

**Characterizing the Temporal Development of  
Cardiovascular Dysfunction and Examining Morphology  
and Function of Resistance Mesenteric Vasculature in a T3  
Experimental Spinal Cord Injury Rodent Model**

**by**

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## **Abstract**

Spinal cord injury (SCI) disrupts autonomic pathways and perturbs cardiovascular homeostasis. High thoracic or cervical SCI may result in a variety of dire cardiovascular consequences such as persistent hypotension, impaired hemodynamic diurnal rhythmicity and autonomic dysreflexia (AD). If left untreated, AD may lead to myocardial infarction, stroke and even death. One of the primary causes underlying impaired arterial blood pressure (ABP) control may be disrupted sympathetic control of the splanchnic resistance circulation. Thus the goal of the thesis is to (1) characterize the temporal development of cardiovascular dysfunction post SCI and (2) to investigate the time course of structural and functional changes in splanchnic resistance vasculature in a T3 SCI experimental rodent model. First, male Wistar rats were implanted with telemetry devices, and subjected to a T3 complete transection surgery. Beat by beat ABP, heart rate and core body temperature was continuously monitored and development of AD was assessed using the *in-house* AD detection algorithm. Second, primary mesenteric arteries were assessed in our SCI rodent model for structure and function *ex-vivo* using pressure myography. Vasoconstrictive properties were assessed using  $\alpha$ 1-adrenoceptor agonists: phenylephrine (PE) and methoxamine (MET). Results suggest that AD is present in acute SCI and there is a significant reduction in AD events between days 6 and 10 post SCI followed by a substantial increase in AD event frequency and severity at day 14 post SCI. Nocturnal dip in blood pressure and core body temperature was absent up to 14 days post SCI and partially restored after 21 days post SCI. There was a significant reduction in PMA wall to lumen ratio in the 1 month-SCI group compared to the 1 week SCI and control group. In addition, there was an increased sensitivity (reduced  $EC_{50}$ ) to PE in the 1 week SCI group compared to all groups ( $p < 0.05$ ), without significant changes in sensitivity to MET. This is the first study to show that experimental SCI exerts maladaptive hypertrophic remodelling and dysfunction at the level of the

splanchnic resistance vasculature, which may underlie the primary cause of chronic ABP dysregulation in SCI individuals.

## **Preface**

All experiments were conducted in strict accordance with the Canadian Council for Animal Care.

Ethics approval was granted by the University of British Columbia. University of British

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Telemetry and T3 complete transection surgeries for Chapter 2 were completed with aid of Dr.

Krassioukov and Dr. West, respectively.

Methods and Results section from Chapter 2 contain excerpts from the manuscript entitled:

“Characterizing the temporal development of cardiovascular dysfunction in response to spinal cord injury”.

T3 complete transection surgeries for Chapter 3 were completed with aid of Dr. Frias.

Pressure myography experiments for Chapter 3 were completed in collaboration with Dr. Laher.

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## List of Abbreviations

AB	Able Bodied
ABP	Arterial Blood Pressure
Ach	Acetylcholine
AD	Autonomic Dysreflexia
ANOVA	Analysis of Variance
BP	Blood Pressure
CBT	Core Body Temperature
cGMP	Cyclic Guanosine Monophosphate
CGRP	Calcitonin Gene Related Protein
COL I	Collagen Type I
COL III	Collagen Type III
CRD	Colorectal Distension
CVLM	Caudal Ventral Lateral Medulla
DRG	Dorsal Root Ganglion
E	Epinephrine
eNOS	Nitric Oxide Synthase
FG	FluroGold
FMD	Flow Mediated Dilation
GABA	$\gamma$ -aminobutyric Acid
HR	Heart Rate
IML	Intermediolateral
Kv	Ca <sup>2+</sup> Activated Potassium Channels
MAP	Mean Arterial Pressure
MET	Methoxamine
NE	Norepinephrine
NPY	Neuropeptide Y
NTS	Nucleus Solitarius
OCT	Optical Cutting Temperature
OH	Orthostatic Hypotension
PBS	Phosphate Buffer Saline Solution
PE	Phenylephrine
PMA	Primary Mesenteric Artery
PNMT	Phenylethanolamine-N-Methyltransferase
PSS	Physiological Saline Solution
PVN	Paraventricular Nucleus
RVLM	Rostral Ventral Lateral Medulla
SBP	Systolic Blood Pressure
SCI	Spinal Cord Injury
SMA	Smooth Muscle Actin
SMC	Smooth Muscle Cells

SR        Sacroplasmic Reticulum  
VGC      Voltage Gated Ca<sup>2+</sup> Channel  
VSM      Vascular Smooth Muscle

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## **Dedication**

To my family, friends and mentors.

## **Chapter 1: Introduction**

### **Overview**

Spinal Cord Injury (SCI) is considered one of the most devastating and debilitating conditions that an individual may experience. In addition to severe motor and sensory dysfunction, SCI disrupts autonomic pathways and consequently perturbs cardiovascular homeostasis. In comparison with able-bodied (AB) individuals, people with chronic SCI have a greater propensity to develop cardiovascular dysfunction[1]. Cardiovascular complications in the early stages of thoracic or cervical SCI can be life threatening, and include chronic, persistent hypotension, and cardiac arrest[2]. High thoracic or cervical SCI individuals have an increased propensity for impaired blood pressure control. In addition to chronic hypotension, individuals living with high thoracic or cervical SCI may suffer from recurrent bouts of autonomic dysreflexia or AD (a life threatening condition characterized by extreme reflexive spikes in SBP which may result in myocardial infarction, stroke and even sudden death)[3]. Autonomic Dysreflexia and blood pressure control after high thoracic/cervical SCI is the primary focus of the dissertation. Specifically, there is a gap in the knowledge in regards to the temporal development of cardiovascular dysfunction post SCI, specifically with regards to diurnal variations in hemodynamics and the development and progression of these spontaneous AD events. Further, resistance vasculature plays a vital role in control of arterial blood pressure, yet there is a gap in knowledge in regards to the time course of morphological and functional adaptations of resistance vasculature after SCI. It would be invaluable to delineate how this swinging of arterial blood pressure post SCI due to chronic, persistent hypotension and spontaneous AD, might influence the structural and functional characteristics of resistance vasculature post SCI.

My dissertation examines the effects of a complete high thoracic SCI in an animal model on diurnal variations in hemodynamics and onset of spontaneous AD. In addition, we look to examine

the time course of morphological and functional changes in resistance vasculature after high thoracic SCI. I will review the current knowledge in regards to the autonomic nervous system and autonomic cardiovascular control after SCI. I will also review the structural and functional parameters of peripheral vasculature in the cardiovascular system and the effects of SCI on peripheral vasculature structure and function.

## **1.1 Cardiovascular Dysfunction Post SCI**

### **1.1.1 Epidemiology**

From a study by Noonan et al in 2012, there is an estimated 85 556 people living with SCI in Canada and 4300 new cases of SCI each year[4]. Among this population 43 974 cases are traumatic SCI and it is estimated by 2030 that there will 121 000 people living with SCI and 5800 new cases of SCI each year in Canada[5]. The worldwide prevalence of traumatic SCI ranges from 3.6 to 195.4 patients per million[6]. This an estimate from recent systematic review by Jazayeri et al with an appreciation that there is more readily available epidemiological data from Canada, Australia, US and other wealthier European countries but lack of appropriate epidemiological data from countries in Asia and Africa. Currently, there about 300000 people living with an acute traumatic SCI in Canada and the United States and 2.5 million worldwide[7]. Though the prevalence of SCI is quite low it does however have substantial lifetime economic (ie monetary) and social consequences[7]. A large fraction of these costs are due to the treatment and control of secondary complications after SCI[8].

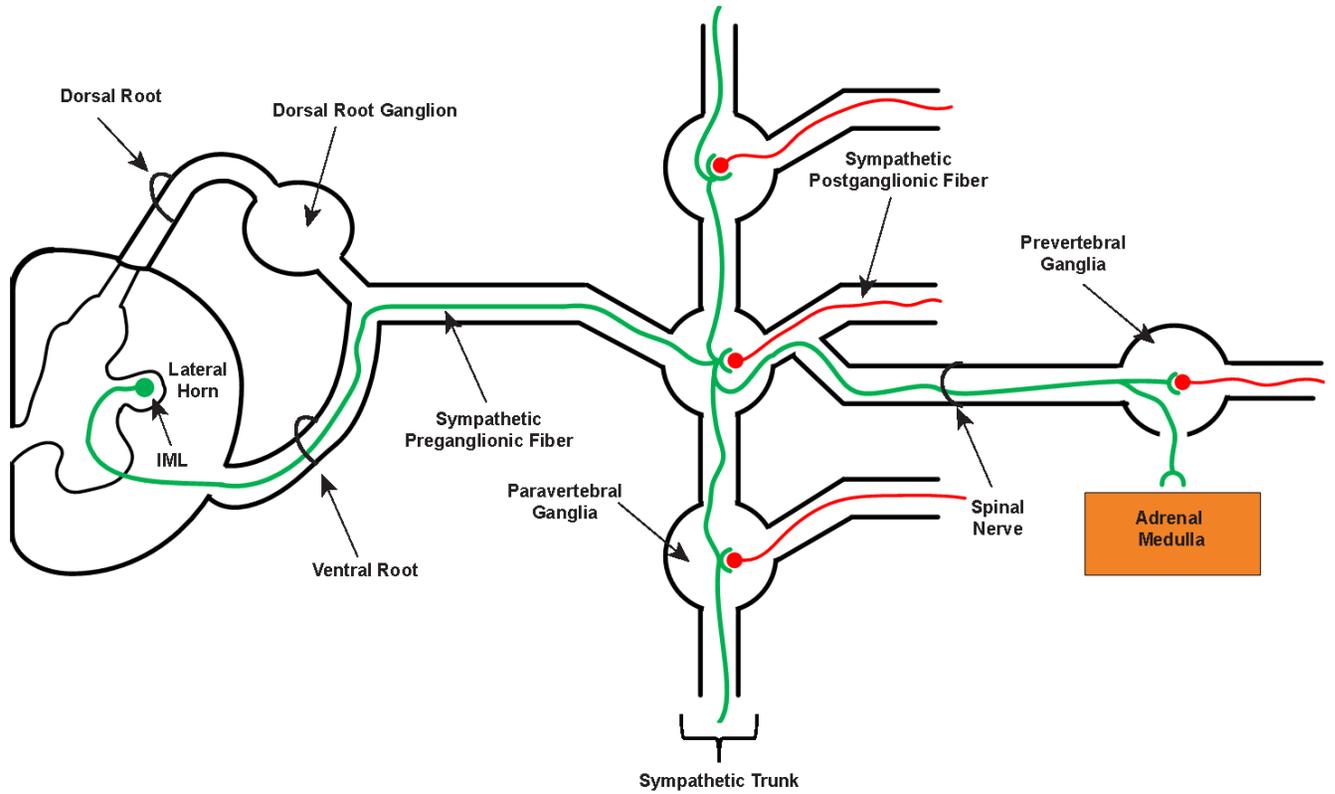
Along with motor dysfunction, there are variety of secondary complications that result in a diminished quality of life in individuals living with SCI. These secondary complications include cardiovascular disease, bladder/pulmonary infections and osteoporosis[9]. Studies have revealed that older patients living with SCI have a higher rate of secondary complications and increased hospital stay[10]. In a study by Anderson individuals with SCI ranked cardiovascular autonomic function among the highest priority even above their locomotor recovery in order to improve their quality of

life[11]. Cardiovascular disease is one of the leading causes of morbidity and mortality in chronic SCI[12]. After adjusting for age and sex, individuals with SCI are at a significant increased risk of heart disease and stroke[13]. The probability of developing a cardiovascular disease rises dramatically after injury[1, 12, 14] and individuals experience these complications at a younger age and more frequently than those without SCI[1]. Impaired blood pressure control, heart rate variability, impaired response to exercise and arrhythmias due to loss of autonomic cardiovascular control contribute to the increased risk of cardiovascular disease and stroke in chronic SCI patients[15, 16].

### **1.1.2 Overview of the Autonomic Nervous System**

The autonomic nervous system consists of the sympathetic and parasympathetic divisions, with exception of the enteric nervous system[17]. Cell bodies of sympathetic preganglionic neurons lie in the gray matter of the lateral horn and in the gray matter in between the central canal and lateral horn of the spinal cord. Preganglionic efferent neurons make up the intermediolateral (IML) column of the spinal cord's thoracolumbar region (T1-L2)[18]. Parasympathetic preganglionic efferent neuronal cell bodies are located within the nuclei of four cranial nerves in the brain stem (oculomotor, facial, glossopharyngeal and vagus nerve) and project from the sacral regions of the spinal cord[19].

Preganglionic efferent fibres (with exception of the innervation of the adrenal medulla) synaptically end on neurons located in autonomic ganglia. Sympathetic ganglia are divided into two major groups: the paravertebral and prevertebral ganglia[20]. The paravertebral ganglia form the sympathetic trunk, which consists of a series of ganglia located on both sides of the vertebral column, extending from the base of the skull to the sacrum[17, 20]. Prevertebral ganglia lie anterior to the spinal column and in close proximity to the major abdominal arteries[20]. There are four routes the preganglionic sympathetic neurons may take to act on a target[21] (refer to Figure 1):



**Figure 1:** Schematic representation of four routes sympathetic preganglionic neurons may take to act on a target. Preganglionic axons may terminate in the paravertebral ganglia (ganglia within the sympathetic trunk) at its level of departure in the spinal cord, it may descend or ascend the sympathetic trunk and synapse on different paravertebral ganglia, or it may move through paravertebral ganglia without synapsing and synapse within the prevertebral ganglia (ganglia outside of the sympathetic trunk). Finally sympathetic preganglionic neurons may also directly innervate target organ (e.g chromaffin cells of adrenal medulla).

Preganglionic axons may terminate in the paravertebral ganglia at its level of departure in the spinal cord, it may descend or ascend the sympathetic chain and synapse on different ganglia, or it may move through paravertebral ganglia without synapsing and synapse prevertebral ganglia.

Preganglionic sympathetic neurons may also directly innervate chromaffin cells of adrenal medulla. Chromaffin cells are neural crest in origin and many consider these cells as post ganglionic neurons that have shed their processes[22].

