"Development and Characterization of an Acute Impact-Compression Lumbar Spinal Cord Injury Model in the Rat"

by

"Gray Moonen"

A thesis submitted in conformity with the requirements for the degree of "Master of Science"

"Institute of Medical Science"
"University of Toronto"

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« Master of Science »

« Institute of Medical Science »
University of Toronto

« 2014 »

Abstract

« Traumatic injury to the lumbar spinal cord results in complex nervous tissue damage causing severe neurobehavioural deficits and significant personal/social adversity. Despite this, there are few clinically relevant models of lumbar spinal cord injury (SCI), even though lumbar cord injuries are common in humans. The following work characterizes a lumbar SCI model in the rat. The effects of moderate (20g) to severe (26g, 35g, and 56g) clip-compression injuries at the lumbar spinal cord level L1-L2 (vertebral level T11-T12) were assessed using a multitude of neurobehavioural, neuroanatomical and electrophysiological outcome measures. Analysis of the lesional tissue demonstrated significant tissue alterations after injury and electrophysiological outcomes confirmed these deficits. Neurobehavioural testing revealed severe deficits in hindlimb locomotion and highly pathological alterations in sensory outcomes. In summary, we have generated a unique lumbar SCI model that should be useful for a variety of pre-clinical translational oriented therapies. »
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Throughout the completion of this graduate degree, I have matured in ways I did not think were possible in such a short timeframe. The level of dedication, perseverance and adaptability required to complete the work for this Masters degree have made me grow as an individual and this journey has been the most challenging; yet rewarding experience of my life. I am deeply grateful to my supervisor, Dr. Charles Tator. It was an honor to have someone that I admire so greatly accept me into his lab and believe strongly in the work that I was capable of doing. This faith has been a guiding force that has stuck with me and driven me to work hard and develop meaningful results in this project. His experience and expertise are incomparable, only matched by his patience and ability to motivate effectively.

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Last but certainly not least I would like to thank my parents and girlfriend whose support and patience have allowed me to follow my dreams and pursue my goals.
Contributions

My supervisor Dr. Charles Tator and myself designed this Masters project. Dr. Michael Fehlings and Dr. Andrea Mothe gave their expert advice in properly designing experiments.

I performed/completed:

- All of the surgeries for all experiments
- All the histological sectioning
- All immunohistochemical stains
- Sensory and neurobehavioural testing
- Data acquisition and analysis

Rita van Bendegem and Linda Lee assisted in blinded neurobehavioural scoring.

Dr. Kajana Satkunendrarajah completed the electrophysiological recordings with my assistance.

Anna Badner completed the in vivo ultrasound recordings with my assistance.

Dr. Warren Foltz performed the MRI acquisitions with my assistance.

Deborah Scollard performed the CT acquisition.
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<th>Description</th>
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<tbody>
<tr>
<td>AREZ</td>
<td>Anterior Root Entry Zone</td>
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<tr>
<td>AIS</td>
<td>American Spinal Injury Association</td>
</tr>
<tr>
<td>BBB</td>
<td>Basso, Beattie, Bresnahan</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline Acetyltransferase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CPG</td>
<td>Central Pattern Generator</td>
</tr>
<tr>
<td>CST</td>
<td>Corticospinal Tract</td>
</tr>
<tr>
<td>DAPI</td>
<td>49, 6-diamidino-2-phenyl-indole</td>
</tr>
<tr>
<td>DREZ</td>
<td>Dorsal Root Entry Zone</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal Root Ganglion</td>
</tr>
<tr>
<td>ESCID</td>
<td>Ohio State University Impactor</td>
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<tr>
<td>H&amp;E</td>
<td>Haemotoxylin and Eosin</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>LFB</td>
<td>Luxol Fast Blue</td>
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<tr>
<td>MASCIS</td>
<td>Multicenter Animal Spinal Cord Injury Study</td>
</tr>
<tr>
<td>MEP</td>
<td>Motor Evoked Potential</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>NDS</td>
<td>Normal Donkey Serum</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>NYU</td>
<td>New York University</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Solution</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>RECA1</td>
<td>Rat Endothelial Cell Antigen 1</td>
</tr>
<tr>
<td>VHRUS</td>
<td>Very High Resolution Ultrasound</td>
</tr>
<tr>
<td>VFF</td>
<td>Von Frey Filaments</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal Cord Injury</td>
</tr>
<tr>
<td>SEP</td>
<td>Sensory Evoked Potential</td>
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</table>
1 Introduction

1.1 Rationale

Spinal cord injury (SCI) is a devastating affliction associated with not only motor and sensory deficits, but bowel and bladder dysfunction, emotional and social disturbances, neuropathic pain and cardiovascular complications. Human traumatic SCI typically occurs when the spinal cord is mechanically crushed, compressed, contused, lacerated, stretched or injured in any way. The resulting injury to the central nervous tissue (CNS) after major SCI results in irreversible damage to the ascending and descending pathways connecting and coordinating the brain and body. The highest incidence of SCI is in patients between the ages of 18 and 35 years, with total lifetime expenses in the millions of dollars (Sekhon & Fehlings, 2001; Farry & Baxter, 2010). Presently, approximately 1.2 million North Americans suffer from paralysis directly caused by damage to the spinal cord, with an annual incidence of 11,000 per year (Farry & Baxter, 2010). Outcomes have improved considerably in recent decades, largely due to advances made in acute care and the minimization of secondary complications (Fehlings et al, 2012). Despite these advances, critical gaps in knowledge remain in accurately modeling SCI in different regions of the spinal cord. Numerous studies have indicated that seeking a single therapy is a disadvantageous approach, since the etiology of the SCI as well as the region of the spinal cord that is injured will respond in varying degrees to different therapies (Tator, 1995; Onifer et al, 2007; Kim et al, 2011). For example, injuries to the cervical and lumbar enlargements will likely require a neuronal replacement strategy because the injured structures causing the local functional deficits are predominantly due
to damage to neuronal cell bodies (Abematsu et al, 2010; Kim et al, 2011; van Gorp et al, 2013). Conversely, repair of the thoracic spinal cord will likely require an axonal approach, since it is the disruption of ascending and descending tracts that are causing dysfunction (Bonner et al, 2010; Fujimoto et al, 2012; Kumagai et al, 2009; Lu et al, 2012; Parr et al, 2008). These considerations necessitate precise, clinically relevant models of injury for each spinal cord region (cervical, thoracic, lumbar and sacral), which will allow tailored therapeutic interventions. There is a need for clinically relevant lumbar cord models since 20-30% of SCIs occur in this region (Tator, 2006; Knop et al, 1999; Sixta et al, 2012), but only a small number of models have been described for this location (Magnuson et al, 1998, 2005; Collazos-Castro et al, 2005; Garcia-Alias et al, 2006). The following work will describe the characterization of an impact-compression lumbar spinal cord injury model in the rat to address this crucial gap in knowledge.

1.2 Anatomy of the Spinal Cord

In addition to providing a pathway from the brain to the body, the spinal cord also possesses neural circuitry that can control certain functions and patterns independently from the brain, such as the central pattern generators (CPG) (Cazalets et al, 1995; Antri et al, 2011). In mammals, the central nervous system is composed of the spinal cord and the brain. The spinal cord is positioned in the middle of the back and is encased within the vertebral canal and is protected by hard bony vertebrae (The Spinal Cord, 2008). The spinal cord consists of nervous tissue (neurons and glial cells) and is organized into a bundle of tracts that originates directly distal to the brainstem. In humans, the cord ends between the L1 and L2 lumbar vertebral level, whereas in the rat it terminates between
L3 and L4 lumbar vertebral level (Gelderd & Chopin, 1977). This disparity in neurological spinal level versus bony vertebral level is due to the fact that the vertebral column develops more quickly than the spinal cord embryologically (Altman & Bayer, 1984) (Figure 3). The expanded lumbar segments containing the cell bodies innervating the hind limbs which then tapers towards its termination is termed the conus medullaris. The spinal roots that continue distal to the conus medullaris into a bundle are called the cauda equina. The spinal cord is organized into five morphologically distinct regions, starting with the most rostral: cervical, thoracic, lumbar, sacral and coccygeal. These distinctions are established partly by morphology and partly by functional innervation of the skin and muscle groups. There are 31 total pairs of spinal nerves in the human (8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal) and 34 in the rat (8 cervical, 13 thoracic, 6 lumbar, 4 sacral and 3 coccygeal) (Ginsberg, 2011). Spinal segments are determined by the emergence of bundles of spinal rootlets (roughly 15 dorsal and ventral in the rat) that converge to form each spinal nerve, which exits from the spinal canal at the intervertebral foramen (Gelderd & Chopin, 1977).

Each spinal nerve is bilateral and consists of both a sensory (afferent) and motor (efferent) component. The ventral rootlets contain axons of motor neurons, whereas dorsal rootlets contain axons from sensory neurons. Schwann cells ensheathe axons in spinal nerves peripherally whereas oligodendrocytes ensheathe axons beyond the transition zone into the CNS, which can be observed microscopically. The neuronal body that gives rise to the sensory axons resides directly adjacent to the spinal cord in a group of sensory cell bodies termed the dorsal root ganglion (DRG, Figure 1). The DRG sends axons both centrally to the spinal cord into the dorsal root entry zone (DREZ, Figure 1) and peripherally to the corresponding peripheral sensory target. Due to the discrepancy
in length of the spinal cord and vertebral column, spinal nerve length increases caudally in the spinal cord (Figure 3). The ventral roots are comprised of thick efferent motor axons arising from alpha motor neurons in the anterior (ventral) horn of the spinal cord. In the lumbar spinal cord segments there are additional preganglionic motor fibers derived from the intermediolateral column exiting the anterior root exit zone (AREZ). In addition to motor fibers, there are a significant number of C-fibers, which code for sensory pain stimuli and can exacerbate neuropathic pain after either spinal cord injury or peripheral nerve injury (Bigbee et al, 2013).

The ventral and dorsal roots combine to form a unified spinal root that exits the spinal canal at the intervertebral foramen caudal to the originating spinal segment (for example the L1 spinal nerve exits the spinal canal caudal to the L1 vertebral segment). From the intervertebral foramen, the spinal root bifurcates into the dorsal and ventral rami. The dorsal ramus innervates the fascia, muscles, ligaments and skin of the dorsal part of the body. The ventral ramus innervates fascia, ligaments, muscles and skin of the ventrolateral area of the body consisting of the trunk, limbs and perineum. Collections of ventral rami in the cervical and lumbar spinal cord form groups of interconnecting nerves called plexi. In the human the ventral rami from C1-C4 form the cervical plexus, rami from C5-C8 form the brachial plexus and the lumbar plexus arises from T12-L4 spinal levels (The Spinal Cord, 2008). The lumbar plexus in the human and the rat is composed of the iliohypogastric (L1), inguinal (L1), genitofemoral (L1-L2), femoral (L2-L4) and obturator nerves (L2-L4) (The Spinal Cord, 2008). These nerves directly supply the large quadratus lumborum (T12-L4) and psoas (L1-L3) muscle groups (The Spinal Cord, 2008). Injuries to these nerves produce a peripheral nerve injury, which can result in muscle weakness, pain and numbness in the effected myotomes and dermatomes. In the
injury being produced in the following work, there is a significant peripheral nerve injury component in addition to a central nervous system injury.

The lumbar spinal cord consists of many important neuronal circuits required for locomotion, bladder control and sexual function. In the rat, there is considerable evidence of a central pattern generator for hindlimb locomotion located in the rostral lumbar spinal cord, particularly in L1 and L2 spinal cord segments (Cazalets et al, 1995; Kiehn, 2006). This circuitry consists of interneurons that produce the rhythmic activity between multiple groups of muscles for the appropriate flexor/extensor pattern of locomotion (Kiehn, 2006). In both bipeds and quadrupeds, the CPG is responsible for coordinated hindlimb locomotion and when it is destroyed, this function is lost. Anatomically, in the axial plane, the interneurons comprising the CPG are located in Lamina VII, VIII and X (Dai et al, 2005).

The lumbar spinal cord also possesses Onuf's nucleus. Onuf's nucleus is a small, distinct group of neurons involved in the maintenance of micturition, defecatory incontinence and muscular function during orgasm (Mannen, 2000). In rats, these neurons are located in the L5/L6 spinal cord segments longitudinally, and lamina IX in the ventral horn axially. In the rat, bladder continence is controlled by sympathetic pre-ganglionic neurons in L1 and L2 spinal cord segment, whereas parasympathetic preganglionic and somatic neurons are grouped in L5/L6 and S1 spinal cord segments (Mannen, 2000).

Within the vertebral canal, three protective meninges cover the spinal cord: the dura, arachnoid and pia mater. The dura mater is the outermost layer and is a thick, tough fibrous layer of cells that acts as a conduit of vascular supply between the brain and spinal cord, while also providing considerable protection to the spinal cord. The
arachnoid mater is a web-like collection of elastic and collagen fibers that act as a barrier for cerebrospinal fluid due to the presence of tight-junctions. The pia mater is the innermost layer and acts as another layer of support for the spinal cord.

Microscopically, the spinal cord can be divided into two distinct regions. A transverse (cross-sectional) slice of the spinal cord reveals the cytoarchitecture and allows observation of the distinction between grey and white matter. White matter is composed largely of motor and sensory axonal connections. The ‘white’ colour observed is due to myelin, which is composed of oligodendrocytes with high lipid content. In addition to oligodendrocytes, the white matter also contains microglia and astrocytes, which serve as support cells. Grey matter is composed primarily of neuronal cell bodies. Due to its appearance, the butterfly, or H-shaped inner region also contains neuropil, glial cells, dendrites and unmyelinated axons. The dorsally projecting arms of the “H” are the dorsal horns and they receive afferent sensory information from the periphery. The ventrally projecting arms are the ventral horns and contain many neuronal populations, of which one is the alpha motor neuron pool, which send motor signals to muscles in the periphery. Centrally in the spinal cord is the central canal, which is surrounded by ependymal cells. This canal is attached to the fourth ventricle in the brain and contains cerebrospinal fluid to nourish the spinal cord.

