SENSORY EFFECTS OF PASSIVE HIND-LIMB CYCLING AFTER SPINAL CORD INJURY

by

Mark Alexander Crawford

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Abstract

Spinal cord injury (SCI) is a devastating insult to the nervous system with implications for locomotor, autonomic and sensory function. Past studies indicate that passively moving the lower limbs may be beneficial for locomotor recovery after SCI, however the literature lacks in studies addressing autonomic and sensory ramifications of passive exercise. I used a well-established passive exercise model, which consists of cycling the hind-limbs of adult male Wistar rats with complete transection SCI at the third thoracic segment (T3), beginning 5 days after injury and continuing for 4 weeks (5 days / week, 1 hour total cycling/day). I measured Hoffman (H)-reflex latency and motoneuron recruitment after the cycling intervention. Latency of the H-wave was shorter in duration and motoneuron recruitment was enhanced after SCI when compared to uninjured controls. Exercise did not affect these properties. I performed histological analysis of parvalbumin-expressing neurons of lumbar (L) and sacral (S) dorsal root ganaglia (DRGs). Proprioceptive neurons at L1/L2 and L4/L5 levels demonstrated somal size decreases after SCI and further decreases with exercise, while there was no change at the L6/S1 level. This effect may be due to exercise-induced changes in neurotrophic support of proprioceptive neurons by target tissues.

The autonomic/cardiovascular effects of passive exercise are largely unknown. I focused on two common cardiovascular conditions associated with SCI, autonomic dysreflexia (AD) and orthostatic hypotension (OH). AD occurs in individuals with an injury above T6, and is marked by massive spikes in blood pressure (BP) due to a normally-innocuous stimulus below the injury level. OH is a large drop in BP upon being seated upright, assessed via tilting the animal to a 90 degree head-up position. Passive exercise led
to a 50% reduction in AD severity, as measured by beat-to-beat BP measurements and an established method for inducing AD. In contrast, I found no change in OH severity with exercise. Lumbosacral nociceptors expressing the capsaicin receptor (TRPV1), which have previously been implicated in AD and demonstrate hypertrophy after SCI, decrease in soma size after the exercise intervention. This may also indicate exercise-induced altered neurotrophic support.
Preface

For both data chapters I performed animal surgeries and care in collaboration with Jessica Yuen, Drs. Leanne Ramer and Chris West. In Chapter 2 I present a detailed animal care summary, which follows the animal care procedures outlined in (Ramsey et al., 2010).

In Chapter 2, I collected all electrophysiological data, which I analyzed in collaboration with Joel Ho and Dr. Matt Ramer. I performed all tissue harvesting, immunohistochemistry and imaging. Image processing was split approximately equally by Sujin Im, Austin Bellantoni and myself. I performed all recursive translation analysis and I wrote the chapter, with edits by Drs. Matt Ramer and Chris West.

Dr. Leanne Ramer performed the cannulations in Chapter 3. I assisted Dr. Chris West with all physiological measurements in Chapter 3. I analyzed physiological data with the assistance of Jessica Inskip and Dr. Chris West. I performed all tissue harvesting, immunohistochemistry and imaging. Image processing was split approximately equally between Joel Ho, Austin Bellantoni and myself. I performed all recursive translation analysis and I wrote the chapter, with edits by Drs. Matt Ramer and Chris West.

All experiments were approved by the University of British Columbia’s Animal Care Committee under the certificate A10-0129.
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<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Autonomic dysreflexia</td>
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<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>AV node</td>
<td>Atrioventricular node</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
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<tr>
<td>C#</td>
<td>Cervical level</td>
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<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRD</td>
<td>Colo-rectal distension</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FES</td>
<td>Functional electrical stimulation</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<tr>
<td>H-reflex</td>
<td>Hoffman reflex</td>
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<tr>
<td>IML</td>
<td>Intermediolateral</td>
</tr>
<tr>
<td>L#</td>
<td>Lumbar level</td>
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<tr>
<td>METs</td>
<td>Metabolic equivalents</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light-chain kinase</td>
</tr>
<tr>
<td>MP</td>
<td>Myosin phosphatase</td>
</tr>
<tr>
<td>NF200</td>
<td>Neurofilament 200</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>NFAT</td>
<td>Nuclear factors of activated T cells</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>NT-4</td>
<td>Neurotrophin-4</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus of the solitary tract</td>
</tr>
<tr>
<td>OH</td>
<td>Orthostatic hypotension</td>
</tr>
<tr>
<td>P2X3</td>
<td>P2X purinoceptor 3, a ligand-gated ion channel activated by ATP</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>RVLM</td>
<td>Rostral ventrolateral medulla</td>
</tr>
<tr>
<td>S#</td>
<td>Sacral level</td>
</tr>
<tr>
<td>SA node</td>
<td>Sinoatrial node</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SCI-Ex</td>
<td>Spinal cord-injured animals with a 4 week exercise intervention</td>
</tr>
<tr>
<td>T#</td>
<td>Thoracic level</td>
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<tr>
<td>TrkA</td>
<td>Tyrosine kinase receptor A</td>
</tr>
<tr>
<td>TrkB</td>
<td>Tyrosine kinase receptor B</td>
</tr>
<tr>
<td>TrkC</td>
<td>Tyrosine kinase receptor C</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid 1 (the capsaicin receptor)</td>
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Dedication

To my family.
Chapter 1: Introduction

1.1 Spinal cord injury

Paralysis is often considered the defining and most devastating feature of spinal cord injury (SCI). The goal of prompting regeneration of damaged axons within the spinal cord and reinstating motor control has traditionally been paramount in SCI research, a notion which is reflected in the names of institutes (i.e., The Miami Project to Cure Paralysis, The Center for Paralysis Research at Purdue University) and service organizations (i.e., Paralyzed Veterans of America). These highly visible public institutions, combined with the pervasive imagery of an individual’s wheelchair in society, places the inability to locomote (and the urgency to reinstate locomotion) above any other health concern in the public’s perception of those living with an injury. Lying beneath the surface, however, are tremendous and devastating changes to sensory and autonomic function. Fittingly, the number of publications examining motor control outcomes after SCI far outweighs the number examining sensory or autonomic function (Inskip et al., 2009). Until the panacea of spinal cord regeneration and complete functional recovery arrives, it is important to tip the scales of research focus towards investigating the health outcomes of SCI that are currently most pressing.

The principal cause of mortality after SCI is cardiovascular dysfunction (DeVivo et al., 1999; Krassioukov, 2009; Krassioukov et al., 2010; West et al., 2012). The probability of developing a cardiovascular disease rises dramatically after injury, and individuals experience these complications at a younger age and more frequently than those without an SCI (DeVivo et al., 1999; Inskip et al., 2012).

To add to the list of non-locomotor concerns following SCI, there are also
debilitating sensory consequences, including chronic pain and spasticity.

Chronic neuropathic pain is one of the most severe results of SCI and arguably the direst sensory concern (Siddall et al., 2003; Siddall, 2009). In Canada, over 40,000 people have sustained an SCI (Canadian Paraplegic Association, 2009) and approximately 1000 individuals per year are injured, many under the age of 30 years (Rick Hansen Registry, 2010). Roughly 65% of the SCI population have chronic pain (Siddall and Loeser, 2001) and 10-15% of those rank pain reduction as their greatest priority (Anderson, 2004). In attempting to control the sensory sequelae of SCI, eradicating chronic pain is increasingly becoming a top clinical priority.

Spasticity is a significant health consequence associated with SCI. There are four central and measurable parameters of SCI-related spasticity: enhanced muscle tone, enhanced tendon reflexes, enhanced flexor reflexes and increased spread of inappropriate reflexes between spinal segments and over time (Skold et al., 1999). The pathologic reflexes elicited during spastic episodes can be extremely painful, compromising both quality of life and potential for recovery (Bose et al., 2012).

Here I provide an overview of cardiovascular control by the nervous system and the interruptions to cardiovascular regulation that may occur after SCI. I then discuss sensory input to the spinal cord and how this changes after SCI, both for a subset of neurons responsible for transmitting pain (nociceptive) information and limb position (proprioceptive) information. The experiments in this thesis examine changes to these neuronal subsets after a passive hind-limb cycling locomotor training regime. I also examine cardiovascular and spinal reflex physiological measures related to these neurons. I then examine the implications of these data for cardiovascular health, spasticity and pain for
individuals with SCI.

1.2 Neural cardiovascular control

At the level of the heart, cardiovascular activity is mediated through a dynamic interplay between the parasympathetic (“rest and digest”) and sympathetic (“fight or flight”) branches of the autonomic nervous system (the multilevel interactions of the sympathetic and parasympathetic systems are reviewed in Ondicova and Mravec, 2010). Autonomic vascular innervation, however, is almost entirely sympathetic with very few blood vessels receiving parasympathetic input.

1.2.1 The arterial baroreflex and brain centres implicated in cardiovascular control

A key cardiovascular control centre in the brain is the nucleus of the solitary tract (NTS), an important medullary relay station. The NTS receives input from baroreceptors, or neurons which sense stretch within the aorta and carotid arteries. When a stretch signal is propagated, or when BP is seemingly high, NTS neurons integrate this signal which is relayed to cells in the dorsal vagal nucleus (Haines and Ard, 2002). As a prime example of negative control, these cells project to the heart’s terminal ganglia to effectively slow heart rate. Conversely, a BP below normal quickens the heart rate (Ondicova and Mravec, 2010).

The paraventricular nucleus (PVN) of the hypothalamus is largely involved in cardiovascular control (Benarroch, 2005). The PVN’s role was revealed in early experiments with intracranial delivery of nitrous oxide (NO): upon delivery of NO-containing artificial cerebrospinal fluid (CSF) directly to the PVN (Horn et al., 1994), there was a significant reduction in systemic BP. Additionally, significant increases in the
amino acids aspartate, glutamate, γ-aminobutyric acid (GABA, a principal CNS inhibitory neurotransmitter) and taurine were observed in the PVN (Horn et al., 1994). This manipulation ultimately supported two hypotheses: that NO is a neurotransmitter directly involved in BP modulation, and that the PVN is an important centre in cardiovascular control and responds metabolically to NO stimulation.

Ventral (in humans, caudal in rodents) to the PVN is another primary CNS region of cardiovascular control, the rostral ventrolateral medulla (RVLM). The RVLM is a brainstem structure containing the sympathetic premotor neurons which regulate tonic activity of sympathetic preganglionic neurons (Guyenet, 2006). Classical experiments applying various drugs to defined regions of the ventral surface of the cat brainstem illuminated the significant role of this structure in BP regulation (Guertzenstein and Silver, 1973; Guertzenstein and Silver, 1974; Feldberg and Guertzenstein, 1976). More recently, one group genetically overexpressed the enzyme endothelial NO synthase (eNOS) in a small number of RVLM neurons to increase RVLM NO production (Sakai et al., 2005). This resulted in an increase in RVLM GABA release and the physiological result of hypotension and bradycardia, demonstrating that the RVLM is a key cardiovascular control centre and, like the PVN, responds to NO signals (Sakai et al., 2005).
1.2.2 Peripheral autonomic nervous system anatomy and cardiovascular regulation

Cell bodies of preganglionic sympathetic neurons lie in the intermediolateral column of the spinal cord’s thoracolumbar region (Amendt et al., 1979), while cell bodies of parasympathetic preganglionic neurons are housed in brainstem and sacral spinal cord (Thomas, 2011). Sympathetic preganglionic neurons typically (but not always, see below) synapse on sympathetic post-ganglionic neurons residing in well-compartmented ganglia relatively close to the spinal cord (paravertebral ganglia), while their parasympathetic counterparts synapse on post-ganglionic parasympathetic neurons in ganglia rather distal from the spinal cord and close to target organs. Correspondingly, sympathetic post-ganglionic neurons tend to have longer axons to reach targets further away from sympathetic ganglia and parasympathetic ganglionic axons tend to be shorter as their targets are nearby. All preganglionic neurons, whether parasympathetic or sympathetic, are cholinergic (Kimura and Tooyama, 1998); the primary neurotransmitter released by post-ganglionic sympathetic fibers is norepinephrine (von Euler, 1951) and that of post-ganglionic parasympathetic fibers is acetylcholine (Perry and Talesnik, 1953).

There are three general routes that a preganglionic sympathetic neuron can take to reach a target (outlined in Haines and Ard, 2002). In one scenario, a preganglionic axon can terminate in a paravertebral ganglion at its level of exit from the cord (Figure 1.1a), in another a preganglionic axon can ascends or descend the sympathetic chain to synapse on different ganglia (Figure 1.1b). Additionally, preganglionic sympathetic axons can move through paravertebral ganglia without synapsing and innervate prevertebral ganglia (Figure 1.1c). Although they release hormones instead of neurotransmitter, the chromaffin cells of the adrenal medulla are considered a ganglion and are also innervated by sympathetic
fibres.

The adrenal medulla is a key cardiovascular control centre. By releasing epinephrine into the bloodstream to act on distant targets, hormone-secreting medullary chromaffin cells can exert a systemic effect on the cardiovascular system during periods of stress. Like sympathetic ganglia, chromaffin cells of the adrenal medulla are neural crest in origin, and mutations to factors important to sympathetic ganglion development similarly affect formation of the adrenal medulla (Britsch et al., 1998). A unique cell type, chromaffin cells are essentially postganglionic sympathetic neurons that have shed their processes.
Figure 1.1. Peripheral sympathetic pathways can take multiple routes.
Preganglionic fibres can terminate in the sympathetic ganglion at the level of origin (a),
move up or down in the sympathetic chain to synapse in other ganglia (b) or move through
the ganglion, entering a splanchnic ganglion and synapsing in a prevertebral ganglion (c).
Postganglionic axons are able to exit directly from a sympathetic chain ganglion via nerves
such as the cardiac nerve, rather than following the path beginning with the gray ramus
(adapted from Haines and Ard, 2002).
1.2.3  Autonomic control of the heart: a balance between parasympathetic and sympathetic input

Four parameters of control over the heart are modulated by the autonomic nervous system: inotropy (contraction), lusitropy (relaxation), dromotropy (conduction velocity) and chronotropy (cardiac rate) (Thomas, 2011). Inotropy and lusitropy are principally controlled by sympathetic axons innervating cardiac myocytes of the atria and ventricles, while both sympathetic and parasympathetic innervation of the sinoatrial (SA) and atrioventricular (AV) nodes control dromotropy and chronotropy. Interestingly, tonic autonomic cardiac innervation sets heart rate at approximately 30% lower than the intrinsic rate (90-100 beats/minute). Additionally, without sympathetic input at rest cardiac output would be about 30% lower (Thomas, 2011).

