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Stabilization of the Cardiac Nervous System During Cardiac Stress Induces Cardioprotection

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by

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ABSTRACT

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The cardiac nervous system consists of nested reflex feedback loops that interact to regulate regional heart function. Cardiac disease affects multiple components of the cardiac nervous system and the myocytes themselves. This study aims to determine: 1) how select components of the cardiac nervous system respond to acute cardiac stress, including myocardial ischemia (MI) and induced neural imbalance leading to cardiac electrical instability, and 2) how neuromodulation can affect neural-myocyte interactions to induce cardioprotection. Thoracic spinal cord stimulation (SCS) is recognized for its anti-anginal effects and ability to reduce apoptosis in response to acute MI, primarily via modulation of adrenergic efferent systems. The data presented here suggest that cervical SCS exerts similar cardioprotective effects in response to MI, but in contradistinction to thoracic SCS, uses both adrenergic and cholinergic efferent mechanisms to stabilize cardiomyocytes and the arrhythmogenic potential. SCS potentially can use efferent and/or anti-dromically activated cardiac afferents to mediate its cardioprotection. Thoracic SCS mitigates the MI-induced activation of both nodose and dorsal root ganglia cardiac-related afferents, doing so without antidromic activation of the primary cardiac afferents. Instead, thoracic SCS acts through altering the cardiac milieu thereby secondarily affecting the primary afferent sensory transduction. In response to cardiac stressors, reflex activation of efferent activity modifies mechanical and electrical functions of the heart. Excessive activation of neuronal input to the cardiac nervous system can induce arrhythmias. Stimulation of intrathoracic mediastinal nerves directly activates subpopulations of intrinsic cardiac neurons, thereby inducing atrial arrhythmias. Neuromodulation, either thoracic SCS

or hexamethonium, suppressed mediastinal nerve stimulation (MSNS)-induced activation of intrinsic cardiac neurons and correspondingly reduced the arrhythmogenic potential. SCS exerted its stabilizing effects on neural processing and subsequent effects on atrial electrical function by selectively targeting local circuit neurons within the intrinsic cardiac nervous system. Together these data indicate that neuromodulation therapy, using SCS, can mitigate the imbalances in cardiac reflex control arising from acute cardiac stress and thereby has the potential to slow the progression of chronic heart disease.

DEDICATION

This dissertation is dedicated to my loving and supportive wife Alison and our children David Mark and Anthony Gibbons.

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LIST OF ABBREVIATIONS

ABC	avidin-biotin complex
AF	atrial fibrillation
AFl	atrial flutter
ATF	atrial tachyarrhythmias/fibrillation
BSA	bovine serum albumin
CAO	coronary artery occlusion
ChAT	Choline acetyltransferase
CNS	central nerve system
CV	cardiovascular
DCNx	dorsal column transection
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethyllindocarbocyanine perchlorate
DRG	dorsal root ganglia
EPs	evoked potentials
HR	heart rate
ICN	intrinsic cardiac nervous system
ICNs	intrinsic cardiac neurons
IS	infarct size
LAD	left anterior descending
LVP	left ventricular pressure
MAPK	mitogen-activated protein kinases
MI	myocardial ischemia
MSN	mediastinal nerves
MSNS	mediastinal nerve stimulation
MT	motor threshold

OVL	overlay
PBS	phosphate buffered saline
PGP 9.5	protein gene product 9.5
PKA	protein kinase A
PKC	protein kinase C
RAGP	right atrial ganglionated plexus
SA	sinoatrial
SCD	sudden cardiac death
SCS	spinal cord stimulation
SVC	superior vena cava
TH	tyrosine hydroxylase
TTC	triphenyltetrazolium chloride
UV	ultraviolet
VAChT	vesicular acetylcholine transferase
VF	ventricular fibrillation

CHAPTER 1

INTRODUCTION

The Cardiac Nervous System

The cardiac nervous system is a subdivision of the autonomic nervous system, which includes the sympathetic and parasympathetic efferent branches. It functions to reflexly control physiological functions like heart rate, cardiac contractility, and more. The cardiac nervous system consists of afferent, efferent, and multiple processing neuronal networks, including those located within the central nervous system (CNS) and within peripheral ganglia (Armour 1999; 2004). The afferent neurons transduce cardiac sensory information to the cardiac nervous system using intracardiac (intrinsic cardiac nervous system) and extracardiac (stellate, middle cervical, nodose, and dorsal root) ganglia (Armour 1986a; 1986b; Bosnjak and Kampine 1989; Brown 1967; Hopkins and Armour 1989; Horackova and others 1996; Kuo and others 1984). Afferent input provided to the cardiac nervous system includes that from arterial baroreceptors, chemoreceptors (cardiac and peripheral), and multimodal cardiopulmonary and somatic afferents (Armour 1999; 2004). For cardiac control, the parasympathetic preganglionic neurons originate in the nucleus ambiguus and the dorsal motor nucleus in the medulla oblongata and travel through the cranial nerve to synapse on postganglionic parasympathetic neurons contained within the intrinsic cardiac ganglia (Cheng and others 1999; Hopkins and Armour 1984; Plecha and others 1988). For cardiac control, the sympathetic preganglionic neurons originate from the intermediolateral cell column of the spinal cord, exiting at the T1-T5 levels, to synapse with postganglionic neurons in the intrathoracic-extracardiac ganglia and the intrinsic cardiac ganglionated plexuses (Forsgren and others 1990; Hopkins and Armour 1984; Horackova and others 1999; Moravec and Moravec 1987).

The intrinsic cardiac and other intrathoracic autonomic ganglia contain local circuit neurons (LCN). The primary function of this neural population is to interconnect afferent and efferent neural components within and between intrathoracic autonomic ganglia (Armour 1999; 2004; 2008). Peripheral neural interconnections are thought to contribute to organ level processing and functional control (Armour 1986a; Armour 1991; Armour and Janes 1988; Armour and others 1997; Darvesh and others 1987; Yuan and others 1994). Cardiac function is regulated by the interactions between central and peripheral neuronal components of the cardiac nervous system (Armour 2004). These interactions are manifest as interdependent feedback loops between intracardiac and extracardiac ganglia, spinal cord, brainstem, and higher centers of the central nervous system (Armour 2004). The intrinsic cardiac nervous system is thought to control short-term function, with longer-term function being controlled by progressively higher neural networks (Armour 1999; 2004; 2008). These interdependent networks interact ultimately to control the autonomic efferent outflows via the sympathetic and parasympathetic nervous systems (Armour 2004).

Parasympathetic activation decreases cardiac chronotropism (rate), dromotropism (conduction), and inotropism (contractility), while sympathetic activation exerts opposite effects (Burwash and others 1993; Randall and others 1996). Normal cardiac function is also regulated through the influence of circulating hormones, such as angiotensin II, and circulating catecholamines acting on both neural components and the cardiac end-effectors (Ardell 2004). Figure 1 summarizes many of the salient neurohumoral interactions that ultimately result in control of cardiac function in the normal and stressed heart.

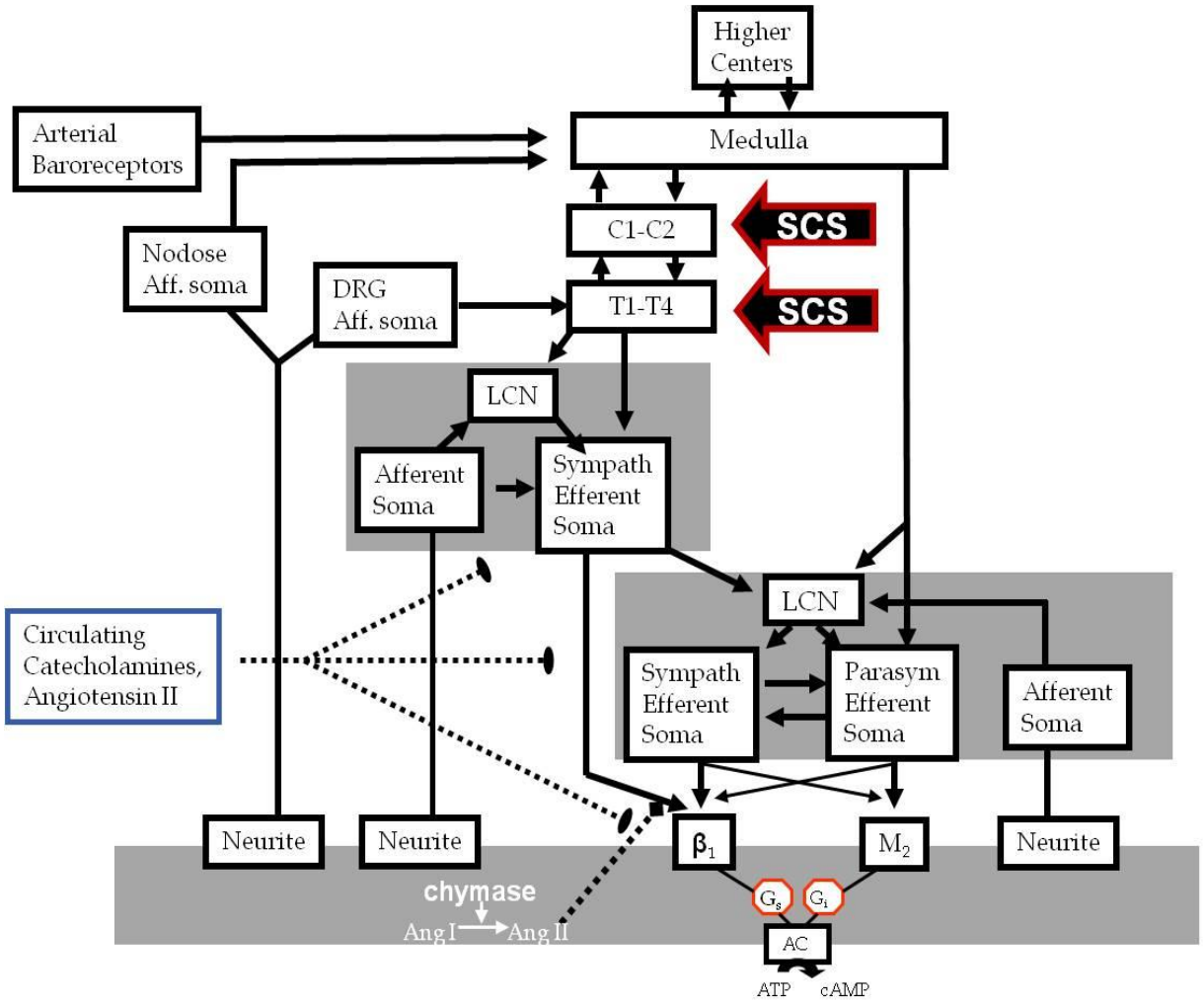

Figure 1.1. A schematic of the neurohumoral components and their interactions that contribute to the control of heart function. DRG – dorsal root ganglia. LCN – local circuit neurons. Aff. – afferent. C1-C2 – Cervical spinal segments 1-2. T1-T4 – Thoracic spinal segments 1-4. Ang I – Angiotensin I. Ang II – Angiotension II. β_1 – Beta 1-adrenergic receptors. M_2 – Muscarinic receptors subtype 2. ATP – Adenosine triphosphate. cAMP – Cyclic adenosine monophosphate. G_s – G_s-protein subunit. G_i – G_i-protein subunit. AC – Adenylate cyclase. (adapted from Ardell and Foreman 2009)

Ischemic Cardiac Disease and Its Effect on the Cardiac Nervous System

Approximately every minute an American dies from a coronary event making coronary heart disease a significant health concern for a large portion of the U.S. population (Lloyd-Jones and others 2009). The incidence of coronary heart disease, including acute coronary syndrome, is estimated at 935,000 new and recurrent attacks in the U.S. per year (Lloyd-Jones and others 2009). Restricting the supply of blood and oxygen to the heart leads to myocardial ischemia (Thygesen and

others 2007). Acute coronary syndrome consists of MI and angina pectoris (Thygesen and others 2007). If severe enough, MI induces changes in the cardiac environment including increasing the propensity for arrhythmia formation, cardiac cell death, and the potential for progression into heart failure (Thygesen and others 2007).

Disease states alter multiple components of the cardiac nervous system (Armour 1999). Cardiac afferent neurons are thought to be the first component affected. For example, regional ventricular ischemia increases cardiac sensory activity via activating multimodal cardiac sensory neurites (Armour 1999; Huang and others 1996). MI reflexly activates intrinsic cardiac neurons leading to adverse function such as arrhythmia formation and compromised mechanical activity (Foreman and others 2000). MI likewise causes the reflex activation of efferent neurons in the stellate and middle cervical ganglia, contributing to the increase in postganglionic sympathetic activity (Ardell and others 2009). Central processing of afferent inputs at the level of the spinal cord and brainstem contribute to reflex evoked changes in efferent outflows (Armour 1999; 2004). Research into the effects of MI on the heart and the cardiac nervous system has identified two distinct sets of effects, short-term and long-term (Armour 1999; 2004). In the short term, excessive reflex activation of parasympathetic and sympathetic efferent neurons can elicit atrial or ventricular arrhythmias (Armour 1976; Armour and others 2005; Cardinal 2004; Carlson and others 1992; Huang and others 1994; Schwartz and others 1978). Excessive catecholamine release, in reflex response to ischemia, is also cardio-toxic (Armour 1999). Over longer periods of time, cardiac disease induces neural remodeling of the cardiac nervous system (and heart tissues) and this contributes to the progression of cardiac pathologies (Armour 1999; 2004). Examples of neural remodeling include focal denervation of nerve projections through the infarct zone and hyperinnervation in the border zone surrounding the region of ischemic insult (Chen and others 2001). Changes in innervation density can be accompanied by focal changes in receptor density,

receptor coupling and neurotransmitter release (Chen and others 2007; Chen and others 2001; Zipes 2008). Heterogeneity in efferent inputs to the heart are a major contributor to adverse consequences of cardiac disease (Armour 2004).

Cardiac Arrhythmias and the Cardiac Nervous System

Heterogeneous efferent input to the heart is a major risk factor for cardiac arrhythmias (Armour 2004; Zipes 2008). In normal hearts, the sympathetic and parasympathetic nervous systems form a interdependent neural substrate to regulate regional cardiac electrical activity (Armour 2004). MI-induced alterations in cardiac tissues by themselves directly increase the arrhythmogenic substrate of the heart (Armour 2004). For example, myocardial infarct disrupts electrical pathways within the heart, leading to arrhythmia formation through reentrant circuits (Iwasaki and others 2011). These reentrant circuits are self-perpetuating waves of cardiac electrical activity that result in the formation of cardiac arrhythmias, including tachycardia and fibrillation (Armour 1999; Iwasaki and others 2011). Superimposed on the alterations of electrical substrate, imbalances of neuronal control can facilitate arrhythmia formation (Armour 1999). Recent evidence suggests that nerve sprouting in and near the infarct site can contribute to this imbalance of neural control (Chen and others 2001). MI-induced nerve sprouting is found predominantly in sympathetic nerves (Chen and others 2001), likely contributing to imbalanced neurotransmitter release during MI stress (Zipes 2008). Heterogeneous adrenergic neural activity evokes imbalances in myocyte excitability and refractoriness, both contributing to cardiac electrical instability (Zipes 2008). These data point to the interdependence of neural and local myocyte factors in the electrical stability of normal and ischemic stressed hearts (Zipes 2008). Therapies that reduce the heterogeneity, either by targeting the myocyte substrate or the neural mechanisms that modulate it, should translate into decreased risk of cardiac arrhythmias (Zipes 2008).

Recent studies have used an experimental model of electrically-induced atrial fibrillation (AF) to study cardiac arrhythmia formation and mechanisms to control them (Armour and others 2005; Cardinal and others 2006; Richer and others 2008). One model involves stimulating mediastinal nerves that course over the ventral and ventrolateral surfaces of the superior vena cava (Armour and others 2005). Stimulation of these nerves creates self-terminating periods of AF (Armour and others 2005). While this model differs in some respect from cardiac disease-created AF, it allows for the study of how neuronal imbalances within the cardiac nervous system can influence the formation of arrhythmias and potentially how aspects of that neural population can be targeted to modulate the potential for AF (Armour and others 2005; Cardinal and others 2006; Richer and others 2008).

Neuromodulation as a Novel Approach to Treat Heart Disease

Cardiac disease is a multi-factored process that involves cardiac tissues and the neurohumoral factors that modulate the heart (Armour 1999; 2004). Cardiac dysfunction, as occurs in acute coronary syndrome, can be targeted by therapies directed at cardiomyocytes, the neurohumoral components that regulate them or by physical means as with restoration of blood flow (Armour 1999; 2004). Coronary artery bypass graft and balloon angioplasty are the primary mechanical therapies that restore blood flow and are effective in a majority of patients (Andrade and others 2011; Mannheimer and others 1998). However, a subset of patients are not candidates for such an approach or conversely, exhibit refractory angina even after revascularization (Mannheimer and others 1998).

Neuromodulation-based therapies for cardiac disease focus on targeting neuronal components within the cardiac nervous system to achieve effects. One such neuromodulatory approach involves cryo- or electrical ablation of specific neuronal targets of the intrinsic cardiac nervous system as an adjunct therapy to traditional ablation of cardiomyocytes to control atrial

arrhythmias (Burkhardt and Natale 2009). This therapy is predicated on the hypothesis that disruption of select parts of the cardiac nervous system can stabilize the heart and reduce the arrhythmogenic potential of the heart (Armour 2008). The resulting lesions eliminate subcomponents of the neural networks that influence cardiac function, but the potential for sudden cardiac death (SCD) still remains (Armour 2008). Importantly, while ablation therapy has shown some positive short-term effects, the interconnectivity and processing ability of remaining parts of the cardiac nervous system along with their remodeling post-ablation allows for those remaining components of the cardiac nervous system to still promote the formation of arrhythmias in the long term (Armour 2008).

Neuromodulation therapy can use pharmacological blockade of receptors to impact neuronal activity (Armour 1999; Association 2007; Marieb 2004). These pharmacological approaches can consist of agents like non-selective β-adrenergic antagonists or specific agents like α- or β1-selective adrenergic receptor blockers (Association 2007). While pharmacological therapies can be effective in combating cardiac dysfunction including progression into congestive heart failure and potential for SCD (Association 2007), the cardiac nervous system isn't the only target of these pharmacologically based therapies (Marieb 2004). The neuropharmacologically-based therapies often result in numerous unwanted side effects, such as diarrhea, rash, slow heart rate, and impotence, reducing the indications for use and are also subject to patient non-compliance (Association 2007).

An emerging neuromodulation therapy involves electrical stimulation of various aspects of the cardiac nervous system to treat cardiac pathophysiology. For example, vagal nerve stimulation is currently being evaluated to treat heart failure (Schwartz and De Ferrari 2009). This approach is predicated on the hypothesis that increasing parasympathetic tone will counteract the increase in sympathetic activity seen in heart failure and thereby slow progression of congestive heart failure

(Schwartz and De Ferrari 2009). In one study of animals with healed infarcts, vagal stimulation reduced heart rate by 75 beats per minute during exercise and reduced the propensity for ventricular fibrillation from 92% in control to 12% during the exercise stress (Vanoli and others 1991). Recent studies also have shown that low-level vagal nerve stimulation reduces the arrhythmogenic potential of the atria in response to rapid atrial pacing while also reducing sympathetic nerve activity (Sha and others 2011; Shen and others 2011b). These studies indicate that induced alterations in autonomic efferent outflows can be moderated by electrical neuromodulation to affect cardiac electrical stability (Zipes 2008).

Another approach for neuromodulation involves direct electrical stimulation of the carotid baroreceptors. This therapy is predicated on the hypothesis that increased baroreceptor activity will reflexly increase parasympathetic activity and decrease sympathetic activation to the heart (Sabbah and others 2011; Wang and others 1991; Zucker and others 2007). This therapy elicited a reduction in the progression of congestive heart failure in response to the cardiac stress of rapid ventricular pacing (Zucker and others 2007). It remains to be determined if such an integrated baroreflex type approach is effective against other models of cardiac disease (e.g. MI, mitral regurgitation, etc.).

A third major approach using electrical neuromodulation involves spinal cord stimulation (SCS). In the treatment of heart disease, SCS is traditionally performed at the T1-T3 levels with electrical stimuli applied to the dorsal aspects of the spinal cord. This level includes the spinal segments where the sympathetic efferent nerve fibers exit and the cardiac sympathetic afferent nerve fibers enter the spinal cord from the heart (Armour 1999; 2004). Clinical research has demonstrated that high thoracic SCS significantly reduces the presentation of anginal symptoms, the onset of angina and increases exercise tolerance (Mannheimer and others 1993; Mannheimer and others 1998; Sanderson and others 1992; Sanderson and others 1994). In addition, SCS decreased the ST-segment depression, a marker of MI, and increased the time before ST-segment depression onset

during a progressive exercise stress test (Eliasson and others 1993; Mannheimer and others 1993; Sanderson and others 1992; Sanderson and others 1994). Clinical investigators have suggested that the cardiac benefits of SCS result from improved oxygen supply/demand balance in the stressed heart (Mobilia and others 1998), but little hard evidence has been put forth to support this concept (Kingma and others 2001).

Basic science research has confirmed and extended the potential cardioprotective effects evoked by SCS (Ardell and others 2009; Armour and others 2002; Armour and others 2005; Cardinal and others 2006; Foreman and others 2000; Qin and others 2008; Richer and others 2008; Southerland and others 2007). High thoracic SCS has been shown to decrease MI-induced sympathetic activation in reflex response to ischemic stress and to reduce the arrhythmogenic potential resulting from ischemic stress (Ardell and others 2009; Cardinal and others 2006; Southerland and others 2007). Other studies have demonstrated that SCS modulates activity in the middle cervical and intrinsic cardiac ganglia and within the spinal cord, attenuating their reflex response to ischemic stress (Ardell and others 2009; Ding and others 2008a; Ding and others 2008b; Foreman and others 2000; Qin and others 2008; Southerland and others 2007). Pre-emptive SCS likewise activates protein kinase C in ventricular myocytes, contributing to the cardioprotective effects against the stress of acute MI (Southerland and others 2007). High thoracic SCS-induced cardioprotection to ischemic stress occurs without significant changes in cardiac blood flow and without significant changes in basic hemodynamic function (Kingma and others 2001). Transection of the ansae subclavian, eliminating afferent and efferent interconnections between the spinal cord and peripheral aspects of the cardiac nervous system, prevents SCS-induced changes in middle cervical and intrinsic cardiac neuronal activity (Cardinal and others 2006; Foreman and others 2000). These data demonstrate that thoracic SCS exerts many of its beneficial effects on cardiac function by; 1) targeting the cardiac nervous system and 2) directly impacting cardiac tissue (Ardell and others

2009; Armour 2008; Armour and others 2002; Armour and others 2005; Cardinal and others 2006; Foreman and others 2000; Southerland and others 2007).

The targeting of neural elements that regulate cardiac function was initially evaluated at the high thoracic spinal cord level, where the sympathetic efferent and afferent neurons enter and exit the cord (Ding and others 2008a; Ding and others 2008b; Qin and others 2008). This high thoracic location mitigates numerous deleterious effects induced by cardiac ischemic pathologies (Ardell and others 2009; Armour and others 2002; Cardinal and others 2006; Foreman and others 2000; Southerland and others 2007). Recent anatomical and functional studies have indicated that there may be other potential sites within the neural hierarchy for cardiac control where SCS may exert cardioprotective effects in response to cardiac stress (Ding and others 2008b; Qin and others 2008). The high cervical region of the spinal cord is one such site. The high cervical spinal segments receive parasympathetic and sympathetic afferent nociceptive inputs (Chandler and others 1996; Chandler and others 2000; Foreman 1999). It is also reciprocally interconnected with lower levels of the spinal cord and with supraspinal elements of the cardiac nervous system (Marieb 2004). Previous work has indicated that high cervical SCS attenuates afferent transduction of cardiac ischemia (Ding and others 2008b; Gonzalez-Darder and others 1991; Qin and others 2008). The high cervical spinal segments also modulate spinal cord reflex processing of autonomic outflow for thoracic and visceral tissues (Ding and others 2008a; Ding and others 2008b; Qin and others 2007a; Qin and others 2008; Qin and others 2007b). These findings indicate that high cervical spinal segments may therefore provide a site higher in the cardiac neural hierarchy for SCS to act upon (Ding and others 2008b; Qin and others 2008).

To investigate the specific neuronal targets of neuromodulation, this dissertation is predicated on the following hypotheses: **1)** The cardiac nervous system contains a complex hierarchy of reflex control systems that contribute to control of regional cardiac function; **2)** The cardiac

nervous system is disrupted by cardiac stressors contributing to deleterious effects on that neural control system and the myocardium it modulates; and **3)** Stabilization of select aspects of the cardiac nervous system through neuromodulation can mitigate the deleterious effects of cardiac stressors on the neural components of the cardiac nervous system and the myocardium thereby inducing states of cardioprotection. To test these hypotheses, this dissertation was divided into three specific aims which are as follows.

Specific Aims

Specific Aim 1: To determine whether high cervical cord neurons can modify infarct size and potential for SCD in response to ischemic stress.

Myocardial ischemia is known to impact cardiomyocytes directly and the cardiac nervous system that controls them (Armour 2008). High thoracic SCS has recently been shown to have anti-ischemic properties that improve the ability of the heart to function following MI (Cardinal and others 2004; Eliasson and others 1993; Mannheimer and others 1993). Pre-emptive thoracic SCS reduces infarct size as well as activates cardiomyocyte protein kinase C pathways (Southerland and others 2007). It also stabilizes the electrical properties of the ventricle in response to transient MI (Cardinal and others 2004; Southerland and others 2007). Neurons in the upper cervical spinal cord are known to modify sensory transduction from the periphery, including those arising from the thorax and viscera (Chandler and others 1993; Ding and others 2008b; Foreman and others 2004). Pre-emptive SCS from the high cervical region modulates dorsal horn sensory afferent signaling from the ischemic myocardium (Ding and others 2008a). This effect may reflect activity of cervical propriospinal pathways that release dynorphin at the thoracic spinal cord to modulate nociceptive signaling (Ding and others 2008b). The C1-C2 region is interconnected with lower levels of the cord and with cardiovascular regions of the brainstem, including the nucleus tractus solitarii (Chandler and others 1993; Ding and others 2008b; Foreman and others 2004). These data indicate

the potential of the upper cervical spinal segments to act as a processing center within the cardiac neuronal hierarchy for cardiac control and a site for potentially modifying the deleterious cardiac effects of MI (Ding and others 2008a; Qin and others 2008). Chapter 2 will focus on the hypothesis that high cervical SCS neuromodulation imparts cardioprotective effects in response to MI by modulating sympathetic and parasympathetic outflows to the heart. Cardioprotective effects evaluated will include infarct size and the potential for MI-induced SCD. This aim will determine if those cardioprotective effects involve 1) descending projections from the cervical to the thoracic spinal cord and 2) the differential roles of high cervical SCS-mediated changes in sympathetic versus parasympathetic efferent outflows and their response to acute MI.

Specific Aim 2: To determine if SCS modulates primary afferent neural transduction of MI.

SCS has been shown to modulate the MI-induced increase in activity of neurons in intrathoracic ganglia (Ardell and others 2009). These ganglia, including intrinsic and extracardiac ones, contain cardiac efferent and afferent and local circuit neurons (Ardell and others 2009; Foreman and others 2000). SCS does not interrupt primary efferent projections to the heart (Armour and others 2002). Within peripheral ganglia of the cardiac nervous system, data indicate that SCS may target the local circuit neurons to blunt reflex activation of autonomic efferent outflow (Ardell and others 2009; Armour 2008; Foreman and others 2000; Qin and others 2008). Primary cardiac afferent neurons and their response to MI in association with SCS neuromodulation have yet to be studied.

Previous studies identifying SCS effects on cutaneous blood flow have suggested that SCS can impose an "efferent" function on afferent neurons (White and Helme 1985). Whether such antidromic evoked responses are manifested in cardiac afferents in response to SCS have yet to be determined. Chapter 3 will examine the hypothesis that high thoracic SCS modifies the ability of primary cardiac afferent neurons to respond to the stress of MI, either by direct antidromic

activation of primary cardiac afferent neurons or through altering the cardiac sensory milieu. To investigate this hypothesis, the activity of primary cardiac afferent neurons (nodose and DRG) will be examined in response to transient MI, with and without pre-emptive SCS neuromodulation.

Specific Aim 3: To determine if SCS targets intrinsic cardiac neurons to attenuate neuronally-mediated atrial arrhythmias.

Excessive activation of discrete inputs to the intrinsic cardiac nervous system have been demonstrated to create atrial arrhythmias in normal and diseased states (Armour and others 2005; Choi and others 2011; Richer and others 2008; Shen and others 2011a). As described previously, the stimulation of select intrathoracic mediastinal nerves evokes reproducible and self-terminating periods of AF (Armour and others 2005). This model of AF is known to involve both parasympathetic and sympathetic efferent mechanisms (Armour and others 2005; Richer and others 2008). SCS has previously been shown to decrease the induction of AF following mediastinal nerve stimulation (MSNS), but the neural mechanisms underlying this response have not been determined (Cardinal and others 2006). Therefore, Chapter 4 will investigate the hypotheses that: 1) MSNS activates select neuronal subpopulations within the intrinsic cardiac nervous system (ICN) in association with the induction of AF; 2) stabilization of those populations within the intrinsic cardiac nervous system via neuromodulation (electrical or pharmacological) blunts the capacity of the ICN to respond to excessive inputs; and 3) stabilization of intrinsic cardiac neurons suppresses the atrial arrhythmogenic potential. The neurochemical phenotype and activity of right atrial ganglionated plexus neurons along with AF induction characteristics in response to MSNS and neuromodulation, electrical or pharmacological, will be examined to test this hypothesis.

CHAPTER 2

Activated cranial cervical cord neurons affect left ventricular infarct size and the potential for sudden cardiac death

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Abstract

To evaluate whether cervical spinal neurons can influence cardiac indices and myocyte viability in the acutely ischemic heart, the hearts of anesthetized rabbits subjected to 30 min of left anterior descending (LAD) coronary arterial occlusion (CAO) were studied 3 hours after reperfusion. Control animals were compared to those exposed to pre-emptive high cervical cord stimulation (SCS; the dorsal aspect of the C1-C2 spinal cord was stimulated electrically at 50 Hz; 0.2 ms; 90% of motor threshold, starting 15 min. prior to and continuing throughout CAO). Four groups of animals were so tested: 1) neuroaxis intact; 2) prior cervical vagotomy; 3) prior transection of the dorsal spinal columns at C6; and 4) following pharmacological treatment [muscarinic (atropine) or adrenergic (atenolol, prazosin or yohimbine) receptor blockade]. Infarct size (IS) was measured by tetrazolium expressed as percentage of risk zone. C1-C2 SCS reduced acute ischemia induced IS by 43%, without changing the incidence of sudden cardiac death (SCD). While SCS-induced reduction in IS was unaffected by vagotomy, it was no longer evident following transection of C6 dorsal columns or atropinization. Beta-adrenoceptor blockade eliminated ischemia-induced SCD, while alpha-receptor blockade doubled its incidence. During SCS, myocardial ischemia induced SCD was eliminated following vagotomy while remaining unaffected by atropinization. These data indicate that, in contrast to thoracic spinal neurons, 1) cranial cervical spinal neurons affect both adrenergic and cholinergic motor outflows to the heart such that 2) their activation modifies ventricular infarct size and lethal arrhythmogenesis.