At the cellular level, the spinal cord is composed of neurons and glial cells (oligodendrocytes and astrocytes). Oligodendrocytes act to sheath or “myelinate” neurons in many fatty layers in order to insulate and therefore enhance conductivity and the speed of cell signaling. Neurons, which are the primary component of the central nervous system, process electrical signals and communicates with other networks of
neurons to coordinate complex tasks. Astrocytes act to maintain homeostasis in the spinal cord and are the cellular component of the blood-spinal cord barrier.

**Figure 1.** Cross-section of the spinal cord displaying the relevant bony anatomy, connective and nervous tissue in the human (Copyright Gray Moonen).
1.3 Traumatic Injury to the Spinal Cord

The vast majority of spinal cord injuries occur traumatically. Traumatic injuries to the spinal cord can occur as a result of a number of injury mechanisms such as contusion, compression and or laceration, with the majority occurring via high impact trauma to the spinal column resulting in a contusion-compression injury to the spinal cord (Rick Hansen Institute 2010; Christopher and Dana Reeve 2010). Depending on the degree of trauma and neurological level of injury, motor and sensory tracts can be completely disrupted resulting in no distal voluntary functional activity, or they can spare some functionality in the sensory or motor tracts (Curt et al, 2004 & 2008). Injuries to the spinal cord are categorized according to the guidelines described by the American Spinal Injury Impairment Scale (AIS), which is based on functional completeness of injury (American Spinal Injury Association, 1992). AIS-A injuries represent the most severe end of the injury spectrum with patients having no preserved motor or sensory function in the S4/S5 spinal cord segments after injury. AIS-B patients are considered motor complete, defined by no motor preservation but some sensory preservation in the S4/S5 spinal cord segments. AIS-C and AIS-D patients are considered motor incomplete, where some motor and sensory activity is preserved in the S4/S5 segments. AIS-E represents completely normal function in both the motor and sensory tracts. After the primary insult, tissue in the spinal cord undergoes a series of secondary injury mechanisms that lead to degeneration and apoptosis of spinal cord tissue exacerbating the initial injury. Producing an injury model that can produce graded injuries correlating to either AIS-A or AIS-B, C or D is extremely beneficial (Onifer et al, 2007; Navarro et al, 2012).
In the U.S., approximately 15,000 lumbar fractures occur every year with over 1/3 of these fractures including a neurological injury resulting in paralysis (Hu et al, 1996; Knop et al, 1999; Sixta et al, 2012). The vast majority of these injuries are to the T10-L1 vertebral level, which represents the transitional zone between the immobile thoracic spine and the flexible lumbar spine. The thoracic spinal cord is immobilized and stabilized by the rib cage, whereas at the thoracolumbar junction the T11 and T12 vertebral levels are stabilized by weaker free floating ribs (not connected to the sternum). Complete neurological injury only occurs in 5% of patients with a neurological component of thoracolumbar fracture, therefore the goal of our model was to produce an incomplete lumbar spinal cord injury as opposed to a complete AIS-A classification injury (Knop et al, 1999). It should be noted that there is a high degree of spontaneous recovery observed in rodents even after very severe spinal cord injury. Thus, a functionally complete injury can be difficult to achieve with the impact-compression injury, although is easily achievable with complete transection of the cord.

The initial trauma of thoracolumbar SCI can be classified into five different relevant forces: axial compression, flexion/distraction, hyperextension, rotation and shear (Heinzelmann & Wanner, 2008). Many of these forces can work together to cause mechanical injury to the spinal cord. Axial compression injuries can result from a very high axial load causing complete or incomplete fracture of the anterior component of the vertebrae, which then impact and compress the spinal cord. Flexion/distraction injuries occur from tension in the posterior aspect of the spinal column causing a horizontal fracture beginning in the spinous process and proceeding through the lamina, transverse process, pedicle and vertebral body. Hyperextension results when the upper portion of the trunk is pushed posteriorly and the anterior component of the spinal column is impacted, which has the opposite orientation to a flexion/distraction injury. Rotational
injuries combine flexion and extension injury mechanisms and shear injuries produce
disruption of the intervertebral ligaments and are often associated with traumatic SCI
(Heinzelmann & Wanner, 2008).

Subsequent to a primary mechanical injury by any of the mechanisms described in the
paragraph above, the pathophysiology of spinal cord injury occurs in a biphasic pattern,
beginning with the initial primary trauma followed by an exacerbating secondary injury
cascade. The initial high-velocity mechanical impact to the spinal column can cause
fracture and displacement of the vertebrae into the spinal cord, bone fragments
compressing the spinal cord tissue and swelling and hematoma, causing a deleterious rise
in pressure within the vertebral canal (Heinzelmann & Wanner, 2008). This displacement
causes physical injury to central nervous tissue resulting in necrosis at the injury
epicenter accompanied by significant vascular disruption (Tator & Koyanagi, 1997).
Subsequent to primary tissue necrosis, physical trauma results in an imbalance of ions
and neurotransmitters in the spinal cord milieu (Beattie et al, 2000). Cellular debris and
edema cause a heightened inflammatory response and an infiltration of many immune
cells, which perpetuate further release of pro-inflammatory cytokines (Krysko et al,
2008). The secondary injury cascade consists of the combination of vascular disruption,
loss of neurotransmitter and ion regulation and the release of pro-inflammatory cytokines
among other mechanisms (Oyinbo et al, 2012). These work in harmony to exacerbate the
primary injury and extend the extent of the primary injury. The onset of secondary injury
mechanisms begins almost immediately after the primary injury and can last several
months. Secondary mechanisms will not be discussed in detail but consist of the
following: glutamate influx, calcium influx, inflammation, demyelination, synthesis of
free radicals, apoptosis and glial scar formation (Dietz & Muller, 2004; Oyinbo et al,
2012). Each of these steps in the secondary injury cascade represents an opportunity for
therapeutic intervention. This secondary pathology can span millimeters in the rat spinal cord and centimeters in the human spinal cord both rostral and caudal to the injury epicenter (Ko et al, 2004; Kwon et al, 2004). White matter is more likely to be spared, whereas the centrally located grey matter is damaged more extensively after cystic cavitation. A summary of the time course of primary and secondary injury with their associated pathologies is described in Figure 2.
Figure 2. Primary and secondary injury pathology time course (copyright Howard Kim).
1.4 Animal Models of Spinal Cord Injury

1.4.1 Neurological Level of Injury

The degree of functional loss is correlated not only to injury severity but also to the neurological level of injury. Losses range from tetraplegia (lost function of arms and legs), paraplegia (lost function of legs) to loss of neural control of a particular organ, weakness in the limbs or neuropathic pain alone (Christopher and Dana Reeve, 2010).

Patients with high cervical injuries from C1-C4 experience the lowest levels of independence and often lose sensation and motor control of the hands, arms trunk and legs resulting in tetraplegia. Individuals with low cervical injuries from C5-C8 will lose specific functions in the arms and hands depending on the segment injured. Cervical injury models have been developed and described extensively in the literature as these injuries are the most common and represent the highest mortality and morbidity in the SCI population (Forgione et al, 2014).

Thoracic injuries from T2-T12 do not impact the arms or hands, which leave patients with far greater quality of life scores when compared to patients with cervical injuries. Their ability to walk depends on the AIS severity, and they have significant bladder, bowel and sexual dysfunction (Rick Hansen, 2010). Models in the thoracic cord are designed to investigate functional deficits arising from disruption in ascending and descending motor tracts. Grey matter loss in the thoracic spinal cord is of low functional significance, as one would lose only the corresponding segmental dermatome and myotome in the thorax. Clinical trials in SCI frequently begin with a Phase I safety trial in patients with AIS-A thoracic cord injury since it represents the lowest risk of inflicting
additional harm (Tator, 2006).

Patients with major lumbar SCI result in a complex mix of symptoms, primarily a loss of the voluntary control of the legs, hips, bladder, bowel and sexual dysfunction. In addition to the upper motor neuron SCI, many spinal nerves are also injured due to the obliquity of the spinal nerves, producing a conus medullaris injury. In contrast to thoracic injuries, it has been shown in previous work that the grey matter in the lumbar spinal cord has critical importance for locomotion (Magnuson et al, 1998 & 2005). In rats, grey matter damage to the T12-L2 spinal cord region results in marked locomotor deficits due to the presence of the interneurons of the central pattern generator. There is some evidence of these circuits in humans (Cazalets et al, 1995; Dimitrijevic & Gerasimenko, 1998; Dominici et al, 2011). In contrast, injuries at lower lumbar cord levels at L3/4/5 can results in dramatic recovery, even though many important neuronal circuits are presumably impacted whereas injuring higher at the T11-T13 spinal cord level results in a tract injury and not an injury that correlates well to lumbar SCI in humans (Magnuson et al, 2005).

Injury to the sacral spinal cord results in a different pattern of CNS injury impacting only the bladder and bowels. Patients with injuries to the sacral cord also experience significant PNS injury due to damage of the roots but tend to recover to a higher degree than cervical, thoracic or lumbar patients (Sixta et al, 2012).

1.4.2 Models of Spinal Cord Injury
The ultimate goal of attempting to mimic human SCI with an animal model is to accurately represent the pathology of clinical SCI in order to test and improve therapies and eventually improve functional outcomes for patients. Beyond the primary goal, there can be many incremental benefits to studying animal models, such as gaining a better understanding of the acute and chronic cellular and molecular consequences of SCI of varying severities to different spinal regions. Being able to properly model human SCI allows scientists to better tailor therapies according to the specific pathologies and functional deficits experienced by particular groups of patients (Courtine et al, 2007; Onifer, 2007). Animal models have proven invaluable for evaluating injury mechanisms and assessing whether or not therapies are efficacious and should be translated to human trials. Lower mammals are typically used such as mice and rats, however larger animal models have been developed in the minipig, cats and non-human primates (described briefly in Table 1) (Courtine et al, 2007; Lee et al, 2013; Rossignol et al, 1998). Rats appear to be the ideal animal model due to their similarity to humans in injury profile and pathology after SCI (Robins & Fehlings, 2008). They are also widely accessible, easy to handle and well described anatomically. Due to these benefits, rats were chosen for all the experiments described in this thesis. Rats have been used in the context of SCI since Allen experimented on dogs in 1911 with a weight-drop injury mechanism (Allen, 1911). Since that time, a large number of different experimental injury models have been developed, many of which are briefly described in Table 2. Literature exists on numerous injury models of traumatic SCI, each utilizing a different mechanism of trauma, such as weight-drop, contusion, transection, excitotoxicity and clip-compression among others (Onifer et al, 2007; Poon et al, 2007). Each model attempts to mimic either the primary trauma, or an aspect of the secondary injury cascade seen in humans.
**Table 1.** Commonly used animals in spinal cord injury research.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Method of Injury</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Rat    | Contusion, compression, NYU, Ohio State University Impactor, Infinite Horizons, chemical, laceration, transection | - Pathomechanism of injury is similar to humans  
- Most well-studied  
- Many reliable outcome measures  
- Develop typical cystic cavitation seen in human SCI  
- Readily available and accessible with reputable breeders | - High degree of spontaneous recovery  
- Significant Neuroanatomical differences  
- CST less functionally important  
- Difficult to manipulate surgically |
| Mouse  | Contusion, compression, NYU, Ohio State University Impactor, Infinite Horizons, chemical, laceration, transection | - Can manipulate genetically  
- Pathomechanism of injury is relatively similar to humans | - Large size and metabolic differences  
- Very high degree of spontaneous recovery  
- Significant Neuroanatomical differences |
| Porcine          | Contusion, laceration | - Spinal cord is closer in length to humans  
|                 |                       | - Closer metabolically to humans  
|                 |                       | - Important intermediary between rat and human  
|                 |                       | - Less spontaneous recovery  
| Non-human primate | Contusion, laceration | - Most relevant to man  
|                 |                       | - Can assess fine dexterity of hand movements making it sensitive to therapies in the cervical cord  
|                 |                       | - CST similar to humans in functional importance  
|                 |                       | - Bladder and sexual function more similar to humans  
|                 |                       | - Very expensive  
|                 |                       | - Inconsistent model  
|                 |                       | - Do not develop cystic cavitation  
|                 |                       | - Few species specific outcome measures  
|                 |                       | - Extremely expensive  
|                 |                       | - Ethical concerns  
|                 |                       | - Inconsistent model  
|                 |                       | - Lack of availability |
Table 2. Description of numerous injury models for spinal cord injury.