Acetylcholine released by parasympathetic fibers of the vagus nerve activate M2 receptors on cells of the SA and AV nodes, in turn increasing $K^+$ conductance of these cells and hyperpolarizing the membrane (Haines and Ard, 2002). This effectively slows the intrinsic heart rate by decreasing SA node spontaneous firing and slowing AV node conduction. Conversely, norepinephrine is released by sympathetic axons and binds to β-adrenergic receptors to activate adenylate cyclase, raise cAMP levels intracellularly and activate protein kinase A (PKA) (Bouvier et al., 1987; Thomas, 2011). This effectively increases the rate of SA node diastolic depolarization and increases AV node conduction leading to an increase in heart rate. β-adrenergic receptor activation also increases membrane $Ca^{2+}$ currents and action potential-mediated release of $Ca^{2+}$ from the sarcoplasmic reticulum, ultimately increasing myocardial force production (Kho et al.,
Relaxation is also enhanced by quickened re-uptake of Ca\textsuperscript{2+} by the sarcoplasmic reticulum (Kho et al., 2012; Thomas, 2011).

1.2.4 Autonomic control of the peripheral vasculature

Although parasympathetic neurons innervate a small number of blood vessels, sympathetic neurons are the dominant modulators of the peripheral vasculature (Guyenet, 2006; Thomas, 2011): in addition to the heart, the kidneys, adrenal glands and the majority of smooth muscle-containing blood vessels are all under sympathetic control: this scheme allows for system-wide direct and indirect control of cardiovascular function by sympathetic activity. Here I focus mainly on the sympathetic branch of the autonomic nervous system, as this is also the type of autonomic input that changes most drastically after high thoracic/cervical SCI (Krassioukov, 2009).

The sympathetic nervous system directly controls the peripheral vasculature through activation of smooth muscle. Arteries, arterioles and veins are directly contacted by postganglionic sympathetic axons which interact with the adventitial-medial border of vessels. There is no sympathetic innervation of venules or capillaries as these structures lack smooth muscle (Thomas, 2011).

Ca\textsuperscript{2+} signaling mediates coordinated contraction of vascular smooth muscle cells in peripheral vasculature (Zang et al., 2006; Erne et al., 1987; Bohr and Webb, 1988). Sympathetic axon terminals release norepinephrine which binds \(\alpha_1\) or \(\alpha_2\) adrenoreceptors on vascular smooth muscle cells, in turn boosting intracellular Ca\textsuperscript{2+} concentrations by inducing flux through membrane Ca\textsuperscript{2+} channels or by releasing Ca\textsuperscript{2+} from the sarcoplasmic reticulum.
Interestingly, many pharmacological targets for BP regulation rely on modulating the activity of two enzymes involved in muscle cross-bridge cycling: calcium/calmodulin-activated myosin light chain kinase (MLCK) and myosin phosphatase (MP) (Fisher, 2010). For smooth muscle contraction to occur, many vasoconstricting agents boost levels of activated MLCK or inhibit MP, allowing myofilaments to become sensitized to Ca\(^{2+}\) activation. In contrast, several vasodilatory agents inhibit MLCK and increase MP activation, thereby desensitizing myofilaments to Ca\(^{2+}\) (Fisher, 2010).

1.3 SCI and cardiovascular dysfunction

There is a demonstrated increased risk of cardiovascular dysfunction in humans with SCI (DeVivo et al., 1999; Garshick et al., 2005; Nash and Mendez, 2007). This appears to be due to several factors, but the overarching problem is a dissociation of the vasculature below the injury site from descending neural control. In particular, injury above the sixth thoracic vertebra (T6) can disrupt descending autonomic pathways from the cardiovascular system, including glands, smooth muscle and cardiac muscle (Krassioukov et al., 1999). It is widely held that as severity and level of the injury increase, so too does cardiovascular dysfunction after SCI (Helkowski et al., 2003).

Cardiovascular dysfunction after SCI has two prevalent players, namely autonomic dysreflexia (AD) and orthostatic hypotension (OH). AD is a severe form of episodic hypertension which occurs following SCI above the sixth thoracic vertebra (T6), and is characterized by spikes in BP (an elevation of 40 mmHg over baseline systolic) due to a normally innocuous stimulus (such as a tight garment or a full bowel). It is thought to develop over time after supraspinal regulatory centres are cut off from spinal circuits.
controlling the splanchnic vascular bed (Lindan et al., 1980; Mathias and Frankel, 1983; Karlsson, 1999). On the other end of the BP spectrum, OH is a hypotensive response, marked by a decrease in diastolic BP of 10 mmHg or more and/or a decrease in systolic BP of 20 mmHg or more when the body is tilted from a supine to upright posture (Harkema et al., 2012).

There is limited understanding of the mechanisms that contribute to cardiovascular dysfunction after SCI (Krassioukov et al., 1999). Although rats with SCI are susceptible to the same cardiometabolic risk factors as humans after SCI, such as higher visceral adiposity (Inskip et al., 2009) and blood vessel changes such as phenylephrine (PE) hypersensitivity (Alan et al., 2010), the physiological mechanisms behind these changes remain poorly understood in both animal models and humans.

1.3.1 Orthostatic hypotension

Orthostatic hypotension is increasingly symptomatic as the level of injury becomes higher: hence, a person with a cervical SCI is more likely to experience OH symptoms than an individual with a lower thoracic SCI (Claydon and Krassioukov, 2006). Symptoms of OH, as in able-bodied individuals, include restlessness, blurred vision, fatigue or weakness, light-headedness and dizziness (Weaver et al., 2012). It is possible, however, for OH to be silent (asymptomatic) for many individuals: even if no outward symptoms are apparent, an individual with SCI may silently experience major reductions in BP (Illman et al., 2000).

The cause of OH is thought to be a weakening of short term reflex BP regulation (Claydon et al., 2006). Understandably, if sympathetic innervation of blood vessels is altered, the typical reflexive vasoconstrictive response to the unloading of baroreceptors in
the aortic arch and carotid body (Mathias, 1995) is compromised as well. Instead of blood moving towards the brain and upper extremities upon sitting upright, it instead pools in the lower extremities and abdomen (Faghri et al., 2001)

1.3.2 Autonomic dysreflexia

AD strikes individuals with an SCI at or above the sixth thoracic vertebra (Gunduz and Binak, 2012). With interference of descending control from the brain to sympathetic preganglionic neurons in the intermediolateral column of the thoracic spinal cord, normal mediation of sympathetic output is lost. The “runaway train” aspect of autonomic dysreflexia, the notion that there is a rapid and uncontrolled rise in BP to accompany a normally innocuous stimulus (which only typically subsides with removal of the stimulus) is thought largely to be due to aberrant central sympathetic reflex activity (McLachlan, 2007). The outcomes of AD can be grave, including myocardial infarction, hemorrhage, seizures, retinal damage and death (Krassioukov et al., 2009b).

The circuit believed to be involved in AD begins with somatic afferent input to lower lumbar spinal cord segments, typically due to rectum or bladder stimulation. This information is relayed via dorsal root ganglion (DRG) sensory neurons which synapse on neurons in the dorsal horn. These neurons relay sensory information through propriospinal circuits to sympathetic preganglionic neurons, which project to postganglionic sympathetic neurons. Postganglionic neurons cause vasoconstriction of the mesenteric arterial bed, leading to spikes in systemic BP associated with AD (Inskip et al., 2009) (Figure 1.2).

It has been found that sprouting of calcitonin gene-related peptide (CGRP)-positive afferent fibres within the spinal cord mimics the time course for AD development after SCI
(Krenz et al., 1999), an effect which may be due to increased presence of intraspinal nerve growth factor (NGF) (Marsh et al., 2002), as well as several other spinal cord-centric phenomena (Gris et al., 2005; Cameron et al., 2006; Hou et al., 2008). To assess the system-wide causes of AD, however, it is imperative to also consider changes that occur outside of CNS circuits.

Both human and animal studies have demonstrated peripheral neural and vascular changes accompanying AD. In one human study, bladder percussion was used to assess spillover of the sympathetic neurotransmitter noradrenaline (Gao et al., 2002). The authors showed a 3-fold increase in plasma noradrenaline, indicating a significantly enhanced sympathetic response to visceral afferent stimulation after SCI (Gao et al., 2002). An enhanced vasopressor response to noradrenaline in completely transected cervical patients has also been reported (Mathias et al., 1976), which fits with more recent animal experiments demonstrating increased sensitivity of peripheral vasculature to the α-adrenoreceptor agonists phenylephrine (Alan et al., 2010; Yeoh et al., 2004) and clonidine (Yeoh et al., 2004) after complete transection SCI in the rat.
Figure 1.2. The circuit involved in AD.

Sensory stimulation below the injury level (typically the urinary bladder or colon/rectum) results in a sensory signal being propagated along primary afferent neurons, the cell bodies of which reside in the DRG. Afferent neurons synapse on propriospinal circuits which relay this sensory information through interneurons to sympathetic preganglionic neurons in the thoracolumbar spinal cord, neurons which are disconnected from descending regulatory control due to SCI at or above T6 (adapted from Inskip et al., 2009).
1.4 The dorsal root ganglion (DRG)

The DRG contains the somata of an astoundingly heterogenous population of sensory cells, which are responsible for relaying all modalities of afferent input from the periphery to the central nervous system (CNS). Although primarily viewed as a cellular way station for sensory input, it is imperative to view these neurons as constituents of a ganglion that is a chemosensory organ in its own rite: lacking a blood-nerve barrier, DRG neurons can be affected by their chemical surroundings and modified by neurotrauma in several ways (Devor, 1999).

One unique modification of DRG neurons by neurotrauma is the phenomenon of sympathetic sprouting and neuronal basketing, first described by Santiago Ramon y Cajal and later characterized by others (McLachlan et al., 1993; Ramer and Bisby, 1997; Ramer and Bisby, 1998a; Ramer and Bisby, 1998b; Ramer and Bisby, 1998c; Ramer et al., 1998a; Ramer et al., 1998b). Postganglionic sympathetic fibres normally exit the spinal nerve for peripheral targets. After peripheral nerve injury, and more recently demonstrated after T3 complete spinal cord transection (Ramer LM, unpublished findings), these axons stray from their normal path. Noradrenergic sympathetic axons, which are aberrant sprouts of postganglionic axons, instead turn centrally upon exiting the gray ramus (see Figure 1) and form pericellular baskets around neuropeptide-negative large DRG neurons (Ramer and Bisby, 1998c). This unique boundary violation by sympathetic axons into sensory territory may well be implicated in chronic pain and autonomic sequelae associated with SCI, although firm evidence for this is still lacking.
1.4.1 DRG development

In development, the trunk neural crest is capable of generating many cell types of the peripheral nervous system (PNS), including enteric, sympathetic and sensory neurons (Dupin et al., 2006). After highly specific spatiotemporal signals are received, neural crest cells are permitted to delaminate from the neural tube. DRGs are ultimately formed when a portion of these neural crest cells migrate ventrally between the neural tube and dermamyotome (Raible and Ungos, 2006). Throughout this process, there is a dynamic and highly coordinated specification system taking place: signals from the spinal cord and adjacent somites lead to the generation of multiple DRG sensory neuron subtypes. The resulting “mosaic” of sensory neurons within the ganglion allows for the sensation of limb spatial position, limb movements, pain, cold, warmth and touch (extensively reviewed in Marmigere and Ernfors, 2007).

The anatomy of DRG neurons is unique in that they do not possess a singular uninterrupted axon or a dendritic arbor. DRG neurons are pseudounipolar: they have one process extending to the periphery to sense a stimulus and another process extending centrally to relay this information to the CNS (Devor, 1999). The body of the ganglion can be divided into a fibre (axonal) layer and a cell (sensory neuron somata) layer.

As each subset of DRG neuron is specialized to relay a specific sensory modality, there are accompanying modality-specific structural features of these neurons. To allow for exacting function, specific ion channels and unique molecular characteristics are found within each subset of DRG neuron. Nociceptive neurons, which respond to noxious stimuli and transmit pain sensation centrally, have thinly myelinated or unmyelinated axons (Willis and Coggeshall, 1991). These neurons may be neuropeptide positive (peptidergic,
neurotrophic tyrosine receptor kinase A (TrkA)-containing) or neuropeptide negative (non-
peptidergic, TrkA"). Mechanical sensations are conveyed by large-diameter low-threshold
mechanoreceptive neurons, including those that sense limb position and movement (TrkC+)
and those that convey touch (TrkB+ and/or TrkC+) (Willis and Coggeshall, 1991;
Marmigere and Ernfors, 2007).

A defining feature of sensory neuron function is where its central projection
terminates in the spinal cord (Willis and Coggeshall, 1991). After DRG neuronal subtype is
established during development, modality-specific terminations are established:
proprioceptive neurons terminate in the ventral horn (muscle spindle Ia afferents) and the
intermediate zone (muscle spindle Ia afferents, Golgi tendon organ Ib afferents),
mechanoreceptors in deeper dorsal spinal cord laminae and nociceptive neurons in the
dorsal horn (Marmigere and Ernfors, 2007).

