DEVELOPMENT AND CHARACTERIZATION OF A GRADED, IN VIVO, COMPRESSIVE, MURINE MODEL OF SPINAL CORD INJURY

Mital Joshi

A thesis submitted in conformity with the requirements for the degree of Master of Science at the Institute of Medical Science, University of Toronto

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COMPRESSIVE, MURINE MODEL OF SPINAL CORD INJURY

By Mital Joshi, November 2000, Master of Science, Institute of Medical Science, 
University of Toronto

ABSTRACT

In order to take advantage of various genetically manipulated mice available to study the pathophysiology of spinal cord injury (SCI) we adapted an extradural clip compression injury model to the mouse. The dimensions of the modified aneurysm clip blades were customized for application to the mouse spinal cord. Three clips with different springs were made to produce differing magnitudes of closing force (3g, 8g and 24g). The clips were calibrated regularly to ensure that the closing force remained constant. The surgical procedure involved a laminectomy at T3 and T4, followed by extradural application of the clip at this level for one minute to produce SCI. Three injury severities (3g, 8g and 24g), sham (passage of dissector extradurally at T3-4) and transection control groups were examined (n=12/group). Quantitative behavioural assessments using the Basso, Beattie and Bresnahan (BBB) and Inclined Plane (IP) tests showed a significant graded increase in neurological deficits with increasing severity of injury (p<0.0001, two-way repeated measures ANOVA). By Day 14, the motor recovery of the mice plateaued. Morphometric analyses of H&E/Luxol Fast Blue stained sections at every 50μm from the injury epicenter indicated that with greater injury severity there was a progressive decrease in residual tissue (p<0.0001, two-way ANOVA). Counts of retrogradely labeled neurons in the brainstem, midbrain and cortex also indicated decreased integrity of descending axons through the injury epicenter with increasing injury severity (p<0.0001, one-way ANOVA). Correlations between the functional deficits and anatomical outcome measure were identified. Specifically, the BBB openfield locomotor test was found to preferentially assess the integrity of raphespinal and corticospinal tracts, whereas the inclined plane preferentially assessed the integrity of vestibulospinal and rubrospinal tracts in mice. This novel, graded compressive model of SCI will facilitate future studies of the pathological mechanisms of SCI using transgenic and knockout murine systems.
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<th>Definition</th>
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<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Basso, Beattie and Bresnahan</td>
</tr>
<tr>
<td>C</td>
<td>cervical</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>cc</td>
<td>cubic centimeter</td>
</tr>
<tr>
<td>CBS</td>
<td>combined behavioural score</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acid protein</td>
</tr>
<tr>
<td>GluR</td>
<td>non-NMDA ionotropic glutamate receptor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>IP</td>
<td>Inclined plane</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>L</td>
<td>lumbar</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>MEP</td>
<td>motor evoked potential</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>msec</td>
<td>millisecond</td>
</tr>
<tr>
<td>N</td>
<td>Newton</td>
</tr>
<tr>
<td>NF200</td>
<td>neurofilament protein (200kDa)</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NYU</td>
<td>New York University</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>R</td>
<td>Pearson correlation coefficient</td>
</tr>
<tr>
<td>R²</td>
<td>stepwise multiple linear correlation coefficient</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SNK</td>
<td>Student-Newman-Keuls test</td>
</tr>
<tr>
<td>SSEP</td>
<td>somatosensory evoked potential</td>
</tr>
<tr>
<td>T</td>
<td>thoracic</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>+/-</td>
<td>wildtype allele pair</td>
</tr>
<tr>
<td>-/-</td>
<td>mutant allele pair</td>
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</table>
INTRODUCTION

Rationale

Spinal cord injury is an important cause of morbidity and mortality, particularly in young adults and children. In North America more than 10 000 people/year sustain a spinal cord injury (SCI). Over 200,000 people live with serious and permanent disabilities as a result of these injuries, resulting in profound socioeconomic costs. Current treatments for SCI include methylprednisolone and GM-1 ganglioside but these result in only minimal neurological improvements. Improved neuroprotective strategies should be directed at the mechanisms involved in the loss of tissue and the neurological deficits sustained. In order to ascertain which molecules should be targeted, the role of specific genes in the pathophysiology of spinal cord injury must first be examined. However, one of the limitations to rapid progress is the tardy development of pharmaceutical agents specific for individual gene products. Furthermore, the pharmaceutical agents which have been developed have been relatively non-specific in their targets, thus not facilitating studies to identify key genes in the pathophysiology of SCI. An ideal solution would be to apply recent breakthroughs in mouse genetics to target a given gene in mice and create mice that lack a detectable gene product. These knockout mice for specific gene product(s) could then be tested for their neurological, physiological, cellular and molecular responses to SCI. In addition to applying targeted knockout mice to study the pathophysiological mechanisms of SCI, mice with naturally occurring/spontaneous mutations could also be studied. In order to facilitate SCI studies with genetically altered mouse systems, a reliable, reproducible, graded model of SCI must first be established in the mouse.

Statement of the Hypotheses

Overall Hypothesis:

A graded, murine model of spinal cord injury can be established that is reliable and reproducible which will ultimately facilitate studies of spinal cord injury in mutant mice.
Specific Hypotheses:

1. Increasing levels of compressive spinal cord injury severity will result in increasing neurological deficits following injury.

2. Increasing levels of compressive spinal cord injury severity will result in increased tissue loss and descending supraspinal neuronal projections involved in motor function.

Objectives

In order to develop and characterize a reliable, reproducible, graded model of spinal cord injury in the murine spinal cord the following specific aims were examined:

1. To design a custom modified aneurysm clip for application to the murine spinal cord to produce three severities of SCI (3g, 8g and 24g).

2. To perform neurological analyses (Basso, Beattie and Bresnahan Hindlimb Locomotor Test and Inclined Plane Test) in order to assess the degree of injury severity following three levels of SCI (3g, 8g and 24g), sham and transection controls.

3. To perform histopathological examinations by the retrograde tracing of descending motor tracts using the fluorescent tracer, Fluoro-Gold, in order to assess the integrity of descending motor fibers through the injury epicenter in three injury severities (3g, 8g and 24g), sham and transection controls.

4. To perform morphometric injury site tissue analyses in order to quantify lesion size and neuronal tissue sparing at the injury epicenter, rostral and caudal to the injury epicenter in the three injury severities (3g, 8g and 24g) and sham controls.

Pathology of Spinal Cord Injury

Spinal cord injury is initiated by a primary mechanical insult to the spinal cord. The primary injury mechanisms include: compression, contusion, stretch and laceration, though most often, SCI has a component of impact plus sustained compression. The primary injury is mediated by vertebral burst fractures, vertebral fracture dislocation, missile injuries and
ruptured discs leading to tissue distortion (Tator, 1995). Following the primary injury, a sequelae of pathophysiological events occur at and adjacent to the injury site.

Initially following SCI, edema occurs and many petechial hemorrhages are observed, especially in the gray matter (Tator, 1995). At the injury epicenter, where maximal tissue damage occurs, there is an infiltration and activation of macrophages/microglia and extravasation of neutrophils as the blood-central nervous system barrier is compromised (Bunge et al., 1997; Kakulas, 1984). These invading immune cells then proceed to phagocytose debris from necrotic tissue and after they leave, a cystic cavity remains. Around the central necrotic zone, a translational zone of damage and a peripheral subpial rim of residual white matter are found. There exists a gradient of necrosis extending both rostrally and caudally from the focal epicenter (Balentine, 1978; Basso et al., 1996; Bresnahan et al., 1976; Bunge et al., 1997; Griffiths and McCulloch, 1983; Kakulas, 1984; Tator, 1995). Astroglial scar tissue, often termed gliosis, is found to serve as a boundary between the central cavity and adjacent tissue (Kakulas, 1984). Gliosis is marked by the hypertrophy of astrocytes, as shown by the immunohistochemical labeling of GFAP (Bunge et al., 1997; Iizuka et al., 1987).

Furthermore, it has been shown that with injuries of increasing magnitude, the amount of tissue damage also increases (Basso et al., 1996; Bresnahan et al., 1976; Fehlings and Tator, 1995; Griffiths and McCulloch, 1983). In penetrating laceration injuries, or injuries of massive compression, where the glia limitans (pial tissue on the cord surface) is breached as dense mass of connective scar tissue remains in the damaged region (Bunge et al., 1997).

A progressive axonal pathology has been carefully examined following SCI using electron microscopy (Anthes et al., 1995; Balentine, 1978; Bresnahan, 1978; Griffiths and McCulloch, 1983). Shortly after SCI, investigators have described periaxonal space formation between the axon and its myelin sheath (Griffiths and McCulloch, 1983) (Balentine, 1978) (Anthes et al., 1995; Bresnahan, 1978). Distal fibres undergo Wallerian degeneration and, at the proximal stump, there is an accumulation of organelles, presumably due to the interruption of anterograde transport (Balentine, 1978). Notably, axons from injured tissue have a
characteristic appearance as swollen or giant and have thin or absent myelin sheaths (Anthes et al., 1995; Balentine, 1978; Bresnahan, 1978; Griffiths and McCulloch, 1983). A time course study by Bresnahan (Bresnahan, 1978) indicated that small diameter fibers degenerate first, followed by large diameter fibres, but a chronic study by Fehlings (Fehlings and Tator, 1995) indicated a preferential loss of large diameter fibres. Investigators have described a vesicular degeneration, rupture and phagocytosis of myelin sheaths (Anthes et al., 1995; Balentine, 1978). Griffiths and McCullouch (Griffiths and McCulloch, 1983) described remyelination of surviving axons by oligodendrocytes or Schwann cells. Thus, partially myelinated axons in addition to completely demyelinated axons are found chronically following SCI.

**Pathophysiology of Spinal Cord Injury**

Ensuing the initial mechanical insult to the spinal cord, are host of secondary injury processes which exacerbate the primary injury. This cascade of pathophysiological mechanisms includes: ischemia, altered ionic homeostasis, free-radical release/lipid peroxidation, glutamate-mediated excitotoxicity, inflammation and apoptosis. It is also important to note that a complex interplay exists between the primary and secondary injury processes which ultimately leads to paralysis. Figure 1 (pg.24) summarizes the major processes of primary and secondary injury implicated in SCI.

**Ischemia**

Ischemia is thought to be a key mechanism of secondary injury propagating further cellular damage following SCI by rendering tissue anoxic and thus depleting cellular energy stores. The reduction of cellular ATP by ischemia results in an imbalance of energy demand and need. Acidosis ensues as the cell undergoes anaerobic respiration leading to an accumulation of lactate and hydrogen ions within the cell (Du et al., 1999; Hovda et al., 1992).

Microangiographic and electron microscopic studies have shown a reduction in the microcirculation and spinal cord blood flow after SCI (Koyanagi et al., 1993; Koyanagi et al.,
1993; Koyanagi et al., 1993; Tator and Fehlings, 1991; Tator and Koyanagi, 1997). This ischemia seems to persist and does not involve the larger blood vessels, but rather entails a loss of microcirculation involving capillaries, arterioles and venules at, and adjacent to, the injury site (Koyanagi et al., 1993; Koyanagi et al., 1993; Koyanagi et al., 1993; Tator, 1995; Tator and Fehlings, 1991; Tator and Koyanagi, 1997). Vasospasm by the release of vasoactive amines, cord compression and edema are thought to be among the mechanisms that mediate ischemia in SCI (Amar and Levy, 1999; Anthes et al., 1996; Tator and Fehlings, 1991). Also hemorrhage, thrombosis and platelet aggregation might contribute to ischemia following SCI. Functionally, post-traumatic ischemia has been linked to axonal dysfunction after spinal cord trauma and is proportional to the amount of ischemia sustained by the spinal cord (Fehlings et al., 1989). Systemically, neurogenic shock results; there is a loss of autoregulation, an initial increase in heart rate followed by a subsequent decline in the arterial pressure and cardiac output of rats after SCI (Tator, 1995; Tator and Fehlings, 1991). Post-traumatic ischemia has been of major research interest because it precedes many of the other secondary injury mechanisms and is postulated to mediate many of them (i.e. excitotoxicity, altered ionic homeostasis) (Tator and Fehlings, 1991).

