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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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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 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, i) cranial cervical spinal neurons affect both adrenergic and cholinergic motor outflows to the heart such that ii) their activation modifies ventricular infarct size and lethal arrhythmogenesis.

Keywords

adrenergic blockade; myocardial infarction; muscarinic blockade; neuromodulation; sudden cardiac death; vagotomy

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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 due 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 manifest by changes in infarct size and the potential for sudden cardiac death, we also sought to elucidate whether such cardioprotection involves not only i) cervical spinal descending projections to the thoracic cord, ii) 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 due to 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% O₂. 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 was 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 (2×3 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 (Southernland *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 2-3mm 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 dyskinesia 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-h reperfusion period.

Animals in protocol 1 ($n = 40$) were subjected to 30 min of regional ventricular ischemia followed by a 3-h 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*).

Preemptive 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 preemptive 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 (Southernland *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 preemptive C1-C2 SCS-induced cardioprotection, 40 additional animals were subjected to *protocol 2* fifteen 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 μ m, 1 ml). The dose of atenolol blocked the tachycardiac response to intravenous isoproterenol (100 μ m, 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*) one hour 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 T₂ spinal cord (identified by placing a small dot of Pontamine blue dye on the dorsal surface of the C₈ spinal cord prior to its removal) was excised and postfixed as previously described (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 previously described (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 H₂O₂ (Fisher, Pittsburgh, PA) to quench endogenous peroxidase activity. The sections were then 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 then 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 then 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, the fos was then visualized by incubation in a solution of 10 mg 3',3'-diaminobenzidine with 7 µL of 30% H₂O₂ in Tris buffer. The reaction was stopped after 4.5 min by transfer of the sections to Tris buffer. The sections

were then mounted on chrome-alum gelatin coated slides, dehydrated through successive ethanol washes followed by a wash in xyline. 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 T₂ 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 immunoreactivity cells in the dorsal horn was expressed as cells per square micrometers.

Statistical analysis

All data are presented as means ($\pm$ 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 transection 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 variable

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 1hr reperfusion.

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.

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).

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 preemptive C1-C2 SCS. These data were compared to data obtained from similar groups following C6 dorsal column transection 1 hour prior to the onset of SCS-coronary artery occlusion (Protocols 1-3). There was a significant change in infarct size when comparing different cord status (intact versus C6 dorsal column transection) vs the absence or presence of SCS ($p=0.036$) with cord status.

In sham animals, ventricular infarct size averaged $43.8 \pm 6.9\%$ of tissue volume. This index was unaffected by prior C6 dorsal column transection ($47.6 \pm 14.7\%$; Fig. 2, column 2). With the spinal cord intact, pre-emptive C1-C2 SCS decreased infarct size to $25.2 \pm 10.7\%$ (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 = $42.8 \pm 9.4\%$). The ability for preemptive C1-C2 SCS to reduce infarct size was also eliminated by pretreatment with prazosin ($40.0 \pm 9.4\%$), atenolol ($40.2 \pm 11.1\%$), or yohimbine ($36.8 \pm 10.3\%$) (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.

Neither atropine nor cervical vagotomy alone (c.f., in the absence of SCS) affected infarct size induced by CAO/ 3hr 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 ($23.6 \pm 11.1\%$) compared to vehicle control ($25.2 \pm 10.7\%$) (Fig. 4). In contrast, pretreatment with atropine 15-min prior to the onset of preemptive SCS (*protocol 2*) eliminated its infarct reducing effects ($51.6 \pm 8.6\%$).

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 $\frac{1}{2}$ 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.

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 an 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 β_1 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 T₂ spinal cord were examined to determine how spinal cord neurons transduce the effect of CAO in the absence and presence of C1-C2 SCS. 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 ; $p=0.028$).

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 preemptive 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 effects 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 increase 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 solitarius (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 eliminate 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 solitarius (Ding *et al.*, 2008a; Ding *et al.*, 2008b), thereby minimally effecting parasympathetic efferent neuronal outflow to the heart; 2) maintenance of parasympathetic efferent neuronal outflows to 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 arrhythmia

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 manifest, 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 outflows but also cholinergic ones 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.

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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, 200 μ s 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.

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 $\pm$ 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.

Figure 3.

Infarct size (percentage of risk zone) for sham controls compared to rabbits with pre-emptive C1-C2 SCS with intact neuraxis (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 $\pm$ SD data for each group (open triangle and vertical bar). * $p < 0.05$ from all other groups.

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 i) intact neuraxis (control SCS), ii) following cervical vagotomy, or iii) muscarinic receptor blockade (atropine). For each group, individual animal data are shown along with mean $\pm$ 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.

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. 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.

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 (Parasymp) 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; β_1 adrenergic receptor; M_2 -muscarinic receptor.

Table 1

Hemodynamic data for non-SCS control groups

	<i>Heart rate, beats/min</i>		
	Baseline	CAO	1 Hr Reperfusion
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
	<i>Blood pressure, mmHg</i>		
	Baseline	CAO	1 Hr Reperfusion
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

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

	<i>Heart rate, beats/min</i>				
	Preblock Baseline	Postblock Baseline	C1-C2 SCS	SCS+CAO	1 Hr Reperfusion
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 [#]
<i>Blood pressure, mmHg</i>					
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

Table 3

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

	<i>Heart rate, beats/min</i>			
	Baseline	C1-C2 SCS	C1-C2 SCS+CAO	1 Hr Reperfusion
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
	<i>Blood pressure, mmHg</i>			
	Baseline	C1-C2 SCS	C1-C2 SCS+CAO	1 Hr Reperfusion
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

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