Neuroprotection Following Acute Spinal Cord Injury Through Inhibition of the Fas Apoptotic Pathway

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Abstract

Neuroprotection Following Acute Spinal Cord Injury Through Inhibition of the Fas Apoptotic Pathway

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Background: Evidence has shown an association between Fas receptor (FasR) expression and apoptosis following acute spinal cord injury (SCI); moreover, genetic deletion of the Fas receptor results in improved functional recovery.

Hypothesis: Administration of soluble Fas receptor (sFasR) will inhibit FasL/FasR interactions and result in significant neuroprotection after SCI.

Methods: sFasR was applied to *in vitro* and *in vivo* models of SCI. Experiments were performed to assess total cell death, apoptosis, behavioural recovery and tissue preservation.

Results: sFasR administration in the organotypic model of SCI resulted in attenuated total cell death following injury. sFasR administration following SCI showed an attenuation of activated caspase-3. Behavioural studies demonstrated improved performance of animals treated with sFasR. Also, sFasR administration increased retrogradely labeled red nucleus neurons and preserved greater amounts of tissue.

Discussion: sFasR inhibition of the Fas ligand/Fas receptor interactions appears to significantly reduce apoptosis and improve behavioural and functional recovery following SCI.

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"I'd like to think the best of me is still hiding up my sleeve..."

Abbreviations

AIF	Apoptosis inducing factor
AMPA	(RS)-alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Apaf-1	Apoptotic protease activating factor-1
Caspase	Cysteine protease, which cleaves at an aspartate residue
Ca^{2+}	Calcium ions
CNS	Central Nervous System
CNQX	6-cyano-7-nitro-2,3-dihydroxyquinoxaline: kainate/AMPA receptor antagonist
CPR	Cardiopulmonary resuscitation
Cyto c	Cytochrome c
DISC	Death-inducing signal complex
FasR	Fas receptor (CD-95, Apo1)
FasL	Fas ligand
FADD	Fas-associated death domain
FAP-1	Fas-associated phosphatase
FLICE	FADD interleukin-1beta-converting enzyme
FLIP	FLICE (Caspase-8) inhibitory protein
H&E/LFB	Hematoxylin & eosin/ luxol fast blue
IAP	Inhibitors of apoptosis
IC	Injured Control
IP	Immunoprecipitation
JNK	Jun amino-terminal kinase
K^+	Potassium ions
KA	Kainate receptor
MTT	Tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MPSS	Methylprednisolone
Na^+	Sodium ions
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NMDA	n-methyl-d-aspartate
NO	Nitric oxide
p75	low affinity neurotrophin receptor
PBS	Phosphate buffer saline
PI	Propidium Iodide
SCI	Spinal Cord Injury
sFasR	Soluble Fas receptor
TNF	Tumor necrosis family
TRADD	TNF receptor-associated death domain
Trk	Tyrosine kinase receptor
TUNEL	terminal-deoxy-transferase mediated dUTP nick end labelling

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Chapter 1- Introduction

Epidemiology of Spinal Cord Injury

Spinal cord injury (SCI) is a devastating traumatic condition that creates enormous physical and financial costs for families throughout North America and the world. Each year there are approximately 12,000 new SCI cases in the United States, while the Canadian National Trauma Registry (1998-99) reported 1347 new SCI cases over a similar 12-month period[1, 2]. The literature suggests that 1-2.5% of all admitted trauma cases suffer from an acute spinal cord injury[1-3].

A majority of these injuries arise from motor vehicle accidents (40-50%); while work accidents, sports & recreation injuries, falls, and violence each claim 10-25% of the incidence of SCI (depending on the global region being sampled) [1]. Although SCI predominantly occurs in younger populations (Ages 11-30 account for 45% of all SCIs.), there has been an increased prevalence of SCI in the elderly population due to increased senescent independence and life expectancy [4]. Furthermore, there is a large discrepancy between gender and SCI. Men have a much higher incidence of SCI as compared to women (3-4:1)[1].

Improvements in first aid, CPR, triage and surgical interventions have greatly increased the success in treatment of SCI. However, the estimates of covering medical treatment and for lost earnings as a result of this debilitating condition are approximately $1.5 million dollars per individual [5]. Minor neurological improvements result in drastic ameliorations on quality of life and on management of SCI. It is important that new

pharmacological, surgical and rehabilitative solutions are further pursued to help amend SCI and all the hardships that encompass it.

Pathophysiology of Spinal Cord Injury: Primary and Secondary Injury

Much has been discerned in the past twenty years regarding the pathobiology of SCI. Spinal cord injury has two phases; the first being the "primary" injury, which is followed by a longer and not as well understood "secondary" injury (Table 1). The primary injury is a combination of mechanical perturbations on the cord arising from fracture dislocations, burst fractures, missile injuries and acutely ruptured discs that create an initial and frequently persisting compression[1]. Lacerations from sharp bone fragments can also be part of the primary injury. However, complete spinal cord transections in clinical cases are rare. Approximately two thirds of all SCIs arise in the cervical region[5]. The cervical region contains smaller and weaker vertebrae, which results in increased mobility but less strength to stabilize the surrounding ligaments and musculature creating a more susceptible region of injury[1].

Secondary injury is a veritable mixture of systemic and local affects that begin acutely (minutes and hours) following SCI and can last for weeks and months. These changes arise from the initial insult and alter the biochemical function of cells localized at the injury epicenter and adjacent sites while also activating certain systemic pathways. In 1911, A.R Allen was the first to document these secondary mechanisms by removing post-traumatic hematomyelia following acute spinal cord injury in an animal model. This removal resulted in improvements in neurological function, which led Allen to conclude

Table 1. Primary and Secondary Mechanisms of Spinal Cord Injury

Primary Injury
• Mechanical perturbations • Compression (both Acute & Chronic) • Laceration • Shearing • Missile

Secondary Injury Mechanisms	
Systemic Effects • Brief ↑, then ↓ of heart rate • Brief ↑, then ↓ blood pressure • ↓ Cardiac output • Hypoxia and Hyperthermia	Vascular Changes • Reduced blood flow • Hemorrhage • ↓ autoregulation • loss of microcirculation
Electrolyte Changes • ↑ $[Na^+]_I$ • ↑ $[Ca^{2+}]_I$ • ↑ $[K^+]_I$	Biochemical Changes • Glutamate excitotoxicity • Free radical production • Eicosanoid production • Calpain activation & lipid peroxidation • Opioid production
Apoptosis • Caspase dependent activation • Mitochondrial permeabilization pores • Caspase-independent activation • Endoplasmic reticulum activation	Loss of ATP production Edema Neurotransmitter Accumulation

there were "biochemical factors" that played a role in heightening the self-destruction of the cells[5, 6].