### **1.1.3 Autonomic Control of the Cardiovascular System**

The autonomic cardiovascular control of the heart is mediated by the action of both the parasympathetic and sympathetic divisions of the autonomic nervous system[19]. Atrial and ventricular cardiac myocytes are innervated by sympathetic axons, while sinoatrial and atrioventricular myocytes receive both parasympathetic and sympathetic innervations. Sympathetic preganglionic efferent fibres stem from spinal segments T1 to T4 and innervate paravertebral ganglia. Sympathetic post ganglionic fibres originating in paravertebral sympathetic ganglia innervate cardiac myocytes and pacemaker cells in the heart.

Blood vessels also receive innervations from the autonomic nervous system and a majority of these blood vessels in the body are heavily innervated by sympathetic neurons. Activation of blood vessels by sympathetic neurons leads to vasoconstriction of arterial beds, increased systemic vascular resistance (increased arterial blood pressure) and reduced blood flow. Sympathetic preganglionic projections from the IML column modify peripheral vasculature resistance via activation of smooth muscle[19]. These include arteries, arterioles and veins with post ganglionic sympathetic axons innervating the adventitial media border of the vessels[19]. These sympathetic axon terminals release norepinephrine, which binds  $\alpha_1$  or  $\alpha_2$  adrenoreceptors on vascular smooth muscle cells. This mediates both increase in  $\text{Ca}^{2+}$  entering the cell or the amount of  $\text{Ca}^{2+}$  released from the sarcoplasmic reticulum of the smooth muscle, ultimately increasing the force of contraction[23].

There are variety of cardiovascular control centers that help mediate basal hemodynamics through local intracranial delivery of NO[24]. These include the paraventricular nucleus of the thalamus and the rostral ventral lateral medulla (RVLM). They mediate these responses via NO, a neurotransmitter stimulant involved in blood pressure modulation. The paraventricular nucleus of the thalamus is a key modulator of cardiovascular control, as seen by early experiments exposing the PVN to increasing doses of NO via artificial cerebrospinal fluid delivery to the PVN[24]. In addition, up-regulation of amino acids aspartate, glutamate,  $\gamma$ -aminobutyric acid (GABA) and taurine were observed in the PVN[24]. The RVLM neurons regulate tonic activity of sympathetic preganglionic neurons[25]. When increasing NO production by genetically over expressing enzyme endothelial NO synthase (eNOS), there is an increase in GABA release that inhibits tonic excitation of the RVLM[26]. This acts on the cell bodies of preganglionic sympathetic neurons in the IML of the thoracolumbar region of the spinal cord, culminating in hypotension and bradychardia[26] .

GABA acting on axons of the RVLM inhibit excitatory projections that innervate the preganglionic sympathetic neurons in the IML[27]. These axons are located within the dorsolateral funiculus of the spinal cord in humans[28]. In the rodent model, the RVLM neurons at the rostrocaudal level of the medulla are bordered rostrally and laterally by the facial nucleus, dorsally by the nucleus ambiguus and medially by the gigantocellular reticular formation and the inferior olivary nuclei[18, 29-32] . RVLM cardiovascular neurons are members of the C1 group of adrenergic neurons that are uniquely distinguished by the presence of the enzyme phenylethanolamine-N-methyltransferase (PNMT)[32].

The nucleus tract of the solitarius or nucleus solitarius (NTS) is a key coordinator of cardiovascular autonomic control. The NTS is a series of nuclei consisting of a column of grey matter within the medulla oblongata. The NTS receives input from baroreceptors that sense stretch (i.e. increase in blood pressure) within the walls of the carotid sinus, aortic arch and auricles of the heart

and vena cava. Baroreceptors send afferent inputs to rostral NTS, which is located in the dorsomedial medulla and ipsilateral portion of the commissural sub-nucleus of NTS[33]. Through a negative feedback mechanism, excitatory inputs from NTS activate caudal ventral lateral medulla (CVLM) through glutamatergic neurons, which inhibit the activity of RVLM through GABAergic inhibitory fibres[34]. Inhibition of the excitatory projections to the IML of the spinal cord results in a decrease in sympathetic activity and reduction in arterial blood pressure[35]. NTS neurons also relay this signal to the dorsal nucleus of the vagus nerve[34]. These parasympathetic vagal neurons project to the heart's terminal ganglia, slowing the heart rate through pacemaker cells in the heart[17].

Blood pressure is regulated through two important factors: cardiac output and peripheral vascular resistance. The contribution to peripheral vascular resistance is not the same among all the arterial beds in the periphery. One of the key determinants of arterial blood pressure is associated with the splanchnic circulation. This region is one of the major blood reservoirs of the circulatory system and 25 % of the blood in the body is constricted to this region[36]. Factors such as relative density of  $\alpha$  and  $\beta$  adrenoreceptors, affinity for the neurotransmitter to the adrenoceptor, catecholamine concentration of the blood plasma, vascular tone, and the blood volume play a major role in how neurotransmitters may induce vascular changes within the splanchnic region[36]. A majority of the sympathetic innervations of the splanchnic organs stem from spinal segments T4-T9 as cholinergic preganglionic neurons. These neurons pass through the *greater* splanchnic nerves and synapse in the celiac ganglion plexus with post ganglionic adrenergic fibres, which ultimately innervate the target[36, 37]. A smaller number of the sympathetic innervations of the splanchnic organs originate in spinal segments T10-T12, pass through the *lesser* and *least* splanchnic nerves and innervate prevertebral ganglia[36].

#### **1.1.4 Cardiovascular Dysfunction Post SCI**

There can be a variety of reasons for this increased risk for cardiovascular dysfunction in individuals with SCI, but one of the major challenges is the lack of descending supraspinal control of peripheral vasculature below the level of injury. Particularly, injury at the sixth thoracic spinal cord segment and above may disrupt descending autonomic pathways of the cardiovascular system that innervate glands, smooth muscle of blood vessels and cardiac muscle[38]. The degree of cardiovascular dysfunction is determined by the level and severity of SCI[39]. There are two major disorders associated with cardiovascular dysfunction post SCI: Autonomic Dysreflexia and Orthostatic Hypotension. Orthostatic Hypotension is a dysregulation of reflexive blood pressure control when shifting from a seated to a standing position[40]. Autonomic Dysreflexia is a life threatening condition characterized by episodes of extreme hypertension due to noxious and non-noxious peripheral stimuli below the level of injury[41-43].

Injury above the sixth thoracic spinal cord segment is associated with disruption of descending autonomic pathways of the cardiovascular system. Inhibition of the excitatory bulbospinal projections that innervate preganglionic sympathetic neurons in the IML of the spinal cord reduce sympathetic activity. This reduction of sympathetic activity may lead to decreased tone in arterial blood vessels and activation of vagal nuclei. An experiment examining the effect of varying severity of T1 SCI (clip compression model) on the neuron counts of the RVLM was implemented by retrograde axonal tracing with FluoroGold (FG) and PNMT staining[44]. FG allows the visualization of those neuronal projections from the RVLM. There is a linear relationship between the decreased number of FG labelled neurons and increased severity of SCI. After 50g clip compression in a rodent model, approximately 20% PNMT labelled neurons within the RVLM underwent degeneration[44]. The lost traction of spinally projecting PNMT positive neurons after 50g clip compression SCI is 42% of all spinally projecting joint FG and PNMT labelled RVLM neurons in non-injured

animals[44]. This large drop in projecting bulbospinal RVLM projections can be attributed to cell death or degeneration, a well known response to axotomy of various neuron populations. There is also a close correlation with the number of FG retrogradely labelled neurons in the red nucleus, vestibular nucleus and reticular formation after graded clip compression injury[45, 46] and contusion injury[47].

### **1.1.5 Acute SCI and Cardiovascular Dysfunction**

Following an acute injury above the T6 level, there is disruption in the central nervous system generated neurogenic tone that is crucial for function of the peripheral vasculature. This loss or decrease in central and peripheral neurogenic tone is commonly associated with acute SCI and referred to as neurogenic shock (i.e. originates from supraspinal neuronal centres)[37]. Even though there is some preservation in the myogenic tone, it is not sufficient in maintaining blood pressure following high thoracic or cervical SCI[37]. Of major importance is the loss of sympathetic activity to a significant portion of the peripheral blood vessels in the lower extremities, including the splanchnic circulation. This acute period post SCI is characterized by profound hypotension and disrupted diurnal rhythmicity[48, 49]. The higher the level and severity of the SCI, the more severe the hypotension[50]. If injury occurs at T6 or higher, sympathetic outflow to the splanchnic circulation may be disturbed[37]. This may result in the loss of tonic excitation to the sympathetic pre-ganglionic neurons that innervate these splanchnic vascular beds.

### **1.1.6 Orthostatic Hypotension**

In addition to chronic hypotension, individuals with SCI exhibit orthostatic hypotension (OH) when shifting from a seated to a standing position, from a supine to a seated position or during transfer[40, 42, 51-53]. When the body is planning to alter the posture to a stand up position, there is a baroreflex mediated sympathetic response to increase peripheral vascular resistance for a short amount of time, in order to sustain blood pressure and cerebral perfusion[54]. Due to sympathetic

hypoactivity and altered baroreceptor sensitivity in individuals with high thoracic or cervical SCI, this response is inhibited[55, 56]. Instead of blood being relayed toward the brain and upper extremities upon sitting upright, there is pooling of blood in the lower extremities and abdomen level[57]. OH is characterized by a decrease in systolic blood pressure by 20mmHg or a decrease in diastolic blood pressure of 10mmHg[40]. Symptoms of OH include restlessness, blurred vision, fatigue or weakness, light headedness and dizziness[40].

### **1.1.7 Autonomic Dysreflexia**

In addition to the persistent hypotension, patients with high thoracic or cervical SCI experience a life threatening condition called autonomic dysreflexia (AD). AD is characterized by reflex evoked bouts of extreme hypertension (systolic blood pressure spikes to 300mmHg). AD results from both noxious and non-noxious visceral or somatic stimuli (such as bladder or bowel distension), which cause exaggerated activation of the spinal circuits caudal to the injury that project to the sympathetic pre-ganglionic neurons[58]. This will result in sympathetically mediated systemic hypertension and baroreflex mediated pronounced bradychardia. Symptoms associated with AD include pounding headaches, profuse sweating and upper body flushing[3]. If left untreated, persistent AD despite chronic hypotension may cause serious fatal complications including intracranial hemorrhage, myocardial infarction and even death[59-61]. The loss of supraspinal input to the spinal sympathetic circuits, disruption of spinal reflexes and plastic changes in the spinal and peripheral autonomic circuits may contribute to the development of AD.

The circuitry associated with AD initiates from noxious or non-noxious afferent input to lumbar-sacral segments of the spinal cord, most commonly bladder or bowel distension. The afferent stimuli are relayed to the dorsal horn of the spinal cord, where sensory neurons synapse with propriospinal circuitry. Sensory information propagates through propriospinal circuitry to the sympathetic preganglionic neurons via interneurons. These sympathetic preganglionic neurons

synapse with post ganglionic neurons, which cause vasoconstriction of the mesenteric vasculature, leading to significant spikes in ABP[62]. Modulation of sympathetic preganglionic neurons are suppressed due to degeneration of descending RVLM bulbospinal projections[63]. The exaggerated sympathetic response is accompanied by more pronounced vagal activity through baroreceptor mediated mechanisms[58]. This would elucidate the observed bradychardia upon the onset of AD.

The development of AD is commonly attributed to plasticity in afferent fibres interacting with sympathetic preganglionic neurons through interneurons via a "reflex arch". These unmyelinated C-type afferent fibres are small in diameter and relay thermal and nociceptive information[64]. A common marker of these unmyelinated C-type fibres is calcitonin gene related protein (CGRP)[64]. CGRP immunoreactivity in the DRG, along with the posterior horn of the spinal cord indicates sprouting of these unmyelinated C type afferent fibres. The time course of sprouting of these CGRP immunoreactive afferent fibres has been found to mimic the time course associated with the development of AD[64].

### **1.1.8 Thermoregulation post SCI**

High thoracic or cervical SCI results in impaired thermoregulation due to loss of descending supraspinal control from thermoregulatory centers. SCI individuals have a greater propensity for extreme core body temperature variations derived from environmental temperature changes, exercise or infection[65-68]. In addition there are numerous studies that report chronic SCI individuals exhibit poikilothermia[69, 70]. Poikilothermia is defined as the inability of an individual to regulate core body temperature upon exposure to hot or cold environments. One of the hallmark signs upon onset of AD, is cold surface temperature in addition to pale lower body extremities due to massive vasoconstriction occurring below the level of injury [52, 71]. There is also sweating and hyperemia observed above the level of injury due to vasodilation[52].

Core body temperature is maintained within a narrow range of 37°C (i.e. +/-0.6) by behavioral adjustments to thermal discomfort via hypothalamic mediated thermoregulatory mechanisms in response to a wide range of hot or cold ambient temperatures[72]. Exposure to hot ambient temperatures may result in profuse sweating and cutaneous vasodilatation via a thermolytic response[73, 74]. Cardiac output may double as skin blood flow increases as much as 60% during periods of hyperthermia[73]. High thoracic/cervical SCI impairs thermal sensation and disrupted sympathetic pathways may disrupt hypothalamic control of sweating[75] and cutaneous vasodilatation below the level of lesion[70]. Maximal sweat responses to heat exposure are only 20-30% in persons with high level SCI which may lead to a rise in core body temperature[75]. In addition upon exposure to cold temperatures there is an impaired response in SCI individuals due to disruption of hypothalamic regulation (i.e. via disruption of incoming sensory and sympathetic pathways) of cutaneous vasoconstriction and “shivering” below the lesion level[76]. The inability to increase circulating NE due to the impaired supraspinal sympathetic response may result in a steadily decreased core body temperature[70, 77]. Deviation in core body temperature may result in adverse effects to cognition, hemodynamic stability, muscle and organ system function[78]. It is interesting to note that there is a study that showed that tetraplegic individuals, upon cold exposure (and subsequent drop in core body temperature) may have a negative impact on working memory and executive function[77].

## **1.2 Peripheral Vasculature Post SCI**

### **1.2.1 Morphology of Blood Vessels**

Majority of knowledge of vascular morphology is derived from histological cross sectional images of vascular beds. From these images, five structural components may be

identified from the exterior toward the lumen of the vessel. These include the tunica adventitia, external elastic lamina, tunica media, internal elastic lamina and tunica intima (refer to Figure 2).