### 1.4.2 A simplified DRG model

The phenotype of DRG neurons is astoundingly complex, and to describe DRG cell
populations in the context of neurotrauma is even more confusing as these phenotypes are
not static. For the purpose of this thesis, I present a highly simplified model of DRG
phenotype and innervation of the spinal cord. To generalize, DRG nociceptive C-fibres can
be divided into cells expressing CGRP or P2X purinoceptor 3 (P2X3, a member of the P2X
family of ATP-gated ion channels), and DRG mechanoreceptive and proprioceptive Aβ
fibres express neurofilament 200 (NF200) (Figure 1.3a). C fibres innervate the spinal cord
more dorsally while Aβ fibres innervate deeper, more ventral laminae (Bradbury et al.,
2000)(Figure 1.3b).
Figure 1.3. DRG afferent input.

Aβ fibres express NF200 and convey mechanical and proprioceptive information. C fibres of small-diameter neurons can be separated into those which express CGRP or P₂X₃ (a). C fibres terminate dorsally in the spinal cord and Aβ fibres terminate more ventrally (b).

Adapted from Bradbury et al., 2000.
1.4.3 Nociceptors

Nociceptors have unencapsulated nerve endings and their afferent fibers are, broadly speaking, either small and unmyelinated (C fibers) or larger and lightly myelinated (Aδ fibers). As degree of myelination and axon caliber are directly related to the conduction velocity of an action potential along an axon, the type of sensory information that is transmitted along nociceptive C or Aδ fibers differs. C fibers transmit sensory information including itch, burning or dull pain, while Aδ fibers transmit sharp pain. Recordings from animal experiments have been compared to recordings from cutaneous and mixed nerves in humans, leading to the classification of distinct nociceptor subtypes in vertebrates, including C heat, C mechanoheat, C polymodal, Aδ mechanical and Aδ mechanoheat nociceptors (Willis and Coggeshall, 2004).

1.4.4 TRPV1+ nociceptors and autonomic dysreflexia

Originally investigated as a relative of a channel family in the Drosophila phototransduction pathway (Montell and Rubin, 1989) and first cloned in 1997 (Caterina et al., 1997), the transient receptor potential vanilloid (TRPV1) cation channel is activated by extracellular acidosis, painful heat and capsaicin, a compound derived by plants in the Capsicum family (Rosenbaum et al., 2004). This channel is expressed in a discrete subset of nociceptive sensory neurons in the DRG to transmit these noxious stimuli from the periphery to the spinal cord (Baumann et al., 2004). Correspondingly, mice lacking this channel are deficient in their response to extracellular acidosis, painful heat and capsaicin (Caterina et al., 2000). With peripheral thermosensation absent in TRPV1−/− mice there is an accompanying drastic reduction in the ability to regulate body temperature in response to
cold or hot temperatures, while normal mechanical pain sensation and touch are retained (Mishra et al., 2011).

A fundamental role has been described for TRPV1-expressing nociceptors in SCI pathology, particularly with respect to AD. After discovering that DRG neurons hypertrophy when stained with a pan-neuronal marker after T3 complete transection SCI, it was found that the TRPV1 subset hypertrophied independently while other DRG neuronal populations did not (Ramer et al., 2012). As this population clearly responded to SCI, the hypothesis driving the next set of experiments – that TRPV1-expressing sensory neurons are implicated in AD pathology – was pursued through established physiological methods of assessing AD, including carotid cannulation and colorectal distension (Inskip et al., 2009; Ramer et al., 2012). After administering capsaicin intrathecally to abolish TRPV1+ afferent input to the spinal cord, AD was dramatically reduced (Ramer et al., 2012). TRPV1 is thus clearly implicated in the pathology of AD.

1.4.5 DRG proprioceptors

With their cell bodies residing in the DRG, proprioceptive sensory neurons innervate skeletal muscle and relay information about muscle length and tension, which is critical to coordinating motor function (Patel et al., 2003). There are three types of proprioceptive neurons relaying this information: Golgi tendon organs are innervated in the periphery by group Ib afferent axons, while muscle spindles are innervated by group Ia and II afferent axons. Group Ia afferent axons terminate in the spinal cord ventral horn’s motor nucleus and group Ib and II afferent axons terminate in the ventral horn (Inoue et al., 2002).
1.4.6 The Hoffman reflex: humans versus rats

The Hoffmann reflex (H-reflex) is the compound muscle action potential (AP) that results from stimulating a motor nerve electrically, and can be seen as the electrophysiological counterpart of the spinal stretch reflex (Chen and Wolpaw, 1995) (Figure 1.4).

In one example of the H-reflex, monosynaptic activation of motoneurons and a resulting soleus muscle twitch (and recorded H-wave) occur after the human tibial nerve is stimulated at low intensity (activating Ia spindle afferents). As the threshold of activation for motor axons is higher, only as the stimulus intensity increases is there direct recruitment of motor axons at the site of stimulation, which results in an M-wave characterized by relatively early contraction (Ramer MS, personal communication). Additionally, as the stimulus intensity increases there is an increase in the number of directly recruited motor axons. In humans, the increasing number of antidromic action potentials (APs traveling from the stimulus site toward the spinal cord) that accompany increased stimulus intensity (and motor axon recruitment) results in a collision between these APs and reflexively-evoked orthodromic (away from the spinal cord and towards the recording electrodes) APs. This results in a reduced H-wave at higher stimulus intensities and no H-wave when M is maximal (Ramer MS, personal communication). The common method for measuring the H-reflex is to average a number of trials, as H-reflex variability in healthy humans is typically high (Ramer MS, preliminary findings).

There is a substantial difference in H-reflex modulation between humans and rats. Central to this difference are differential activation thresholds for Ia primary afferent axons versus motor axons: the activation threshold of rat Ia primary afferents is about equal to, or
in some cases higher than, motor axons, while in humans Ia primary afferent activation thresholds are much lower than activation thresholds for motor axons (Thompson et al., 1992; Cliffer et al., 1998; Gozariu et al., 1998; Ho and Waite, 2002; Cote et al., 2011). In rats, the stimulus threshold at which an H-wave can be generated is on par with the threshold for generating an M-wave (if not slightly higher), and this therefore leads to input/output curves in rats that look dramatically different from those recorded in humans. In rats, several motoneurons are anti-dromically activated before reflex activation by Ia afferents. Additionally, as stimulus intensity is increased in the rat, H-wave amplitude is not reduced to zero as in humans.
Figure 1.4. H-reflex recording.

The H-reflex is the electrophysiological analogue of the spinal stretch reflex. The isolated tibial nerve (a mixed nerve) is stimulated with bipolar hook stimulating electrodes. The stimulus either travels through the DRG-mediated afferent spinal circuit (the longer path), resulting in a muscle twitch recorded by bipolar recording electrodes in the interosseus muscle of the foot (the H-wave), or results in immediate firing of the motoneuron and a muscle twitch without traveling through the spinal circuit (the M-wave).
1.5 Effects of locomotor training after SCI

1.5.1 Cardiovascular studies in humans

Several human studies have highlighted the potential cardiovascular benefits of locomotor training after SCI. In one case study, a functional electrical stimulation (FES) system was surgically implanted to recruit key hindlimb muscles required for stepping in a 22 year old man with an incomplete SCI at C6-7 (American Spinal Injury Association grade C) (Hardin et al., 2007). After a 12 week training regimen involving FES and walker- and physiotherapist-supported walking, the individual demonstrated improvements in several cardiovascular parameters: metabolic equivalents (METs), representing rate of energy expenditure at the resting state, were increased, indicating enhanced aerobic fitness. The individual’s resting and working heart rate decreased, further suggesting enhanced cardiovascular function (Hardin et al., 2007).

Other human studies have utilized treadmill training and demonstrated cardiovascular improvements: (Ditor et al., 2005) showed that autonomic control of the heart can be modulated by exercise after SCI by assessing changes in heart-rate variability, a measure which uses electrocardiogram-derived ratios of high and low frequency oscillations to determine the contributions of parasympathetic and sympathetic input. Locomotor training has also been shown to have beneficial effects on orthostatic hypotension, a hallmark cardiovascular deficit of SCI (Harkema et al., 2008), and on key cardiovascular parameters such as coronary flow reserve, left ventricular function and endothelial function (Turiel et al., 2011).

It is evident that studies in humans have guided the SCI field towards an appreciation of the potential benefits of exercise training on cardiovascular health.
However, to study neuronal effects of an exercise intervention, I believe it is necessary to examine the effects of exercise training in an animal model to standardize the location and severity of the injury and to eliminate other potential effects and comorbidities typically associated with human studies.

1.5.2 H-reflex modulation by exercise after SCI

Regions of the spinal cord caudal to an SCI demonstrate exaggeration of the H-reflex, an effect that may be coupled to increased spasticity (i.e. aberrant increases in muscle tone) after SCI (Burke, 1988; Skinner et al., 1996). As the stimulating frequency is increased during H-reflex recording in uninjured animals, the amplitude of the H-reflex is depressed at higher stimulation frequencies. With a lack of descending control over spinal circuits after SCI, frequency-dependent depression is decreased, leading to aberrant reflex behavior (Little and Halar, 1985; Little et al., 1999; Thompson et al., 1992).

Passive hind-limb cycling has a normalizing effect on frequency dependent depression (Reese et al., 2006) and the authors of one particular study propose that intrasegmental reorganization of inhibitory interneurons and their activity, and increased depression of motoneuron activity within the segment, could be the result of passive cycling training (Skinner et al., 1996). This study equated the effect of passive cycling to fetal tissue transplantation into the injured spinal cord, which also normalized H-reflex frequency-dependent depression, perhaps also by restoring segmental inhibitory control (Thompson et al., 1993).

Another study has demonstrated that proprioceptive neuropathy alters H-reflex normalization by exercise after SCI (Ollivier-Lanvin et al., 2010). The role of
proprioceptive neurons in H-reflex modulation by passive exercise was investigated using pyridoxine, a drug that is toxic to large DRG neurons (Ollivier-Lanvin et al., 2010). Passive cycling after complete transection SCI at the ninth thoracic vertebra restored frequency-dependent depression. This effect was lost, however, when cycling was coupled with pyridoxine treatment (Ollivier-Lanvin et al., 2010).

Additionally, increases in specific neurotrophic factor levels are correlated with frequency-dependent depression recovery by passive hind-limb cycling after SCI. As brain-derived neurotrophic factor (BDNF), neurotrophin-4 (NT-4) and neurotrophin-3 (NT-3) levels rise in the lumbar (L4-L6) spinal cord during passive cycling, H-reflex modulation increases as well (Cote et al., 2011) such that these neurotrophic factors may play a role in restoring normal reflex activity in the spinal cord segments involved in hind-limb locomotion.

Ultimately, the H-reflex can be used to assess changes in spinal cord circuitry resulting from passive exercise after SCI. Previous findings demonstrate that passive exercise may impose beneficial intrasegmental changes to H-reflex modulation, restoring reflexes to a more normal state.
1.5.3 Passive hind-limb cycling and SCI

Due to their injury, individuals with SCI are mainly limited to upper limb exercise. Unfortunately, this type of voluntary exercise does not enhance lower limb circulation (Kinzer and Convertino, 1989; Hopman et al., 1993). Thrombosis, lymphedema and pressure sores are all considered risks of decreased blood flow in the lower limbs (Walden et al., 1991). The importance of investigating strategies to improve lower limb blood flow, such as passive hind-limb cycling, is therefore quite clear.

The effects of passive hind limb cycling after SCI have been investigated for the past 15 years, primarily by John Houlé’s group (first at the University of Arkansas and more recently at Drexel University in Philadelphia). Topics have included modulation of spinal reflex excitability after SCI and exercise (Skinner et al., 1996; Ollivier-Lanvin et al., 2010; Cote et al., 2011), studies to determine the effects of passive cycling on muscle (Dupont-Versteegden et al., 1998; Dupont-Versteegden et al., 1999; Houle et al., 1999; Dupont-Versteegden et al., 2000; Dupont-Versteegden et al., 2002; Dupont-Versteegden et al., 2004) and motoneuronal properties (Beaumont et al., 2004; Keeler et al., 2012). No direct measures of the cardiovascular effects of passive hind-limb cycling in rats after SCI have been published to date.
1.6 Research objectives

By performing these experiments, I sought to determine changes to DRG sensory neurons following SCI and passive hind-limb cycling and to describe physiological responses which may be related to these changes.

I chose to investigate properties related to proprioception, as this type of sensory input is clearly altered as the hind-limbs are passively cycled. Until now, no one has assessed somal size changes of proprioceptive neurons in response to a passive cycling intervention, a measure which may reflect changes to trophic support of these neurons. Based on somal size alterations of proprioceptive neurons after SCI and also after passive hind-limb cycling, I finished this study with the hypothesis that the chemical milieu of proprioceptive target tissues is altered by both the injury and the cycling intervention.

Passive hind-limb cycling after SCI is under investigation by others for its ability to alter locomotor properties in injured rats (Liu et al., 2010; Ollivier-Lanvin et al., 2010; Cote et al., 2011; Liu et al., 2012). Up until this point, there have been no animal studies examining the possible cardiovascular benefits of this type of exercise with respect to AD and OH. I discovered that after a bout of passive hind-limb cycling, the severity of AD, a hallmark condition of cardiovascular dysfunction in SCI, is reduced by approximately 50%.

This led to further experiments to investigate properties of neurons related to nociception, and in particular neurons expressing the capsaicin receptor TRPV1. This particular subset of sensory neurons has previously been shown to be involved in the pathology of AD (Ramer et al., 2012). I found that these neurons also were altered by SCI and the passive hind-limb cycling intervention, indicating a potential link between TRPV1 and reduction in AD severity by passive hind-limb cycling.
Chapter 2: H-reflex modulation and somal size changes of proprioceptive neurons

In this chapter I explore the consequences of passive cycling exercise on modulation of the spinal H-reflex. I also investigate the relationship between the afferent arm of the H-reflex and its anatomical substrate, the Ia spindle afferent. I test the hypothesis that since somal size and axon diameter co-vary, so too should changes in the H-reflex and Ia afferent somal size. I demonstrate that even though soma size is reduced with SCI and even further with passive hind-limb cycling, there is a change only to H-reflex modulation with SCI when compared to uninjured controls. I did not detect a difference in H-reflex modulation due to the cycling intervention.