Systemic effects have been well characterized following SCI, in that there is an initial increased tachycardia followed by prolonged bradycardia. These coincide with an acute hypertension that is followed by a chronic hypotensive state[7]. The chronic loss of blood pressure control can be attributed to a loss of descending sympathetic pre-ganglionic neurons within the rostro-ventro-lateral medulla (RVLM)[8]. Decreases in cardiac output and in peripheral vascular resistance also coincide with the above-mentioned effects and are one of the reasons for hypoxic conditions following SCI[1, 5]. Thermoregulatory dysfunction is another systemic effect that has been implicated in SCI, and hyperthermia may play a role in some of the detrimental events that occur to patients with SCI[9].

Local blood supply near the injury epicenter is considerably altered following SCI due to mechanical disruption of the arteries and venules[10]. Ischemic zones appear in a large portion of the grey and white matter following SCI, especially in white matter adjacent to grey matter hemorrhages[11, 12]. Moreover, this secondary injury is further exacerbated through an increased incidence of vasospasm, thrombosis and loss of autoregulation of spinal cord blood flow[5, 11, 13].

At a cellular level, there are large shifts in ionic gradients with changes in intracellular Ca^{2+}, K^{+} and increased Na^{+} permeability[14-22]. Moreover, excitotoxicity (through glutamate), inflammation, free radical production, lipid peroxidation, opiate mediated cell death and apoptosis have been shown to play a role in the secondary injury[23-28].

All the above mechanisms contribute to the secondary injury creating a progressive degeneration of cells. The temporal and spatial distribution of cell death can occur both rostral and caudal to the injury epicenter and can continue for weeks following the initial insult. These mechanisms provide unique opportunities to assess neuroprotection methods of the cord following injury, and this is important due to the steady incidence of SCI [1, 29].

Secondary Injury Mechanisms

The following section will highlight and expand on some of the better understood pathways involved in secondary injury. Although these mechanisms are explained independently, it is important to remember that a majority of these pathways cross-talk and can affect each other's function. However, to maintain conciseness, interactions will only be mentioned once and not repeated under different secondary mechanisms.

Glutamate Neurotoxicity

Glutamate is the main excitatory amino acid transmitter of the CNS. The glutamate receptor family is divided into two groups: NMDA and non-NMDA. The NMDA receptors are found exclusively in grey matter, while the non-NMDA receptors are divided into AMPA, KA and metabotropic (G protein-coupled) groups and are found both in grey and white matter tissue[30].

It has been shown that following SCI, glutamate levels are increased dramatically and subsequently are neurotoxic[24, 31, 32]. This neurotoxicity is an acute response to trauma and is activated through an influx of $Ca^{2+}$and Na^{+}, depending on the receptors ion

gating properties. This influx of ions intimately interacts with a variety of intracellular processes, such as, release of intracellular Ca^{2+}, activation of protein kinases & proteases, and free radical production[24]. These interactions activate both necrotic and apoptotic mechanisms which results in the death of neurons and glial cells. It is generally thought that NMDA receptor activation is responsible for neuronal excitotoxicity, while oligodendrocytes, astrocytes and microglia have been shown to be susceptible to non-NMDA glutamate excitotoxicity thus contributing to white matter destruction[26, 30, 33-35].

Calpain Mediated Proteolysis

There has been substantial evidence of breakdown of cytoskeletal proteins like neurofilaments and myelin basic proteins in SCI[28, 36-38]. Cytoskeletal proteolysis happens rapidly (as early as 15 minutes) following SCI and this degeneration progresses further for several hours. Moreover, the loss of cytoskeletal proteins correlates directly with the degeneration of axons and myelin, as well as with the activation of neutral proteinases[38].

Calpain, a calcium-activated neutral proteinase, is thought to play a regulatory role on cytoskeletal proteins[38]. However, following SCI calpain has been implicated in the breakdown of certain cytoskeletal proteins specifically those that have the cytoskeletal calpain substrate, such as dephosphorylated neurofilament 200 which is present in axons[28]. It is hypothesized that increased levels of intracellular Ca^{2+} due to the initial insult results in activation of calpain, which directly breaks down certain

cytoskeletal proteins. Calpain has also been implicated in initiating apoptosis and astrocytic scar formation[39].

Free Radical Production & Lipid Peroxidation

Following SCI, there is a buildup of highly reactive free radicals. These radicals are predominantly formed from superoxide (O_2), which cascades through a variety of intracellular steps (ie. Hydrogen peroxide) to form harmful hydroxy radicals (HO). In uninjured tissue, these free radicals are scavenged by a variety of antioxidants, in particular vitamin E, ascorbic acid, and glutathione. Antioxidants convert these potentially harmful substances into water and oxygen[1, 24].

Free radical production has been shown to play a detrimental role following SCI[23, 25, 40]. Minutes following injury there is evidence of free radical build-up due to the loss of antioxidants near and at the injury site[25]. Free radical build up initiates lipid peroxidation, which inhibits the phosphorylation of cytosolic proteins that can affect ion channels and transport mechanisms destroying ion gradients, and ultimately leading to membrane lysis[1, 24]. Free radical formation is also increased from free fatty acids, and results directly from the lipid dependent Na^+-K^+-adenosine triphosphatase (ATPase) dysfunction, which also contributes to ionic gradient imbalances[1, 24, 41].

Free radical damage arises in mechanical disturbances to the cord and as well in ischemic conditions. Moreover, free radicals not only trigger necrotic cell death, but can also trigger the formation of more free radicals, initiate apoptosis and inhibit synthesis and repair mechanisms[42].

Role of Inflammation in Spinal Cord Injury

The role of inflammation following SCI is seen as a dichotomy, which has triggered an abundance of traumatic inflammatory research. It has long been thought that inflammatory mechanisms that are triggered through the initial insult on the cord are detrimental to the cord[24, 43-46]. However, much has been learned of the beneficial effects that inflammation can have on regeneration and repair[47-51]. Whether an inflammatory response is detrimental or beneficial is dependent on a variety of factors, such as the magnitude, the temporal & spatial distribution and duration of the response.