### **Adventitia**

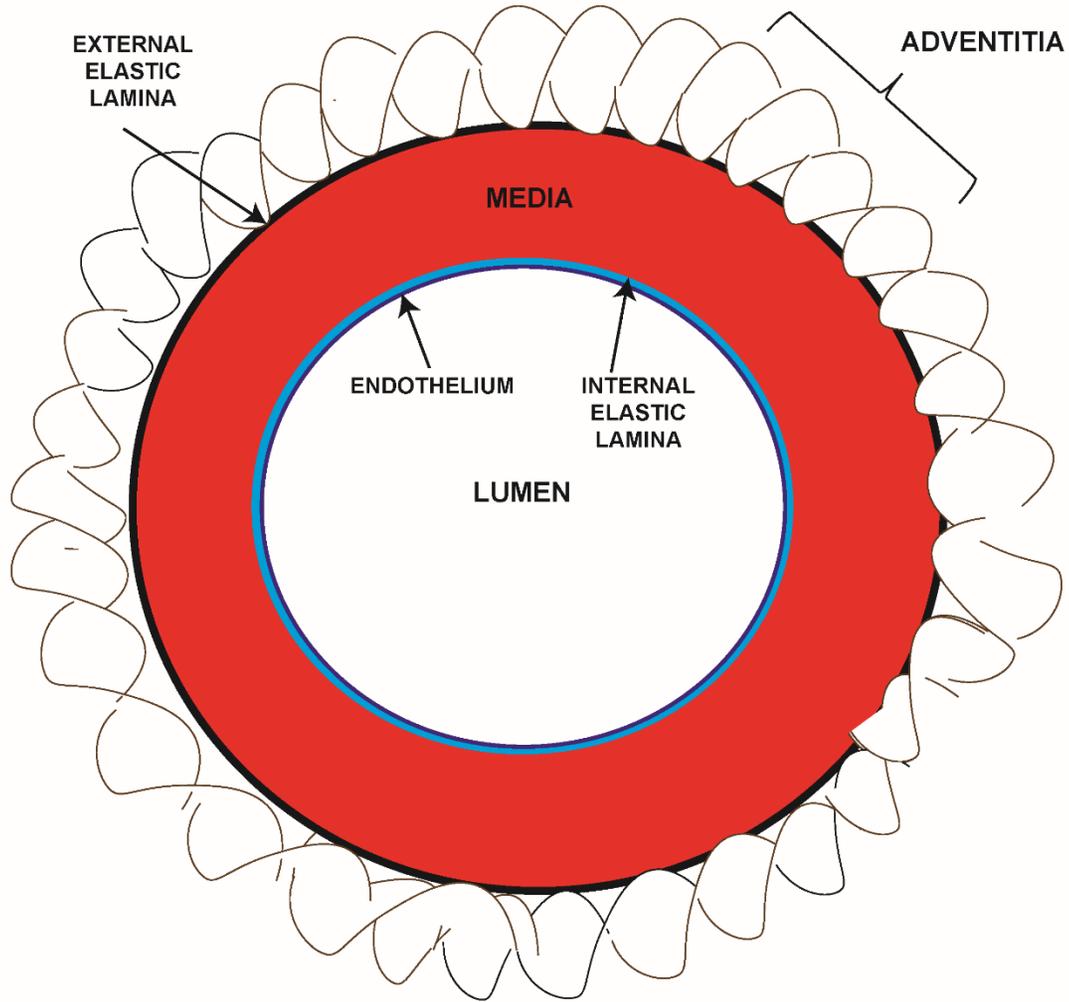
The outer perimeter of the vessel which is composed mostly of collagen and elastin, is the adventitia. Fibroblasts (cells involved in collagen synthesis), mast cells and macrophages reside within this layer[79]. Primary adrenergic neurons also innervate this layer and the density of  $\alpha 1$  adrenoceptors has been shown to be equal to that found in the SMC segment of vascular beds[80]. The presence of these adrenoceptors within adventitial cells may suggest a physiological trophic effect following vascular damage[81].

### **Media**

The media layer of the cell primarily consists of SMCs. There are no fibroblasts present in this layer and SMCs take over production of collagen and proteoglycans[82]. During hypertension, atherosclerosis, angiogenesis and early development, the production of matrix components changes in SMCs[83-85]. The number of SMC layers depends on the size of the artery (varies from 6 in 300 $\mu$ m arteries to 1 in arterioles)[86]. Tensile stress exerted upon vessels is dispersed between cells within the media via anchor points in the plasma membrane that interact with myofilaments[87, 88].

### **Intima**

The intima layer consists primarily of endothelial cells which folds inwards toward the plasma membrane to communicate with the lumen. The basement membrane upon which endothelial cells reside, sit on the internal elastic lamina. The internal elastic lamina is present in larger and medium sized arteries but disappears in smaller arteries and arterioles. Endothelial cells within the intima layer interact with SMCs via myoendothelial junction. The dynamic nature of the internal elastic lamina play a prominent role in altering myoendothelial junctions, which may lead to changes in vascular function as a whole[89].



**Figure 2:** Diagram of an arterial cross-section with main structural components. Five structural components may be identified from the exterior toward the lumen of the vessel. These include the adventitia, external elastic lamina, media, internal elastic lamina and endothelium.

Endothelial cells have previously found to mediate the contraction of SMCs. Evidence to support this was exhibited by denuding the endothelium mechanically in rat aorta which increased the contractile response to  $\alpha$ -adrenoceptor agonists [90].

### **1.2.2 Resistance Vasculature and Arterial Blood Pressure Control**

Resistance arteries consist of smaller muscular arteries, arterioles and capillaries. Resistance arteries are the site where a drastic drop in hydrostatic pressure occurs. The lumen diameter of small arteries is less than 350 $\mu$ m and for arterioles is less than 100  $\mu$ m[91, 92]. The media layer of the resistance vasculature consists of 1-3 layers of SMCs and normally possess an internal elastic laminae but lack an external elastic lamina. Resistance arteries possess structural elements to facilitate changes in vascular length in response to BP fluctuations and reduce pulse wave velocity in the arterial tree. These arteries have an inherent wider lumen with SMCs oriented perpendicular with a diagonal offset along the axis of flow in order to create a spring like organization. Structural properties for dispersion of tensile strength upon vessel wall is still unknown as these vessels do not seem to possess similar anchor points that interact with myofilaments as larger arteries[93].

Resistance arteries play key role in regulating regional blood flow (ie peripheral resistance) and in order to appreciate the robust effect that constriction of resistance vessels can have on blood flow one may refer to Poiseuille's Law. Flow varies directly (inversely with resistance) to the 4<sup>th</sup> power of the vessel radius. Blood pressure is dependent on the cardiac output and the total peripheral resistance. Since resistance arteries are a large contributing factor to total peripheral resistance, they play a pivotal role in regulation of systemic blood pressure. Resistance arteries play an integral role in maintaining MAP and venous pressure within a narrow range (3-8mmHg) and allow for distribution of blood to pre-capillary vessels. This is extremely pertinent to the control of perfusion pressure and amount of blood being relayed to the capillaries. In order for the circulatory system to overcome resistance to blood flow, there are variety of local and systemic factors associated with maintenance

of vascular tone. Thus regulation of ABP via peripheral resistance arteries is mediated through sympathetic nervous system, inherent characteristics of the vessel wall (i.e. myogenic tone) and endothelial factors[94-96].

### **Anatomy of Resistance Mesenteric Vasculature**

Primary mesenteric resistance vasculature extends perpendicular to the superior mesenteric artery toward the gut wall. These vessels supply blood to the intestine, transverse colon and pancreas. The superior mesenteric artery extends from the anterior surface of the abdominal aorta and is responsible for supplying blood to the intestine and pancreas[97].

### **1.2.3 Intrinsic Vascular Response to Stretch and Pressure**

Changes in stretch and shear stress are constantly amplified in blood vessels. Blood vessels adapt to these changes via structural remodelling. Specifically, to maintain basal tensile and shear forces, blood vessels modify their diameters. When changes in stretch and shear stress are maintained for longer periods of time, there is hyperplasticity in endothelial cells and SMCs which lead to functional and structural alterations in vasculature.

Stretch response is dependent on BP, leading primarily to changes in diameter and wall thickness of the vasculature. BP fluctuations cause an increase in circumferential wall stress which acts tangentially on the vessel wall[98]. It is still unknown how this stretch signal is transduced to SMCs but this may be due activation of SMC ion channels, changes in contractile protein function, detection of mechanical stretch via endothelial cells and communication between endothelial cells and SMCs. Endothelial cells respond to stretch by activating calcium ion channels which eventually lead to depolarization of endothelial cell membranes[99]. Endothelial cells transduce these electrical signals to SMCs[100]. Further, SMCs also possess stretch-activated cation channels and depolarization of the cell membrane is achieved through an influx of  $\text{Ca}^{2+}$  or activation of intracellular  $\text{Ca}^{2+}$  stores[101, 102]. The stretch is primarily dependent upon extracellular availability

of  $\text{Ca}^{2+}$ , as a previous in vitro study on small arteries isolated from a rabbit ear, showed that calcium blockade may inhibit the intrinsic stretch response[101-106].

Fluctuations in BP put forth forces that act perpendicular to the luminal surface of vasculature. This form of strain induced by BP fluctuations, is in opposition to other tangential forces on the vessel wall such as in the circumferential or longitudinal directions[98]. These forces due to BP fluctuations have been studied in arteries and arterioles with regards to pressure induced vasocontractility or myogenic tone. This intrinsic vascular response to increasing BP is mediated via ion gated channels in the SMC and is dependent on intracellular  $\text{Ca}^{2+}$  concentration. Recent studies have reported that myogenic tone in arterial beds isolated from rats, is independent of endothelial function and denuding the endothelial cell layer is representative of a true myogenic response[104, 107-109].

#### **1.2.4 Mechanisms of Generation Myogenic Tone**

In the vascular system, the myogenic response are associated with two important physiological functions: 1. Aid in establishment of basal vascular tone, in addition to neurogenic tone and 2. Autoregulation of blood flow and capillary hydrostatic pressure. Myogenic response is most pronounced in smaller arteries and arterioles but may be present in venules, veins and lymphatics[110]. Artery and arteriole preparations (i.e. pressure myography) ex vivo have shown a similar level of tone that what would otherwise be found in vivo and the myogenic response does not develop unless pressurized to a physiological level. Myogenic contraction is mediated by vascular smooth muscle depolarization which regulates  $\text{Ca}^{2+}$  entry through voltage-gated  $\text{Ca}^{2+}$  channels. Mechanosensitive cation (i.e.  $\text{Na}^{+}$  for rat mesenteric arteries) channels are proposed to initiate contraction by depolarizing SMCs past the threshold for activation of voltage gated channels and allowing  $\text{Ca}^{2+}$  entry through these voltage gated channels to activate contractile proteins[111]. Lines of evidence to support this include: 1. Intracellular  $\text{Ca}^{2+}$  concentration is increased when increasing

stretch in vascular smooth muscle (VSM) cells[112]. 2. Pressure elevates SMC intracellular Ca<sup>2+</sup> concentration in isolated arterioles. 3. Voltage gated channel antagonists produce only a partial block of stretch induced increase in intracellular Ca<sup>2+</sup> concentration[112], where as Gd<sup>3+</sup> (voltage gated channel and mechanosensitive channel blocker) inhibits increase in intracellular Ca<sup>2+</sup> concentration. Voltage gated Ca<sup>2+</sup> channels (VGC) may play a role in propagation of the depolarization process when brought to threshold via the mechanosensitive cation channels. Lines of evidence to support this include: 1. The open probability of the VGC is increased 10 to 15 fold upon depolarization of VGC[113]. 2. Dihydropyridines (VGC blocker) attenuates myogenic response[114-119] and reduces intracellular Ca<sup>2+</sup> in isolated arterioles[120]. Elevated levels of extracellular Ca<sup>2+</sup> enhance both myogenic responsiveness and the degree of pressure-induced depolarization[115]. Ca<sup>2+</sup> activated Potassium channels (K<sub>v</sub>) can provide potentially powerful repolarizing mechanism to counteract stimuli resulting from VSM stretch. Lines of evidence to support this include: 1. K<sub>v</sub> channels exhibit a massive increase in open probability upon depolarization[113] 2. K<sub>v</sub> channel blockers depolarize VSM cells in pressurized arterioles and augment myogenic tone[121].

### **1.2.5 Endothelial Factors and Vascular Tone**

In addition to neurogenic tone, another local factor associated with myogenic tone is the endothelium. The endothelial cell layer is a functional barrier between the lumen and vascular wall. The endothelium mediates production of a variety of other vasoconstrictor and vasodilators (NO, prostacyclin, endothelin-1 and endothelium-derived hyperpolarizing factor). Shear stress is one of the more potent stimuli associated with release of NO through stretch gated cationic channels in SMC and endothelium. Increased intracellular Ca<sup>2+</sup> allows for calmodulin dependent activation of eNOS and increased NO production. The vasodilator diffuses to the SMC layers and stimulated production of cyclic guanosine monophosphate (cGMP) which culminates in reduced intracellular Ca<sup>2+</sup> and relaxation of SMCs. It is well known that when blood flow increases, vessels increase their diameter

in response to increase NO release through a process called Flow Mediated Dilation (FMD) (26). Under turbulent blood flow, there is decreased expression of eNOS (enzyme associated with NO production) and increased vasoconstriction and pro-inflammatory responses. Pathologies such as diabetes, hypertension and hypercholesterolemia are associated with reduced FMD.

### **1.2.6 Adrenergic Receptors and Sympathetic Neural Control of Vasculature**

Post ganglionic sympathetic nerves are localized to the adventitial medial border of most arteries and veins throughout the body. Only the outermost layer of smooth muscle receives sympathetic innervation. Inner portions of the medial layer contract due to diffusion of NE and cell to cell communication via gap junctions. Norepinephrine is released from sympathetic nerve terminals and bind to  $\alpha 1$  or  $\alpha 2$  adrenergic receptors. This allows the release of  $Ca^{2+}$  from the sarcoplasmic reticulum (SR) or by increasing influx through voltage gated calcium channels. Rise in  $Ca^{2+}$  mediates calmodulin dependent activation of light chain kinase and phosphorylation of myosin light chain which is required for the activation of myosin ATPase and binding of myosin to actin filaments[122].  $\alpha 1$ -adrenoceptors are coupled to  $G_{q/11}$  protein to stimulate phospholipase C activity, which promotes enzymatic hydrolysis of phosphatidylinositol bisphosphate producing inositol trisphosphate and diacylglycerol[122]. This mediates intracellular  $Ca^{2+}$  release from non-mitochondrial pools and activate protein kinase C. This may trigger further activation of voltage gated calcium channels and increase the slope associated with depolarization to threshold.