2.1 Materials and methods

2.1.1 Spinal cord injury surgery

Adult male Wistar rats (n=24) were on a 12 hour reversed light-dark cycle and housed in a secure, conventional rodent facility at the Blusson Spinal Cord Centre (Vancouver, BC, Canada). Three days prior to surgery, rats (250-350 g.) were placed on an enriched diet (for diet habituation) consisting of meal replacement shake (Ensure; Abbott, Saint-Laurent, Canada), rat treats/kibble (LabDiet, Rodent Diet 5001), cereals, fruit, and nutritive transport gel (Charles River).

Animals prepared for SCI surgery (n=16) were administered prophylactic enrofloxacin (Baytril; 10 mg/kg, s.c., Associated Veterinary Purchsing; AVP, Langley, Canada) on three days before surgery and immediately before surgery. Anesthetic agents were medetomidine hydrochloride (Domitor®; 0.5 mg/kg, i.p., AVP) and ketamine hydrochloride (Vetalar®; 70 mg/kg, i.p., McGill University Animal Resources Centre,
Montreal, Canada). The non-steroidal anti-inflammatory drug ketoprofen (Anafen®, 5 mg/kg, s.c., AVP) and the semi-synthentic opioid analgesic buprenorphine (Temgesic®; 0.02 mg/kg, s.c., McGill University) were both given ten minutes pre-operatively while under anesthesia. The surgical site was prepared by shaving, scrubbing with HibiHibitane® detergent (Mölnlycke Health Care, Gothenburg, Sweden), 70% ethanol and treatment with iodine.

The animal was placed in the prone position for surgery. A midline incision of the skin and superficial muscles was made, followed by blunt dissection of the muscles above the C8-T3 vertebrae. Connective tissues were excised at the T2-T3 intervertebral space, the dura was opened with a syringe needle and surgical scissors were used to completely transect the spinal cord. Complete transection of the spinal cord was confirmed under the surgical microscope: the severed ends of the spinal cord were clearly discontinuous and retracted, with the ventral portion of the vertebral column clearly visible and unobstructed by spinal cord. Throughout the procedure, bleeding was controlled with Gelfoam® absorbable gelatin compressed sponge (Pfizer, New York, NY). Continuous 4-0 Vicryl sutures were used to close the muscle layer, and interrupted 4-0 Prolene sutures were used to close the skin.

2.1.2 Post-operative care

Animals recovered in a warmed environment (Animal Intensive Care Unit, Hotspot for Birds, Los Angeles, CA) and were administered warmed lactated Ringer’s solution (5 mL s.c.). Atipamezole hydrochloride (Antisedan; 1mg/kg, s.c., Novartis, Mississauga, Canada) was administered to reverse anesthesia. Ketoprofen (5 mg/kg, s.c.), enrofloxacin
(10 mg/kg, s.c.) and buprenorphine (0.02 mg/kg, s.c.) were given once per day for three
days after surgery.

Post-surgically, rats were housed with their usual cage-mates and maintained on the
same enriched diet. Cages were fitted with low-reaching water bottles, while rubber
matting was placed under woodchips and food was scattered on the cage bottom (both to
encourage movement). Bladders were manually expressed 3-4 times daily until
spontaneous voiding returned. A systematic monitoring procedure was used to assess
animal health, which included objective measures of clinical morbidity signs, body weight,
activity level, surgical site healing and social behavior.

Pre-operative, surgical and post-operative care were carried out as previously
described (Ramsey et al., 2010).

2.1.3 Passive hind-limb cycling

Eight animals were randomly-assigned to undergo passive cycling (SCI-Ex). These
recovered for five days (as described by Houle et al., 1999) before beginning the exercise
program after SCI surgery. Animals were placed in a leather full-body sling and their legs
were fed through an opening that is wide enough to permit the cycling motion (Figure 2.1).
was used to attach the feet to the pedals of a motorized cycling machine, which functions to
move both legs through a full cycling motion (45 rpm with flexion and extension of ankle,
knee and hip joints). The hind-limbs were not hyperextended. To minimize friction, water-
based lubricant was applied to the cycling apparatus and hind-limbs (K-Y®, Markham, ON,
Canada). The exercise regimen consisted of 30 minutes of cycling, 10 minutes of rest,
followed by a second round of 30 minutes of cycling. This occurred 5 days per week for 4 weeks.
Figure 2.1. The cycling apparatus employed in passive hind-limb cycling after SCI.

The feet are secured to the pedals using Parafilm (Pechiney Plastic Packaging Company, Chicago, Illinois). A leather strap secures the animal into the leather restraint. Water-based lubricant was applied to the cycling apparatus and hind-limbs (K-Y®, Markham, ON, Canada) to reduce friction and prevent hind-limb sores from occurring.
2.1.4 Electrophysiology

Rats were urethane-anesthetized (1.5 g/kg, intraperitoneally; Sigma-Aldrich) and core body temperature was maintained between 36.5°C and 37.5°C with a heating pad. The ankle was dissected to reveal the tibial nerve, which was placed onto bipolar hook electrodes for stimulation. Bipolar needle recording electrodes were placed into the interosseus muscle of the foot. Input/output curves were generated by applying square pulses (100 μs pulse width) at set intervals with an AMPI Master-9 stimulator (AMP Instruments Ltd., Jerusalem, Israel) and recorded with an AD Instruments Powerlab 1630 amplifier (AD Instruments, Bella Vista, Australia).

Latency analysis was performed by extracting the time delay between stimulation and peak amplitude of either the M or H wave for each animal, and averaged over the number of traces obtained from input/output curves (approximately 10 per animal) (Uninjured n=11, SCI n=5, SCI-Ex n=8). Motoneuron recruitment analysis was performed by measuring area under the curve for both the M- and H-wave. \( M/M_{\text{max}} \) and \( H/M_{\text{max}} \) values from each individual trace from each animal’s input output curve were obtained. As a measure of motoneuron recruitment, \( H_{\text{max}}/M_{\text{max}} \) values were also obtained from area under the curve measurements for each animal (Uninjured n=11, SCI n=5, SCI-Ex n=8).
2.1.5 Tissue processing

Rats were killed with chloral hydrate (1 g/kg, i.p.) and perfused first with phosphate-buffered saline (PBS, room temperature), then 4% paraformaldehyde (PF, 4º C). DRGs were removed and placed in 4% PF to post-fix for 12 hours, after which they were transferred to 20% sucrose in 0.1M PB and kept at 4º C. DRGs were embedded in Tissue Tek (Fisher Scientific, Ottawa, Canada), frozen over liquid nitrogen and stored in a -80º C freezer until sectioning. DRGs were sectioned at 20 μm on a cryostat (Micron), thaw-mounted onto superfrost-plus glass slides and stored at -80º C.

2.1.6 Immunohistochemistry

Slides were thawed and then incubated in blocking solution (10% normal donkey serum) in PBS plus Triton X-100 (0.1%) for 20 min. Parvalbumin antibody raised in mouse (Millipore, Billerica, MA, USA, 1:1000) was diluted with PBS plus Triton X and applied to slides overnight. The slides were then washed three times in PBS and incubated in secondary antibodies raised in donkey and conjugated to Cy3 (Jackson ImmunoResearch, West Grove, USA) at 1:200 in PBS plus Triton X-100 (0.1%) for 2 hours. Epifluorescent images were obtained with an Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with a digital camera (Q Imaging, Burnaby, Canada) and Northern Eclipse software (Empix Imaging Inc., Mississauga, Canada).
2.1.7 Image analysis and recursive translation

SigmaScan Pro 5.0 (SPSS Inc., Systat Software Inc., San Jose, California) was used to analyze all images. DRGs were pooled for analysis: L1 and L2 DRGs (referred to as L1/L2 throughout), L4 and L5 DRGs (L4/L5) and L6 and S1 DRGs (L6/S1). To assess changes in soma size, recursive translation (Rose and Rohrlich, 1988) was employed to reconstruct the cell population from cell profiles (as described in (Ramer et al., 2001)). 400-600 cell profiles were generated per animal per DRG level (L1/2, L4/5, L6/S1) using SigmaScan Pro 5.0 (SPSS Inc.). Cell profiles were converted from circumference to diameter measurements as required for the algorithm and cell diameter bin sizes (20 bins) were set at 5 μm within the range of 0 to 100 μm. Bin sizes and overall range were held constant across all size comparisons to allow for accurate comparisons of somal size changes.

2.1.8 Statistics

For electrophysiological data, a one-way ANOVA was used to assess differences in H-wave latency and motoneuron recruitment between uninjured, SCI and SCI-Ex groups. From cumulative size-frequency (Q-sum) plots generated by recursive translation, the Kolmogorov-Smirnov test was used to determine the significance of shift in soma size between groups (uninjured versus SCI, SCI versus SCI-Ex). Test statistics were doubled to account for multiple comparisons. The D-statistic must be greater than the doubled test statistic for the shift in soma size to be significant. All values are presented in Table 2.1.

For all tests, P values <0.05 were considered significant. Where appropriate, results
are expressed as mean±standard error of the mean (SEM). Image analyses were performed in a blinded fashion with coded image files. For the recursive translation component of this study, the same microscope, objective and experimenter were employed for each separate comparison.
2.2 Results

2.2.1 Electrophysiology

The H-reflex is the electrical analogue of the monosynaptic stretch reflex and can be studied to determine changes in proprioceptive circuitry. I performed electrophysiological H-reflex measurements on rats from all groups. This involved stimulating the tibial nerve with bipolar hook electrodes and recording from the interosseous muscles of the foot with bipolar needle recording electrodes. Input/output curves were generated for each animal and then analyzed for H-wave latency and motoneuron recruitment.

2.2.2 M-wave latency

As the value of M-wave latency represents orthodromic action potential propagation from the stimulating electrode directly to the interosseous muscles, there is no central reflex component involved. M-wave latency was unchanged by passive hind-limb cycling (Figure 2.2a), indicating that there were no measurable changes in motoneuron conduction velocity (uninjured=2.66 ± 0.11 ms, SCI=2.17 ± 0.19 ms, and SCI-Ex=2.40 ± 0.16 ms).
2.2.3 H-wave latency

The monosynaptic circuit involved in the H-reflex consists of an afferent fibre which synapses on the soma of a motoneuron residing in the spinal cord gray matter, and this motoneuron’s efferent motor axon which synapses on a muscle cell’s motor end plate. H-wave latency reflects the amount of time taken by a compound action potential, or the sum of action potentials of individual axons in a nerve, to propagate along the length of this circuit. H-wave latency decreased in SCI rats and this effect remained unchanged with passive hind-limb cycling (Fig 2.2b, uninjured=10.22 ± 0.256 ms, SCI=9.07 ± 0.14 ms, SCI-Ex=8.61 ± 0.21 ms). Differences were determined with a one-way ANOVA (P<0.05).
Figure 2.2. H-wave latency is decreased after SCI and after SCI with passive hind-limb cycling.

M-wave latency was unaffected by either SCI or SCI-Ex (a) and H-wave latency was reduced by SCI and unaffected by SCI-Ex (b). *P<0.05, one-way ANOVA.
2.2.4 Reflex motoneuron recruitment

The measure of H-wave amplitude in relation to amplitude of $M_{\text{max}}$ reflects the degree to which reflex motoneurons are recruited upon stimulation. This can reflect the ability of a population of afferent axons to propagate an action potential or of the ability of motoneurons involved in the circuit to fire.

I observed increased reflex motoneuron recruitment in both SCI (Figure 2.3b,d, $H_{\text{max}}/M_{\text{max}} = 0.32 \pm 0.050$) and SCI-Ex (Figure 2.3c,d, $H_{\text{max}}/M_{\text{max}} = 0.36 \pm 0.032$) groups when compared to uninjured controls (Figure 2.3a,d, $H_{\text{max}}/M_{\text{max}} = 0.20 \pm 0.021$). There was no effect of exercise on motoneuron recruitment when comparing SCI versus SCI-Ex rats (Figure 2.3d). Differences were determined with a one-way ANOVA ($P<0.05$, uninjured n=11, SCI n=5, SCI-Ex n=8).
Figure 2.3. Reflex motoneuron recruitment is increased after SCI and after SCI with passive hind-limb cycling.

Scatterplots represent M/M_{max} and H/M_{max} values for all traces of input/output curves for uninjured (a), SCI (b) and SCI-Ex (c) animals. Enhanced motoneuron recruitment after H-reflex recording occurred in SCI and SCI-Ex animals (d). *P<0.05, one-way ANOVA.
2.2.5 Proprioceptive sensory neuronal somata responded to SCI and SCI-Ex in a level-dependent fashion

I next used recursive translation to examine somal size changes of proprioceptive sensory neurons in the DRG. I used an established marker for proprioceptive sensory neurons, the calcium-binding protein parvalbumin (Celio, 1990; Copray et al., 1994; Ernfors et al., 1994; Inoue et al., 2002)(sample immunostaining shown in Figure 2.4). I found that SCI leads to somal atrophy of proprioceptive neurons in L1/2 (Figure 2.5a) and L4/5 (Figure 2.5b) ganglia and that this atrophy is enhanced further in rats subjected to passive hind-limb cycling. In contrast, proprioceptive neurons at the L6/S1 DRG level (Figure 2.5c) did not change in soma size in response to SCI or SCI-Ex. All shifts in cumulative frequency (Q) plots were significantly different after applying the K-S goodness-of-fit test (values displayed in Table 1).
Figure 2.4. Sample parvalbumin staining.

Sample staining of the calcium-binding protein parvalbumin, a marker of large-diameter proprioceptive neurons, in L4/L5 DRG of an uninjured animal.
Figure 2.5. T3 SCI leads to level-dependent somal size changes in Parvalbumin⁺ DRG sensory neurons.

(a) L1/2 and (b) L4/5 parvalbuming-expressing proprioceptive DRG neurons underwent atrophy following SCI. Passive cycling increased the effect of SCI, leading to further somal size reduction. (c) In L6/S1 DRGs, parvalbumin-expressing proprioceptive DRG neurons did not demonstrate a somal size shift. All shifts in Q plots were statistically significant (K-S goodness-of-fit test, *P<0.05).
Table 2.1. D-statistics and calculated test statistics for the K-S test of somal size shifts using recursive translation analysis of parvalbumin-expressing neurons.