**Altered Ionic Homeostasis**

The ischemia resulting from SCI causes many perturbations in the cell and one of the earliest is the depletion of cellular ATP which disrupts energy-dependent mechanisms, such as cellular ionic homeostasis. With this loss of control, sodium, potassium, chloride and calcium pass across the cell membrane according to their respective concentration gradients (Amar and Levy, 1999). There is a net efflux of potassium and magnesium, an influx of sodium, calcium and chloride accompanied by a movement of water into the cell, thus resulting in cellular edema (Amar and Levy, 1999; Du et al., 1999; LoPachin et al., 1999; Stys and Lopachin, 1998).
The altered ionic homeostasis also produces conduction abnormalities in axons, such as conduction block, mediated by the altered expression and distribution of potassium channels (Fehlings and Nashmi, 1996; Hansebout et al., 1993; Nashmi et al., 2000). Studies have shown that sodium influx from the extracellular space may occur through a variety of mechanisms including: voltage-gated sodium channels, sodium-potassium pump dysfunction, glutamate receptors and the sodium-hydrogen exchanger (Agrawal and Fehlings, 1997; Agrawal and Fehlings, 1996; Fehlings and Agrawal, 1995; LoPachin et al., 1999; Stys and Lopachin, 1998). Increased intracellular sodium concentrations may then result in acidosis, membrane depolarization and the release of excitotoxic amino acid neurotransmitters, such as glutamate, thereby precipitating further secondary injury cascades (Amar and Levy, 1999). Also, the rise in intracellular sodium levels elicits a rise in intracellular calcium by the reverse activation of the sodium-calcium exchanger (Amar and Levy, 1999; LoPachin and Lehning, 1997; Stys and Lopachin, 1998).

Reverse action of the sodium-calcium exchanger is not the sole mechanism for an increased cellular load of calcium following SCI. Voltage-gated calcium channels, dysfunction of calcium pumps, glutamate receptors, release from intracellular calcium stores and leak through voltage-sensitive sodium channels all mediate rises in intracellular calcium also (Agrawal et al., 2000; Du et al., 1999; Stys and Lopachin, 1998; Tymianski and Tator, 1996; Young, 1992). This rise in intracellular calcium concentrations produces devastating effects for the cell which are stereotypical regardless of mechanism of central nervous system trauma. Elevated calcium potentiates further increases in calcium, by the release of neurotransmitters, thus increasing cell excitability (Young, 1992). The electron transport chain is interrupted by calcium binding to mitochondrial membrane, further depleting cellular energy stores (Young, 1992). Furthermore, calcium activates a host of enzymes such as: calpains, other proteases, phospholipases, protein kinases, and endonucleases (Agrawal et al., 2000; Du et al., 1999; LoPachin and Lehning, 1997; Schumacher et al., 1999; Schumacher et al., 2000; Tymianski and Tator, 1996; Young, 1992). Activation of these enzymes will result in an increase in free
radicals, cytoskeletal breakdown, apoptosis and necrosis (Tymianski and Tator, 1996; Young, 1992).

**Lipid Hydrolysis, Lipid Peroxidation and Free Radical Release**

Lipid hydrolysis leads to the release of fatty acids by the activation of membrane phospholipases and lipases. Early after SCI, there is a marked increase in the release of arachadonic acid which is a precursor to the cyclooxygenase pathway which produces eicosanoids (Anderson and Hall, 1993). High levels of eicosanoids (prostaglandins and thromboxane) and, in some species leukotrienes, are detected in spinal cord tissue following trauma (Anderson and Hall, 1993). Eicosanoids and leukotrienes can then in turn mediate the inflammatory response, exacerbating tissue damage (Hsu et al., 1996).

Following SCI, there is a rise in the oxygen free radical-mediated fatty acid peroxidation products, such as malonyldialdehyde, as well as a decline in the levels of antioxidants, and an inhibition of lipid peroxidation-sensitive proteins, such as the Na⁺/K⁺ ATPase (Hall, 1996). Methylprednisolone, 21-aminosteroids and vitamin E have been shown to improve neurological function in spinal cord injured cats through their antioxidant actions (Hall, 1996). In particular, methylprednisolone and U-74006F (a 21-aminosteroid) have also decreased tissue damage after SCI (Hall, 1996). Furthermore, it has been hypothesized that lipid peroxidation might play a role in ischemia as studies of methylprenisolone have associated decreased lipid peroxidation with decreased tissue ischemia following spinal cord trauma (Hall, 1996).

**Glutamate Excitotoxicity**

Glutamate is a ubiquitous neurotransmitter that mediates its excitotoxic actions through a variety of ionotropic (NMDA/AMPA/Kainate) and metabotropic (group I, II and III) receptors. Both ionotropic and metabotropic receptors have been implicated in the secondary injury after SCI. It has been shown that the levels of glutamate released during SCI contribute to the pathophysiology of SCI (Liu et al., 1999). The excitotoxic effects of glutamate have been
demonstrated by an increase in the influx of Ca\textsuperscript{2+} prior to secondary damage (Mills et al., 1995; Regan and Choi, 1991). Furthermore, glutamate has also been linked in mediating other secondary injury processes such as inflammation and apoptosis (Matute, 1998; Matute et al., 1997; Nakai et al., 2000; Sanchez-Gomez and Matute, 1999; Tenneti and Lipton, 2000; Wada et al., 1999).

Studies have shown that the application of ionotropic glutamate (NMDA/AMPA/kainate) receptor antagonists, both in vitro and in vivo, have reduced tissue loss and functional deficits associated with SCI (Agrawal and Fehlings, 1997; Amar and Levy, 1999; Faden et al., 1988; Faden and Simon, 1988; Gaviria et al., 2000; Li and Stys, 2000; Rosenberg et al., 1999; Tator, 1995; Wrathall et al., 1994; Wrathall et al., 1996; Wrathall et al., 1997). It has also been shown that ionotropic glutamate receptor-mediated excitotoxicity was Ca\textsuperscript{2+} dependent in the white matter of the rat spinal cord (Li and Stys, 2000). Furthermore, the antagonism of group I mGluR's and of phospholipase C has been shown to improve the recovery of compound action potentials. In contrast, the selective activation of group I mGluR receptors has led to impairments in the compound action potential recovery following a clip compression injury to the in vitro rat dorsal column (Agrawal et al., 1998).

**Inflammation**

Immediately after SCI, an inflammatory reaction is elicited with two waves of invading leukocytes: neutrophils followed by macrophages. Polymorphonuclear neutrophils (PMN's) infiltrate damaged tissue through the recognition of surface adhesion molecules on PMN's and endothelial cells, thus mediating further damage (Hsu et al., 1996; Taoka et al., 1997). Studies with double knockout mice for the adhesion molecules, ICAM-1 and P-selectin, showed improved neurological recovery following spinal cord injury further implicating PMN's in secondary injury processes (Farooque et al., 1999). PMN's generate free radicals, release proteases, cytokines, nitric oxide, eicosanoids and kinins thus potentiating other injury mechanisms such as free-radical mediated lipid peroxidation and cellular protein degradation.
Platelet deposition has also been observed following SCI (Hsu et al., 1996). Platelet degranulation results in the release of inflammatory mediators and proteolytic enzymes, activating PMN's (Hsu et al., 1996). Blight (Blight, 1985) described a secondary pathology of invading macrophages which phagocytosed cellular debris at the injury site. Furthermore, the reduction of macrophages has been shown to improve neurological function and neuronal survival following SCI (Hsu et al., 1996). Studies indicate that mice which have a delayed Wallerian degeneration response, also have a delayed activation of macrophages/microglia after spinal cord crush injury (Fujiki et al., 1996).

**Apoptosis**

Apoptosis is a highly organized form of programmed cell death that is characterized by the activation of specific genes leading to DNA fragmentation, chromatin condensation and cytosolic condensation. Recent reports have indicated that apoptotic cascades contribute to the secondary injury mechanisms following SCI and occur in macrophages/microglia, neurons and glia (Cash et al., 2000; Crowe et al., 1997; Li et al., 1999; Liu et al., 1997; Shuman et al., 1997; Springer et al., 1999; Yong et al., 1998). Oligodendroglial apoptosis has been demonstrated in apposition to degenerating axons (Cash et al., 2000; Emery et al., 1998; Li et al., 1999; Liu et al., 1997). Furthermore, specific cell death signalling molecules have been identified are associated with the apoptosis of oligodendrocytes, through the expression of FAS and p75 on the cell surface (Cash et al., 2000). In addition, caspases, which are cysteine proteases that cleave at aspartate residues, are key downstream effectors of apoptosis. These enzymes have been implicated in apoptosis following SCI, including caspase, 3, 8 and 9 (Cash et al., 2000; Emery et al., 1998; Springer et al., 1999). Another cell death pathway which has been implicated in apoptosis after spinal cord trauma is the p53 pathway (Saito et al., 2000).
In Vivo Experimental Spinal Cord Injury Models

The goals of modeling SCI in vivo are: to produce transient and/or permanent paraplegia; to screen potential pharmacological therapies for SCI and; to study the mechanisms which underlie and augment neurological deficits following SCI. Important criteria for modeling SCI are that a potential model must be relevant, reliable and reproducible in order to enable researchers to draw conclusions from studies using a particular model.

Kinetic Impact Models

All kinetic impact models have a component of contusion where a force is imparted to the cord rapidly, usually in less than 1 second, producing an impulse (energy) (Fehlings and Tator, 1988). Models that utilize this strategy include: the weight-drop models, the clip compression model, the forceps crush model, screw compression model, balloon compression models and vertebral displacement models. All of these models are applied to study pathophysiological mechanisms of SCI as well as to screen potential therapeutic agents because kinetic models mimic the acute mechanical trauma that is sustained by the spinal cord.

Weight-Drop Models

In modeling SCI, the trend has been to take the complex situation of SCI and simplify it by using a quantifiable method. The first modern experimental model of SCI was the Allen weight-drop model in dogs where, after a laminectomy, a given weight could be dropped from a known height through a hollow tube, producing a quantifiable and standardized contusive injury (Allen, 1911). Since Allen’s original model, many researchers have adapted the original weight-drop model to many different species, including non-human primates, sheep, pigs, cats, ferrets and rats, making it the most widely used model of SCI (Anderson, 1982; Balentine, 1978; Balentine, 1978; Basso et al., 1996; Beattie, 1992; Behrmann et al., 1992; Black et al., 1988; Bresnahan, 1978; Bresnahan et al., 1976; Dohrmann et al., 1976; Ford, 1983; Griffiths and
McCulloch, 1983; Gruner, 1992; Kearney et al., 1988; Metz et al., Neurotrauma 2000 Jan; Noyes, 1987; Southern et al., 1990; Stokes, 1992; Stokes et al., 1992; Wrathall et al., 1985; Yeo et al., 1975). A number of groups have modified it further to obtain gradations of injury that are reliable and reproducible in the rat (Basso et al., 1996; Behrmann et al., 1992; Gruner, 1992; Stokes, 1992; Stokes et al., 1992; Wrathall et al., 1985).

A common variation of the weight-drop model has been to drop a known weight from a given height along a metal rod. The weight falls onto a gauge which measures the force of impact which subsequently hits an impounder resting on the dorsal surface of the spinal cord (Black et al., 1988; Dohrmann et al., 1976; Wrathall et al., 1985). In order to ensure reproducibility, this method requires that the animal be placed in a stereotaxic frame and that the spinal column is stabilized by clamps or forceps (Black et al., 1988; Dohrmann et al., 1976; Wrathall et al., 1985). In order to achieve different grades of injury, the height and/or the weight can be varied. The units of injury quantification were described in gram-centimeters, which was the product of the weight and height, however this was later shown to be an inappropriate measure as different weights and heights that produced the same gram-centimeter product did not inflict the same grade of injury severity (Dohrmann et al., 1976).

In addition to the device described above, other variations of the weight-drop method have been developed. For example, to ensure that ventral displacement of the spinal column does not absorb the energy of the impact, a model which places an anvil under the ventral aspect of the cord has been presented (Ford, 1983). Furthermore, a model less invasive than the version of the weight-drop model above has been developed. A weight is dropped through a plexiglass guide tube which impacts an impounder that rests on the dorsal surface of the vertebrae (no laminectomy) and can be placed to produce three different types of trauma: compression, flexion-compression and extension-compression (Southern et al., 1990).

With the demonstration that inherent sources of variability existed in the biomechanical parameters of weight-drop techniques, especially in small animals (multiple impacts, differences in force of impact, velocity of impact, rib and spinal column movements,
reproducibility) investigators sought to engineer devices to control for these problems (Das, 1989; Khan and Griebel, 1983; Khan et al., 1985; Koozekanani et al., 1976). Pneumatic techniques have been applied to weight-drop models of spinal cord injury which attempt to control certain biomechanical parameters such as the amount and velocity of compression sustained by the spinal cord during trauma (Anderson, 1982; Kearney et al., 1988). Another refined variation of the weight-drop technique is the NYU contusion model in rats (Gruner, 1992). This model monitors biomechanical parameters such as rate of tissue compression, vertebral movement and impact velocity through digital optical potentiometers sensors when a 10g rod falls along a guiding rod from a set height above the surface of the spinal cord (Basso et al., 1996; Gruner, 1992). Monitoring of biomechanical parameters allows the experimenter to discard an animal whose impact does not fall within a set range of parameters for a particular grade of injury (Gruner, 1992). This model has produced behaviourally and anatomically graded, reproducible injuries (Basso et al., 1996).