<table>
<thead>
<tr>
<th>Model</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Clip-Compression (Poon et al, 2007; Mothe et al, 2008, 2011; Parr et al, 2007) | Modified aneurysm clips are calibrated into ordered forces by weight i.e. 20g, 35g etc. The two blades of the clip are opened around the exposed spinal cord, and subsequently rapidly closed, therefore compressing the cord bilaterally and in the dorsal and ventral aspect simultaneously. | - Generates varying degrees of injury based on calibration of compression force and duration of compression  
- Bilateral injury  
- Simulates clinical condition of both ventral and dorsal compression  
- Low cost | - Possibility of injuring the spinal cord during placement of the blades of the clip  
- High degree of surgical skill required to place the clip  
- Only two parameters recorded: closing force and duration of compression  
- Variability due to clip orientation can arise |
| Weight-drop (NYU / MASCIS). (Gruner, 1992; Magnuson et al, 2005; | Graded weights are dropped through a guiding tube directly onto the dorsal surface of the exposed spinal cord from different heights. | - Reasonably mimics blunt force trauma observed in human injury  
- Capable of producing varying degrees of injury  
- Mimics many aspects of the secondary injury cascade | - Does not replicate extended compression  
- Does not replicate simultaneous dorsal and ventral compression  
- Variations in extent of injury due to multiple impacts due to “weight bounce” of rod after initial hit |
<p>| Ohio State University     | Similar to the NYU/MASCIS device. | - Reasonably mimics some aspects of blunt force | - Does not replicate simultaneous dorsal |</p>
<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Impact</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Impactor (ESCID). (Stokes et al, 1992; Pearse et al, 2005). | An impactor tip is pressed onto the dorsal surface of the spinal cord after laminectomy. Allows monitoring of kinematic impact of the tip. | trauma observed in human injury  
- Generates varying degrees of injury  
- Allows monitoring of kinematics (e.g. force, velocity, or tissue displacement) in order to eliminate outliers and stay consistent | and ventral compression  
- Not commercially available  
- Cannot vary the duration of compression for clinically relevant times |
| Infinite Horizons Impactor. (Scheff et al, 2003).    | Impactor tip displaces the dorsal surface of the exposed spinal cord with the assistance of a computer. Allows the user to set pre-defined displacement in order to grade the injury. | - Reasonably mimics blunt force trauma observed in human injury  
- Generates varying degrees of injury  
- Allows monitoring of kinematics (e.g. force, velocity, or tissue displacement) | - Does not replicate simultaneous dorsal and ventral compression  
- Cannot vary the duration of compression for clinically relevant times  
- Variability due to inconsistent clamping of the spinal column can arise |
| Chemical-mediated injury. (Magnuson et al, 1999). | Typically involves an excitotoxic injection directly into the spinal cord or onto the dorsal surface of the exposed spinal cord | - Allows modeling of specific part of secondary injury cascade  
- Can be white or grey matter specific | - Not observed in humans  
- Does not replicate the forces or mechanism of human traumatic injury |
<p>| Ischemic                                         | Aortic occlusion models                                                     | - Models ischemic part of | - Only one part of |</p>
<table>
<thead>
<tr>
<th>Injury Type</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury (Garcia-Alias et al, 2006)</td>
<td>Mimic rare operative complication where the aorta is occluded and results in oxygen deprivation of the lower body. Rose Bengal dye can also produce ischemic injury.</td>
<td>The secondary injury cascade</td>
<td>Injury cascade - Rarely observed in isolation</td>
</tr>
<tr>
<td>Hemisection (Cloud et al, 2012)</td>
<td>Dorsal or lateral aspect of the spinal cord is surgically severed with a blade.</td>
<td>Ideal model for mechanistic studies of axonal regeneration and “bridging the gap” studies - Allows comparison of the intact contralateral side of the spinal cord and body as a control - Requires considerable animal care</td>
<td>Very rare injury mechanism in humans - More significant loss of function than compression or contusion - Does not replicate the forces or mechanism of human traumatic injury - Does not replicate secondary injury cascade</td>
</tr>
<tr>
<td>Transection (Silver et al, 2004; Bradbury et al, 2012)</td>
<td>Spinal cord is completely severed surgically with a blade.</td>
<td>Ideal model for mechanistic studies of axonal regeneration and “bridging the gap” studies. Generates a consistent injury across various studies - Requires considerable animal care</td>
<td>Very rare in humans - Does not replicate the forces or mechanism of human traumatic injury - Does not replicate secondary injury cascade</td>
</tr>
<tr>
<td>Focal Myelotomy</td>
<td>The central region of the spinal cord is severed with a blade.</td>
<td>-- Ideal model for mechanistic studies of axonal regeneration and “bridging the gap” studies.</td>
<td>- Not typically observed in humans - Does not replicate the forces or mechanism of human traumatic injury</td>
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</tr>
<tr>
<td>Root Avulsion (Bigbee et al, 2007; Chew et al, 2013).</td>
<td>Cutting or pulling root from spinal cord either dorsally, ventrally or both dorsally and ventrally.</td>
<td>- Allows specific modeling of lumbar or brachial plexus injury - Allows modeling of neuropathic pain observed after traumatic SCI</td>
<td>- Very rare clinically - Does not replicate common human spinal cord injury</td>
</tr>
</tbody>
</table>

SCI models can be categorized into in vitro models and in vivo models. In-vitro models can be beneficial to study specific cellular responses to specific parts of the secondary injury cascade. In vitro studies allow for a reduction in complexity and possible confounding effects in the diverse in-vivo injury milieu. These models also allow for precise manipulation of specific cell types to investigate specific questions about cell-cell interactions or single cell responses (Krassioukov et al, 2002). Primary cultures of neurons, astrocytes or oligodendrocytes represent the least clinically relevant in-vitro models and are restricted to investigating cell-cell interactions. A slightly more clinically relevant in-vitro model is the use of organotypic slice culture models, which are also used commonly in traumatic brain injury investigations. One example of an in vitro model consists of very thin cross-sections of mice spinal cord that are cultured and exposed to a weight-drop injury (Krassioukov et al, 2002). Neurons, glial cells and
supportive tissue are all present in this culture, which relates closer to an in-vivo model than primary cultures. The benefit of this model is that it is significantly less expensive than in-vivo work, allows separate time-point analysis with the same spinal tissue and requires less surgical skill. The obvious limitation is its considerable difference from an in-vivo injury model and the fact that mechanical trauma to exposed tissue will not replicate the forces and mechanisms as an in-vivo injury.

Knife-cut injury models such as a full or partial transection, lateral hemisection, overhemisection and resection all aim to sever spinal cord axons in the spinal cord (Tator et al, 1995; Silver et al, 2004; Bradbury et al, 2011). These models are ideal for investigating axonal regeneration, sprouting or axonal transplant strategies since axons are required to “bridge the gap” created by the transection. These models can help examine the efficacy of axonal regeneration therapies: however these injuries are exceptionally rare in the clinical population and do not replicate the secondary injury cascade observed in humans.

Of the many injury models developed in the last 100 years, dynamic impact models such as the clip-compression and contusion devices have the most relevance and similarity to injuries seen clinically. The New York University impactor / Multicenter Animal Spinal Cord Injury Study (NYU / MASCIS) device is an extension of the model developed by Allen in 1911 (Allen, 1911; Tator et al, 1995; Anderson et al, 2009; Ferguson et al, 2013). Developed specifically for rats, this device impacts the exposed dorsal surface of the spinal cord after laminectomy. The spinal column is fixed and stabilized during the procedure to control the impact. Weights of varying mass are dropped down a tube onto the spinal cord from various heights to manipulate the force and subsequent severity of the injury. Histological and behavioural analyses indicate that this model produces an
injury severity that correlates well with the two variables of weight and height. One of the primary advantages of this model is the use of intraoperative monitoring of tissue displacement so that outliers can be readily removed from the experiment. The primary disadvantage of this model is the lack of a circumferential (simultaneous dorsal and ventral) compression on the spinal cord, which is the most common form of traumatic SCI in humans. Another drawback is the inability to alter the duration of contusion, since clinically the spinal cord is compressed for a longer duration than a transient hit. Despite many key benefits, this model does not mimic a key aspect of the clinical injury mechanism, which is problematic to overcome in the context of translational applicability.

Following the success of the NYU impactor, the Infinite Horizons impactor was developed and made commercially available. This device is similar in a number of ways to the NYU impactor described above. The key difference is the use of force, as opposed to tissue displacement (NYU impactor) as the variable for measuring impact. This device follows the same intraoperative approach as the NYU impactor, however a steel rod produces the injury as opposed to a weight dropped from a given height. The Infinite Horizons impactor shares the same drawbacks as the NYU impactor of not producing a circumferential injury and not being able to vary the duration of contusion.

Tator and Rivlin (1978) developed a clip-compression injury model that replicates both the bilateral and simultaneous dorsal and ventral mechanism of SCI, thus overcoming the strictly unilateral mechanism of the currently described contusive SCI models. This model addresses the issue of continued cord compression that occurs in combination with vertebral displacement and dislocation seen in traumatic SCI patients. In this model, a
modified aneurysm clip is calibrated to a specific closing force in accordance with the spring inside the clip allowing for a wide range of clip strengths and corresponding injury severities. The rat undergoes a laminectomy and the clip is applied ventrally and extradurally around the spinal cord and rapidly released to impact the spinal cord for 60 seconds. This injury produces both an immediate contusion due to the rapid closing of both clip blades and a variable duration of compressive traumatic injury. The primary advantage of this injury model is the fact that it simulates the simultaneous ventral/dorsal forces associated with most clinical SCI. Secondly, it is very consistent and reliable, produces a graded injury based on the clip strength and duration of compression which correlates very well with histological, behavioural and electrophysiological outcomes in many different labs. The clip is easy to maintain and inexpensive as well. Furthermore, for the lumbar spinal cord the clip-compression technique presents a major advantage. This method is ideal since the spinal cord and all of its contents are compressed extradurally, therefore intradural spinal roots are intentionally included in the compression, thus relating to the human clinical issue when an individual is injured in this region. The weight drop model is more applicable for the thoracic or cervical spinal cord where the spinal roots exit perpendicularly out of the arising spinal segment. In the lumbar spinal cord, the roots are far longer, traveling far greater distances and are oblique along the spinal canal. Due to this anatomy, the roots are included in a mechanical crush or fracture dislocation of the vertebral canal in human thoracolumbar junction injuries (Figure 3). Despite these benefits there are shortcomings associated with the clip-compression technique, chiefly the degree of surgical difficulty associated with the technique. This can be overcome by rigorous practice and teaching from a skilled surgeon.
As described above, the pathological events after SCI follow a biphasic pattern beginning with the primary mechanical insult followed by a series of secondary events termed the secondary injury cascade. The ideal model of SCI is one that accurately simulates this biphasic pattern of injury. A key aspect of the secondary injury cascade is vascular disruption caused by the initial mechanical impact, which exacerbates the primary injury and initiates a series of deleterious events. The clip impact-compression produces significant ischemia and haemorrhage due to its prolonged constriction of the cord, which unequivocally produces vascular disruption. The contusion devices described above (NYU and Infinite Horizons) mimic primarily the blunt mechanical force. Due to its accurate replication of the human clinical scenario and several key advantages when modeling lumbar SCI, the clip compression model was selected for the injury model in this thesis work.

1.4.3 Effective Injury Models

One model cannot accurately replicate all injury mechanisms simultaneously (compression, laceration, contusion), but the quality of an injury model can be determined by its replication of several key clinical features. This can be examined by: (1) how similar the model is to the clinical representation of human injury, (2) how similar the model is to the histopathology of human SCI, (3) how reproducible and consistent the injury is between individual animals, (4) the degree and timing of spontaneous motor/sensory recovery, (5) the ability to generate different degrees of functional outcome after different severities of injury (adapted from Navarro, 2012). The aneurysm clip impact-compression technique developed by Rivlin and Tator (1978)
fulfills all of those requirements making it a powerful model of human lumbar cord SCI and the injury technique used in this thesis.

1.4.4 Injury Model Limitations

Despite the popularity and benefits of using rodents in modeling SCI, there are several key interspecific differences in the anatomy between humans and rats that need to be noted. There are metabolic, size, gait, neurophysiological, anatomical and behavioural differences as well as many biochemical cascades that differ both qualitatively and quantitatively between species. Size differences have important translational implications especially for modeling therapies in the thoracic or cervical cord, where regenerating axons need to regenerate distances that are orders of magnitude farther in humans versus rodents. The lumbar spinal cord escapes this limitation to a degree since an axonal therapy is likely not the most efficacious strategy and neuronal transplant is the therapy likely to work.

A significant hurdle in rodent research is the high degree of spontaneous recovery observed in rodents, especially in rats. Even after very severe injuries where over 90% of axons are destroyed, rats can recover to scores of over 10 on the BBB locomotor scale in only 2-4 weeks, representing occasional plantar stepping. This degree of spontaneous locomotor recovery is poorly understood and creates issues with interpreting the efficacy of therapies (Basso et al, 1996; Ek et al, 2010; Fischer & Peduzzi, 2007).

There are also significantly different anatomical locations of the white matter tracts in the spinal cord; specifically the corticospinal tract in the human is located laterally in the
lateral column, whereas in the rat it is located in the ventral part of the dorsal funiculus. In the rat, these projections connect primarily to dorsal horn neurons and pre-motor neurons in the ventral horn, whereas the human CST has more complex interactions within the spinal cord (Courtine et al., 2007).

More pronounced differences between humans and rodents are seen in hand function as humans have evolved exquisitely precise control of many muscles in the hand that produce very fine motor behaviours (Lemon et al., 2004; Courtine et al., 2007). These differences are less prominent when investigating hindlimb control in the lumbar spinal cord since there are fewer fine muscles in the hindlimbs of both species.

It is important to note these limitations when assessing the accuracy and efficacy of an injury model as well as translatable therapeutic outcomes.