There are 3 subtypes of  $\alpha 1$  adrenergic receptors:  $\alpha 1A$ ,  $\alpha 1B$  and  $\alpha 1D$  found in the rat mesenteric artery and these are characterized with different mRNA expression profiles ( $\alpha 1A$ ,  $\alpha 1B$  and  $\alpha 1D$ ) and vasoconstrictive efficiencies[123]. The rat  $\alpha 1A$  adrenoceptors play the most prominent role in the regulation of blood pressure of the 3 receptor subtypes[123] and the pressor response to phenylephrine is mediated by  $\alpha 1A$  and  $\alpha 1D$  adrenoceptors[124].  $\alpha 1D$  adrenoceptors are thought to play a role in the development and maintenance of hypertension in rat mesenteric arteries[125].  $\alpha 1B$

are functionally present and participate in the response to exogenous agonists but it is still uncharacterized with regards to the receptor's role in blood pressure regulation[123].  $\alpha_2$  adrenergic receptors are located on the presynaptic sympathetic nerve terminal and reduce sympathetic vasoconstriction. These receptors provide feedback inhibition of NE release. The  $\alpha_2$  adrenergic receptors are predominantly coupled to the inhibitory heterotrimeric GTP binding protein, which then inactivate adenylyl cyclase and voltage gated calcium channels[126].

Sympathetic nerves also release co-transmitters: Neuropeptide Y (NPY) and ATP. These co-transmitters may also constrict vessels by activating NPY Y1 and purinergic P2X receptors, respectively, resulting in an increase in intracellular  $Ca^{2+}$ . NPY seems to play a more prominent role in vasoconstriction of the mesenteric veins rather than arteries[127]. ATP works in conjunction with NE to induce vasoconstriction in mesenteric arteries[128]. ATP activates P2X<sub>1</sub> receptors which cause depolarization via opening of  $Ca^{2+}$  gated channels[127].

### **1.2.7 Blood Flow and Vascular Changes in Able-Bodied vs. SCI Individuals**

Depending on the level of injury, there is varying degrees of cardiac deconditioning and vasomotor dysregulation[129, 130]. Changes in blood flow post SCI may result in structural remodelling of vasculature, and the level of restructuring is dependent on the level and severity of injury. Tetraplegic individuals showed that blood flow and femoral artery diameter is reduced following SCI compared to AB individuals[131, 132]. It is interesting to note, that if you normalize the reduction of muscle mass following SCI to remodelling in vasculature there would be no significant structural differences in response to the decreased blood flow[133, 134]. Other studies showed no significant change in resting hyperaemic blood flow of lower limbs between SCI and AB individuals[133-135]. Studies have also found that vascular structural plasticity is not exhibited in vasculature above the level of injury. These studies have shown no significant differences in the diameters of common carotid, brachial and femoral arteries between SCI and AB individuals [136-

138]. Remodelling of vasculature below the level of injury may be attributed to a drastic drop in lower limb movements post SCI and the resulting reduced metabolic demands within this region[139].

### **1.2.8 Shear Stress and Vascular Changes Post SCI**

Blood flow and shear stress are closely interconnected. As mentioned earlier, shear stress is a tangential drag force of blood moving across the endoluminal surface and this increased circumferential stress may cause damage to the vessel wall. Changes in blood flow may result in remodelling of vasculature to reduce shear stress. An increase in blood flow may result in increased vessel diameter or outward remodelling and a decrease in blood flow may result in a decreased vessel diameter or inward remodelling. A study by Schmidt – Trucksass et al on changes in common femoral artery diameter, compliance and shear stress found that femoral artery diameter and compliance was lowest in paraplegic individuals when compared to control groups[140]. In addition, the same study reported mean peak shear stress values in common femoral artery almost doubled in the SCI group[140]. In a study by Boot et al on assessing vascular diameter, blood viscosity and shear stress using echo-doppler ultrasound also corroborated that mean peak shear stress was significantly greater in the common femoral artery[141]. No significant changes were reported for diameter and shear stress changes in common carotid artery. Boot et al argues that increased blood velocity, increased blood viscosity, inward remodelling of vasculature and a reduction in diameter of the vessels following SCI could explain the increased shear stress levels below the level of injury. De Groot et al. also showed that at 3 and 6 weeks post injury there is increased basal and peak shear stress rates in femoral artery[141]. De Groot argued that the inward remodelling of vasculature following SCI is a response to changes in peak shear stress rates rather than blood flow.

### **1.2.9 Peripheral Resistance and SCI**

Depending on the level and severity of injury, loss of descending sympathetic control of the peripheral circulation may result in lower peripheral resistance and profound vasodilation. The structural and functional properties of peripheral vasculature may be influenced by a lack of sympathetic tonus and drop in metabolic demand. This lack of activity at the level of the peripheral vasculature may culminate in vascular atrophy[132]. In regards to changes in peripheral resistance following SCI, there is variability among different studies. Some studies have shown that leg arterial inflow increases in chronic SCI individuals and peripheral resistance is reduced in the legs and arms of SCI individuals[142-144]. There are also studies that have shown an increase in peripheral resistance in the vasculature below the level of injury following SCI[139, 145-147]. Specifically, some studies have shown increased leg arterial blood inflow and reduced peripheral resistance[142, 143]. Further, the normal difference in peripheral resistance between arm and legs in AB subjects (lower peripheral resistance in arms than legs) is absent in SCI subjects[143]. Similar reduction in lower extremity peripheral resistance was found in paraplegic individuals. In addition, onset of upper limb exercise may acutely enhance vasodilatory response in the upper extremity peripheral resistance vessels[144].

Conversely, there are number of studies that show an increase in peripheral vascular resistance below the level of injury[148, 149]. In order to underlie why there is increased peripheral resistance following SCI, we would have to refer to studies on long term sympathectomised rodents. In these studies there is decrease activity of eNOS and a substantial reduction in the NO level (endothelial vasodilator)[150]. A reduction in NO is preceded by an upregulation of endothelin-1 (potent vasoconstrictor)[150]. The increase in circulating endothelin-1 may increase BP and could partially explain the increased peripheral resistance following SCI[150]. One study using an endothelin receptor blockade showed an increase in femoral artery blood flow following SCI[147].

### 1.2.10 Adrenoceptor Response Post SCI

SCI results in perturbed sympathetic pathways which may play a prominent role in the amount of NE and epinephrine (E) circulating in the blood[151, 152]. Tetraplegic individuals exhibit reduced levels of NE and E and even during the hypertensive phase (i.e. AD) of SCI the level of NE never increases above the threshold of normal NE levels in resting AB individuals[153]. This leads to the hypothesis that it is not at the substrate level but rather receptor hyper-responsiveness that may be responsible for the pressor response associated with AD.

Using NE as the primary approach to study receptor hyper-responsiveness is not valid. This is primarily due to 2 reasons: 1) There is non-specific binding of NE to post synaptic  $\alpha$ -adrenergic receptor and 2) Post synaptic reuptake of NE frequently occurs[154]. Thus a NE homolog such as PE is used a substitute. PE is a selective agonist for  $\alpha$ 1-adrenergic receptors which is not subject to reuptake. A study infusing PE in tetraplegic subjects, showed an increased pressor response when compared to AB subjects.

There are couple of studies that have assessed hypersensitivity of  $\alpha$ 1-adrenergic receptors post SCI. In a study assessing AD in response to colorectal distension (i.e. a potent stimulus to induce AD), partially attributed the pressor response to hyper-responsiveness of  $\alpha$ -adrenergic receptors. Brock et al (2006) assessed high responsiveness to PE in second order mesenteric arteries in T4 complete SCI rodent models. Spinalized rodents exhibited increased sensitivity to PE and this finding was attributed to a lower rate of PE removal by NE transporters at the post synaptic junction[155]. Alan et al assessed the role of endothelial dysfunction in hyper-responsiveness to PE in mesenteric arteries and found that the vasodilatory response to acetylcholine (Ach) was not different between SCI and SCI+CRD (i.e. repetitive induced AD) group[95]. Another study by Laird et al (2008) assessed hypersensitivity to PE in upper (brachial and carotid arteries) and lower (femoral and renal arteries) body peripheral vasculature after ganglionic blockade[156]. They found no differences

between SCI and control groups in regards to hypersensitivity to PE, suggesting the suppression of sympathetic activity below the level of lesion may play a prominent role in hyper-responsiveness in  $\alpha$ -adrenergic receptors[96, 155].

## **Chapter 2: Characterizing the Temporal Development of Cardiovascular Dysfunction in a T3 Complete Transection Rodent Model.**

The aim of this study is to characterize oscillations in hemodynamic variables and core body temperature from the acute to chronic stages of high thoracic experimental SCI. Using high fidelity implantable telemetric blood pressure monitoring, 24 hour beat by beat arterial blood pressure, heart rate and core body temperature was assessed. The progression of spontaneous AD was assessed via our novel AD detection algorithm from the acute to chronic stages of SCI using telemetric arterial blood pressure and heart rate data. In addition, daily averages and diurnal rhythmicity of multiple hemodynamic variables and core body temperature was assessed in order to fully characterize the temporal development of cardiovascular dysfunction following SCI in an experimental SCI rodent model.

### **2.1 Methods**

#### **2.1.1 Experimental Design**

We conducted a within animal, pre and post-SCI design, to specifically investigate the cardiovascular and autonomic changes that accompany SCI. Experiments were conducted on 6 Male Wistar rats (250-300g; Harlan Laboratories). Three weeks prior to SCI surgery, rats were fitted with a radiotelemetric devices for the subsequent continuous (24hr/day) assessment of arterial BP, HR, and core body temperature. To ensure rats had adequately recovered from telemetry implantation, pre-injury values were averaged between days 14-17 post-implantation (i.e., days 7-to-4 pre-SCI). These time points also ensured that any SCI surgery medication did not interfere with intact cardiovascular indices (see also ‘SCI surgery and animal care’ below). Responses at various time points post-SCI were compared to pre-SCI values. All procedures

were conducted in strict accordance with the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee.

### **2.1.2 Telemetry Implantation Surgery**

The telemetry device (TRM54P, Millar, Auckland, New Zealand) was prepared for implantation according to manufacturer guidelines. Rats were pre-treated for 3 days with prophylactic enrofloxacin (Baytril; 10 mg/kg, s.c., Associated Veterinary Purchasing; AVP, Langley, Canada). On the day of surgery, rats were anaesthetized with Isoflurane (initial chamber induction at 5% Isoflurane with 2 L/min Oxygen, followed by maintenance on a Bain's system at 1.5% Isoflurane with 1.5-2 L/min Oxygen). The abdomen was shaved and rubbed with iodine. A midline abdominal incision was made and the descending aorta was exposed *via* blunt dissection. The aorta was briefly occluded with 4-0 silk and punctured 1-2 mm anterior to the iliac bifurcation. A 20-Gauge curved needle was used to guide the tip of the telemetry device into the aorta, after which it was advanced rostrally such that the tip was just distal to the renal arteries. The catheter was fixed in place using a small amount of tissue adhesive. The body of the telemetry device was secured to the abdominal wall using 4-0 silk sutures. The abdominal wall was then closed using 4-0 Vicryl subcuticular sutures. Animals received warmed Lactated Ringer's solution (5 ml, s.c.) and recovered in a temperature-controlled environment (Animal Intensive Care Unit, Los Angeles, CA, USA). Enrofloxacin (10 mg/kg, s.c.), buprenorphine (0.02 mg/kg, s.c.) and ketoprofen (5 mg/kg, s.c.) were administered once a day for 3 days post-operatively.

### **2.1.3 T3 Complete Transection Surgery and Animal Care**

Rats were pre-treated for 3 days with prophylactic enrofloxacin (Baytril; 10 mg/kg, s.c., AVP). On the day of surgery, rats were anaesthetized with ketamine hydrochloride (70 mg/kg,

i.p.; Vetalar; AVP) and medetomidine hydrochloride (0.5 mg/kg, i.p.; Domitor; AVP). A dorsal midline incision was made in the superficial muscle overlying the C8-T3 vertebrae. The dura was opened at the T2-T3 intervertebral gap and the spinal cord was completely transected using microscissors. Complete transection was confirmed by two surgeons via visual separation of the rostral and caudal spinal cord stumps, and Gelfoam (Pharmacia & Upjohn Company, Pfizer, New York) was placed between the stumps to achieve hemostasis. The muscle and skin were closed with 4-0 Vicryl and 4-0 Prolene sutures, respectively. Animals received warmed Lactated Ringer's solution (5 ml, s.c.) and recovered in a temperature-controlled environment (Animal Intensive Care Unit, Los Angeles, CA, USA). Enrofloxacin (10 mg/kg, s.c.), buprenorphine (0.02 mg/kg, s.c.), and ketoprofen (5 mg/kg, s.c.) were administered once a day for 3 days post-operatively. The bladder was manually expressed three times (8am, 2pm, 10pm) daily until spontaneous voiding returned (about 10 days post-injury). Daily animal monitoring was conducted between 8am and 10am.

#### **2.1.4 24-hour Continuous Hemodynamic Monitoring**

Beat-by-beat arterial BP, HR, and body temperature were continuously monitored 24 hr/day, for 3 days pre-SCI (days 14-17 post transmitter implantation) and every second day post-SCI until day 28. This protocol was designed to reduce the enormous amount of data generated from beat-to-beat analyses. Hemodynamic variables were monitored at 1000Hz during each 24 hr period and the mean for the entire day was reported. Due to the need for animal monitoring we deemed it necessary to remove the following hemodynamic data from all subsequent analyses. For the first 10 days post-SCI, rats were monitored 3 times per day (see also above), but the bulk of the monitoring (e.g., medications, cage changes, weighing) was conducted in the morning (8-9:30am); hence we completely eliminated this time from the analyses. The other monitoring

times were bladder expression only and lasted no longer than 2-3 minutes per animal. For these checks the 10 min before and the 30 min after bladder expression were also removed. From day 10 onwards reflexive micturition occurred and rats were only checked once per day in the morning.