_Electromechanical Contusion Model_

Recently, a very sophisticated electromechanical spinal injury device has been created that attempts to minimize many of the sources of error that exist in the weight-drop technique (Behrmann et al., 1992; Stokes, 1992; Stokes et al., 1992). In this device (Ohio State University SCI device), an electromagnetic driver and impact pattern generator compress the spinal cord, producing trauma in less than 25 msec to the rat spinal cord (Stokes et al., 1992). The key elements of this model are: 1) the elimination of multiple impacts; 2) the use of sensitive transducers to measure biomechanical parameters; 3) elimination of errors in the calculation of the force due to the mass of the injury probe and 4) the measurement of a starting point by measuring the touch sensitivity (Stokes, 1992). During injury, either the force of impact or spinal cord displacement is the controlled variable and a rat is discarded, should either of the variables exceed the acceptable range. This injury paradigm has produced a consistent, graded injury in rats (Behrmann et al., 1992).
Clip Compression Model

Other kinetic experimental SCI models involve not only contusion, but also a persisting compression which is similar to what frequently occurs in clinical SCI (Tator, 1995) thus, modeling both the primary mechanical trauma and the subsequent ischemia. The Rivlin and Tator rat clip compression model is an example of such a technique. A modified Kerr-Lougheed aneurysm clip with curved blades is used to impact and compress the spinal cord extradurally. After laminectomy, the lower blade is passed anteriorly between the spinal cord and the vertebral bone and the upper blade is allowed to close rapidly on the spinal cord, producing both dorsal and ventral compression (Dolan and Tator, 1979; Dolan et al., 1980; Rivlin and Tator, 1978). The grade of injury in this method can also be varied when the spring of the clip is changed, resulting in different forces of blade closure. Regular calibration of clip closing forces must be performed to ensure that a reproducible injury can be obtained with this method (Dolan and Tator, 1979). This technique has been shown to produce a graded severities of SCI based on anatomical, behavioural and electrophysiological outcomes (Fehlings and Tator, 1995; Fehlings et al., 1989; Fehlings et al., 1987; Khan and Griebel, 1983; Khan et al., 1985; Krassioukov and Fehlings, 1999; Maiorov et al., 1998; Midha et al., 1987; Nashmi et al., 1997). A variation of the Rivlin and Tator rat clip compression injury model has been recently reported (von Euler et al., 1997).

Crush Models

Another SCI model that produces kinetic impact injury is the modified forceps crush model developed by Blight in guinea pigs (Blight, 1991). In this technique, the degree of injury is controlled by compressing the spinal cord to a set thickness with a pair of modified forceps. After laminectomy, the flat tips of the forceps are inserted so that they are on either side of the spinal cord and compressed the spinal cord laterally towards the midline. Compression occurs over 1 second and persists for 15 seconds (Blight, 1991; Gruner et al., 1996). This model has been applied to the rat spinal cord producing a graded injury depending on the thickness of the
compressed spinal cord when behavioural and histological outcome measures were examined (Gruner et al., 1996). Hashimoto and Fukuda (Hashimoto and Fukuda, 1990) have developed a model whereby screws are inserted into a small hole made in the vertebrae of rats. The principle of injury is similar to the modified forceps crush model in that the controlled variable is the extent of spinal cord compression. Screws of different lengths are used to impart compression from 5 min to 1 hour thus producing a graded injury. The model takes advantage of an intact vertebrae exerting pressure onto the spinal cord during injury.

**Inflatable Balloon/Cuff**

Rapid compression by an inflatable balloon/cuff has also been described (Khan and Griebel, 1983; Khan et al., 1985; Martin et al., 1992; Martin and Bloedel, 1973; Tarlov, 1957; Tator, 1973). Circumferential compression by an inflatable cuff was produced in monkeys by passing a Silastic cuff around the exposed spinal cord in the extradural space and rapidly inflating the cuff to a set pressure by a sphygmomanometer. The duration of compression could also be varied from 2 to 5 minutes, resulting in an injury that resembles the fracture-dislocation injury observed in man (Tator, 1973). However, application of this model to smaller animals, such as the rat, using a Fogarty balloon catheter resulted in a steep, dose response curve, making graded injuries difficult (Khan and Griebel, 1983). This is compounded by the fact that the pressure exerted on the spinal cord is extremely difficult to measure with accuracy (Fehlings and Tator, 1988). Recently, it has been shown that inflatable balloons inserted into the subarachnoid space can produce graded injuries in rats however a disadvantage of this technique is the uncertainty of the spinal injury level when the balloon is inserted (Martin et al., 1992).

**Other Kinetic Models**

A vertebral displacement model has been described which offers the advantage of not requiring a laminectomy to produce injury (Fialho et al., 1982). This technique produces spinal cord trauma by luxation between the L1 and L2 vertebrae or fractures of L1 and L2 in dogs.
Unfortunately, this method varies in the force that is applied to the spinal column (i.e. 1848N to 2666N), thus adding additional variability to the system.

**Static Load Models**

Static load models apply compression slowly to the spinal cord (Wada et al., 1999). In contrast to the kinetic impact models, static load models do not replicate the primary mechanical insult to the spinal cord. However, the static load models do contribute information about the secondary injury processes which follow primary mechanical insults to the spinal cord (Wada et al., 1999; Yu et al., 1999). Eidelberg (Eidelberg et al., 1976) (Eidelberg et al., 1977) produced static load SC1 in ferrets by adding weights to a pressor device which resulted in submaximal SCI. From static balloon inflation techniques in monkeys, it was concluded that blood flow changes do not appear to be significant pathological mechanisms in this type of injury (Kobrine et al., 1978; Kobrine et al., 1979). Furthermore, other investigators concluded that duration of load compression did not affect outcome and that load force did not correlate with the inclined plane test for neurological outcome (Black et al., 1986; Kushner et al., 1987). Unfortunately, the static load models have the limitation of not mimicking the pathology of human spinal cord injury (Black et al., 1986; Fehlings and Tator, 1988). Specifically, static load injury models do not produce the same vascular damage as is observed in kinetic models (Fehlings and Tator, 1988); moreover Black and colleagues (Black et al., 1986) concluded that the damage is primarily located in the posterior half of the spinal cord.

**Transection Models**

Transection and hemisection models all involve cutting the spinal cord with a knife or blade. Complete transection models are useful in studies of regeneration, provided that the experimenter is certain that they have achieved a complete transection. Incomplete lesions can lead to incorrect conclusions of regenerating axons (Das, 1989). Complete transection studies can also permit studies of segmental reflexes, serve as controls for studies of
retrograde tracing and locomotor recovery (Basso et al., 1996; Edgerton et al., 1992; Ritz et al., 1992). For studies of descending pathways, hemisections are also important, when done with precision, because they may provide information about the role of specific pathways in locomotion (Fernandes et al., 1999; Jenkins et al., 1993; Little et al., 1988; Ritz et al., 1992; Windle et al., 1951).

Other SCI Models

A number of other mechanical and non-mechanical approaches have been used to create experimental SCI. All of the mechanical approaches outlined above utilize posterior approaches to producing injury, however, one study has detailed an anterior approach (Benzel et al., 1990). In this method, an aneurysm clamp is inserted into the upper lumbar, retroperitoneal space, ensuring that the clamp was immediately anterior to the vertebral column using an operating microscope. The flat end of a knife handle is placed between the clamp and the dorsal part of the spinal column before the clamp is closed. This model minimizes focal injuries to the dorsal spinal cord so that a focal injury is from the ventral side (Benzel et al., 1990). This type of injury, although clinically appealing, is not ideal because the ventrally applied force is not graded. Another closed model of acceleration-deceleration producing a C1-2 subluxation has been reviewed, although the variability in injury severity and location preclude its use in controlled experimental studies (Fehlings and Tator, 1988).

Among the non-mechanical, non-graded approaches, freezing injury has been described as a method for studying spinal cord tissue injury and repair in the rat (Collins et al., 1986). An interesting finding was that the experimental conditions demonstrated axonal outgrowths and may provide an alternate model for studying regeneration (Collins et al., 1986). Photochemical induced vascular stasis is another non-mechanical, non-graded injury procedure that has been developed (Gaviria et al., 2000; Watson et al., 1986). A photosensitizing dye (rose bengal) is injected into a rat before the exposed spinal cord is subjected to irradiation at a wavelength of 560nm for 40 min. The dye generates an oxygen
free-radical thus peroxidizing endothelial cells, stimulating platelet adherence and producing vascular occlusion. The pathology described by the investigators included hemorrhagic necrosis of the gray matter, edema and vascular congestion (Watson et al., 1986). The element of induced vascular stasis may make this model useful in studying vascular dysfunction following SCI. Microwave hyperthermia has also been applied to the rabbit spinal cord to produce non-invasive graded injuries (Sutton, 1988). Somatosensory evoked potentials were measured following microwave irradiation at 915MHz which raised intraspinal temperatures from 40 to 43°C for time periods ranging from 15 to 60 minutes and complete paraplegia was obtained after irradiation for 30 minutes at 42°C (Sutton, 1988). Finally, lesioning by radiofrequency in the cat spinal cord produces a non-graded, consistent injury that does not spread like the mechanical approaches discussed previously (Haghighi et al., 1996).

**Outcome Measures to Assess Severity of Chronic Spinal Cord Injury In Vivo**

**Neurological**

Openfield locomotor function has been widely used to assess recovery from *in vivo* SCI. Modifications to the Tarlov locomotor scale were the most commonly used indicators of hindlimb motor recovery. The modified Tarlov scale ranges from no spontaneous movement to normal walking (Blight, 1996; Fehlings et al., 1989; Kerasidis et al., 1987; Stokes and Homer, 1996). Recently, a more sensitive and reliable method for assessing openfield walking in rats has been reported, termed the BBB (Basso, Beattie and Bresnahan) Locomotor Rating Scale (Basso et al., 1995). This scale discriminates between slight and extensive joint movements and; between occasional, frequent and consistent coordination and stepping in rats and has been applied to mice (Basso et al., 1995; Jakeman et al., 2000; Ma et al., 1999).

In addition to testing openfield walking, postural function can also be reliably tested using the Inclined Plane Test developed by Rivlin and Tator (Rivlin and Tator, 1977) which has been shown to correlate with anatomical outcome measures in rats (Fehlings and Tator, 1995).
The Inclined Plane Test requires that the rat is placed horizontally on a rubber mat and the maximal angle which a rat could maintain its balance for 5 seconds is recorded (Rivlin and Tator, 1977).

It has been proposed that a combination of locomotor and reflex tests can be applied to assess behavioural function in rats following SCI (Kunkel-Bagden et al., 1993). The Combined Behavioural Score (CBS), introduced by Wrathall, has attempted to use this principle to assess functional deficits in both rats and mice (Kerasidis et al., 1987; Kuhn and Wrathall, 1998; Wrathall et al., 1985). This testing paradigm consists of a battery of behavioural tests including: motor score, toe spread reflex, righting reflex, withdrawal reflexes, placing reflex, swim test, Inclined Plane test, platform hang, wire mesh descent, rope walk and tail flick response. The scores from each test are combined, resulting in the combined behavioural score for an animal. Footprint analyses, rotarod tests and grid walking tests are also often used by investigators to assess motor function in rats and mice (Behrmann et al., 1992; Crawley and Paylor, 1997). However, these tend to detect deficits in coordination among rodents with slight to moderate neurological deficits and often do not detect differences between rodents with complete injuries. In general, when behavioural tests are applied, two independent, blinded observers are required in order to ensure that objectivity is maintained and that bias is eliminated.

**Neurophysiological**

The measurement of evoked potentials is also useful to study functional tissue responses following SCI. Specifically, somatosensory and motor evoked potentials are the most predominantly used tests (Fehlings et al., 1989; Fehlings et al., 1987; Nashmi et al., 1997). The attraction of using electrophysiological recordings measures is their objectivity in quantifying the integrity of sensory and motor tracts in the spinal cord. Despite these attributes, SSEP’s and MEP’s have important limitations. For example, in a recent report myoelectric motor evoked potentials (MEP’s) were concluded to not differentiate well between different severities of SCI (Nashmi et al., 1997). Also, evoked potentials are technically challenging,
selective for specific tracts and the responses are non-linearly related to the tissue damage in those tracts (Blight, 1992).

**Histopathological/Anatomical**

There are many histopathological outcome measures that are suitable for quantifying the degree of injury severity and present the opportunity to infer structure/function relationships following SCI when combined with neurological and/or neurophysiological techniques. Digital morphometry provides an accurate method of measuring residual tissue at and adjacent to the injury site (Agrawal and Fehlings, 1997; Basso et al., 1996; Beattie, 1992; Behrmann et al., 1992; Noble and Wrathall, 1985; Schumacher et al., 2000). This requires staining the fixed spinal cord tissue with Luxol Fast Blue for myelin and counterstaining with Nissl stain, Cresyl Violet or Hematoxylin and Eosin. Usually every fifth or tenth transverse section is analyzed under brightfield microscopy for the cross-sectional area of cavity, neuron sparing, white and gray matter and lesion length. Furthermore, three-dimensional reconstructions can be done of the injury site to describe the shape of the lesion.