In the unperturbed spinal cord, inflammation has been shown to play a small regulatory role because the blood-brain barrier limits the inflammatory response. Following SCI, the blood-brain barrier is damaged, which allows infiltration of various inflammatory molecules and leukocytes into the newly damaged area[24, 52]. Neutrophils, B & T lymphocytes penetrate the cord releasing a variety of cytokines, interleukins, complement, chemokines and other cytotoxic molecules (ie. 'death receptor ligands' TNFα, FasL and NO) at and adjacent to the injury site while macrophages phagocytose the damaged tissue. This immunological response further augments the inflammatory cascade through activation, proliferation and differentiation of dormant inflammatory cells recruited to and found in the spinal cord[24, 43, 46].

Microglia are the prominent immune cells found in the spinal cord. Resident microglia become activated following SCI and respond by phagocytosing the damaged area and releasing soluble factors that further exacerbate the damage. However, they also possess a 'double edge sword' in that they have been implicated in being beneficial to the CNS[51].

Some inflammatory molecules and cells that are suspected to be beneficial to the CNS following trauma were thought to initially be detrimental to CNS tissue following trauma. TNFα is released by a variety of cells (ie. activated astrocytes, microglia, macrophages and T cells) that elicit an inflammatory response that may lead to neuronal death and affecting CNS metabolism that decreases oxygen uptake, and reduces blood flow. However, experiments have shown that deletion of TNFα receptor resulted in reduced functional recovery, increased apoptosis and decreased tissue preservation following SCI, which would suggest a beneficial role for TNFα[53]. Furthermore, NO has been shown to be both detrimental and neuroprotective to the CNS. It is acutely involved in free radical production yet at later stages may play a reparative role[24]. Lastly, interleukin-10 (IL-10) is an anti-inflammatory cytokine that suppresses a variety of the inflammatory mechanisms by inhibiting a majority of the macrophage, astrocytic and microglial immune responses. It has also been implicated in decreasing apoptotic cell death in neurons[48].

Calcium, Sodium, and Potassium: Roles Following Spinal Cord Injury

The role of calcium following trauma and stress is complex and although much has been discerned about its mechanisms, it involves and associates (as denoted by the above sections) with many different pathways. The complexity of these interactions in the context of SCI will be briefly reviewed here.

Following trauma, calcium enters the cell from the extracellular space and is also released intracellularly into the cytoplasm. Voltage-gated Ca^{2+} channels, glutamate receptors and reversed Na^{+}-Ca^{2+} exchange are some of the known ways of Ca^{2+} entry in

which intracellular Ca^{2+}concentrations are increased following trauma[16, 17, 39, 54-56]. Moreover, the endoplasmic reticulum releases Ca^{2+} from its stores further exacerbating the intracellular Ca^{2+} concentrations. This rise in intracellular Ca^{2+} affects mitochondrial function, activates fragmentation of nuclear proteins, affects transcription of a number of essential genes and, through increased association with calmodulin, modulates the activity of a large number of protein kinases and phosphatases[54]. Along with the above mentioned effects, intracellular Ca^{2+} increase invariably plays a large role in determining whether glial cells survive following SCI.

In uninjured spinal cord axons, Na^+ channels are found in the nodes of Ranvier, and in cooperation with K^+ channels propagate action potentials throughout the CNS. However, following SCI these channels can have detrimental effects on neurons. Following SCI, Na^+ channels increase intracellular Na^+, which promotes acidosis (through increased Na^+/H^+ exchange), stimulates phospholipase activity, accumulates calcium (through the Na^+-Ca^{2+} exchanger), depletes ATP and reverses the Na^+-K^+ ATPase, all in an attempt to extrude Na^+ from the cytoplasm[17-19, 55, 57-60]. Furthermore, trauma causes increased extracellular K^+ accumulation and altered K^+ channel distribution and function resulting in chronic conduction deficits[15, 61].

Apoptotic Pathways Following Spinal Cord Injury

Although apoptosis is a secondary mechanism after SCI, it is essential to discuss its mechanisms and pathophysiology independently because it is the focus of the thesis and therefore requires more specific detail. Furthermore, apoptosis is a complex process

that involves multiple overlapping pathways (Figure 1) and in an attempt to be succinct we will mention interactions between different pathways only once.

Characterization of Necrosis and Apoptosis

Necrosis and apoptosis are two forms of cell death found following SCI. Necrosis or 'accidental cell death' arises when cells are subjected to an extreme physical or chemical environment or insult[62-64]. This exposure damages the plasma membrane resulting in a compromised membrane integrity leading to the cells inability to maintain homeostasis, dysfunction of organelles and eventually the lysis and extrusion of the cytoplasmic contents into the extracellular space (Table 2)[62, 64]. This spontaneous passive process (no ATP needed) elicits an inflammatory response, activated by the release of intracellular contents into the outside of the cell, which could potentially affect neighbouring areas[64].

Apoptosis, also known as 'programmed cell death', is a process that eliminates superfluous or compromised cells [62, 64-66] and has been strongly implicated in SCI[1, 24, 67-76]. Apoptosis is an active (requires ATP) event that has been observed in developmental, aging, and traumatic environments. Cells receive extracellular and/or intracellular signals that initiate apoptosis and result in cytoskeletal rearrangement, cell membrane disruption and blebbing, nuclear condensation and DNA fragmentation (Table 2)[62, 64, 66].

Apoptosis can be deficient or excessive in a variety of diseases, such as cancer and multiple sclerosis[77, 78]. Moreover, it has been implicated in a variety of traumatic conditions, such as stroke, spinal cord injury and traumatic brain injury[24, 79].