### 1.5 Lumbar Spinal Cord Injury Models

As noted above, injuries to the thoracolumbar region of the spinal cord are significantly different than injuries to the thoracic or cervical region and result in distinct motor and sensory complications (Tator, 1983; Sixta et al., 2012). Patients with thoracolumbar injuries (T10-L1) typically suffer from paraplegia, intractable pain and a loss of bladder and bowel function (Sixta et al., 2012). Furthermore, they often lose the circuits of neurons that are essential for locomotion (Kiehn, 2006; Sixta et al., 2012; van Gorp et al., 2013). In addition to a central nervous system (CNS) injury, the spinal nerves are often involved due to their obliquity and positioning in the lumbar spinal cord, thereby producing a peripheral nervous system (PNS) injury as well (Figure 3). Thus, this injury
level represents a large clinical population and to our knowledge, there is currently no clinically relevant thoracolumbar SCI model that mimics both the central and peripheral injury simultaneously. It is important to note that there is some uncertainty surrounding the notion of “clinical relevance” with regards to spinal cord injury models. We are defining clinical relevance as the similarity of the circumferential and extended compression that occurs in patients after burst-fracture injury in the thoracolumbar junction.

**Table 3.** Description of current lumbar spinal cord injury models in animals.

<table>
<thead>
<tr>
<th>Injury Model</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Contusion (Magnuson et al, 2005) | Weight-drop technique with NYU impactor. 12.5g cm and 25g cm weights dropped from 25mm produced moderate and moderately-severe injuries at T13/L1, L2, L3/4 spinal cord segments. | - Mimics many of the aspects of the secondary injury cascade
- Able to create differing severities of injury
- Targeted different lumbar segments and compared functional and histological outcomes | - Does not replicate ventral/dorsal compression
- Did not include peripheral component
- Did not assess sensory outcomes
- Did not use imaging techniques to confirm operative level |
<p>| Chemical                   | Kainic acid injection into                                                 | - Selective injury of                                                      | - Not observed                                                               |</p>
<table>
<thead>
<tr>
<th>Injury Type</th>
<th>Details</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnuson et al, 1999</td>
<td>The spinal cord at L2 spinal cord segment grey matter, sparing most white matter</td>
<td>Clinically</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Inability to vary injury severity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Does not include peripheral component</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Does not replicate ventral/dorsal compression</td>
</tr>
<tr>
<td>Garcia-Alias et al, 2006</td>
<td>Photochemical ischemic injury with rose bengal performed topically at L2 spinal cord segment</td>
<td>Investigated multiple electrophysiological outcomes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Compared thoracic versus lumbar</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not observed clinically</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Inability to vary injury severity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Does not include peripheral component</td>
</tr>
<tr>
<td>van Gorp et al, 2013</td>
<td>35g compression with 2.9mm diameter rod on L3 spinal segment for 15 minutes</td>
<td>Produces cystic cavitation which is consistent with human injuries</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Investigated spasticity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No verification of model</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Unilateral compression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Small sample size in ‘survey’ study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Only mimicked ischemic injury, not traumatic primary injury</td>
</tr>
</tbody>
</table>
### Root avulsion

(Bigbee et al, 2007; Chew et al, 2013)

- Ventral and dorsal roots are torn from the spinal cord at T13 and L1 spinal segments
- Selective peripheral nerve injury
- Produced significant neuropathic pain
- Very rare clinically
- Typically observed in combination with spinal cord injury
- Does not include spinal cord component

### Contusion

(Collazos-Castro, 2005)

- Adapted weight-drop technique (from Allen). 10g weight dropped from 25mm on L2 spinal cord segment
- Mimics many of the aspects of the secondary injury cascade
- Only used very light injury – BBB > 15
- Variations in extent of injury

Magnuson *et al* (2005) investigated behavioural and histological outcomes after weight-drop contusion injury to various lumbar spinal cord segments in the rat, which was the first description of a comparison of traumatic SCI to the lumbar cord. The authors described moderately severe (12.5g-cm) versus severe injuries (25g-cm) with the NYU impactor at T13/L1 segments, L2 segment and L3/4 segments of the spinal cord. An analysis of spared white and grey matter determined that grey matter loss is critical in the lumbar spinal cord and a considerable amount of white matter can be spared but functional locomotor outcomes are still very dysfunctional.

They accomplished an important first step in describing the functional consequences after loss of both grey and white matter in the lumbar spinal cord. This work has
commendable scientific merit, but has the following limitations: a) imaging was not utilized to confirm the operative level, b) the weight-drop model does not replicate the dorsal/ventral compression and duration of human injury, c) the electrophysiological outcomes do not describe the injury or the functionality of the spared white matter and d) there was no sensory component to behavioural testing. These shortcomings are addressed in the injury model described in this thesis.

Magnuson et al (1999) also described a comparison of the NYU impactor versus an excitotoxic injury in the L2 and T9 spinal cord of adult rats using kainic acid, which selectively ablates neurons. They found that selective grey matter damage to the thoracic cord by a kainic acid injection resulted in very minor locomotor deficits as measured by the BBB scale whereas grey matter damage to the lumbar cord resulted in severe locomotor deficits. The authors posited that the neuronal circuitry in the lumbar cord is crucial to functional locomotion in comparison to a similar injury in the thoracic spinal cord. This study was stimulating because it led to a discussion about important interneurons in the lumbar spinal cord comprising the CPG. The kainic acid injury is not a clinically relevant injury model however and has similar drawbacks to the Magnuson (2005) work.

Garcia-Alias et al (2006) induced a similar chemical/ischemic injury with rose bengal at either L2 or T8 spinal cord segment in rats to compare the electrophysiological and behavioural profile after ischemic injury to these two regions. Confirming previous reports, injury to the lumbar spinal cord produced far more profound behavioural deficits when compared to an identical thoracic injury. BBB scores in the L2 injury were on average 10, in comparison to 18 with a T8 injury. The Hoffmann reflex ratio was enhanced in the lumbar injury, indicating higher degrees of spasticity. The study by
Garcia-Alias described several clinically relevant behavioural outcomes after lumbar SCI, however there are significant drawbacks to this study. The main shortcoming is the use of an ischemic injury as opposed to a more relevant model of trauma such as a contusion or compression. Further, the authors did not describe any sensory alterations, which are a principal component of any injury to the thoracolumbar junction in humans. The clear limitation in all chemical injury studies is the inability of a chemical injury to produce a comprehensive, clinically relevant SCI.

Van Gorp et al (2013) investigated a human neural stem cell transplant therapy in the lumbar spinal cord after rod-compression injury at L3 spinal cord segment. The injury was produced by a Teflon coated 35g static pressure with a diameter of 2.9mm. It is held on the dorsal surface on the spinal cord for a minimum of 15 minutes thereby creating an injury that replicates an ischemic pathology as opposed to an impact-compressive traumatic SCI. Despite the use of an ischemic injury, the histological and behavioural results correlated well with a traumatic injury such as severe locomotor deficits and below level hyper reflexia (spasticity). A benefit of this technique is the replication of the ischemic aspect of the secondary injury cascade. The drawback of this technique is that it has not been verified by a study describing the rod compression injury model. The authors cite a “pilot study” but do not offer data on the variability and specifics of the model. Also, a unilateral compression does not replicate the simultaneous dorsal/ventral compression observed in human injuries. This model likely only mimics the ischemic aspect of the secondary injury cascade and not the blunt trauma and associated side effects observed in the traumatic human injury.
Collazos-Castro et al (2005) compared kinetic measurements and retrograde motoneuron labeling after moderate lumbar cord (L2) and cervical (C7) enlargements after contusion injury with a modified weight-drop device. Their principle finding was that 30% of quadratus femoris motoneurons are destroyed after L2 contusion and related motoneuron loss to locomotor recovery. A disadvantage of this study is that the authors only investigated a weak injury where animals recovered to over 15 on the BBB scale, which correlates to only minor behavioural deficits. Another limitation is the use of this contusion model, which has been described above.

When designing the current injury model we considered all of the limitations of using a weight-drop, contusion, transection or chemical injury and decided that the only acceptable model would be to use the aneurysm clip impact-compression technique. The injury produced in this thesis is inflicted at the L1-L2 spinal cord segments because severe deficits can be produced in this region as opposed to lower lumbar spinal cord injuries where spontaneous recovery is dramatic (Magnuson et al, 1999, 2005). Application of the clip-compression technique results in inclusion of all of the adjacent roots in the injury. This is a benefit when modeling proximal conus medullaris syndrome, but can result in severe neuropathic pain if too many roots are included in the injury.

Therefore, the decision was made to injure the L1 spinal cord segment with a slight bias towards the L2 spinal cord segment in order to avoid centering the injury too close to the T13 spinal cord segment (Figure 3). The L1-L2 spinal cord is an ideal combination of a mixed injury producing a significant upper motor neuron SCI and lower motor neuron peripheral injury seen in humans with conus medullaris injuries. It also damages critical neuronal structures such as the CPG in the L1 and L2 spinal cord, which opens up many
opportunities for therapies attempting to remodel neuronal circuitries. The SCI we have characterized is an improvement over current models due to the clinically relevant injury the clip impact-compression produces, inclusion of spinal roots to produce a mixed injury and imaging and electrophysiological techniques to further describe the lumbar SCI.
Figure 3. Schematic diagram of the thoracolumbar rat spinal cord (Copyright Gray Moonen). The disparity between the vertebral levels and spinal cord segments is exemplified in this diagram. Lumbar spinal cord segments begin at the junction between the T11 and T12 vertebrae and end midway through L1 vertebral level. Also worth noting is that three pairs of intra-dural roots (T12, T13 and L1) are involved in the clip-compression injury we are producing. The lumbar enlargement is clearly indicated throughout the lumbar spinal cord segments (outlined in blue) and the location of the injury is marked accordingly at the L1 spinal cord segment.
1.6 Hypothesis and Objectives

As described above, patients with injuries to the thoracolumbar spinal cord represent a significant portion of patients with SCI and suffer deficits that are distinct from cervical or thoracic injuries. Currently there are few clinically relevant lumbar SCI models in the literature and due to the heterogeneity of human SCI there is a need for a model that replicates both the central and peripheral nervous system injury. In this study, we characterize an impact-compression injury to the rostral lumbar spinal cord segments. The main aim of this work is to directly address the lack of lumbar SCI models in the literature.

The primary goals were:

(1) To demonstrate consistent and reproducible paralysis after SCI

Perhaps the most important feature of an animal injury model is how reproducible and consistent the injury is between individual animals. If a model is highly variable between animals, it is difficult to interpret therapeutic results and draw any conclusions. Although the clip impact-compression model is one of the best characterized and clinically relevant SCI techniques, no studies currently exist investigating the application of the clip to the lumbar spinal cord segments, thus we set out to prove that injury to the L1-L2 lumbar spinal cord segment can be reproducible. For example, the inclusion of several spinal roots in the clip could interfere with the application of the clip. Therefore, in this project it was necessary to determine if the variability was acceptable with the clip technique.

The majority of patients with injuries to the thoracolumbar junction incur a spinal cord injury that often results in paralysis of the lower limbs. Thus, it was necessary to prove

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that clip impact-compression injury to the lumbar spinal cord produces hindlimb
dysfunction consistent with paralysis observed in the human population.

(2) To demonstrate sensory alterations after SCI and their similarity to proximal
conus medullaris injury in humans

Patients with thoracolumbar SCI experience both a SCI and a peripheral nerve injury due
to the anatomy of where the spinal nerves exit the spinal cord (Figure 3). This combined
injury has never been modeled before in a rodent, and it is important to determine
whether the rats experience at-level neuropathic pain in order to mimic at-level root
damage seen in patients.

The conus medullaris syndrome is a debilitating syndrome caused by injury to the spinal
nerves entering and exiting the spinal cord near the caudal tapering end of the spinal
cord, and to the spinal cord itself. By injuring three-four pairs of nerve roots bilateral to
the compression site at L1-L2 spinal cord segment, we hypothesize that we have created
a proximal conus medullaris injury model. Models of conus medullaris syndrome do
exist, but are typically modeled as an avulsion injury in isolation. Avulsion injuries in
isolation are extremely rare and the more commonly observed injury is impact-
compression injury of many nerves concurrently, which we have produced in this injury
model.

(3) To demonstrate the dynamic alterations to neural tissue both in vivo and ex vivo

In humans, cystic cavitation is a key pathological characteristic in the majority of
traumatic SCI cases. Thus, it was important to demonstrate cavitation in the injuries produced to accurately model not only the behavioural characteristics but also the histological aspects of the injury.

The majority of human SCI results in some sparing of connectivity through the injury site in both motor and sensory tracts, such as seen in AIS-B, C, and D patients. It is not possible to determine functionality of tissue through histological outcomes, and therefore, electrophysiological techniques are used to characterize the functional extent of damage inflicted after clip impact-compression injury. A complete loss of motor and sensory connectivity correlates to an AIS-A class of injury, which was not the goal of this injury model. Therefore, to produce an injury of clinical relevance, some preservation of motor and sensory evoked potentials at the 6-week end point was important.
2 Methods

2.1 Animals and Experimental Numbers

All experiments were performed on adult female Wistar rats (Charles River, Quebec, Canada) weighing 250-280g. Animal procedures were approved by the Animal Care Committee of the University Health Network in accordance with the policies established in the Guide to the Care and Use of Experimental Animals made by the Canadian Council on Animal Care. The rats were housed singly in a temperature-controlled room at 26°C for 42 days (unless otherwise indicated) with a 12-hour light/dark cycle. Sentinels were housed in the same room and periodically assessed for pathogens. Bladders were expressed 3 times daily until spontaneous voiding. Food and water was provided ad libitum. Post-operative Buprenorphine (0.03 mg/kg, Temgesic) was given subcutaneously before the animals awakened, and then every 12 hours for 7 days. Rats were given Clavamox (amoxicillin trihydrate/clavulanate potassium) in their drinking water for 7 days post-injury to prevent urinary tract infection.

