-71.
172. Yu, L., et al., *Spinal cord stimulation suppresses focal rapid firing-induced atrial fibrillation by inhibiting atrial ganglionated plexus activity.* J Cardiovasc Pharmacol, 2014. **64**(6): p. 554-9.
173. Etzion, Y., *A stimulating environment for the atrial kick: spinal cord stimulation can inhibit atrial fibrillation.* Heart Rhythm, 2012. **9**(9): p. 1434-5.
174. Bernstein, S.A., et al., *Spinal cord stimulation protects against atrial fibrillation induced by tachypacing.* Heart Rhythm, 2012. **9**(9): p. 1426-33 e3.
175. Ardell, J.L., et al., *Chronic spinal cord stimulation modifies intrinsic cardiac synaptic efficacy in the suppression of atrial fibrillation.* Auton Neurosci, 2014. **186**: p. 38-44.
176. Gibbons, D.D., et al., *Neuromodulation targets intrinsic cardiac neurons to attenuate neuronally mediated atrial arrhythmias.* Am J Physiol Regul Integr Comp Physiol, 2012. **302**(3): p. R357-64.
177. Tewfik, T.L. and A. Meyers, *Vagus nerve anatomy*. 2014.
178. Grimm, S. and M. Bajbouj, *Efficacy of vagus nerve stimulation in the treatment of depression.* Expert Rev Neurother, 2010. **10**(1): p. 87-92.
179. Ben-Menachem, E., *Vagus-nerve stimulation for the treatment of epilepsy.* Lancet Neurol, 2002. **1**(8): p. 477-82.
180. Schwartz, P.J., et al., *Long term vagal stimulation in patients with advanced heart failure: first experience in man.* Eur J Heart Fail, 2008. **10**(9): p. 884-91.
181. Li, M., et al., *Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats.* Circulation, 2004. **109**(1): p. 120-4.
182. Zhang, Y., et al., *Chronic vagus nerve stimulation improves autonomic control and attenuates systemic inflammation and heart failure progression in a canine high-rate pacing model.* Circ Heart Fail, 2009. **2**(6): p. 692-9.
183. Van Wagoner, D.R., *Chronic vagal nerve stimulation for the treatment of human heart failure: progress in translating a vision into reality.* Eur Heart J, 2011. **32**(7): p. 788-90.
184. De Ferrari, G.M., *Vagal Stimulation in Heart Failure.* J Cardiovasc Transl Res, 2014.
185. Sabbah, H.N., et al., *Vagus nerve stimulation in experimental heart failure.* Heart Fail Rev, 2011. **16**(2): p. 171-8.

186. Zhang, Y., et al., *Relationship between right cervical vagus nerve stimulation and atrial fibrillation inducibility: therapeutic intensities do not increase arrhythmogenesis.* Heart Rhythm, 2009. **6**(2): p. 244-250.
187. Stavrakis, S., et al., *Inhibition of atrial fibrillation by low-level vagus nerve stimulation: the role of the nitric oxide signaling pathway.* Journal of Interventional Cardiac Electrophysiology, 2013. **36**(3): p. 199-208.
188. Yu, L., et al., *Low-level vagosympathetic nerve stimulation inhibits atrial fibrillation inducibility: direct evidence by neural recordings from intrinsic cardiac ganglia.* J Cardiovasc Electrophysiol, 2011. **22**(4): p. 455-63.
189. Ansari, S., K. Chaudhri, and K.A. Al Moutaery, *Vagus nerve stimulation: indications and limitations.* Acta Neurochir Suppl, 2007. **97**(Pt 2): p. 281-6.
190. Milby, A.H., C.H. Halpern, and G.H. Baltuch, *Vagus nerve stimulation for epilepsy and depression.* Neurotherapeutics, 2008. **5**(1): p. 75-85.
191. Yu, L., et al., *Low-level transcutaneous electrical stimulation of the auricular branch of the vagus nerve: a noninvasive approach to treat the initial phase of atrial fibrillation.* Heart Rhythm, 2013. **10**(3): p. 428-35.