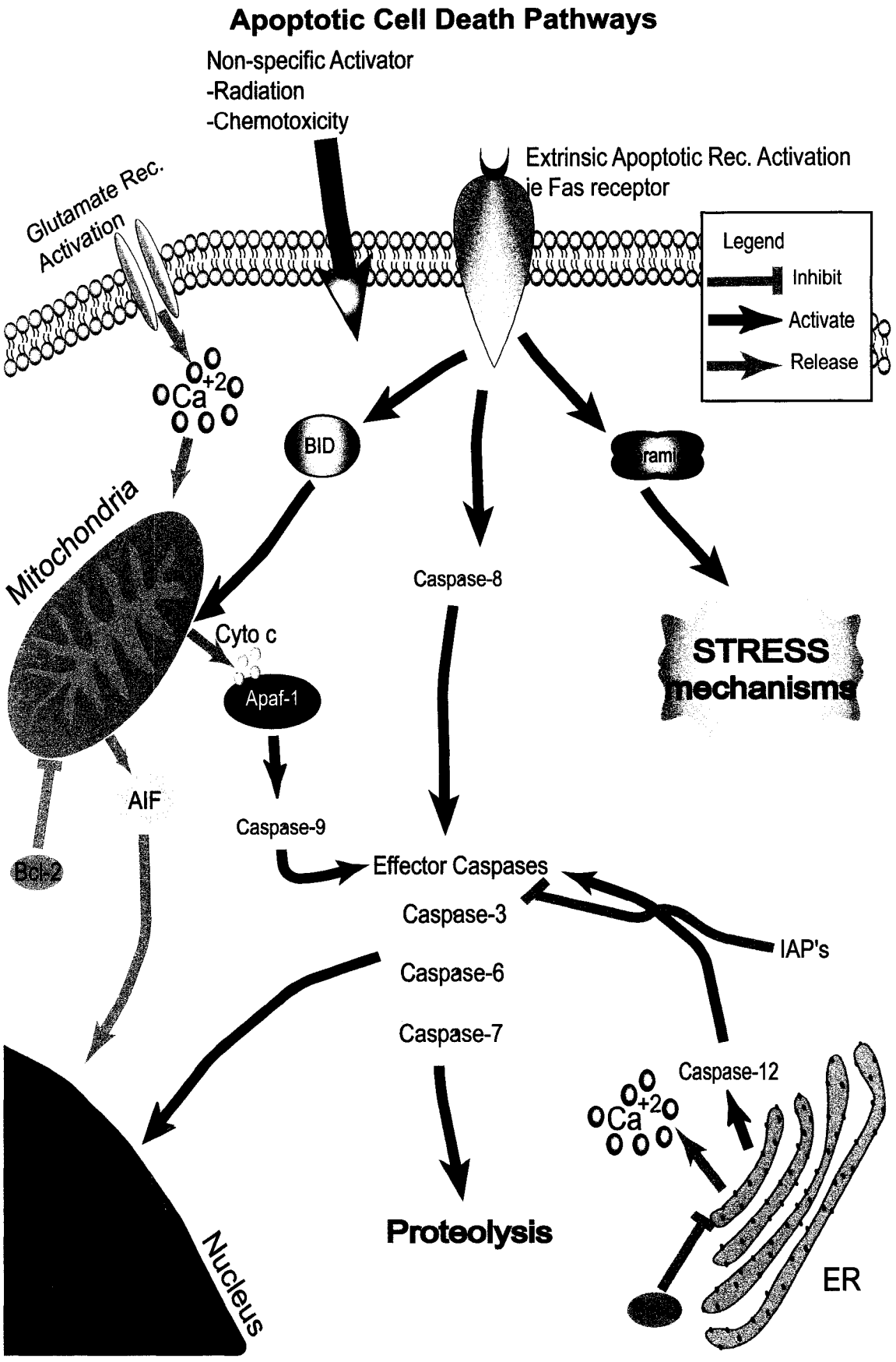


Figure 1. Schematic illustrating various apoptotic pathways thought to be involved following spinal cord injury and other neurotraumatic events.

The extracellular and intracellular factors involved in SCI-induced apoptotic activation include receptor activation, growth factor starvation, toxic agents, and oxidative and mechanical stress[66]. The apoptotic pathways that are activated following SCI are described below.

The Caspases

The caspases are a vital family of cysteine proteases that organize, initiate, and act as apoptotic mechanisms. These cytosolic proteins have the amino acid cysteine in their active site and act upon other proteins by cleaving specific aspartic acids[65]. To date, there are 14 members of the caspase family (numbered accordingly, i.e. caspase-1, 2, etc...) that have been identified. Like most enzymes, these proteases are synthesized as inactive precursors or zymogens, allowing them to associate with their target proteins, while not inducing any damage[79, 80].

There are two types of caspases: initiator and effector/executor caspases. The initiator caspases associate with accessory molecules, like death receptors and their adaptor proteins or mitochondrial associating proteins[66, 79]. These caspases remain in the cytosol, becoming activated through proteolytic cleavage and releasing their pro-domains. This cleavage is followed by subsequent activation of effector/executor caspases, which activate downstream molecules affecting nuclear, mitochondrial, and plasma membrane function (Figure 1.). Lastly, caspases also have the capacity to be modulated, enhancing or repressing their function, and thus changing the apoptotic potential[79].

Table 2. Characteristics of Apoptotic versus Necrotic Cell Death

Necrosis	Apoptosis
• Passive Process (no ATP required) • Cell membrane lysis due to swelling. • Random DNA breakdown and fragmentation. • Mitochondrial dysfunction and electron transport chain damage. • Expulsion of organelles into intracellular compartments • Affects neighbouring cells and induces inflammatory response.	• Active Process (requires ATP) • Cell membrane blebbing. • Organized inter-nucleosomal DNA fragmentation • Chromatin condensation • Mitochondrial condensation • Intact cytoplasmic organelles and cell membranes. • Doesn't induce inflammatory response.

Caspase activation has been implicated following SCI[81]. Strategies involved in targeting caspase inhibition following SCI have been performed using the caspase inhibitor z-VAD-fmk, although minimal physiological and functional improvement was seen[82].

Extrinsic Apoptotic Pathway - Death Receptors

This section will give a brief overview of the extrinsic apoptotic mechanisms and the roles they play in SCI with further elucidation on the details of the Fas receptor in a later section. Activation of the extrinsic apoptotic pathway is in large part through ligation of Fas ligand to Fas receptor, TNFα to TNFR1, or activation of any one of the 18 other members of the TNF receptor family. The TNF receptors are transmembrane proteins that contain a varying number of cysteine-rich domains that are interspersed within the cytoplasmic tail. The receptors function in trimeric form and aggregate their cytoplasmic adaptor proteins as a result[79, 83].