In several experiments the numbers of animals in the experimental groups are uneven. We chose to focus our efforts on the 20g injury versus sham operation, and in these experiments the group numbers were larger and more evenly distributed. We chose to examine locomotor recovery between all groups in order to determine any significant differences in this important outcome. Subsequent to the behavioural analysis we analyzed several other outcomes including electrophysiological, imaging and histological parameters partly based on the numbers used in previous similar projects and
power analysis. Attrition also contributed to some of the disparities observed in group numbers.

2.2 Spinal Cord Injury Model

The rats were anesthetized with 2% isofluorane and a 2:1 mixture of nitrous oxide and oxygen. We determined that palpation of the iliac crests is a useful landmark to locate the adjacent L6 vertebra and spinous process, which lie immediately between the iliac crests. Then, the last rib is palpated to locate the junction of the last rib and the adjacent T13 vertebra. Counting the spinous processes sequentially cephalad from L6 to T13 was then used to confirm the location of the T13 vertebra (Figures 1-3). Under aseptic conditions, the spinal cord is then exposed through a midline incision and T9, T10, T11 and T12 vertebrae are exposed. An important intraoperative landmark is the relative angulation of the T9, T10 and T11 vertebrae where the spinous process of T9 is pointed caudally, T10 is pointed directly upwards and T11 is pointed rostrally. This produces a “triangle” of spinous process orientation that can be readily seen if the animal is positioned flatly on the operative table. After T11 and T12 have been identified a laminectomy is performed at levels T11 and T12 with a rongeur (Figures 1-3). The clip was then held in its opened position and the lower blade of the clip was passed extradurally completely around the cord and nerve roots and then rapidly released from the applicator to produce a bilateral impact-compression injury simultaneously by dorsal-ventral compression. The spinal cord is compressed at the junction between the T12 and T11 vertebral level, corresponding to the L1-L2 spinal cord level (Figure 2). The clip was left compressing the spinal cord for 60 seconds before removal with the applicator. Before use, the force
of the clip was measured with a clip calibrator that measures the closing force of the clip and any deviation from the desired force can be corrected. After injury, the muscles were sutured using 3–0 vicryl sutures and the skin was closed with Michel clips.

**Figure 4. Operative anatomy of the lumbar injury model.** CT imaging was used to verify that we are operating consistently at the T11 and T12 vertebral level. Panel (A) displays a lateral view, while panel (B) displays an AP view. The last rib (13th rib) articulates onto the last thoracic (T13) vertebrae and is a useful anatomical landmark. Laminectomy is performed directly rostral to T13 to remove the T12 and T11 lamina, revealing the T13, L1 and L2 spinal cord segments. Panel A shows the absence of the spinous processes of T11 and T12.
Figure 5. Intraoperative approach. After midline incision and clearance of connective tissue, 4-5 vertebrae are exposed. Panel (A): A useful landmark is the “triangle” of spinous process orientation involving T9, T10 and T11, where T9 is directed caudal, T10 is directed dorsal and T11 is directed rostral. Panel (B) Removal of T11 and T12 lamina exposes three spinal cord segments, T13, L1 and L2. The experimental SCI is made roughly halfway between both vertebrae and is indicated with a dotted red line. Panel (C) a durotomy was performed and the T12 and T13 roots are splayed over the injury site, confirming that three nerve roots are included in the injury model (L1 cannot be seen in
Injury to these nerve roots will produce a significant root injury, which relates to the human conus medullaris syndrome.

### 2.3 Behavioural Outcomes

#### 2.3.1 Open-field locomotion (BBB)

Locomotor function was evaluated using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale weekly for 6 weeks (Basso et al., 1995). Rats were placed in an open field and hindlimb movements were video recorded for 4 minutes to assess the animal’s motor function including joint movements, coordination, paw placement, and toe clearance. A score of 0 indicates no hindlimb movement whereas a score of 21 indicates unimpaired locomotion as observed in normal uninjured rats. Rating of the BBB score was completed by two blinded evaluators. Five groups of rats were used in this study with one group undergoing laminectomy only (sham) and four groups undergoing injury with clips of four different closing forces (clip strength): 20, 26, 35 and 56 g (n = 52 total). Clips of varying closing force were used to determine the sensitivity of the lumbar spinal cord to impact-compression injury since the clip-compression model has not been applied previously in the lumbar spinal cord.

#### 2.3.2 Sensory Testing

Assessment of at-level Mechanical Allodynia: Von Frey Filaments
Cutaneous sensitivity to normally innocuous mechanical stimulation was evaluated weekly post-injury using Von Frey filaments (VFF). We compared animals in the 20g injury group versus the sham injury group. A series of VFF’s with stiffness’ of 1.4g, 2.0g and 4.0g was applied to T13, L1 and L2 dermatomes as described by Takahashi (2003) to determine at-level mechanical allodynia. Probing was performed when the animals were calm and not mobile. A negative response occurs when the VFF bends and the animal does not respond with any aversive action. In contrast, a positive response occurs when the filament bends and the animal does respond with an aversive action i.e. vocalizing, flinching, licking, turning away, or any other behavioural cue corresponding to discomfort. A total of ten applications of each VFF were scored as positive or negative to obtain a total percentage out of 100% (i.e. 6 positive responses = 60% response rate).

Assessment of Thermal Hyperalgesia: Tail-Flick

To measure the thermal nociceptive response, we assessed animals in the 20g injury and sham injury groups biweekly post-injury for 6 weeks. Animals were wrapped in a soft, dark material to calm and distract them. Subsequently, the dorsal surface of the tail between 4 and 6cm from the tip was exposed to a beam of light calibrated to 50 °C generated from an automated analgesia meter (IITC Life Science, Woodland Hills, CA). The timer was stopped when the animal flicked its tail away from the beam of light, indicating an aversive response. Latency was measured at 5-minute intervals until a
stable baseline was obtained over 3 consecutive trials. The mean latency was used as a measure to indicate thermal hyperalgesia.

2.4 Histological Outcome Measures

Animals underwent one of two separate tissue fixation protocols depending on whether the tissue was intended for histopathological or immunohistochemical analysis. In both protocols animals were first deeply anesthetized by intraperitoneal injection of sodium pentobarbital.

2.4.1 Histopathological analysis of cavitation and spared grey/white matter

For the cavitation and spared grey/white matter analysis animals were perfused with 500mL of 10% neutral buffered formalin after intravascular injection of 1mL heparin (1000 IU/mL). A 1.5cm segment of the spinal cord centered on the injury site was excised and post-fixed in 10% neutral buffered formalin at room temperature. All tissue was processed with alcohol and chloroform in a tissue processor and then embedded in paraffin blocks. Spinal cords from each of the four injury groups were randomly selected, and 8um serial cross-sections were made. The sections were stained with Luxol Fast Blue (LFB) and counterstained with hematoxylin and eosin (H&E) to visualize white and grey matter.
Image analysis was performed with a Nikon TE300 inverted bright field microscope and images of LFB/H&E stained sections were captured with an Optronic CCD camera connected to the microscope. To assess the impact of a clip compression injury at the L1-L2 spinal cord segment, tissue sections for each animal were systematically sampled every 240-µm over a distance of 1-cm. Tissue section areas were obtained using the Cavalieri method. Any necrotic tissue within the cavities was counted as part of the lesion. The percentage of scar tissue and cavity area for each section was calculated using the following formula: % of scar tissue and cavity of tissue section = area of scar tissue and cavity of tissue section / total area of section. The percentage of the preserved gray matter and that of the white matter was calculated using the following formulas: % of preserved gray matter of tissue section = area of gray matter of tissue section / total area of tissue section. Cavity length was determined by the following calculation: \( \text{Length} = n \times d \), where \( n \) is the number of sections with a cavity and \( d \) is the intersection distance.

### 2.4.2 Immunohistochemistry

For immunohistochemical analysis, rats underwent fixation with 4% transcardial perfusion in 0.1M PBS, pH 7.4 and the tissue was excised and maintained in 30% sucrose at 4°C. A 1cm segment of the spinal cord centered on the injury site was dissected and used for cryosectioning. The tissue was embedded in OCT and cryosectioned in 20um serial cross sections collected on Superfrost slides. Sections were then rehydrated in 0.1 M PBS and permeabilized with 0.1% Triton-X 100, blocked with either 10% normal goat (NGS) or normal donkey serum (NDS) for 1hr at room
temperature and then incubated with the primary antibody overnight at 4°C. The following primary antibodies were used: anti-ChAT (1:500, Millipore, AB144P) for motoneurons and anti-RECA1 (1:200, AbD Serotec, MCA970R). After three washes, cells were incubated for 1hr with one of the following secondary antibodies: donkey-anti-goat Alexa 564 IgG or goat-anti-mouse Alexa 564 IgG, both at a concentration of 1:500.Slides were washed with PBS and then coverslipped with Vectashield mounting medium containing DAPI (49, 6-diamidino-2-phenyl-indole) to counterstain the nuclei. Immunofluorescent tissue was examined using a Nikon TE300 inverted fluorescent microscope.

2.4.3 Quantification of microvasculature

For blood vessel quantification we used sections immunostained with the monoclonal antibody RECA1, which is specific to rat endothelial cell antigen. Counting was performed at 20X magnification on 4 selected fields (ventral horn, dorsal horn, left and right lateral columns). The obtained values were pooled and the number of vessels was calculated at epicenter, 1mm, 2mm and 3mm rostral and caudal to injury site.

2.4.4 Motoneuron Quantification

For motoneuron quantification we used sections immunostained with the polyclonal antibody ChAT, which is specific against choline acetyltransferase found in large cholinergic motor neurons in the anterior horn of the spinal cord. Motoneurons counts
were performed at 20X magnification on both anterior horns. The obtained values were pooled and the number of motoneurons was calculated every 1mm for a total of 6 counts (6mm).

2.5 Electrophysiology

Animals underwent electrophysiological procedures to determine if a) animals are spastic or flaccid after injury to the rostral lumbar spinal cord segments (Hoffmann reflex) and b) if there is any signal transduction through the injury site (sensory and motor evoked potentials).

2.5.1 Hoffmann Reflex

The Hoffmann reflex (H-reflex) is one of the most well studied reflexes in humans and rats and is the electrophysiological equivalent to the monosynaptic stretch. As opposed to a mechanical stretch of the muscle spindle, instead the H-reflex is evoked by electric stimulation of the afferent tibial nerve resulting in monosynaptic excitation of alpha-motoneurons. In addition to clinical observation, the H-reflex can be used as a tool to examine spasticity after spinal cord injury. Under isofluorane anesthesia, the left hindlimb was secured and electrodes were inserted transcutaneously. A pair of stimulating electrodes was inserted into the region of the tibial nerve slightly above the ankle. For recording, a pair of silver needle electrodes was placed into the plantar muscles of the left hind paw. The tibial nerve was stimulated using a 0.1 ms duration
square wave pulse at a frequency of 1 Hz. The recordings were filtered between 10 and 10,000 Hz. Recordings of the H-reflex typically consist of two EMG responses – an initial M-wave and a later H-wave. The M-wave is the result of direct activation of the motor axons and does not involve the spinal circuits. The later H-wave, or H-reflex, is a compound EMG response in the plantar muscle elicited by the synaptic activation of motoneurons by muscle afferents. The threshold for both the M and H waves was determined and the Hmax/Mmax ratio was calculated.

2.5.2 Motor and Sensory Evoked Potentials

In addition to several neurobehavioural outcomes, motor (MEP) and sensory evoked potentials (SEP) were examined to evaluate the electrophysiological integrity of the spinal cord after clip-compression injury to the lumbar spinal cord. Both recordings were completed at 6 weeks post-injury with the rats under isofluorane anesthesia. The rats were immobilized in the prone position and fixed in a stereotaxic holder. The presence and functional conductivity of both descending motor tracts and ascending sensory tracts were analyzed.

SEPs: The intervertebral ligaments between C1 and C2 were removed surgically using fine forceps. Two pairs of 1.0 mm ball electrodes were positioned extradurally over the spinal cord at C1 and C2 for recording evoked potentials. The sciatic nerve was exposed, desheathed, and placed on bipolar silver electrodes. A constant current stimulus of 0.1 ms in duration and 2.0 mA in intensity was applied at a rate of 5.7 Hz to the sciatic
nerve. At a bandwidth of 10 to 3000 Hz, a total of 2000 SEPs were averaged and replicated. SEPs peak latency was measured from the start of the stimulus to the peak of first negative peak (N1). The evoked potential amplitudes were measured as the voltage difference from the peak of first positive peak to the peak of the negative peak (N1) (Wu et al, 2013). Recordings were acquired using Keypoint Portable (Dantec Biomed, Denmark).

MEPs: Stainless steel subdermal needle recording electrodes were inserted into the biceps femoris muscle in the hindlimb. Stimulation was applied to the midline of the cervical spinal cord between C1 and C2 vertebrae using a ball electrode as described above with the following specifications: 0.13 Hz; 0.1 ms; 2 mA; 200 sweeps. The amplitude was determined by the difference between the first positive and negative peak. Recordings were acquired using Keypoint Portable (Dantec Biomed, Denmark).