Light microscopic studies can also be performed to measure the surviving axons and the degree of myelination through the injury site (Blight, 1991; Blight and Decrescito, 1986; Eidelberg et al., 1977; Fehlings and Tator, 1995; Gruner et al., 1996). One method involves staining transverse sections with toluidine blue and observing the sections under oil immersion at 1000x magnification. Visualized axons can then be counted by a line-sampling technique as well, myelination indices can be calculated (Blight, 1991; Blight and Decrescito, 1986; Fehlings and Tator, 1995).

Another histopathological outcome measure which assesses the integrity of axons through the injury site is retrograde tracing by molecules such as horse-radish peroxidase (HRP) or Fluoro-Gold (Eidelberg et al., 1981; Fehlings and Tator, 1995; Hogan, 1992; Midha et al., 1987; Naso et al., 1993). This can be accomplished by implanting the retrograde tracer several segments caudal to the injury site and allowing uptake and transport of the tracer to the
Following fixation, the brain can be extracted, sectioned, processed and examined for labeled neurons in regions of the brain stem that are involved in motor function (i.e. raphe nuclei, reticular nuclei, vestibular nuclei, red nuclei, rostro-ventrolateral medulla and motor cortex). Ideally histopathological analyses should be performed in a blinded fashion in order to minimize observer bias in experimental studies, especially in those of a pre-clinical nature.

In Vivo Murine Models of Spinal Cord Injury

In recent years, mice have been used to study spinal cord injury in vivo. Table 1 (pg.25) outlines the different models and outcome measures used to produce experimental SCI in mice. Inherent physiological differences of mice compared to other animals used to study SCI, along with their small size, present technical challenges as it may not be feasible to apply complex or large apparatuses reliably to produce graded injuries in mice. Thus, transection studies have been commonplace to study spinal cord pathophysiology and repair in mice (Bartholdi and Schwab, 1997; Bjugn et al., 1997; Huang et al., 1999; Pekny et al., 1999; Wang et al., 1997; Zhang et al., 1998). Wang et al. (Wang et al., 1997) studied GFAP knockout mice following dorsal hemisections at T8 using immunofluorescence for extracellular matrix molecules and found that the absence of GFAP in reactive astrocytes does not have an effect on axonal sprouting or regeneration. Pekny et al. (Pekny et al., 1999) studied GFAP and vimentin double knockout mice following an upper thoracic hemisection and a 5mm longitudinal section of the spinal cord through the dorsal funiculus for nestin immunofluorescence and in situ hybridization, as well as, morphological assessment of blood vessel diameter and glial scar formation. This group found that both GFAP and vimentin are necessary for glial scar formation following CNS trauma (Pekny et al., 1999). Lateral transections were performed at T8 in C57Bl/6 mice and mutants for a delayed onset of Wallerian degeneration (Zhang et al., 1998). It was found that mice with a delayed Wallerian degeneration response had a deficit in tissue repair responses as measured by the quantitative morphometry of cavity and motor recovery (Zhang et al., 1998). Bartholdi and Schwab (Bartholdi and Schwab, 1997) hemisectioned the
spinal cord of C57Bl/6 mice at T9 in order to study the expression of pro-inflammatory cytokines and chemokines following SCI using immunofluorescence and in situ hybridization. This study indicated that TNFα, IL-1, MIP-1α and MIP-1β are upregulated within 1 hour following SCI and that resident microglia are the likely source of the mRNA for these molecules (Bartholdi and Schwab, 1997). In another experiment, the number of distal ventral horn neurons were not found to change after spinal cord transection at T9-T12 (Bjugn et al., 1997).

Most recently, extensive spinal cord regeneration was reported by investigators, using the techniques of retrograde tracing, anterograde tracing and motor function, following complete spinal cord transection in BALB/c mice immunized against myelin and inhibitory proteoglycans (Huang et al., 1999).

An ischemia model of SCI was reported in C57Bl/6NcrlBR mice following aortic arch, left subclavian artery and internal mammary artery cross-clamping for 9 or 11 minutes (Lang-Lazdunski et al., 2000). In this model, motor deficit and spinal cord tissue damage were the outcomes chosen to evaluate the injury severity. Radiation has also been used to generate SCI in C3Hf/Sed/Kam mice (Lavey et al., 1994; Lo et al., 1993; Lo et al., 1992). In these studies it was determined that onset and recovery from paralysis depended on the extent, timing and fraction size of radiation delivered to the lower thoracic/upper lumbar spinal cord.

Non-graded, crush injuries have also been applied to the mouse spinal cord using forceps. The degree of cavitation, GFAP immunohistochemistry and the activation of microglia/macrophages was compared in mice with a mutation for delayed Wallerian degeneration were against C57Bl/6 mice following crush injury with forceps at T8 in a time course study (Fujiki et al., 1996; Zhang et al., 1996). The mice with delayed Wallerian degeneration exhibited a delayed activation of microglia/macrophages and astrocytes, and deficient tissue repair (Fujiki et al., 1996; Zhang et al., 1996). The transport system for TNFα across the blood-brain barrier was upregulated and functional deficits were observed after forceps crush injury at L1-L2 in ICR mice (Pan et al., 1997; Pan et al., 1999). Improved open-field walking ability was observed after CD1 mice were treated with an antiangiogenic
polysaccharide (CM101) following spinal cord crush injury with forceps at T10-T11 (Wamil et al., 1998).

Graded, weight-drop models of SCI have also been recently applied to murine systems (Jakeman et al., 2000; Kuhn and Wrathall, 1998; Ma et al., 1999; Ma et al., 1998). The classic weight drop model has been developed and characterized using neurological tests (the combined behavioural score) and anatomical assessments of the injury site following two graded injuries (and sham injury) at T8 in C57Bl/6 mice (Kuhn and Wrathall, 1998). In addition, correlations between behavioural indices (inclined plane, mesh descent, rope walk, bar grab and motor score with anatomical indices (residual white matter and lesion length) were done to further quantify this model (Kuhn and Wrathall, 1998). The Ohio State University SCI device was also adapted in order to produce a controlled contusion injury in C57Bl/6, Balb/C and B10/PL mice (Jakeman et al., 2000; Ma et al., 1999; Ma et al., 1998). Data from these studies indicate that graded injury can be produced using this device and that injury severity and BBB scores are correlated with the percentage of spared white matter and biomechanical parameters of injury (i.e. impulse) (Jakeman et al., 2000; Ma et al., 1999).

A graded, static load model has also been adapted to examine double knockout mice for P-selectin and ICAM-1 (Farooque et al., 1999). Following two severities of injury at T8, these mice were compared to wild-type mice for behavioural differences in response to static load SCI (Farooque et al., 1999). This group concluded that knockout of these genes resulted in improved neurological recovery following SCI, however uninjured shams were not examined in this study, thus evidence was not provided to ensure that the operative procedures did not induce injury. A follow-up study with ICAM-1 knockout mice indicated that, this molecule does not mediate secondary tissue damage alone following traumatic SCI (Isaksson et al., 2000).

There are important limitations of the present techniques used in murine systems. Firstly, the transection, ischemia, irradiation and crush mouse models of SCI are not graded. Transection models have the additional drawback of not mimicking the elements of clinical SCI, typically involving an injury mediated by burst fracture of the vertebral body followed by a
persisting circumferential compression (Fehlings and Tator, 1988; Tator, 1973; Tator, 1983).

Secondly, the graded contusive models lack a rigorous quantitation of anatomical and neurological outcomes and the relationships between them in the mouse. Moreover, animal stability, low variability and ease of injury production are still not fully assessed in the mouse contusion models.

The selection of strains is an important issue in the development of a murine model of SCI. There are two general categories of mice: inbred and outbred strains. An inbred strain is one where brother-sister matings have been performed for at least twenty generations, and the line can be traced back to a single breeding pair (Picciotto and Wickman, 1998). As a result of this breeding strategy, inbred mice are homozygous at every gene allele (Picciotto and Wickman, 1998). In contrast, outbred mice have not undergone such mating schemes and thus have more genetic variability and resilience among its members. Selection of a strain is important factor because different inbred strains have different physiological characteristics and, in some cases, direct comparisons cannot be made between different inbred strains (Cutler, 1993; Davis et al., 1995; Gao and Cutler, 1992; Gerlai, 1998; Gerlai, 1998; Homanics et al., 1999; Picciotto and Wickman, 1998; Tsao et al., 1999).

In the present study, the adaptation of an existing, well-developed rat model of SCI to a resilient, outbred strain (CD1) has been used to develop and characterize the general response of the murine spinal cord to injury at T3-4.
FIGURE 1: Mechanisms of Injury Involved in Spinal Cord Trauma

**PRIMARY INSULTS**
- Contusion
- Stretch
- Laceration
- Persisting Compression

**SECONDARY INJURY PROCESSES**

- **Ischemia**
  - anoxia
  - ↓ energy supply
  - acidosis
  - free radical release

- **Lipid Hydrolysis**
  - ↑ arachadonic acid, eicosanoids, leukotrienes

- **Glutamate Excitotoxicity**
  - ↑ cellular sodium, calcium

- **Ionic Disruptions**
  - ↑ cellular sodium, calcium and chloride
  - ↓ cellular potassium and magnesium
  - edema, membrane depolarization, release of EAA's
  - activation of proteases, phospholipases and endonucleases

- **Inflammation**
  - activation of macrophages and neutrophils
  - release of proteases, free radical, cytokines, NO, eicosanoids

- **Apoptosis**
  - oligodendroglial, neuronal death
# TABLE 1: Murine Spinal Cord Injury Models

<table>
<thead>
<tr>
<th>Injury Model</th>
<th>References</th>
<th>Level of Injury</th>
<th>Outcome Measures</th>
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<tbody>
<tr>
<td>Transection/Hemisection</td>
<td>• Bartholdi and Schwab, 1997</td>
<td>T9</td>
<td>• Immunohistochemistry, <em>in situ</em> hybridization</td>
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<tr>
<td></td>
<td>• Bjugn et al., 1997</td>
<td>T9-T12</td>
<td>• Cell counts, cell morphometry</td>
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<td></td>
<td>• Wang et al., 1997</td>
<td>T8</td>
<td>• Immunohistochemistry</td>
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<td></td>
<td>• Zhang et al., 1998</td>
<td>T8</td>
<td>• Motor function, lesion morphometry</td>
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<tr>
<td></td>
<td>• Huang et al., 1999</td>
<td>Lower thoracic</td>
<td>• Motor function, retrograde &amp; anterograde tracing</td>
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<tr>
<td></td>
<td>• Pekny et al., 1999</td>
<td>Upper-mid thoracic</td>
<td>• Immunohistochemistry, <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>Ischemia</td>
<td>• Lang-Lazdunski et al., 2000</td>
<td>Lower thoracic/Upper lumbar</td>
<td>• Motor function, lesion size</td>
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| Radiation                    | • Lo et al., 1992
• Lo et al., 1993
• Lavey et al., 1994       | Lower thoracic/Upper lumbar     | • Motor function, lesion size                                                   |
| Forceps Crush                | • Fujiki et al., 1996
• Zhang et al., 1996
• Pan et al., 1997
• Pan et al., 1999
• Wamit et al., 1998      | T8                           | • Lesion morphometry, immunohistochemistry                                   |
|                              |                                  | L1-L2          | • Motor function, serum protein radiolabeling                                   |
|                              |                                  | T10-T11        | • Motor function, MRI scan                                                       |
| Weight Drop Contusion        | • Kuhn and Wrathall, 1998
• Ma et al., 1998
• Ma et al., 1999
• Jakeman et al., 2000    | T8                           | • Motor function, lesion size                                                   |
|                              |                                  | T9             | • Motor function, immunohistochemistry, lesion size, lesion site reconstruction |
| Static Load Compression      | • Farooque et al., 1999
• Farooque, 2000
• Isaksson, 2000        | T8                           | • Motor function, lesion histopathology                                         |
METHODS

**Clip Design and Calibration**

The clip used to produce spinal cord injury was a modified Kerr-Lougheed aneurysm clip, which has already been applied to produce SCI in rats (Dolan et al., 1980). However, in order to adapt the clip to the mouse spinal cord, several design modifications were made which are illustrated in Figures 2A and 2B (pg.33). The clip consists of two curved, flattened blades that are 1 mm in width. The curved ends of the blades are tapered so that thickness at the tips is 0.3 mm to a maximal thickness of 0.6 mm. The length of the blades was also shortened to 13 mm in order to allow application to the murine spinal cord. A C-shaped spring closes the blades of the clip, which rotate about a ball, serving as a fulcrum. The closing force of the blades can be calculated by the following relationship of Hooke’s Law: $F = kx$, where $F$ is the closing force, $k$ is the spring constant and $x$ is the displacement of the blades. Thus, at maximal blade displacement, the closing force is maximal. A modified pair of forceps, with tips to fit the flanges of the blades, was used as the applicator. Clips of three magnitudes of closing force were used to produce traumatic SCI in mice: 3g, 8g and 24g. These closing forces were selected by testing intermediate forces in pilot experiments on 24 mice until three distinct severities of injury were achieved based on neurological assessments (BBB and Inclined Plane tests).