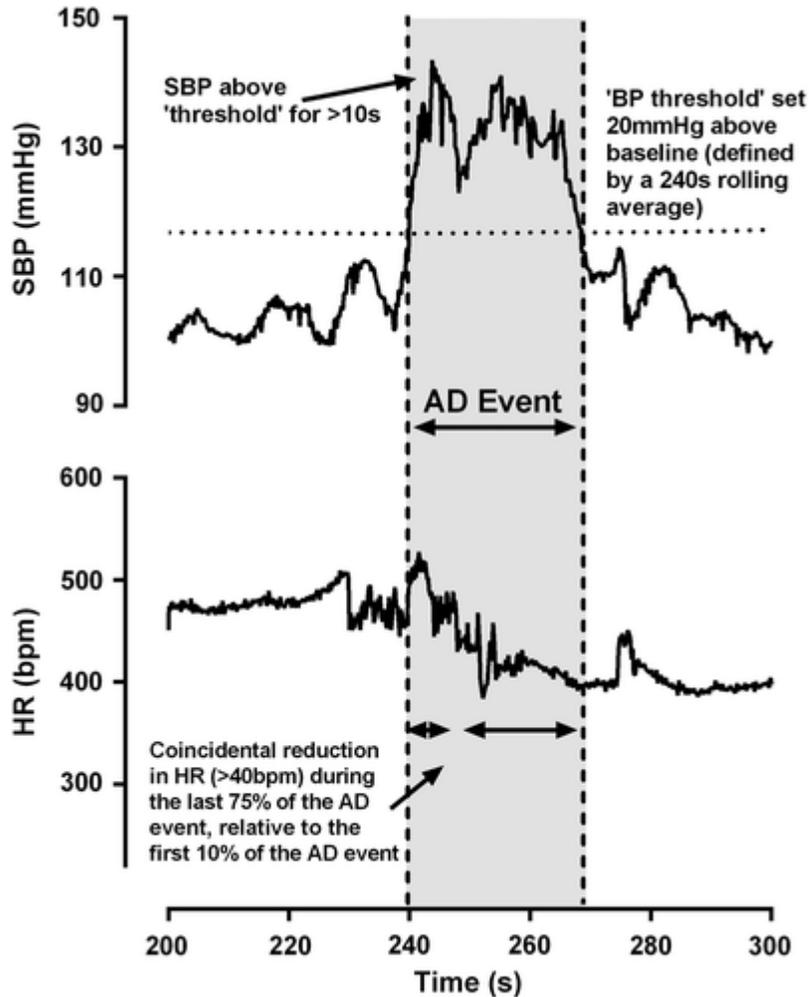
### **2.1.5 Assessment of Diurnal Rhythmicity in Hemodynamics**

On day 4 pre-SCI, day 4 and 8 (acute SCI) and on days 14, 20 and 28 (chronic SCI), hemodynamic variables were additionally averaged on an hour-by-hour basis such that the diurnal rhythmicity in hemodynamic variables could be assessed. For these analyses, hemodynamic variables during night hours were compared against hemodynamic variables during daytime hours. For core-body temperature, we also calculated the peak-to-trough variation.[157]

### **2.1.6 Spontaneously Occurring Autonomic Dysreflexia**

We developed a novel AD detection algorithm that was capable of screening beat-by-beat BP and HR data to detect the incidence, severity and duration of spontaneously occurring AD (MATLAB, The MathWorks, Inc., Natick, MA). Our algorithm extended previous work by Rabchevsky *et al.*,[158] and Zhang *et al.*,[159] who previously used a basic algorithm to detect abnormal spikes in BP and troughs in HR that resembled the hypertension and bradycardia typically associated with AD. A potential limitation of the precedent approaches however, is that BP and HR raw data were down-sampled from their initial sampling frequency, which may result in an underestimation of the true peak pressor response during AD. Furthermore, AD was defined as a 10mmHg increase in mean arterial pressure (MAP) above baseline coupled with a 10 bpm drop in HR. The latter of which seems inappropriate to define bradycardia given the high resting

HR of a rodent. We used the clinically accepted definition of autonomic dysreflexia, a condition that is characterized by a spike of SBP greater than 20mmHg above baseline[52]. We circumvented these potential limitations by recording beat-by-beat systolic BP (SBP) and HR telemetry recordings 24-hr/day for 3 days pre-SCI and every second day post-SCI until day 28 (Lab Chart; AD Instruments. Inc., Dunedin, New Zealand). Beat-by-beat SBP and HR (calculated from R-R interval) were extracted from the raw data using commercially available software (Lab Chart) and imported into MATLAB. SBP and HR were screened for non-physiological values and any data point that had a HR <180 or >625bpm were excluded from the analyses. Next, a moving average window of 240 seconds was created. An 'AD threshold' was then set at 20mmHg above the moving average baseline in order to detect sudden spikes in SBP. A cluster of SBP spikes was defined as an AD event when the following conditions were met: (1) SBP peaks exceed the 'AD threshold' for a duration greater than 10 seconds, and (2) There was a corresponding mean HR drop of 40 bpm or greater during the last 75% of the AD event relative to the mean HR during the first 10% of time during the potential spontaneous AD event. The 40 bpm was chosen to reflect a similar percentage drop from baseline to that typically observed clinically. If more than one AD event was identified within 120s of the preceding event, these events were amalgamated and counted as one AD event. For each identified AD event we extracted the pressor response, maximum SBP, average SBP, minimum HR, average HR, duration of the event, and time of day. An example AD event detected using this algorithm is presented in Figure 3.



**Figure 3:** Example of detection of an autonomic dysreflexia (AD) event using our algorithm. First, a 240 sec rolling average baseline is fitted to the systolic blood pressure (SBP) and heart rate (HR) data. Next, a SBP and HR “threshold” is transposed 20 mm Hg above the moving baseline or 40 bpm below the moving baseline, respectively. To be considered an AD event, the SBP must rise above the threshold and remain elevated above threshold for at least 10 sec. During this time, there must also be a coincidental reduction in HR below the threshold.

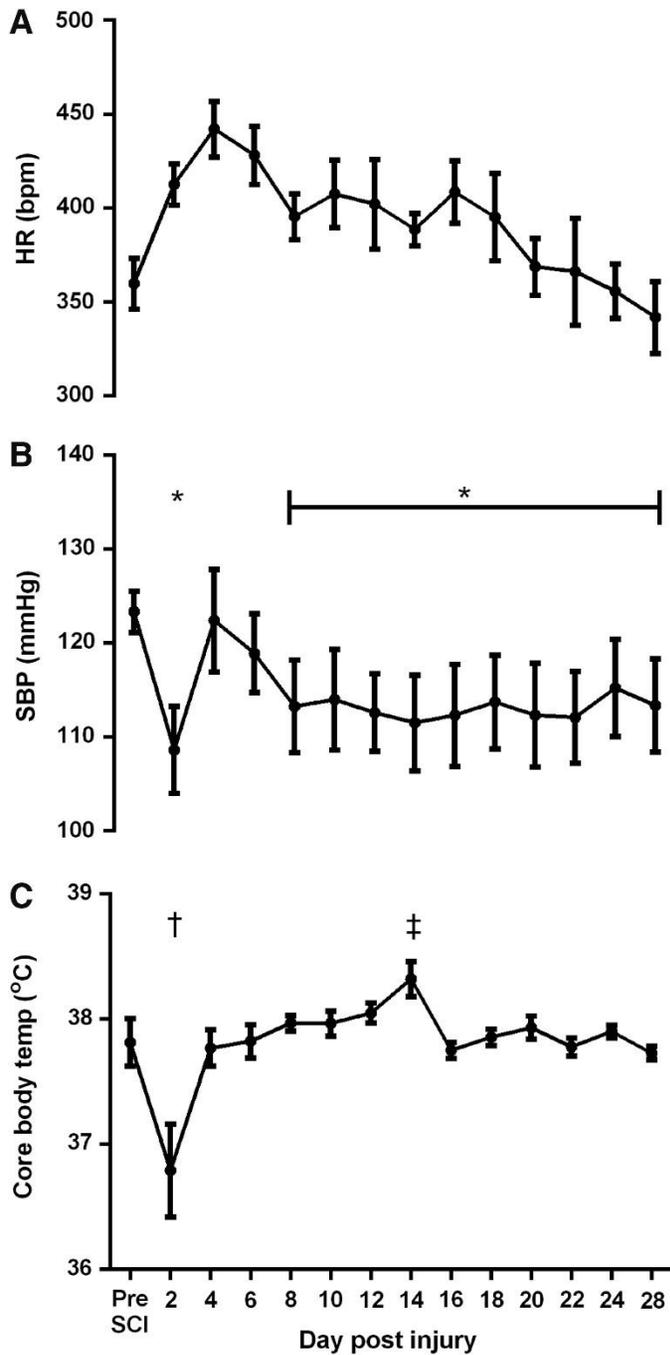
### **2.1.7 Statistical Analyses**

Dependent variables were assessed for assumptions of parametric testing. Alterations in hemodynamics across time were assessed using a one-way repeated measures ANOVA with Bonferroni corrected post-hoc comparisons. The presence or absence of a nocturnal dip in hemodynamics along with the incidence of AD during day and night cycles were assessed using a two factor repeated measures ANOVA, with one factor for time of day and one factor for time post-injury. Statistical analyses were conducted using STATA v12.1. Statistical significance was accepted at  $p < 0.05$ .

## **2.2 Results**

### **2.2.1 Time-course of Blood Pressure, Heart Rate and Core Body Temperature**

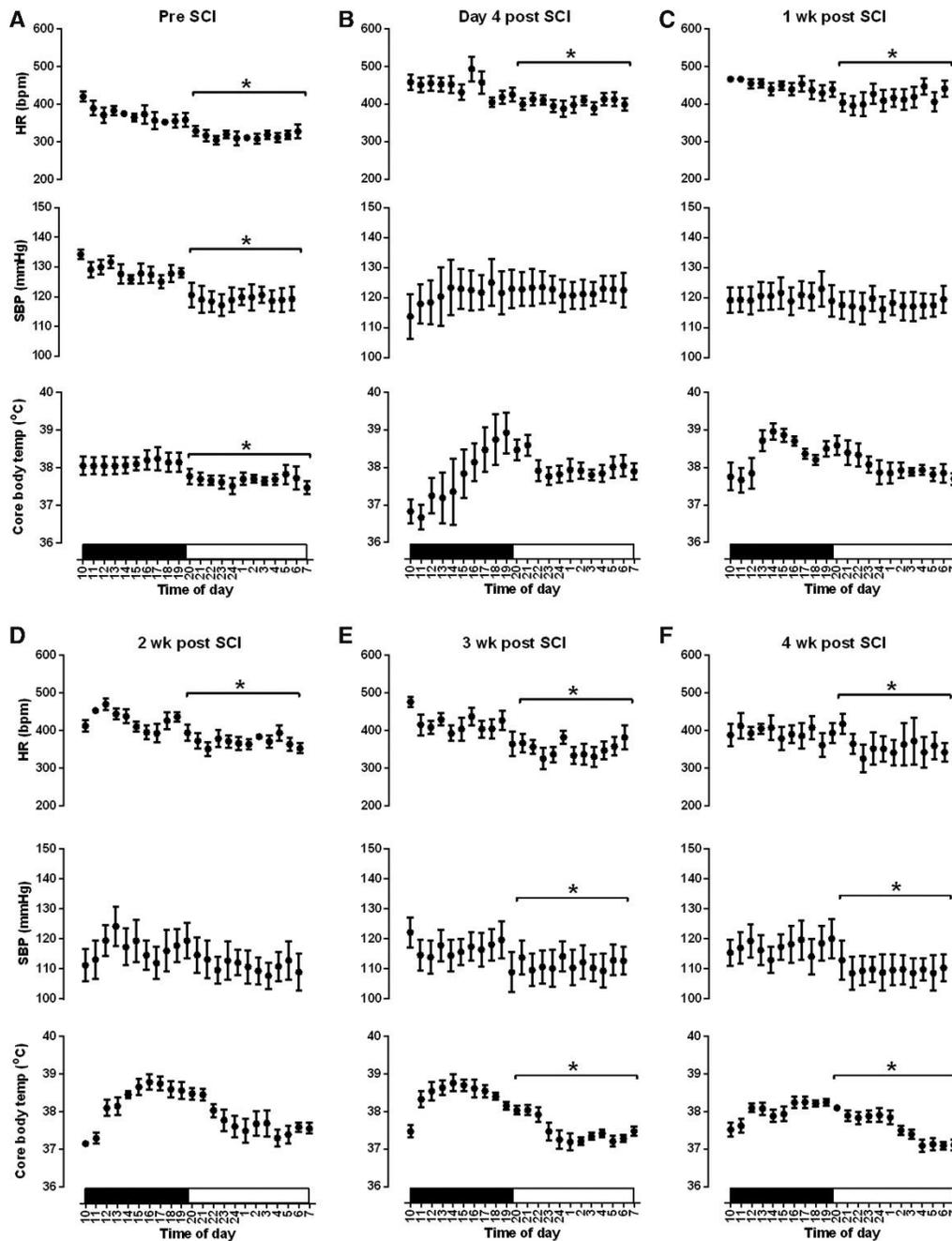
SCI is associated with time-dependent changes in blood pressure, heart rate, and core body temperature. SBP was reduced at day 2 post-SCI vs. pre-SCI ( $p = 0.001$ ; Figure 4). There was a transient recovery of SBP at days 4 and 6 post-injury, such that SBP was not different at these time-points vs. pre-SCI (both  $p > 0.092$ ). From day 8 onwards, there was a chronic reduction in SBP vs. pre-SCI (all  $p < 0.002$ ). Core body temperature was reduced at day 2 post-SCI vs. pre-SCI ( $p = 0.001$ ), but recovered by day 4. There was a spurious increase in core body temperature at day 14 post-SCI vs. all other days (all  $p < 0.047$ ). There was a trend towards an increase in HR at day 2 and 4 post-SCI vs. pre-SCI, but this just failed to reach statistical significance (both  $p = 0.056$ ).



**Figure 4:** Temporal changes in heart rate (HR) (A), systolic blood pressure (SBP) (panel B), and core body temperature (C) in response to spinal cord injury (SCI). Note that there was an initial reduction in SBP during the first 2 days post-injury that was partially compensated for by tachycardia. From day 7 onwards, rodents exhibited persistent hypotension compared with pre-SCI. Data are presented as mean $\pm$ SEM. \*P<0.05, significant difference between post SCI days vs. pre-SCI. †P<0.05, significant difference between post SCI days vs. pre-SCI. ‡P<0.05, significant difference between day 14 post SCI vs. all other days.