One of the important adaptor protein families is the associated death domain. TNF receptor-associated death domain (TRADD) and the Fas-associated death domain (FADD or MORT1) are both involved in the death inducing signaling complex (DISC) for their respective receptors. They perpetuate the apoptotic signal by activating mitochondrial, stress and caspase pathways (Figure 1.)[79, 83].

It was initially believed that TNFα secretion signals apoptotic cell death of oligodendrocytes following trauma and exacerbates white matter destruction through delayed degenerative mechanisms[75, 84]. TNFα is elevated and has increased transport across the blood-brain barrier following SCI. Thus many studies reported that after

traumatic or ischemic insults, TNFα is a potent inducer of apoptotic cell death[85-88]. However, Kim *et al.* showed that TNF receptor activation can be neuroprotective through activation of nuclear factor-κB (NF-κB), which is an anti-apoptotic protein, and that deletion of the TNF receptor can decrease functional recovery following SCI[53].

The p75 low affinity neurotrophin receptor is an exception, in that this 'death receptor' does not bind a ligand from the TNF family. p75 binds NGF, as well as other neurotrophins and has a role as co-receptor with the Trk receptors. The biological roles of p75 have implicated it as both a pro-survival molecule and as a death-inducing receptor. Moreover, p75 has been shown to activate caspase cascades (although not clearly understood), and is upregulated on glial cells following SCI. Casha *et al.* [68] further showed that the p75 receptor had a delayed upregulation and co-localized with apoptotic (TUNEL positive) cells. Also, Beattie *et al.*[89] have shown that the p75 receptor is required for cell death in oligodendrocytes following SCI and that it helps mediate oligodendrocyte cell death through proNGF/p75 binding regardless of the presence of the TrkA receptor.

Mitochondria

The disruption of mitochondrial function following SCI plays an important role in apoptotic and necrotic cell death following SCI (Figure 1.). The mitochondria regulate energy metabolism, control Ca^{2+} homeostasis, and are a source of reactive oxygen species that arise due to their oxidation-reduction reactions[90, 91]. These functions are highly controlled, and during neurotrauma are quite susceptible to dysfunction through excitotoxicity, inflammatory reactions, mechanical stress, and ischemic conditions[90].

Following neurotraumatic events, mitochondria also release apoptogenic proteins from mitochondrial permeability transition pores[90].

As a result of mitochondrial membrane permeabilization, cytochrome c and apoptosis inducing factor (AIF) are two of the important apoptogenic proteins that are released into the cytosol[92, 93]. Upon release, cytochrome c binds with Apaf-1 and pro-caspase-9 to activate caspase-9, which continues downstream to activate the effector caspase-3[93, 94]. AIF is a protein that is released into the cytoplasm and nucleus, triggering a caspase-independent pathway that induces chromatin condensation and DNA fragmentation[92, 93, 95, 96]. Moreover, AIF has been shown to specifically mediate neuronal and microglial apoptosis following certain insults, however, further research is needed to confirm its role in SCI[97].

The Bcl-2 family of proteins has been shown to modulate the mitochondrial apoptogenic function by controlling the release of mitochondrial proteins (ie. cytochrome-c and AIF). Bcl-2 and Bcl-X_L are anti-apoptotic proteins, while BAX, Bcl-X_s and BID are pro-apoptotic upon activation[90, 93]. The relationship between the pro and anti-apoptotic proteins is not completely understood, but it is thought that it is the ratio between the two that determines whether apoptotic proteins, like AIF and cytochrome c, are released from the mitochondria. Studies have shown conflicting results in the expression of Bcl-2 family proteins following trauma. The literature showed findings of an increased Bcl-2 expression following traumatic brain injury, and protective effects of Bcl-X_L after middle cerebral artery occlusion. Moreover, following SCI Bcl-2 did not seem to be upregulated in oligodendrocytes and has the potential to be a neuroprotective target pathway[98-100].

Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a network of tubules, vesicles, and sacs/histones that has recently been implicated in cell trauma and stress. In uninjured cells, the ER is involved in protein synthesis, sequestration of calcium, production of steroids, and production and storage of glycogen. Following insult to the cell, the ER releases Ca^{2+} into the cytosol, which can cause mitochondrial dysfunction. Also, following stress the ER activates caspase-12, which can activate the caspase-9 apoptotic-initiating pathway(Figure 1)[101-105].

The Fas Receptor

The Fas receptor (FasR) is an extrinsic apoptotic pathway which has been a focus of our laboratory in the context of SCI (Figure 2). This thesis work continues this approach by examining and manipulating the interactions of Fas ligand and Fas receptor following injury. Accordingly, the following section describes the mechanisms that underlie the actions of Fas ligand and the Fas receptor.

Fas Receptor and Fas Ligand Characterization, Conformation, and Expression.

Fas Ligand (FasL) is a 40 kDa transmembrane protein belonging to the TNF family[106-115]. It is believed that following activation of cells through stress signals or injury, FasL is upregulated by an increase in the Jun amino-terminal kinase (JNK) pathway that phosphorylates c-jun, which activates the transcription of FasL by binding to AP-1 sites in FasL's promoter[116]. Furthermore, FasL is brought to the cell surface from the trans-Golgi network by secretory lysosomes and the SH3-domain-