Introduction

Myocardial ischemia represents a complex cardiovascular stress that involves cardiomyocytes as well as neurohumoral control of cardiomyocytes (Kajstura *et al.*, 2006; Armour, 2008). Imbalances in such neurohumoral control, especially those leading to excessive sympathetic efferent neuronal activation, have been associated with an enhanced arrhythmogenic substrate (Schwartz, 2001) as well as deterioration of pump function (Armour, 2008; Dell'Italia & Ardell, 2004). Conversely, endogenous or exogenous mechanisms that maintain parasympathetic efferent neuronal control during transient myocardial ischemia have been proposed to exert cardioprotective effects - thereby reducing any risk of sudden cardiac death (Vanoli *et al.*, 2008).

Neuromodulation-based therapies that underlie the emerging field of neurocardiology are known to affect the progression of cardiac pathologies. For instance, pharmacological therapies that employ β-adrenoceptor blockade and/or angiotensin-converting enzyme inhibition (Hankes *et al.*, 2006; Perry *et al.*, 2002) modulate not only cardiomyocyte function directly, but also indirectly by mitigating adverse remodeling of the cardiac neuronal hierarchy (Armour, 2008; Hankes *et al.*, 2006; Tallaj *et al.*, 2003).

Spinal cord stimulation (SCS) has been reported to exhibit anti-ischemic properties that include increased exercise tolerance (Hautvast *et al.*, 1998), diminished ST segment deviation during stress (Cardinal *et al.*, 2004; Hautvast *et al.*, 1998; Odenstedt *et al.*, 2011), and improved lactate metabolism (Mannheimer *et al.*, 2002). Yet, anginal pain can still be evoked in the presence of SCS when the stress (e.g. exercise) is of sufficient magnitude to induce critical levels of myocardial ischemia (Eddicks *et al.*, 2007; Mannheimer *et al.*, 2002).

Evidence indicates that neurons in the thoracic spinal cord play a role in processing afferent inputs arising from the stressed heart (Ding *et al.*, 2008a; Ding *et al.*, 2008b). In fact, electrical stimuli

delivered to neurons within the thoracic spinal cord have been shown to reduce electrical instability of the stressed ventricle (Cardinal *et al.*, 2004; Cardinal *et al.*, 2006; Odenstedt *et al.*, 2011). We have proposed that the cardioprotection elicited by delivering pre-emptive electrical stimuli to the thoracic spinal cord acts to stabilize excessively active intrinsic cardiac local circuit neurons involved in transducing the ischemic event (Armour, 1997; Armour *et al.*, 2002). Such therapy also activates cardiomyocyte protein kinase C (PKC) pathways and reduces infarct size in response to transient periods of myocardial ischemia (Southerland *et al.*, 2007).

Neurons in the C1-C2 spinal cord are known to be involved in modifying pain transmission arising from intrathoracic and abdominal viscera (González-Darder *et al.*, 1991) presumably because to the fact that they receive afferent neuronal inputs from sympathetic ascending projections and nodose ganglion neurons (Chandler *et al.*, 1993; Ding *et al.*, 2008a). In addition, the C1-C2 spinal cord region is known to be interconnected reciprocally with cardiovascular-related medullary neurons (Foreman *et al.*, 2004). As it remains to be established whether neurons in the cervical spine are involved in cardiac neuromodulation and, if so, in what manner, this study was devised to determine whether neuromodulation of cervical spinal neurons can affect cardiac indices, particularly in the presence of myocardial ischemia.

If cervical SCS does indeed impart cardioprotection, as manifested by changes in infarct size and the potential for sudden cardiac death, we also sought to elucidate whether such cardioprotection involves not only 1) cervical spinal descending projections to the thoracic cord, 2) but also whether supraspinal projections arising from the C1-C2 region modify parasympathetic outflow to impact cardiac function in response to ischemic stress – something not evident from thoracic SCS. Thus we sought to determine whether such a therapeutic intervention might involve

adrenergic versus cholinergic efferent neurons from high cervical spinal cord neuronal inputs and whether such modulation imparts potential therapeutic benefits to the ischemic ventricle.

Materials and Methods

Subjects. One hundred eighty two New Zealand White rabbits of either sex, weighing between 1.7 and 3.8 kg (2.7±0.4 kg), were used in these studies. Seventeen of these were excluded from final analysis for technical reasons. Because the rabbit heart has minimal collateral blood flow, this animal model produces a distinct, homogenous ventricular risk zone, which is ideal to directly evaluate therapeutic interventions for transient myocardial ischemia (Maxwell *et al.*, 1987). 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.

Surgical preparation. Rabbits were anesthetized with intravenous pentobarbital sodium (30 mg/kg iv via ear access, supplemented as needed with 2 mg/kg iv if the animal responded to noxious stimuli or an increase in arterial blood pressure was observed). The trachea was intubated via a cervical incision and mechanical ventilation initiated and maintained with a positive pressure ventilator (MD Industries, Mobile, AL) using 100% O_2. Core body temperature was maintained at 38°C via a circulating water heating pad. The right carotid artery was cannulated for monitoring blood pressure and the right jugular vein cannulated to administer anesthetic agents and drugs. Heart rate was assessed from a Lead II electrocardiogram. All hemodynamic data were recorded concurrently on a Gould model TA6000 recorder. For protocols 1d and 3b (fig. 1), both cervical

vagi were exposed by a midline cervical skin incision and isolated in order that bilateral vagotomy could be performed.

Animals were placed in ventral recumbency and a laminectomy was performed at the C2 level followed by the subdural placement of two plate electrodes (2 x 3 mm) slightly to the left of the midline at the C1-C2 level. Spinal cord stimulation (SCS) was performed via these indwelling electrodes which were connected to a Grass S88 stimulator (Grass Instruments, Quincy, MA) using a constant current stimulus isolation unit (Grass PSIU 6G). The parameters used to stimulate the spinal cord were 50 Hz, 0.2 ms duration and at an intensity of 90% of motor threshold. SCS stimulation parameters were chosen because of their proven therapeutic benefits (Mannheimer *et al.*, 2002) including its documented ability to reduce infarct size in response to transient myocardial ischemia (Southerland *et al.*, 2007). To determine the adequate stimulus intensity, the current intensity was progressively increased until minor muscle contractions were observed in the cervical neck region and/or left upper shoulder (motor threshold). Current intensity was set at 90% of motor threshold for the experimental protocols; this intensity averaged 0.27 $\pm$ .07 (SD) mA. The rostral and caudal poles were chosen as cathode and anode, respectively, according to current clinical practice (Mannheimer *et al.*, 2002). Motor threshold determinations were repeated at the end of the experimental protocols to determine the stability of the stimuli intensity. For protocols 1c and 3a (Fig. 1), an additional laminectomy was performed at the C6 level, and a midline incision was made in the spinal cord and extended approximately 2-3 mm laterally on each side to transect the dorsal columns of the spinal cord at that level.

Animals were rotated to their right-side and a thoracotomy was performed in the left fourth intercostal space. The pericardium was opened to expose the heart. A 2-0 silk suture on a curved, tapered needle was passed around the left anterior descending coronary artery one-third of the

distance from the left ventricular base to apex. Regional cyanosis and bulging were observed when the ends of the suture were pulled through a small polyethylene tube to form a snare, which was then secured by clamping the tube with a hemostat. Cyanosis and regional diskinesia were observed in the region downstream to the occluded vessel and both disappeared immediately upon reperfusion. The risk zone consisted of the myocardial tissue perfused by the snared coronary artery; the non-risk zone consisted of the remainder of the left ventricle. A 20-minute stabilization period preceded the onset of each experimental protocol.

Experimental protocols. Figure 1 summarizes the experimental protocols used for each of the 11 groups of rabbits studied. Each group underwent a dorsal laminectomy at the C2 level prior to being subjected to 30 min. of coronary arterial occlusion (CAO) followed by a 3-hr reperfusion period.

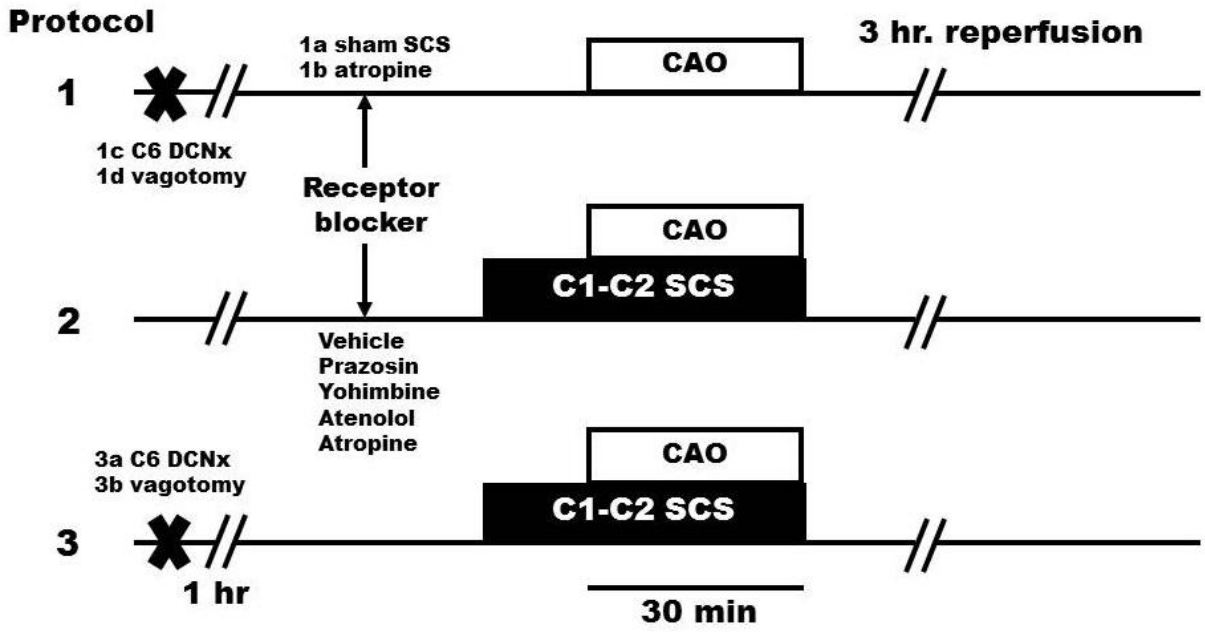

Figure 1. Protocols for the *in situ* rabbit heart experiments. The ventricles in each respective group were exposed to regional ischemia (open boxes) with or without pre-emptive C1-C2 spinal cord stimulation (SCS: filled boxes). The control group (Protocol 1) consisted of 30 min. of left coronary artery occlusion (CAO) followed by a 3-hr reperfusion period. A similar 30 min. CAO and 3 hr reperfusion stress was utilized to evaluate all neuromodulation treatments (protocols 2 and 3). For pre-emptive SCS, SCS was delivered at frequencies of 50 Hz, 200μs and 90% motor threshold. For protocols 1 and 2, the arrow indicates the time when pretreatment with the vehicle (control), adrenoceptor blocking agents (prazosin, yohimbine or atenolol) or the muscarinic blocking agent atropine occurred. For protocols 1 and 3, X indicates the time of dorsal column transection at the C6 spinal segment (C6 DCNx) or bilateral transection of the cervical vagosympathetic trunk (vagotomy). Specific subsets for protocols 1 (a to d) and 3 (a and b) are indicated on the left. Protocol 2 has 5 subsets, as determined by indicated vehicle or drug injection.

Animals in protocol 1 (n = 40) were subjected to 30 min. of regional ventricular ischemia followed by a 3-hr reperfusion period. Nine of these animals were in the control coronary occlusion group (*protocol* 1a: sham SCS), 9 animals were pretreated with atropine (0.2mg/kg, iv) 30 min. prior to the onset of regional ischemia (*protocol* 1b). This dose of atropine was sufficient to block the bradycardic responses to vagal stimulation. Eleven animals underwent bilateral transection of the dorsal columns at the C6 spinal level 1 hour prior to the onset of regional ischemia (protocol 1c).

Selective transection of C6 dorsal columns was confirmed post-mortem. Eleven animals had their cervical vagi transected 1 hour prior to the onset of regional ischemia (protocol 1d).

Pre-emptive electrical neuromodulation. Animals in this part of the study (*protocols 2-3*) were subdivided into seven separate groups. Each group was subjected to 46 min. of SCS initiated 15 min. prior to the onset of the 30 min. coronary artery occlusion and terminated 1 min. after release of the coronary artery occlusion. The ischemic period was followed by a 3-hr reperfusion period. The duration and time point for the onset of pre-emptive SCS applied to upper cervical segments of the spinal cord were based upon its effectiveness to reduce infarct size when delivered to the high thoracic cord (Southerland *et al.*, 2007).

Animals in *protocol 2* (n = 94) were subjected to 46 min. of C1-C2 SCS, as described above. Fifty-four of these animals underwent C1-C2 SCS with vehicle control, run in a blinded fashion with each of the protocols using selective autonomic receptor blockade. To determine whether adrenergic or muscarinic receptor blockade affected pre-emptive C1-C2 SCS-induced cardioprotection, 40 additional animals were subjected to *protocol 2* 15 min. after either the α_1-adrenoceptor blocking agent prazosin (0.15mg/kg iv; n = 11), the α_2-adrenoceptor blocking agent yohimbine (1mg/kg iv, n = 12), the β_1-adrenoceptor blocking agent atenolol (2mg/kg iv; n = 8), or the muscarinic receptor blocking agent atropine (0.2mg/kg, iv; n = 9) was administered. The prazosin dose was selected because of its effectiveness in blocking the hypertensive response to intravenous injections of phenylephrine (100 μm/1 ml). The dose of atenolol blocked the tachycardiac response to intravenous isoproterenol (100 μm/1 ml). The dose of yohimbine was selected according to its effectiveness in stabilizing neuronal processing within the intrinsic cardiac nervous system (Richer *et al.*, 2008). The dose of atropine was sufficient to block any bradycardia responses elicited by vagal stimulation.

Protocol 3 (n=31) evaluated the potential contributions of intersegmental propriospinal and supra-spinal/parasympathetic interconnections in mediating the cardioprotection to C1-C2 SCS. To determine whether C1-C2 SCS effects on myocyte cell death and electrical stability involved intersegmental spinal cord connections, we subjected 15 animals to bilateral transection of the dorsal columns at C6 *(protocol 3a)* 1 hr prior to the pre-emptive SCS-coronary artery occlusion stress. Selective transection of C6 dorsal columns was confirmed post-mortem. To determine whether C1-C2 SCS mediated effects on cardiac viability and electrical stability involved supraspinal pathways, reflected back to the heart via the parasympathetic efferent pathways, 16 animals in *protocol 3b* were subjected to bilateral cervical vagotomy 1 hr prior to the SCS-coronary artery occlusion stress.

Infarct measurement. At the end of each experiment, the hearts were rapidly excised, mounted on a modified Langendorff apparatus, and perfused with 0.9% saline at 37°C to remove blood from the coronary circulation. Following re-occlusion of the coronary artery that had been occluded previously, 2- to 9-μm fluorescent polymer microspheres (Duke Scientific, Palo Alto, CA) were injected into the aortic perfusion fluid to demarcate the ventricular region at risk. After removing both atria, the rest of the heart was weighed and then frozen at -20°C. The heart was then cut into 2-mm-thick slices parallel to the atrioventricular groove. Tissue slices were incubated for 20 min. at 37°C in 1% triphenyltetrazolium chloride (TTC) and sodium phosphate buffer (pH 7.4). Tissue slices were then placed in 10% formalin to improve the contrast between stained and unstained tissue. Areas of infarction (TTC negative), risk zone (negative fluorescence under UV light), and non-risk zone (positive fluorescence under UV light) were traced onto plastic overlays. These areas were measured using computer-assisted planimetry (Image Research). Infarct and risk zones were calculated by multiplying each area by tissue thickness and their products summed. Infarct size is expressed as a percentage of risk zone.

Immunohistochemistry. A total of 24 animals were used for this section of the study. Following the 3 hr reperfusion, animals were given a large dose of pentobarbital and then perfused transcardially with 2 L of normal saline followed by 2 L of 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4. The T_2 spinal cord (identified by placing a small dot of Pontamine blue dye on the dorsal surface of the C_8 spinal cord prior to its removal) was excised and postfixed as described previously (Hua *et al.*, 2004). Consecutive 50 μm sections were cut with a cryostat (Leica Microsystems Inc., Bannockburn, IL) at -20°C and placed immediately into wells of polypropylene plates containing PBS. Sections were processed for the presence of c-Fos immunoreactivity as described previously (Hua *et al.*, 2004). Briefly, tissues were rinsed in PBS for 15 min. and then permeabilized in PBS containing normal donkey serum (NDS; Millipore, Billerica, MA) and Triton-X-100 (Sigma) for 20 min. The sections were then treated with H_2O_2 (Fisher, Pittsburgh, PA) to quench endogenous peroxidase activity. The sections were rinsed with PBS and permeabilized again. The sections were then incubated in a 10% NDS blocking solution. Following blocking, the sections were incubated in c-Fos antibody from goat (1:15000 in PBS; Cat. # sc-52-G, Santa Cruz Biotechnology, Santa Cruz, CA) for 48 hrs at 4°C with constant gentle shaking. Following the completion of this incubation, the sections were rinsed and permeabilized followed by incubation in biotinylated donkey anti-goat IgG secondary antibodies (1:200, Cat. # 708-065-003, Jackson Immunoresearch, West Grove, PA) for 2.5 hrs at room temperature. The sections were rinsed and permeabilized and incubated in ABC solution (1:100, Cat. # PK-4000, Vector Labs, Burlingame, CA) for 1 hr at room temperature with constant gentle shaking. Following incubation with ABC solution, the sections were rinsed three times in Tris buffer for 15 min. each. Following these rinses, fos was visualized by incubation in a solution of 10 mg 3',3'-diaminobenzidine with 7 μL of 30% H_2O_2 in Tris buffer. The reaction was stopped after 4.5 min by transfer of the sections to Tris buffer. The sections were mounted on chrome-alum gelatin coated slides and dehydrated

through successive ethanol washes followed by a wash in xylene. The slides were coverslipped using Permount mounting medium (Fisher, Pittsburgh, PA).

Localization of c-fos immunoreactivity was evaluated using bright-field microscopy using an Olympus BH2 microscope. Quantification of Fos-positive cells in the T_2 dorsal horn (Laminae I-V) was performed by counting dark brown nuclei in a 200 μm^2 area of each animal. Data were then grouped and averaged for each experimental category. The number of immunoreactive cells in the dorsal horn was expressed as cells per square micrometers.

Statistical analysis. All data are presented as means (±SD). SigmaStat 3.1 (Systat Software) with two-way analysis of variance with post hoc comparisons (Holm-Sidak test) was used to test for interactions between SCS and neural ablation (C6 dorsal column transaction or cervical vagotomy) and for interactions between SCS and cervical vagotomy versus atropine blockade for modulation of infarct size. One-way analysis of variance with post hoc comparisons (Holm-Sidak test) was used for hemodynamic data and for effects of selective adrenergic blockade on infarct size. A significance of $P < 0.05$ was used. Summary data for Fos-positive cells were compared using a two-way ANOVA with the Holm-Sidak test for pair-wise comparisons (significance at $p<0.05$).

RESULTS

Hemodynamic variables. Table 1 summarizes heart rate and blood pressure changes identified in each of the four non-SCS experimental groups. For sham SCS control, dorsal column transection control and cervical vagotomy control groups, heart rate was no different during baseline, coronary artery occlusion or reperfusion. For the atropine control group (without C1-C2 SCS), heart rate decreased from baseline only in the later stages of reperfusion. Baseline blood pressures were

similar in all four groups of animals. During coronary artery occlusion, blood pressure was significantly decreased from baseline for all groups except vehicle control. For all four groups, blood pressure was decreased significantly from baseline at 1 hour reperfusion.

Table 1. Hemodynamic data for non-SCS control groups

	Baseline	CAO	1 Hr Reperfusion
		Heart rate, beats/min	
Sham SCS	253.8 ± 21.1	253.7 ± 24.0	252.0 ± 22.4
Atropine control	292.0 ± 20.1	286.4 ± 17.1	278.8 ± 20.3*
C6 Dorsal column transection	269.3 ± 33.0	267.6 ± 37.6	263.6 ± 32.7
Cervical Vagotomy	254.8 ± 33.0	250.6 ± 25.1	255.6 ± 31.5
		Blood pressure, mmHg	
Sham SCS	81.3 ± 9.4	76.7 ± 6.7	75.5 ± 5.4*
Atropine control	84.6 ± 10.3	76.9 ± 11.1*	71.5 ± 11.2*
C6 Dorsal column transection	87.2 ± 7.3	78.1 ± 8.3*	76.3 ± 3.2*
Cervical Vagotomy	81.2 ± 6.9	78.4± 5.9*	72.2 ± 5.3*#

within group comparison
* versus baseline
# versus CAO

Table 2 summarizes heart rate and blood pressure for animals with pre-emptive C1-C2 SCS with selective autonomic receptor blockade. For the vehicle-treated group, there were minor increases in heart rate associated with C1-C2 SCS and during the subsequent addition of the cardiac ischemic stress. In the vehicle control group, blood pressure was reduced significantly from post-"block" baseline values during coronary artery occlusion and reperfusion. For selective autonomic blockade, pretreatment with prazosin reduced basal mean blood pressure, which was sustained throughout the succeeding observation periods. Pretreatment with atenolol reduced heart rate significantly and pretreatment with atropine increased heart rate significantly; these changes were sustained throughout the subsequent observation periods. With the exception of the prazosin group, blood pressure decreased during coronary artery occlusion and reperfusion.

Table 2. Hemodynamic data for pre-emptive C1-C2 neuromodulation with autonomic receptor blockade

	Preblock Baseline	Postblock Baseline	C1-C2 SCS	SCS+CAO	1 Hr Reperfusion
			Heart rate, beats/min		
C1- C2 SCS + vehicle	251.9 ± 23.6	257.0 ± 22.3	258.2 ± 22.9*	261.6 ± 22.7*+	260.9 ± 24.9*
C1-C2 SCS + prazosin	243.0 ± 21.4	250.5 ± 24.7	243.3 ± 21.4	246.1 ± 18.6	235.5 ± 25.7+
C1-C2 SCS + yohimbine	250.7 ± 20.3	241.0 ± 23.3	236.9 ± 18.3	238.7 ± 17.3	229.0 ± 21.8*
C1-C2 SCS + atenolol	272.5 ± 17.7	207.7 ± 14.8*#	202.7 ± 16.9*#	206.4 ± 16.3*#	204.5 ± 15.8*#
C1-C2 SCS + atropine	291.5 ± 21.1	299.5 ± 28.7#	301.0 ± 26.6#	291.2 ± 11.1#	289.5 ± 32.1#
			Blood pressure, mmHg		
C1- C2 SCS + vehicle	78.6 ± 10.3	80.9 ± 10.0*	80.4 ± 9.8*	76.8 ± 9.3+	72.6 ± 8.9*+
C1-C2 SCS + prazosin	86.6 ± 8.2	65.7 ± 5.9*#	65.8 ± 4.9*#	65.6 ± 5.0*	66.5 ± 4.8*
C1-C2 SCS + yohimbine	83.7 ± 6.1	76.8 ± 6.4*	79.4 ± 7.2	72.1 ± 1.9*	64.8 ± 7.1*+
C1-C2 SCS + atenolol	94.0 ± 4.2	88.8 ± 4.5*	85.4 ± 6.4*	76.3 ± 9.5*+	70.9 ± 6.0*+
C1-C2 SCS + atropine	89.3 ± 9.6	86.5 ± 9.8	87.2 ± 9.3	77.9 ± 10.9*	77.6 ± 12.6*

within group comparison
* versus pre-block baseline
+ versus postblock baseline

between group comparison
versus all other groups

Heart rate was minimally affected from baseline values during C1-C2 SCS (Tables 2 and 3). Neither was it different during coronary artery occlusion or reperfusion in animals undergoing either C6 dorsal column transection or cervical vagotomy (Table 3). Within group comparisons indicate that C1-C2 SCS by itself had no significant effect on heart rate or blood pressure. In all groups, blood pressure declined slightly from baseline during the C1-C2 SCS + coronary occlusion, with significant reductions from baseline occurring during reperfusion in all three groups (Table 3).

Table 3. Hemodynamic data for pre-emptive C1-C2 neuromodulation with neural ablation

	Baseline	C1-C2 SCS	C1-C2 SCS+CAO	1 Hr Reperfusion
		Heart rate, beats/min		
Intact	257.0 ± 22.3	258.2 ± 22.9	261.6 ± 22.7*	260.9 ± 24.9
C6 Dorsal Column section	258.8 ± 24.9	259.6 ± 25.8	261.1 ± 23.1	260.0 ± 18.9
Cervical vagotomy	270.5 ± 23.7	273.3 ± 20.2	274.2 ± 21.1	275.3 ± 19.0
		Blood pressure, mmHg		
Intact	80.9 ± 10.0	80.4 ± 9.8	76.8 ± 9.3*#	72.6 ± 8.9*#
C6 Dorsal Column section	88.9 ± 6.4	87.5 ± 6.9	84.5 ± 5.2*	82.1 ± 6.0*
Cervical vagotomy	87.5 ± 11.9	85.1 ± 12.0	83.9 ± 10.1	81.8 ± 11.3*

within group comparison
* versus baseline

between group comparison
versus all other groups

Effects of C1-C2 SCS on Infarct size. Body weight and left ventricular risk zones were similar in all experimental groups (data not shown). Figure 2 summarizes infarct sizes (expressed as a percentage of the zone at risk) quantified in rabbits with intact neuroaxis (cord intact) that were subjected to 30-min. periods of regional ischemia in the absence of SCS (Sham SCS) vs. those with pre-emptive C1-C2 SCS. These data were compared to data obtained from similar groups following C6 dorsal column transection 1 hr prior to the onset of SCS-coronary artery occlusion (Protocols 1-3 in Figure 1). There was a significant change in infarct size when comparing different cord status (intact versus C6 dorsal column transaction) vs. the absence or presence of SCS ($p=0.036$) with cord status.

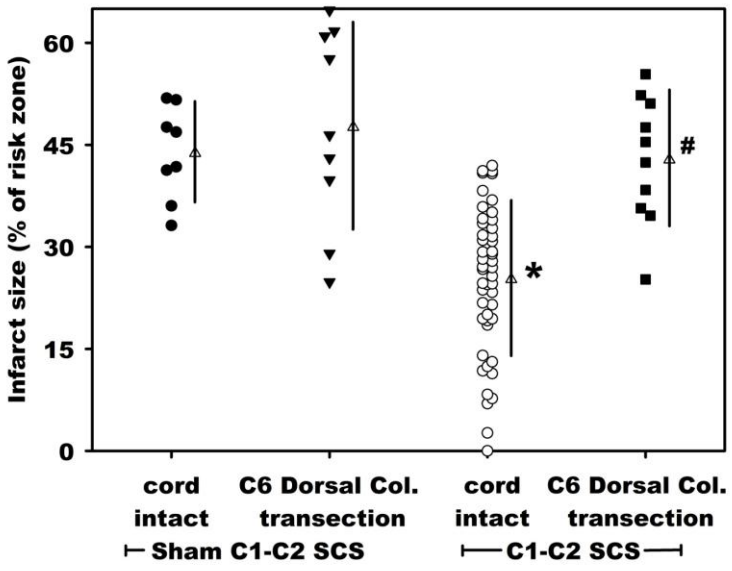

Figure 2. Infarct size (percentage of risk zone) for sham SCS rabbits without (cord intact) and with C6 dorsal column transection compared to rabbits with pre-emptive C1-C2 SCS without (cord intact) and with C6 dorsal column transection. For each group, individual animals are shown along with mean ± SD data for each group (open triangle and vertical bar). # $p<0.05$ for innervations state (cord intact versus C6 dorsal column transection), without (sham) and with pre-emptive C1-C2 SCS. * $p<0.05$ for SCS factor, intact versus C6 dorsal column transection.

In sham animals, ventricular infarct size averaged 43.8 ± 6.9% of tissue volume. This index was unaffected by prior C6 dorsal column transection (47.6 ± 14.7%; Fig. 2, column 2). With the spinal cord intact, pre-emptive C1-C2 SCS decreased infarct size to 25.2 ± 10.7% (Fig. 2, column 3; significant reduction compared to Sham SCS controls). The efficacy of pre-emptive C1-C2 SCS to reduce infarct size in response to the 30 min. ischemic stress was eliminated after C6 dorsal column transection (Fig. 2, right hand columns infarct size = 42.8 ± 9.4%). The ability for pre-emptive C1-

C2 SCS to reduce infarct size was also eliminated by pretreatment with prazosin (40.0 ± 9.4%), atenolol (40.2 ± 11.1%), or yohimbine (36.8 ± 10.3%) (c.f., Fig. 3). In total, these data indicate the ability of pre-emptive C1-C2 SCS to modify sympathetic efferent function via intersegmental projections to the thoracic cord.

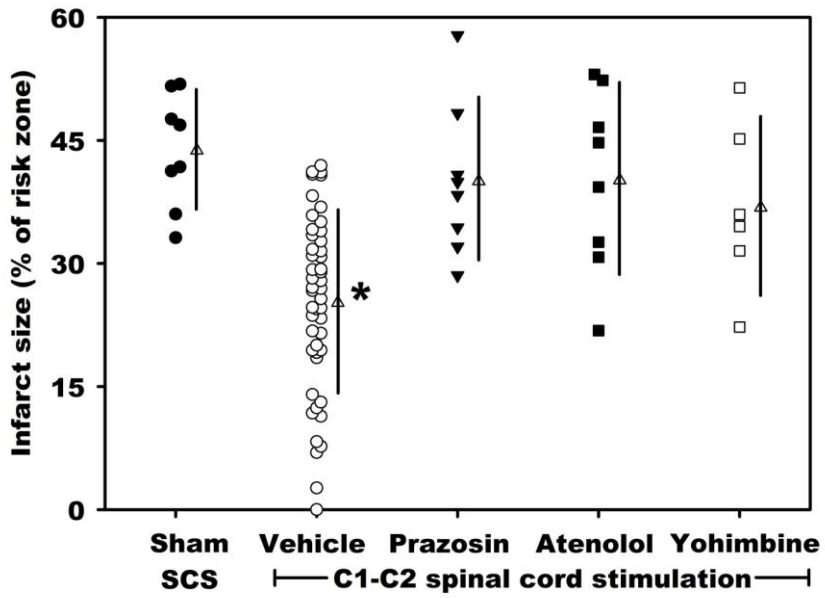

Figure 3. Infarct size (percentage of risk zone) for sham controls compared to rabbits with pre-emptive C1-C2 SCS with intact neuroaxis (vehicle) or following selective adrenergic receptor blockade with α_1 (prazosin), β_1 (atenolol) or α_2 (yohimbine) pretreatment (c.f. Fig. 1, protocol 2). For each group, data for individual animals are shown along with mean ± SD data for each group (open triangle and vertical bar). * $p<0.05$ from all other groups.