### 2.2.2 Diurnal Rhythmicity

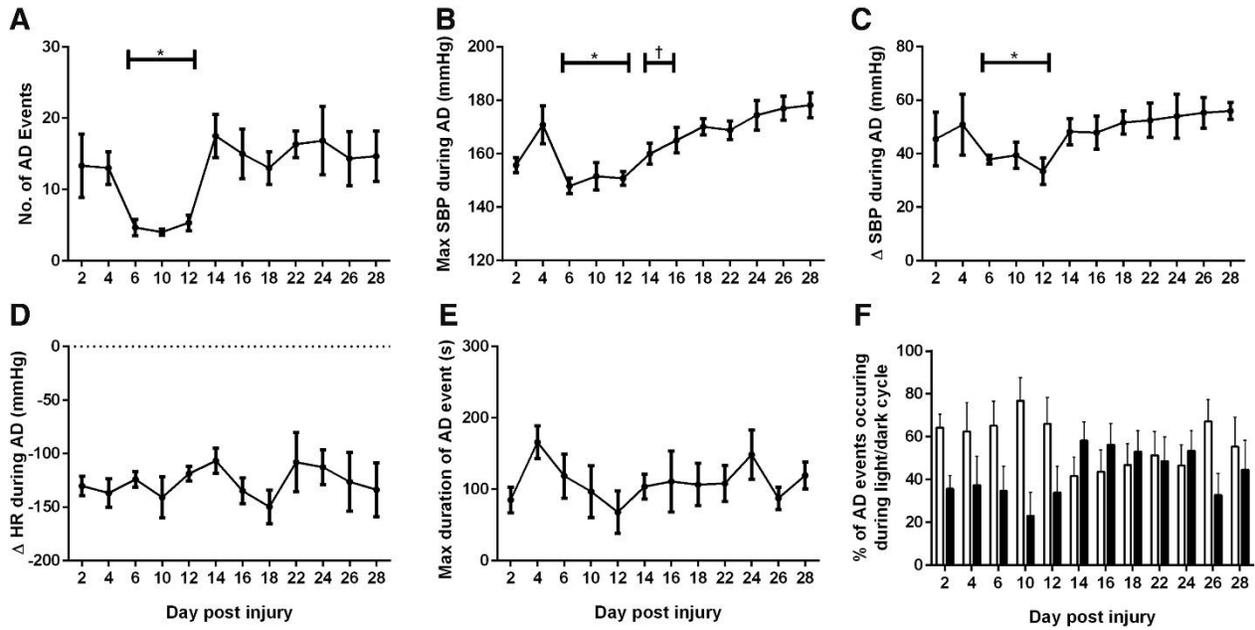
Loss of rhythmicity in cardiovascular function in response to SCI is partially reversible with time. Prior to SCI surgery, all rats exhibited typical diurnal rhythmicity in cardiovascular control, as characterised by the presence of nocturnal dip (all  $p < 0.012$ ; Figure 5). During the first 14 days post-SCI, there was no difference in SBP or core body temperature between day and night (all  $p > 0.323$ ), implying the normal diurnal rhythmicity in BP and core-body temperature was disrupted. In particular, animals struggled to increase core body temperature during the day, and the peak-to-trough value was substantially higher at days 4 ( $3.84 \pm 1.87^\circ$ ), 7 ( $2.09 \pm 0.75^\circ$ ) and 14 ( $2.21 \pm 0.52^\circ$ ) post-SCI compared with pre-SCI ( $1.26 \pm 0.23^\circ$ ; all  $p < 0.038$ ). Conversely, the nocturnal dip in HR remained stable after SCI, as evidenced by a significantly lower HR during night vs. day (all  $p < 0.019$ ). At 21 and 28 days post-SCI, rats regained some degree of cardiovascular control, as evidenced by the recurrence of a nocturnal dip in SBP and core body temperature (all  $p < 0.024$ ). Similarly, the peak-to-trough value for core-body temperature was no longer statistically different at day 28 post-SCI ( $1.68 \pm 0.39^\circ$ ) vs. pre-SCI.



**Figure 5:** Diurnal rhythms for heart rate (HR), systolic blood pressure (SBP), and core body temperature stratified by time after spinal cord injury (SCI). Black and white rectangles represent night and day cycles, respectively. Note that there was substantial disruption in typical diurnal rhythms during the first 2 weeks post-SCI (A–C). Although diurnal rhythmicity returned from 3 weeks post-SCI onwards (E–F), all cardiovascular indices appeared to exhibit greater daily variation post-SCI. Data are presented as mean±SEM. \*P<0.05, significant difference between day vs. night.

### 2.2.3 Development of Autonomic Dysreflexia

SCI is associated with time-dependent development of autonomic dysreflexia. We used a custom-built algorithm to detect the incidence, severity and duration of spontaneously occurring AD, along with the time of day at which AD occurred (Figure 3). During the three weeks of data collection pre-SCI, our algorithm picked up an average of one 'AD event' in each of the animals (data not shown). It should be noted that these patterns of BP that resembled an AD event pre-SCI never exceeded 12s in duration. Post-SCI, we found that animals exhibited AD as early as days 2 and 4 post-SCI (Figure 6). During this acute period, animals exhibited up to 14 AD events per day, with an average pressor response of  $48.5 \pm 11.1$  mmHg. Between day 6 and 12 post-SCI, AD incidence was lower than day 4 and day 14 onwards (all  $p < 0.035$ ). Both the maximal SBP and magnitude of the AD pressor response was lower between days 6 and 12 vs. day 14 onwards (all  $p < 0.006$ ). From day 22 onwards, the AD pressor response exceeded all previous time points (all  $p < 0.0022$ ), except for day 4 where there were no statistical differences. There were no differences across time for the degree of AD-induced bradycardia, the duration of AD events, or whether AD occurred predominantly during the light or dark period. By day 28 post-SCI, rodents exhibited up to 39 AD events per day with a maximum pressor response of 68 mmHg.



**Figure 6:** Data collected from each of the spontaneous autonomic dysreflexia (AD) events stratified by time post-injury. During the first 4 days post-injury there was a high daily incidence of spontaneously detected AD (panel A) events that were characterized by a pressor response similar to that in the chronic phase after spinal cord injury (SCI) (panels B and C). From days 6–12, there was a large reduction in both the number of AD events and the pressor response to AD. By day 14 post-injury, there was a high daily incidence of AD events, but the maximum pressor response was not apparent until day 18 post-injury onwards. There were no differences in the degree of bradycardia (panel D), the duration of AD events (panel E), or the time of day at which the AD events occurred (panel F). Data presented as mean±SEM. \*P<0.05 day 6-12 post SCI vs. day 14 onwards. †P<0.05 day 14-16 post SCI vs. day 18 onwards.

## **Chapter 3: Time course of functional and structural adaptations of resistance vasculature after T3 complete transection.**

The aim of this study is to examine the time course of structural and functional adaptations of primary mesenteric arteries (PMA) in a complete T3 transection rodent model. Using an ex vivo technique called pressure myography, morphological features of the vessel may be assessed, in addition to myogenic and neurogenic tone. Further, morphological assessments were corroborated with immunofluorescence staining of key structural proteins such as collagen and smooth muscle actin.

### **3.1 Methods**

#### **3.1.1 Experimental Design**

Experiments were conducted on thirty-three male Wistar rats (age = 9 weeks, mass = 250-350g; Harlan Laboratories, Indianapolis, IN, USA). Animals were assigned to three groups: T3 1 week complete spinal cord transection group (T3 SCI 1 week; n=11), T3 1 month complete spinal cord transection group (T3 SCI 1 month; n=11) and uninjured age-matched 1 month control group (control; n=11). At 7 days (T3 SCI 1 week; n=5) and 28 days post T3 complete SCI (T3 SCI 1 month n=5 and aged-matched 1-month control n=5) primary mesenteric artery (PMA) passive structure and vasoconstrictive responsiveness were assessed using pressure myography. An additional six animals in each group were assessed for collagen and smooth muscle cell density using immunofluorescence staining.

#### **3.1.2 Surgery and Animal Care**

For 3 days prior to the SCI surgery, animals were administered enrofloxacin (Baytril 10mg kg<sup>-1</sup>, s.c., Associated Veterinary Purchasing (AVP), Langley, Canada). On the day of surgery, animals were administered enrofloxacin (10mg kg<sup>-1</sup>, s.c.), buprenorphine (Temgesic,

0.02 mg kg<sup>-1</sup>, s.c., McGill University) and ketoprofen (Anafen, 5mg kg<sup>-1</sup>, s.c., AVP) injections. Animals were anesthetized with ketamine hydrochloride (70mg kg<sup>-1</sup>, I.P., Vetalar; AVP) and medetomidine hydrochloride (0.5 mg kg<sup>-1</sup>, I.P.; Domitor; AVP). A dorsal midline incision was made in the superficial muscle overlying the C8-T3 vertebrae. The dura was isolated at the T2-T3 intervertebral gap and using micro-scissors, the spinal cord was completely transected. Two surgeons confirmed the complete transection via visual separation of the rostral and caudal spinal cord stumps. In order to achieve hemostasis Gelfoam (Pharmacia & Upjohn Company, Pfizer, New York, USA) was placed between the stumps. Using 4-0 vicryl and 4-0 prolene sutures, the muscle and skin were closed respectively. Animals were placed in a temperature-controlled environment for post-surgical recovery (Animal Intensive Care Unit, Los Angeles, CA, USA), received warmed lactated Ringer's solution (5mL, s.c.) and continued to receive enrofloxacin (10mg/kg, s.c.) and buprenorphine (0.02 mg/kg, s.c.) for three more days. Bladders were manually expressed 3 to 4 times daily until such time that spontaneous voiding returned (approximately 10 days post injury).

### **3.1.3 Pressure Myography**

At both 1 week (T3 SCI 1 week; n=5) and 1 month time points (T3 SCI 1 month; n=5 and control; n=5), rats were anesthetized with 3% isoflurane before ex vivo pressure myography studies of PMA structure and function (Living Systems, St. Albans, Vermont). A proximal quarter segment (isolated at the transition point between the small intestine and large intestine) of the PMA was isolated from the gut (first branch of the superior mesenteric artery). The artery segment was mounted between two glass cannulas in a pressure myograph chamber which was then placed in an inverted microscope coupled to a camera (Olympus SZ30, Center Valley, PA, USA) and auto-detection dimension analyser (Living Systems, VDA-10) under a 10x objective

(Nikon E plan LWD, 10x/0.25). The PMA was immersed in  $\text{Ca}^{2+}$  free PSS solution at 4°C. The composition of the PSS solution was as follows (in mM): NaCl (141.9), KCl (4.7),  $\text{KH}_2\text{PO}_4$  (1.12),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (1.7),  $\text{CaCl}_2$  (2.8), HEPES (10), dextrose (5) and EDTA (0.5) at a pH of 7.4 and was bubbled with a mixture of  $\text{O}_2$  (95%) and  $\text{CO}_2$  (5%). Passive structure was assessed using  $\text{Ca}^{2+}$  free PSS (PSS without 2.8mM  $\text{CaCl}_2$ ) starting at an intraluminal pressure of 20mmHg and increased in a stepwise manner to 140mmHg. Wall to lumen ratio, lumen diameter, wall thickness and vessel external diameter was assessed. Circumferential wall stress and strain was calculated as described previously by Biones *et al.*, 2003 with exception that  $D_{00\text{Ca}}$  was calculated as the intraluminal diameter at 20mmHg[160]. The elastic modulus (B-coefficient) was calculated from stress/strain curves using the exponential model ( $y=ae^{\beta x}$ ), where  $\beta$  is the slope of the curve and is directly correlated to vascular stiffness. Myogenic tone was subsequently assessed using PSS with  $\text{Ca}^{2+}$  (PSS with 2.8mM  $\text{CaCl}_2$ ) starting at an intraluminal pressure of 20mmHg and then proceeding to 140mmHg. Intraluminal pressure was reduced to 40mmHg to avoid generation of myogenic tone and contractile responsiveness assessed in response to increasing concentrations of phenylephrine (PE) and methoxamine (MET) (1nM to 10 $\mu$ M) added to the PSS. MET is a PE analog that is not a substrate of the neuronal norepinephrine transporter[155]. A thorough wash was completed using PSS with  $\text{Ca}^{2+}$  after PE functional assessment prior to the MET functional assessment.

### **3.1.4 Tissue Collection and Immunofluorescence Staining**

Animals used for histological studies were sacrificed with an overdose of chloral hydrate (1gm/kg, i.p.) which, was perfused transcardially with 0.1M PBS and fixed with 4% formaldehyde solution. The gut was collected and post-fixed for 24 hours and then transferred to a 20% sucrose solution for a further 24 hours. A proximal quarter segment of the PMA isolated at

the transition point between the small and large intestine, as previously described, was embedded in optical cutting temperature (OCT) freezing medium. Using a cryostat, 10 $\mu$ m transverse PMA sections were collected on Superfrost Plus slides and stored at -80°C.

Morphological assessment of smooth muscle cell (SMC) nuclei density and collagen deposition was determined using immunofluorescence staining. Sections were allowed to air dry and sequentially washed with 0.1M PBS followed by 0.1M PBS-T and subsequently incubated in 10% NDS. Primary antibodies used for the immunohistochemical morphological assessment were: rabbit  $\alpha$  collagen 1 (1:1000, Abcam, ab292), mouse  $\alpha$  collagen 3 (1:300, Abcam, ab6310) and rabbit  $\alpha$  smooth muscle actin (1:400, Abcam, ab19134). Sections were incubated in primary antibodies for 24 hours at room temperature. Sections were washed in PBS-T prior to incubation with Alexa<sup>TM</sup>-fluor 488 donkey  $\alpha$  mouse (1:1000: Molecular Probes, USA) and/or donkey  $\alpha$  rabbit conjugated Cy3 (1:1000, Jackson ImmunoResearch, USA) for 3 hours. All sections were washed with 0.1M PBS-T and PBS, and mounted using Prolong Gold mounting medium (Molecular Probes, USA). Sections assessed for smooth muscle actin (SMA) were also stained with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, California) to allow co-localization of SMC nuclei with SMA in the medial layer of the vessel. Immunofluorescence staining on transverse PMA sections was observed using AxioImager.M2.Zeiss microscope (HR R3; objective magnification 20X, ocular magnification, 10X) using Zen 2 Pro. For assessment of collagen type I (COL I) and collagen type III (COL III), integrative density (fluorescence intensity normalized to area of interest) of fluorescent markers within the PMA were assessed using Image J. Integrative density measures were averaged from 4 sections per animal within the proximal quarter of the PMA. The inner and outer boundaries of the analysis for COL I and COL III were the luminal interface and the adventitial border,

respectively. For assessment of SMC density, co-labelled cells within the media layer of the vessel (visualized by immunofluorescence staining of SMA and DAPI) were counted using Image J. Cell density measures were averaged for 4 sections within the proximal quarter of the PMA. A negative control (absence of primary antibody in the presence of secondary antibody) was used to ensure specific binding of secondary antibody to primary antibody.