The mouse clips were calibrated regularly during the course of the experiments in order to ensure that the closing force of the clip remained constant. Clip calibration was performed by hooking wires onto the grooves of the blades, and one of the wires was attached to a double arm balance. Increasing weights were applied to the balance and resulted in increasing blade displacements, thereby measuring the opening forces of the clip. Applying linear regression to the measured blade displacements at increasing weights the relationship of Hooke’s Law was derived for the clip and the force applied perpendicular to the cord was determined. See Dolan and Tator (Dolan and Tator, 1979) and Fehlings and Nashmi (Fehlings and Nashmi, 1997) for further details.
**Mouse Spinal Cord Injuries**

All experimental protocols of this study were approved by the animal care committee of the Toronto Western Hospital Research Institute in accordance with the policies established in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council of Animal Care.

Female, adult CD1 mice (20-24g) (Charles River, Canada) were deeply anesthetized with halothane (2%), nitrous oxide and oxygen (1:1/min), as evidenced by lack of response to a nociceptive stimulus. A 0.5cc injection of saline was given subcutaneously and during surgery, the mice were placed on a heating pad at 37°C. Under aseptic conditions, a longitudinal incision was made on the midline of the back exposing the superficial muscle layers. These muscle layers were bluntly dissected away and the muscle attached to the vertebrae was removed using a No. 15 scalpel blade, exposing the T2-T5 vertebrae. Using the large spinous process of the T2 vertebrae as a landmark, a laminectomy was performed on the T3 and T4 vertebrae with a pair of microscissors. In addition, part of the pedicles of the T3 and T4 vertebrae were also removed. A dissecting hook was then applied to clear an extradural path between the spinal cord and the vertebral body that remained on the ventral side. Gelfoam, saturated with saline, was used to control excessive bleeding during the laminectomy procedure.

The spinal cord was compressed anteriorly and posteriorly at T3-T4 by extradurally applying a modified Kerr-Lougheed aneurysm clip for 1 minute (Walsh Manufacturing Ltd., Mississauga, Canada). One minute was chosen as the length of clip compressive injury based on pilot studies with differing times of clip compression. The one minute duration of clip compression produced the most reliable neurological injury/recovery profile. The lower blade was passed underneath the cord, in the path cleared by the dissecting hook, and the upper blade was released and allowed to close rapidly by release of the applicator designed for the mouse clips (Walsh Manufacturing Ltd., Mississauga, Canada), thus producing the compressive injury (Figure 3, pg.35). The clip was allowed to compress the spinal cord for 1
minute before removal. Five groups of mice (n=12 per group) were injured for the chronic
behavioural and anatomical data in this study. Three groups were subjected to varying degrees
of clip compression: 3g, 8g and 24g closing forces. In order to produce three levels of injury
severities, three identical clips were manufactured with different springs, thus producing three
levels of closing force. The remaining two groups studied were subjected to sham and
transection control injuries. The sham control mice were subjected to a laminectomy and hook
dissection, but the clip was not applied to the spinal cord. The transection control animals were
also subjected to a laminectomy, the dura mater was slit at T3 and T4 and a No. 11 scalpel
blade was used to completely transect the spinal cord. Complete transection was confirmed by
direct visual inspection. When the injury procedure was completed, the superficial muscle
layers were sutured using a 6.0 continuous suture and the skin incisions were closed using
small Michel clips. An additional eight animals were injured (four at 8g clip compression and
four at 24g clip compression) for 1 day and 7 day lesion site analysis after SCI.

Following surgery, 1.0cc of saline was administered subcutaneously in order to replace
the blood volume lost during the surgery. During recovery from anesthesia, the mice placed on
a warm heating pad and covered with a warm towel. The mice were singly housed in a
temperature-controlled room at 27°C for a survival period of 28 days. Food and water were
provided to the mice *ad libitum*. During this time period, the animals’ bladders were manually
voided twice a day until the mice were able to regain normal bladder function. The incidence of
hematuria was low (6 cases) and gentamycin was administered once daily in the event of
hematuria (20mg/kg) subcutaneously once a day for 5 days. Concomitantly 0.5cc of saline was
administered everyday that gentamycin was given.

**Quantitative Assessments of Motor Function**

Prior to injury, all of the mice were pre-conditioned to the openfield environment used to
test locomotor function. All neurological assessments were performed on Days 1, 4, 7, 10, 14,
21 and 28 post injury during the active phase of the rodent sleep/wake cycle. The five groups of
mice were blindly assessed for functional recovery by adapting the Basso, Beattie and Bresnahan (BBB) hindlimb locomotor test using two independent observers (Basso et al., 1995). The BBB scale used is shown in Table 2 (pg.37). Neurological function was also assessed using the Inclined Plane (IP) test developed by Rivlin and Tator (Rivlin and Tator, 1977). This test involved placing the mice horizontally on a rubber mat and recording the maximal angle of the board at which the animal could maintain balance for 5 seconds. The rubber mat used for the IP test was modified for the mice such that the grooves in the mat were thinner, shallower and closer together.

**Retrograde Labeling of Neurons**

On day 28, the mice were deeply anesthesized again with halothane, nitrous oxide and oxygen. Another incision was made on the back exposing the superficial muscle layers. These muscle layers were bluntly dissected away and the muscle attached to the vertebrae was removed using a No. 15 scalpel blade, exposing the dorsal vertebral surface at T9 (approximately 5-6 mm caudal to the clip compression injury site). The mice underwent a T9 laminectomy and subsequently the dura was cut open using a No. 11 scalpel blade and the spinal cord was transected with a single slice using the No. 11 scalpel blade. Once the bleeding had ceased, a Gelfoam pledget (3mm x 1mm) saturated with 4% Fluoro-Gold (FG, Fluorochrome Inc., Colorado, USA) in sterile saline was introduced at the transection site and placed at the rostral cut end of the spinal cord. The transection site was covered with petroleum jelly (Vaseline) in order to prevent diffusion of Fluoro-Gold. The superficial muscle layers were sutured using a 6.0 continuous suture and the skin incisions were closed using small Michel clips.

**Tissue Extraction and Processing**

Five days following Fluoro-Gold introduction, all of the mice from the chronic subset were sacrificed. From the eight acutely injured mice, two 8g and two 24g mice were sacrificed
at 1 day post injury, and the other two 8g and two 24g mice were sacrificed at 7 days post injury. All of the mice were perfused after deep anesthesia with 0.2cc of sodium pentobarbital (Somnotol), as evidenced by lack of response to a nociceptive stimulus. After ventricular injection of heparin, the mice were perfused transcardially with 125ml of 4% paraformaldehyde. The injury site and adjacent spinal cord tissue was extracted and post-fixed in 4% paraformaldehyde. The spinal cords underwent manual processing through increasing alcohols, chloroform and paraffin wax for paraffin embedding of spinal cord blocks. The brains were also removed and post-fixed in 4% paraformaldehyde and then placed in 30% sucrose for cryopreservation.

**Morphometric Assessments of Spinal Cord Tissue**

Paraffin embedded blocks of spinal cord tissue from twenty four randomly selected animals (n=6/group) 5 weeks after injury and the eight spinal cords at 1 day and 7 days post injury were cut into 10μm transverse sections and stained with Luxol Fast Blue (LFB), to identify residual white matter, and counter-stained with Hematoxylin and Eosin (H&E), to identify non white matter. Spinal cords taken from the sham, 3g, 8g and 24g injury severity groups 5 weeks after injury underwent quantitative morphometric analysis in a blinded fashion. The injury epicenter and sections at every 50μm until 1500μm rostral and caudal to the injury epicenter were examined under 40x (4x objective, 10x secondary) magnification using brightfield microscopy (Nikon E800 Microscope) with an ImagePro Plus image analysis system for quantification of spared white matter, spared gray matter and cavity size after clip compressive SCI. A low power microscopic image of each transverse section to be analyzed was digitalized using the image analysis software and calibrated in mm². The image of the section was outlined and the total cross-sectional area was measured with the image analysis software. Areas of cavity within the section were next identified by the technique of colour selection, using the background colour of the slide as the criterion for areas of cavity formation. The total cavity area was expressed and also recorded. The areas of spared gray and white
matter were identified by their cytoarchitectural appearance, as well as, by their histochemical staining properties. The regions of residual gray and white matter were then outlined, and the enclosed area was also measured.

**Counts of Retrogradely Labeled Neurons in Key Motor Areas of the Cerebrum and Brainstem**

Serial 35µm frozen coronal sections were cut from the brains of thirty randomly selected animals (n=6/group) in a cryostat. All five experimental groups (sham, 3g, 8g, 24g and transection control) were analysed for retrograde labeling of descending spinal motor tracts. Alternate sections were mounted and stained with cresyl violet for the identification of the nuclei of interest (raphe nuclei, reticular nuclei, vestibular nuclei, red nuclei and motor cortex). The remaining unstained sections were mounted and coverslipped with Mowiol for the detection of retrogradely labeled neurons with Fluoro-Gold after graded, compressive SCI. Counts were taken, by a blinded observer, of Fluoro-Gold labeled neurons under 100x (10x objective, 10x secondary) magnification fluorescence microscopy (Nikon E800 Microscope) with a UV 2A filter (330-380 nm excitation wavelength).

**Statistical Analysis**

Differences in motor performance following SCI were determined by two-way repeated measures analysis of variance (ANOVA). The subsamples chosen for histopathological analysis from the experimental groups were tested to ensure that they were representative of the animals tested for behavioural function. Differences in the morphometric assessments between groups were determined by two-way analysis of variance (ANOVA). Differences in cell counts in brain nuclei between groups were determined by one-way analysis of variance (ANOVA). For behavioural and histopathological data, all pairwise comparisons were made between groups using a Student Newman-Keuls multiple range post-hoc test. Correlation coefficients were calculated as Pearson Correlation Coefficients (R) and as multiple linear
regression correlation coefficients ($R^2$). A variance stabilizing square root transformation was performed on the counts of neurons retrogradely labeled with Fluoro-Gold before analysis of variance was applied. Data are presented in all graphs as mean ± S.E.M. and differences were reported as statistically significant at p<0.05.
FIGURE 2: (A) Side view of modified mouse aneurysm clip depicting the shortening of blade length to 13mm and tapering of blade thickness from 0.6mm to 0.3mm at the tips. (B) Front view of modified aneurysm clip depicting a blade width of 1.0mm.
FIGURE 3: A view of the mouse spinal cord (A) before and (B) during 8g *in vivo* compressive injury following a laminectomy at T3 and T4.
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</tr>
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</tr>
<tr>
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<td>Weight-supported plantar steps, frequent coordination</td>
</tr>
<tr>
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</tr>
<tr>
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<td>5</td>
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<td>21</td>
<td>Consistent plantar stepping, coordination and toe clearance, tail up, trunk stable</td>
</tr>
</tbody>
</table>

*< 50% range of motion

**> 50% range of motion

***50-94% of the time

****95-100% of the time

†0-50% of the time

‡front/hind limb coordination
RESULTS

Effects of Compressive Spinal Cord Injury Severity on Mortality and Neurological Function

Mortality was less than 10% during the injury procedure and, usually occurred during the laminectomy due to excessive blood loss and/or respiratory failure due to the anaesthetic sensitivities of the mice. The incidence of mortality during the post-injury neurological assessment period was zero. Following Fluoro-Gold introduction, two animals from the 24g injury group and two animals from the transection control group died. Following traumatic spinal cord injury all mice exhibited varying levels of paraplegia and a progressive recovery of function was observed until Day 14 post injury, where BBB and IP scores reached a plateau.

BBB Openfield Locomotor test scores are shown in Figure 4A (pg.45) as the mean combined BBB score for each injury severity ± S.E.M. Sham control mice displayed no neurological deficits, thus indicating that any observed deficits were attributable to the force of clip compression on the spinal cord. A graded decrease in locomotor performance was observed with increasing severities of injury immediately post injury and continued for the duration of the 28 day neurological assessment period. At the end of the 28 day post injury assessment period, the sham control mice performed to a mean score of 21 on the BBB scale, presenting no deficits. The 3g injured mice performed to a mean score of 17 on the BBB scale, presenting mild deficits. The 8g injured mice performed to a mean score of 13 on the BBB, presenting moderate deficits. The 24g injured mice performed to a mean score of 7 on the BBB, presenting severe deficits and; the transection control mice performed to a mean score of 6 on the BBB scale (See Table 2, pg.37, for a description of neurological presentation associated with a given BBB score). A two-way repeated measures ANOVA indicated significant differences with respect to injury severity (p<0.0001), time (p<0.0001) and the injury severity x time interaction (p<0.0001). Post-hoc pairwise comparisons indicated significant differences between all groups at all time points (p<0.05, Student-Newman-Keuls multiple
range test) except between the 3g injury and the 8g injury at Day 7 and no significant differences are found between the 24g injury and transection controls (Table 3, pg.47).