Neither atropine nor cervical vagotomy alone (c.f., in the absence of SCS) affected infarct size induced by CAO/ 3 hr reperfusion (Fig. 4, c.f., middle columns of both groups). Moreover, the capacity for pre-emptive C1-C2 SCS to reduce infarct size was unaffected by cervical vagotomy (23.6 ± 11.1%) compared to vehicle control (25.2 ± 10.7%) (Fig. 4). In contrast, pretreatment with atropine 15 min. prior to the onset of pre-emptive SCS *(protocol 2)* eliminated its infarct reducing effects (51.6 ± 8.6%).

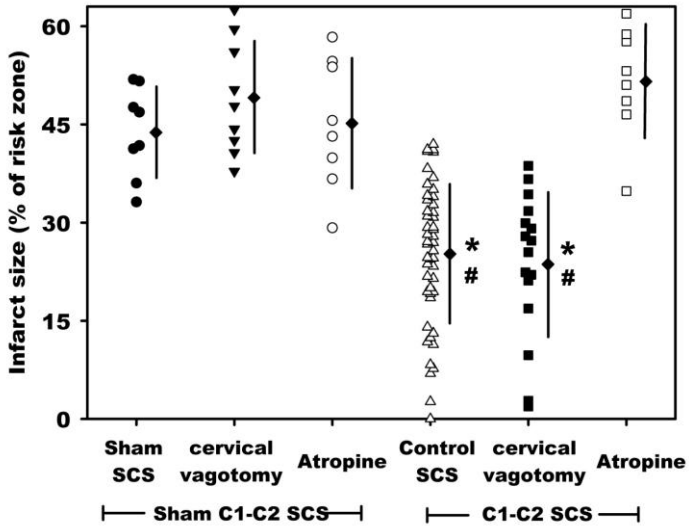

Figure 4. Infarct size (percentage of risk zone) in sham controls compared to that of rabbits pretreated with the muscarinic blocker atropine or cervical vagotomy, as compared to animals exposed to pre-emptive C1-C2 SCS with 1) intact neuraxis (control SCS), 2) following cervical vagotomy, or 3) muscarinic receptor blockade (atropine). For each group, individual animal data are shown along with mean ± SD data for each group (closed diamond and vertical bar). # $p<0.05$ for treatment (none, cervical vagotomy or atropine) with and without pre-emptive C1-C2 SCS. * $p<0.05$ (with pre-emptive C1-C2 SCS) for atropine pre-treatment versus control or cervical vagotomy.

Effects of C1-C2 SCS on ventricular arrhythmogenic potential. Ventricular fibrillation secondary to myocardial ischemia occurred in 22% of control animals (sham SCS) with 11% experiencing sudden cardiac death (SCD) (Table 4). Across all groups, VF occurred preferentially in the first ½ of the ischemic insult with no stratification with respect to those that showed spontaneous conversion back to sinus rhythm versus those that did not recover, e.g. SCD. Incidence of CAO-induced VF increased to 36% in animals with prior vagotomy or C6 dorsal column transection, with incidence of SCD of 18% for both groups (Table 4). Pretreatment with atropine showed similar responses to control.

Table 4. Neuromodulation and the arrhythmogenic potential to Ventricular Fibrillation (VF)

	# ANIMALS	# VF	VF (SCD)	VF (spontaneous conversion)	# VF in CAO	# VF reper	% VF	% VF-SCD
Sham SCS	9	2	1	1	2	0	22.2%	11.1%
DCNx + Sham SCS	11	4	2	2	4	0	36.4%	18.2%
Vagotomy + Sham SCS	11	4	2	2	3	1	36.4%	18.2%
Atropine + Sham SCS	9	2	1	1	2	0	22.2%	11.1%
SCS	54	11	6	5	10	1	20.4%	11.1%
DCNx + SCS	15	5	5	0	4	1	33.3%	33.3%
Vagotomy + SCS	16	0	0	0	0	0	0.0%	0.0%
Prazosin + SCS	11	4	3	1	4	0	36.4%	27.3%
Atenolol + SCS	8	0	0	0	0	0	0.0%	0.0%
Yohimbine + SCS	12	11	6	5	10	1	91.7%	50.0%
Atropine + SCS	9	3	1	2	3	0	33.3%	11.1%

SCD: Sudden cardiac death
DCNx: C6 dorsal column transection
CAO: Coronary artery occlusion
reper: reperfusion phase post-CAO

In animals with C1-C2 SCS, pretreatment with cholinergic or adrenergic antagonists, along with selective neuroablation, modified the incidence of CAO-induced VF and subsequent events of SCD (Table 4). C1-C2 SCS by itself did not alter the incidence of either VF or SCD. Prior C6 dorsal column transection however, increased the lethality of CAO. In contradistinction to sham SCS animals, prior cervical vagotomy completely eliminated the incidence of CAO-induced VF. Such was not case with atropine pretreatment where CAO applied during SCS resulted in a 33% CAO-induced VF with 11% subsequent SCD. Pretreatment with alpha adrenergic blockade was associated with substantial increases in events, especially following α_2 blockade, with the incidence of CAO-induced VF increasing to 92%; 50% of such animals having SCD. In contrast, prior treatment with the β1 adrenergic selective blockade (atenolol) completely eliminated CAO-induced VF. These data point to the dynamic interactions between adrenergic and cholinergic control of cardiac electrical stability in ischemic stress.

Effects of C1-C2 SCS on thoracic dorsal horn activity. The effects of CAO on cFos-immunoreactivity in the dorsal horns (Laminae I-V) of the T_2 spinal cord were examined to determine how spinal cord neurons transduce the effect of CAO in the absence and presence of C1-C2 SCS (Figure 5). Coronary artery occlusion (CAO) increased activation of the laminae I-V neurons in the high thoracic spinal cord. CAO increased this index from 7.19±3.50 Fos-positive cells per 200 μm^2 in control states to 19.36±5.46 cells per 200 μm^2. C1-C2 SCS alone increased this activation index in dorsal horn neurons of the high thoracic spinal cord to a lesser degree when compared to control states (9.73±4.82 cells per 200 μm^2). In the presence of C1-C2 SCS, CAO-induced activation of dorsal horn neurons was reduced when compared to animals subjected to CAO alone (3.18±1.83 cells per 200 μm^2; p=0.028).

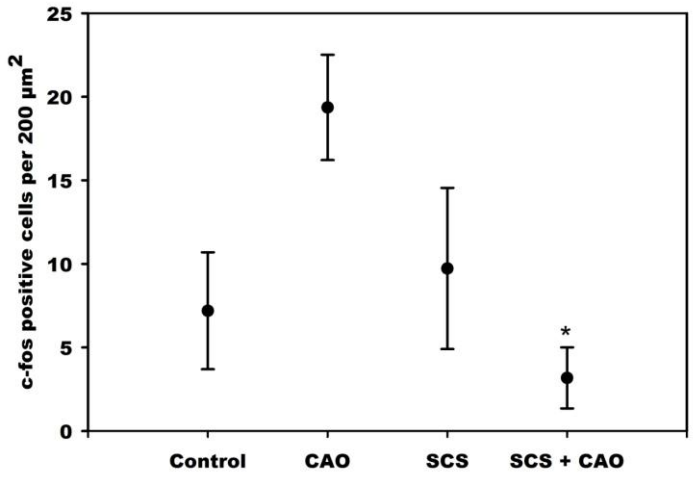

Figure 5. Activation of neurons in the thoracic spinal cord in response to ischemia. C-fos expression was measured in dorsal horns (laminae I-V) of the upper thoracic spinal cord. Coronary artery occlusion (CAO) caused an increase in the activation of spinal neurons. Following SCS, this increase was lessened indicating reduction in cardiac afferent neuronal activation. SCS applied prior to and during CAO caused a greater (significant) reduction in dorsal horn spinal neuronal activation. * indicates $p<0.05$ using two-way ANOVA with the Holm-Sidak post hoc comparison.

Discussion

This study demonstrates for the first time that pre-emptive electrical neuromodulation therapy applied to the cranial cervical spinal cord reduces the size of infarcts induced by transient myocardial ischemia. It further indicates that disruption of intersegmental communication from C1-C2 to T1-T4 cord neurons, by transection of the C6 dorsal column, prevents the infarct reducing effect of C1-C2 electrical stimulation. Thus, a necessary difference in control is exerted by thoracic cord neurons (Foreman *et al.*, 2004; Foreman *et al.*, 2000; Wu *et al.*, 2008) versus cervical neurons, given the potential of high cervical cord neurons to modify not only thoracic cardiac efferent adrenergic neuronal outputs, but also vagal neuronal outflows to modulate infarct size in response to the regional ischemia (Fig. 3).

Although cervical vagotomy did not affect the capacity of pre-emptive high cervical SCS to modify infarct size, this does not preclude some involvement of peripheral cardiac cholinergic neurons in these events since atropinization completely abolished the capacity of C1-C2 SCS to reduce infarct size. Cervical vagotomy decentralizes parasympathetic inputs to the intrinsic cardiac

nervous system (ICN) but still allows for spontaneous neuronal activity and intracardiac reflex function (Ardell *et al.*, 1991), whereas atropine interferes with both end-organ and neuronal processing within the ICN (Armour, 2008). In other words, high cervical SCS affects ventricular electrical stability via both sympathetic and parasympathetic efferent neurons.

There appears to be concurrent influences of alpha- and beta-adrenoceptor neurons in such events since blockade of either receptor subgroup elicited diametrically opposed responses on the incidence of sudden cardiac death. While β_1-adrenoceptor blockade mitigated the potential for sudden cardiac death, α_1- and α_2-adrenoceptor blockade enhanced (doubled or tripled, respectively) that potential. In contrast, following cervical vagotomy while coronary artery occlusion alone was associated with an increased incidence of sudden cardiac death, concurrent high cervical SCS (post vagotomy) completely abolished ventricular fibrillation initiated during transient myocardial ischemia.

With regards to neural responses to cardiac stress, electrical stimulation of the C1-C2 dorsal column region is known to decrease the activity of spinothalamic tract neurons receiving cardiac nociceptive sensitive inputs (Chandler *et al.*, 1993; Foreman *et al.*, 2004). It also decreases myocardial ischemia evoked dorsal horn neuronal activation (Ding *et al.*, 2008a; Ding *et al.*, 2008b) and substance P release from ischemia sensitive cardiac afferent neurons, while recruiting thoracic spinal sympathetic neurons (Ding *et al.*, 2008a; Ding *et al.*, 2008b).

Cranial cervical cord information is transmitted to the lower segments of the spinal cord via the dorsal columns, propriospinal pathways and/or supraspinal loops. C1-C2 SCS does not activate supraspinal neurons, including those in brainstem nucleus tractus solitaries (Ding *et al.*, 2008a; Ding *et al.*, 2008b; Qin *et al.*, 2007). In fact, C1-C2 SCS or chemically-activated C1-C2 propriospinal neurons suppress the responses of upper thoracic spinal neurons to noxious cardiac afferent input

(Qin *et al.*, 2004; Qin *et al.*, 2008). C1-C2 SCS also inhibits substance P release from activated ischemia sensitive cardiac afferent neurons (Ding *et al.*, 2008b; Ding *et al.*, 2008a). Thus, caudal cervical dorsal column transection mitigates cranial cervical-mediated processing of noxious visceral afferent inputs (Qin *et al.*, 2007). These results are in accord with the finding that C6 dorsal columns transection mitigates C1-C2 SCS ventricular infarct size reduction secondary to transient myocardial ischemia. Such data indicate that spinal cord propriospinal descending projections or antidromic activation of ascending fibers in the dorsal column influence high cervical inputs to the thoracic spinal cord neurons.

Cervical cord neuromodulation of ischemia-induced myocardial infarction. Adrenergic receptors modulate both cardiomyocytes and neurons that regulate them (Armour, 2008; Ardell, 2004). Thus, infarct size can be influenced via α-receptor coupled PKC pathways as well as β-adenoreceptor coupled PKA and p38 MAPK pathways (Yellon & Downey, 2003; Tsuchida *et al.*, 1994; Sanada & Kitakaze, 2004). In fact, high thoracic SCS activates cardiomyocyte PKC (Southerland *et al.*, 2007). Furthermore, the capacity of C1-C2 SCS to reduce infarct size can be eliminated by α or β_1 adrenergic blockade. In other words, intersegmental spinal cord interactions may impart cardioprotection, in part, via cardiac adrenergic efferent neurons.

In the current study, cervical vagotomy did not change C1-C2 SCS-mediated reduction in infarct size while atropine eliminated it. Cholinergic receptors influence cardiomyocytes directly and indirectly via the cardiac neuroaxis (Armour, 2008; Ardell, 2004). The minimal impact of vagotomy on C1-C2 SCS-induced infarct size reduction may reflect at least three factors: 1) the lack of contribution of C1-C2 SCS evoked activity on supraspinal projections to the nucleus tractus solitaries (Ding *et al.*, 2008a; Ding *et al.*, 2008b), thereby minimally effecting parasympathetic efferent neuronal outflow to the heart; 2) maintenance of parasympathetic efferent neuronal outflow changes

from the intrinsic cardiac nervous system post-cervical vagotomy (Ardell *et al.*, 1991; Huang *et al.*, 1993; Murphy *et al.*, 2000); and 3) the function of intrathoracic reflexes, including those on the heart (Foreman *et al.*, 2000; Armour *et al.*, 2002; Ardell *et al.*, 2009).

Neuromodulation therapy and arrhythmias. These data provide important insights into putative interactions that occur among peripheral and central aspects of cardiac neuroaxis and its impact on electrical stability of the ischemic heart. Pre-emptive thoracic SCS is known to impact afferent (Foreman *et al.*, 2004; Chandler *et al.*, 1993; Ding *et al.*, 2008a; Ding *et al.*, 2008b), efferent (Southerland *et al.*, 2007; Olgin *et al.*, 2002; Ardell *et al.*, 2009) and local circuit neuronal (Cardinal *et al.*, 2006; Armour *et al.*, 2002; Ardell *et al.*, 2009) processing within brainstem, spinal cord, and peripheral nodes of the cardiac neuroaxis. When specific elements of this neuronal hierarchy for cardiac control are altered, control of cardiac function may become compromised (Armour, 2008). For instance, the increased incidence of myocardial ischemia-induced sudden cardiac death identified following C6 dorsal column transection may reflect a loss of descending inhibitory restraints on spinal cord sympathoexcitatory reflexes (Ardell *et al.*, 1982). Correspondingly, the increase in myocardial ischemia-induced sudden cardiac death post α_2-adrenoceptor blockade may be reflective of imbalances in neurotransmitter release secondary to loss of negative feedback on neurotransmitter release from autonomic efferent postganglionic neurons (Xu & Adams, 1993; Adams & Cuevas, 2004). With respect to α_1-adrenoceptor blockade and a resultant loss of background excitation of subpopulations of intrinsic cardiac neurons (Armour, 1997; Ishibashi *et al.*, 2003; Richer *et al.*, 2008), subsequent reflex activation derived from ischemia-sensitive afferent neuronal inputs may asymmetrically modify efferent neuronal outputs such that any arrhythmogenic substrate becomes enhanced.

The abolition of myocardial ischemia-induced sudden cardiac death post-vagotomy in the presence of high cervical SCS seems to be counter-intuitive to the documented anti-arrhythmogenic effects of enhanced parasympathetic efferent neuronal activity (Vanoli *et al.*, 2008; Schwartz, 2001). It should be noted that imbalances in cardiac sympathetic and parasympathetic control initiated by ischemia can be mitigated by: 1) interrupting parasympathetic preganglionic neuronal inputs (post-vagotomy) (Huang *et al.*, 1993); and 2) the stabilizing effects of SCS on the intrinsic cardiac nervous system (Armour *et al.*, 2002; Foreman *et al.*, 2000).

Fos expression is an indicator of cellular activation in the spinal cord as manifested, in this instance by cardiac afferent neuronal activation secondary to cardiac ischemia transduction (Hua *et al.*, 2004; Ding *et al.*, 2008a). Ischemia activates populations of the cardiac afferent neurons, as indicated by increased Fos expression in the high thoracic spinal cord dorsal horn, specifically laminae I-V, as reported in other studies (Hua *et al.*, 2004). Such afferent neuronal activation to the spinal cord, secondary to myocardial ischemia transduction, also occurs following application of noxious stimuli to afferent neuronal inputs to lower thoracic and lumbar spine (Qin *et al.*, 2007). Myocardial ischemia activation of cardiac afferent neuronal inputs to the spinal cord and higher centers (Hua *et al.*, 2004; Ding *et al.*, 2008a) may initiate deleterious reflexes (Hua *et al.*, 2004; Ding *et al.*, 2008a), thereby indirectly modifying cardiac function. Any ability to moderate such reflexes may moderate such adverse effects.

Neuromodulation of the spinal cord at a level higher (C1-C2 SCS) than the incoming cardiac sympathetic afferent neuronal inputs (T1-T5) demonstrates that intersegmental interactions that sub-serve reflex processing can occur within and between various levels of the spinal cord. Thus, these data support the thesis that high cervical spinal segments of the spinal cord may not only act as a coordinating center for visceral organ afferent inputs traveling centrally but also display a capacity

to modify sympathetic efferent control of visceral organs (Qin *et al.*, 2007; Ding *et al.*, 2008b; Ding *et al.*, 2008a).

Perspectives and significance

High cervical SCS stabilizes reflex processing within the cardiac neuroaxis to reduce infarct size and the potential for ischemia-induced sudden cardiac death, doing so via modulating not only efferent adrenergic outflow, but also cholinergic outflow to the heart (Fig. 6). These data also indicate that cervical SCS neuromodulation therapy targets multiple nodes of the cardiac neuroaxis, namely both sympathetic and parasympathetic efferent outflows to the heart. That C6 dorsal column transection and cervical vagotomy exerted differing effects on myocyte viability post-ischemia or even evoked sudden cardiac death point to the importance of understanding the multiple interactions existing within the various nodes of the cardiac neuroaxis. This was made evident by the fact that β-adrenergic blockade, while reducing the risk for myocardial ischemia-induced sudden cardiac death, obtunded any infarct size reduction capability of SCS. It is only by understanding the interactions that occur among peripheral and central nodes of the cardiac neuroaxis that appropriate therapies targeting select neuroaxis components can evolve.

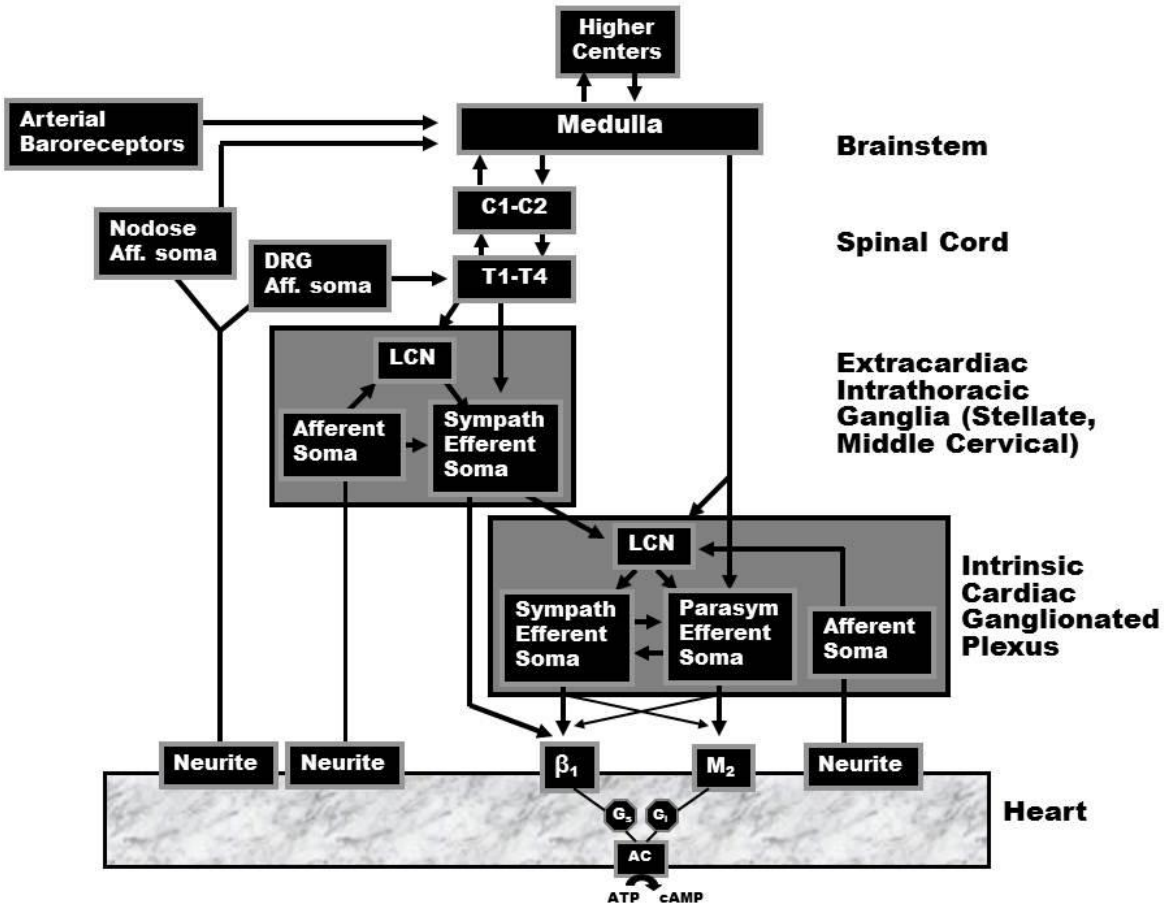

Figure 6. Schematic of proposed interactions that occur within and among intrathoracic autonomic neurons and between them and central neurons. Intrinsic cardiac ganglia possess afferent neurons, sympathetic (Sympath) and parasympathetic (Parasym) efferent neurons and interconnecting local circuit neurons (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. Symbols: Aff., afferent; DRG, dorsal root ganglia; G_s, stimulatory guanine nucleotide binding protein; G_i, inhibitory guanine nucleotide binding protein; AC, adenylate cyclase; beta-$_1$ adrenergic receptor; M_2- muscarinic receptor.

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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.

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, Barman SM, & Gebber GL (1982). Sympathetic nerve discharge in chronic spinal cat. *Am J Physiol* **243**, H1463-H1470.

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.

Armour JA (1997). Intrinsic cardiac neurons involved in cardiac regulation possess α1, α2, β1 and β2-adrenoceptors. *Can J Cardiol* **13**, 277-284.

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

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.

Cardinal R, Ardell JL, Linderoth B, Vermeulen M, Foreman RD, & Armour JA (2004). Spinal cord activation differentially modulates ischemic electrical responses to different stressors in canine ventricles. *Autonomic Neuroscience: Basic and Clinical* **111**, 37-47.

Cardinal R, Pagé PL, Vermeulen M, Bouchard C, Ardell JL, Foreman RD, & Armour JA (2006). Spinal cord stimulation suppresses bradycardias and atrial tachyarrhythmias induced by mediastinal nerve stimulation in dogs. *Am J Physiol Regulatory Integrative Comp Physiol* **291**, R1369-R1375.

Chandler MJ, Brennan TJ, Garrison DW, Kim KS, Schwartz PJ, & Foreman RD (1993). A mechanism of cardiac pain suppression by spinal cord stimulation: implications for patients with angina pectoris. *Eur Heart J* **14**, 96-105.

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.

Ding XH, Ardell JL, Hua F, McAuley RJ, Sutherly K, Daniel JJ, & Williams CA (2008a). Modulation of cardiac ischemia-sensitive afferent neuron signaling by preemptive C2 spinal cord stimulation: effect on substance P release. *Am J Physiol Regulatory Integrative Comp Physiol* **294**, R93-R101.

Ding XH, Hau F, Sutherly K, Ardell JL, & Williams CA (2008b). C2 spinal cord stimulation induces dynorphin release from the rat T4 spinal cord: potential modulation of ischemia-sensitive neurons. *Am J Physiol Regulatory Integrative Comp Physiol* **295**, R1519-R1528.

Eddicks S, Maier-Hauff K, Schenk M, Müller A, Baumann G, & Theres H (2007). Thoracic spinal cord stimulation improves functional status and relieves symptoms in patients with refractory angina pectoris: the first placebo-controlled randomized study. *Heart* **93**, 585-590.

Foreman RD, DeJongste MJL, & Linderoth B (2004). Integrative control of cardiac function by cervical and thoracic spinal neurons. In *Basic and Clinical Neurocardiology*, eds. Armour JA & Ardell JL, pp. 153-186. Oxford Univ. Press, NY.

Foreman RD, Linderoth B, Ardell JL, Barron KW, Chandler MJ, Hull SS, TerHorst GJ, DeJongste MJL, & Armour JA (2000). Modulation of intrinsic cardiac neurons by spinal cord stimulation: implications for therapeutic use in angina pectoris. *Cardiov Res* **47**, 367-375.

González-Darder JM, Canela P, & González-Martinez V (1991). High cervical spinal cord stimulation for unstable angina pectoris. *Stereotact Funct Neurosurg* **56**, 20-27.

Hankes GH, Ardell JL, Tallaj J, Wei CC, Aban I, Holland M, Rynders P, Dillon AR, Cardinal R, Hoover DB, Armour JA, Husain A, & Dell'Italia LJ (2006). β1-Adrenoceptor blockade mitigates excessive norepinephrine release into cardiac interstitium in mitral regurgitation in dog. *Am J Physiol Heart Circ Physiol* **291**, H1-H5.

Hautvast RW, DeJongste MJL, Staal MJ, Van Gilst VH, & Lie KI (1998). Spinal cord stimulation in chronic intractable angina pectoris: a randomized, controlled efficacy study. *Am Heart J* **136**, 114-120.

Hua F, Harrison R, Qin C, Reifsteck A, Ricketts B, Carnel C, & Williams CA (2004). c-Fos expression in rat brainstem and spinal cord in response to activation of cardiac ischemic-sensitive afferent neurons and electrostimulatory modulation. *Am J Physiol Heart Circ Physiol* **287**, H2728-H2738.

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.

Ishibashi H, Umezu M, Jang IS, Ito Y, & Akaike N (2003). α_1-Adrenoceptor-activated cation currents in neurons acutely isolated from rat cardiac parasympathetic ganglia. *J Physiol* **548**, 111-120.

Kajstura J, Bolli R, Sonnenblick EH, Anversa P, & Leri A (2006). Cause of death: suicide. *J Mol Cell Cardiol* **40**, 425-437.

Mannheimer C, Camici P, Chester MR, Collins A, DeJongste MJL, Eliasson T, Follath F, Hellemans I, Herlitz J, Lüscher T, Pasic M, & Thelle D (2002). The problem of chronic refractory angina. Report from the ESC joint study group on the treatment of refractory angina. *Eur Heart J* **23**, 355-370.

Maxwell M, Hearse D, & Yellon D (1987). Species variation in the coronary collateral circulation during regional myocardial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction. *Cardiov Res* **21**, 737-746.

Murphy DA, Thompson GW, Ardell JL, McCraty R, Stevenson R, Sangalang VE, Cardinal R, Wilkinson M, Craig S, Smith FM, Kingma JG, & Armour JA (2000). The heart reinnervates after transplant. *Ann Thorac Surg* **69**, 1769-1781.

Odenstedt J, Linderoth B, Bergfeldt L, Erke O, Grip L, Mannheimer C, & Andrell P (2011). Spinal cord stimulation effects on myocardial ischemia, infarct size, ventricular arrhythmia, and noninvasive electrophysiology in a porcine ischemia-reperfusion model. *Heart Rhythm* **8**, 892-898.

Olgin JE, Takahashi T, Wilson E, Vereckei A, Steinberg H, & Zipes DP (2002). Effects of thoracic spinal cord stimulation on cardiac autonomic regulation of the sinus and atrioventricular nodes. *J Cardiov Electrophysiol* **13**, 475-481.

Perry GJ, Wei CC, Hankes GH, Dillon SR, Rynders P, Mukherjee R, Spinale FG, & Dell'Italia LJ (2002). Angiotensin II receptor blockade does not improve left ventricular function and remodeling in subacute mitral regurgitation in the dog. *J Am Coll Cardiol* **39**, 1374-1379.

Qin C, Farber JP, Linderoth B, Shahid A, & Foreman RD (2008). Neuromodulation of thoracic intraspinal visceroreceptive transmission by electrical stimulation of spinal cord dorsal column and somatic afferents in rats. *J of Pain* **9**, 71-78.

Qin C, Kranenburg A, & Foreman RD (2004). Descending modulation of thoracic visceroreceptive transmission by C1-C2 spinal neurons. *Autonomic Neuroscience: Basic and Clinical* **11**.

Qin C, Lehew RT, Khan KA, Wienecke GM, & Foreman RD (2007). Spinal cord stimulation modulates intraspinal colorectal visceroreceptive transmission in rats. *Neurosci Res* **58**, 58-66.

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

Sanada S & Kitakaze M (2004). Ischemic preconditioning: emerging evidence, controversy, and translational trials. *International J Cardiol* **97**, 263-276.

Schwartz PJ (2001). QT prolongation, sudden cardiac death, and sympathetic imbalance: the pendulum swings. *J Cardiov Electrophysiol* **12**, 1074-1077.

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.

Tallaj J, Wei CC, Hankes GH, Holland M, Rynders P, Dillon AR, Ardell JL, Armour JA, Lucchesi PA, & Dell'Italia LJ (2003). β1-adrenergic receptor blockade attenuates angiotensin II-mediated catecholamine release into the cardiac interstitium in mitral regurgitation. *Circ* **108**, 225-230.

Tsuchida A, Liu Y, Liu GS, Cohen MV, & Downey JM (1994). α1-adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C. *Circ Res* **75**, 576-585.

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.

Wu M, Linderoth B, & Foreman RD (2008). Putative mechanisms behind effects of spinal cord stimulation on vascular diseases: A review of experimental studies. *Autonomic Neuroscience: Basic and Clinical* **138**, 9-23.