### **3.1.5 Statistical Analysis**

Between group differences for pressure myography structural measures of wall thickness, lumen diameter, wall to lumen ratio, vessel diameter and myogenic tone were determined using a two- way repeated measures analysis of variance (ANOVA), with one factor for injury groups (T3 SCI 1 week group/T3 SCI 1 month group/control group) and one factor for transmural pressures. Immunofluorescence morphology measures were analyzed using one-way ANOVA and Tukey post-hoc comparison test. Concentration response curves of PE and MET were further analyzed using nonlinear regression (Hill equation) to calculate EC<sub>50</sub>. All statistical analysis was carried out using GraphPad Prism 6.0 software (Graphpad, San Diego, CA) and statistical significance accepted at  $p < 0.05$ .

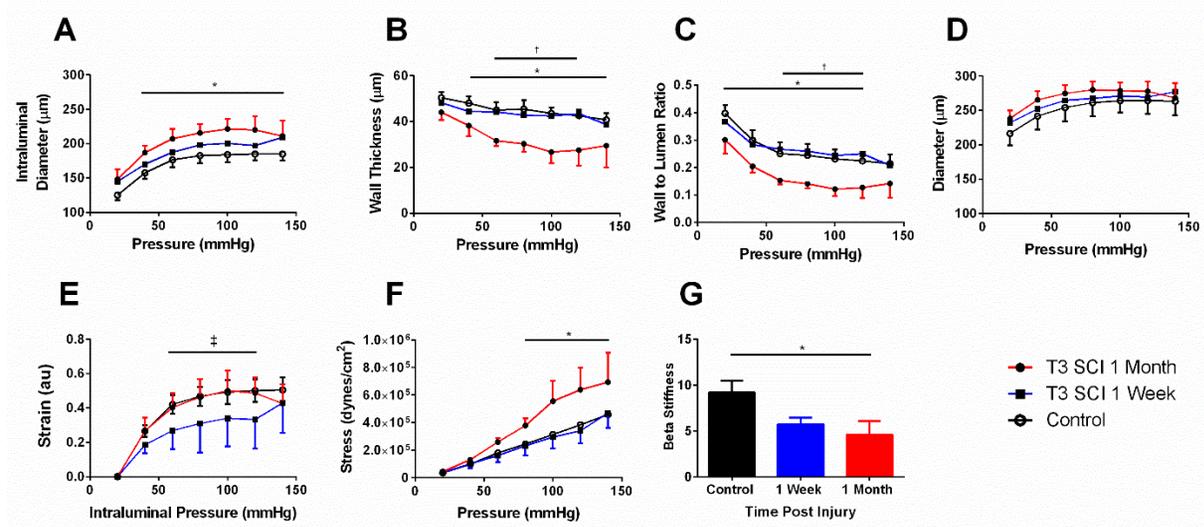
## **3.2 Results**

### **3.2.1 PMA Pressure Myography**

Structural assessment of the PMA using pressure myography suggests hypotrophic remodelling in PMA after SCI. Intraluminal diameter was significantly higher in the T3 SCI 1 month group compared to control at intraluminal pressures of 40mmHg to 140mHg (all  $p < 0.05$ ; Figure 7). Wall thickness was significantly lower in the T3 SCI 1 month group compared to T3 SCI 1 week and control groups at intraluminal pressures of 60mmHg to 120mmHg (all  $p < 0.05$ ).

Wall to lumen ratio was significantly lower in the T3 SCI 1 month group compared to T3 SCI 1 week and control groups at intraluminal pressures of 60mmHg to 120mmHg (all  $p < 0.05$ ).

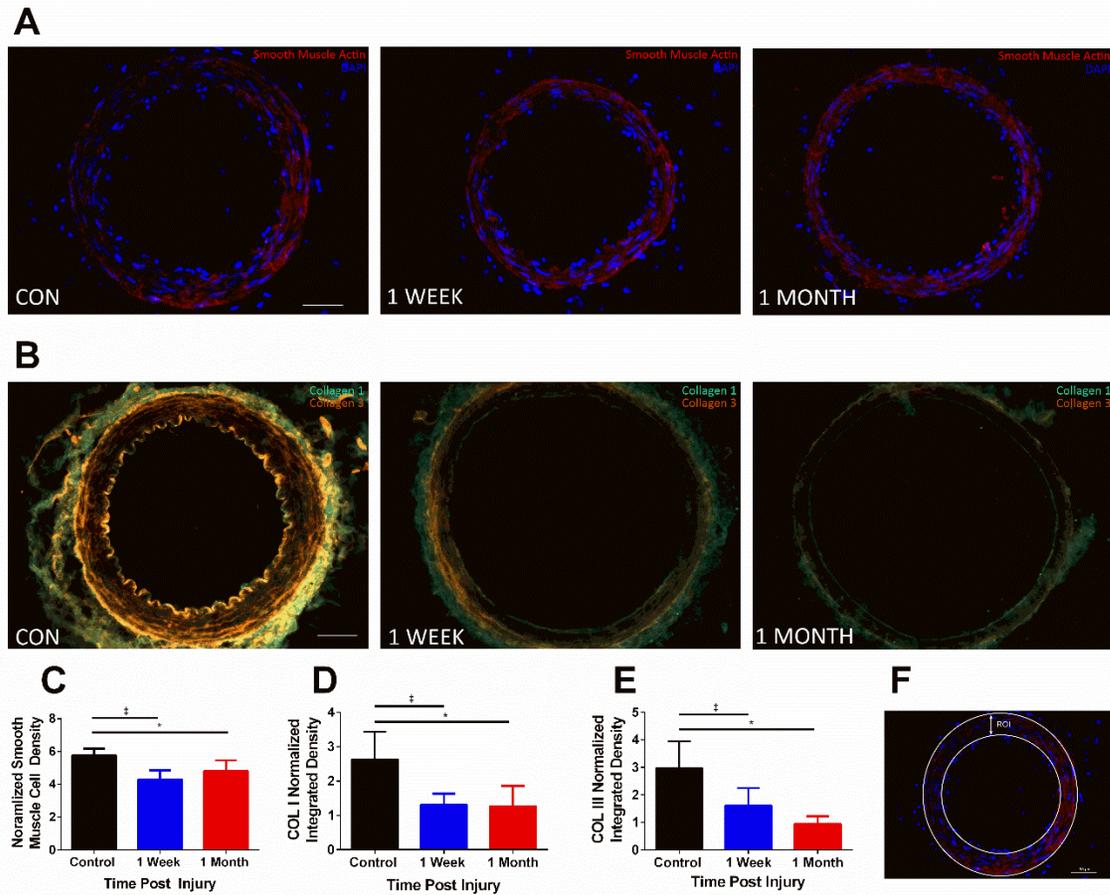
Further, there was a 50% increase in vessel stress in the T3 SCI 1 month group compared to control and T3 SCI 1 week group at intraluminal pressures of 80mmHg to 140mmHg (all  $p < 0.05$  for both comparisons). This increase in stress in the T3 SCI 1 month group was compounded by a decrease in  $\beta$  stiffness (elastic modulus) values compared to the control group ( $p = 0.0382$ ). There were significant decreases in strain in the T3 SCI 1 week group compared to the T3 SCI 1 month and control groups at intraluminal pressures of 80mmHg to 120mmHg (all  $p < 0.05$ ). There were no significant differences in total vessel diameter between groups.



**Figure 7:** Chronic SCI induces hypotrophic remodelling in PMA, characterized by a decrease in wall to lumen ratio and reduced stiffness. Mean  $\pm$ SD are reported. Post-hoc comparisons of structural (A-D) and mechanical measures (E-G) were made using physiologically relevant intraluminal pressures (20mmHg-140mmHg). \* $P < 0.05$ , significant difference between T3 SCI 1 month vs. control groups. † $P < 0.05$ , significant difference between T3 SCI 1 month vs. T3 SCI 1 week groups. ‡ $P < 0.05$  significant difference between T3 SCI 1 week vs. control groups.

### **3.2.2 PMA Morphology Immunofluorescence Assessment**

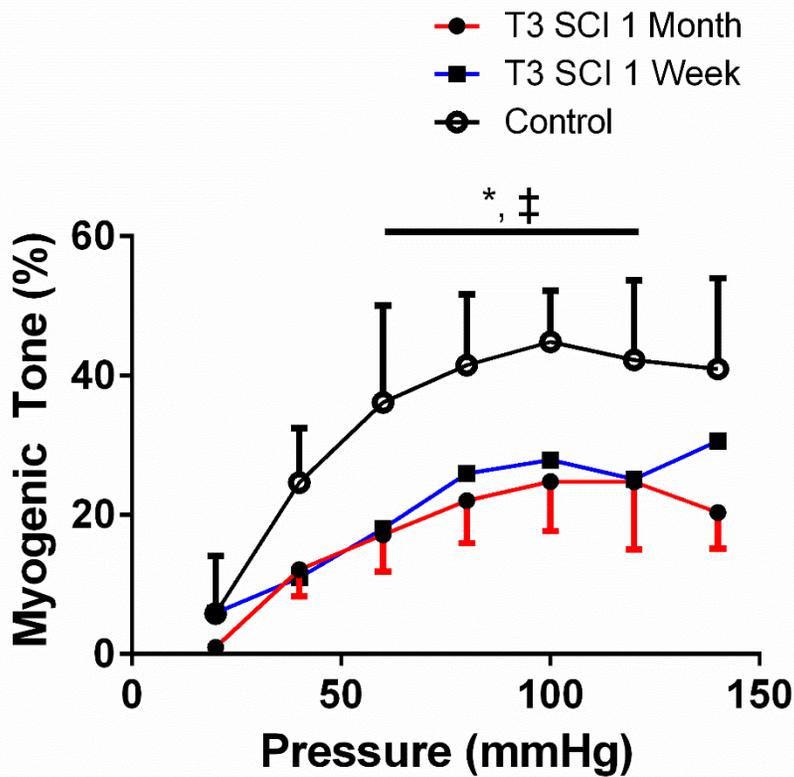
SCI groups exhibited smooth muscle atrophy and decreased collagen deposition within the medial layer of PMA. The T3 SCI 1 week and 1 month groups exhibited significant reductions in smooth muscle cell density compared to control ( $p=0.003$  and  $p=0.0146$ , respectively; Figure 8). There was a significant decrease in collagen type I ( $p=0.0111$  and  $p=0.0067$ , respectively) and collagen type III ( $p=0.0112$  and  $p=0.004$ , respectively) deposition within the media layer of PMA in both 1 week and 1 month SCI groups.



**Figure 8:** High thoracic SCI may result in increased smooth muscle atrophy and decreased collagen deposition. Nuclei of the smooth muscle cells were stained with DAPI and then counted (C). Mean normalized smooth muscle cell (SMC) density  $\pm$  SD is determined from smooth muscle actin (SMA) immunofluorescent region of interest (F). Mean normalized integrated density  $\pm$  SD are measured for COL I (collagen type I) and COL III (collagen type III) within region of interest bounded by the outer adventitial border and internal elastic lamina (D and E). Example images for immunofluorescence measures are presented at 20x magnification for SMA co-stained with DAPI (A) and co-labelled images with COL I and COL III (B). \* $P < 0.05$ , significant difference between T3 SCI 1 month vs control groups. ‡ $P < 0.05$ , significant difference between T3 SCI 1 week vs control groups.

### **3.2.3 PMA Myogenic Tone Assessment**

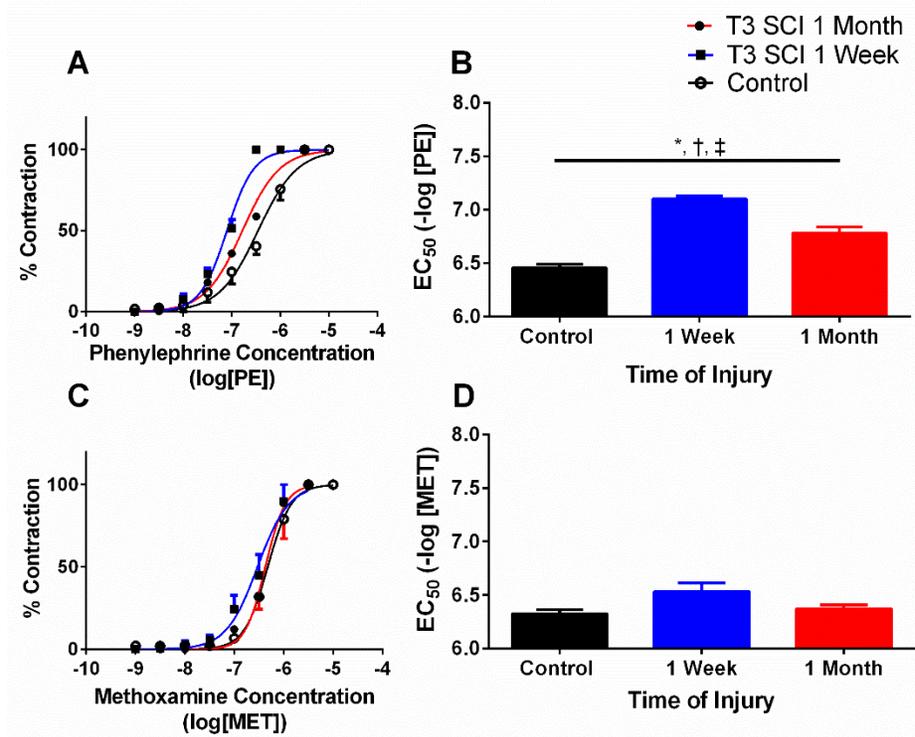
We assessed pressure induced constriction (i.e. myogenic constriction) of the PMA over a series of intramural pressures. There were significant reductions of myogenic tone generation in T3 SCI 1 week and 1 month groups at intraluminal pressures of 40mmHg to 120 mmHg (all  $p < 0.05$ ; Figure 9).



**Figure 9:** High thoracic SCI results in impaired maintenance of myogenic tone in PMA. Generation of myogenic tone was monitored in physiologically relevant pressures of 20mmHg to 240mmHg. Myogenic tone is assessed as a percentage of the lumen diameter measured initially using Ca<sup>2+</sup> free PSS at the same intraluminal pressure. \*P < 0.05, significant difference between T3 SCI 1 month vs. control groups. ‡P < 0.05, significant difference between T3 SCI 1 week vs. control groups.