Inclined plane test scores are shown in Figure 4B (pg.45) as the mean inclined plane angle for each injury severity ± S.E.M. There was also a graded decrease in inclined plane performance with increasing severities of injury immediately following injury and continued for the duration of the IP assessment period. Maximal IP performance was observed in the sham control group at an angle of 60° at 28 days. The 3g injury group presented with a mean angle of 56° (mild deficits). The 8g injury group presented with a mean angle of 53° (mild-moderate deficits). The 24g injury group presented with a mean angle of 33° (severe deficits) and; the transection control group presented with a mean angle of 32° on the IP 28 days post injury. A two-way repeated measures ANOVA indicated significant differences with respect to injury severity (p<0.0001), time (p<0.0001) and the injury severity x time interaction (p<0.0001). Post-hoc pairwise comparisons (Table 4, pg.47) indicated significant differences between all groups at all time points (p<0.05, Student-Newman-Keuls multiple range test) except between the 3g injury and the 8g injury at Day 4, 10, 14, 21, 28 and between the 3g injury and sham controls at Day 7, 10, 14, 21, 28. A significant difference was only found between the 24g injury and transection controls at Day 10.

Qualitative Examination of Injury Site Pathology Following Compressive Spinal Cord Injury

Acute

The injury epicenters were examined 1 day and 7 days after 8g and 24g clip compression injury (Figure 5, pg.48). One day following 8g injury, sparing of dorsal, lateral and to a lesser extent, ventral, white matter, central hemorrhagic necrosis in the grey matter and medial white matter was evident. At 7 days following 8g injury, necrotic regions were diminished, white matter vacuolization, sparing of dorsal and lateral white matter and an
Inflammatory infiltrate (mononuclear leukocytes) appeared to be present. In the 24g injury, at 1 day and 7 days, there was more extensive hemorrhagic necrosis and white matter sparing was only seen in the subpial rim. By 7 days following 24g injury, a central cyst had formed. In all of the injuries, cells that appeared to be mononuclear leukocytes, were observed at the injury site and there appeared to be preferential damage in the ventral regions of the spinal cord.

**Chronic**

The injury epicenters in all three compressive injury severities (3g, 8g and 24g), at 5 weeks, were most notably characterized by an extensive network of scar tissue that may be of glial (astrocytes, oligodendrocytes, Schwann cells) or fibrous origin (Figures 6A, B, C and F, pg.50) which extended both rostrally and caudally. Diffuse microcystic cavitation was also evident in the three compressive injuries and occurred to a greater degree in the ventral regions of the spinal cord (Figure 6D, pg.50). The appearance of a robust infiltration of inflammatory cells (mononuclear leukocytes) was frequently observed (Figure 6E, pg.50). Large, central zones of cavitation which are commonly seen in rats, monkeys, and humans were not found (Bresnahan et al., 1976; Bunge et al., 1997; Fehlings and Tator, 1995; Tator, 1995). However, two animals (one 8g and one 24g injury) displayed a smaller, but not microcystic, central cavity (Figure 6C, pg.50). The extent of cavitation and scar tissue progressively diminished with increasing distance from the injury epicenter. Examination of tissue adjacent to the injury epicenter indicated a preferential loss of ventral horns and ventral columns. All but one sham control appeared normal with no damage to the spinal cord.

**Quantitative Morphometric Examination of the Compressive Spinal Cord Injury Site**

Digital morphometric spinal cord analyses of the three compressive injury severities (3g, 8g and 24g) and the sham control involved quantification of the cross-sectional area of residual tissue (normal appearing gray matter and white matter) and cavitation of sections sampled at 50μm increments from the injury epicenter to a distance of 1500μm rostral and 1500μm caudal
from the injury epicenter. The injury epicenter was defined as the section with the least amount of normalized residual tissue. Measurements of residual tissue cross-sectional area and cavity cross-sectional area were expressed as a percent of the total cross-sectional area of the section according to the following equations in order to account for biological differences in spinal cord dimensions between animals.

Percent residual tissue = (Cross-sectional residual tissue area/Total cross-sectional area) x 100

Percent cavity = (Cross-sectional cavity area/Total cross-sectional area) x 100

The following equation could be applied to calculate the cross-sectional area of residual tissue, cavity and scar tissue/inflammatory infiltrate:

Total cross-sectional area = Cross-sectional area of residual tissue + Cross-sectional area of cavity + Cross-sectional area of scar/inflammatory tissue

Figures 6A, B and C (pg.50) show representative sections of the injury epicenter from each injury severity. There was a significant difference in the preservation of percent residual tissue between the three injury severity and the sham control groups (p<0.0001, two-way ANOVA), at discrete 50μm distances from the epicenter (p<0.0001, two-way ANOVA) and the group x distance interaction (p<0.0001, two-way ANOVA). Figure 7 (pg.52) depicts the mean percent residual tissue at every sampled interval. The sham control displayed a very minor loss of intact tissue at the epicenter which will be further explored in the Discussion section. The injury resulting from the 3g clip compression extended for a total of 1000μm (mild injury). The injury from the 8g clip compression extended for a total of 1500μm (moderate injury) and; the injury from the 24g clip compression extended for more than 2000μm (severe injury). The severe 24g injury most clearly displayed a non-symmetrical spread of injury, with a greater extent of injury expansion in the caudal direction. A similar non-symmetrical pattern of injury distribution was not observed in the 3g and 8g injury groups. In the 8g and 24g injuries, the injury spread for a distance longitudinally that was greater than the actual portion which was in contact with the clip (1000μm). However, the spread of 3g injury did not exceed the width of the
clip. At the injury epicenter the percent cross-sectional area of residual tissue was found to be significantly different between all groups (3g, 8g, 24g and sham) by post hoc pairwise testing (p<0.05, Student-Newman-Keuls multiple range test) as illustrated in Figure 8 (pg.54).

Further analyses of the percent cross-sectional area of cavitation also indicated significant differences between the injury severity and sham control groups (p<0.0001, two-way ANOVA), at distances from the epicenter (p<0.0001, two-way ANOVA) and the group x distance interaction (p<0.01, two-way ANOVA). Post hoc pairwise testing demonstrated that at the injury epicenter, the percent cross-sectional area of cavitation was significantly different between all groups except the 3g and 8g, and the 3g and sham controls (p<0.05, Student-Newman-Keuls multiple range test), as shown in Figure 8 (pg.54).

**Effects of Compressive Spinal Cord Injury Severity on the Integrity of Descending Motor Tracts**

Retrograde labeling of brainstem, midbrain and cortical neurons with Fluoro-Gold was applied to assess the integrity of axons that descended through the injury epicenter. The origin of spared axons through the injury epicenter were labeled with Fluoro-Gold and neuron counts were taken of the raphe nuclei, reticular formation, vestibular nuclei, red nucleus and the motor cortex in the 3g, 8g, 24g, sham and transection control groups. Examples of Fluoro-Gold labeled neurons from the red nucleus are shown in Figure 9 (pg.56). The mean raw counts are shown with the corresponding S.E.M. in Figure 10 (pg.58) and range of raw counts in Table 5 (pg.60). Due to unequal variances, one-way ANOVA was performed on the square root transformation of the raw count data and indicated that there were statistically significant differences between the injury severity, sham and transection control groups in all five areas that were examined (p<0.0001). Results detailing all pairwise Student-Newman-Keuls multiple range test post hoc tests between all the injury groups in each of motor regions examined are shown in Table 5 (pg.60) by postscripts for the sake of simplicity. Mean neuron counts repeated the trends seen with the behavioural and morphometric assessments; 3g clip
compression produced mild injury, 8g clip compression produced moderate injury and 24g clip compression produced severe injury.

**Relationships Between Neurological Function, Histopathology and Injury Severity**

The counts of Fluoro-Gold labeled neurons and residual tissue were significantly correlated with neurological function when analyzed independently (Table 6, pg.61). The amount of normalized area of residual tissue at the injury epicenter was very strongly correlated with the BBB scores at Day 28, using the Pearson Correlation Coefficient (R) (R=0.946, p<0.0001). The counts of neurons retrogradely labeled with Fluoro-Gold were also strongly correlated with the BBB scores at Day 28. The extrapyramidal tracts: raphespinal (R=0.814, p<0.0001), reticulospinal (R=0.812, p<0.0001) and vestibulospinal (R=0.813, p<0.0001) tracts were correlated with BBB scores at Day 28 post SCI. The extrapyramidal rubrospinal (R=0.747, p<0.0001) tracts, as well as the pyramidal corticospinal (R=0.776, p<0.0001) tract were correlated with the BBB scores at Day 28.

Strong, positive correlations were identified between the IP scores (28 days post SCI) and number of retrogradely labeled cells in the extrapyramidal tracts: rubrospinal tract (R=0.836, p<0.0001), raphespinal tract (R=0.801, p<0.0001), reticulospinal tract (R=0.782, p<0.0001) and vestibulospinal tract (R=0.790, p<0.0001). A weaker, positive correlation was found between the IP scores at Day 28 following SCI and the number of Fluoro-Gold labeled cells in the pyramidal corticospinal tract (R=0.583, p<0.0001). In addition, the percent area of cross-sectional residual tissue at the injury epicenter was significantly correlated with IP function at Day 28 post SCI (R=0.781, p<0.0001).

In order to ascertain which motor tracts were contributed most significantly to the neurological outcomes, the neuron counts from all the brain regions were analyzed together for correlation with the BBB scores and IP scores at day 28 post-injury, using stepwise multiple linear regression (Table 7, pg.61). The neuron counts from the raphe nuclei (p<0.0001) and the motor cortex (p<0.0001) were found to be significantly related to the BBB scores. The R$^2$
(multiple correlation coefficient) was determined to be 0.805 (F=55.89, p<0.0001) when the counts from the raphe nuclei and motor cortex were correlated with BBB scores. Similarly, the counts from all the brain regions were analyzed together, using stepwise multiple linear regression, for determining which regions correlated significantly with the IP scores at day 28 post-injury. The counts from the vestibular nuclei (p<0.05) and the red nucleus (p<0.001) were found to correlate significantly with the IP scores at day 28 post-injury. The $R^2$ (multiple correlation coefficient) was calculated to be 0.754 (F=41.34, p<0.0001).
Functional Recovery Assessed By BBB Locomotor Performance Following Spinal Cord Injury

A

Functional Recovery Assessed By Inclined Plane Performance Following Spinal Cord Injury

B
FIGURE 4: (A) BBB Openfield Locomotor test scores are shown as mean combined BBB score for each injury group ± S.E.M. (B) Inclined Plane test scores are shown as mean inclined plane angle for each injury group ± S.E.M.
TABLE 3: Summary of Student-Newman-Keuls Post-Hoc Comparisons of BBB Tests

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<th></th>
<th>Sham*</th>
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* means are significantly different by one-way ANOVA (p<0.0001)
α means are significantly different from the 3 g injury mean (p<0.05)
β means are significantly different from the 8 g injury mean (p<0.05)
γ means are significantly different from the 24 g injury mean (p<0.05)
δ means are significantly different from the sham control mean (p<0.05)
ε means are significantly different from the transection control mean (p<0.05)

TABLE 4: Summary of Student-Newman-Keuls Post-Hoc Comparisons of IP Tests

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<td>γδε</td>
<td>γδε</td>
<td>αβδε</td>
<td>αβδε</td>
</tr>
<tr>
<td>Day 7</td>
<td>αβγε</td>
<td>γδε</td>
<td>γδε</td>
<td>αβδε</td>
<td>αβδε</td>
</tr>
<tr>
<td>Day 10</td>
<td>αβγε</td>
<td>γδε</td>
<td>γδε</td>
<td>αβδε</td>
<td>αβδε</td>
</tr>
<tr>
<td>Day 14</td>
<td>αβγε</td>
<td>γδε</td>
<td>γδε</td>
<td>αβδε</td>
<td>αβδε</td>
</tr>
<tr>
<td>Day 21</td>
<td>αβγε</td>
<td>γδε</td>
<td>γδε</td>
<td>αβδε</td>
<td>αβδε</td>
</tr>
<tr>
<td>Day 28</td>
<td>αβγε</td>
<td>γδε</td>
<td>γδε</td>
<td>αβδε</td>
<td>αβδε</td>
</tr>
</tbody>
</table>

* means are significantly different by one-way ANOVA (p<0.0001)
α means are significantly different from the 3 g injury mean (p<0.05)
β means are significantly different from the 8 g injury mean (p<0.05)
γ means are significantly different from the 24 g injury mean (p<0.05)
δ means are significantly different from the sham control mean (p<0.05)
ε means are significantly different from the transection control mean (p<0.05)
FIGURE 5: Cross-sections of mouse spinal cords, stained with Luxol Fast Blue and H&E, at the injury epicenter following clip compression (A) 1 day after 8g injury, (B) 7 days after 8g injury, (C) 1 day after 24g injury and (D) 7 days after 24g injury. Closed arrows indicate areas of white matter sparing, open arrows indicate regions of hemorrhage and double arrows indicate zones of cavity. Scale bar is equal to 100 microns.
FIGURE 6: Cross-sections of mouse spinal cords, stained with Luxol Fast Blue and H&E, 5 weeks following (A) 3g, (B) 8g, (C) 24g clip compression spinal cord injury viewed under low power magnification. Examples of (D) ventral microcystic cavity (E) clusters of inflammatory cells (mononuclear leukocytes) and (F) swirls of scar tissue (astrocytes, oligodendrocytes, Schwann cells or fibroblasts) 5 weeks after SCI viewed under high power magnification. Scale bars are equal to 100 microns.
Distribution of Residual Tissue Following SCI
FIGURE 7: Extent of tissue damage at 50μm rostral-caudal increments from injury epicenter shown as by the measurement of percent cross-sectional area of residual tissue. Injury epicenter is denoted by 0 with positive (+) values indicating caudal to the injury epicenter and negative (-) values indicating rostral to the epicenter.
Cross-Sectional Area of Cavitation and Residual Tissue at the Injury Epicenter

![Graph showing the cross-sectional area of cavitation and residual tissue for different injury groups.