2.6 Imaging

2.6.1 Ultrasound Imaging

Very High-Resolution Ultrasound Imaging (VHRUS) was utilized to measure in vivo cavity volume and morphology. Ultrasound imaging followed the protocols developed by Soubeyrand et al (2014). At 6 weeks post-injury, animals were placed under 2% isofluorane anesthesia as described above and a midline incision was made to the skin to reveal the surgical site and spinal cord. Ultrasound gel was then applied on the dorsal aspect of the dura mater. The VHRUS probe (44MHz, Vevo 770, Visualsonics, Toronto,
Canada) was attached to the rail mount of the Vevo Integrated Rail system III (Visualsonics, Toronto, Canada) by an integrated clamp. A motor stage attached to the rail allowed three-dimensional acquisitions. The rail mount was then pulled down until the distance between the acoustic window (top of the real-time image) and the middle of the spinal cord (identified by the central canal) was 6mm (the focal length of the transducer). The scanhead was rotated and tilted in order to obtain a strict sagittal view and its position was locked. VHRUS acquisitions were made either in B-mode or in Power Doppler mode. For each mode, two-dimensional (2D) and three-dimensional (3D) acquisitions were performed. To perform 3D acquisitions, the 3D motor stage travelled a distance of 6mm in a series of steps from the left to the right of the spinal cord. At each step, the scanhead took a 2D image (i.e. slice) and the distance between each slice was 102 um. Each slice was stacked and assembled together to form a 3D file. Each 3D acquisition took 7 seconds in B-mode and 4 minutes in Power Doppler mode.

The 3D files were analysed with ImageJ software. The analysis was then performed on a stack of 19 sagittal slices centered on the midline. On each slice the dark space (known to be the spinal cord cavity based on previous experience) was delineated to obtain the area, subsequently the cavity area of each slice was combined to get a cavity volume.

### 2.6.2 Computed Tomography

Computed Tomography (CT) imaging was utilized to confirm the correct operative level (Figure 1). The 13\textsuperscript{th} rib articulates at the anterior end of the 13\textsuperscript{th} thoracic vertebral body, and the L6 vertebra lies directly adjacent to the iliac crests, which can clearly be seen in
the images. The bony anatomy was imaged with the GE Locus Ultra MicroCT system at the STTARR facility, University of Toronto. The following technical specifications were used: 16 second anatomical scan time with 150µm³ resolution, a 14cm maximum transaxial field of view, 10.2cm/16s maximum longitudinal field of view with 1000 volumes per rotation. The X-ray tube used an 80kV voltage, 50mA current and 0.15mm Cu filter with a “standard” reconstruction filter. Animals were induced under 2% isofluorane to avoid movement during scan acquisition and imaged at 7 days post-surgery.

2.6.3 Magnetic Resonance Imaging

Animals were placed under 2% isofluorane anaesthesia for MRI imaging. The images were acquired on a 7 Tesla Biospec USR 70/30 (Bruker Corporation, Ettlingen, DE), with the B-GA12 gradient coil insert. A 7.2 cm inner diameter cylindrical linearly polarized RF coil was used for RF transmission, and a 20 mm flat surface coil taped above the lesion was used for RF signal reception.

Coronal 2D-RARE T1-weighted images were acquired with the following parameters: echo time 12 ms, echo train length of 2 and repetition time was gated to the respiratory cycle (TR ~1300 ms). Images were acquired over a 40 x 40 mm field-of-view with a 320 x 320 matrix for 125-micron in-plane resolution. The effective readout bandwidth was 44.6 kHz, slice thickness was 1.1 mm for a total of 5 slices. The scan time was approximately 24 minutes.
Axial 2D-RARE T1-weighted images were acquired with the following parameters: echo time 14.4 ms, echo train length of 4 and repetition time was gated to the respiratory cycle (TR ~1300 ms). Images were acquired over a 16 x 16 mm field-of-view with a 128 x 128 matrix for 125-micron in-plane resolution. Out-of-FOV signal was suppressed using saturation bands. The effective readout bandwidth was 48 k Hz, with a slice thickness of 1.1 mm for a total of 15 slices. The scan time was approximately 22 minutes.

2.7 Statistics

Comparisons involving two factors such as the tail-flick test, VFF and lesion analysis were analyzed using two-way analysis of variance (ANOVA), followed by post hoc pair wise multiple comparisons. Two-way repeated measures ANOVA was used to compare BBB scores between different injury groups and t-tests were used to compare ultrasound quantification, H-reflex scores and evoked potentials. P-values less than 0.05 were used as the criteria for statistical significance and all statistical analysis was performed using SigmaStat software. All values are represented as mean ± standard error of the mean (SEM) except BBB scores, which are calculated as mean ± standard deviation (SD).
3 Results

3.1 Rats do not recover coordinated hindlimb locomotion

Figure 6. Open-field locomotor recovery. Injuries created by the application of the 35g (n=10) and 56g (n=8) clips produce severe locomotor deficits and rats only recover to the level of slight movement of three joints in the hindlimb. Application of the 20g (n=20) clip produces a moderately-severe injury and rats regain extensive movement of all three hindlimb joints. Application of the 26g (n=14) clip produces a severe injury, however these animals recover significantly more than animals injured with the 35g or 56g clips. It is important to note that none of the groups recover the ability to bear weight or to coordinate hindlimb motion. Data are represented as mean ± SD. Statistical significance was accepted for values of p<0.05.
SCI to the rostral lumbar segments produces profound deficits in hindlimb locomotion. BBB scores of both hind limbs were averaged and are shown in Figure 4. The only distinction in spontaneous functional recovery is seen between the moderate clip strength (20g) BBB = 8.1±1.5, versus the severe clip strengths (26g, 35g and 56g) BBB = 4.6±3.4, 3.3±1.7 and 2.3±1.9 respectively, p<0.05, n=50. The more forceful clip strengths all produce severe cavitation that results in an injury that is functionally similar to a complete transection since there is no ascending or descending activity. Scores on the BBB appear to plateau around 4-5 weeks, with severe injuries reaching plateau earlier than the moderate injury since less spontaneous recovery is observed. Clinically, rats had atrophic hip muscles and the hindlimbs were continually extended, displaying a lack of flexor/extensor coordination. This can be expected when injuring the iliopsoas motoneuron pools in the L1 and L2 spinal segments.
3.2 Injury to the rostral lumbar spinal cord produces pronounced sensory alterations

**Figure 7. Sensory outcome measures.** Effect of 20g impact-compression injury on thermal and mechanical sensory outcome measures (n=12 injury, n=6 sham). The above graphs illustrate withdrawal latency to noxious stimuli (A) and percent avoidance responses to mechanical stimuli (B). Data are represented as mean ± SEM. Statistical significance was accepted for values of p<0.05.
Marked sensory alterations were observed in both the tail-flick and VFF tests. There was no difference in VFF response between the sham (n = 6) and the 20g impact-compression group (n = 12) 1-week post-injury (Bonferroni post-hoc, p=0.62). Evidence of pathological changes in sensation occurs as early as 2 weeks post-injury, which is consistent with other studies (Karadimas et al, 2012). The spinal cord injury group demonstrated significant progressive increases in both sensitivity to mechanical stimuli and heat over the 6-week period and are most severe at end-point (two-way ANOVA, Bonferroni post-hoc, p <0.001 for each time point). Sensory alterations were not observed in sham-operated animals at any time point.
3.3 Motor and sensory conduction through the injury site is reduced but remains physiologically intact after the 20g injury.

Figure 8. Electrophysiological evoked potential recordings. Analysis of sensory evoked potentials in panel (A) revealed that latency is increased and the peak amplitude was significantly decreased after the 20 g injury (n=8) compared to the sham group (n=4). The same result is seen in motor evoked potentials in panel (B). Data are represented as mean ± SEM. Statistical significance was accepted for values of p<0.05.
Rostral lumbar SCI causes a significant, but not complete decrease in hindlimb sensory and motor evoked potentials (SEP and MEP respectively). SEPs and MEPs were analyzed by peak amplitude measurements from the first positive peak to the first negative peak. At 6-weeks post-injury, peak amplitude was significantly lower in injured rats versus sham rats, n = 4 in sham group and n=8 in injury group, t-test; p<0.05.
3.4 Rostral lumbar spinal cord injury at 20 grams results in an enhanced H-reflex

![Figure 9. Hoffmann Reflex recordings.](image)

We tested the Hoffmann-reflex (H-reflex) in our animals as an indicator of spasticity. The maximal plantar H-reflex/maximal plantar M-response (HMAX/MMAX) ratios determined the excitability of the H-reflex. Analysis of the recordings revealed that the mean value obtained for maximal H-reflexes in sham-operated animals (n=4) was significantly lower than those that underwent traumatic spinal cord injury (n=8). The H-reflex is heightened as early as one-week post injury and is maintained after injury for 6 weeks. Data are represented as mean ± SEM. Statistical significance was accepted for values of p<0.05.
Animals with rostral lumbar spinal cord injuries display enhanced excitability of the H-reflex as measured by the (HMAX / MMAX) ratio as early as 1-week post injury. All animals were tested for a baseline H-reflex before injury. Significant differences between sham and injury groups are observed as early as 1-week post-injury (t-test, p<0.05), n=12. This heightened H-reflex is maintained weekly until end-point at 6 weeks. Animals that underwent laminectomy only (sham) showed no heightened H-reflex after injury compared to measurements before injury.

3.5 Impact-compression injury produces significant cavitation and loss of grey and white matter

At 6-weeks post-injury, ultrasound imaging revealed no cavitation in the sham injury group (laminectomy only) versus an average cavity volume of 2.64mm$^3$ for animals injured with the 20g clip.
Figure 10. Very High Resolution Ultrasound Imaging (VHRUS). Shown above are representative images taken with the VHRUS system in rats injured with a 20g clip “SCI” versus animals that underwent laminectomy only “Sham”. The cavity is fusiform in shape and has a mean volume of 2.64mm$^3$, n=6. There is no cavity or damage to the spinal cord in the laminectomy only group (sham), n=4.
Figure 11. Histology. Representative images from each clip strength (sham n=7, 20g n=10, 26g n=7, 35g n=6 and 56g n=5) stained with luxol fast blue and haemotoxylin/eosin (LFB, H&E) after 6 weeks post-injury. As clip strength was increased, the total lesional tissue extended progressively with less preserved tissue.
**Figure 12. Lesion morphometry.** Analysis of grey/white matter sparing and lesional analysis displayed as a linear relationship between clip strength and volume of both grey and white matter and lesional tissue (sham n=7, 20g n=10, 26g n=7, 35g n=6 and 56g n=5). Data are represented as mean ± SEM.
We observed a progressive lengthening of the cavity and increase in lesional tissue as clip strength was increased, which confirms previous reports relating to injury strength and degree of cavitation (Poon, 2007). Only the 20g clip strength resulted in fusiform cystic cavitation. The more severe injuries showed marked atrophy of both the grey and white matter at the lesion site. Diffuse lesional tissue and extensive grey/white matter degeneration was seen in all clip strengths. The moderate clip strength (20g) produced a lesion measuring 4.2±1.7mm in length whereas more severe injuries (26g, 35g and 56g) measured 6.2±2.2mm, 8.5±2.6mm and 10.2±3.1mm respectively, n=18 (Figure 10). Grey matter was largely obliterated, but only in the 20g injury was there an identifiable rim of spared white matter. In the more severe injuries, the white matter rim was either absent or almost entirely demyelinated at the epicenter. Figure 11 shows representative images from each experimental injury group. Intact control animals were “sham operated”, i.e., the animals received a laminectomy of T12 and T11, but no clip-compression was applied. These animals displayed no cavitation and tissue was completely preserved.

Two-way ANOVA showed significant differences in lesion, grey and white matter volumes between 20g versus severe injuries (26g, 35g and 56g) p<0.05 and all injuries are statistically significant versus sham p<0.05, n=18.
Figure 13. Magnetic Resonance Imaging. Serial T1-weighted 2D-RARE MRI images were taken to view dynamic changes in vivo after SCI. Shown in panel (A) are axial acquisitions after 20g clip-compression injury at 4 weeks post-injury. Panel (B) displays a sagittal view of the same animal with arrows corresponding to the numbered axial images in panel A. Panel (C) shows sagittal views both before SCI (naive) and 48hrs and 4 weeks after. Vertebral bodies are labeled and the injury can clearly be seen at the T12 vertebral level.
Serial T1-weighted 2D-RARE MRI images were taken in both the sagittal and axial plane allowing complete observation of dynamic changes occurring both before, 48hr and 4 weeks after injury, n=6. As depicted in Figure 13, MRI images reveal significant edema and cavitation 48hr post-injury. Axial MRI images display clear dorsal column degeneration rostral to the lesion epicenter and a loss in the distinction between grey and white matter at the lesion epicenter. At the caudal portion of the injury there is a decrease in signal intensity, which could reflect edema and haemorrhage. These in vivo images corroborate the ex vivo histological techniques described in Figure 11 and 12.