192. Sha, Y., et al., *Low-level right vagal stimulation: anticholinergic and antiadrenergic effects.* J Cardiovasc Electrophysiol, 2011. **22**(10): p. 1147-53.
193. Go, A.S., et al., *Prevalence of diagnosed atrial fibrillation in adults: national implications for rhythm management and stroke prevention: the AnTicoagulation and Risk Factors in Atrial Fibrillation (ATRIA) Study.* JAMA, 2001. **285**: p. 2370-5.
194. Arora, R. and H.K. Koduri, *Mechanisms of Atrial Fibrillation*, in *Cardiac Arrhythmias*. 2014, Springer. p. 401-412.
195. Schotten, U., et al., *Pathophysiological mechanisms of atrial fibrillation: a translational appraisal.* Physiol Rev. **91**(1): p. 265-325.
196. Pappone, C., et al., *Atrial electroanatomic remodeling after circumferential radiofrequency pulmonary vein ablation: efficacy of an anatomic approach in a large cohort of patients with atrial fibrillation.* Circulation, 2001. **104**(21): p. 2539-44.
197. Pappone, C., et al., *Circumferential radiofrequency ablation of pulmonary vein ostia: A new anatomic approach for curing atrial fibrillation.* Circulation, 2000. **102**(21): p. 2619-28.
198. Lemola, K., et al., *Pulmonary vein isolation as an end point for left atrial circumferential ablation of atrial fibrillation.* J Am Coll Cardiol, 2005. **46**(6): p. 1060-6.
199. Calo, L., et al., *Catheter ablation of right atrial ganglionated plexi in patients with vagal paroxysmal atrial fibrillation.* Circ Arrhythm Electrophysiol, 2011. **5**(1): p. 22-31.
200. Chang, H., et al., *Relationship Between Intrinsic Cardiac Autonomic Ganglionated Plexi and the Atrial Fibrillation Nest.* Circulation journal: official journal of the Japanese Circulation Society, 2014.
201. Scherlag, B.J., et al., *The Autonomic Nervous System and Atrial Fibrillation: The Roles of Pulmonary Vein Isolation and Ganglionated Plexi Ablation.* JAFIB: Journal of Atrial Fibrillation, 2009. **1**(8).
202. Wang, H., et al., *Epicardial ablation of right pulmonary artery ganglionated plexi for the prevention of atrial fibrillation originating in the pulmonary veins.* J Electrocardiol, 2010. **43**(4): p. 367-72.
203. Sakamoto, S., et al., *Vagal denervation and reinnervation after ablation of ganglionated plexi.* J Thorac Cardiovasc Surg, 2010. **139**(2): p. 444-52.
204. Moers, A.M., et al., *Pharmacological prevention and termination of focal atrial fibrillation.* Europace, 2012. **14**(3): p. 426-30.
205. Moss, E., et al., *Bilateral atrial ganglionated plexus involvement in atrial responses to left-sided plexus stimulation in canines.* Cardiovasc Res, 2013. **99**(1): p. 194-202.
206. Zhang, Y. and T.N. Mazgalev, *Arrhythmias and vagus nerve stimulation.* Heart failure reviews, 2011. **16**(2): p. 147-161.

207. Morrison, D., *Multivariate statistical methods*. 1976, New York: McGraw-Hill.
208. Rey, H.G., C. Pedreira, and R. Quian Quiroga, *Past, present and future of spike sorting techniques.* Brain Res Bull, 2015.
209. Longpre, J.P., et al., *Measure of synchrony in the activity of intrinsic cardiac neurons.* Physiol Meas, 2014. **35**(4): p. 549-66.
210. Beaumont, E., et al., *Network interactions within the canine intrinsic cardiac nervous system: implications for reflex control of regional cardiac function.* J Physiol, 2013. **591**(Pt 18): p. 4515-33.