<table>
<thead>
<tr>
<th>DRG level</th>
<th>Uninjured vs. SCI</th>
<th>SCI vs. SCI-Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1/L2</td>
<td>D=0.092, test=0.045, test*2=0.090</td>
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<td>L4/L5</td>
<td>D=0.081, test=0.031, test*2=0.062</td>
<td>D=0.093, test=0.032, test*2=0.064</td>
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<tr>
<td>L6/S1</td>
<td>D=0.073, test=0.056, test*2=0.112</td>
<td>D=0.12, test=0.060, test*2=0.12</td>
</tr>
</tbody>
</table>
2.3 Discussion

2.3.1 M- and H-wave latency

Exercise had no effect on M-wave latency or H-wave latency. M-wave latency was unaffected by either SCI or SCI-Ex, demonstrating that excitability of α-motoneurons was unaffected by SCI or the exercise intervention. SCI led to decreased latency of the H wave after SCI, most likely reflecting increased conduction velocity along muscle spindle afferents. This is puzzling, however, as at L1/L2 and L4/L5 levels proprioceptive soma size decreased after SCI and further again after SCI-Ex. One would argue that if soma size decreases, so should axon calibre and conduction velocity. Perhaps some other feature of proprioceptive afferents that could increase axonal conduction velocity, such as membrane channel composition or degree of myelination (less likely), is changing in response to SCI and SCI-Ex.

2.3.2 Motoneuron recruitment

Motoneuron recruitment, as measured by $H_{\text{max}}/M_{\text{max}}$ was increased after SCI and exercise had no effect. This fits with data describing decreased inhibitory control of spinal circuits, and therefore enhanced reflex activity and motoneuron recruitment, in individuals with SCI. Our data does not match that of others who show decreased motoneuron recruitment after passive hind-limb cycling. The difference in my results may be ascribed to the chosen anesthetic: I used urethane anesthesia, while others have used a mixture of ketamine and xylazine for H-reflex recordings (Cote et al., 2011).
2.3.3 Response of parvalbumin-expressing neurons to SCI and SCI-Ex

In L1/L2 and L4/L5 pooled ganglia, there was a reduction in soma size associated with SCI and SCI-Ex. In L6/S1 ganglia, there was no change in soma size associated with SCI or SCI-Ex. When considering potential neurotrophic factors that could be involved in this response, neurotrophin-3 (NT-3) seems a fitting candidate.

2.3.4 A potential role for NT-3

Although the role of neurotrophic factors in development has been studied extensively, they also play a large role in neuronal maintenance, axonal plasticity and growth in the adult (Terenghi, 1999; Jones et al., 2001; Lu and Tuszynski, 2008). NT-3 belongs to the neurotrophin family of neurotrophic factors, which includes BDNF (Barde et al., 1982; Leibrock et al., 1989), NGF (Levi-Montalcini, 1964) and NT-4/5 (Ip et al., 1992). NT-3 is critical for normal proprioceptive neuron development, as homozygous null NT-3 mice completely lack proprioceptive neurons (Ernfors et al., 1994) and other neurotrophins are not able to rescue limb proprioceptive neuronal loss after NT-3 knockout (Fan et al., 2000). Additionally, muscle spindles are lacking in NT-3\(-/-\) mice, which results in the behavior of abnormal limb positioning and reduced movements (Farinas et al., 1994). The very few NT-3\(-/-\) mice that survive longer than 24 hours after birth display athetotic walking patterns, marked by impaired muscle coordination and slow writhing movements (Farinas et al., 1994).

NT-3 is expressed in peripheral targets of sympathetic postganglionic axons in the adult (Randolph et al., 2007). When the superior cervical ganglion (SCG) is removed, and sympathetic input of SCG neurons is lost in target tissues, there is a marked increase in
NGF in the pineal gland and extracerebral blood vessels, while there is a marked decrease in NT-3 (Randolph et al., 2007). The effect of SCG removal is different, however, in external carotid artery where NGF levels are unchanged and NT-3 levels increase (Randolph et al., 2007).

There is severe disruption to sympathetic outflow after complete transection SCI at T3. Due to changes in sympathetic input to targets of lumbar DRGs after T3 SCI, there may be a change in NT-3 production in target tissue. This could lead to an alteration of neurotrophic support by NT-3 of parvalbumin-expressing DRG neurons, leading to the observed changes in soma size. As removal of SCG neurons led to variable responses of NT-3 expression in target tissues (i.e., NT-3 levels increased significantly in the carotid but decreased significantly in other targets), this may be the case with the targets of parvalbumin-expressing neurons as well. L6/S1 neurons did not change in soma size after SCI or SCI-Ex, and therefore it may be that SCI does not change NT-3 production in bladder/bowel afferent targets, while the atrophy of parvalbumin-expressing neurons at L1/L2 and L4/L5 levels may be coupled to decreased NT-3 production in skeletal muscle. Furthermore, passively moving the hind-limbs may generally decrease NT-3 production in muscle and lead to system-wide decreases in NT-3 production.

One study measured mRNA expression of NT-3 and its receptor TrkC in large DRG neurons after T9 complete SCI and after T9 complete SCI with passive hind-limb cycling (Keeler et al., 2012). There was no difference found between intact, SCI and SCI-Ex groups, but this study in no way rules out further exploration into NT-3 expression level changes after SCI and SCI-Ex: the target tissues of proprioceptive neurons have yet to be examined, and the level of injury (T9) leads to remarkably different changes in the
periphery than would occur after injury at T3 (Ramer et al., 2012).

2.3.5 Previously described effects of passive hind-limb cycling on motoneurons

Passive hind-limb cycling has shown promise in altering pathological motoneuron activity after SCI. Thoracic SCI results in altered hind-limb motoneuron electrophysiological properties: resting potentials of motoneurons are more positive, or in a more depolarized state, after transection (Beaumont et al., 2004). Intuitively, this has implications for spasticity after SCI: a motoneuron whose resting potential is closer to firing potential will require less external stimulation to fire, resulting in increased muscle activity. One report demonstrates normalization of motoneuron resting potential with passive hind-limb cycling, which is apparently similar in effect to fetal tissue spinal cord transplants at the transection site (Beaumont et al., 2004). Another study has shown gene expression changes in motoneurons due to thoracic spinal cord transection and passive hind-limb cycling (Keeler et al., 2012). After laser capture microdissection and quantitative polymerase chain reaction (PCR) of roughly 300 L4-L6 motoneurons per rat in uninjured, transected and transected plus passive hind-limb cycling rat groups, changes in mRNA expression of several motoneuron neurotrophic factors increased significantly in response to exercise, including BDNF, GDNF, NT-3 and NT-4/5 (Keeler et al., 2012). Although not reflected in my data, it is clear that motoneurons are influenced by and respond to passive cycling of the hind-limbs after SCI.
Previously described effects of passive hind-limb cycling on muscle

Muscle properties after SCI and passive hind-limb cycling have been studied extensively to uncover the rehabilitative potential of this type of exercise (Dupont-Versteegden et al., 1998; Dupont-Versteegden et al., 1999; Houle et al., 1999; Dupont-Versteegden et al., 2000; Dupont-Versteegden et al., 2002; Dupont-Versteegden et al., 2004). The greatest discovery to come from these experiments is that passive hind-limb cycling can reverse SCI-induced muscle atrophy (Dupont-Versteegden et al., 1998).

After spinal cord transection and loss of normal muscle innervation, the dominant muscle fibre type rapidly shifts from type I myosin heavy chain (MyHC) (“slow” fibres) to type II MyHC (“fast” fibres) (Jiang et al., 1990; Lieber et al., 1986; Roy and Acosta, 1986). In an initial survey of muscle gene expression and fibre size in a short duration (1-5 day) passive hind-limb cycling intervention, atrophy of both soleus and extensor digitorum longus muscles was reversed by cycling but alteration of muscle fibre type by SCI was unaffected (Dupont-Versteegden et al., 1998). Another experiment demonstrated the inability of passive cycling to affect any change to the “slow to fast” muscle phenotype switch that SCI incurs: after a 3 month passive hind-limb cycling intervention coupled to fetal SCI site graft implants, muscle atrophy was greatly reduced but fibre type remained in the pathological “fast-” or type II-dominant state (Houle et al., 1999). After making the SCI-induced switch to high levels of type II fibre type, hind-limb muscle seems to be exceptionally resilient to phenotypic shift.

Muscle satellite cells are found between the sarcolemma and the basal lamina, and are undifferentiated myogenic stem cells (Dupont-Versteegden et al., 1999). Muscle myonuclei in adult muscle, on the other hand, are plentiful within the cytoplasm: one
cytoplasmic unit contains multiple myonuclei. Reduction in the number of skeletal muscle nuclei was investigated by one group as a potential mechanism for muscle atrophy after SCI, as well as the potential mechanism for restoration of muscle mass after passive cycling by increasing myonuclear number (Dupont-Versteegden et al., 1999). Additionally, because muscle satellite cells contribute to regeneration and growth of skeletal muscle (Dupont-Versteegden et al., 1999), the same study investigated whether satellite cell number changes with injury and again with a cycling intervention. This study found that muscle satellite cells are indeed activated after spinal cord transection, but changes in their number or activation are not altered by a cycling intervention (Dupont-Versteegden et al., 1999). In a similar fashion, myofiber nuclei number is reduced after SCI but no change is witnessed to this reduction in nuclei after passive hind-limb cycling (Dupont-Versteegden et al., 1999). Contrary to this, in a similar study of the same muscle (soleus) with the same exercise intervention, increased myonuclear number and increased satellite cell fusion did indeed seem to drive atrophy reversal (Dupont-Versteegden et al., 2000).

In another attempt to understand the mechanisms of skeletal muscle atrophy after SCI and reversal of atrophy with passive exercise, a pathway previously implicated in skeletal and cardiac muscle hypertrophy – the calcineurin-NFAT (nuclear factor of activated T cells) pathway – was assessed (Dupont-Versteegden et al., 2002). Using cyclosporin A (CsA) to block calcineurin activity throughout the post-SCI passive hind-limb cycling intervention, the group found no change in the atrophy-reversing properties of the cycling intervention. The calcineurin-NFAT pathway, therefore, is not implicated in the atrophy-reversing properties of passive hind-limb cycling (Dupont-Versteegden et al., 2002). To summarize, muscle fibre type is not seemingly malleable in the context of...
passive hind-limb cycling after SCI. Increasing the size of remaining muscle fibres is a more likely mechanism for atrophy reversal due to passive exercise intervention. Based on my own experimental proprioceptor somal size change findings, muscle neurotrophic factor expression may change due to SCI and SCI-Ex in an atrophy/hypertrophy-dependent manner, rather than in a way that is dependent upon muscle phenotype.
Chapter 3: Cardiovascular outcomes of passive hind-limb cycling and TRPV1

In this chapter, I examine the relationship between capsaicin-sensitive nociceptive neurons and two hallmarks of cardiovascular dysfunction in SCI, OH and AD. Passive exercise had no effect on the severity of OH. However, I demonstrate that a 4 week cycling intervention after T3 complete spinal cord transection can reduce the severity of AD by approximately 50%.

Additionally, I find that while SCI leads to hypertrophy of TRPV1⁺ nociceptors, this hypertrophy is reversed or prevented with passive cycling. I discuss the possibility that neurotrophic support of nociceptive neurons in target tissues is affected by the cycling intervention.

3.1 Materials and Methods

3.1.1 Spinal cord injury surgery, post-operative care, passive hind-limb cycling and tissue processing

The same protocols were followed for SCI surgery, post-operative care, passive hind-limb cycling and tissue processing as in Chapter 2.

3.1.2 Cardiovascular assessment

Isoflurane anaesthesia was used for carotid cannulations. Each cannula (PU-30) contained a lock solution of 1:10 heparin (Hepalean®, AVP) and Lactated Ringer’s with 5% dextrose. One end was inserted 20 mm into the left carotid artery. The other end was externalized through a small opening in the back skin. Cannulations were performed on the
SCI-Ex group one day after the last cycling training session, at one month post-SCI for SCI controls, and at the appropriate time in age-matched uninjured controls.

A system consisting of the cannula, a fluid-filled pressure transducer (SP844, MEMScAP, Norway) and the monitoring suite PowerLab / Chart™ 5 for Windows (ADInstruments, Colorado Springs, USA) was used to record beat-to-beat BP beginning two hours after cannulation. A BP reading that was stable for five minutes was recorded as baseline.

A previously devised protocol for inducing AD was employed (Alan et al., 2010). A balloon-tipped catheter (Coloplast, Denmark) was inserted 1.5 cm. into the rat’s rectum and fastened to the tail with surgical tape. After arterial pressure stabilized and a consistent baseline was achieved, 2 mL of air was injected into the balloon over a period of 10 seconds (colorectal distension, CRD). The balloon was kept inflated for a period of one minute and was then deflated inside the rectum for a recovery period. BP during two separate AD events was recorded for each animal with a minimum of 5 minutes recovery between each event. The rats were awake and roaming freely while measurements were taking place. Beat-to-beat data was averaged over 1 second intervals. For each AD event, average baseline SAP was compared to maximum stimulus-evoked SAP. The mean of both AD events per animal is reported.

Orthostatic hypotension (OH) was also assessed. Rats were placed on the experimenter’s forearm and allowed to rest normally. After baseline BP was achieved, the rat was tilted 90 degrees vertically with its head held upwards (head-up tilt, HUT) and was maintained in this position for one minute. BP changes during two separate OH events were obtained for each animal with a minimum of 5 minutes recovery between each event.
The rats were awake and roaming freely while measurements were taking place. Beat-to-beat BP data was averaged over 1 second intervals. For each HUT event, average baseline SAP was compared to maximum stimulus-evoked SAP. The mean of both OH events per animal is reported.