- **Residual Tissue**
  - Sham: High percentage
  - 3g: Moderate percentage
  - 8g: Lower percentage
  - 24g: Lowest percentage

- **Cavitation**
  - Sham: Low percentage
  - 3g: Higher percentage
  - 8g: Highest percentage
  - 24g: Minimal percentage

The graph indicates a significant increase in residual tissue and cavitation in the injury groups compared to the sham group, with the 24g group showing the least residual tissue and cavitation.
FIGURE 8: Percent cross-sectional area of residual tissue and cavitation ± S.E.M. at the injury epicenter. An overall significant difference was found in the area of residual tissue and cavitation (p<0.0001, two-way ANOVA). The percent cross-sectional area of residual tissue was significantly different between all groups (p<0.05, Student-Newman-Keuls test). The percent cross-sectional area of cavitation was significantly different between all groups except between the 3g and 8g, and the 3g and sham controls (p<0.05, Student-Newman-Keuls test).
FIGURE 9: Neurons in the red nucleus retrogradely labeled by fluorogold (introduced into the cord at T9) 5 weeks following (A) 3g, (B) 8g and (C) 24g compressive spinal cord injury at T3-4. Scale bar is equal to 100 microns.
Mean Counts of Fluoro-Gold Labeled Neurons

![Graph showing the mean counts of Fluoro-Gold labeled neurons across different injury groups. The graph displays the number of neurons for different brain regions (Raphe Nuclei, Reticular Formation, Vestibular Nuclei, Red Nucleus, Motor Cortex) for different injury groups (Sham, 3g, 8g, 24g, Transection). The error bars indicate the variability in the counts.](image-url)
FIGURE 10: Mean number of neurons retrogradely labeled with Fluoro-Gold in the raphe nuclei, reticular formation, vestibular nuclei, red nucleus and the motor cortex in each injury group ± S.E.M. An overall significant difference was found between the groups (p<0.0001, one-way ANOVA).
<table>
<thead>
<tr>
<th></th>
<th>Raphe Nuclei</th>
<th>Reticular Formation</th>
<th>Vestibular Nuclei</th>
<th>Red Nucleus</th>
<th>Motor Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean: 921</td>
<td>Mean: 4241</td>
<td>Mean: 1062</td>
<td>Mean: 605</td>
<td>Mean: 940</td>
</tr>
<tr>
<td></td>
<td>Mean: 557</td>
<td>Mean: 2320</td>
<td>Mean: 561</td>
<td>Mean: 660</td>
<td>Mean: 13</td>
</tr>
<tr>
<td><strong>24g</strong></td>
<td>Range: 1-331</td>
<td>Range: 7-674</td>
<td>Range: 1-177</td>
<td>Range: 7-325</td>
<td>Range: 0-3</td>
</tr>
<tr>
<td></td>
<td>Mean: 114</td>
<td>Mean: 270</td>
<td>Mean: 56</td>
<td>Mean: 124</td>
<td>Mean: 1</td>
</tr>
<tr>
<td></td>
<td>Mean: 767</td>
<td>Mean: 3847</td>
<td>Mean: 1343</td>
<td>Mean: 746</td>
<td>Mean: 2041</td>
</tr>
<tr>
<td><strong>Transection</strong></td>
<td>Range: 0-0</td>
<td>Range: 0-0</td>
<td>Range: 0-0</td>
<td>Range: 0-0</td>
<td>Range: 0-0</td>
</tr>
<tr>
<td></td>
<td>Mean: 0</td>
<td>Mean: 0</td>
<td>Mean: 0</td>
<td>Mean: 0</td>
<td>Mean: 0</td>
</tr>
</tbody>
</table>

* means are significantly different by one-way ANOVA (p<0.0001)
α means are significantly different from the 3 g injury mean by Student-Newman-Keuls post hoc test set at p<0.05.
β means are significantly different from the 8 g injury mean by Student-Newman-Keuls post hoc test set at p<0.05.
γ means are significantly different from the 24 g injury mean by Student-Newman-Keuls post hoc test set at p<0.05.
δ means are significantly different from the sham control mean by Student-Newman-Keuls post hoc test set at p<0.05.
ε means are significantly different from the transection control mean by Student-Newman-Keuls post test set at p<0.05.
### TABLE 6: Independent Correlations Between Histopathological Outcome Measures and Neurological Outcome Measures

<table>
<thead>
<tr>
<th>Number of Cells Labeled in</th>
<th>BBB Score at Day 28</th>
<th>IP Score at Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raphe Nuclei</td>
<td>R=0.814*</td>
<td>R=0.801*</td>
</tr>
<tr>
<td>Number of Cells Labeled in Reticular Nuclei</td>
<td>R=0.812*</td>
<td>R=0.782*</td>
</tr>
<tr>
<td>Number of Cells Labeled in Vestibular Nuclei</td>
<td>R=0.813*</td>
<td>R=0.790*</td>
</tr>
<tr>
<td>Number of Cells Labeled in Red Nucleus</td>
<td>R=0.747*</td>
<td>R=0.836*</td>
</tr>
<tr>
<td>Number of Cells Labeled in Motor Cortex</td>
<td>R=0.776*</td>
<td>R=0.583**</td>
</tr>
<tr>
<td>Normalized Cross-Sectional Area of Residual Tissue at Epicenter</td>
<td>R=0.945*</td>
<td>R=0.781*</td>
</tr>
</tbody>
</table>

*indicates p<0.0001  
**indicates p<0.001

### TABLE 7: Summary of Correlations of Cell Counts from Brain Regions using Multiple Linear Regression

<table>
<thead>
<tr>
<th>Neurological Outcomes</th>
<th>Brain Regions which Correlated with Neurological Outcomes</th>
<th>Regression Correlation Coefficient (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBB scores at Day 28</td>
<td>Raphe Nuclei, Motor Cortex</td>
<td>0.805*</td>
</tr>
<tr>
<td>IP scores at Day 28</td>
<td>Vestibular Nuclei, Red Nucleus</td>
<td>0.754*</td>
</tr>
</tbody>
</table>

* indicates p<0.0001
DISCUSSION

The results of this study demonstrate that SCI can be modeled in the murine spinal cord using rapid compression induced by a modified aneurysm clip. Quantitative behavioural and histopathological analyses characterized three distinct grades of injury severity (3g, 8g and 24g) in a dose-response style. Moreover, the behavioral and histopathological parameters were found to correlate with each other. In particular, the counts of Fluoro-Gold labeled neurons in the raphe nuclei and the motor cortex correlated with BBB scores and counts in the vestibular nuclei and red nucleus correlated with IP scores.

Advantages and Disadvantages of the Clip Compressive Model of SCI

This technique of producing injury is an ideal method due to its simplicity in application and clinical relevance. The modified aneurysm clip has the following advantages compared to other kinetic models of injury:

1. The injury consists of an initial contusion, plus a persisting compression, which is a common feature of clinical SCI.

2. The spinal cord sustains both anterior and posterior mechanical damage, which is also a feature of clinical SCI.

3. The concern of spinal column stabilization during injury is abolished due to the presence of the lower blade of the clip.

4. Low variability in the injury event due to regular maintenance and calibration of the clip.

5. The relatively low cost and ease of use of the clip.

Some disadvantages of the clip compressive model of SCI in mice exist. These limitations include:

1. The surgical technique required to produce injury requires a laminectomy procedure, thus reducing the pressure exerted by the bone following traumatic SCI. After human SCI, the spinal cord sustains added pressure from the vertebrae.
2. The laminectomy and hook dissection prior to clip application requires practice to ensure that the surgical procedure does not produce additional injury to the spinal cord.

3. The absence of large central cavities in the mouse spinal cord following injury, compared to SCI in other mammals (including humans), may indicate that there are important pathophysiological differences between mice and other species which need to be considered when results from mouse SCI studies are extrapolated to other species, such as humans.

**Behavioural Responses of Mice to Spinal Cord Injury**

The BBB scores from the three clip compression injury groups were well distributed along the scale, demonstrating light, moderate and severe injury severities. However, the IP scores did not exhibit a moderate injury severity after Day 28, rather the scores appeared to cluster either above an angle of 50° or below 35°. This finding may indicate that mice recover postural stability more rapidly than locomotor function since moderate IP scores were attained at earlier time points following injury. Also, this bimodal distribution in IP scores may highlight the need for another injury severity between 8g and 24g to be tested for IP performance.

Neurological recovery is non-linear in mice and plateaus in half the time that rats recover (by Day 14 post-injury) as illustrated by BBB and IP (Agrawal and Fehlings, 1997; Basso et al., 1995; Nashmi et al., 1997; Schumacher et al., 2000). This decreased length of recovery to plateau could be due to a number of factors including: a decreased length of time for the resolution of primary and secondary injury events, different cellular repair responses, synaptic plasticity, sprouting and/or differences in compensatory pathways (Blight, 1993; Tator, 1998).

An expeditious reorganization of spared pathways after incomplete injury could explain the earlier neurological recovery plateau seen in mice compared to rats. Another recovery mechanism that could account for the earlier plateau of motor function is an earlier denouement of conduction block of descending fibres by a reduction in edema, hematomyelia and remyelination of axons following spinal cord trauma (Little et al., 1999). Moreover,
differences in the central pattern generators between rats and mice might contribute to the differences seen in the rate of recovery between these rodent species.

Transection control mice also underwent recovery from early timepoints following injury until the end of the neurological assessment period. This recovery exhibited by the transection control mice could also be attributable to a number of events, including the resolution of primary and secondary injury events, resolution of spinal shock, transition from a hyporeflexic state to one of hyperreflexia and spasticity (Blight, 1993; Little et al., 1999; Tator, 1998). Indeed, the lack of supraspinal input has been thought to be an important mechanism of producing hyperreflexia because the local motoneuron pools have an increased excitability due to a lack of descending inhibition.

**Pathological Responses of the Mouse Spinal Cord to Injury**

Both methods (morphometric analyses and counts of Fluoro-Gold labeled neurons) used to assess histopathological differences between the clip compression injury severities established three distinct injury severities: light, moderate and severe. At acute time points following injury, central cavities appeared in the mouse spinal cord following clip compression injury. The cavities were not well-defined and were found in the 8g group 1 day after injury, as well as in the 24g group 1 day and 7 days after injury.

The response of the murine spinal cord to compressive injury at chronic time points verified previous accounts of injury site morphology (Jakeman et al., 2000; Kuhn and Wrathall, 1998; Ma et al., 1999; Wang et al., 1997; Zhang et al., 1996; Zhang et al., 1998). A lack of central cavitation in the chronically injured mouse spinal cord and microcystic cavitation appears to characterize the response of the murine spinal cord to injury. In place of this cavitation, a robust response of reactive non-neuronal cells and glial cells seems to occur (Fujiki et al., 1996; Kuhn and Wrathall, 1998; Ma et al., 1999; Wang et al., 1997; Zhang et al., 1996; Zhang et al., 1998). The reasons for these differences have been postulated to be attributable to differences in secondary injury processes and cellular responses of the tissue.
(Kuhn and Wrathall, 1998; Wang et al., 1997). This may be mediated by differences in the immune system or vascular structure of the mouse spinal cord compared to other species that undergo central cavitation following SCI. Indeed, studies of vascular changes in the rat and human spinal cord have been shown to mediate ischemia and result in hemorrhagic necrosis following SCI (Koyanagi et al., 1993; Koyanagi et al., 1993; Koyanagi et al., 1993; Tator and Koyanagi, 1997). There may also be other genetic differences between rats and mice, and between strains of mice (Steward et al., 1999). For example, unpublished results have shown that the GluR2 receptor is localized to the white matter of the CD1 mouse spinal cord, contrary to what has been found in the rat (Joshi and Fehlings, unpublished data). This difference in glutamate receptor distribution may contribute to mechanisms of excitotoxic cell death and lead to the differing pathologies seen in the rat and the mouse following spinal cord trauma.