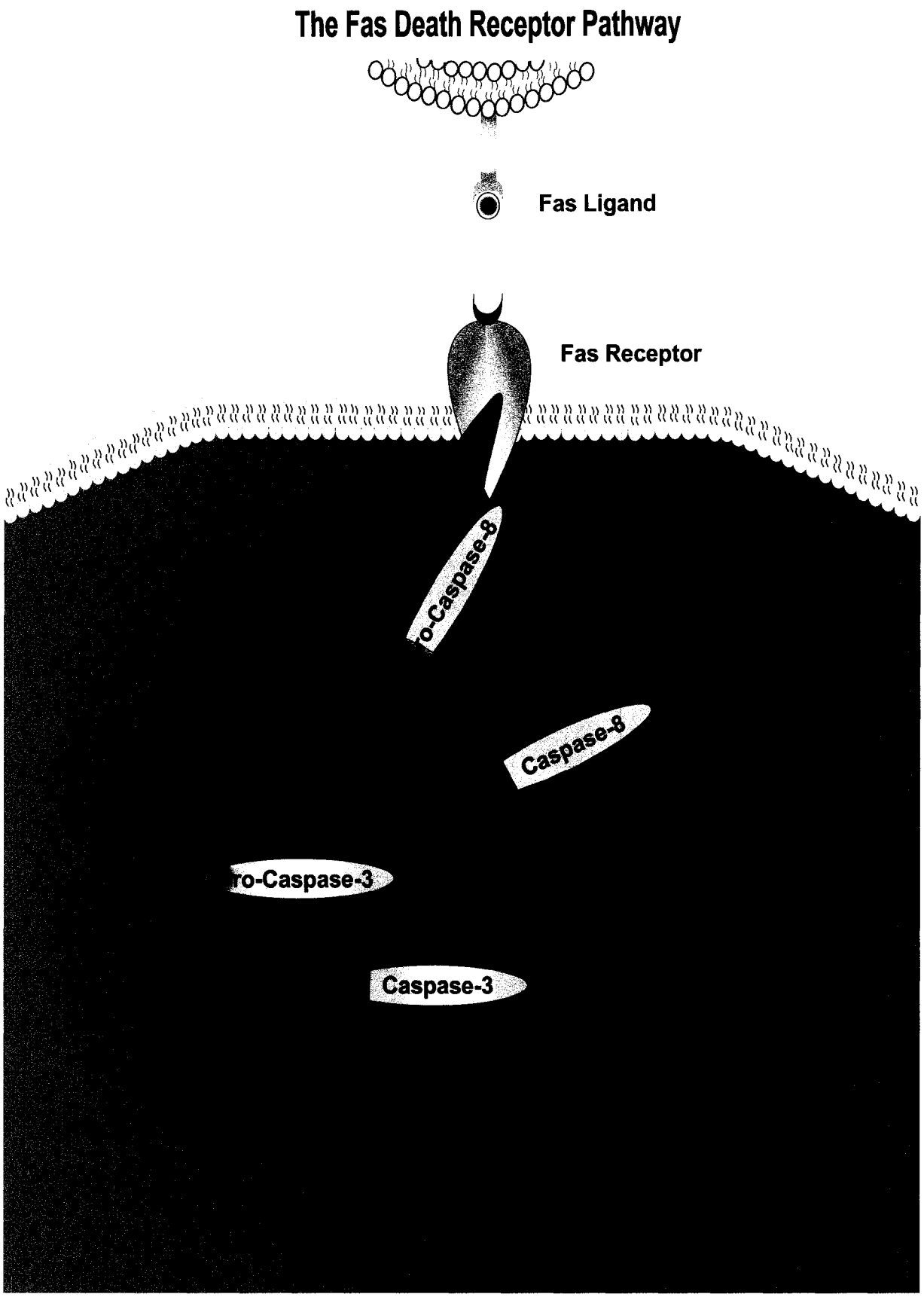


Figure 2. Schematic of the Fas receptor with activation of selected caspase-8 mediated intracellular pathways.

containing chaperone protein[113]. The SH3-domain-containing chaperone attaches to the proline-rich domain on FasL's cytoplasmic tail, which enables it to be exocytosed onto the cell surface[113].

FasL is expressed and upregulated by a variety of cells, with activated T-cells, macrophages, microglia and neurons being of particular importance[108, 110, 111, 116, 117]. It has a high affinity for the Fas receptor (FasR) when it is membrane bound, presented on the cell surface and associated in trimeric form. Moreover, the FasL monomers (within the trimer) possess separate binding domains for the FasR.

The FasL membrane trimers can bind FasR, inducing a death signal. However, the membrane bound FasL can be cleaved by a metalloproteinases. Upon cleavage, the newly formed trimeric soluble FasL doesn't lose its ability to bind FasR, but does lose its ability to activate the death signal acting in an intrinsic manner to help regulate the death signal[99, 107, 110, 111, 115]. Also, soluble FasL has a much lower binding affinity for FasR than its membrane bound counterpart[107]. Lastly, the literature has shown evidence of stored granules of intracellular FasL that can be released in the need for rapid responses[111].

The FasR (also known as APO-1, CD95) was originally thought to be mainly restricted to the immune system and weakly expressed in many tissues[118]. However, much work has been done to observe the upregulation of Fas and to delineate its roles in development, oncology, ischemia, trauma and a variety of diseases[68, 78, 119-126]. FasR is a 45 kDa transmembrane protein composed of 325 amino acids[111, 118]. It aggregates in trimeric form so as to bind the trimeric FasL. The FasR binds FasL

independently but has an eighty amino acid cytoplasmic death domain that self-clusters upon ligation [83, 109].

Fas Receptor Intracellular Pathways

With FasL/FasR ligation and cross-linking, the Fas receptor becomes competent to form its death-inducing signal complex (DISC) with Fas associated death domain (FADD, or Mort 1) and caspase-8 (also known as FLICE)(Figure 2). FADD is an adaptor protein (23kDa) that joins its death domain with FasR's death domain[109, 127, 128]. FADD has another site at its N-terminus, called a death effector domain (DED) that allows caspase-8 binding. DISC formation creates high local concentrations of caspase-8. Normally an inactive zymogen in the cytoplasm, the high concentrations lead to autoproteolytic cleavage and activation of caspase-8[128]. Additionally, recent work has shown that caspase-10 can also be part of the DISC, however, additional work needs to be conducted to elucidate its true function[109, 129, 130].

The activation of caspase-8 perpetuates the apoptotic signal and activates multiple cascades that induce cell death (Figure 2). The proteolytic cleavage releases caspase-8, which then cleaves and activates effector caspases-3, 6 and 7 and initiates the termination of the cell through DNA fragmentation. Activated caspase-8 also activates and cleaves Bid, a member of the Bcl family. Upon cleavage, truncated Bid translocates to the mitochondria, stimulating the function of Bax and Bak. These proteins perturb the mitochondrial membrane potential by creating mitochondrial permeability transition pores, which results in the release of cytochrome c. Cytochrome c complexes itself with pro-caspase 9 and apoptotic protease-activating factor 1 (Apaf-1) initiating the activation of caspase-9, a caspase that can activate the effector caspases (Figure 1) [83, 109, 116,

131]. Other proteins associating with DISC and the FasR include Daxx activation of the JNK pathway[109], SMase activation and ceramide production[120], second mitochondria-derived activator of caspases (SMAC, also known as Diablo) is released in the same manner as cytochrome c[106, 132], and RIP, a death domain containing kinase whose mechanism is not fully understood[109].