Xu ZJ & Adams DJ (1993). α-adrenergic modulation of ionic currents in cultured parasympathetic neurons from rat intracardiac ganglia. *J Neurophysiol* **69**, 1060-1070.

Yellon D & Downey JM (2003). Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev* **83**, 1113-1151.

CHAPTER 3

Spinal cord stimulation mitigates cardiac afferent neuronal transduction of myocardial ischemia without antidromic activation

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Key words: dorsal root ganglia; myocardial ischemia; nodose ganglia; cardiac sensory neurons; spinal cord stimulation; ventricle

Abstract

This study evaluated whether electrical neuromodulation therapy applied to the rostral thoracic spinal cord affects the capacity of cardiac first order afferent neurons to transduce myocardial ischemia. We recorded the transduction characteristics of left ventricular primary afferent neuronal somata in nodose and dorsal root ganglia (DRG) *in situ* in response to exposing their sensory neurite fields to chemical stimuli versus regional ventricular ischemia (transient left anterior descending [LAD] coronary artery occlusion). Epicardially applied veratridine activated both sensory populations, as did transient ventricular ischemia. After 20 min. of spinal cord stimulation (SCS: 50 Hz, 200 ms duration, 90% motor threshold applied to dorsal columns of T1-T3 spinal segments) the ischemia-induced depression of left ventricular chamber dp/dt was mitigated. SCS likewise attenuated the capacity of both sensory populations to transduce local ischemia. Yet, SCS did not directly (antidromically) activate somata of either population. It is concluded that while SCS does attenuate ischemia-induced enhancement of nodose and dorsal root ganglion ventricular sensory neurons, this is reflective of an altered milieu surrounding their neurites rather than direct (antidromic) somata effects. These data further imply that while such neuromodulation therapy can alter cardiac afferent response to transient MI, such alterations are secondary to changes in the transduction of cardiac milieu rather than suppression of the afferents' capacity to transduce that milieu.

Introduction

A pressing issue with respect to spinal cord stimulation therapy (SCS) in the treatment of myocardial ischemia (MI) remains whether if such therapy obtunds the capacity of cardiac primary afferent neurons to transduce MI (Foreman and others 2004; Mannheimer and others 2002). If blunting of the transduction capabilities of the cardiac neuroaxis to transduce an ischemic event does indeed occur, that might negate the clinical usefulness of SCS because the patient would no longer become aware of critical ischemic episodes (Armour 2008). Cardiac afferent neurons, distributed throughout nodose and cranial thoracic dorsal root ganglia (DRG), transduce a constantly changing cardiac mechanical and chemical milieu (Armour 1999; 2004). The neurites of cardiac afferent neurons are activated by a number of chemicals, including those known to be released from the ischemic myocardium: c.f., adenosine and peptides such as bradykinin and substance P (Armour and Kember 2004). It is well recognized that nodose and DRG cardiac afferent neurons exert major influences via the cardiac neuroaxis on regional cardiac dynamics via reflex modification of both sympathetic and parasympathetic efferent outflows (Ardell 2004; Armour 1999; 2004).

Neuromodulation applied to select nodes of the cardiac neuroaxis (e.g. SCS applied at T1-T3 spinal levels) is known to mitigate excessive MI-induced reflex activation of extracardiac (Ardell and others 2009) and intrinsic cardiac neurons (Foreman and others 2000). SCS does so without interrupting direct autonomic efferent projections to the heart (Cardinal and others 2006). It has been proposed that SCS modulates sensory transduction of the ischemic myocardium to neurons in the thoracic spinal cord dorsal horn (Qin and others 2008). SCS has been demonstrated to modulate the response of second order neurons within the spinal cord to ischemia (Qin and others 2008). Conversely, recent evidence has suggested that SCS directly activates DRG afferent neurons

to increase their release of neurotransmitters into peripheral tissues from their sensory neurites, imposing an efferent function on afferent neurons (Linderoth and others 1994). In fact, it has been proposed that this afferent/efferent function could be a primary mechanism whereby SCS exerts its clinical benefits to the cardiac patient (Linderoth and others 1994). However to date, the effects of SCS on first order cardiac afferent neurons has not been assessed.

To evaluate whether such SCS neuromodulation therapy does indeed blunt a patient's capacity to sense an ischemic event, we sought to determine: 1) whether SCS alters the capacity of first order cardiac sensory neurons to transduce MI and, if that is the case, 2) whether that occurs via direct antidromic modification of first order afferent neuronal somata or 3) indirectly by altering the milieu surrounding their cardiac sensory neurites.

Materials and Methods

Animal preparation: Mongrel dogs (n=41; either sex), weighing 17.1-28.0 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 Animal and Human Beings" (Society 2002). The Institutional Animal Care and Use Committee of the East Tennessee State University approved these experiments.

Animals were pre-medicated with sodium thiopental (25 mg/kg, i.v., supplemented as needed), intubated and maintained under positive-pressure ventilation. Isoflurane (2%) was used as the anesthetic agent during surgery. Following surgery, the anesthesia was switched to α-chloralose (75 mg/kg i.v. bolus, with repeat doses of 12.5 mg/kg i.v., as required). Heart rate and blood pressure were continuously monitored with depth of anesthesia determined by monitoring corneal

reflex, jaw tone, and hemodynamic parameters. Body temperature was maintained via a circulating water heating pad (Gaymar T/Pump, Gaymar Industries Inc., Orchard Park, NY).

The left femoral artery and vein were catheterized to monitor blood pressure and deliver anesthetic agents throughout the experiment. The right femoral artery was catheterized and a Mikro-Tip Pressure Transducer Catheter (Millar Instruments, Houston, TX) was advanced into the left ventricular (LV) chamber. Heart rate was monitored via a Lead II electrocardiogram. These indices, along with concurrent afferent neuronal activity (see below), were digitalized (Power 1401, Cambridge Electronic Design, Cambridge, England), stored and analyzed offline by the Spike2 (Cambridge Electronic Design, Cambridge, England).

Spinal cord stimulation (SCS): Two different protocols for SCS electrode placement were used, depending on the site of afferent recording. With respect to experiments for recording nodose activity, animals were first placed in the prone position and the spinal epidural space was penetrated percutaneously with a Touhy needle through a small skin incision in the T_6 spinal level. An eight-pole lead (Octrode, Advanced Neuromodulation Systems, Plano, TX) was advanced rostrally in the epidural space to the T_1-T_3 spinal cord level. The tip of the lead was positioned slightly to the left of midline under fluoroscopy. Consistent with current clinical practice (Augustinsson and others 1995), the rostral pole was positioned at T_1 and the caudal pole was positioned at T_3 level. With respect to DRG neuronal activity recordings, a laminectomy was performed removing the dorsal spinal processes of the C8-T6 vertebrae and the eight-pole lead was laid directly on the dorsal aspect of the T_1-T_3 spinal cord. In both cases, proper electrode placement was determined by delivering electrical current to the spinal cord dorsal horn via the rostral (cathode) and caudal (anode) poles of the electrode using a PSIU6 constant current isolation unit (Grass Instruments, Quincy, MA) connected to a Grass S88 stimulator (Grass Instruments, Quincy, MA). Motor threshold (MT)

intensity was determined as the lowest current that induced muscle contractions in the proximal forepaw and shoulder. For nodose ganglion recordings, following SCS electrode implantation the animals were rotated to the supine position, with MT being rechecked in this position. In each experimental protocol, SCS was delivered for 20 min. at 50 Hz, 200 μs duration and at a current intensity of 90% MT (range 0.25-2.8 mA, mean 0.98±0.19 mA). MT was checked periodically during the experiments. MT did not vary significantly from initial levels throughout each experiment.

Afferent neuronal activity recording: Activity generated by neuronal somata in the nodose ganglia of 20 animals vs. neuronal somata in the DRGs in 21 animals was recorded *in situ*, using methods as reported previously (Gagliardi and others 1988). Briefly, for nodose ganglion recordings, the animals were placed in the prone position and an incision was made in the ventral neck to expose the nodose ganglia. The tissue surrounding the nodose ganglia was left intact to stabilize it during prolonged recording of the activity generated by neuronal somata therein. With respect to recording DRG somata activity, animals were placed in a supine position and after laminectomy (C8-T6) the T1-T4 DRG ganglia were exposed.

For either DRG or nodose neurons, a tungsten microelectrode (250 μm diameter and exposed tip of 1 μm; impedance of 9-11 MΩ at 1000 Hz), mounted on a micromanipulator, was lowered into respective ganglia using a microdrive. Each ganglion was explored and electrical signals so derived were led into a differential preamplifier (BMA-831, CWE Inc., Ardmore, PA) with a high impedance head stage (band width set at 300 Hz to 10 kHz). Signals were further amplified by a battery-driven pre-amplifier (5113 Pre-Amp, Signal Recovery, Oak Ridge, TN) (band width 100 Hz to 2 kHz). Amplified neuronal signals, together with recorded cardiovascular indices, were digitized

(Cambridge Electronics Design, power 1401 data acquisition system), stored, and analyzed using the Spike 2 software package (Cambridge Electronics Design).

Neuronal activity was identified as action potentials with signal to noise ratios greater than 3:1. The activity generated by individual neuronal somata in either the nodose or DRG ganglia was identified by the amplitude and configuration (waveform recognition) of these recorded action potentials using the Spike 2 program. Using these techniques and criteria, action potentials generated by individual somata and/or dendrites, rather than axons of passage, can be recorded for extended periods of time (Gagliardi and others 1988).

Protocols employed: Gauze soaked in solutions of the following chemicals: veratridine (100 μM), bradykinin (100 μM), adenosine (100 μM), or substance P (10 μM) were first applied to the surface of the ventral left ventricle to test whether recorded nodose or DRG soma transduced cardiac stimuli. After waiting 1 min., the chemical-soaked pledge was removed and the pericardium flushed with normal saline. If none of the DRG or nodose ganglion neurons were activated by these chemicals, the microelectrode was moved until an active site was identified that consistently responded to chemical activation of the LV sensory neurites. Once such a subset of nodose or DRG neurons was identified, the left anterior descending (LAD) coronary artery was occluded for 1 or 2 min by means of a silk ligature snare placed around that vessel at the level of its first diagonal branch. If coronary artery occlusion (CAO) did not evoke changes in primary afferent activity, the electrode was moved to another recording site. Once a population of LV afferent neurons was identified which responded first to chemicals and then to LAD CAO, animals were randomized to receive either pre-emptive SCS (50 Hz, 200 μs, 90% MT) for 20 min or sham SCS for 20 min and the LAD CAO stress repeated. In a separate group of 3 animals with DRG recordings, the dorsal roots were sectioned between identified ganglia and the spinal cord; all other DRG were left intact.

Thereafter, the capacity of neuronal somata therein to transduce myocardial ischemia was studied before and after SCS.

Suprathreshold SCS: In a subset of animals (n=5), we evaluated the potential of spinal cord stimulation to modify identified DRG neural activity antidromically. A short-acting competitive neuromuscular blocker, rocuronium bromide (1 mg/kg; time to onset 2-4 min. with clinical duration of action of 20 min. (Sparr and others 2001)), was used to temporarily prevent spinal cord electrically induced motor movement. For these experiments, the frequency of spinal cord stimulation ranged from 0.1 to 5 Hz and the voltage employed ranged from motor threshold to 2.5x motor threshold. For these experiments, alpha chloralose (8-12 mg/kg) was temporarily increased prior to neuromuscular blockade and maintained at that level during subsequent supra-motor threshold stimuli.

Data analysis: Action potentials arising from a nodose or dorsal root ganglion site were characterized by means of differences in their amplitudes and configurations, employing waveform recognition analysis (Spike2 program). Neuronal activity changes elicited during each intervention were evaluated by comparing activity generated immediately before chemical application or occlusion with data obtained during and following (e.g. reperfusion) those interventions. Average activity data are presented as impulses per second. Antidromic responses to SCS were quantified by spike-triggered averages of afferent neuronal activity during the progressive suprathreshold stimulation protocols, the spike derived from delivery of electrical pulses to the SCS electrode. All data are expressed as means ± SEM. SigmaStat 3.1 (Systat software) with 2-way ANOVA with post-hoc comparisons (Holm-Sidak method) was used to test for differences between groups. A significance of $p < 0.05$ was used for these data analyses.

Results

Hemodynamic data: Since both groups generated similar hemodynamic changes during LAD coronary artery occlusion (CAO), CV indices obtained during nodose and DRG studies were grouped together. Before SCS, MI induced a significant increase in heart rate (115±9 to 130±9 beats/min; $p<0.05$). CAO in control states likewise did not significantly change left ventricular systolic pressure (140±4 - 134±4 mm Hg; ns) or mean arterial blood pressure (134±6 – 129±6 mm Hg; ns). However, CAO did reduce +LV dp/dt (1999±98 to 1852±96 mmHg/sec; $p<0.01$) and – LV dp/dt (-2755±340 to -2159±320 mmHg/sec; $p<0.02$). SCS by itself did not change any recorded CV variable. Following SCS, the major change in CAO-induced hemodynamic responses was that +LV dp/dt (control: 2037±83 mmHg/s vs. 1976±53 mmHg/s; ns) was significantly altered during the ischemic stress. In contrast, the LV dp/dt depression was maintained with repeated occlusions.

Dorsal root ganglion afferent neuronal activity: The activity generated by identified DRG neurons increased during epicardial veratridine application (0.4±0.2 to 2.9±0.6 imp/s). Subsequent ischemic challenge evoked a brisk increase in neuronal activity, especially at the transition points between normal flow and ischemia (e.g. onset and release). Fig. 1 demonstrated that the duration of transient MI-induced activity exceeds the duration of the ischemic insult. Overall, LAD CAO increased average DRG activity (0.6±0.2 to 2.7±0.8 imp/s; $p<0.02$) with even greater activity levels observed during reperfusion (4.9±2.6 imp/s; $p>0.05$ from control and ischemic states; c.f., Figs. 1 & 2). The average recovery from peak activity to baseline of the evoked response in DRG activity to a 1 min LAD CAO was 207±35 s. Enhancement of DRG activity in response to repeated LAD CAO (time control) remained stable (415 % increase, LAD CAO 1 versus 570% increase, LAD CAO 2).

Recovery of evoked responses from peak activity to baseline was likewise maintained in time controls (180±49 s for first LAD to 220±135 s for second LAD).

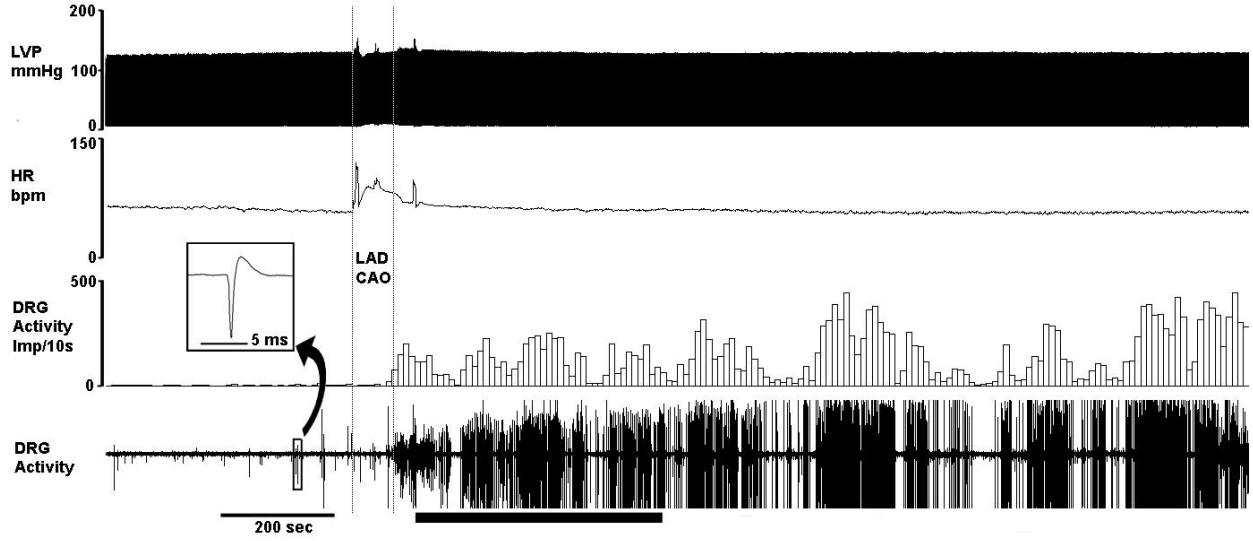

Figure 1: DRG neuronal activity recorded in response to 1 min. LAD CAO. LAD CAO increased DRG afferent neuronal activity, activity increasing further during reperfusion. DRG neuronal activity was also grouped into 10 second segments to display average rate changes over time (3rd trace). LVP = Left ventricular pressure. HR = Heart rate.

SCS mitigated increases in DRG afferent neuronal activity evoked by LAD CAO (Fig. 2). While in control states LAD CAO increased their activity (0.6±0.2 imp/s to 2.7±0.8 imp/s ($p<0.02$)), following SCS the MI-induced stressor no longer changed DRG activity (0.4±0.3 to 0.5±0.2 imp/s (ns)). After SCS, the reperfusion phase was also associated with reduced activity (0.6±0.4 imp/s). In the 3 dogs in which the recorded dorsal root ganglia was acutely decentralized from the spinal cord, SCS still reduced the LAD CAO-induced activation of the primary cardiac afferent neurons within these ganglia (290% increase from baseline of 0.5±0.3 imp/s to 33% increase from baseline of 0.3±0.2 imp/s post SCS). For this experiment, DRG located rostral and caudal to the recorded ganglia and all contralateral DRG remained connected to the spinal cord.

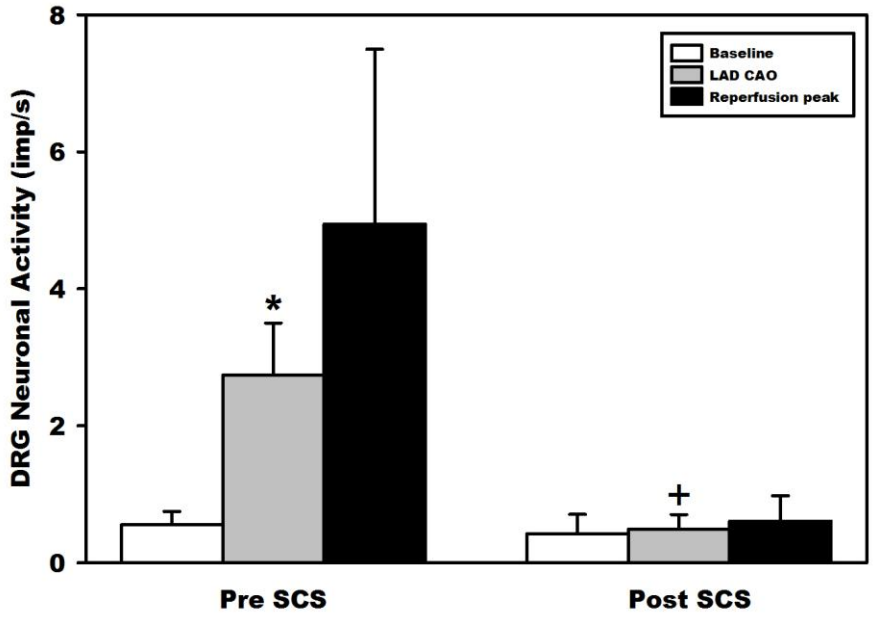

Figure 2: Activity generated by DRG neurons recorded during baseline, LAD CAO and reperfusion, peak activity being achieved during reperfusion. Following SCS, the capacity of these neurons to transduce ventricular ischemia and subsequent reperfusion was reduced. * $p<0.05$ comparing baseline and ischemia data during before SCS; + $p<0.05$ post-SCS, comparing control and ischemia data.

Nodose ganglion afferent neuronal activity: The activity generated by identified nodose ganglion afferent neuronal somata increased during LAD CAO from 1.35±0.49 to 2.94±0.87 imp/s ($p<0.001$), maintaining their enhanced state during reperfusion (Fig.3). Repeat LAD CAO elicited similar neuronal responses. Nodose ganglion afferent neurons that responded to transient ventricular ischemia were initially identified by their evoked response to chemical stimuli applied to the LV epicardial ventral surface. Of the 34 ischemic-sensitive afferent neurons evaluated, 44% responded to epicardial application of veratridine (0.8±0.2 to 3.5±1.2 imp/s; $p<0.05$), 44% responded to bradykinin (0.7±0.5 to 3.2±1.0 imp/s; $p<0.01$), 24% to adenosine (0.6±0.1 to 1.3±0.3 imp/s; p0.01) and 35% to substance P (0.3±0.1 to 1.6±0.6 imp/s; $p<0.03$).

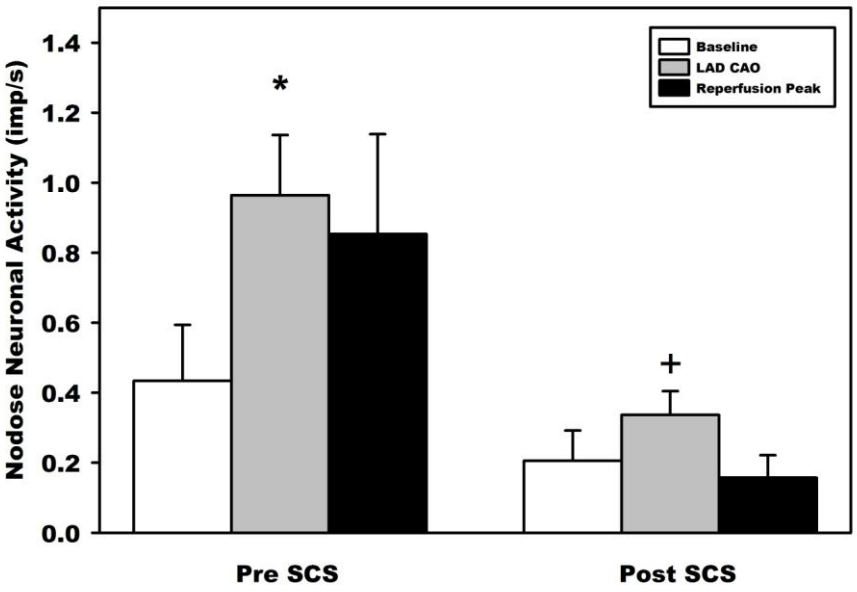

Figure 3: Activity of nodose ganglion afferent neurons prior to, during and after (reperfusion) 2 min. of LAD CAO. Following SCS (right columns) the ability of ischemia to activate nodose cardiac afferent neurons decreased significantly. * $p<0.05$ comparing ischemia to baseline data in control.; + $p<0.05$ comparing post- to pre-SCS data.

Following SCS, the capacity of nodose ganglion afferent neurons to transduce regional left ventricular ischemia was reduced compared to control states (Fig. 3). During control states ischemia enhanced nodose ganglion neuronal activity from 0.4±0.2 to 0.9±0.2 imp/s ($p<0.05$). Following SCS, ischemia elicited only minor enhancement of their activity (0.2±0.1 to 0.3±0.1 imp/s; ns) (Fig. 3). Similarly, SCS obtunded nodose ganglion neuronal activation during the reperfusion phase (0.9±0.3 imp/s versus 0.2±0.1 imp/s; ns). SCS also truncated the duration of nodose afferent neuronal excitation in response to the 1 min ischemic stress (200±28 sec control vs 90±10 sec post SCS; $p=0.01$).

DRG activity during supra-threshold SCS: To determine whether identified dorsal root ganglion afferent neuronal somata could be antidromically activated by SCS, their activity was recorded as SCS was applied at varying frequencies (0.5, 1 and 5 Hz) at intensities ranging from 90% MT to 2.5x MT (Fig. 4). Spike-triggered averages of DRG activity, based upon delivered SCS pulses, were used to quantify the potential for antidromic activation. No combination of frequencies or intensities of stimuli evoked action potentials time-locked to the SCS pulses in identified ventricular afferent

neurons indicating no antidromic activation of cardiac-related DRG neurons by SCS parameter sets utilized.

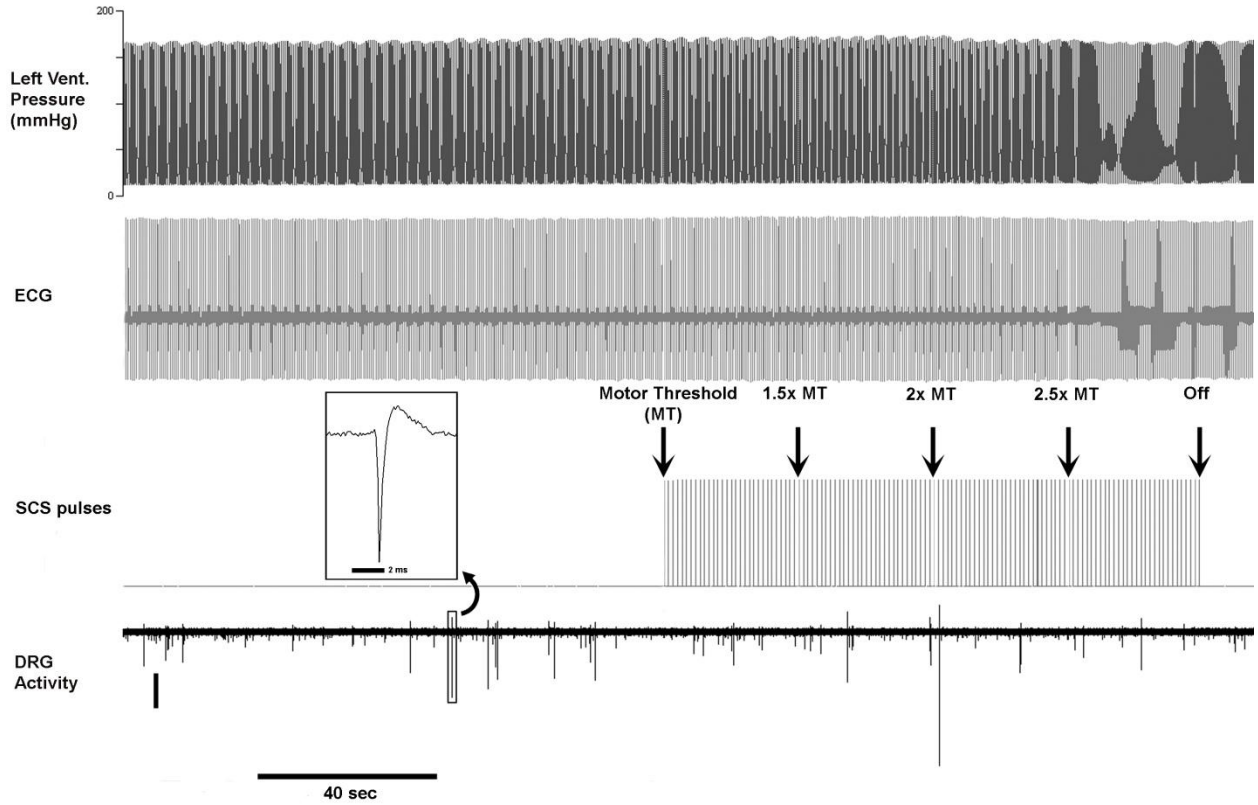

Figure 4: In intact preparations, stimulation (frequency of 0.5 Hz) of the dorsal spinal cord with different current intensities (MT, MT, 1.5x MT, 2x MT, and 2.5x MT) failed to activate identified DRG cardiac afferent neurons (bottom trace). SCS = spinal cord stimulation. DRG activity = dorsal root ganglion activity.

Discussion:

The results of this study demonstrate that the capacity of first order cardiac afferent neurons (nodose and dorsal root ganglia) to transduce an ischemic myocardium is obtunded following application of SCS. Previous work has indicated that SCS can suppress local circuit neurons in peripheral ganglia without (Foreman and others 2000) interfering flow-through efferent pathways of the sympathetic and parasympathetic nervous systems (Gibbons and others 2011). SCS likewise

blunts ischemia-induced reflex activation of central and peripheral aspects of the hierarchy for cardiac control (Ardell and others 2009; Foreman and others 2000; Qin and others 2008). This is the first demonstration of SCS-mediated suppression of first order cardiac-related afferent inputs and these effects are reflected on both DRG and nodose neurons.

Previous studies have demonstrated that SCS modulates the responsiveness of ischemia-sensitive neurons located within the spinal cord related to both pain perception (Krames and Foreman 2007; Qin and others 2008) and cardiovascular control (Ding and others 2008a; Ding and others 2008b). The spinothalamic tracts carry nociceptive inputs from cardiac-related DRG neurons to higher centers of the central nervous system (Krames and Foreman 2007; Qin and others 2008). SCS likewise modulates reflex activation of peripheral aspects of the cardiac nervous system (Armour and others 2002; Foreman and others 2000), a response likely related to suppression of local circuit neurons (Gibbons and others 2011).

Cardiac nociceptive inputs project centrally via multiple pathways (Marieb 2004). Vagal cardiac afferent neurons have their soma in nodose ganglia and project to nucleus tractus solitarius neurons of the medulla (Marieb 2004) Dorsal root ganglia neurons project centrally to the dorsal horn of the spinal cord and from there second order neurons project to other regions of the spinal cord and supraspinal sites (Kuo and others 1984). As both populations (DRG and nodose) were suppressed by SCS, it is unlikely that any suppressor effects can be ascribed to anti-dromic activation of either population. That concept was confirmed by direct recording of the activity generated by DRG neurons in the presence of low frequency SCS (c.f., no antidromic activation).

Ischemia is known to cause a decrease in myocardial contractility as indicated by a decrease in LV dp/dt (Khalil and others 2005). The transient period of ischemia used in this study was sufficient to cause a significant decrease in LV dp/dt. Following SCS, while basal contractile

function was unaffected, transient LAD CAO failed to evoke a decrease in LV dp/dt. This finding indicates that SCS reduced the stress to the myocardium resulting from transient myocardial ischemia, a fact likely related to pre-emptive SCS rendering the myocytes downstream from the occlusion ischemia-resistant.

In support of this concept, prior studies from our laboratory have demonstrated that both high thoracic (Southerland and others 2007) and high cervical SCS (paper in press) reduce infarct size to transient MI. Previous studies have demonstrated several intracellular mechanisms, i.e. α_1–PKC pathway and ß-PKA pathway, that cause the protection of myocytes against ischemic stress (Southerland and others 2007; Yellon and Downey 2003). These intracellular mechanisms themselves may play a significant role in the SCS-induced protection of the myocardium against ischemic stress that was observed in this study (Wu and others 2008).

Previous investigators have suggested that SCS-mediated protection of peripheral tissues is related to imposed efferent function on afferent projections, specifically to antidromic activation of primary afferents with resultant release of neuropeptides to target tissues (Linderoth and others 1991). We found no corresponding data for first order cardiac-related afferents projecting centrally in either the DRG or nodose ganglia. We further determined that if central projections to the recorded DRG populations were selectively interrupted while maintaining all other DRG intact, high thoracic SCS was still capable of truncating the MI-induced increase in neural activity. Together with the antidromic experiment described above and the induced changes in LV dp/dt, these data point to the importance of SCS-induced changes in myocytes themselves (e.g. rendering them ischemic-resistant) as the primary mechanism for decreased afferent response to ischemic stress.