### 3.2.4 PMA $\alpha$ -Adrenoceptor Function Assessment

Acute high thoracic SCI results in increased sensitivity of PMA to PE but not MET. The concentration response curves to PE demonstrated increased sensitivity to PE in the T3 SCI 1 week group compared to T3 SCI 1-month and control groups. There was a lower EC<sub>50</sub> value for PE at T3 SCI 1 week compared to both T3 SCI 1 month and control groups (p=0.004 and p<0.001, respectively; Figure 10). The EC<sub>50</sub> value for PE in 1 month T3 SCI PMA was also significantly lower than control group (p=0.004). There were no significant differences in EC<sub>50</sub> values for MET in T3 SCI 1 week, T3 SCI 1 month and control PMAs.



**Figure 10:** Increased sensitivity of PMA to phenylephrine (PE) but not methoxamine (MET) after high thoracic SCI. Percent Contraction  $\pm$  SEM, are determined relative to PMA maximum constriction dose. Concentration response curves for phenylephrine (A) and methoxamine (C) in PMA for T3 SCI 1 week, T3 SCI 1 month and control groups. EC<sub>50</sub>  $\pm$  SEM (B and D) values are determined from best fit curves from Hill Equation. \*†‡ P < 0.05, significant difference between all groups.

## Chapter 4: Discussion

### 4.1 Basal Hemodynamics and Core Body Temperature

T3 complete transection results in acute reduction in ABP, return to pre injury indices and then a chronic period of persistent hypotension. This acute hypotensive period may be attributed to neurogenic shock, which is characterized by stifled sympathetic activity and spinal reflexes. This acute reduction in BP is in agreement with previous studies in animal models of high thoracic SCI[161, 162]. Persistent chronic hypotension is a result of loss of supraspinal control over the majority of spinal sympathetic circuitry particularly those that innervate the splanchnic circulation (critical capacitance vasculature integral for ABP control). The rationale for chronic hypotension cannot be attributed to neurogenic shock as this acute depression of sympathetic activity lasts only a few days in rodent models of SCI[161]. Chronic period of hypotension is in agreement with Laird et al. who assessed hemodynamic variations in rodents using a high thoracic complete SCI model[162]. Another study assessing a mid-thoracic 50g clip compression incomplete SCI model found that BP recovers to pre-SCI levels within 5-10 days and does not present with chronic hypotension [161, 163]. These findings are different from our complete transection due to the lower severity of injury as a result of the 50g clip compression and the partial preservation of bulbospinal sympatho-excitatory inputs to sympathetic preganglionic neurons.

Depending on the level and severity of SCI, there is a greater loss of supraspinal control of sympathetic activity and in turn a greater degree of cardiac autonomic dysfunction[163-165]. As a compensatory baroreceptor mediated mechanism to acute hypotension, animals exhibit tachycardia which is likely a balance of vagal input withdrawal and increased supraspinal sympathetic drive to intact T1 and T2 spinal segments. In our complete transection model T1 and T2 sympathetic circuitry is left intact, which are imperative for sympathetic control over the heart. In a study assessing a mid-

thoracic SCI model and sympathetic innervation of the heart found that there is an increased in left ventricular sympathetic innervation and emphasised arborisation of cardiac sympathetic preganglionic neurones[166]. There is no evidence of tachycardia in our T3 complete transection model which based on our previous finding may be attributed to reduction in cardiac output[167].

There is a substantial drop in core body temperature at 4 days post injury which is in agreement with previous studies in high thoracic SCI rodent models[162]. Although there are no studies that examined CBT in humans during acute SCI, there are studies of chronic SCI individuals with injuries above T6 exhibiting poikilothermia [69, 70]. The acute period post SCI (i.e. neurogenic shock) results in disrupted afferent pathways from skin receptors to temperature regulation centres below the level of injury. This impairs the ability to vasoconstrict, vasodilate or sweat in peripheral vascular beds below the level of injury [66, 168]. Due to decreased vascular tone and increased vasodilation, there is increased skin blood flow which allows for increased dissipation of heat to the surroundings[162]. CBT recovers to pre injury levels by day 4 post SCI which may be attributed to subsidence of neurogenic shock.

#### **4.2 Diurnal Rhythms**

Within the first 2 weeks post SCI, there is disrupted diurnal variations in BP which recovers by 3 weeks post SCI. Clinical studies assessing diurnal control of BP in chronic SCI individuals found the autonomic completeness of injury (i.e. complete loss of descending sympathetic input to sympathetic preganglionic neurons) determines the degree of disruption of diurnal rhythmicity[169-172]. Tetraplegic individuals exhibit impaired diurnal rhythmicity compared to high thoracic (T2-T5) SCI individuals as characterized by the presence or absence of a nocturnal dip[171, 172]. At 2 weeks post SCI, neurogenic shock subsides and there is a re-emergence of the nocturnal dip. The gradual recovery of the nocturnal dip is surprising due to complete disruption of the supraspinal control of spinal sympathetic neurons which are crucial for the generation of

sympathetic rhythmic activity. The partial recovery of diurnal rhythmicity may be due to amplified sympathetic activity through rostral spinal sympathetic circuits, in addition to spontaneous firing of sympathetic circuitry below the level of lesion. As expected, diurnal rhythms in HR did not significantly differ post SCI. This is due to preservation of supraspinal sympathetic control, in addition to full vagal control in our T3 complete transection rodent model.

As previously stated CBT appears to normalize by day 4 post SCI and onwards due to subsidence of neurogenic shock. Surprisingly, within the first 2 weeks post SCI, disruptions to normal diurnal control are characterized by a “slow” kinetic response and an emphasized increase in peak to trough variation. By 2 week post SCI and onwards, there is a return of the nocturnal dip but the substantial increase in peak to trough variation is still present. The increased peak to trough variation in CBT at 2 weeks post SCI is consistent with studies assessing circadian CBT rhythms in tetraplegic individuals[173]. It is still unknown the reason for the impaired diurnal rhythmicity in CBT but one may attribute this to disrupted nighttime melatonin release[174]. Complete lesions of the cervical spinal cord have an absence of nighttime melatonin which demonstrates that the neural pathway for the endogenous production of melatonin passes through the cervical spinal cord segment[175]. Exogenous production of melatonin can shorten sleep onset, improve sleep maintenance and increase REM sleep[174, 176, 177]. Disruption of endogenous melatonin production may play an imperative role in the disruption of normal sleep patterns and subsequently circadian rhythms in CBT, BP and HR in cervical SCI individuals. Alterations in nocturnal melatonin release seem to resemble closely to that of diurnal variations in CBT in humans[178] yet is still unknown if disrupted nighttime melatonin release is directly responsible for impaired diurnal variations in CBT.

### 4.3 Spontaneous AD

Rodents with high thoracic (T3) SCI were found to develop AD as early as 2-4 days after SCI (refer to Figure 3a and 3b). Even though AD is typically considered a chronic condition, there have been clinical reports that confirmed the presence of AD in humans as soon as 24 hours post SCI[179]. Due to the lack of supraspinal control of sympathetic preganglionic neurons below the lesion, there is no descending inhibition of RVLM adrenergic bulbospinal projections. Lack of suppression of sympatho-excitatory projections from the RVLM, results in an inability to suppress increased sympathetic activity in response to noxious or non-noxious afferent stimuli via the “AD reflex arch”. The frequency and intensity of these spontaneous AD events significantly decrease at day 6 to day 12 post SCI. This decrease in sympathetic activity may match the time scale, where atrophy of sympathetic preganglionic neurons was observed as an acute (1 week) response to SCI in a rodent[180]. Krassioukov et al. have shown that after SCI, there is deafferentation of spinal cord neurons, which leads to the significant atrophy of sympathetic preganglionic neurons caudal to the injury[180]. The size and number of dendrites of the sympathetic preganglionic neurons significantly decreases[180]. These sympathetic preganglionic neurons lose their connectivity via their dendritic arbors to the interneurons that relay the afferent stimuli through the reflex arch. This disrupts the reflex mediated increase in sympathetic activity, in response to noxious and non-noxious afferent stimuli.

Around Day 14, there is a spike in the frequency and intensity of spontaneous AD that persists to Day 28 post SCI (Figure 3A and 3B). The two week marker matches the timescale where previous studies have reported sprouting of unmyelinated primary afferent fibres within the dorsal horn of the spinal cord, in response to T5 SCI in a rodent[64]. Krenz et al. observed increased CGRP immunoreactivity within the dorsal horn of thoracic and lumbar spinal cord segments, below the lesion, two weeks after T5 complete transection. Additionally, CGRP immunoreactivity was found in

the IML of the spinal cord in thoracic segments above the transected area and in lower lumbar segments[64]. This increased CGRP immunoreactivity may represent sprouting of unmyelinated afferent and interneuron neurites. As well, after two weeks, anterograde degeneration of the sympathetic preganglionic neurons reverses[180]. Sympathetic preganglionic neurons form an increased number of novel aberrant connections with primary afferent fibres, via interneuron neurites[180]. These novel connections could contribute to the increased sympathetic reflex response to sensory stimulation in the lower lumbar spinal segments (afferent connectivity associated with bladder and bowel distension), which is observed at this 2 week time point.

#### **4.4 PMA Morphology**

Reduction in PMA wall thickness, wall to lumen ratio and increased intraluminal diameter in chronic SCI is indicative of hypotrophic remodeling, and is similar to that which occurs in neonatal sympathectomy [181]. SCI result in significant reduction in sympathetic activity that is associated with neurogenic shock immediately after the injury and chronic hypotension. These likely plays pivotal roles in remodelling of resistance mesenteric vasculature noted in our study. It is likely that reduced trophic effects of the sympathetic nervous system, as a possible compensatory mechanism to normalize strain in acute SCI, triggers remodelling of the SMC layer of resistance vasculature. The morphological changes we observed in the mesenteric resistance vasculature are similar to those reported in other studies on the effects of chronic hypotension on the morphology of resistance arteries. For example, regional hypotension (by partially constricting the femoral resistance artery) caused hypotrophic remodelling similar to the changes we report in our model of chronic SCI [182]. It is important to note that chronic hindlimb unloading (a model of chronic physical inactivity) has no effect on mesenteric vasculature morphology [183], suggesting that hypotrophic remodelling of mesenteric vasculature is likely

due to loss of descending sympathetic control as opposed to physical inactivity that occurs in chronic SCI.

There is reduced stiffness of mesenteric resistance arteries in acute and chronic SCI. This finding is in agreement with a previous study of the mechanical properties of carotid and femoral arteries in chemically sympathectomized rodents [184]. We assessed collagen content to gauge the relative compliance of these arteries. We found decreased collagen deposition at 1 week and 1 month post SCI and our pressure myography study revealed reduced beta-stiffness which, suggests the development of a compliant vasculature. As previously stated, there is decreased vascular strain in acute SCI, and our results support the concept that hypotrophic remodelling may occur as a compensatory mechanism. This hyperplasticity in resistance arteries may also be maladaptive as stress forces act in opposition to strain forces, thus making the vessel more susceptible to circumferential stress acting tangentially to the vessel wall in chronic SCI. This is confirmed by the increased circumferential wall stress in response to increasing intraluminal pressures in chronic SCI. The SMC density relative to cross sectional media area is lower in both acute and chronic SCI, relative to control, suggesting that hypotrophic remodelling of resistance vasculature may be due to atrophy. This is similar to findings in neonatal sympathectomy where decreases in SMC layers occurs in smaller mesenteric arteries [181]. This is the first study to show maladaptive chronic hypotrophic remodelling post SCI, which may be compensatory to acute reduction in strain. In addition, reductions in wall to lumen ratio in chronic SCI may result in increased circumferential wall stress. Thus therapeutic strategies to normalize strain in acute SCI may be essential to prevent chronic hypotrophic remodelling in resistance vasculature post SCI.

#### **4.5 Functional Assessment of PMA**

Myogenic tone, a pressure induced vasoconstriction characteristic of small arteries, is impaired in both acute and chronic SCI. These changes occur in parallel with atrophy of these arteries in acute and chronic SCI, possibly due to a reduced number of SMC and the resultant loss of Ca<sup>2+</sup> sensitive ion channels and disrupted smooth muscle gap junctions. We assessed sensitivity to PE and MET using two agonists of  $\alpha$ 1 adrenoceptors, where only PE is a substrate of synaptic reuptake. Differences in responsiveness to these agents could provide mechanistic insight into the underlying cause of changes in receptor activation [155]. We observed hypersensitivity to PE in both acute and chronic SCI, without any significant changes in constriction to MET. This is an extension of a previous study by Brock et al., who found increased responsiveness to PE but not MET in second order mesenteric arteries after chronic high thoracic SCI [155], where the differential responses were ascribed to the lower rate of PE removal by NE transporters at the synaptic junction. A novel finding of our study is that this same phenomenon is present in the primary mesenteric resistance arteries and is amplified in the acute stages of SCI. This exaggerated responsiveness to PE at the 1 week time point is likely a result of the decreased basal level of sympathetic activity in acute SCI (ie neurogenic shock) as opposed to the development of sympathetic reflexive activity in chronic SCI (ie AD) [155].

## Prospective and Conclusive Remarks

High thoracic SCI may result in perturbed autonomic cardiovascular homeostasis particularly due to lack of descending supraspinal control of sympathetic activity below the level of lesion. This is the first comprehensive study to concurrently examine resting hemodynamics, thermoregulation and spontaneously occurring AD events across time pre and post-SCI. We showed here that the acute period post SCI is characterized by hypotension, hypothermia and disrupted diurnal rhythms. We have developed a novel AD Detection algorithm that is able to analyse oscillations in continuous arterial blood pressure and heart rate to detect spontaneous AD events. Using the novel AD Detection algorithm we detected spontaneous AD events as early as 2 days post SCI and the frequency and severity of AD events subside by day 6-12 post SCI. By day 14 post SCI, diurnal rhythms recovered and temperature control had improved (although there is an increased peak to trough variation). At day 14 post SCI there is a twofold increase in the number and severity of spontaneous AD events. Upon advent of new continuous hemodynamic monitoring technologies, the *AD Detection* algorithm may be applied as a vital tool clinically to monitor the onset of these events.

This is the first study to show hypotrophic remodelling and reduced stiffness in resistance mesenteric vasculature following SCI. Further, this is the first study to show hypersensitivity to PE in acute SCI which suggests impairment in synaptic reuptake of NE may be a by-product of the complete disruption of sympathetic activity in acute SCI (ie neurogenic shock). Most structural and functional changes in resistance vasculature starts in the acute stages of SCI. Future studies need to explore acute therapeutic strategies to prevent structural and functional maladaptive plasticity in splanchnic resistance vasculature in order to improve arterial blood pressure control in individuals living with high thoracic or cervical SCI.

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