The DISC has endogenous inhibitory proteins that help mediate the Fas apoptotic signal. Fas-associated phophatase 1 (Fap-1), FLICE inhibitory protein (FLIP), inhibitory apoptosis proteins (IAPs), and the Bcl-2 family anti-apoptotic proteins are all involved in inhibiting various pathways activated by the Fas receptor (Figure 2)[131]. The most intriguing are FLIP, which has the ability to dock onto FADD and hinder caspase-8 binding and activation, and Fap-1 which interacts physically with the intracytoplasmic C-terminal 15 amino acids thus inhibiting FasR activation[131]. Moreover, upregulation of anti-apoptotic proteins appears to be tightly regulated by transcription factors like NF-κB[131]. Recent literature on FasR has exhibited evidence that the FasR pathway may induce cell proliferation, peripheral neurite growth and improve cell survival[133-135].

Endogenous Soluble Fas Receptor

The Fas pathway has an endogenous negative feedback system that helps modulate its apoptotic response. A truncated form of the FasR, which has been termed "soluble Fas receptor" (sFasR), is produced through FasR cleavage and/or through alternative splicing to form a protein that binds FasL, yet lacks the transmembrane domain, is not membrane bound, and does not produce any of the apoptotic signals regularly involved with FasL/FasR binding(Figure 3)[136-141].

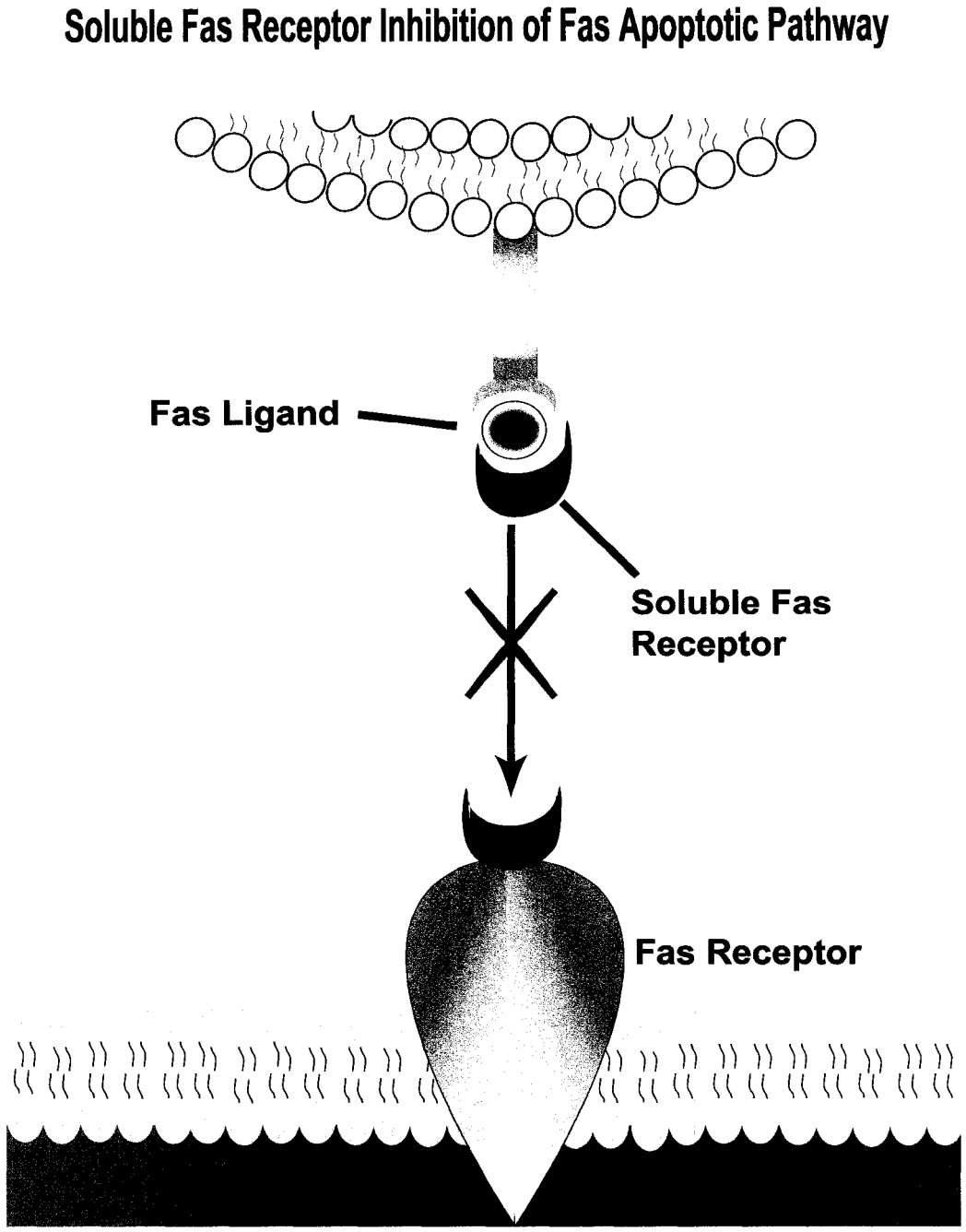


Figure 3. Simplified schematic showing decreased Fas receptor activation with soluble Fas receptor administration. The soluble Fas receptor chelates Fas ligand, inhibiting the Fas ligand/Fas receptor binding interactions.

The presence of sFasR is not normally found in human cerebrospinal fluid. However, prolonged endogenous release of sFasR has been observed following traumatic brain injury in humans[142]. Furthermore, the production of sFasR has already been shown to be an effective method to inhibit the Fas pathway[138, 141]. Cheng *et al.* [141] injected sFasR *in vivo* and altered immune function by inhibiting the Fas-mediated apoptosis. The elucidation of sFasR function offers the potential of targeting diseases and/or conditions that involve Fas pathways (Figure 3).