Perspectives and significance: Data derived from this study indicate that while SCS can modify the stress of transient myocardial ischemia, as evidenced by its capacity to reduce the influence of

myocardial ischemia on +LVdp/dt, it does not directly affect the capacity of cardiac afferent neurons to transduce an ischemic event. We conclude that any neuronal modification so induced was a consequence of their transduction of an altered ventricular milieu. Pain studies have suggested that pre-emptive SCS renders cardiomyocytes ischemic resistant and this process involve alterations in sympathetic efferent neuronal inputs to the heart (Southerland and others 2007). In the clinical setting, patients are capable of tolerating increased levels of stress with SCS therapies (Mannheimer and others 1993). However, when stressors reach a critical threshold, as indicated by indices such as ST segment shift during a progressive exercise test (Mannheimer and others 1993), pain is perceived. These data, in conjunction with that presented herein, demonstrate the increased ischemia resistance exerted by neuromodulation therapies. This effect is likely reflective of induced changes in the myocytes (Southerland and others 2007) and in the blunting of excessive MI-induced reflex activation of efferents within the cardiac nervous system (Ardell and others 2009; Armour and others 2002; Foreman and others 2000).

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References

Ardell JL. 2004. Intrathoracic neuronal regulation of cardiac function. In: Armour JA, Ardell JL, editors. Basic and clinical neurocardiology. New York: Oxford University Press. p. 118–152.

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 Regul Integr Comp Physiol 297(2):R470-7.

Armour JA. 2004. Cardiac neuronal hierarchy in health and disease. Am J Physiol Regul Integr Comp Physiol 287(2):R262-71.

Armour JA. 2008. Potential clinical relevance of the 'little brain' on the mammalian heart. Exp Physiol 93(2):165-76.

Armour JA, Kember GC. 2004. Cardiac sensory neurons. In: Armour JA, Ardell JL, editors. Basic and clinical neurocardiology. New York: Oxford University Press. p. 79-117.

Armour JA, Linderoth B, Arora RC, DeJongste MJ, Ardell JL, Kingma JG, Jr., Hill M, Foreman RD. 2002. Long-term modulation of the intrinsic cardiac nervous system by spinal cord neurons in normal and ischaemic hearts. Autonomic Neuroscience 95(1-2):71-9.

Augustinsson LE, Linderoth B, Mannheimer C, Eliasson T. 1995. Spinal cord stimulation in cardiovascular disease. Neurosurg Clin N Am 6(1):157-65.

Cardinal R, Page P, Vermeulen M, Bouchard C, Ardell JL, Foreman RD, Armour JA. 2006. Spinal cord stimulation suppresses bradycardias and atrial tachyarrhythmias induced by mediastinal nerve stimulation in dogs. Am J Physiol Regul Integr Comp Physiol 291(5):R1369-75.

Ding X, Ardell JL, Hua F, McAuley RJ, Sutherly K, Daniel JJ, Williams CA. 2008. Modulation of cardiac ischemia-sensitive afferent neuron signaling by preemptive C2 spinal cord stimulation: effect on substance P release from rat spinal cord. Am J Physiol Regul Integr Comp Physiol 294(1):R93-101.

Ding X, Hua F, Sutherly K, Ardell JL, Williams CA. 2008. C2 spinal cord stimulation induces dynorphin release from rat T4 spinal cord: potential modulation of myocardial ischemia-sensitive neurons. Am J Physiol Regul Integr Comp Physiol 295(5):R1519-28.

Foreman RD, Linderoth B, Ardell JL, Barron KW, Chandler MJ, Hull SS, Jr., TerHorst GJ, DeJongste MJ, Armour JA. 2000. Modulation of intrinsic cardiac neurons by spinal cord stimulation: implications for its therapeutic use in angina pectoris. Cardiovasc Res 47(2):367-75.

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(4 Pt 2):H789-800.

Gibbons DD, Southerland EM, Hoover DB, Beaumont E, Armour JA, Ardell JL. 2011. Neuromodulation targets intrinsic cardiac neurons to attenuate neuronally mediated atrial arrhythmias. Am J Physiol Regul Integr Comp Physiol 302(3):R357-64.

Khalil PN, Neuhof C, Huss R, Pollhammer M, Khalil MN, Neuhof H, Fritz H, Siebeck M. 2005. Calpain inhibition reduces infarct size and improves global hemodynamics and left ventricular contractility in a porcine myocardial ischemia/reperfusion model. Eur J Pharmacol 528(1-3):124-31.

Krames ES, Foreman R. 2007. Spinal Cord Stimulation Modulates Visceral Nociception and Hyperalgesia via the Spinothalamic Tracts and the Postsynaptic Dorsal Column Pathways: A Literature Review and Hypothesis. Neuromodulation 10(3):224-37.

Kuo DC, Oravitz JJ, DeGroat WC. 1984. Tracing of afferent and efferent pathways in the left inferior cardiac nerve of the cat using retrograde and transganglionic transport of horseradish peroxidase. Brain Res 321(1):111-8.

Linderoth B, Fedorcsak I, Meyerson BA. 1991. Peripheral vasodilatation after spinal cord stimulation: animal studies of putative effector mechanisms. Neurosurgery 28(2):187-95.

Linderoth B, Herregodts P, Meyerson BA. 1994. Sympathetic mediation of peripheral vasodilation induced by spinal cord stimulation: animal studies of the role of cholinergic and adrenergic receptor subtypes. Neurosurgery 35(4):711-9.

Mannheimer C, Camici P, Chester MR, Collins A, DeJongste M, Eliasson T, Follath F, Hellemans I, Herlitz J, Luscher T et al. . 2002. The problem of chronic refractory angina; report from the ESC Joint Study Group on the Treatment of Refractory Angina. Eur Heart J 23(5):355-70.

Mannheimer C, Eliasson T, Andersson B, Bergh CH, Augustinsson LE, Emanuelsson H, Waagstein F. 1993. Effects of spinal cord stimulation in angina pectoris induced by pacing and possible mechanisms of action. Bmj 307(6902):477-80.

Marieb EN. 2004. Human anatomy & physiology. New York: Pearson Education.

Qin C, Farber JP, Linderoth B, Shahid A, Foreman RD. 2008. Neuromodulation of thoracic intraspinal visceroreceptive transmission by electrical stimulation of spinal dorsal column and somatic afferents in rats. J Pain 9(1):71-8.

Society A. 2002. Guiding principles for research involving animals and human beings. Am J Physiol Cell Physiol 282(6):3 p following instructions for authors.

Southerland EM, Milhorn DM, Foreman RD, Linderoth B, DeJongste MJ, Armour JA, Subramanian V, Singh M, Singh K, Ardell JL. 2007. Preemptive, but not reactive, spinal cord stimulation mitigates transient ischemia-induced myocardial infarction via cardiac adrenergic neurons. Am J Physiol Heart Circ Physiol 292(1):H311-7.

Sparr HJ, Beaufort TM, Fuchs-Buder T. 2001. Newer neuromuscular blocking agents: how do they compare with established agents? Drugs 61(7):919-42.

Wu M, Linderoth B, Foreman RD. 2008. Putative mechanisms behind effects of spinal cord stimulation on vascular diseases: a review of experimental studies. Autonomic Neuroscience 138(1-2):9-23.

Yellon DM, Downey JM. 2003. Preconditioning the myocardium: from cellular physiology to clinical cardiology. Physiol Rev 83(4):1113-51.

CHAPTER 4

Neuromodulation targets intrinsic cardiac neurons to attenuate neuronally-mediated atrial arrhythmias

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Key words: Neurocardiology, atrial fibrillation, cardiac nervous system, spinal cord stimulation, ganglionic blockade

Abstract

Our objective was to determine if atrial fibrillation (AF) results from excessive activation of intrinsic cardiac neurons (ICNs) and, if so, whether select subpopulations of neurons therein represent therapeutic targets for suppression of this arrhythmogenic potential. Trains of 5 electrical stimuli (0.3-1.2mA, 1ms) were delivered during the atrial refractory period to mediastinal nerves (MSN) on the superior vena cava to evoke AF. Neuroanatomical studies were performed by injecting the neuronal tracer DiI into MSN sites that induced AF. Functional studies involved recording of neuronal activity *in situ* from the right atrial ganglionated plexus (RAGP) in response to MSN stimulation (MSNS) prior to and following neuromodulation involving either pre-emptive spinal cord stimulation (SCS, T1-T3, 50 Hz, 200 ms duration) or ganglionic blockade (hexamethonium, 5 mg/kg). The DiI neuronal tracer labeled a subset (13.2%) of RAGP neurons, which also co-localized with cholinergic or adrenergic markers. A subset of Dil labeled RAGP neurons were non-cholinergic/non-adrenergic. MSNS evoked a ~4-fold increase in RAGP neuronal activity from baseline which SCS reduced by 43%. Hexamethonium blocked MSNS evoked increases in neuronal activity. MSNS evoked AF in 78% of right-sided MSN sites, which SCS reduced to 33% and hexamethonium reduced to 7%. MSNS induced bradycardia was maintained with SCS, but mitigated by hexamethonium. We conclude that MSNS activates subpopulations of intrinsic cardiac neurons, thereby resulting in the formation of atrial arrhythmias leading to atrial fibrillation. Stabilization of ICN local circuit neurons by SCS or the local circuit and autonomic efferent neurons with hexamethonium reduces the arrhythmogenic potential.

Introduction

Excessive activation of select inputs to the intrinsic cardiac nervous system (ICN) are known to elicit atrial arrhythmias in normal (9; 22; 28) and pathophysiological states (11; 30). Discrete activation of the axons in select mediastinal nerves can reproducibly elicit self-terminating periods of atrial tachyarrhythmias/fibrillation (ATF) (9). Mediastinal nerves are made up of sympathetic and parasympathetic efferent neuronal inputs into the intrinsic cardiac nervous system, as well as afferent axons arising from cardiac tissues (9). They likewise contain interganglionic connections mediated via local circuit neuronal projections (15) which sub-serve, in part, to coordinate peripheral reflex function (7; 34). Because excessive activation of the axons in select mediastinal nerves reproducibly elicits self-terminating periods of atrial tachyarrhythmias/fibrillation (ATF), this animal model has been employed to study the neuropharmacological basis of neurally-evoked atrial arrhythmias (9; 28).

The extent of involvement of various ICN neuronal populations in mediating AF has yet to be determined. Secondly, whether neuronally-induced atrial arrhythmias involve excessive activation of select populations within the intrinsic cardiac neurons remains unknown. Since neuromodulation therapy has been shown to suppress the ability of mediastinal nerves to induce AF (10), it has been postulated that spinal cord stimulation (SCS) acts to suppress the responsiveness of the ICN to excessive sensory inputs arising from the diseased myocardium in the induction of such arrhythmias (10; 13). Likewise, pharmacological neuromodulation that blocks neuronal transmission within the ICN has been shown to decrease the propensity of ATF formation secondary to mediastinal nerve stimulation (9; 28). While these neuromodulation therapies are known to act upon the peripheral autonomic nervous system (4; 13), how they target the ICN to reduce ATF formation initiated by excessive mediastinal nerve stimulation has yet to be determined.

To understand how neurons within the ICN respond to excessive inputs from extracardiac sources (as mimicked by mediastinal nerve stimulation) in the induction of ATF, the present study was designed to test the following hypotheses: 1) mediastinal nerve stimulation activates select neuronal populations within the ICN in the induction of ATF; 2) stabilization of those populations within the intrinsic cardiac nervous system via neuromodulation (electrical or pharmacological) blunts the capacity of the ICN to respond to excessive inputs and thereby suppress its potential to induce atrial tachyarrhythmias. This study identified the fact that excessive inputs to the ICNs in the induction of ATF does indeed primarily involve excessive activation of local circuit neurons therein, sparing, for the most part, direct efferent outflows to the heart.

Methods

Animal Preparation: Thirty mongrel dogs (either sex), weighing 18.6-26.9 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 Animal and Human Beings" (1). The Institutional Animal Care and Use Committee of the East Tennessee State University approved these experiments.

Neuronal Tracer Injection: Animals (n=2) were premedicated with sodium thiopental (15 mg/kg, i.v.), intubated and anesthetized using 2% isoflurane. Heart rate and blood pressure were continuously monitored (Surgivet Advisor Monitor, Smiths Medical) with depth of anesthesia determined by corneal reflex, jaw tone, and hemodynamic parameters. Body temperature was maintained via a circulating water heating pad (Gaymar T/Pump, Gaymar Industries Inc., Orchard Park, NY). Using aseptic techniques, a right thoracotomy was performed at the 4th intercostal space; an incision was made into the pericardial sac to expose the superior vena cava at the pericardial reflection into the right atrium, and a pericardial cradle formed. Using techniques described previously (9; 28),

mediastinal nerve projection sites were identified on the superior vena cava at the pericardial reflection in which train electrical stimuli delivered during the atrial refractory period reproducibly induced transient periods of atrial fibrillation. Ten μL boluses of 0.1 M solution of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) were then injected via a 1 cc Hamilton syringe into that site. Following tracer injection, the pericardium and thoracic cavity were closed in layers and residual air removed from the chest via a chest tube. Buprenorphine (0.01 mg/kg, i.m. or i.v.) was administered pre- and post-operatively for pain management. Antibiotic therapy (intramuscular cefazolin or oral cephalexin, 22 mg/kg) was administered twice daily prior to surgery and continued for 2 days postoperatively. Animals were recovered for 2 days following surgery to allow for transport of the tracer. The animals were re-anesthetized in the manner described above and a thoracotomy performed to collect the right atrial ganglionated plexus (RAGP), an aggregate of intrinsic cardiac neurons associated with control of atrial electrical and mechanical function (5; 27).

Immunohistochemistry: The RAGP cardiac ganglia were briefly washed in saline and then transferred to a 4°C fixative solution containing 4% paraformaldehyde and 0.2% picric acid in phosphate buffered saline (PBS, pH 7.3). Tissues were fixed at 4°C for 24 hr. They were then transferred to 20% sucrose in PBS (4°C, 4 days) for cryo-protection, frozen on powdered dry ice, and stored in sealed tubes at -80°C until processed. The ganglia were sectioned at a thickness of 16 μm using a Leica CM 3050S cryostat (Leica Microsystems Inc., Bannockburn, IL). Adjacent sections were collected on separate chrome-alum gelatin coated slides such that six representative sets of tissue sections were obtained from each tissue sample. Slide-mounted sections were stored in closed slide boxes at -20°C prior to immunohistochemistry. Single- and double-labeling of sections were studied at room temperature using standard methods previously described (19). Digitonin was used in solution instead of triton X-100 to minimize loss of DiI during processing (24). Briefly, slides were rinsed with 0.1 M PBS (pH 7.3), permeabilized in PBS containing 1000 μg/ml digitonin (Cat # 300411, EMD Biosciences,

San Diego, CA) and 0.5% bovine serum albumin (BSA), and blocked for 2 hr in PBS containing 10% normal donkey serum (Cat # S30, Millipore, Billerica, MA), 1% BSA and 1000 μg/ml digitonin. Tissues were then incubated overnight in a buffer containing either rabbit anti-Protein Gene Product 9.5 (PGP 9.5, AB1761, Millipore, Billerica, MA) or various combinations of sheep anti-tyrosine hydroxylase (TH, AB1542, Millipore, Billerica, MA), goat/rabbit anti-choline acetyltransferase (ChAT, AB144P, Millipore, Billerica, MA /AB-N34, Advanced Targeting Systems, San Diego, CA) or guinea pig anti-vesicular acetylcholine transporter (VAChT, AB1588, Millipore, Billerica, MA). After washing with PBS, tissues were incubated for 2 hr with donkey anti-rabbit secondary antibodies conjugated to AlexaFluor 488 and donkey anti-sheep secondary antibodies conjugated to AlexaFluor 647 (A-21206, A-21448, Invitrogen, Carlsbad, CA) and washed with PBS. Coverglasses were attached with Citifluor mounting medium (Ted Pella, Redding, CA) and sealed with clear nail polish. Negative control slides were processed similarly without using primary antibodies. The selectivity of fluorescence filters and the presence of co-localized markers were confirmed in several sections obtained from each animal by sequential scanning with a Leica TCS SP2 confocal microscope (Leica Microsystems Inc., Bannockburn, IL). The software used to discriminate DiI-positive neuronal somata from background was ImageJ. This was performed on images obtained by confocal microscopy. The neurochemical phenotype of DiI labeled neurons was identified based on overlay confocal images.

Hemodynamic recording: In the remaining canines (n=28), animals were anesthetized and instrumented for recording of neuronal activity from the RAGP ganglia. Responses (neural and atrial electrical activity) to mediastinal nerve stimulation were evaluated prior to and following neuromodulation using either ganglionic blockade (n=12) or spinal cord stimulation (n=16) (see below). Anesthesia was maintained during surgery using 2% isoflurane with depth of anesthesia determined by corneal reflex, jaw tone, and hemodynamic parameters. Following completion of the surgery, anesthesia was

changed to α-chloralose (75 mg/kg i.v. bolus), with repeat doses (12.5 mg/kg i.v.) administered as required throughout the duration of each study. Body temperature was maintained via a circulating water heating pad (Gaymar T/Pump, Gaymar Industries Inc., Orchard Park, NY). The left femoral artery and vein were catheterized to record arterial blood pressure and to allow for anesthetic, fluid replacement, and pharmacological agent delivery, respectively. The right femoral artery was catheterized and left ventricular pressure was measured via a Mikro-Tip Pressure Transducer Catheter (Millar Instruments, Houston, TX). Heart rate was monitored via a Lead II electrocardiogram.

Spinal cord stimulation (SCS): For the SCS treatment group, each animal was placed in the prone position and the spinal epidural space was penetrated percutaneously with a Touhy needle through a small skin incision in the caudal dorsal thorax as detailed previously (4). An eight-pole lead (Octrode, Advanced Neuromodulation Systems, Plano, TX) was advanced rostrally in the epidural space to the T1-T3 spinal cord level. The tip of the lead was positioned slightly to the left of midline using anterior-posterior fluoroscopy. The rostral pole was positioned at T1 while the caudal pole was positioned at T3 level. Proper electrode placement was determined via electrical current delivered to the rostral and caudal poles using a stimulus isolation unit and constant current generator (PSIU6, Grass Instruments, Quincy, MA) connected to a stimulator (S88, Grass Instruments, Quincy, MA). SCS was delivered at 50 Hz and 200 μs duration, stimulus parameters that have previously been shown to induce neuromodulation of extracardiac intrathoracic and intrinsic cardiac ganglia neurons during periods of acute cardiac stress (4; 13). Motor threshold (MT) intensity was determined as the lowest current that induced muscle contractions in the proximal forepaw and shoulder. Animals were rotated to the supine position for the experimental protocols with MT rechecked in this position. MT was checked periodically during the experiments and did not vary significantly from initial levels (0.25±0.02 mAmp).

Mediastinal nerve stimulation - MSNS: Following thoracotomy, an incision was made into 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 atrial electrograms. Right-sided mediastinal nerves were identified visually on the ventral and ventro-lateral surface of the intrapericardial aspects of the superior vena cava and then stimulated using techniques detailed elsewhere (9; 28). Briefly, trains of five electrical stimuli (0.3-1.2 mA, 1 ms duration, 5 ms pulse interval) were delivered for up to 20 seconds to select mediastinal sites during the refractory period on each atrial beat, as triggered off the reference atrial electrogram. This was done 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 (S88, Grass Instruments, Quincy, MA). The stimulator was externally controlled by a computer running LabChart5 (ADInstruments, Colorado Springs, CO) interfacing with a Powerlab 4/30 (ADInstruments, Colorado Springs, CO) and triggered by atrial wavefront detections. Active sites were identified by the creation of atrial tachyarrhythmias (including atrial fibrillation) when exposed to focal electrical stimuli. Each active site was marked with India ink for repeat stimulation. Two to four active sites were identified in each animal. Contact between the bipolar electrodes and tissue was discontinued immediately after the onset of the atrial tachyarrhythmia.

Neuronal Recording: Activity generated by neurons in the right atrial ganglionated plexus (RAGP) was recorded *in situ*, as reported previously (3; 14). Briefly, to decrease epicardial motion during each cardiac cycle, a circular ring of stiff wire was placed gently on the epicardial fat on the ventral surface of the right atrium - a region known to contain the RAGP. A tungsten microelectrode (250 μm diameter and exposed tip of 1 μm; impedance of 9-11 MΩ at 1000 Hz), mounted on a micromanipulator, was lowered into this fat using a microdrive. The ganglionated plexus therein was explored and electrical signals so derived were led into a differential preamplifier (BMA-831, CWE

Inc., Ardmore, PA) with a high impedance head stage (band width set at 300 Hz to 10 kHz). Signals were further amplified by a battery-driven pre-amplifier (5113 Pre-Amp, Signal Recovery, Oak Ridge, TN) (band width 100 Hz to 2 kHz). Amplified neuronal signals, together with recorded cardiovascular indices, were digitized (Cambridge Electronics Design, power 1401 data acquisition system) and analyzed using the Spike 2 software package (Cambridge Electronics Design).

Neuronal activity was identified as action potentials with signal to noise ratios greater than 3:1. The activity generated by individual neuronal somata was identified by the amplitude and configuration (waveform recognition) of these recorded action potentials, using the Spike 2 program. Using these techniques and criteria, action potentials generated by individual somata and/or dendrites, rather than axons of passage, can be recorded for extended periods of time (3; 4; 14).

Treatment Groups: Animals were divided randomly into two neuromodulation treatment groups, one that had SCS electrode implantation (n=16) and another that evaluated the effects of ganglionic blockade on arrhythmia responses evoked by MSNS (n=12). For each of these animals, 2-3 mediastinal sites were first identified that reproducibly evoked atrial arrhythmias. In 8 of these animals (4 from each group), time controls were done to verify reproducibility of evoked responses to MSNS over time. For the SCS treatment group, electrical stimuli (50 Hz, 200 μs duration, 90% Motor threshold) were delivered to the T1-T3 spinal level for 20 min. Responses to MSNS stimulation were evaluated prior to and 5-30 min. following SCS. For the hexamethonium treatment group, responses to MSNS stimulation were evaluated prior to and 15 min after hexamethonium administration (5 mg/kg, i.v.).

Data Analysis: Action potentials arising from a site within each RAGP studied were characterized by means of their differing amplitudes and configurations (waveform recognition via the Spike2 program: Cambridge Electronic Design, Cambridge, England CED). Neuronal activity responses

elicited by MSNS were evaluated by comparing activity generated immediately before stimulation with data obtained during nerve stimulation; neuronal activity data are presented as impulses per second (imp/sec). Characterization of atrial rhythms evoked by MSNS included latency and duration of atrial arrhythmias; this analysis was performed offline using Spike 2. Data are expressed as means ± SEM. Sigmastat 3.1 (Systat software) was used to test for differences between groups by ANOVA with post-hoc comparisons (Holm-Sidak or Tukey method for comparisons, depending on results of normality tests). A significance of $p < 0.05$ was used for these experiments.

Results:

Neuroanatomical data: Select mediastinal nerve projections to the right atrial ganglionated plexus were identified that, when subjected to trains of focal electrical stimuli delivered during the atrial refractory period, reproducibly induce transient periods of atrial fibrillation. DiI was then micro-injected into the wall of the superior vena cava at that nerve site. RAGP tissues harvested 2 days after application of Dil to that mediastinal nerve site identified 13.2% of RAGP neuronal soma labeled. PGP 9.5 (green) labeled all neuron soma in these RAGP. Some neuronal somata demonstrated co-localization of PGP 9.5 and Dil (Fig. 1). The neurochemical profile of Dil labeled neurons within these RAGP demonstrated that 16.7% of Dil labeled neurons also labeled for cholinergic marker choline acetyltransferase (ChAT) (Fig. 2). 22.2% of Dil labeled neurons labeled for the noradrenergic marker tyrosine hydroxylase (TH); 55.6% of Dil labeled neurons co-localized with markers for ChAT and TH. The remaining 5.5% of Dil labeled neurons did not exhibit either noradrenergic or cholinergic markers.

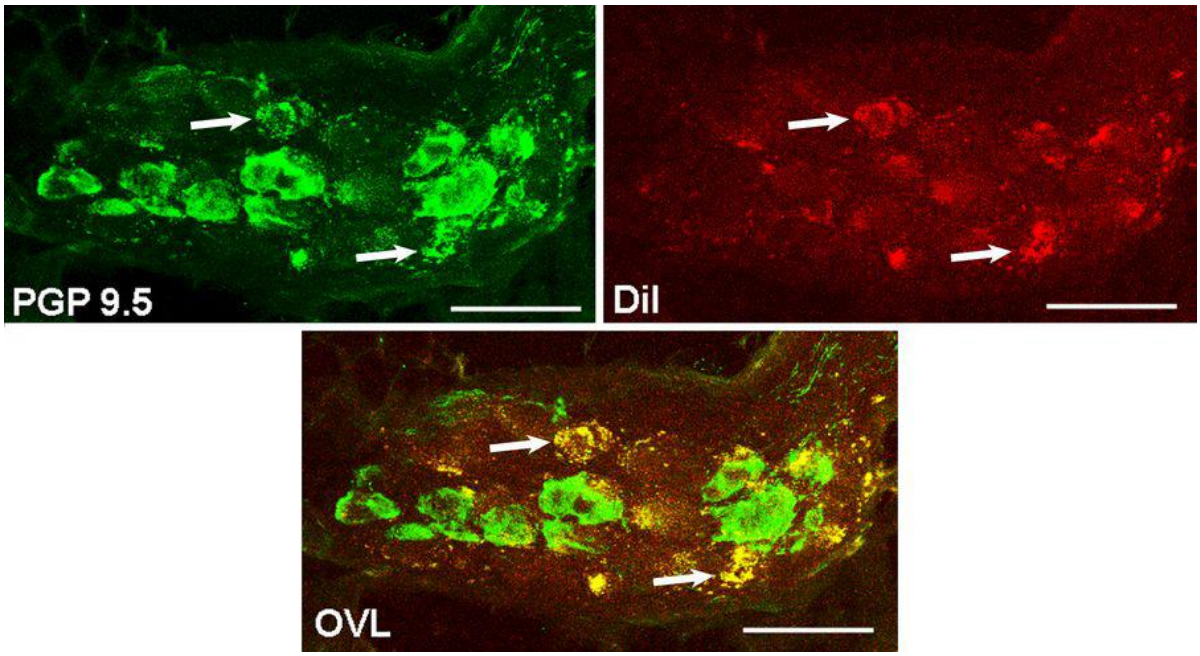

Figure 1. Characterization of neuronal cell bodies in the RAGP, labeled with the tracer (DiI) injected into right-sided mediastinal nerve sites where electrical stimulation (MSNS) induced AF. PGP 9.5 labeled all intrinsic cardiac neurons (ICN) green and DiI labeled neurons projecting from MSNS responsive sites in red. Co-localization of PGP 9.5 and DiI is indicated by yellow (arrows indicate double-labeled soma) in the overlap figure (OVL). 13.2% of PGP 9.5 labeled neurons within the RAGP co-localized with DiI. Scale bar equals 75 um.

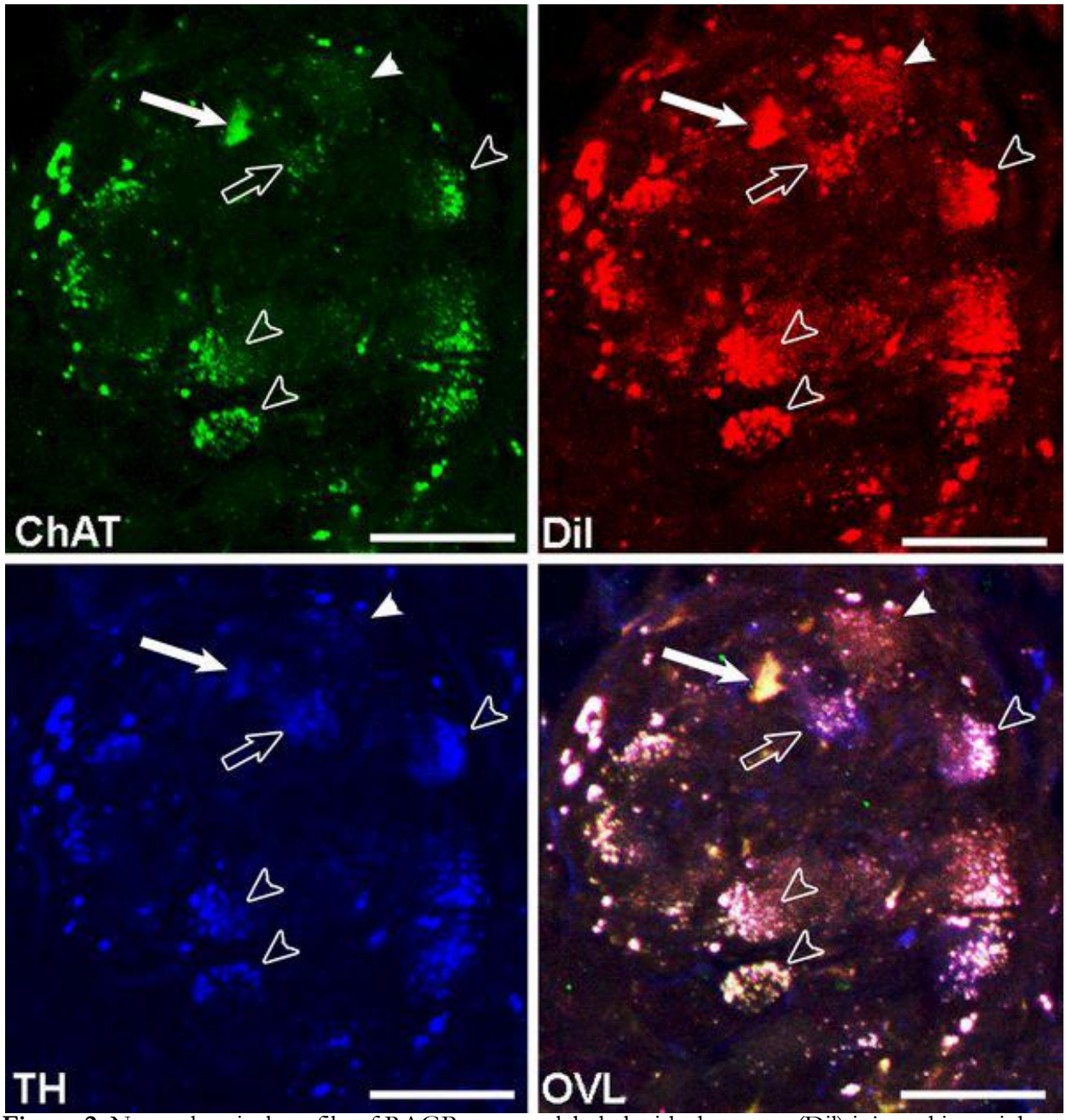

Figure 2. Neurochemical profile of RAGP neurons labeled with the tracer (DiI) injected into right-sided mediastinal nerve sites where electrical stimulation (MSNS) induced AF. Neurons were immunolabeled for choline acetyltransferase (ChAT; green label), tyrosine hydroxylase (TH; blue label) and DiI (red label). Solid arrow indicates co-localization of ChAT and DiI (16.7% of neurons). Open arrow indicates co-localization of TH and DiI (22% of neurons). Open arrowheads indicate co-localization of DiI with both ChAT and TH (55.6% of neurons). Solid arrowhead indicates a DiI neuron that did not label with either TH or ChAT markers (5.5% of neurons). Scale bar equals 40 um.