3.1.3 Immunohistochemistry

Slides were thawed and then incubated in blocking solution (10% normal donkey serum) in PBS plus Triton X-100 (0.1%) for 20 min. TRPV1 antibody raised in goat (Neuromics, Edina, MN, USA; 1:2000) was diluted with PBS plus Triton X and applied to slides overnight. The slides were then washed three times in PBS and incubated in secondary antibodies raised in donkey and conjugated to Cy3 (Jackson ImmunoResearch, West Grove, USA) at 1:200 in PBS plus Triton X-100 (0.1%) for 2 hours. Epifluorescent images were obtained with an Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with a digital camera (Q Imaging, Burnaby, Canada) and Northern Eclipse software (Empix Imaging Inc., Mississauga, Canada). Identical imaging settings were utilized for each antigen.

3.1.4 Image analysis and recursive translation

SigmaScan Pro 5.0 (SPSS Inc., Systat Software Inc., San Jose, California) was used to analyze all images. DRGs were pooled into 3 separate groups for analysis: L1 + L2 (referred to as L1/L2 throughout), L4 + L5 (L4/L5) and L6 + S1 DRGs (L6/S1). To assess changes in soma size, recursive translation (Rose and Rohrlich, 1988) was employed to
reconstruct the cell population from cell profiles (as described in (Ramer et al., 2001), see Appendix 1.1 and 1.2). 400-600 cell profiles were generated per animal per DRG level (L1/2, L4/5, L6/S1) using SigmaScan Pro 5.0 (SPSS Inc.). Cell profiles were converted from circumference to diameter measurements as required for the algorithm and cell diameter bin sizes (20 bins) were set at 5 μm within the range of 0 to 100 μm. Bin sizes and overall range were held constant across all groups to allow for accurate comparisons of somal size changes.

3.1.5 Statistics

An independent samples t-test was used to compare the change in systolic arterial pressure (ΔSAP = stimulus-evoked maximum systolic arterial pressure minus baseline arterial pressure, average of two CRD events) between the SCI control group and SCI-Ex.

A one-way analysis of variance (ANOVA) was used to assess head-up tilt (HUT)-induced BP changes across three groups: uninjured, SCI and SCI-Ex.

From cumulative size-frequency (Q-sum) plots generated by recursive translation, the Kolmogorov-Smirnov test was used to determine the significance of shift in soma size between groups (uninjured versus SCI, SCI versus SCI-Ex). The test statistic was calculated according to the formula 1.36 / √n , where n is total number of cells of the reconstructed cell population from recursive translation. Test statistics were doubled to account for multiple comparisons. The D-statistic must be greater than the doubled test statistic for the shift in soma size to be significant.

For all tests, P values <0.05 were considered significant. Where appropriate, results are expressed as mean±standard error of the mean (SEM). Image analyses were performed
in a blinded fashion with coded image files. For the recursive translation component of this study, the same microscope, objective and experimenter were employed for each separate comparison.
3.2 Results

3.2.1 Cardiovascular assessment

I measured BP changes in response to colo-rectal distension (CRD) and head-up tilt (HUT). Rats received carotid cannulations after 4 weeks of passive hind-limb cycling training and were assessed twice for AD (2x CRD separated by at least 5 minutes, change in systolic arterial pressure (SAP) for both events averaged upon analysis) and twice for OH (2x HUT separated by at least 5 minutes, change in systolic arterial pressure (SAP) for both events averaged upon analysis). I did not perform CRD on uninjured animals as this stimulus (understandably) provokes an escape response in uninjured animals that is not analogous to an AD response.

3.2.1.1 Orthostatic hypotension was unaffected by passive hind-limb cycling

Systolic BP was decreased upon HUT in both SCI and SCI-Ex rats when compared to uninjured controls (uninjured = −2.4 ± 3.4 mmHg, SCI = −32.2 ± 4.1 mmHg, SCI-Ex = −26.2 ± 3.6 mmHg, minus sign denotes negative drop from baseline) (Figure 3.1a). HR was static with HUT in uninjured (32.4 ± 5.7 bpm), SCI (30.3 ± 6.3 bpm) and SCI-Ex (27.1 ± 7.8 bpm) rats (Figure 3.1b). Importantly, there was no difference in BP (and therefore the OH response) between SCI and SCI-Ex rats during HUT (Figure 3.1a)(n=8 animals per group, *P<0.05, one-way ANOVA). Sample traces are displayed in Figure 3.1c.
Figure 3.1. Severity of orthostatic hypotension is unaffected by passive hind-limb cycling.

(a) Beat-to-beat systolic arterial BP (SAP) was recorded during two bouts of OH-eliciting head-up tilt (HUT). (b) Average change in heart rate in response to HUT did not change between groups. (c) Sample traces of the OH response in uninjured (n=8), SCI (n=8) and SCI-Ex (n=8) animals. In all cases a one-way ANOVA was used to compare groups for significant differences. *P<0.05.
3.2.1.2 Severity of autonomic dysreflexia is attenuated with passive hind-limb cycling

When compared with SCI controls, SCI-Ex rats demonstrated a significantly smaller change in systolic arterial pressure (stimulus-evoked SAP minus baseline SAP, Figure 3.2a) (SCI = 49.1 ± 5.3 mmHg, SCI-Ex = 28.2 ± 4.9 mmHg) in response to CRD. Exercise animals demonstrated a decreased drop in HR when compared to SCI controls (Figure 3.2b)(SCI = −63.0 ± 7.9 bpm, SCI-Ex = −20.6 ± 6.9 bpm, minus sign denotes negative drop from baseline, n= 8 animals per group, P<0.05, independent samples t-test). Sample traces are displayed in Figure 3.2c.
Figure 3.2. Severity of autonomic dysreflexia is attenuated by passive hind-limb exercise.

(a) Rise in beat-to-beat systolic arterial BP (SAP), (b) heart rate drop (beats per minute, bpm) and (c) sample traces of the AD response in SCI and SCI-Ex animals. *P<0.05, independent samples t-test (n=8 animals / group).
3.2.2 TRPV1-expressing sensory neurons decreased in soma size after SCI but not in exercised SCI rats

I next sought to determine a potential mechanism by which passive hind-limb cycling could lead to AD reduction. Our laboratory recently showed that sensory neurons expressing the transient receptor potential cation channel subfamily V member 1 (TRPV1) undergo selective hypertrophy after SCI and that abolishing afferent input of TRPV1-expressing sensory neurons leads to an attenuation of AD (Ramer et al., 2012). With this in mind, I hypothesized that there would be an association between passive hind-limb cycling and change in soma size of this subset of sensory neurons.

Accompanying the reduction of AD with passive hind-limb cycling was a decrease in soma size of TRPV1-expressing DRG neurons at all levels assessed (sample staining shown in Figure 3.3). I assessed TRPV1 soma size at L1/2 (Figure 3.4a), L4/5 (Figure 3.4b) and L6/S1 (Figure 3.4c) DRG levels. The previous findings of (Ramer et al., 2012) were reproduced in this study: in addition to significant hypertrophy demonstrated at the L4/5 and L6/S1 DRG levels as per Ramer et al. (2012), I demonstrated SCI-induced hypertrophy of TRPV1-expressing neurons at the L1/2 level.

All shifts in cumulative frequency (Q) plots were significantly different after applying the K-S goodness-of-fit test (P<0.05). All values are presented in Table 3.1.
Figure 3.3. TRPV1 staining.

Sample staining of the capsaicin receptor TRPV1, a marker of a subset of small-diameter nociceptive primary afferent neurons, in L4/L5 DRG of an uninjured animal.
Figure 3.4. Complete spinal cord transection at T3 leads to somal hypertrophy of
TRPV1⁺ DRG sensory neurons, which is prevented or reversed after passive hind-
limb cycling.

(a-c) Size-frequency histograms and cumulative size-frequency (Q) plots of L1/2 (a), L4/5
(b), and L6/S1 (c) pooled DRGs. All shifts in Q plots were statistically significant (K-S
goodness-of-fit test, *P<0.05).
Table 3.1. D-statistics and calculated test statistics for the K-S test of somal size shifts using recursive translation analysis of TRPV1-expressing neurons.

<table>
<thead>
<tr>
<th>DRG level</th>
<th>Uninjured vs. SCI</th>
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<td>L1/L2</td>
<td>D=0.24, test=0.032, test*2=0.064</td>
<td>D=0.46, test=0.037, test*2=0.074</td>
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<tr>
<td>L4/L5</td>
<td>D=0.072, test=0.026, test*2=0.052</td>
<td>D=0.40, test=0.028, test*2=0.056</td>
</tr>
<tr>
<td>L6/S1</td>
<td>D=0.097, test=0.036, test*2=0.072</td>
<td>D=0.25, test=0.039, test*2=0.078</td>
</tr>
</tbody>
</table>
3.3 Discussion

3.3.1 Passive hind-limb cycling and OH

High thoracic SCI results in loss of reflex vasoconstriction and decreased efferent sympathetic output to blood vessels in the lower extremities and abdominal viscera (Claydon and Krassioukov, 2006; Krassioukov and Claydon, 2006). When blood pools in lower vessels and is unable to reach the brain, the symptoms of OH, including light-headedness and dizziness, are present (Lahrmann et al., 2006). Similarly, when blood does not reach the intrathoracic veins, end diastolic volume and therefore left ventricular stroke volume are also reduced. The body responds with reflex tachycardia (ten Harkel et al., 1993; Ten Harkel et al., 1994), which is typically not sufficient to increase BP to relieve OH symptoms (Krassioukov et al., 2009a).

Passive hind-limb cycling did not alter OH or associated reflex tachycardia. As the main baroreceptors sensing postural changes are in the aortic arch and the carotid arteries (Robertson et al., 1956), medullary afferents normally relay postural information to the RVLM and through to the IML column of the thoracic spinal cord. In an individual with cervical or high-thoracic SCI, descending input from the RVLM in response to baroreceptor unloading is unable to traverse the injury site and communicate with sympathetic preganglionic neurons. Since afferent input below the injury does not alter normal descending control to sympathetic preganglionic neurons, it is perhaps unsurprising that we saw no change in OH severity with exercise.

There was, however, sound rationale for assessing whether OH is altered after passive exercise. Before these experiments, the possibility that passive cycling may have
changed properties related to peripheral vessels and capacity for vasoconstriction was still viable. Eliminating this possibility was an important part of the study.

3.3.2 Passive hind-limb cycling and AD

These data demonstrate that a bout of passive hind-limb cycling can reduce the severity of AD by approximately 50%. Interestingly, when we used telemetry devices connected to the abdominal aorta to assess the BP response during passive cycling, we found that there was no BP change during cycling (unpublished observations). If passive hind-limb cycling does not raise BP as would typically occur with aerobic exercise (Fadel and Raven, 2012), it is likely that passive cycling somehow modifies the chemical environment of the hind-limbs and viscera to alter afferent input to the spinal cord. These data suggest a significant role for an altered sensory contribution of afferents in both the hind-limbs and viscera in AD reduction by the cycling intervention.

In the AD circuit (see Figure 2), aberrant sympathetic vasoconstriction of vasculature in the splanchnic vascular bed is the primary explanation for AD-induced BP increases. If afferent input to this circuit is altered at lumbar spinal segments in a way that dampens reflex activity, input to ascending propriospinal circuits which interact with below-injury sympathetic preganglionic neurons would be altered as well. Outflow to the splanchnic circulation would likely be decreased, in turn decreasing AD severity. In the context of my experiments and by considering this circuit, if afferent input from TRPV1-expressing nociceptors is changing due to exercise, the degree of vasoconstriction in the splanchnic vascular bed could be decreased as well, ultimately lessening the severity of spikes in BP associated with AD.
Reduction of the bradycardic response with SCI-Ex is likely due to changes in baroreceptor loading. If the AD BP response is decreased by decreased reflex circuit activity, there will be less stretching of baroreceptors in the aortic arch and carotid arteries, and therefore less afferent input to the RVLM. In this case, RVLM-mediated sympathetic drive to the heart is closer to baseline when compared to SCI controls, and vagal parasympathetic input to the heart is closer to baseline if AD severity is reduced.

The passive cycling intervention likely alters primary cardiac components as well. Previous studies in humans with SCI have demonstrated that passive cycling can increase venous return to the heart, thereby raising stroke volume and cardiac output (Muraki et al., 1996; Muraki et al., 2000). To achieve this effect, it is probable that passive cycling increases action of the myogenic pump in passively moved muscles.

3.3.3 TRPV1 neuronal hypertrophy and reversal with passive hind-limb cycling

Passive hind-limb exercise prevented or reversed neuronal hypertrophy of TRPV1-expressing nociceptive neurons. As the cycling intervention also decreased the severity of AD by approximately 50%, there exists a potential role for this neuronal subset in AD pathology.

The previous findings demonstrating neuronal hypertrophy of the TRPV1 subset in L4/L5 and L6/S1 DRGs after SCI (Ramer et al., 2012) were reproduced in this study, and these experiments involving ablation of TRPV1+ afferents (see Introduction and Ramer et al., 2012) may offer insight into the role of TRPV1 neurons in AD. Further experiments combining a bout of passive hind-limb cycling and intrathecal capsaicin injection will be instructive in determining whether there is an additive effect of capsaicin and exercise: in
the case that there is not, and that there is a 50% reduction in AD severity as witnessed in both abolishment of TRPV1+ afferents (Ramer et al., 2012) and after passive exercise, the sole sensory contribution of afferent input to AD pathology would be from those afferents expressing TRPV1. An effect of cycling and capsaicin that surpasses the approximately 50% reduction would suggest a potential role of other sensory mechanisms or adaptive changes to spinal cord circuitry resulting from cycling the hind-limbs, leaving other avenues to be explored.