### 3.6 Clip-compression injury to the lumbar spinal cord caused significant motor neuron death

Animals from the 20g compression group (n = 7) and laminectomy only (n = 4) were used to analyze motoneuron loss after clip-compression injury. The total number of motoneurons (ChAT positive cells) within a 7mm length of spinal cord tissue (3mm rostral and caudal to the epicenter) was determined by counting. As depicted in figure 14, the total estimated number of motoneurons was significantly decreased after clip-compression injury to the lumbar spinal cord (t-test, p < 0.05).
Figure 14. Choline Acetyltransferase (ChAT) positive cells were significantly reduced in the injury group (n=7) compared to sham (n=4) over a distance of 7mm centered on the injury epicenter. SCI represents spinal cord injury (clip-compression) whereas sham represents laminectomy-only of T11 and T12 vertebral levels. VH represents ventral horn. Data are represented as ± SEM.
3.7 Compromise of spinal cord microvasculature

Figure 15. Rat endothelial cell antibody 1 (RECA-1) positive cells were significantly decreased after 20g clip-compression injury (n=7) to the lumbar spinal cord compared to sham (n=4) over a distance of 7mm centered on the injury epicenter. SCI represents spinal cord injury (clip-compression) whereas sham represents laminectomy-only of T11 and T12 vertebral levels. DH represents dorsal horn. Data are represented as ± SEM.
In order to quantify the effect of 20g clip-compression injury on the L1-L2 spinal cord segment in the rat, tissue was taken at 6 weeks post-injury and immunostained with rat endothelial cell antibody 1 (RECA1), which is specific for blood vessels. Counts were performed on 4 selected fields (ventral horn, dorsal horn, left and right lateral columns) in each section under 20X magnification in both injury (n = 7) and laminectomy-only (n = 4). These counts were completed at 3mm and 1mm both rostral and caudal to the injury site including the injury epicenter. A significant reduction in blood vessel density is observed after 20g injury compared to sham (two-way ANOVA, p < 0.05).
4 Discussion

The current project describes an important clinically relevant lumbar spinal cord injury model in the rat and adds significant knowledge to the SCI field. With the primary aim of creating a clinically relevant and translatable SCI model, a variety of techniques were employed to relate the current experimental lumbar injury to injuries seen in humans. Tissue alterations were reliably observed both histologically and with in-vivo ultrasound imaging. Ultrasound quantification revealed a mean cavity volume of 2.64mm$^3$ for 20g injury in comparison to no cavitation in the sham group. Neurobehavioural testing revealed lasting paralysis and no evidence of weight bearing or hindlimb coordination in any of the injury groups. Evaluation of sensory outcomes revealed highly pathological alterations in at-level mechanical allodynia and below-level thermal hyperalgesia, consistent with SCI including the adjacent roots, such as seen in the conus medullaris syndrome in humans. Deficits in hindlimb function were confirmed by electrophysiological outcomes that showed increased latency and decreased amplitude of both sensory and motor evoked potentials, confirming that the injury is neurologically incomplete as opposed to complete. An increase in the plantar H-reflex was indicative of hindlimb spasticity, further highlighting the pathological changes after SCI.

4.1 Operating at the correct spinal cord level
One of the primary goals in this project was to produce an injury model that was consistent and reproducible. In order to accomplish this goal, it was essential to establish a reliable landmarking protocol. We developed several steps to confirm the correct injury level in the vertebral column and spinal cord. In general, operating at the target anatomical level is critical in every injury model attempting to replicate the human condition, but particularly so at the thoracolumbar junction. For example, if the SCI was performed one level rostral at the T10 – T11 vertebral level, the injury would result in a lower thoracic SCI, which would not replicate a lumbar spinal cord injury. If the injury was made one vertebral level caudal at the T12 – T13 level, the injury would represent a caudal lumbar SCI, with animals spontaneously recovering almost full function of the hindlimbs (Magnuson et al, 2005). Therefore, efforts were taken to ensure a consistent and accurate landmarking procedure. As mentioned previously, by using the 13th rib, iliac crests and the “triangle” of spinous process orientation intra-operatively, we generated a reliable landmarking protocol that allowed consistent location of the correct operative site. CT imaging was invaluable in confirming the operative level, and thus, we can conclude with confidence that the rats in these experiments were operated at the target spinal cord level of L1-L2. In our experience, the spinal cord segments reliably aligned with the corresponding vertebral levels. In the human population there is more variability in the number of lumbar vertebra and where the spinal cord ends.

There is a significant learning curve with respect to the surgical techniques required for this operation. Ensuring consistent and reliable gross anatomical landmarks is critical to the success of the operation as well as proper positioning of the animal. In order to observe the angulation of the spinous process, the animal is required to be placed flat on the operative table or else kyphosis can cause the spinous process orientation to be altered.
In a pilot study we performed a T12 and T13 laminectomy and placed the clip around the L3/4 spinal cord. This injury produced severe neuropathic pain with 40% of animals reaching humane end-point due to autophagia (self-biting). This injury level also produced a highly variable injury (n = 10, BBB = 13±4.4) since the peripheral nerves produced a lower motor neuron injury for many of the large leg muscles, whereas it produced an upper motor neuron injury for the foot and ankle muscles and some calf muscles. It was difficult to determine which pathology was produced by the central spinal cord injury and which functional deficits were due to a peripheral nerve injury (data not shown).

### 4.2 Clip-compression injury to the lumbar spinal cord is reproducible and results in significant hindlimb deficits and paralysis

Hindlimb activity is highly affected after clip-compression injury to the rostral lumbar spinal cord, resulting in persistent paralysis (Figure 6). No evidence of coordinated hindlimb movement was observed in any of the injury groups, nor weight-supported stepping (Figure 6). Lumbar SCI also produced clear dysfunction in the flexor/extensor pattern by selectively eliminating the flexor motoneuron pools in L1 and L2, producing constantly extended hindlimbs (Watson & Paxinos, 2008; Takahashi et al, 2010). Based on these results, it appears that an anatomically intact lumbar spinal cord is a prerequisite to achieving weight-supported hindlimb stepping and coordinated hindlimb motion.
Creating an injury model that follows a dose-dependent curve can be an advantage in being able to precisely model injuries of varying severities (Navarro et al, 2012). In order to create a dynamic SCI model, we investigated the effects of a multitude of clip strengths, which enabled us to reasonably mimic both the clinical outcome of a severe AIS-B and incomplete AIS-C/D SCI. Based on both the functional and neuroanatomical outcomes (Figures 6, 8, 9, 11 and 15) the 20g clip injury may have produced a clinical injury similar to (AIS-C/D) where some functional connections persist and there is not a complete loss of motor and sensory tracts. The 26g clip produces injuries intermediate between the 20g and 35g and 56g clip, with better recovery than the 35g or 56g injuries but significantly less recovery than the 20g clip. The more forceful 35g and 56g clips produced a more severe injury, which may be similar to AIS-A or AIS-B categories of injury. This relationship however is speculative since BBB scores and their corresponding functional recovery in the rat do not correspond completely to human functional recovery. This limitation is described in the limitations section below.

The relationship between functional locomotor deficits and graded clip-compression injury to the rostral lumbar spinal cord is not linear. In general, there were three identifiable types of functional deficits as discussed above. This came as a surprise as injuries produced in our laboratory in the thoracic and cervical spinal cord with the 20g, 26g and 35g produce “moderately severe” injuries with BBB scores reaching well into the double-digits (Summarized in Table 4) (Parr et al, 2007; Poon et al, 2007; Mothe et al, 2013). Therefore it appears that the lumbar spinal cord is more sensitive to injury than the thoracic or cervical spinal cord. In the lumbar spinal cord, a clip strength of 20g produces a “moderate” injury, the 26g clip produces a “moderately severe” injury and the 35g and 56g clips produce “severe” injuries. The BBB scale is sensitive to hindlimb deficits and the injury we are producing is selective for the motoneurons innervating the
hips and quadriceps (Figure 14), whereas thoracic and cervical injuries produce tract injuries with regards to the hindlimbs and the underlying circuitry encoding the hindlimbs. Moreover, more rostral injuries do not impact the CPG containing the interneurons responsible for overall hindlimb pattern generation. There is a significant literature on remodeling of the lumbar spinal cord after higher injury in the cervical or thoracic spinal cord, which is thought to be responsible for the heightened behavioural recovery (high BBB scores) observed after tract injuries (reviewed by Kiehn, 2006). In contrast to other injury levels, we have selectively destroyed the circuits responsible for this plastic recovery and therefore the injury produced is more severe as judged by the BBB locomotor scale.

Table 4. Comparison of functional outcomes between lumbar, thoracic and cervical clip-compression injuries.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Injury level</th>
<th>Injury strength</th>
<th>End point</th>
<th>BBB score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poon et al, 2007</td>
<td>T2 spinal cord level</td>
<td>20g, 26g, 35g</td>
<td>4 weeks</td>
<td>20g = 12, 26g = 12, 35g = 10</td>
</tr>
<tr>
<td>Forgione et al, 2014</td>
<td>C6 spinal cord level</td>
<td>20g</td>
<td>8 weeks</td>
<td>18g = 11.3</td>
</tr>
<tr>
<td>Figley et al,</td>
<td>T7 spinal cord</td>
<td>35g</td>
<td>8 weeks</td>
<td>35g = 9</td>
</tr>
</tbody>
</table>
### 4.3 Clip-compression injury to the lumbar spinal cord produces pathological alterations in sensation relating to a proximal conus medullaris injury in humans

Over two-thirds of patients develop debilitating neuropathic pain after traumatic SCI, with a higher proportion developing pain after injury to the thoracolumbar junction (T10-L1 vertebral level) (Crozier et al, 1991; Finnerup et al, 2001). In humans, traumatic injuries from T10 to L1 vertebral level and lower can produce either conus medullaris or cauda equina syndrome (below L1-L2 vertebral level). Traumatic cases involving both a SCI in the conus medullaris injury (T10 – L1) are very complicated, poorly understood and very resistant to treatment with very poor outcomes (Finnerup et al, 2012). In the animal literature neuropathic pain cannot be directly tested and is instead assessed with indirect measures such as mechanical allodynia (with VFF) and thermal hyperalgesia (tail-flick), both of which can be components of neuropathic pain in humans. Mechanical allodynia is a common form of pain experienced by patients post-SCI and occurs when a
normally innocuous mechanical stimuli produces pain. Thermal hyperalgesia is an exaggerated pain response to innocuous thermal stimuli.

In our model, we investigated both at-level and below level sensory alterations and identified pathological changes in both at-level and below-level sensation. By producing a significant peripheral nerve injury with clip compression at the L1-2 spinal cord level, it is no surprise that our animals experienced mechanical allodynia at the T12, T13 and L1 dermatomes (Takahashi, et al, 2003). Denervation of spinal roots results in pain and loss of sensation in the effected dermatomes, which is clinically observed in the conus medullaris syndrome when a large component of peripheral nerves are traumatically injured (Finnerup et al, 2012; Sixta et al, 2012). In comparison to models of thoracic injury, a 35g clip applied to the thoracic cord at T6-T7 produced fewer than 30% positive responses after 4 weeks, in comparison to 60% of responses in animals injured with the 20g clip after 4 weeks (Figley et al, 2014). There is a significant increase in at-level mechanical allodynia in the lumbar injury model that is probably due to the inclusion of spinal roots in the impact-compression injury.

The at-level alterations in sensation observed in our injury model replicates similar pathological alterations observed in many root avulsion models (Bigbee et al, 2007; Wieseler et al, 2010; Chew et al, 2013). The drawback with avulsion models is that they replicate a very rare clinical scenario where the root is avulsed in isolation. More relevant in human situations are combinations of spinal nerve injuries with a cord injury as seen in injuries to the thoracolumbar spinal cord at T10-L1 vertebral level (Wieseler et al, 2010; Chew et al, 2013).
Increased sensitivity to thermal stimuli was confirmed by measuring the latency of withdrawal after application of a beam of light to the tip of the tail. This pattern of increased sensitivity to thermal stimuli over the course of 6 weeks is consistent with clinical reports of below-level pain and models the central aspect of sensitization after SCI (Baastrup & Finnerup, 2008).

If we produced an injury to the caudal lumbar spinal cord at segments L4/L5, the resulting conus medullaris syndrome would be very severe due to the greater number of peripheral nerves adjacent to the L4/L5 segments. The rationale for not injuring lower is that the neuropathic pain resulting from this injury is very severe with rats reaching humane end-point due to autophagia commonly within 2 weeks post-injury (data not shown).

4.4 Moderate 20g injury to the lumbar spinal cord results in spared functional white matter and significant loss of grey matter

Interestingly, the 20g clip was the only clip strength that reliably produced cystic cavitation, which is a common feature in traumatic SCI patients both with radiological and histological observations. With the 26g clip cystic cavitation with a preserved subpial rim was inconsistent. Severe injuries caused by the 35g and 56g clips produced injuries with collapsed thin subpial rims and extensive atrophy of lesional tissue involving most of the cross section of the spinal cord at the level of injury. In contrast, a significant amount of white matter was spared in the 20g injury (35%), and therefore, as other studies have shown, it appears that the grey matter plays a more important role in
determining the clinical effects of injuries to the lumbar spinal cord (Magnuson et al; 2005). To produce a clinically relevant injury to the lumbar spinal cord, the optimal clip strength should be 20g or lower. The use of high-resolution in-vivo ultrasound imaging allowed us a detailed view of the spinal cord, as it appears in situ. Histological techniques require harvesting of the spinal cord thereby precluding any in-vivo observation of the spinal tissue. In addition, perfusion of the spinal cord during processing for histological study causes shrinkage of the tissue and excision releases the tension that is inherently present in the parenchyma and modifies the morphology of the tissue (Carlo & Stevens, 2011).

Cavity formation can be observed as early as 48 hours after injury as observed by T1 weighted MRI. The decrease in intensity is thought to be a fluid filled cavity and a more defined syrinx can be observed at 4 weeks post-injury in the same animal with MRI (Figure 13). The in-vivo MRI correlates well with histological sectioning and ultrasound imaging, showing areas of both haemorrhage, edema and cavity formation, which are all commonly observed clinically.

The primary goal was to produce a clinically relevant SCI model, and we have shown that both the motor and sensory evoked potentials, although abnormal, are still capable of being evoked through the spinal cord. Sensory evoked potentials assess function in the afferent or ascending tracts of the spinal cord, whereas motor evoked potentials represent function in the efferent or descending tracts in the spinal cord. In humans, only 5% of injuries to the thoracolumbar spinal cord are functionally complete and preservation of motor and sensory evoked potentials after 6 weeks post-injury is indicative of an incomplete lesion (Knop et al, 1999). Evoked potentials are an important complement to histological techniques in SCI research because it is not possible to determine which of
the roughly 35% of spared white matter, if any, is functionally propagating electrical signals. The presence of both SEP and MEPs is proof that some of the spared white matter is indeed functional.