Intrinsic Cardiac Neuronal Activity: The activity generated by RAGP neurons increased in response to MSNS (Fig. 3). Mediastinal nerve stimuli induced an initial bradycardia (average 23.7±3.5% change from baseline cycle length) that transitioned rapidly to atrial fibrillation/flutter (AF/AFl). The average latency of such responses was 1.3±0.2 sec. from stimulus onset; the average duration of AF/AFl was 26.4±6.0 sec. Intrinsic cardiac neuronal activity increased in association with stimulus onset; neuronal activity increased further during the induction of AF. This enhancement of activity persisted during the initial period following sinus rhythm restoration (Fig. 3). On average, MSNS evoked ~4 fold increase in neuronal activity (Fig. 5, left panels; 0.29±0.08 imp/s to 1.20±.16 imp/sec, $p<0.001$).

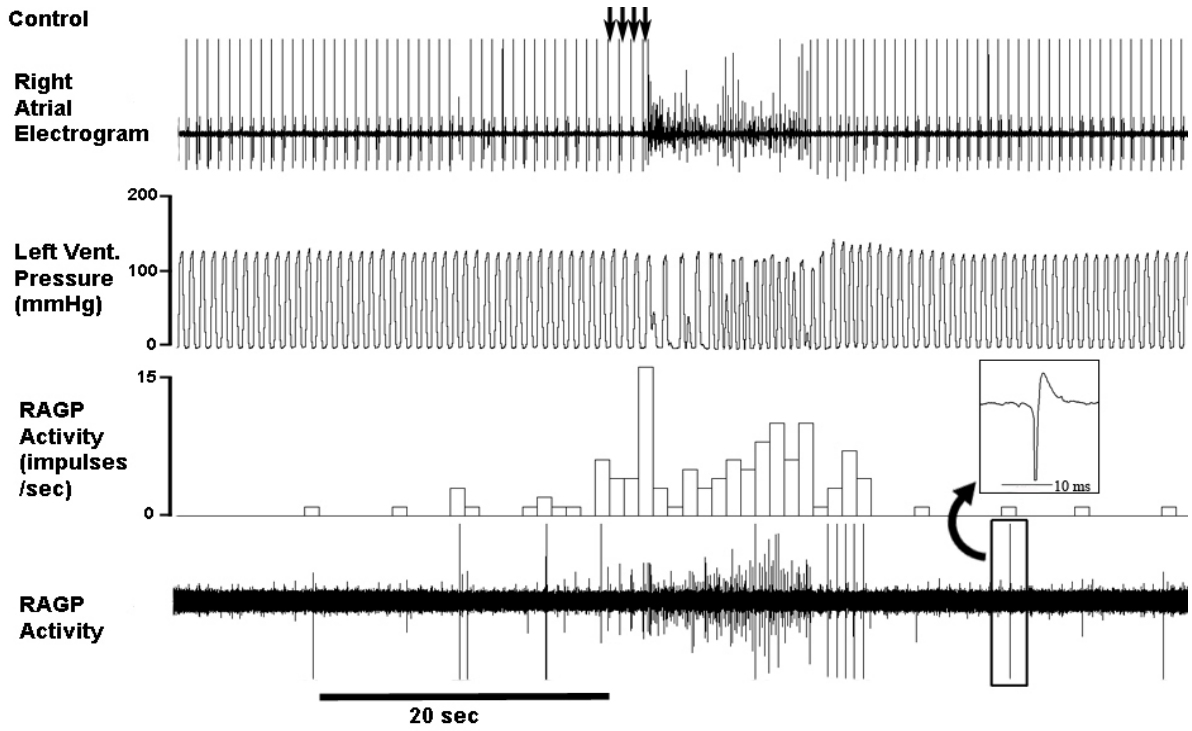

Figure 3. RAGP neuronal activity and corresponding cardiac electrical activity in response to MSNS. Arrows indicate delivery of 4 successive burst stimuli to a right-sided mediastinal nerve located on the SVC at the level of the pericardial reflection. MSNS induced an increase in RAGP neuronal activity, which persisted after stimulus cessation. Neuronal activity was further enhanced once the arrhythmia was induced. Vent. – ventricle, RAGP activity – right atrial ganglionated plexus neuronal activity Trace three (activity in impulses per second) indicates ongoing 1 sec bin summed activities. Note that during burst stimuli heart rate was reduced from 83 to 76 bpm immediately prior to initiation of atrial fibrillation. Insert shows expanded trace for one of RAGP neurons recorded.

Neuronal responses evoked by MSNS were altered by both pre-emptive SCS and nicotinic receptor blockade. While, SCS did not decrease basal neuronal activity (Fig 4 and Fig 5 top panel; 0.27±0.10 imp/sec versus 0.22±0.10 imp/sec), MSNS-induced changes in RAGP activity was suppressed significantly after 20 min. of pre-emptive SCS (1.19±0.17 to 0.68±0.18 imp/sec, $p<0.001$) (Fig. 5, top panel). Hexamethonium administration likewise did not affect basal neuronal activity (Fig. 5, bottom), yet completely suppressed the MSNS-evoked increase in RAGP neuronal activity (0.38±0.20 to 0.55±0.19 imp/sec; $p=.143$) (Fig. 5, bottom panel).

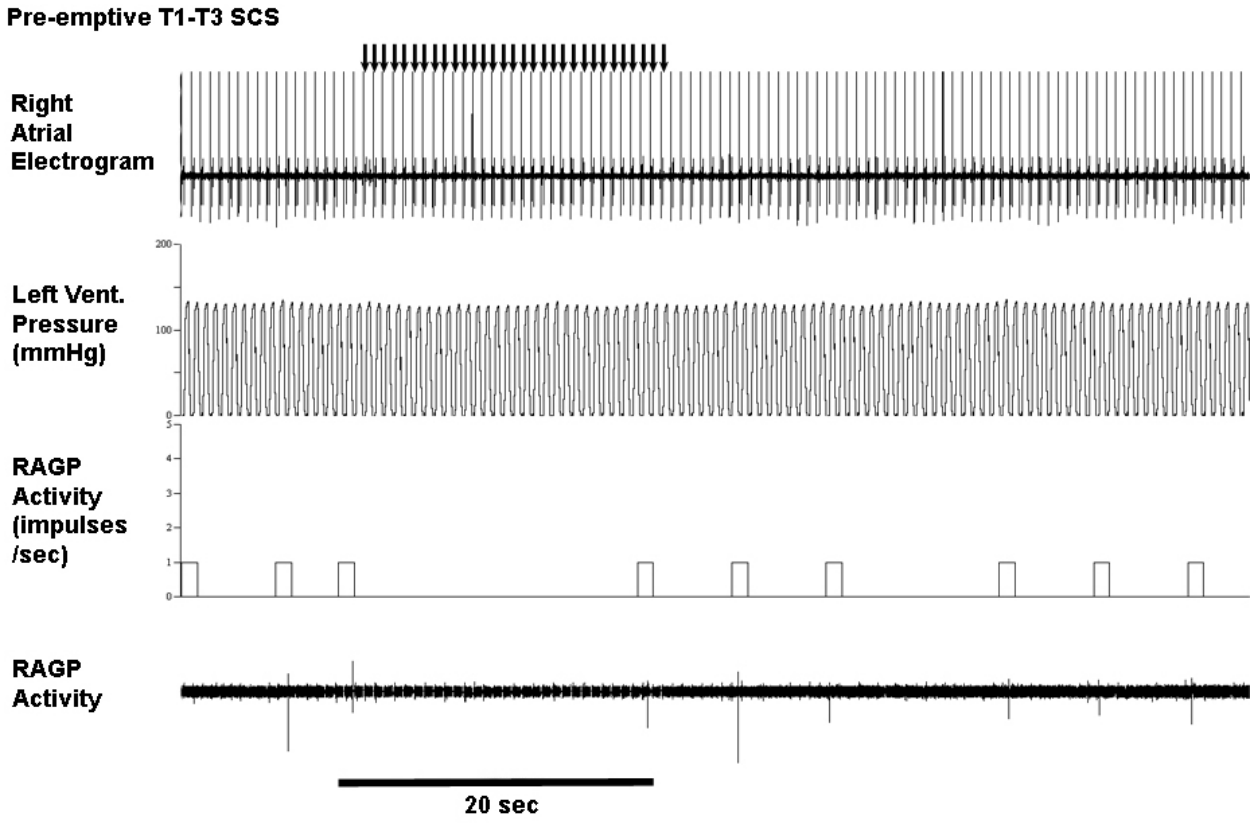

Figure 4. Neuronal activity recorded from the same RAGP site depicted in Fig. 3 demonstrating the effects of MSNS repeated 5 min after 20 min pre-emptive SCS. Note that following SCS, MSNS was ineffectual in evoking atrial arrhythmias.

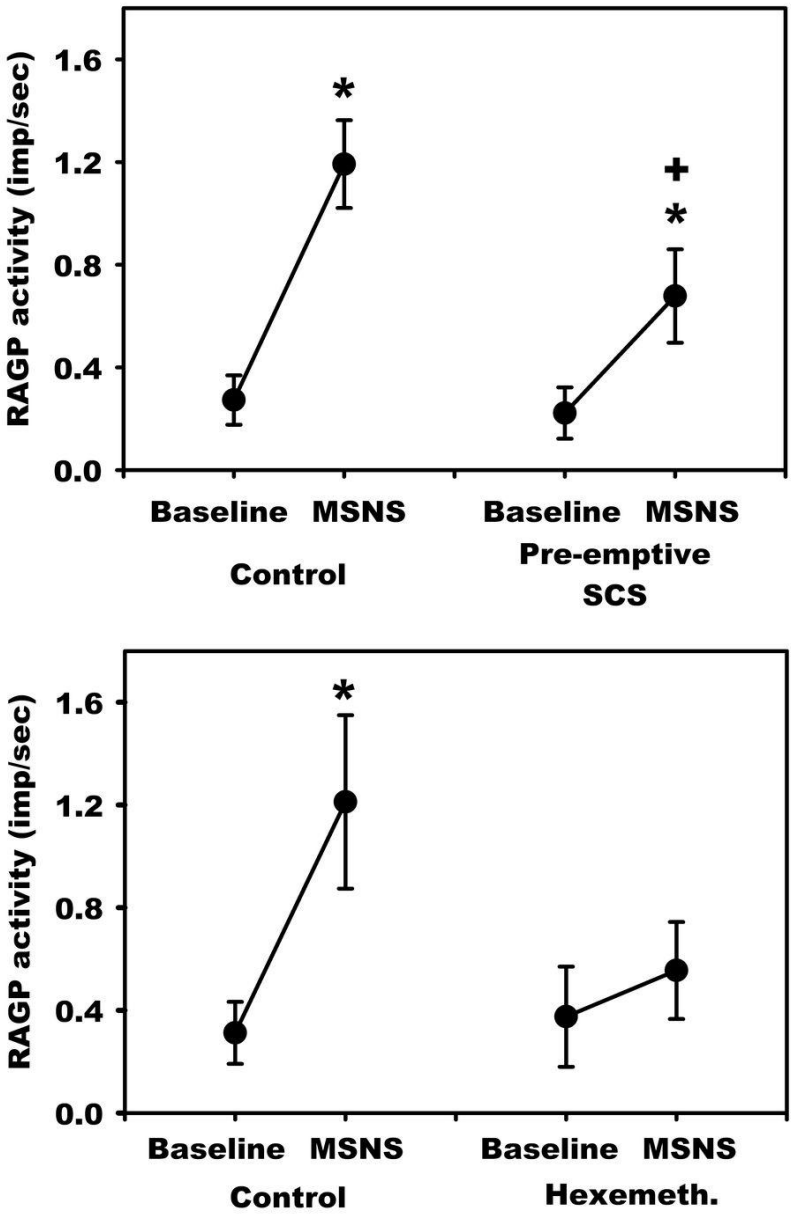

Figure 5: Effects of MSNS on RAGP neuronal activity recorded prior to and following pre-emptive SCS (top panel) or hexamethonium administration (bottom panel). MSNS initiated a ~4 fold increase in neuronal activity. Pre-emptive SCS blunted the ability of MSNS to increase neuronal activity. Hexamethonium blocked MSNS evoked increase in neuronal activity. * $p<.002$ versus baseline; + $p<.001$ versus control.

MSNS-induced bradycardia: Figure 6 summarizes the effects of hexamethonium (top panel) or pre-emptive SCS (bottom panel) on the magnitude of the initial MSNS-evoked bradycardia. In the hexamethonium treated group, while basal cardiac cycle length increased (454.3±16.6 versus 507.9±20.5 ms, p=.009), the absolute MSNS-evoked bradycardia was similar over time (535.6±16.3 versus 540.1±20.5 ms, p=.78). Moreover, even following nicotinic receptor blockade, MSNS

continued to increase cardiac cycle length (507.9±23.8 baseline to 540.1±20.5 MSNS, p<.001), albeit at a lesser magnitude compared to control. In contrast, pre-emptive SCS did not affect the MSNS-evoked bradycardia (23.7±3.5%: 433.6±11.4 to 532.0±11.9 ms, p<0.001).

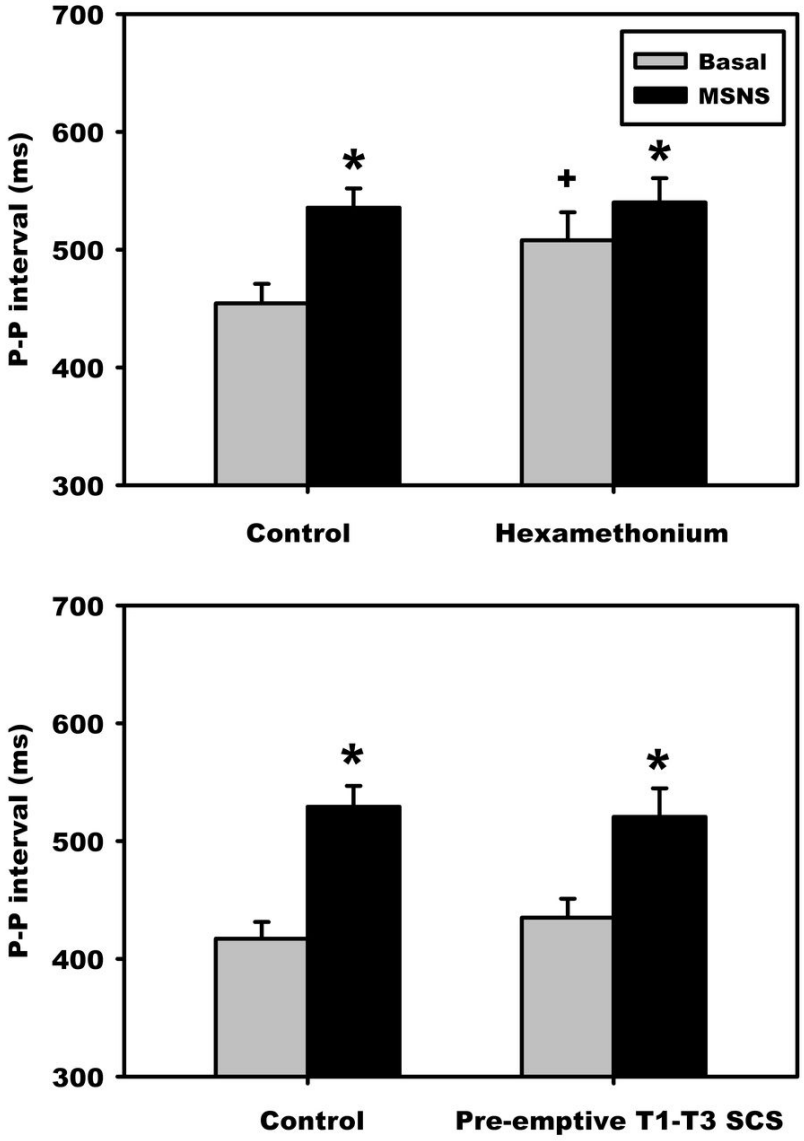

Figure 6. Mediastinal nerve stimulation-induced an initial atrial bradycardia before and following hexamethonium administration (top panel) or pre-emptive SCS (bottom panel). * p<.001 versus basal; + p<.01 versus control.

Time controls: Time controls for both treatment groups exhibited similar MSNS evoked changes in RAGP neuronal activity (0.21±0.05 to 1.00±.40 imp/sec versus 0.15±0.04 to 1.02±.11 imp/sec, basal to MSNS). Time controls also demonstrated consistent responses in basal cycle length

(420.3±9.6 ms versus 415.8±7.9 ms, p=.99) and equivalent evoked changes in cardiac cycle length with MSNS onset (526.1±22.2 ms versus 507.3±20.9ms; p=0.70).

Atrial Rhythms: Atrial rhythm responses to MSNS were studied before (panel A: control 94 active sites) and following either hexamethonium administration (panel B: 28 of the 94 sites) or pre-emptive SCS (panel C: 66 of the 94 sites). In control states, the predominant atrial rhythm evoked by right-sided MSNS was AF/AFl (77.7%); sinus bradycardia (12.8%), sinus tachycardia (4.2%), and other rhythms (5.3%) were induced less frequently. Time controls demonstrated no significant change in these response characteristics. Following ganglionic blockade, MSNS evoked AF/AFl from only 7.1% of the nerve sites stimulated (Fig. 7, panel B); sinus rhythm/bradycardia was the predominant rhythm (82.2% of sites).

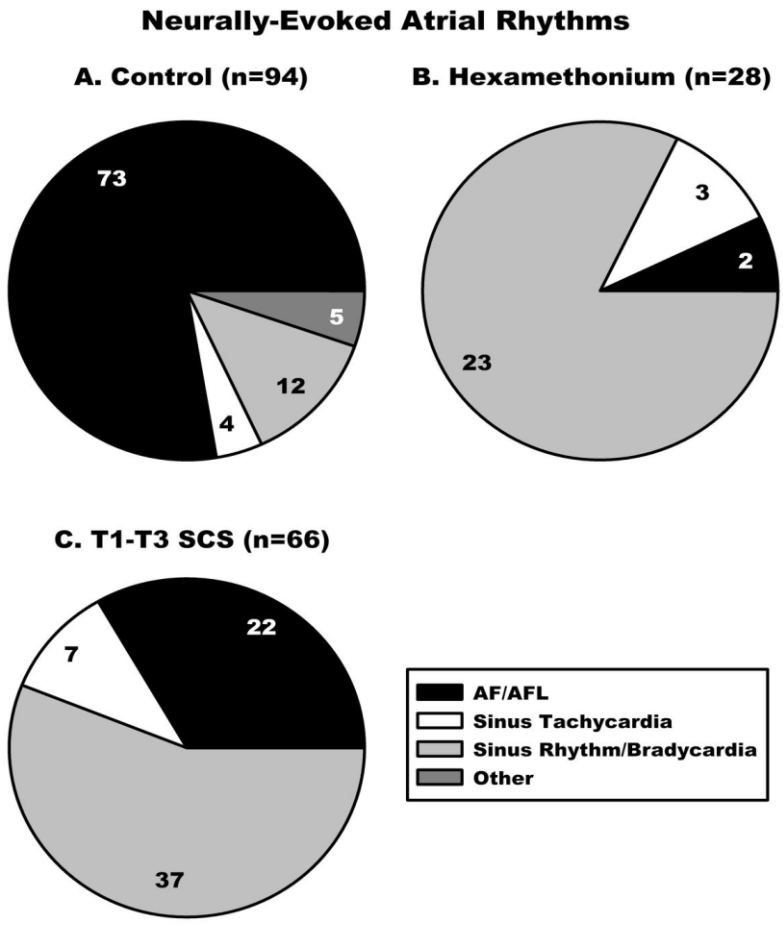

Figure 7. Types of atrial rhythms evoked during mediastinal nerve stimulation prior to (A, control) and following treatment with hexamethonium (panel B) or pre-emptive T1-T3 SCS (panel C). The number of stimulation sites in each group is indicated above each chart; the number of arrhythmias elicited in each subgrouping are also indicated. AF/AFL – atrial fibrillation/atrial flutter.

Following pre-emptive SCS, the predominant atrial rhythm evoked by MSNS shifted to sinus bradycardia (56.2%). Not only did pre-emptive SCS reduce the number of mediastinal sites that evoked AF (77.7% to 33.3%, Fig. 7), but for the residual active sites the latency to evoke such AF increased (1.03±0.12 to 1.63±0.20 sec; p=.004) and duration of such evoked AF decreased (33.8±9.9 to 14.8±3.0 sec; p=.047) (Fig. 8). Time controls exhibited no change in the latency to AF onset (1.26±0.29 to 1.15±0.25 sec; ns) or the duration of MSNS-evoked AF (21.5±13.9 to 22.6±6.8 sec; n.s.).

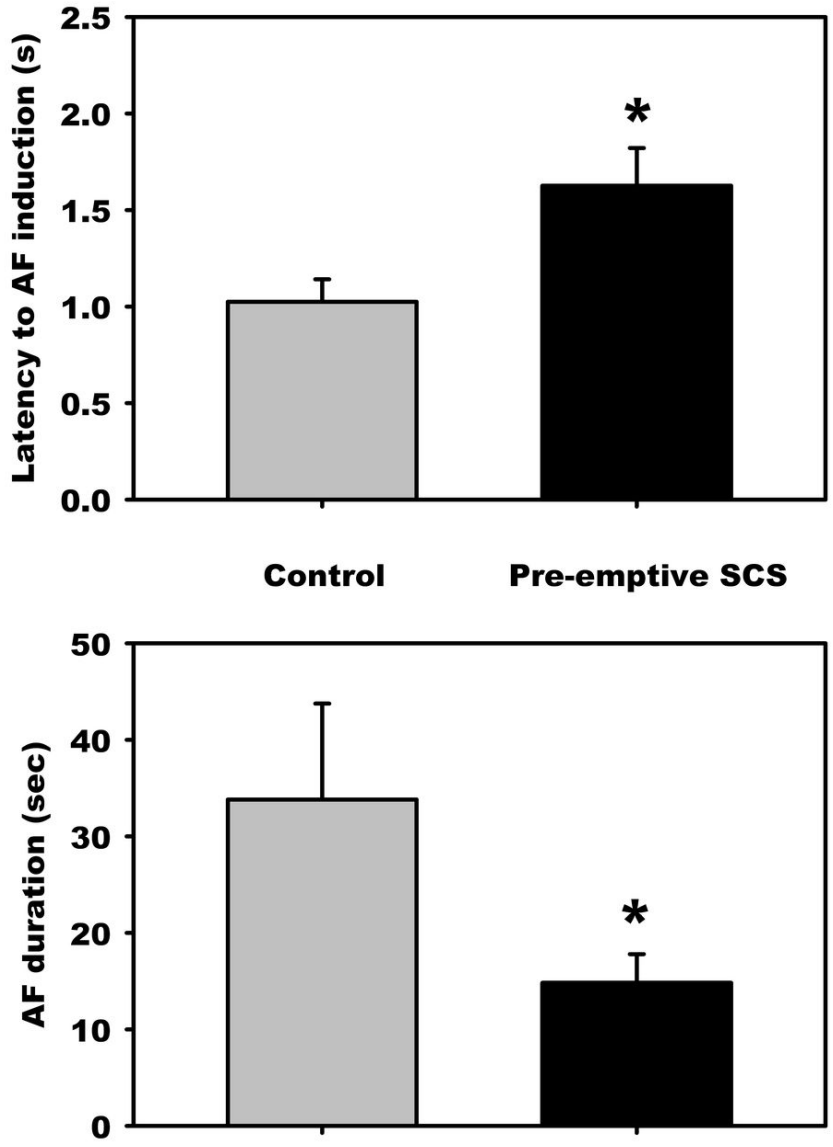

Figure 8. SCS-induced changes in AF characteristics in SCS-resistant stimulation sites. Even when atrial fibrillation persisted from 22 sites following pre-emptive SCS, its latency increased and duration decreased. * p<.05 from control.

Discussion:

Excessive inputs to the intrinsic cardiac nervous system via, for instance, activating select mediastinal nerve inputs to that system, are known to initiate ectopic atrial activation that can lead to alterations in atrial rhythms (9; 10 28). As such, it has been proposed that selective activation of inputs to the ICN leads to excessive and heterogeneous activation within that nervous system to induce atrial arrhythmias (9).

In fact, select neuronal receptors (specifically nicotinic and α-adrenergic ones) have been implicated in mediating neuronally-induced atrial arrhythmias (9; 28). In agreement with that concept, systemic administration of hexamethonium or α-adrenergic receptor blocking agent obtunds the ability of MSNS to generate atrial dysrhythmias (9; 28). In addition to such pharmacological neuromodulation, neuromodulation involving electrical stimulation of the dorsal, thoracic spinal cord can similarly decrease the ability of the intrinsic cardiac nervous system to generate atrial arrhythmias when excessively activated (10). While previous studies have inferred the involvement of specific subpopulations of intrinsic cardiac neurons in neural mechanisms associated with atrial arrhythmias, this study demonstrates the specifics of the neural-substrate so involved and the potential to target it therapeutically to manage atrial arrhythmias.

The ICN has been traditionally thought to represent a simple relay station for parasympathetic efferent innervation of the heart (21). However, recent research has altered this view by elucidating the presence of a variety of intrinsic cardiac neurochemical phenotypes in conjunction with different functional classes of neurons including afferent, efferent and local circuit neurons that interact reflexly to modulation regional cardiac electrical and mechanical activities (6; 16; 26). Most intrinsic cardiac neurons display immunoreactivity to cholinergic markers, some without cholinergic inputs (18). Other intrinsic cardiac neurons, including human intrinsic cardiac ganglia, express an

adrenergic phenotype (17; 18; 32). Moreover, various of these neurons are also innervated by peptidergic, nitrergic, and noradrenergic fibers (16; 18; 29). In accord with such anatomy, in this study immunohistochemistry analysis demonstrated that the populations of ICN neuronal somata receiving inputs from mediastinal nerves that induce arrhythmogenic activity express cholinergic and/or adrenergic markers. In addition, there is another population of intrinsic cardiac neurons that appear to be involved in the genesis of atrial tachyarrhythmias that are neither cholinergic nor adrenergic in function.

It has been reported that the largest population of intrinsic cardiac neurons, represented by local circuit neurons, has the ability to process afferent and efferent inputs, thereby contributing to efferent neuronal control over regional cardiac function (6). As such, this population receives not only cardiac sensory inputs (3) but also efferent inputs from both limbs of the autonomic nervous system (8; 14). The integration of afferent and efferent inputs, combined with the capacity of intrinsic cardiac local circuit neurons to interact with neurons within other intrathoracic ganglia, comprises organ-level neuronal processing for functional control (7; 15; 34; 36).

The present study demonstrates that MSN activation in the induction of atrial arrhythmias is associated with alterations in the activity generated by populations of intrinsic cardiac neurons that represent different phenotypical subtypes (Fig. 3). Furthermore, these data demonstrate that excessive inputs to the ICN can indeed result in activation of neuronal populations therein – such neurons responding with activity rates considerably above control levels. As such, these data confirm the fact that atrial electrical instability can be related to excessive activation of select populations of ICN neurons. When synaptic efficacy within the ICN is compromised by nicotinic blockade, this arrhythmogenic potential is reduced in response to nerve imbalances within the cardiac nervous system. However, such ganglionic blockade is associated with major deficits in

efferent outflow to the periphery (33). On the other hand, SCS apparently imparts an obtunding effect on such excessive excitation of intrinsic cardiac neurons such that in many instances excessive mediastinal nerve inputs failed to enhance intrinsic cardiac neuronal activity (Fig. 4). Yet efferent function to the heart is maintained following SCS (Fig. 6). This, in turn, may explain why SCS appears to influence cardiac indices (12; 23), albeit in a relatively minor fashion (13; 20). It does so by restraining reflex pathways within intrathoracic autonomic ganglia associated with cardiac control (4; 13). SCS likewise does not blunt the chronotropic or dromotropic responses associated with extracardiac stimulation of principal parasympathetic or sympathetic projections to the heart (25). On the other hand, the reduction in ICN neuronal activation induced by excessive mediastinal nerve inputs presumably was the result of SCS modulation/suppression of local circuit neurons, c.f. figure 3. In fact, that population represents the largest target population within the ICN (8; 14). That such neuronal responsiveness to excessive inputs was obtunded is further demonstrated by the fact that more than half of previously responsive neurons identified were no longer activated by MSNS following SCS.

Perspectives and Significance: It is becoming apparent that neuronal control of the heart is significantly more complex than previously thought (6). Multiple tiers of neuronal processing occur within the cardiac neuronal hierarchy that ultimately acts to coordinate neuronal inputs to the heart (4; 7; 13). Stresses, either pathological or physiological, may disrupt the balance of efferent neuronal inputs that can lead to altered cardiac function (6; 22). As such, novel therapies that overcome any such imbalance in cardiac control, as represented by neuromodulation of the ICN via SCS, may moderate excessive activation of the ICN in response to transduction of pathological cardiac stressors. Other electrical neuromodulation-based therapies, such as low-level vagosympathetic stimulation, may target peripheral aspects of the cardiac nervous system to reduce the arrhythmogenic potential (31; 35), but their respective neuronal targets have yet to be established (2).

Indeed, data derived from this study indicate that SCS can 1) stabilize intrinsic cardiac local circuit neurons in the presence of excessive extracardiac inputs, thereby obtunding any capacity to induce atrial arrhythmias and 2) do so without affecting direct autonomic efferent neuronal outflows to the heart to compromise cardiac function. In contrast, nicotinic receptor blockade suppressed not only the capacity of local circuit neurons to become excited but also autonomic efferent projections that influence cardiac indices. Such data delineate the possibility of targeting local as opposed to central derived inputs to the heart. They also support the thesis that SCS may represent a novel therapeutic strategy to counteract pathologically-induced excessive activation of the ICN that would otherwise lead to atrial arrhythmia induction.