Counts of neurons retrogradely labeled with Fluoro-Gold were shown to be a reliable technique to assess injury severity. In particular, neuron counts in the sensory-motor cortex were most susceptible to injury, as labeling was obliterated in this area with moderate SCI. In contrast, robust labeling was observed in the reticular formation in all injury severities.

**Relationship of Tracts to BBB and Inclined Plane Scores**

Strong independent positive correlations were found between the Fluoro-Gold labeled cells in key motor nuclei, residual tissue at injury epicenter and neurological outcomes (BBB and IP tests). The results of the independent correlational analyses implicated that the extrapyramidal tracts, not the pyramidal tracts, were strongly related to IP function, confirming the findings of Fehlings and Tator (1995) in the rat. Furthermore, independent Pearson Correlation Coefficients described correlations of both the extrapyramidal (raphespinal, reticulospinal, vestibulospinal and rubrospinal) and the pyramidal (corticospinal) tracts and, the BBB openfield locomotor test.

The results of stepwise multiple linear regression indicated that the BBB scores were related to counts of retrogradely labeled cells in the raphe nuclei, and the motor cortex. Since
these counts represent the integrity of specific tracts through the injury epicenter, the BBB openfield locomotor test may be an important outcome to assess the survival of the raphespinal and corticospinal tracts in mice.

The finding that the raphespinal pathways correlated with BBB function was an unexpected result given that this pathway has been implicated in the descending control of pain (Paxinos, 1995). Indeed, the raphespinal tracts may also play an important role in the openfield movement by maintaining control of the trunk during locomotion. Also, the mathematical modeling using stepwise multiple linear regression might have caused an artefactual result due to a bystander effect. The raphespinal tract projects to the dorsolateral quadrant of the spinal cord, very close to the rubrospinal projections. This region of the spinal cord may be preferentially spared following SCI, thus involved in mediating locomotor recovery. It is then possible that during the stepwise multiple regression procedure that rubrospinal counts could have been the actual region that correlated with the BBB test. However, because of a higher variance, the neuron counts from the red nucleus were eliminated from the correlation model, leaving the raphe nuclei counts in the model due to a lower variance. It is also conceivable that during the counting procedure, some of the recticular formation neurons labeled with Fluoro-Gold were mistaken and counted as labeled raphe nuclei neurons, as these nuclei are juxtaposed to each other in the medulla.

The corticospinal tracts have been implicated in the control of skilled movements, especially those involving precise foot placement and locomotion (Armstrong, 1988). Locomotor recovery has not been previously reported to wholly rely on the integrity of the corticospinal tract in rats (Eidelberg and Yu, 1981; Little et al., 1988; Muir and Whishaw, 1999). Rather, it was shown that lesioning of the corticospinal tract resulted in temporary locomotor deficits (Eidelberg and Yu, 1981; Muir and Whishaw, 1999). However, it is important to note that these findings do not diminish the importance of the corticospinal tract in locomotion. Alternatively, the initial transient deficits reported by others (Eidelberg and Yu, 1981; Muir and Whishaw, 1999), combined with our results that openfield hindlimb locomotion correlated
significantly with the integrity of corticospinal tracts, may imply that the pyramidal systems are an important source of locomotor control, but that they may be compensated for by other descending motor systems.

Using the same techniques of stepwise multiple linear regression, IP scores were related to counts of retrogradely labeled cells in the vestibular nuclei and the red nucleus. These findings are in agreement with a previous study in our laboratory that implicated the vestibulospinal and rubrospinal tracts in IP performance following clip compression SCI in rats (Fehlings and Tator, 1995). The vestibulospinal tracts have been considered an integral descending motor pathway mediating locomotor recovery, especially in their role in maintaining postural control during locomotion (Eidelberg et al., 1981; Yu and Eidelberg, 1981). It is, therefore, possible that the IP task requires the vestibulospinal pathway for its control of equilibrium as the plane is raised. A recent study indicated that the rubrospinal pathway was important in producing non-skilled movements during locomotion in rats (Muir and Whishaw, 2000). Indeed, it was demonstrated that ablation of the red nucleus produced an asymmetrical gait, even during unperturbed locomotion over a flat surface (Muir and Whishaw, 2000). The present study results suggest that IP performance can predicted by the survival of the vestibulospinal and rubrospinal tracts in mice, and imply that these tracts may be important systems for mediating gross postural recovery.

**Limitations of Tract Tracing Techniques**

When studies of tract tracing are undertaken, important controls must be established to ensure the validity of the results. Positive (sham) and negative (transection) control animals were examined in the present study. The sham controls exhibited the greatest amount of labeling, whereas, the transection control animals exhibited no labeling. These controls provided benchmarks that allowed the comparisons of the graded clip compressive injury severities. Also, the introduction site of the retrograde tracer, Fluoro-Gold, was examined to determine the passive spread of the tracer during implantation. In this study, the diffusion of
Fluoro-Gold, on the proximal stump to the injury was not found to be in excess of two segment. Thus, the Fluoro-Gold introduction site (at T9-10) was sufficiently caudal to the injury site (T3-4), ensuring that none of the tracer diffused to the site of injury and did not result in non-specific labeling of brainstem, midbrain and cortical neurons. Furthermore, the duration of incubation with Fluoro-Gold before tissue extraction was sufficiently short (5 days) to eliminate the possibility of transport of the tracer by gap-junctional coupling of cells.

One caveat that must be considered is that our findings of tract integrity by the uptake of the retrograde tracer, Fluoro-Gold, may not fully represent the functional integrity of these tracts. To fully ascertain which tracts are involved in locomotion, in vivo conduction studies of motor evoked potentials and studies that stimulate specific motor nuclei must be performed, along with corresponding anatomical studies. The execution of such experiments was beyond the scope of the present investigation.

Another limitation of the current study is that the correlations identified between specific motor pathways and behavioural function do not form a causal relationship. Rather the $R^2$ values (multiple correlation coefficient) are a measure of the proportion of the variance in the neurological performance explained by the counts of retrogradely labeled neurons in key motor regions of the brainstem, midbrain and cerebral cortex. In order to confirm the findings that have been presented in the present study, studies which specifically lesion motor nuclei or pathways, combined with detailed behavioural analyses, would address the causal relationships between specific descending motor pathways and neurological function.

**Methodological Considerations**

In the present study, one sham control mouse was found to have minor damage in the spinal cord. This could be ascribed to two possible events. Firstly, in the sham control group, after a two-level laminectomy, the ventral aspect of the spinal cord was dissected with a hook to clear an extradural path; thus the sham control was not simply a laminectomy control. The spinal cord manipulation by the dissecting hook might have caused some grossly unobserved
damage. Secondly, the possibility of a subdural hematoma, causing damage, following Fluoro-
Gold introduction can not be excluded. Although, the contribution of this observation to the
results was minimal since no statistically significant functional or histopathological deficits were
seen in the sham controls, similar controls must be performed when SCI experiments have
manipulation of the spinal cord/spinal column other than the intended injury.

**Application of Murine Models to SCI**

The motivation behind developing a graded murine model of SCI has been the desire to
apply genetically altered murine systems in order to study the pathophysiology of SCI. This
study characterizes a clip compressive injury at T3-4, which is anatomically higher in the spinal
cord than in the mouse SCI models that have been presented to date in the literature. This
level of SCI was chosen because the hook dissection was more easily accomplished at T3-4
compared to the lower thoracic level. Also, an injury at T3-4 will affect the regulation of the
mid-thoracic intermediolateral neurons which are responsible for autonomic (cardiovascular)
control. Thus, an injury at T3-4, also models the potential for cardiovascular disturbances
following SCI including: neurogenic shock, bradycardia and autonomic dysreflexia. In order to
apply the clip compressive injury model to another spinal level, sham controls and injured
(untreated) controls must be examined to enable valid comparisons to be made between
genetically altered animals and their wildtype counterparts.

There are also other important issues that must be considered when applying injury
models to knockout mouse mutants. Physiological differences have been noted between
different inbred and knockout strains of mice. For example, differences in motor function, size
of mouse, response to excitatory amino acid administration, inflammatory responses and
neurogenesis amongst inbred strains have been reviewed (Crawley et al., 1997; Crawley and
Paylor, 1997; Schauwecker and Steward, 1997; Steward et al., 1999).

With these differences in mind, SCI research in knockout mice requires additional
attention when designing studies with knockout mice. Methods to control for such differences
can be incorporated into the project design. Fundamental to any SCI study with mutant mice, relevant and appropriate controls must be tested. This is important because knockout mutant mice are usually a hybrid between two background inbred strains of mice, thus any responses in the background strain to a given stimulus could be propagated into the knockout breed. In order to minimize artefactual findings, control mice (these are also called wildtype (+/+) littermate mice in the literature) that are the same sex, from the same generation and have the same parents as the knockout mutants, (identified as -/- in the literature) should undergo the same experimental procedures as the knockout mutant mice.

Genetic deletion can also present limitations in which outcome measures can be applied to study SCI in mutant mice. Certain knockout mutants have lethal mutations that prohibit their use; or have syndromes which complicate their use in SCI studies. For example, mGluR1 knockout mice display decreased coordination and decreased synaptic plasticity (Conquet et al., 1994) and GluR2 knockout mice also display ataxia (Jia et al., 1996). Such phenotypic abnormalities do not preclude the use of such animals in experimental SCI, rather these traits preclude the use of some behavioural outcome measures (i.e. openfield locomotor testing) to study the functional recovery in the injured mice.

**Future Directions**

The development and characterization of an *in vivo* murine model of SCI has permitted studies to progress with mutant mice as well as, raised many questions which merit further study. To further characterize the model, time course light microscopic and electron microscopic studies should be done of the onset of edema, extravasation of cellular components, necrosis, cavitation, demyelination, remyelination and fibro-gliotic scar formation. Experiments that characterize the molecular events following injury are also important to validate and further characterize this model of spinal cord injury. For example, a detailed account of the apoptotic cascades, and their time course, that are activated in the mouse spinal cord would be useful to compare/contrast with other animals, including humans. Comparative
studies of the differences in inflammatory reactions between species may also be useful in ascertaining the role of certain immune responses following SCI. In addition, the integrity of cytoskeletal protein complexes, such as NF200 and spectrin, might provide data on the secondary injury mechanisms that ensue in the murine spinal cord. Time course studies of the secondary injury events would be very useful in understanding which factors contribute to the earlier recovery plateau in mice.

Differences in the pathology between SCI in mice and in other mammals are especially interesting. A study of the vascular structure of the mouse spinal cord would address the issue that the lack of cavity formation observed in the chronically injured mouse spinal cord might be due to differences in the vascular supply of the murine spinal cord. Microangiographic and electron microscopic techniques, similar to those applied by Koyanagi and Tator (Koyanagi et al., 1993; Koyanagi et al., 1993; Koyanagi et al., 1993) could be applied to the injured and non-injured mouse spinal cord. Furthermore, such studies could also provide insights into murine recovery mechanisms that could then be compared with those in humans. Further questions into the mechanisms of early cavity formation and disappearance at late timepoints need to be posed and the answers might provide neurotrauma research with valuable information about wound-healing and recovery responses.

Finally, application of the compressive, murine model of SCI is already underway. These studies are important in terms of validating the model in addition to testing hypotheses about the role of specific genes in the pathophysiology of SCI. For example, the responses of knockout mutant mice for the GluR2 gene to SCI could result in important information about the role of calcium permeable AMPA receptors in SCI. Thus, the development of a compressive, in vivo, murine model of spinal cord injury has not only provided investigators with a tool to further study the pathophysiological mechanisms of SCI, but it has also evoked novel examinations which can be pursued.
CONCLUSIONS

A reliable, graded, in vivo model of compressive SCI has been established in the mouse by customizing an existing method of producing injury in the rat. Neurological and histopathological outcome measures were found to accurately discriminate between three grades of injury severity: 3g clip compression (light injury), 8g clip compression (moderate injury) and 24g clip compression (severe injury). In addition, the histopathological outcome measures correlated highly with the behavioural outcome measures. Independent correlational analyses implicated that the extrapyramidal tracts, not the pyramidal tracts, were strongly related to IP function. Furthermore, independent correlations were identified between both the extrapyramidal (raphespinal, reticulospinal, vestibulospinal and rubrospinal) and the pyramidal (corticospinal) tracts and, the BBB openfield locomotor test. This model of in vivo SCI establishes, for the first time, the use of the clip compression method to produce a reliable SCI technique in mice. This model will enable future projects to be undertaken to investigate the role of specific genes and molecules in spinal cord injury.
REFERENCES


Nashmi, R., Jones, O. T., and Fehlings, M. G. (2000). Abnormal axonal physiology is associated with altered expression and distribution of kv1.1 and kv1.2 K+ channels after chronic spinal cord injury [In Process Citation]. Eur J Neurosci 12, 491-506.