### 3.3.4 A potential role for Artemin

Artemin is a neurotrophic factor (Baloh et al., 1998) expressed by multiple cell types, including vascular smooth muscle cells (VSMCs) (Honma et al., 2002). Like Glial Cell Line-Derived Neurotrophic Factor (GDNF) (Lin et al., 1993), Persephin (PSPN) (Milbrandt et al., 1998) and Neurturin (NRTN) (Kotzbauer et al., 1996), Artemin belongs to the GDNF family of ligands (Baloh et al., 1998), a group of neurotrophic factors that signals through a receptor complex consisting of the Ret tyrosine kinase and one of the identified GDNF family receptor (GFR)α subunits (Tansey et al., 2000). GDNF, NRTN, ARTN, and PSPN preferentially bind GFRα1-4 subunits, respectively (Honma et al., 2002).

In mouse DRG neurons, TRPV1 expression heavily colocalizes with expression of the GFRα3 subunit of the GFRα3/Ret complex, the subunit that binds Artemin (Bennett et al., 2006). Additionally, GFRα3 neurons undergo selective hypertrophy after SCI alongside TRPV1-expressing neurons (Ramer et al., 2012). With this in mind, a likely mechanism for both TRPV1+ neuronal hypertrophy and hypertrophy reversal with passive hind-limb
cycling could be changes in Artemin expression levels in target tissues.

If Artemin is involved in this process, a blood flow-based mechanism may modulate Artemin expression. Passive hind-limb cycling may mechanically increase blood flow in the exercising hind-limbs, and I demonstrate here that cycling reduces AD severity and prevents or reverses hypertrophy of TRPV1-expressing DRG neurons. Furthermore, a previous study demonstrates that inducing AD on a daily basis in rats with T3 complete SCI, and therefore also increasing flow in the vasculature, also reduces the severity of AD (Alan et al., 2010). Additionally, vascular smooth muscle cells function abnormally after SCI by enhanced phenylephrine sensitivity (Brock et al., 2006; McLachlan and Brock, 2006) and cyclooxygenase-2 expression (LM Ramer unpublished). After passive hind-limb cycling and increasing flow throughout the hind-limb vasculature, including regions where there is a high density of TRPV1⁺ afferents, a flow-coupled mechanism may be attenuating Artemin expression by VSMCs and limiting the amount of this neurotrophic factor in target tissue, while in the case of SCI controls, Artemin expression may be increased by a pathological mechanism based on inadequate blood flow. Studies in humans regarding changes to blood flow after passive cycling in the peripheral vasculature are contradictory, however. In two studies using Doppler flowmetry of the common femoral artery, one group claims that there is indeed an acute increase in blood flow in the lower extremities after passive cycling training (Ballaz et al., 2007) while the other showed no difference (Ter Woerds et al., 2006). Further animal studies to determine the acute and chronic hemodynamic response to passive hind-limb cycling are necessary.

An interesting phenomenon to consider in explaining hypertrophy of TRPV1-expressing DRG neurons after SCI is aberrant sprouting of postganglionic sympathetic
axons into lumbar DRGs after T3 SCI (LM Ramer unpublished, see introduction). If there is enhanced Artemin expression within the DRG itself by DRG arteriolar VSMCs, this could be acting as an attractive guidance cue to sprouting sympathetic axons, as Artemin guides sympathetic axons along blood vessels in development (Honma et al., 2002). Taken together, free Artemin could also be acting on TRPV1 soma in the DRG, leading to hypertrophy. To examine this, it will be important to determine the state of sympathetic sprouting after SCI with a passive hind-limb cycling intervention.

3.3.5 Passive hind-limb cycling, TRPV1 and SCI-related pain

Based on the response of TRPV1-expressing neurons to passively cycling the hind limbs, and because the TRPV1 channel integrates pain stimuli of several types (Tominaga et al., 1998), there is reason to believe that pain mechanisms behind SCI may be affected by the exercise intervention.

Two main components of chronic pain, central sensitization and peripheral sensitization, are modulated by neurotrauma. Central sensitization is a pain mechanism involving increased sensitivity and hyperexcitability of spinal cord dorsal horn neurons to sensory stimulation (Woolf, 1983; Woolf, 1993). Just as central sensitization that can be achieved through increased peripheral C input such as experimental peripheral nerve injury and capsaicin injection (Ren and Dubner, 1999; Willis, 2001; Ren and Dubner, 2010), enhanced dorsal horn excitability has been demonstrated following SCI (Hains et al., 2003a; Hains et al., 2003b; Hains et al., 2003c; Crown et al., 2012). Another component of chronic pain after SCI is peripheral sensitization, a mechanism by which peripheral endings of afferent fibres can become more sensitive to noxious stimulation (Du et al., 2001; Du et
Implicit to tissue injury is high proton concentrations resulting from ischemia, inflammation and infection (Uchida and Murao, 1975; Stevens et al., 1991; Lindahl, 1962; Steen et al., 1995a; Bevan and Geppetti, 1994; Steen et al., 1995b). Higher proton concentrations can sensitize the affected area to noxious stimuli, creating a lower pain threshold. This mechanism is backed by findings demonstrating that the activation rate of TRPV1 capsaicin-mediated currents is increased and the deactivation rate of capsaicin-mediated currents is decreased in single afferent nociceptive neurons following acid application coupled to capsaicin (Neelands et al., 2005).

Passively moving the hind-limbs may change the chemical environment of nociceptive afferents, somehow altering their excitability. As there are several reports of the inflammation-mitigating effects of exercise in humans (Vieira et al., 2007; Tracey, 2009; Teixeira de Lemos et al., 2012), I propose that passive hind-limb cycling may attenuate the inflammatory response to SCI in tissues outside of the spinal cord, including within the DRG and target tissues of TRPV1-expressing nociceptors, thereby reducing the production of H⁺ in tissues and decreasing potentiation of TRPV1-mediated currents. A histological assessment of the inflammatory state of target tissue is warranted, as well as further electrophysiological testing of the response of TRPV1-expressing neurons to the relevant stimuli, such as capsaicin, H⁺ and noxious heat after SCI and SCI-Ex. This would allow for the activity profile and sensory contribution of this neuronal subset to be determined within the context of the exercise intervention.
3.3.6 Spontaneous activity of primary nociceptive neurons after SCI

Spontaneous activity of neurons involves action potential firing when external stimulation is absent (Linley et al., 2010). Spontaneous activity of small, nociceptive DRG neurons has been described after such sensory disruptions as tissue inflammation (Dang et al., 2005; Xiao and Bennett, 2007; Weng et al., 2012), peripheral neuropathy (Kajander and Bennett, 1992; Study and Kral, 1996) and cancer chemotherapy (Xiao and Bennett, 2008). Recent findings indicate that a thoracic contusion SCI (T10) also leads to chronic spontaneous activity of primary nociceptive (TRPV1+ and IB4+) neurons, as measured in vitro in dissociated neuronal DRG cultures and in vivo by recordings from dorsal root axons (Bedi et al., 2010). Lumbar DRG neurons below the injury site are much more spontaneously active after T10 contusion SCI than cervical DRG neurons above the injury level (Bedi et al., 2010), and dissociated nociceptive DRG neurons are spontaneously active both acutely (three days after SCI) and chronically (1-8 months after SCI) (Bedi et al., 2010). Behavioural correlates of spontaneously active nociceptors include thermal hypersensitivity (significantly decreased paw withdrawal response latency to noxious heat) and mechanical hypersensitivity (decreased force threshold for paw withdrawal after von Frey filament application) (Bedi et al., 2010).

Spontaneous activity of TRPV1-expressing nociceptive neurons could play a significant role in the pathology of SCI chronic neuropathic pain. Based on the somal size changes of TRPV1-expressing neurons, further studies to determine whether passive hind-limb cycling can modulate spontaneous activity of nociceptors are necessary.
3.3.7 Gate control theory and cycling

If passive exercise does indeed reduce chronic pain after SCI, there are cord mechanisms which could account for this beyond altering nociceptive afferents themselves. Wall and Melzack first published their gate control theory of pain in 1965, outlining a model that accounts for different modalities of sensory input to the spinal cord and their summative effects leading to the experience of pain (Melzack and Wall, 1965). Their new model suggested that the spinal cord integrates afferent input before it is relayed to brain centres for processing. If input from large diameter proprioceptive/mechanoreceptive afferents is able to effectively silence input from small diameter nociceptive afferents at the level of the cord, they argued, the resulting pain experience would be less than if only nociceptive/small diameter input is conveyed to the spinal cord (Melzack and Wall, 1965). Passive cycling may provide large-diameter input needed to dampen small-diameter nociceptive input; the mechanism by which this could occur is termed primary afferent depolarization (PAD). PAD is thought to act through axoaxonic synapses of GABA-releasing terminals on primary afferent endings in the spinal cord, ultimately resulting in less neurotransmitter release at primary afferent nerve endings. There is uncontested evidence for PAD of large fibres by the plethora of other afferents including those from deep tissue, skeletal muscle and skin, yet PAD of small-diameter afferents by other afferents is contested (Rudomin and Schmidt, 1999). However, the possibility of PAD of nociceptive afferents occurring in an animal with T3 complete transection SCI is intriguing.
Perspective and concluding remarks

Much of the enthusiasm surrounding exercise and the nervous system has stemmed from studies demonstrating a neurogenic effect of exercise. Exercise studies in animals have linked increased activity levels to enhanced performance on cognitive tests such as the radial arm maze (Schweitzer et al., 2006) and Morris water maze (van Praag et al., 2005), while human studies have extensively demonstrated the antidepressant effects of exercise (Babyak et al., 2000; Blumenthal et al., 2007; Mather et al., 2002; Singh et al., 2001). These and other early reports of higher cognitive performance in active individuals versus their inactive peers (Young, 1979) has led to further investigations of neural mechanism. These effects are thought to primarily stem from the generation of new hippocampal neurons (van Praag et al., 1999; van Praag et al., 2005; van Praag, 2009), a phenomenon that in the past (before the early 1960s (see Altman, 1962)) was dismissed as impossible as it was believed that new CNS neurons are only generated in development. Mechanistically, increased mRNA and protein expression levels of BDNF occur after exercise in the hippocampus (Oliff et al., 1998; Berchtold et al., 2001; Berchtold et al., 2002; Cotman and Berchtold, 2002), which is thought to drive exercise-induced adult hippocampal neurogenesis (Cotman and Berchtold, 2002).

There are documented changes to spinal cord neurons after exercise as well. Adaptive (and maladaptive) changes to spinal cord circuitry have been demonstrated in SCI, and indeed the spinal cord is adaptive and “smart” even below an SCI lesion site. In one seminal case study in a 23 year old man with an injury spanning C7 to T1 spinal segments, epidural stimulation of L1-S1 spinal segments by an implanted electrode array was paired with extensive physiotherapy (Harkema et al., 2011). The patient was
locomotor-trained for 2 years before implantation of the electrode array, which included body-weight supported treadmill training and stand training. After 7 months of physical training combined with electrical stimulation, the man gained voluntary supraspinal control of leg movements, but only when the stimulator was on (Harkema et al., 2011). This case study lends credence to the notion that spinal cord locomotor circuits below the lesion site are plastic and trainable, and also the idea that combining therapies (e.g., physiotherapy and electrical stimulation) in appropriate order and magnitude (Oudega et al., 2012) will likely be a desired approach for reinstating function after SCI (Oudega et al., 2012).

Exercise can also induce changes in neuronal sprouting and markers of regeneration. In the CA3 region of the hippocampus, mossy fibre sprouting occurs after an exercise regimen, an effect that may be attributed to enhanced long term memory outcomes (Toscano-Silva et al., 2010). In the peripheral nervous system, treadmill interval training in a mouse model boosts axon regeneration and sprouting (Sabatier et al., 2008). In the realm of SCI, however, the potential benefits of sprouting in the cord after acute exercise training are controversial. One study has claimed that while there is increased sprouting of sensory axons within the spinal cord after thoracic SCI and exercise, these are nociceptive C fibres that contribute largely to allodynia, or pain caused by a stimulus that is not usually painful (Endo et al., 2009). Another study has shown convincing behavioural recovery in mice with low thoracic hemisection after a bout of treadmill training, including significant improvements in climbing, grid walking and an open field locomotor test, which was coupled to enhanced sprouting of axons close to the lesion site (Goldshmit et al., 2008). Exercise alone has never demonstrated regeneration of CNS axons through an SCI injury site, the ultimate goal of SCI research. However, exercise clearly modulates plasticity in
the CNS above and below the injury site, and its role in CNS rehabilitation should continue to be investigated.

As an addition to the body of knowledge surrounding the effects of exercise on the nervous system, my findings support the hypothesis that changes to sensory neurons outside the CNS also occur following passive exercise. These changes may impart beneficial effects to autonomic/cardiovascular function in a pathological state (SCI). Based on my findings describing enhanced cardiovascular function with AD severity reduced by approximately 50% after a post-SCI passive cycling intervention, a therapy involving passively moving the hind-limbs could be a cost-effective, non-invasive approach for reducing the severity of AD in humans. These experiments call for further exploration of the sensory consequences and benefits of post-SCI passive exercise rehabilitation.
References


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Appendix

1.1 Recursive translation overview

To assess somal size changes amongst uninjured, SCI and SCI-Ex groups, the technique known as recursive translation was employed (Rose and Rohrlich, 1988). This analysis does not require localization of subcellular structures, but rather an algorithm is used to reconstruct cell populations from size histograms of cell profiles (Rose and Rohrlich, 1988). When compared to other measurement techniques, the algorithm is relatively robust as it accounts for small somal fragments which are often missed in image analysis (Rose and Rohrlich, 1988). Cell profiles are first grouped into radius profile histograms from raw perimeter measurements. The number of bins and bin sizes are selected by the experimenter; for my experiments the bin range deemed most appropriate was 0-100 μm with a bin size of 5 μm. The algorithm is then applied (see Appendix 1.2) which generates cell numbers within these chosen bins: a certain number of cells will fall into each bin, with the resulting histograms generated as proportional frequency histograms (number of cells in each bin / total number of cells). The Kolmogorov-Smirnov test, which generates a “D” statistic based on the greatest vertical distance between two cumulative frequency plots of the size-frequency histograms, is used to make comparisons between the sizes of two measured groups of cells (Rose and Rohrlich, 1988; Ramer et al., 2001).