Acknowledgments

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References

1. **Am.Physiol.Society**. Guiding principles for research involving animals and human beings. *Am J Physiol Regulatory Integrative Comp Physiol* 283: R281-R283, 2002.
2. **Ardell JL**. The cardiac neuronal hierarchy and susceptibility to arrhythmias. *Heart Rhythm* 8: 590-591, 2011.
3. **Ardell JL, Butler CK, Smith FM, Hopkins DA and Armour JA**. Activity of in vivo atrial and ventricular neurons in chronic decentralized canine hearts. *Am J Physiol* 260: H713-H721, 1991.
4. **Ardell JL, Cardinal R, Vermeulen M and Armour JA**. Dorsal spinal cord stimulation obtunds the capacity of intrathoracic extracardiac neurons to transduce myocardial ischemia. *Am J Physiol Heart Circ Physiol* 297: R470-R477, 2009.
5. **Ardell JL and Randall WC**. Selective vagal innervation of sinoatrial and atrioventricular nodes in canine heart. *Am J Physiol* 251: H764-H773, 1986.
6. **Armour JA**. Potential clincial relevance of the 'little brain' on the mammalian heart. *Exp Physiol* 93.2: 165-176, 2008.
7. **Armour JA, Collier K, Kember G and Ardell JL**. Differential selectivity of cardiac neurons in separate intrathoracic autonomic ganglia. *Am J Physiol* 274: R939-R949, 1998.
8. **Armour JA and Hopkins DA**. Activity of canine in situ left atrial ganglion neurons. *Am J Physiol* 259: H1207-H1215, 1990.
9. **Armour JA, Richer LP, Pagé PL, 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. *Autonomic Neuroscience: Basic and Clinical* 118: 68-78, 2005.

10. **Cardinal R, Pagé PL, Vermeulen M, Bouchard C, Ardell JL, Foreman RD and Armour JA**. Spinal cord stimulation suppresses bradycardias and atrial tachyarrhythmias induced by mediastinal nerve stimulation in dogs. *Am J Physiol Regulatory Integrative Comp Physiol* 291: R1369-R1375, 2006.
11. **Choi E-K, Shen MJ, Han S, Kim D, Hwang S, Sayfo S, Piccirillo G, Frick K, Fishbein MC, Hwang C, Lin S-F and Chen P-S**. Intrinsic cardiac nerve activity and paraoxysmal atrial tachyarrhythmia in ambulatory dogs. *Circulation* 121: 2615-2623, 2010.
12. **Eliasson T, Augustinsson LE and Mannheimer C**. Spinal cord stimulation in severe angina pectoris: Presentation of current studies, indications and clinical experience. *Pain* 65: 169-179, 1996.
13. **Foreman RD, Linderoth B, Ardell JL, Barron KW, Chandler MJ, Hull SS, TerHorst GJ, DeJongste MJL and Armour JA**. Modulation of intrinsic cardiac neurons by spinal cord stimulation: implications for therapeutic use in angina pectoris. *Cardiov Res* 47: 367-375, 2000.
14. **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-H800, 1988.
15. **Gray AL, Johnson CI, Ardell JL and Massari VJ**. Parasympathetic control of the heart. A novel interganglionic intrinsic cardiac circuit mediates neural control of the heart. *J Applied Physiol* 96: 2273-2278, 2004.
16. **Herring N and Paterson DJ**. Neuromodulators of peripheral cardiac sympathovagal balance. *Exp Physiol* 94: 46-53, 2009.
17. **Hoard JL, Hoover DB, Mabe AM, Blakely RD, Feng N and Paolocci N**. Cholinergic neurons of mouse intrinsic cardiac ganglia contain noradrenergic enzymes, norephinephrine

transporters, and the neurotropin receptors tropomyosin-related kinase A and p75. *Neurosci* 156: 129-142, 2008.

18. **Hoover DB, Isaacs ER, Jacques F, Hoard JL, Pagé P and Armour JA**. Localization of multiple neurotransmitters in surgically derived specimens of human atrial ganglia. *Neurosci* 164: 1170-1179, 2009.
19. **Hoover DB, Shepherd AV, Southerland EM, Armour JA and Ardell JL**. Neurochemical diversity of afferent neurons that transduce sensory signals from dog ventricular myocardium. *Autonomic Neuroscience: Basic and Clinical* 141: 38-45, 2008.
20. **Kingma JG, Linderoth B, Ardell JL, Armour JA, DeJongste MJL and Foreman RD**. Neuromodulation therapy does not influence blood flow distribution or leftventricular dynamics during acute myocardial ischemia. *Autonomic Neuroscience: Basic and Clinical* 91: 47-54, 2001.
21. **Langley G**. *The Autonomic Nervous System*. Cambridge, UK: Cambridge University Press, 1921,
22. **Lu Z, Scherlag BJ, Lin J, Yu L, Guo J-H, Niu G, Jackman WM, Lazzara R, Jiang H and Po SS**. Autonomic mechanism for initiation of rapid firing from atria and pulmonary veins: evidence by ablation of ganglionated plexi. *Cardiov Res* 84: 245-252, 2009.
23. **Mannheimer C, Camici P, Chester MR, Collins A, DeJongste MJL, Eliasson T, Follath F, Hellemans I, Herlitz J, Lüscher T, Pasic M and Thelle D**. The problem of chronic refractory angina. Report from the ESC joint study group on the treatment of refractory angina. *Eur Heart J* 23: 355-370, 2002.
24. **Matsubayashi Y, Iwai L and Kawasaki H**. Fluorescent double-labeing with carbocyanine neuronal tracing and immunohistochemistry using a cholesterol-specific deterent digitonin. *J Neurosci Methods* 174: 71-81, 2008.

25. **Olgin JE, Takahashi T, Wilson E, Vereckei A, Steinberg H and Zipes DP**. Effects of thoracic spinal cord stimulation on cardiac autonomic regulation of the sinus and atrioventricular nodes. *J Cardiov Electrophysiol* 13: 475-481, 2002.

26. **Parsons RL**. Mammalian cardiac ganglia as local integration centers histochemical and electrophysiological evidence. In: *Neural Mechanisms of Cardiovascular Regulation*, edited by Dun NJ, Machado BH and Pilowsky PM. Boston: Kluwer Academic Publishers, 2004.

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

28. **Richer LP, Vinet A, Kus T, Cardinal R, Ardell JL and Armour JA**. α−Adrenoceptor blockade modifies neurally induced atrial arrhythmias. *Am J Physiol Regulatory Integrative Comp Physiol* 295: R1175-R1180, 2008.

29. **Rysevaite K, Saburkina I, Pauziene N, Vaitkevicius R, Noujaim SF, Jalife J and Pauza DH**. Immunohistochemical characterization of the intrinsic cardiac neural plexus in whole-mount mouse heart preparations. *Heart Rhythm* 8: 731-738, 2011.

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

31. **Shen MJ, Shinohara T, Park HW, Frick K, Ice D.S., Choi EK, Han S, Maruyama M, Sharma R, Shen C, Fishbein MC, Chen LS, Lopshire JC, Zipes DP and Chen P-S**. Continuous low-level vagus stimulation reduces stellate ganglion nerve activity and paroxysmal atrial tachyarrhythmias in ambulatory canines. *Circulation* 123: 2204-2212, 2011.

32. **Singh S, Johnson PI, Javed A, Gray TS, Lonchyna VA and Wurster RD**. Monamine- and histamine-synthesizing enzymes and neurotransmitters within neurons of adult human cardiac ganglia. *Circulation* 99: 411-419, 1999.

33. **Taylor P**. Agents acting at the neuromuscular junction and autonomic ganglia. In: *Goodman & Gilman's: The Pharmacological Basis of Therapeutics*, edited by Brunton LL, Lazo JS and Parker KL. New York: McGraw-Hill, 2006.

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

35. **Yu L, Scherlag BJ, Li S, Sheng X, Lu Z, Nakagawa H, Zhang Y, Jackman WM, Lazzara R, Jiang H and Po SS**. Low-level vagosympathetic nerve stimulation inhibits atrial fibrillation inducibility: Direct evidence by neural recording from intrinsic cardiac ganglia. *J Cardiovasc Electrophysiol* 22: 455-463, 2011.

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

CHAPTER 5

Summary

Chapter 2 determined that cervical SCS decreased infarct size resulting from MI by using dorsal spinal column projections to modify adrenergic efferent functions. Dorsal column transection or adrenergic receptor blockade eliminated the infarct size reduction effect of cervical SCS. Cervical SCS also decreased the activation of spinal cord neurons in laminae I-V that resulted from MI. In the presence of cervical SCS, β-adrenergic blockade and cervical vagal transection eliminated the incidence of SCD while muscarinic blockade had no effect. The data presented indicate that cervical SCSC utilizes both adrenergic and cholinergic efferent mechanisms to stabilize cardiomyocytes and the arrhythmogenic potential in response to ischemic stress.

Chapter 3 found that cardiac primary afferent neurons in the nodose and DRG were activated by transient myocardial ischemia. High thoracic SCS did not directly activate cardiac primary afferent neurons in the DRG or nodose, yet it did mitigate the MI-induced increase in cardiac primary afferent neuronal activity in both afferent populations. Our data indicate that pre-emptive SCS exerts this effect by altering the sensory milieu of the stressed heart rather than by direct antidromic activation of primary afferent fibers.

Chapter 4 demonstrated that intrinsic cardiac neurons have numerous distinct neurochemical phenotypes including adrenergic, cholinergic, adrenergic/cholinergic, and non-adrenergic/non-adrenergic phenotypes. MSNS activated distinct subpopulations of intrinsic cardiac neurons within associated periods of AF. Neuromodulation therapy, exerted by thoracic SCS or hexamethonium, decreased the activation of the intrinsic cardiac neurons resulting from MSNS and stabilized the atrial arrhythmogenic substrate. The stabilization effects on AF induction of

hexamethonium were greater than those of thoracic SCS. Our data indicate that hexamethonium targets efferent and local circuit neurons while SCS preferentially modulates local circuit neurons.

Organization of the Cardiac Nervous System for Control of Normal and Stressed Hearts

The cardiac nervous system consists of afferent, efferent, and local circuit neurons that interact to control heart function (Armour 1999; 2004). Cardiac-related afferent neurons transmit sensory signals (mechanosensory and/or chemosensory) via the DRG to the spinal cord for sympathetic afferents and via nodose ganglia to the brainstem for parasympathetic afferents (Armour 1999; 2004; Cheng and others 1999; Hopkins and Armour 1984; Marieb 2004). For control of cardiac sympathetic function, brainstem cardiovascular-related centers send projections down to the thoracic spinal cord to the intermediolateral cell column (origin of sympathetic pre-ganglionic neurons)with subsequent projections from these neurons to the intrathoracic/extracardiac ganglia (Marieb 2004). These ganglia, in turn, send efferent sympathetic postganglionic projections to the heart (Marieb 2004). Parasympathetic efferent pathways travel from the brainstem through the vagi to the heart making the pre- to post-ganglionic connections at the intrinsic cardiac ganglia (Armour 1999; 2004; Cheng and others 1999; Hopkins and Armour 1984; Marieb 2004). Contained within each of the autonomic ganglia of the cardiac nervous system are local circuit neurons that serve to connect afferent and efferent neurons within single ganglia and between different intrathoracic ganglia (Armour 2008). The cardiac nervous system is organized in a series of nested feedback control loops (Armour 1999; 2004; 2008). The most basic of these networks is the intrinsic cardiac nervous system, which is composed of numerous ganglia on the heart itself and serve to control regional cardiac function on a beat-to-beat basis (Armour 1999; 2004; 2008). Expanding outward from the intrinsic cardiac nervous system are the reflex control

networks contained within the intrathoracic "sympathetic" ganglia, with subsequent nodes for reflex processing contained within the spinal cord and brainstem (Armour 1999; 2004; 2008). Higher centers of the brain can adjust cardiac control by modulating the cardiac nervous system, e.g. fight or flight, automatic response to pain, etc. (Marieb 2004). Neural interactions between various levels of the cardiac nervous system allow for effective coordination of cardiac function at rest and during periods of cardiac stress (Armour 1999; 2004; 2008). Included in these network interactions are propriospinal pathways within the spinal cord that allow for communication between spinal cord segments with the high cervical cord exerting major modulating effects on thoracic and lumbar reflex processing of organ specific functions (Chandler and others 1996; Ding and others 2008b; Foreman 1999; Marieb 2004).

Research into numerous cardiac pathologies has elucidated characteristics of the cardiac nervous system and how it remodels during such stress (Cardinal and others 2004; Eliasson and others 1996; Mannheimer and others 1998; Southerland and others 2007). One of those cardiac pathologies, MI, causes referred pain that is sensed in the arm or neck (Foreman 1999). Those areas are part of the upper cervical somatic dermatomes, sections of the surface of the body whose sensory information enters the spinal cord at specific levels (Foreman 1999). C1-C3 cervical spinal cord segments also receive parasympathetic and sympathetic afferent inputs, arising from both intrathoracic and visceral organs (Chandler and others 1996; Chandler and others 2000; Ding and others 2008a; Ding and others 2008b; Foreman 1999; Qin and others 2007a; Qin and others 2008; Qin and others 2004; Qin and others 2007b). These findings led to the theory that the upper cervical spinal cord functions as a coordinating and processing center for sensory information passing up the spinal cord (Ding and others 2008a; Ding and others 2008b; Foreman 1999; Qin and others 2008). The upper cervical region of the cord likewise uses intersegmental interconnections to interact with other levels of the cord (Qin and others 2007b). As such, targeting the upper cervical

region with electrical neuromodulation has the potential to modulate reflex control of visceral functions including the heart (Ding and others 2008b; Qin and others 2007a; Qin and others 2008).

Neuromodulation as a Therapeutic Approach for Control of Cardiac Nervous System and Heart

The first experiments to study how SCS affects the cardiac response to MI focused on the spinal segments where the cardiac afferent- and efferent-related pathways enter and exit the spinal cord (Hua and others 2004b; Qin and others 2008) These clinical studies focused on retractable angina pectoris as the disease state to which SCS may have therapeutic benefits (Eliasson and others 1996; Mannheimer and others 1993). The results of those studies indicated that thoracic SCS decreased anginal pain symptoms, increased exercise tolerance, and delayed the onset of anginal symptoms(Eliasson and others 1996; Mannheimer and others 1993) . These clinical observations prompted subsequent basic science research into how SCS exerted its anti-anginal effects (Cardinal and others 2006; Foreman and others 2000; Southerland and others 2007).

Basic science studies have elucidated that thoracic SCS decreases intrinsic cardiac and extracardiac sympathetic neuronal reflex activation in response to MI stress and, if initiated pre-emptively, decreases the infarct size resulting from MI (Ardell and others 2009; Foreman and others 2000; Southerland and others 2007). SCS does so without changing blood flow through coronary vessels at rest or during ischemic stress or by changing pressure-volume ratio within the left ventricle (Ardell and others 2009; Kingma and others 2001). Transection of the ansae subclavian, interruption of afferent and efferent interconnections between the spinal cord and peripheral aspects of the cardiac nervous system, eliminates the cardioprotective effects of thoracic SCS (Cardinal and others 2006; Foreman and others 2000). These data suggest that the SCS-induced effects are mediated by changing neural activity within the cardiac nervous system (Armour 1999; 2004; 2008).

In this regard, SCS has demonstrated efficacy to modify neural processing in multiple levels of the cardiac nervous system hierarchy and the effects are long-lived (Armour 1999; 2004; 2008). Thoracic SCS decreases the cardiac nociception transduction in dorsal laminae (I-V) of the spinal cord, a response that affects not only evoked components of cardiac pain but also induced changes in autonomic functions (Ardell and others 2009; Qin and others 2008). MI causes the release of several chemicals including bradykinin and adenosine into the milieu of the heart (Armour 1999; Herring and Paterson 2009). Exogeneous application of those chemicals can therefore be used to mimic the effects of acute MI (Chandler and others 2000; Gnecchi-Ruscone and others 1995; Huang and others 1996). Research shows that SCS can modulate the effects of MI by decreasing the responsiveness of the cardiac nervous system to those "algogenic" chemicals (Qin and others 2008). The exact nature of the mechanism through which SCS can decrease the responsiveness of the cardiac nervous system and therefore decrease the detrimental effects of MI remains to be determined (Armour 1999; 2004; 2008).

Cardiac stress impacts cardiac electrical and mechanical function and the neurohumoral systems that modulates it (Armour 1999; 2004). SCS reduces electrically-induced arrhythmia formation and naturally-occurring arrhythmias (Cardinal and others 2006; Issa and others 2005; Lopshire and others 2009; Zipes 2008). For example, SCS has been shown to reduce the atrial arrhythmogenic potential arising from induced neural imbalance within the cardiac nervous system (Cardinal and others 2006). Thoracic SCS also decreased the incidence of ventricular arrhythmia formation in response to transient ischemia following rapid ventricular pacing in an animal model with a previous ischemic injury (Issa and others 2005; Lopshire and others 2009). Finally, SCS improves cardiac contractile function during recovery from experimentally-induced heart failure (Lopshire and others 2009). Together, these data demonstrate the overall efficacy of SCS to mitigate adverse consequences of cardiac stress.

In addition to impacting the cardiac nervous system, SCS also exerts cardioprotective effects, in part, by modulating cardiomyocytes and their response to stress (Ardell and others 2010; Southerland and others 2007). SCS activates intracellular signaling pathways, such as protein kinase C (PKC) (Ardell and others 2010; Southerland and others 2007). Activating PKC pathways is a major mediator in preconditioning the heart to reduce the effects of ischemia (Sanada and Kitakaze 2004; Yellon and Downey 2003). These mechanisms serve to protect the myocardium from the insult of MI (Ardell and others 2010; Sanada and Kitakaze 2004; Southerland and others 2007; Yellon and Downey 2003). The specifics of how SCS-induced cardioprotection is imparted on neural and myocyte function is the central focus of this dissertation.

Neuromodulation of the Ischemic Heart

SCS Effects on Afferent Transduction

One theory for the mechanism of action of SCS is that SCS directly activates afferent neurons causing them to act in an efferent fashion. The theory of afferent neurons acting as efferents was put forth based on data that SCS-mediated antidromic activation of afferent neurons causes the release of neuropeptides in the periphery that induce vasodilation (Horsch and others 2004; Lembeck and Holzer 1979; Linderoth and others 1994; Tanaka and others 2003; White and Helme 1985). This vasodilation, in turn, salvaged ischemic tissue in a skin flap preparation (Linderoth and others 1991; Tanaka and others 2003). While antidromic activation of afferent fibers accounts for peripheral vasodilation for cutaneous tissues, Chapter 3 indicates that SCS does not antidromically activate primary cardiac-related afferent neurons (Gherardini and others 1999; Horsch and others 2004). These data point to a different mechanism. Clinical studies put forth the hypothesis that SCS-induced anti-anginal effects by altering supply/demand balance (Linderoth and Foreman 1999). However, subsequent research has demonstrated that thoracic SCS does not induce

coronary blood flow changes, at least in a normal heart, and has minimal effects on pressure-volume characteristics of the normal or ischemia-stressed heart (Kingma and others 2001). Yet, SCS does blunt MI-induced activation of dorsal column neurons receiving inputs from cardiac nociceptors (Ding and others 2008a; Ding and others 2008b). Based on the studies outlined in Chapter 3, we propose the hypothesis that pre-emptive thoracic SCS modifies the milieu of the heart arising in response to stress which secondarily alters the sensory transduction of cardiac stress (Armour 2008). That is to say, pre-emptive SCS renders cardiomyocytes ischemic-resistant and thereby mitigates the MI-induced release of metabolites that activate the cardiac nociceptors.

SCS Effects on Infarct Size

While thoracic neuromodulation induces cardioprotective effects, it remained to be determined if cervical SCS exerts cardioprotection against the effects of acute MI (Southerland and others 2007). Previous studies showed that pre-emptive thoracic SCS decreased infarct size in response to transient MI (Southerland and others 2007). Previous work also demonstrated that high cervical spinal segments modified neural activity in thoracic and lumbar regions of the spinal cord arising from peripheral organs (Ding and others 2008a; Ding and others 2008b; Qin and others 2007a; Qin and others 2008; Qin and others 2007b). Several mechanisms of the potential cardioprotective effects of high cervical SCS were investigated in this dissertation, including the role of spinal cord segmental and supraspinal interconnections and their role in modifying adrenergic and cholinergic efferent functions.

Intersegmental communication underlies the ability of high cervical SCS to exert cardioprotective effects. This requirement of intersegmental communication was demonstrated by how C6 cervical dorsal column transection eliminated that cardioprotection. Chapter 2 determined for the first time that communication between the cervical and thoracic spinal segments is necessary

for cervical SCS to exert its cardioprotective effects (Hua and others 2004a; Hua and others 2004b; Qin and others 2004b; Qin and others 2007b). These data indicate that the upper cervical spinal segments may in fact function as a coordinating center for lower spinal cord reflex function with respect to the cardiac nervous system (Ding and others 2008a; Ding and others 2008b; Qin and others 2007a; Qin and others 2008; Qin and others 2007b).

High cervical neuromodulation uses peripheral ganglia of the cardiac nervous system to induce its cardioprotective effects. Previous research indicates that intrinsic cardiac ganglia contain adrenergic and cholinergic efferents, a fact that Chapter 4 corroborates (Armour 1997; Hoard and others 2008; Hoover and others 2009; Singh and others 1999). Data presented in Chapter 2 demonstrate that selective blockade of adrenergic receptors or the cholinergic muscarinic receptors differentially modifies the ability of C1-C2 SCS to mitigate the MI-induced changes in myocyte viability in response to transient MI. The fact that cervical vagotomy, in contradistinction to atropine, produced no change in the ability of SCS to protect myocytes is likely reflective of the reflex processing that remains in the peripheral aspect of the cardiac nervous system, even when surgically disconnected from higher centers (Ardell and others 1991; Armour 2008; Armour and others 2005).

Previous work has indicated the predominant role of SCS-induced changes in sympathetic reflex function in mediating overall cardioprotection (Armour 1997; Armour and others 2005; Richer and others 2008). The ability of selective adrenergic blockade to eliminate the cardiomyocyte protective effects of SCS in response to MI substantiates that SCS exerts its effects via modulation of adrenergic function using both α- and β-dependent receptors (Southerland and others 2007). This adrenergic mediation of SCS cardioprotective effects is substantiated by previous research where surgical disruption of the sympathetic afferent/efferent pathways, via transection of the ansae

subclavian, eliminates the protective effects of SCS (Cardinal and others 2006; Foreman and others 2000). The fact that intrinsic cardiac neurons, of which a large population is local circuit, also contain adrenergic receptors suggests that SCS mediates its cardiomyocyte protective effects, in part, through modifying neural subtypes at both intracardiac and extracardiac ganglia, as well as at the end effectors of the heart (Armour 2008; Armour and others 2002; Cardinal and others 2006; Foreman and others 2000; Southerland and others 2007). Chapter 4 indicates that SCS preferentially modifies local circuit neuronal activity without affecting efferent outflow to the heart. Together, these findings implicate the local circuit neurons, which are contained throughout the cardiac nervous system, in mediating, in part, the cardiomyocyte protective effects of SCS (Armour 2008).

In addition to modulation of reflex function within the cardiac nervous system, SCS also modulates myocyte function (Southerland and others 2007). For example, SCS-induced activation of the protein kinase C pathways contributes to the infarct reduction associated with pre-emptive SCS (Southerland and others 2007). Other intracellular signaling pathways such as α-adrenoceptor coupled PKC pathways and β-adrenoceptor coupled PKA and p38 MAPK pathways, when activated can also reduce the lethal effects of MI on cardiomyocytes (Sanada and Kitakaze 2004; Tsuchida and others 1994; Yellon and Downey 2003). The fact that both α- and β-receptor blockade blunted SCS-induced reduction in infarct size substantiates this hypothesis.

The ability of pre-emptive SCS to impact the cardiac myocytes is also demonstrated by the modification of the contractility response to acute MI. While SCS has no discernible effects on gross hemodynamics during unstressed states, left ventricular function was significantly improved during transient MI when done in the presence of SCS (Foreman and others 2000; Kingma and others 2001; Southerland and others 2007). It remains to be determined if this effect is produced by induced changes in neural reflex processing in response to transient MI or if the myocytes are

rendered ischemia-resistant by pre-emptive SCS (Kingma and others 2001; Sanada and Kitakaze 2004; Tsuchida and others 1994; Yellon and Downey 2003).

SCS and the Potential for Ischemia-Induced SCD

MI-induced changes in cardiac electrical stability involve both intrinsic and extrinsic factors (Armour 1999). Intrinsic effects include alterations in cardiac conduction and induction of ectopic pacemakers (Armour 1999). Extrinsic factors include alterations in autonomic reflex control involving both parasympathetic and sympathetic efferent projections (Armour 1999; 2004). Without SCS, atropine evoked minimal changes on the potential for MI-induced ventricular fibrillation. Following vagotomy, the potential for MI-induced ventricular fibrillation increased. This is in agreement with previous studies where increased parasympathetic efferent outflow to the heart has anti-arrhythmogenic effects (Schultz 2001; Vanoli and others 2008). By inference therefore, the elimination of all centrally-derived parasympathetic efferent outflow should result in the emergence of an exaggerated sympathetic efferent reflex response to MI, an effect that would increase lethal arrhythmia formation (Chen and others 2001; Schwartz 2001). With high cervical SCS, atropine moderately increased the potential for MI-induced ventricular fibrillation; but cervical vagotomy prevented it. The elimination of lethal arrhythmia formation from cervical vagotomy in the presence of MI and SCS likely reflects the reduction in heterogeneity of autonomic inputs to the heart coupled with neurally- (sympathetic) induced stabilization of the intrinsic cardiac neuronal reflex processing (Armour and others 2002; Foreman and others 2000; Huang and others 1993). These data support the theory that SCS-induced stabilization of the cardiac nervous system is fundamental to the anti-arrhythmic effects of SCS (Zipes 2008). These data further indicate that the level of SCS influences the involvement of sympathetic versus parasympathetic efferent activity so

modulated. Cervical SCS moderation of cardiac electrical function involves both branches of the autonomic nervous system. Thoracic SCS primarily involves changes in efferent function in extrinsic sympathetic and intrinsic cardiac neural systems.

Neuromodulation of arrhythmias

Arrhythmia formation can result from excessive neuronal input to the intrinsic cardiac nervous system (Armour 2004; Armour and others 2005; Cardinal and others 2006; Richer and others 2008). As demonstrated in Chapter 4, excessive neuronal input to the intrinsic cardiac nervous system alters the activity patterns of the intrinsic cardiac neurons (Armour and others 2005). Global suppression of peripheral autonomic ganglia activity with hexamethonium blunts both the neurally evoked response to MSNS and the potential for induction of atrial arrhythmias. However, such a therapeutic approach has limited applicability due to its consequent effect on overall cardiovascular control (Armour and others 2005; Miyazaki and others 1989; Randall and others 1988). Electrical neuromodulation approach has the potential to evoke more discrete alterations in local reflex function without compromising overall cardiovascular control (Armour 2008). For example, modulation of intrinsic cardiac neurons, through blocking either adrenergic or cholinergic receptors, modifies the potential for formation of arrhythmias (Armour and others 2005; Richer and others 2008). We suggest that adrenergic/cholinergic receptor-mediated stabilizing effect to induced nerve imbalances reflects modulation of neural processing within the intrinsic cardiac nervous system. In support of this hypothesis, Chapter 4 indicates that SCS and hexamethonium modulate the intrinsic cardiac neuronal response to MSNS with a corresponding decrease in the potential for atrial arrhythmogenesis (Armour 1997; Cardinal and others 2006). Future studies should focus on

the effects of specific receptor agonists and antagonists to modify these neuronal populations and their response to evoked stress.

Conclusions

This dissertation has determined some of the primary neural mechanisms by which SCS neuromodulation-based therapies induces cardioprotection in the stressed heart. The ability of SCS at the cervical spinal segments to reduce the infarct size or potential for SCD has implications for future uses of cervical SCS as a potential therapeutic target for treatment of ischemic heart disease. The ability of SCS to minimize electrically-induced arrhythmia formation has implications for effective treatment of arrhythmia formation of atrial or ventricular origin. The effects of SCS on afferent transduction of ischemic cardiac stress demonstrate the potential of SCS to render cardiomyocytes resistant to transient periods of MI. Together, these data indicate the effectiveness of SCS to help maintain a state of homeostasis in the face of the destabilizing effects of cardiac disease.

Topics for Future Study

Like all research studies, the current study has answered some questions and also elucidated topics that need to be investigated. This is a list of some of those topics:

- Investigate the effects of selective adrenergic and/or cholinergic receptor blockade on the activity of the intrinsic cardiac neurons in response to MSNS-induced AF.

- Determine how adrenergic receptor systems contribute to the actions of SCS in reducing the reflex activation of the intrinsic cardiac neurons in response to MSNS.

- Determine the specific neurochemical phenotypes of the intrinsic cardiac neurons and how they respond to imbalances in neural inputs, with and without SCS.

- Determine the specific neurochemical phenotypes of the intrinsic cardiac neurons and how they respond to imbalances in the stress of MI, with and without SCS.

REFERENCES

Adams DJ, Cuevas J. 2004. Electrophysiological properties of intrinsic cardiac neurons. In: Armour JA, Ardell JL, editors. Basic and clinical neurocardiology. New York: Oxford University Press. p. 1-60.

Andrade PJ, Medeiros MM, Andrade AT, Lima AA. 2011. Coronary angioplasty versus CABG: review of randomized. Arq Bras Cardiol 97(3):e60-e69.

Ardell JL. 2004. Intrathoracic neuronal regulation of cardiac function. In: Armour JA, Ardell JL, editors. Basic and clinical neurocardiology. New York: Oxford University Press. p. 118–152.

Ardell JL. 2011. The cardiac neuronal hierarchy and susceptibility to arrhythmias. Heart Rhythm 8(4):590-1.

Ardell JL, Barman SM, Gebber GL. 1982. Sympathetic nerve discharge in chronic spinal cat. Am J Physiol 243(3):H463-70.

Ardell JL, Butler CK, Smith FM, Hopkins DA, Armour JA. 1991. Activity of in vivo atrial and ventricular neurons in chronically decentralized canine hearts. Am J Physiol 260(3 Pt 2):H713-21.

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 Regul Integr Comp Physiol 297(2):R470-7.

Ardell JL, Foreman RD. 2009. Neuronal control of the heart. In: Krames E, Peckman PH, Reza A, editors. Textbook of Neuromodulation. Elsevier. p. 793-799.

Ardell JL, Randall WC. 1986. Selective vagal innervation of sinoatrial and atrioventricular nodes in canine heart. Am J Physiol 251(4 Pt 2):H764-73.

Ardell JL, Southerland EM, Armour JA. 2010. Intrathoracic neurons modify myocardial ischemia-induced sudden cardiac death. p. 809.14.