4.5 Compression injury to the lumbar spinal cord results in an upper motor neuron injury for the lower hindlimbs

The heightened Hmax/Mmax ratio, or H-reflex, is indicative of disinhibition of the 1a alpha motor neurons and indicates the presence of clinical spasticity in the hindlimbs. Spasticity is defined as the velocity-sensitive increase in resistance to manipulation in subjects with lesions of descending motor pathways (Nielsen et al, 2006). It is a common side effect of SCI with estimates ranging from 65–78% of patients (Maynard et al. 1990). In humans, spasticity tends to develop over a period of months whereas in our rat experiment spasticity can be observed both upon observation but also with the H-reflex as early as one week post-injury (Nielsen et al, 2006).

It is reasonable to expect that the animals in this model would be spastic when measured from the plantaris muscle since it is innervated by motoneurons in the L4/L5 segments in the rat (Finkelstein et al, 1991). If we stimulated the femoral nerve and recorded from the quadriceps, I would hypothesize that we would not see an enhanced H-reflex since the quadriceps is innervated partly by L1 lumbar segment in the rat and there is a decline in motor neurons after L1 injury for several millimeters caudal where more motor neurons supply the quadriceps muscle. In comparison to thoracic or cervical injuries of similar
severities and injury mechanisms, the H-reflex is higher in this model. This may be due to both a decrease in inhibition from upper motor streams in the brain in addition to segmental inhibition from the L1 and L2 spinal cord segments that inhibit lower lumbar segments (Garcia-Alias et al, 2006).

In human SCI patients, injury to the lumbar spinal cord typically involves many of the lumbar segments L1-L5 because in most people the entire lumbar cord is opposite the T12 vertebral body, a common site of injury, whereas in our experimental injury only one to two lumbar cord segments are injured. Lumbar injury in humans results in flaccidity since most of the motoneurons innervating the muscles of the legs originate in the lumbar segments that are damaged. However, due to anatomical differences between the rat and human, the rat has 13 thoracic segments and 6 lumbar segments, therefore the hindlimb and hip muscles are innervated by a longer motor pool spread throughout more spinal cord segments. Thus, the model we have created is both an upper motor neuron injury for the feet and calf muscles and a lower motor neuron injury for the T12, T13, L1 and L2 spinal cord. The H-reflex confirms the presence of an upper motor neuron injury and is well described in both the human and animal literature.
5 Conclusions

5.1 Summary

In summary, we have produced a lumbar SCI model that reasonably mimics the clinical picture of traumatic SCI to the thoracolumbar vertebral junction in humans. This model addressed a clear gap in the literature in describing a clinically relevant lumbar SCI model including a comprehensive neuroanatomical and neurobehavioural characterization of the injury. Paralysis is consistently produced after injury to the L1-L2 spinal cord segment with significant functional white matter sparing and a centralized cyst forming by 6 weeks post-injury. A significant nerve root component is included to imitate an injury to the proximal conus medullaris, which is partially confirmed by at-level mechanical allodynia in the dermatomes innervated by T12, T13 and L1 spinal cord segments. Blood vessel staining confirms significant vascular disruption, which is key to replicating human SCI. Motor neuron death is highest at the injury epicenter and continues for many millimeters both rostral and caudal to the injury site likely resulting in weakness in the muscles innervated by T13, L1 and L2 spinal cord segments (psoas and quadriceps). Spasticity is observed both clinically and electrophysiologically confirming the presence of an upper motor neuron injury for the most distal muscles of the hindlimbs. Many clinical imaging techniques were used including CT, MRI and ultrasound, which confirmed the operative level and the presence of cavitation. Numerous opportunities exist for applications such as stem cell transplant therapies, rehabilitative studies or pharmacotherapy for neuropathic pain.
5.2 Directions for future research

The impact-compression lumbar SCI model we have described opens up opportunities for future work in a variety of therapeutic contexts. In addition to therapeutic studies, more research is required to determine the mechanism of the exaggerated functional deficits observed after L1 and L2 SCI in the rat in comparison to either cervical or thoracic injuries of similar clip strength. Compared to lower lumbar injuries of similar severity, injury to L1 and L2 segments cause a loss of coordinated hindlimb locomotion and far greater locomotor disruption. As was mentioned previously, it is thought to be due to the presence of the CPG in this region, however there are no definitive mechanisms as to why the lumbar spinal cord is more sensitive to traumatic SCI.

Autonomic Function

Patients with injuries to the conus medullaris can recover good motor function in their limbs but seldom recover full sensory and autonomic function with respect to the bowel and bladder. Injuries to the conus medullaris result in high morbidity partly due to the presence of many circuits subserving autonomic function in the lumbar and sacral spinal cord. In the future, it would be beneficial to investigate outcomes related to autonomic function such as the use of metabolic cages, catheterization to measure urinary output, bladder histology and sexual function among other measures. These techniques would allow the author to investigate recovery more holistically in this level of injury which is of major importance in patients because of the high morbidity.

Neuronal transplant
Due to the important neuronal circuits severely damaged in this injury model, it may represent an opportune model for neuronal transplant studies. Transplant of neurons into the thoracic cord is of little clinical significance with respect to replacement of neurons since one would only recover a dermatome or myotome in the thorax, whereas transplant into the cervical or lumbar enlargement could result in great enhancements in locomotor ability in the hips/legs (lumbar transplant) or arms/hands (cervical transplant). The lumbar cord represents an ideal region to attempt to replace functional neuronal circuitry, and even slight recovery could greatly enhance independence and quality of life. Various cell types and sources have been differentiated into neurons (Reynolds & Weiss, 1992; Pearse et al, 2004; Tarasenko et al, 2007; Yan et al, 2007; Zahir et al, 2009, Kim et al, 2011; Nori et al, 2011; van Gorp et al, 2013) including transplants of the human spinal cord (Mothe et al, 2011). The important combination would be 1) a clinically relevant lumbar SCI model, 2) the correct cell source (probably neural stem / precursor cells differentiated into neurons, and 3) combination with treadmill training, epidural stimulation, environmental enrichment, pharmacological therapy or another complementary therapeutic strategy. Many authors have investigated a neuronal transplant, stem cell transplant or use of a lumbar model but none have used the appropriate combination to observe significant functional recovery (Abematsu et al, 2011; Kim et al, 2012; van Gorp et al 2013, Mothe et al, 2011). Stem cell transplant, particularly neuronal transplant studies offer an abundant opportunity in the model we have described, and such studies are already underway in our laboratory.

**Epidural stimulation**

Recent studies have investigated epidural stimulation of the lumbar spinal cord segments in both the rat and the human (Ichiyama et al, 2005; Courtine et al, 2008; Angeli et al,
2014, reviewed by Gerasimenko et al, 2008). In the human technique, an electrical stimulator device is surgically implanted on the dorsal surface under T11 and T12 vertebra, thereby stimulating the full lumbar circuitry from L1-S1. In rats, the stimulator is implanted below vertebra T11 and T12, thereby stimulating L1-L4 spinal cord levels, with the stimulation being targeted at L2 spinal cord level. Both of these techniques rely on having an intact lumbar spinal cord in order to harness these circuits to enhance locomotion. It is of great scientific and clinical interest to investigate these strategies with a lesioned lumbar spinal cord. The model we have described is the only clinically relevant lumbar SCI model focused exactly at the segments stimulated by the epidural stimulator.

**Environmental Enrichment**

The goal of environmental enrichment strategies is to induce voluntary locomotor training by housing rats in cages that possess stimulating objects such as running wheels, a shelter house, climbing frames, tubes and nesting material in addition to many other objects (Engesser-Cesar et al, 2005; Lankhorst et al, 2001; Koopmans et al, 2012). The crux of functional recovery and plasticity in these studies is thought to be on both serotonergic sprouting and an increase in spinal cord progenitor cells in the lumbar spinal cord at L1 and L2 spinal segment levels in the rat. Currently there are no studies investigating if these strategies will be efficacious if the L1 and L2 spinal cord segments have been impacted by a traumatic injury. It is difficult to conclude that sprouting and plasticity in the L1 and L2 spinal cord segments have causative roles in the recovery of function after thoracic injuries if a positive control group does not exist that ablates the L1 and L2 spinal cord segments. Is it possible to encourage plasticity in motor pathways encouraged by sensory locomotor feedback to areas rostral or caudal to the CPG (which
is abolished in our injury at the L1-L2 spinal cord segment) or to recover function in the residual tissue substrate left after lumbar compression injury? What quantity and phenotype of spared tissue is required to regain function after traumatic injury to the lumbar cord?

**Treadmill training**

Many of the treadmill training studies investigate injuries to the thoracic spinal cord, which leave the lumbar spinal cord and its circuitry structurally and functionally intact (Reviewed by Battistuzzo et al, 2012 and Edgerton et al, 2006; Rossignol et al, 1998; Van Meeteren et al, 2003). Rats are harnessed in either robotic devices, which allow the experimenter to determine the weight-bearing ability of the animals, or in closed off treadmills, which force the animals to run on the treadmill for a given amount of time. Similar to environmental enrichment studies, these strategies harness the potential of the lumbar spinal cord, particularly the L1 and L2 spinal cord segments to enhance functional locomotor recovery (Hutchinson et al, 2004). In contrast to environmental enrichment, the locomotor stimulation is forced.

There have been many clinical trials using rehabilitative strategies involving treadmill training even though the mechanism of recovery is not known (Harkema et al, 1997). Is this training still efficacious after injury to the lumbar neuronal circuitry that are being harnessed after training? Can treadmill training regenerate the damaged lumbar circuitry? What cell types are essential in the lumbar spinal cord to permit this type of recovery? Does serotonergic sprouting occur through the lesion site? Is segmental sensation improved after treadmill training? What quantity of grey or white matter sparing is required for functional improvement after treadmill training?
It would be of interest to examine these techniques in utilizing spared grey/white matter after lumbar SCI to examine if these techniques can improve the functional and/or neuroanatomical outcome measures we have described (Bouyer, 2005).

**Pharmacological interventions**

Several techniques have been employed to harness the spared lumbar circuitry pharmacologically with the use of a variety of agonists and antagonists. Disinhibition of the lumbar spinal cord GABAergic circuits has been proven to be a successful strategy in addition to serotonergic and noradrenergic agonists (Courtine et al, 2008, 2009). The most successful of these strategies have been employed in combination with treadmill training or the use of multiple pharmacological agonists and antagonists. How do pharmacological interventions remodel the lumbar spinal cord after clinically relevant traumatic injury?

**Pain studies**

The model we have characterized may present a unique opportunity for pharmacological therapies attempting to reduce pain after SCI (Hofstetter et al, 2005; Finnerup & Baastrup, 2012; Sindrup & Finnerup, 2012). It is possible that the described model replicates a more complete picture of the pathology occurring after traumatic SCI involving nerve roots, and would therefore represent an opportunity to test drugs in a more clinically relevant manner.

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5.3 Limitations
**BBB locomotor scale**

The BBB locomotor scale has been the “gold standard” for functional assessment for many decades (Basso, Beattie, Bresnahan, 1995). The scale evaluates joint movement of the hips, hindlimb and hindpaw in rats as well as general orientation/stepping to give an indication of locomotor capabilities, such as weight-bearing, coordinated hindlimb movement and plantar stepping. Despite its widespread use in SCI research the BBB has several shortcomings. First, although it has been reported to have low inter-rater variance in the literature, it is subject to the rating ability of two blinded assessors, which makes the scores vulnerable to human error and biases. Secondly, the scores are not linear. An increase from 9-10 on the BBB scale could represent considerable improvements in functional recovery whereas an improvement from 17-18 could represent very minor improvements in recovery. Ferguson et al (2005) attempted to reconcile these differences and pool scores from the lower portion of the scale and essentially eliminate ratings in the upper end of the scale (12-21) to make the scores more linear. Regardless of the scoring system, the BBB scale is based on gross qualitative differences in hindlimb movement and it is not sensitive to minor variations in behavioural recovery or decline.

**Von Frey Filaments**

The VFF technique has been criticized for its lack of objectivity, inter-species and inter-operator variability (Detloff et al, 2010; Kjell et al, 2013). Kjell compared VFF responses after mild contusion injury of three different substrains of Sprague-Dawley rats from Charles River, Harlan and Scanbur. The Harlan substrain never developed mechanical allodynia at any time post-injury in contrast to both the Charles River and Scanbur rats. This study raises significant questions about the validity of the technique and more
research is required to determine the mechanism of this disparity. Currently there are no valid alternatives and the VFF technique has been used exhaustively in the SCI literature.

**MRI**

We were not able to quantify the T1 weighted MRI images since the resolution is not as high as would be required to confidently analyze tissue data. Therefore, the MRI was used qualitatively to show anatomical landmarks and the injury site as it develops in vivo from 48 hours to 4 weeks post-injury with the 20g clip.

**H-reflex**

The H-reflex is one of the most well studied electrophysiological reflexes in the literature (Remy-Neris et al, 1999). However, the H-reflex is subject to many potential confounding factors if the techniques are not executed accurately. In addition to the technical aspects, there are many limitations in the interpretation of results. First, is the assumption that the H-reflex is a pure reflection of type 1a afferents projecting monosynaptically onto type 1a alpha motoneurons. Secondly, is the assumption that the H-reflex is a measure of motoneuron excitability. The potential pitfalls are discussed at length in reviews by Knikou (2008) and Misiaszek (2003).

**Tissue Analysis**

In quantifying tissue there can be great difficulty in distinguishing functional grey/white matter from dysfunctional grey/white matter, which we have defined as lesional tissue (Magnuson et al; 1999, 2005). We decided to describe grey/white matter and lesional tissue in terms of volumes, quantified by the Cavalieri method, with lesional tissue being
defined as tissue that exhibited a fibrous, inconsistent tissue matrix (Karadimas et al, 2013).
6 References