The technique has shown to be robust even in the face of irregularly shaped somata. Rose and Rohrlich (1988) used Long Island potatoes as models of irregularly shaped neuronal somata to determine whether they could accurately count and group potatoes of different sizes using recursive translation - when compared to other types of potatoes, the Long Island variety deviate more from a standard ovoid form. Potatoes were sectioned and
analyzed using profile histograms, the recursive translation algorithm was applied, and the result-
ing frequency distribution was compared with actual potato number. Only at very small and very large bin widths did the reconstructed potato number differ from their actual sizes by more than 5% (Rose and Rohrlich, 1988).

Recursive translation analysis was employed because it is a robust measure of sensory neuron size, and is capable of detecting somal size shifts in specific cell populations.
The Sigmaplot transform for recursive translation analysis to assess somal size changes

The algorithm adapted from Rose and Rohrlich (1988):

\[
\begin{align*}
\text{col}(6,20,20) &= \text{col}(4,20,20)/(1 + (2*\sqrt{(\text{col}(1,1,1) * \text{col}(2,20,20) - ((\text{col}(1,1,1)^2)/4))}/\text{col}(3,1,1))} \\
\text{col}(5) &= \text{col}(4) - 2*\text{col}(6,20,20) * ((\text{sqrt}((\text{col}(2,20,20)^2) - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\text{sqrt}((\text{col}(2,20,20)^2) - (\text{col}(2) + \text{col}(1,1,1)/2)^2)))/\text{col}(3,1,1)) \\
\text{col}(6,19,19) &= \text{col}(5,19,19)/(1 + (2*\sqrt{(\text{col}(1,1,1) * \text{col}(2,19,19) - ((\text{col}(1,1,1)^2)/4))}/\text{col}(3,1,1))} \\
\text{col}(6) &= (\text{col}(6) + \text{abs}(\text{col}(6)))/2 \\
\text{col}(5) &= \text{col}(5) - 2*\text{col}(6,19,19) * ((\text{sqrt}((\text{col}(2,19,19)^2) - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\text{sqrt}((\text{col}(2,19,19)^2) - (\text{col}(2) + \text{col}(1,1,1)/2)^2)))/\text{col}(3,1,1)) \\
\text{col}(6,18,18) &= \text{col}(5,18,18)/(1 + (2*\sqrt{(\text{col}(1,1,1) * \text{col}(2,18,18) - ((\text{col}(1,1,1)^2)/4))}/\text{col}(3,1,1))} \\
\text{col}(6) &= (\text{col}(6) + \text{abs}(\text{col}(6)))/2 \\
\text{col}(5) &= \text{col}(5) - 2*\text{col}(6,18,18) * ((\text{sqrt}((\text{col}(2,18,18)^2) - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\text{sqrt}((\text{col}(2,18,18)^2) - (\text{col}(2) + \text{col}(1,1,1)/2)^2)))/\text{col}(3,1,1)) \\
\text{col}(6,17,17) &= \text{col}(5,17,17)/(1 + (2*\sqrt{(\text{col}(1,1,1) * \text{col}(2,17,17) - ((\text{col}(1,1,1)^2)/4))}/\text{col}(3,1,1))} \\
\text{col}(6) &= (\text{col}(6) + \text{abs}(\text{col}(6)))/2 \\
\text{col}(5) &= \text{col}(5) - 2*\text{col}(6,17,17) * ((\text{sqrt}((\text{col}(2,17,17)^2) - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\text{sqrt}((\text{col}(2,17,17)^2) - (\text{col}(2) + \text{col}(1,1,1)/2)^2)))/\text{col}(3,1,1)) \\
\text{col}(6,16,16) &= \text{col}(5,16,16)/(1 + (2*\sqrt{(\text{col}(1,1,1) * \text{col}(2,16,16) - ((\text{col}(1,1,1)^2)/4))}/\text{col}(3,1,1))} \\
\text{col}(6) &= (\text{col}(6) + \text{abs}(\text{col}(6)))/2 \\
\text{col}(5) &= \text{col}(5) - 2*\text{col}(6,16,16) * ((\text{sqrt}((\text{col}(2,16,16)^2) - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\text{sqrt}((\text{col}(2,16,16)^2) - (\text{col}(2) + \text{col}(1,1,1)/2)^2)))/\text{col}(3,1,1)) \\
\text{col}(6,15,15) &= \text{col}(5,15,15)/(1 + (2*\sqrt{(\text{col}(1,1,1) * \text{col}(2,15,15) - ((\text{col}(1,1,1)^2)/4))}/\text{col}(3,1,1))} \\
\text{col}(6) &= (\text{col}(6) + \text{abs}(\text{col}(6)))/2 \\
\text{col}(5) &= \text{col}(5) - 2*\text{col}(6,15,15) * ((\text{sqrt}((\text{col}(2,15,15)^2) - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\text{sqrt}((\text{col}(2,15,15)^2) - (\text{col}(2) + \text{col}(1,1,1)/2)^2)))/\text{col}(3,1,1)) \\
\text{col}(6,14,14) &= \text{col}(5,14,14)/(1 + (2*\sqrt{(\text{col}(1,1,1) * \text{col}(2,14,14) - ((\text{col}(1,1,1)^2)/4))}/\text{col}(3,1,1))} \\
\text{col}(6) &= (\text{col}(6) + \text{abs}(\text{col}(6)))/2
\end{align*}
\]
\[
\text{col}(5) = \text{col}(5) - 2 \times \text{col}(6,14,14) \times ((\sqrt{(\text{col}(2,14,14) \times 2)^2} - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\sqrt{(\text{col}(2,14,14)^2} - (\text{col}(2) + \text{col}(1,1,1)/2)^2)) / \text{col}(3,1,1)
\]
\[
\text{col}(6,13,13) = \text{col}(5,13,13)/(1+((2^2* \sqrt{(\text{col}(1,1,1) \times \text{col}(2,13,13)) - ((\text{col}(1,1,1)/2))})) / \text{col}(3,1,1))
\]
\[
\text{col}(6) = (\text{col}(6)+\text{abs}(\text{col}(6)))/2
\]
\[
\text{col}(5) = \text{col}(5) - 2 \times \text{col}(6,13,13) \times ((\sqrt{(\text{col}(2,13,13) \times 2)^2} - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\sqrt{(\text{col}(2,13,13)^2} - (\text{col}(2) + \text{col}(1,1,1)/2)^2)) / \text{col}(3,1,1)
\]
\[
\text{col}(6,12,12) = \text{col}(5,12,12)/(1+((2^2* \sqrt{(\text{col}(1,1,1) \times \text{col}(2,12,12)) - ((\text{col}(1,1,1)/2))})) / \text{col}(3,1,1))
\]
\[
\text{col}(6) = (\text{col}(6)+\text{abs}(\text{col}(6)))/2
\]
\[
\text{col}(5) = \text{col}(5) - 2 \times \text{col}(6,12,12) \times ((\sqrt{(\text{col}(2,12,12) \times 2)^2} - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\sqrt{(\text{col}(2,12,12)^2} - (\text{col}(2) + \text{col}(1,1,1)/2)^2)) / \text{col}(3,1,1)
\]
\[
\text{col}(6,11,11) = \text{col}(5,11,11)/(1+((2^2* \sqrt{(\text{col}(1,1,1) \times \text{col}(2,11,11)) - ((\text{col}(1,1,1)/2))})) / \text{col}(3,1,1))
\]
\[
\text{col}(6) = (\text{col}(6)+\text{abs}(\text{col}(6)))/2
\]
\[
\text{col}(5) = \text{col}(5) - 2 \times \text{col}(6,11,11) \times ((\sqrt{(\text{col}(2,11,11) \times 2)^2} - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\sqrt{(\text{col}(2,11,11)^2} - (\text{col}(2) + \text{col}(1,1,1)/2)^2)) / \text{col}(3,1,1)
\]
\[
\text{col}(6,10,10) = \text{col}(5,10,10)/(1+((2^2* \sqrt{(\text{col}(1,1,1) \times \text{col}(2,10,10)) - ((\text{col}(1,1,1)/2))})) / \text{col}(3,1,1))
\]
\[
\text{col}(6) = (\text{col}(6)+\text{abs}(\text{col}(6)))/2
\]
\[
\text{col}(5) = \text{col}(5) - 2 \times \text{col}(6,10,10) \times ((\sqrt{(\text{col}(2,10,10) \times 2)^2} - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\sqrt{(\text{col}(2,10,10)^2} - (\text{col}(2) + \text{col}(1,1,1)/2)^2)) / \text{col}(3,1,1)
\]
\[
\text{col}(6,9,9) = \text{col}(5,9,9)/(1+((2^2* \sqrt{(\text{col}(1,1,1) \times \text{col}(2,9,9)) - ((\text{col}(1,1,1)/2))})) / \text{col}(3,1,1))
\]
\[
\text{col}(6) = (\text{col}(6)+\text{abs}(\text{col}(6)))/2
\]
\[
\text{col}(5) = \text{col}(5) - 2 \times \text{col}(6,9,9) \times ((\sqrt{(\text{col}(2,9,9) \times 2)^2} - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\sqrt{(\text{col}(2,9,9)^2} - (\text{col}(2) + \text{col}(1,1,1)/2)^2)) / \text{col}(3,1,1)
\]
\[
\text{col}(6,8,8) = \text{col}(5,8,8)/(1+((2^2* \sqrt{(\text{col}(1,1,1) \times \text{col}(2,8,8)) - ((\text{col}(1,1,1)/2))})) / \text{col}(3,1,1))
\]
\[
\text{col}(6) = (\text{col}(6)+\text{abs}(\text{col}(6)))/2
\]
\[
\text{col}(5) = \text{col}(5) - 2 \times \text{col}(6,8,8) \times ((\sqrt{(\text{col}(2,8,8) \times 2)^2} - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\sqrt{(\text{col}(2,8,8)^2} - (\text{col}(2) + \text{col}(1,1,1)/2)^2)) / \text{col}(3,1,1)
\]
\[
\text{col}(6,7,7) = \text{col}(5,7,7)/(1+((2^2* \sqrt{(\text{col}(1,1,1) \times \text{col}(2,7,7)) - ((\text{col}(1,1,1)/2))})) / \text{col}(3,1,1))
\]
\[
\text{col}(6) = (\text{col}(6)+\text{abs}(\text{col}(6)))/2
\]
\[
\text{col}(5) = \text{col}(5) - 2 \times \text{col}(6,7,7) \times ((\sqrt{(\text{col}(2,7,7) \times 2)^2} - (\text{col}(2) - \text{col}(1,1,1)/2)^2) - (\sqrt{(\text{col}(2,7,7)^2} - (\text{col}(2) + \text{col}(1,1,1)/2)^2)) / \text{col}(3,1,1)
\]
\[
\text{col}(6,6,6) = \text{col}(5,6,6)/(1+((2^2* \sqrt{(\text{col}(1,1,1) \times \text{col}(2,6,6)) - ((\text{col}(1,1,1)/2))})) / \text{col}(3,1,1))
\]
\[
\text{col}(6) = (\text{col}(6)+\text{abs}(\text{col}(6)))/2
\]
col(5) = col(5) - 2 * col(6, 6) * ((sqrt((col(2, 6, 6)**2) - (col(2) - 
(col(1, 1, 1)/2)**2)) - (sqrt((col(2, 6, 6)**2) - 
(col(2)+col(1, 1, 1)/2)**2)))/col(3, 1, 1)

col(6, 5, 5) = col(5, 5, 5)/(1+((2*(sqrt((col(1, 1, 1)*col(2, 5, 5)) - 
(((col(1, 1, 1))**2)/4))))/col(3, 1, 1))

col(6) = (col(6) + abs(col(6)))/2

col(5) = col(5) - 2 * col(6, 5, 5) * ((sqrt((col(2, 5, 5)**2) - (col(2) - 
(col(1, 1, 1)/2)**2)) - (sqrt((col(2, 5, 5)**2) - 
(col(2)+col(1, 1, 1)/2)**2)))/col(3, 1, 1)

col(6, 4, 4) = col(5, 4, 4)/(1+((2*(sqrt((col(1, 1, 1)*col(2, 4, 4)) - 
(((col(1, 1, 1))**2)/4))))/col(3, 1, 1))

col(6) = (col(6) + abs(col(6)))/2

col(5) = col(5) - 2 * col(6, 4, 4) * ((sqrt((col(2, 4, 4)**2) - (col(2) - 
(col(1, 1, 1)/2)**2)) - (sqrt((col(2, 4, 4)**2) - 
(col(2)+col(1, 1, 1)/2)**2)))/col(3, 1, 1)

col(6, 3, 3) = col(5, 3, 3)/(1+((2*(sqrt((col(1, 1, 1)*col(2, 3, 3)) - 
(((col(1, 1, 1))**2)/4))))/col(3, 1, 1))

col(6) = (col(6) + abs(col(6)))/2

col(5) = col(5) - 2 * col(6, 3, 3) * ((sqrt((col(2, 3, 3)**2) - (col(2) - 
(col(1, 1, 1)/2)**2)) - (sqrt((col(2, 3, 3)**2) - 
(col(2)+col(1, 1, 1)/2)**2)))/col(3, 1, 1)

col(6, 2, 2) = col(5, 2, 2)/(1+((2*(sqrt((col(1, 1, 1)*col(2, 2, 2)) - 
(((col(1, 1, 1))**2)/4))))/col(3, 1, 1))

col(6) = (col(6) + abs(col(6)))/2

col(5) = col(5) - 2 * col(6, 2, 2) * ((sqrt((col(2, 2, 2)**2) - (col(2) - 
(col(1, 1, 1)/2)**2)) - (sqrt((col(2, 2, 2)**2) - 
(col(2)+col(1, 1, 1)/2)**2)))/col(3, 1, 1)

col(6, 1, 1) = col(5, 1, 1)/(1+((2*(sqrt((col(1, 1, 1)*col(2, 1, 1)) - 
(((col(1, 1, 1))**2)/4))))/col(3, 1, 1))

col(6) = (col(6) + abs(col(6)))/2