Armour JA. 1976. Instant-to-instant reflex cardiac regulation. Cardiology 61:309-328.

Armour JA. 1986a. Activity of in situ stellate ganglion neurons of dogs recorded extracellularly. Can J Physiol Pharmacol 64(2):101-11.

Armour JA. 1986b. Neuronal activity recorded extracellularly in chronically decentralized in situ canine middle cervical ganglia. Can J Physiol Pharmacol 64(7):1038-46.

Armour JA. 1991. Anatomy and function of the intrathoracic neurons regulating the mammalian heart. In: Zucker IH, Gilmore JP, editors. Reflex control of the circulation. Boca Raton: CRC Press. p. 1-37.

Armour JA. 1997. Intrinsic cardiac neurons involved in cardiac regulation possess alpha 1-, alpha 2-, beta 1- and beta 2-adrenoceptors. Can J Cardiol 13(3):277-84.

Armour JA. 1999. Myocardial ischaemia and the cardiac nervous system. Cardiovasc Res 41(1):41-54.

Armour JA. 2004. Cardiac neuronal hierarchy in health and disease. Am J Physiol Regul Integr Comp Physiol 287(2):R262-71.

Armour JA. 2008. Potential clinical relevance of the 'little brain' on the mammalian heart. Exp Physiol 93(2):165-76.

Armour JA, Collier K, Kember G, Ardell JL. 1998. Differential selectivity of cardiac neurons in separate intrathoracic autonomic ganglia. Am J Physiol 274(4 Pt 2):R939-49.

Armour JA, Hopkins DA. 1990. Activity of canine in situ left atrial ganglion neurons. Am J Physiol 259(4 Pt 2):H1207-15.

Armour JA, Janes RD. 1988. Neuronal activity recorded extracellularly from in situ canine mediastinal ganglia. Can J Physiol Pharmacol 66(2):119-27.

Armour JA, Kember GC. 2004. Cardiac sensory neurons. In: Armour JA, Ardell JL, editors. Basic and clinical neurocardiology. New York: Oxford University Press. p. 79-117.

Armour JA, Linderoth B, Arora RC, DeJongste MJ, Ardell JL, Kingma JG, Jr., Hill M, Foreman RD. 2002. Long-term modulation of the intrinsic cardiac nervous system by spinal cord neurons in normal and ischaemic hearts. Autonomic Neuroscience 95(1-2):71-9.

Armour JA, Murphy DA, Yuan BX, Macdonald S, Hopkins DA. 1997. Gross and microscopic anatomy of the human intrinsic cardiac nervous system. Anat Rec 247(2):289-98.

Armour JA, Richer LP, Page P, 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 118(1-2):68-78.

Association TBCP. Heart Drugs [Internet]. Available from: http://www.bcpa.co.uk/pdf/HeartDrugs.pdf

Augustinsson LE, Linderoth B, Mannheimer C, Eliasson T. 1995. Spinal cord stimulation in cardiovascular disease. Neurosurg Clin N Am 6(1):157-65.

Bosnjak ZJ, Kampine JP. 1989. Cardiac sympathetic afferent cell bodies are located in the peripheral nervous system of the cat. Circ Res 64(3):554-62.

Brown AM. 1967. Excitation of afferent cardiac sympathetic nerve fibres during myocardial ischaemia. J Physiol 190(1):35-53.

Burkhardt JD, Natale A. 2009. New technologies in atrial fibrillation ablation. Circulation 120(15):1533-41.

Burwash IG, Morgan DE, Koilpillai CJ, Blackmore GL, Johnstone DE, Armour JA. 1993. Sympathetic stimulation alters left ventricular relaxation and chamber size. Am J Physiol 264(1 Pt 2):R1-7.

Cardinal R. 2004. Neuronal modulation of atrial and ventricular electrical properties. In: Armour JA, Ardell JL, editors. Basic and clinical neurocardiology. New York: Oxford University Press. p. 315-339.

Cardinal R, Ardell JL, Linderoth B, Vermeulen M, Foreman RD, Armour JA. 2004. Spinal cord activation differentially modulates ischaemic electrical responses to different stressors in canine ventricles. Autonomic Neuroscience 111(1):37-47.

Cardinal R, Page P, Vermeulen M, Bouchard C, Ardell JL, Foreman RD, Armour JA. 2006. Spinal cord stimulation suppresses bradycardias and atrial tachyarrhythmias induced by mediastinal nerve stimulation in dogs. Am J Physiol Regul Integr Comp Physiol 291(5):R1369-75.

Carlson MD, Smith ML, Thames MD. 1992. Autonomic Influences on Arrhythmia Development. In: Zipes DP, Rowlands DJ, editors. Progress in cardiology. Philadelphia,: Lea & Febiger. p. 59-73.

Chandler MJ, Brennan TJ, Garrison DW, Kim KS, Schwartz PJ, Foreman RD. 1993. A mechanism of cardiac pain suppression by spinal cord stimulation: implications for patients with angina pectoris. Eur Heart J 14(1):96-105.

Chandler MJ, Zhang J, Foreman RD. 1996. Vagal, sympathetic and somatic sensory inputs to upper cervical (C1-C3) spinothalamic tract neurons in monkeys. J Neurophysiol 76(4):2555-67.

Chandler MJ, Zhang J, Qin C, Yuan Y, Foreman RD. 2000. Intrapericardiac injections of algogenic chemicals excite primate C1-C2 spinothalamic tract neurons. Am J Physiol Regul Integr Comp Physiol 279(2):R560-8.

Chen LS, Zhou S, Fishbein MC, Chen PS. 2007. New perspectives on the role of autonomic nervous system in the genesis of arrhythmias. J Cardiovasc Electrophysiol 18(1):123-7.

Chen PS, Chen LS, Cao JM, Sharifi B, Karagueuzian HS, Fishbein MC. 2001. Sympathetic nerve sprouting, electrical remodeling and the mechanisms of sudden cardiac death. Cardiovasc Res 50(2):409-16.

Cheng Z, Powley TL, Schwaber JS, Doyle FJ, 3rd. 1999. Projections of the dorsal motor nucleus of the vagus to cardiac ganglia of rat atria: an anterograde tracing study. J Comp Neurol 410(2):320-41.

Choi EK, Shen MJ, Han S, Kim D, Hwang S, Sayfo S, Piccirillo G, Frick K, Fishbein MC, Hwang C et al. . 2011. Intrinsic cardiac nerve activity and paroxysmal atrial tachyarrhythmia in ambulatory dogs. Circulation 121(24):2615-23.

Darvesh S, Nance DM, Hopkins DA, Armour JA. 1987. Distribution of neuropeptide-like immunoreactivity in intact and chronically decentralized middle cervical and stellate ganglia of dogs. J Auton Nerv Syst 21(2-3):167-80.

Dell'Italia LJ, Ardell JL. 2004. Sympathetic nervous system in the evolution of heart failure. In: Armour JA, Ardell JL, editors. Basic and clinical neurocardiology. New York: Oxford University Press. p. 340–367.

Ding X, Ardell JL, Hua F, McAuley RJ, Sutherly K, Daniel JJ, Williams CA. 2008a. Modulation of cardiac ischemia-sensitive afferent neuron signaling by preemptive C2 spinal cord stimulation: effect on substance P release from rat spinal cord. Am J Physiol Regul Integr Comp Physiol 294(1):R93-101.

Ding X, Hua F, Sutherly K, Ardell JL, Williams CA. 2008b. C2 spinal cord stimulation induces dynorphin release from rat T4 spinal cord: potential modulation of myocardial ischemia-sensitive neurons. Am J Physiol Regul Integr Comp Physiol 295(5):R1519-28.

Eddicks S, Maier-Hauff K, Schenk M, Muller A, Baumann G, Theres H. 2007. Thoracic spinal cord stimulation improves functional status and relieves symptoms in patients with refractory angina pectoris: the first placebo-controlled randomised study. Heart 93(5):585-90.

Eliasson T, Albertsson P, Hardhammar P, Emanuelsson H, Augustinsson LE, Mannheimer C. 1993. Spinal cord stimulation in angina pectoris with normal coronary arteriograms. Coron Artery Dis 4(9):819-27.

Eliasson T, Augustinsson LE, Mannheimer C. 1996. Spinal cord stimulation in severe angina pectoris--presentation of current studies, indications and clinical experience. Pain 65(2-3):169-79.

Foreman RD. 1999. Mechanisms of cardiac pain. Annu Rev Physiol 61:143-67.

Foreman RD, De Jongste MJ, Linderoth B. 2004. Integrative Control of Cardiac Function by Cervical and Thoracic Spinal Neurons. In: Armour JA, Ardell JL, editors. Basic and clinical neurocardiology. New York: Oxford University Press. p. 153-186.

Foreman RD, Linderoth B, Ardell JL, Barron KW, Chandler MJ, Hull SS, Jr., TerHorst GJ, DeJongste MJ, Armour JA. 2000. Modulation of intrinsic cardiac neurons by spinal cord stimulation: implications for its therapeutic use in angina pectoris. Cardiovasc Res 47(2):367-75.

Forsgren S, Moravec M, Moravec J. 1990. Catecholamine-synthesizing enzymes and neuropeptides in rat heart epicardial ganglia; an immunohistochemical study. Histochem J 22(12):667-76.

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(4 Pt 2):H789-800.

Gherardini G, Lundeberg T, Cui JG, Eriksson SV, Trubek S, Linderoth B. 1999. Spinal cord stimulation improves survival in ischemic skin flaps: an experimental study of the possible mediation by calcitonin gene-related peptide. Plast Reconstr Surg 103(4):1221-8.

Gibbons DD, Southerland EM, Hoover DB, Beaumont E, Armour JA, Ardell JL. 2011. Neuromodulation targets intrinsic cardiac neurons to attenuate neuronally mediated atrial arrhythmias. Am J Physiol Regul Integr Comp Physiol 302(3):R357-64.

Gnecchi-Ruscone T, Montano N, Contini M, Guazzi M, Lombardi F, Malliani A. 1995. Adenosine activates cardiac sympathetic afferent fibers and potentiates the excitation induced by coronary occlusion. J Auton Nerv Syst 53(2-3):175-84.

Gonzalez-Darder JM, Canela P, Gonzalez-Martinez V. 1991. High cervical spinal cord stimulation for unstable angina pectoris. Stereotact Funct Neurosurg 56(1):20-7.

Gray AL, Johnson TA, Ardell JL, Massari VJ. 2004. Parasympathetic control of the heart. II. A novel interganglionic intrinsic cardiac circuit mediates neural control of heart rate. J Appl Physiol 96(6):2273-8.

Hankes GH, Ardell JL, Tallaj J, Wei CC, Aban I, Holland M, Rynders P, Dillon R, Cardinal R, Hoover DB et al. . 2006. Beta1-adrenoceptor blockade mitigates excessive norepinephrine release into cardiac interstitium in mitral regurgitation in dog. Am J Physiol Heart Circ Physiol 291(1):H147-51.

Hautvast RW, DeJongste MJ, Staal MJ, van Gilst WH, Lie KI. 1998. Spinal cord stimulation in chronic intractable angina pectoris: a randomized, controlled efficacy study. Am Heart J 136(6):1114-20.

Herring N, Paterson DJ. 2009. Neuromodulators of peripheral cardiac sympatho-vagal balance. Exp Physiol 94(1):46-53.

Hoard JL, Hoover DB, Mabe AM, Blakely RD, Feng N, Paolocci N. 2008. Cholinergic neurons of mouse intrinsic cardiac ganglia contain noradrenergic enzymes, norepinephrine transporters, and the neurotrophin receptors tropomyosin-related kinase A and p75. Neuroscience 156(1):129-42.

Hoover DB, Isaacs ER, Jacques F, Hoard JL, Page P, Armour JA. 2009. Localization of multiple neurotransmitters in surgically derived specimens of human atrial ganglia. Neuroscience 164(3):1170-9.

Hoover DB, Shepherd AV, Southerland EM, Armour JA, Ardell JL. 2008. Neurochemical diversity of afferent neurons that transduce sensory signals from dog ventricular myocardium. Autonomic Neuroscience 141(1-2):38-45.

Hopkins DA, Armour JA. 1984. Localization of sympathetic postganglionic and parasympathetic preganglionic neurons which innervate different regions of the dog heart. J Comp Neurol 229(2):186-98.

Hopkins DA, Armour JA. 1989. Ganglionic distribution of afferent neurons innervating the canine heart and cardiopulmonary nerves. J Auton Nerv Syst 26(3):213-22.

Horackova M, Armour JA, Byczko Z. 1999. Distribution of intrinsic cardiac neurons in whole-mount guinea pig atria identified by multiple neurochemical coding. A confocal microscope study. Cell Tissue Res 297(3):409-21.

Horackova M, Croll RP, Hopkins DA, Losier AM, Armour JA. 1996. Morphological and immunohistochemical properties of primary long-term cultures of adult guinea-pig ventricular cardiomyocytes with peripheral cardiac neurons. Tissue Cell 28(4):411-25.

Horsch S, Schulte S, Hess S. 2004. Spinal cord stimulation in the treatment of peripheral vascular disease: results of a single-center study of 258 patients. Angiology 55(2):111-8.

Hua F, Harrison T, Qin C, Reifsteck A, Ricketts B, Carnel C, Williams CA. 2004a. c-Fos expression in rat brain stem and spinal cord in response to activation of cardiac ischemia-sensitive afferent neurons and electrostimulatory modulation. Am J Physiol Heart Circ Physiol 287(6):H2728-38.

Hua F, Ricketts BA, Reifsteck A, Ardell JL, Williams CA. 2004b. Myocardial ischemia induces the release of substance P from cardiac afferent neurons in rat thoracic spinal cord. Am J Physiol Heart Circ Physiol 286(5):H1654-64.

Huang MH, Ardell JL, Hanna BD, Wolf SG, Armour JA. 1993. Effects of transient coronary artery occlusion on canine intrinsic cardiac neuronal activity. Integr Physiol Behav Sci 28(1):5-21.

Huang MH, Horackova M, Negoescu RM, Wolf S, Armour JA. 1996. Polysensory response characteristics of dorsal root ganglion neurones that may serve sensory functions during myocardial ischaemia. Cardiovasc Res 32(3):503-15.

Huang MH, Wolf SG, Armour JA. 1994. Ventricular arrhythmias induced by chemically modified intrinsic cardiac neurones. Cardiovasc Res 28(5):636-42.

Ishibashi H, Umezu M, Jang IS, Ito Y, Akaike N. 2003. Alpha 1-adrenoceptor-activated cation currents in neurones acutely isolated from rat cardiac parasympathetic ganglia. J Physiol 548(Pt 1):111-20.

Issa ZF, Zhou X, Ujhelyi MR, Rosenberger J, Bhakta D, Groh WJ, Miller JM, Zipes DP. 2005. Thoracic spinal cord stimulation reduces the risk of ischemic ventricular arrhythmias in a postinfarction heart failure canine model. Circulation 111(24):3217-20.

Iwasaki YK, Nishida K, Kato T, Nattel S. 2011. Atrial fibrillation pathophysiology: implications for management. Circulation 124(20):2264-74.

Kajstura J, Bolli R, Sonnenblick EH, Anversa P, Leri A. 2006. Cause of death: suicide. J Mol Cell Cardiol 40(4):425-37.

Khalil PN, Neuhof C, Huss R, Pollhammer M, Khalil MN, Neuhof H, Fritz H, Siebeck M. 2005. Calpain inhibition reduces infarct size and improves global hemodynamics and left ventricular contractility in a porcine myocardial ischemia/reperfusion model. Eur J Pharmacol 528(1-3):124-31.

Kingma JG, Jr., Linderoth B, Ardell JL, Armour JA, DeJongste MJ, Foreman RD. 2001. Neuromodulation therapy does not influence blood flow distribution or left-ventricular dynamics during acute myocardial ischemia. Autonomic Neuroscience 91(1-2):47-54.

Krames ES, Foreman R. 2007. Spinal Cord Stimulation Modulates Visceral Nociception and Hyperalgesia via the Spinothalamic Tracts and the Postsynaptic Dorsal Column Pathways: A Literature Review and Hypothesis. Neuromodulation 10(3):224-37.

Kuo DC, Oravitz JJ, DeGroat WC. 1984. Tracing of afferent and efferent pathways in the left inferior cardiac nerve of the cat using retrograde and transganglionic transport of horseradish peroxidase. Brain Res 321(1):111-8.

Langley G. 1921. The Autonomic Nervous System. Cambridge, UK: Cambridge University Press.

Lembeck F, Holzer P. 1979. Substance P as neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation. Naunyn Schmiedebergs Arch Pharmacol 310(2):175-83.

Linderoth B, Fedorcsak I, Meyerson BA. 1991. Peripheral vasodilatation after spinal cord stimulation: animal studies of putative effector mechanisms. Neurosurgery 28(2):187-95.

Linderoth B, Foreman RD. 1999. Physiology of spinal cord stimulation: review and update. Neuromodulation 2(3):150-64.

Linderoth B, Herregodts P, Meyerson BA. 1994. Sympathetic mediation of peripheral vasodilation induced by spinal cord stimulation: animal studies of the role of cholinergic and adrenergic receptor subtypes. Neurosurgery 35(4):711-9.

Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, Ford E, Furie K, Go A, Greenlund K et al. . 2009. Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 119(3):480-6.

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(4):286-94.

Lu Z, Scherlag BJ, Lin J, Yu L, Guo JH, Niu G, Jackman WM, Lazzara R, Jiang H, Po SS. 2009. Autonomic mechanism for initiation of rapid firing from atria and pulmonary veins: evidence by ablation of ganglionated plexi. Cardiovasc Res 84(2):245-52.

Mannheimer C, Camici P, Chester MR, Collins A, DeJongste M, Eliasson T, Follath F, Hellemans I, Herlitz J, Luscher T et al. . 2002. The problem of chronic refractory angina; report from the ESC Joint Study Group on the Treatment of Refractory Angina. Eur Heart J 23(5):355-70.

Mannheimer C, Eliasson T, Andersson B, Bergh CH, Augustinsson LE, Emanuelsson H, Waagstein F. 1993. Effects of spinal cord stimulation in angina pectoris induced by pacing and possible mechanisms of action. Bmj 307(6902):477-80.

Mannheimer C, Eliasson T, Augustinsson LE, Blomstrand C, Emanuelsson H, Larsson S, Norrsell H, Hjalmarsson A. 1998. Electrical stimulation versus coronary artery bypass surgery in severe angina pectoris: the ESBY study. Circulation 97(12):1157-63.

Marieb EN. 2004. Human anatomy & physiology. New York: Pearson Education.

Matsubayashi Y, Iwai L, Kawasaki H. 2008. Fluorescent double-labeling with carbocyanine neuronal tracing and immunohistochemistry using a cholesterol-specific detergent digitonin. J Neurosci Methods 174(1):71-81.

Maxwell MP, Hearse DJ, Yellon DM. 1987. Species variation in the coronary collateral circulation during regional myocardial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction. Cardiovasc Res 21(10):737-46.

Miyazaki T, Pride HP, Zipes DP. 1989. Modulation of cardiac autonomic neurotransmission by epicardial superfusion. Effects of hexamethonium and tetrodotoxin. Circ Res 65(5):1212-9.

Mobilia G, Zuin G, Zanco P, Di Pede F, Pinato G, Neri G, Cargnel S, Raviele A, Ferlin G, Buchberger R. 1998. [Effects of spinal cord stimulation on regional myocardial blood flow in patients with refractory angina. A positron emission tomography study]. G Ital Cardiol 28(10):1113-9.

Moravec J, Moravec M. 1987. Intrinsic nerve plexus of mammalian heart: morphological basis of cardiac rhythmical activity? Int Rev Cytol 106:89-148.

Murphy DA, Thompson GW, Ardell JL, McCraty R, Stevenson RS, Sangalang VE, Cardinal R, Wilkinson M, Craig S, Smith FM et al. . 2000. The heart reinnervates after transplantation. Ann Thorac Surg 69(6):1769-81.

Odenstedt J, Linderoth B, Bergfeldt L, Ekre O, Grip L, Mannheimer C, Andrell P. 2011. Spinal cord stimulation effects on myocardial ischemia, infarct size, ventricular arrhythmia, and noninvasive electrophysiology in a porcine ischemia-reperfusion model. Heart Rhythm 8(6):892-8.

Olgin JE, Takahashi T, Wilson E, Vereckei A, Steinberg H, Zipes DP. 2002. Effects of thoracic spinal cord stimulation on cardiac autonomic regulation of the sinus and atrioventricular nodes. J Cardiovasc Electrophysiol 13(5):475-81.

Parsons RL. 2004. Mammalian cardiac ganglia as local integration centers: histochemical and electrophysiological evidence. In: Dun NJ, Machado BH, Pilowsky PM, editors. Neural mechanisms of cardiovascular regulation. Boston: Kluwer Academic Publishers. p. 335-356.

Perry GJ, Wei CC, Hankes GH, Dillon SR, Rynders P, Mukherjee R, Spinale FG, Dell'Italia LJ. 2002. Angiotensin II receptor blockade does not improve left ventricular function and remodeling in subacute mitral regurgitation in the dog. J Am Coll Cardiol 39(8):1374-9.

Plecha DM, Randall WC, Geis GS, Wurster RD. 1988. Localization of vagal preganglionic somata controlling sinoatrial and atrioventricular nodes. Am J Physiol 255(5 Pt 2):R703-8.

Qin C, Farber JP, Foreman RD. 2007a. Spinal cord stimulation modulates activity of lumbosacral spinal neurons receiving input from urinary bladder in rats. Neurosci Lett 428(1):38-42.

Qin C, Farber JP, Linderoth B, Shahid A, Foreman RD. 2008. Neuromodulation of thoracic intraspinal visceroreceptive transmission by electrical stimulation of spinal dorsal column and somatic afferents in rats. J Pain 9(1):71-8.

Qin C, Kranenburg A, Foreman RD. 2004. Descending modulation of thoracic visceroreceptive transmission by C1-C2 spinal neurons. Autonomic Neuroscience 114(1-2):11-6.

Qin C, Lehew RT, Khan KA, Wienecke GM, Foreman RD. 2007b. Spinal cord stimulation modulates intraspinal colorectal visceroreceptive transmission in rats. Neurosci Res 58(1):58-66.

Randall DC, Brown DR, McGuirt AS, Thompson GW, Armour JA, Ardell JL. 2003. Interactions within the intrinsic cardiac nervous system contribute to chronotropic regulation. Am J Physiol Regul Integr Comp Physiol 285(5):R1066-75.

Randall WC, Ardell JL, O'Toole MF, Wurster RD. 1988. Differential autonomic control of SAN and AVN regions of the canine heart: structure and function. Prog Clin Biol Res 275:15-31.

Randall WC, Wurster RD, Randall DC, Xi-Moy SX. 1996. From cardioaccelerator and inhibitory nerves to a heart brain: an evolution of concepts. In: Shepherd JT, Vatner SF, editors. Nervous control of the heart. Australia ; United Kingdom: Harwood. p. 173–200.

Richer LP, Vinet A, Kus T, Cardinal R, Ardell JL, Armour JA. 2008. Alpha-adrenoceptor blockade modifies neurally induced atrial arrhythmias. Am J Physiol Regul Integr Comp Physiol 295(4):R1175-80.

Rysevaite K, Saburkina I, Pauziene N, Vaitkevicius R, Noujaim SF, Jalife J, Pauza DH. 2011. Immunohistochemical characterization of the intrinsic cardiac neural plexus in whole-mount mouse heart preparations. Heart Rhythm 8(5):731-8.

Sabbah HN, Gupta RC, Imai M, Irwin ED, Rastogi S, Rossing MA, Kieval RS. 2011. Chronic electrical stimulation of the carotid sinus baroreflex improves left ventricular function and promotes reversal of ventricular remodeling in dogs with advanced heart failure. Circ Heart Fail 4(1):65-70.

Sanada S, Kitakaze M. 2004. Ischemic preconditioning: emerging evidence, controversy, and translational trials. Int J Cardiol 97(2):263-76.

Sanderson JE, Brooksby P, Waterhouse D, Palmer RB, Neubauer K. 1992. Epidural spinal electrical stimulation for severe angina: a study of its effects on symptoms, exercise tolerance and degree of ischaemia. Eur Heart J 13(5):628-33.

Sanderson JE, Ibrahim B, Waterhouse D, Palmer RB. 1994. Spinal electrical stimulation for intractable angina--long-term clinical outcome and safety. Eur Heart J 15(6):810-4.

Schultz HD. 2001. Cardiac vagal chemosensory afferents. Function in pathophysiological states. Ann N Y Acad Sci 940:59-73.

Schwartz PJ. 2001. QT prolongation, sudden death, and sympathetic imbalance: the pendulum swings. J Cardiovasc Electrophysiol 12(9):1074-7.

Schwartz PJ, Brown AM, Malliani A, Zanchetti A. 1978. Neural mechanisms in cardiac arrhythmias. New York: Raven press.

Schwartz PJ, De Ferrari GM. 2009. Vagal stimulation for heart failure: background and first in-man study. Heart Rhythm 6(11 Suppl):S76-81.

Sha Y, Scherlag BJ, Yu L, Sheng X, Jackman WM, Lazzara R, Po SS. 2011. Low-level right vagal stimulation: anticholinergic and antiadrenergic effects. J Cardiovasc Electrophysiol 22(10):1147-53.

Shen MJ, Choi EK, Tan AY, Han S, Shinohara T, Maruyama M, Chen LS, Shen C, Hwang C, Lin SF et al. . 2011a. Patterns of baseline autonomic nerve activity and the development of pacing-induced sustained atrial fibrillation. Heart Rhythm 8(4):583-9.

Shen MJ, Shinohara T, Park HW, Frick K, Ice DS, Choi EK, Han S, Maruyama M, Sharma R, Shen C et al. . 2011b. Continuous low-level vagus nerve stimulation reduces stellate ganglion nerve activity and paroxysmal atrial tachyarrhythmias in ambulatory canines. Circulation 123(20):2204-12.

Singh S, Johnson PI, Javed A, Gray TS, Lonchyna VA, Wurster RD. 1999. Monoamine- and histamine-synthesizing enzymes and neurotransmitters within neurons of adult human cardiac ganglia. Circulation 99(3):411-9.

Society AP. 2002. Guiding principles for research involving animals and human beings. Am J Physiol Regul Integr Comp Physiol 283(2):R281-3.

Southerland EM, Milhorn DM, Foreman RD, Linderoth B, DeJongste MJ, Armour JA, Subramanian V, Singh M, Singh K, Ardell JL. 2007. Preemptive, but not reactive, spinal cord stimulation mitigates transient ischemia-induced myocardial infarction via cardiac adrenergic neurons. Am J Physiol Heart Circ Physiol 292(1):H311-7.

Sparr HJ, Beaufort TM, Fuchs-Buder T. 2001. Newer neuromuscular blocking agents: how do they compare with established agents? Drugs 61(7):919-42.

Tallaj J, Wei CC, Hankes GH, Holland M, Rynders P, Dillon AR, Ardell JL, Armour JA, Lucchesi PA, Dell'Italia LJ. 2003. Beta1-adrenergic receptor blockade attenuates angiotensin II-mediated catecholamine release into the cardiac interstitium in mitral regurgitation. Circulation 108(2):225-30.

Tanaka S, Barron KW, Chandler MJ, Linderoth B, Foreman RD. 2003. Role of primary afferents in spinal cord stimulation-induced vasodilation: characterization of fiber types. Brain Res 959(2):191-8.

Taylor P. 2006. Agents acting at the neuromuscular junction and autonomic ganglia. In: Goodman LS, Gilman A, Brunton LL, Lazo JS, Parker KL, editors. Goodman & Gilman's the pharmacological basis of therapeutics. 11th ed. New York: McGraw-Hill. p. 217-236.

Thygesen K, Alpert JS, White HD, Jaffe AS, Apple FS, Galvani M, Katus HA, Newby LK, Ravkilde J, Chaitman B et al. . 2007. Universal definition of myocardial infarction. Circulation 116(22):2634-53.

Tsuchida A, Liu Y, Liu GS, Cohen MV, Downey JM. 1994. alpha 1-adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C. Circ Res 75(3):576-85.

Vanoli E, Adamson PB, Hull SS, Jr., Foreman RD, Schwartz PJ. 2008. Prediction of unexpected sudden death among healthy dogs by a novel marker of autonomic neural activity. Heart Rhythm 5(2):300-5.

Vanoli E, De Ferrari GM, Stramba-Badiale M, Hull SS, Jr., Foreman RD, Schwartz PJ. 1991. Vagal stimulation and prevention of sudden death in conscious dogs with a healed myocardial infarction. Circ Res 68(5):1471-81.

Waldmann M, Thompson GW, Kember GC, Ardell JL, Armour JA. 2006. Stochastic behavior of atrial and ventricular intrinsic cardiac neurons. J Appl Physiol 101(2):413-9.

Wang W, Chen JS, Zucker IH. 1991. Carotid sinus baroreceptor reflex in dogs with experimental heart failure. Circ Res 68(5):1294-301.

White DM, Helme RD. 1985. Release of substance P from peripheral nerve terminals following electrical stimulation of the sciatic nerve. Brain Res 336(1):27-31.

Wu M, Linderoth B, Foreman RD. 2008. Putative mechanisms behind effects of spinal cord stimulation on vascular diseases: a review of experimental studies. Autonomic Neuroscience 138(1-2):9-23.

Xu ZJ, Adams DJ. 1993. Alpha-adrenergic modulation of ionic currents in cultured parasympathetic neurons from rat intracardiac ganglia. J Neurophysiol 69(4):1060-70.

Yellon DM, Downey JM. 2003. Preconditioning the myocardium: from cellular physiology to clinical cardiology. Physiol Rev 83(4):1113-51.

Yu L, Scherlag BJ, Li S, Sheng X, Lu Z, Nakagawa H, Zhang Y, Jackman WM, Lazzara R, Jiang H et al. . 2011. Low-level vagosympathetic nerve stimulation inhibits atrial fibrillation inducibility: direct evidence by neural recordings from intrinsic cardiac Ganglia. J Cardiovasc Electrophysiol 22(4):455-63.

Yuan BX, Ardell JL, Hopkins DA, Losier AM, Armour JA. 1994. Gross and microscopic anatomy of the canine intrinsic cardiac nervous system. Anat Rec 239(1):75-87.

Zipes DP. 2008. Heart-brain interactions in cardiac arrhythmias: role of the autonomic nervous system. Cleve Clin J Med 75 Suppl 2:S94-6.

Zucker IH, Hackley JF, Cornish KG, Hiser BA, Anderson NR, Kieval R, Irwin ED, Serdar DJ, Peuler JD, Rossing MA. 2007. Chronic baroreceptor activation enhances survival in dogs with pacing-induced heart failure. Hypertension 50(5):904-10.

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Activated cranial cervical cord neurons affect left ventricular infarct size and the potential for sudden cardiac death

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