Pharmacological Characterization of Soluble Fas Receptor

Soluble FasR has been recognized as a potential tool that could inhibit FasL/FasR interactions and minimize FasL induced tissue damage[137, 141]. Mahiou *et al.* performed experiments that analyzed the pharmacological characteristics of murine sFasR. Soluble Fas receptor was expressed in recombinant baculovirus/insect cell system to yield two soluble Fas receptor forms: a monomeric FasL binding site and a homodimeric FasL binding sites fused to an Fc portion of a human IgG heavy chain. They found that although both forms could bind FasL, the homodimeric form had a half-maximal effective concentration of approximately 50nm (inhibiting FasL induced/ FasR-specific lysis) and was also six times more effective than the monomeric form[137].

The Role of the Fas Receptor Following Spinal Cord Injury

The Fas receptor has recently been implicated in the pathogenesis of traumatic and ischemic CNS injury. Qiu *et al.*[123] demonstrated an upregulation of the DISC following traumatic brain injury, inducing cell death that could be markedly reduced with the addition of a pan-caspase inhibitor. Ischemic conditions resulted in increased

activation of FasL, and evidence of increased DISC formation and neuronal cell death[117, 143].

Apoptotic cell death is not restricted to neurons following traumatic and ischemic injury. Glial cells have been shown to be greatly involved in apoptotic cell death, with evidence that oligodendrocytes are especially susceptible to FasR mediated cell death[68, 78, 144]. IFNγ has been shown to induce apoptosis *in vitro* in microglia through simultaneous upregulation of FasR and FasL[112].

Our laboratory has done extensive work examining the role of glial cells following SCI. Casha *et al.*[68] found that oligodendrocytes, and microglia, upregulated the FasR following SCI. The FasR co-localized with TUNEL (a marker for apoptosis) and apoptosis was delayed, maximizing at 7 days following injury. Moreover, Casha *et al.*[121] used mice lacking the FasR (B6.MRL-Fas-lpr mice[145]) and found improved neurological outcome, reduced apoptotic cell death and enhanced axonal survival following SCI.

The delayed death of oligodendrocytes in the white matter long tracts is involved in Wallerian degeneration of axons, and appears to play a significant role in the exacerbation of secondary tissue damage following SCI[68, 69, 75, 121, 146-148]. Axons have been shown to interact with oligodendrocytes providing cell survival signals[146, 149]. Following SCI, degenerating axons and activated microglia/ macrophages have been thought to activate apoptotic cell death mechanisms in oligodendrocytes[75]. The death of one oligodendrocyte results in the demyelination and dysfunction of multiple axons. The Fas receptor is suspected in playing a role in communication between the activated microglia, axons and oligodendrocytes[75].

Targeting oligodendrocyte survival appears to be a promising avenue in the repair of the injured spinal cord.

Neuroprotective Methods Following SCI

In animal models of SCI, it has been observed that a 10% increase of axon survival has profound impact on locomotor recovery[150]. However, to date there has been very little progress in finding successful treatments to help minimize the damage caused by injury to the spinal cord.

In humans and in animal models, improvements following SCI have been seen with administration of methylprednisolone (an anti-inflammatory steroid)[151-155]. Additionally, the administration of GM-1 ganglioside shows trends towards neuroprotection in humans [154, 156, 157]. However, both of these treatments appear to offer only modest neurological improvements, which highlights the need to develop more neuroprotective approaches.

There has been significant progress in animal models studying secondary mechanisms following trauma and SCI. Much research has been done regarding the neuroprotection using Na^+ and K^+ channel blockers, Ca^{2+} channel blockers, glutamate channel blockers, calpain inhibitors, anti-oxidants, opiate inhibitors, and inhibitors of apoptosis[18, 19, 36, 38, 57, 59, 60, 74, 82, 88, 158-161]. It is most likely that a neuroprotective cocktail will eventually be found that will block multiple secondary mechanisms.

Rationale for the Present Investigation

Although much work has been accomplished in understanding the secondary mechanisms following SCI, there is still no clinically relevant treatment that provides convincing neurological improvements. It has been discerned that apoptosis does play a significant role following SCI. Moreover, administration of anti-apoptotic treatments could potentially offer profound results clinically because of the delayed onset of apoptosis.

Our laboratory has found that the extrinsic apoptotic pathways are major players in the apoptotic response and that its role on glial cell death (especially oligodendrocytes) can have severe consequences in exacerbating the white matter injury through Wallerian degeneration[24, 68, 69, 72, 76, 123, 143, 148, 162]. The FasR, one of the extrinsic apoptotic receptors, has been shown to be upregulated on neurons and glial cells following SCI [68]. Genetic deletion of the receptor (using lpr mice) resulted in improved functional and anatomical recovery, as well as decreased apoptotic cell death after SCI[121].

We undertook the task of trying to find a treatment that would manipulate FasR activation and have the potential to be neuroprotective in the clinical setting of SCI. We found literature showing that sFasR was capable of inhibiting the FasL/FasR interaction decreasing apoptotic cell death [136, 141]. Additionally, we found that humans produced an endogenous soluble form of the FasR following traumatic brain injury suspected to modulate the apoptotic response[142]. Based on this background, we targeted the

FasL/FasR interaction, using a soluble truncated form of the FasR, to inhibit the activation of the Fas apoptotic pathway.

Hypothesis

Administration of soluble Fas receptor will inhibit the increased Fas ligand/Fas receptor interaction, decrease apoptosis, and improve functional and behavioural recovery following acute spinal cord injury.

Statement of Objectives

1. To examine the *in vivo* relationship of FasL and FasR and caspase-8 in a severe clip compression model of spinal cord injury.
2. To observe the *in vitro* effects of sFasR in organotypic spinal cord slice following traumatic injury.
3. To observe the *in vivo* apoptotic response and caspase-3 activation after intrathecal sFasR delivery after SCI.
4. To observe the functional neurological outcome and histological recovery following sFasR treatment in the *in vivo* clip compression model of spinal cord injury.

Publications and Presentations

The following is a list of publication and presentations that have emanated from this thesis:

Publications

A.V. Krassioukov, A.Ackery, G. Schwartz, Y. Adamchik, Y. Liu, and M.G. Fehlings. **An *in vitro* model of neurotrauma in organotypic spinal cord cultures from adult mice.** *Brain Res Brain Res Protoc.* 2002 Oct;10(2):60-8.

A. Ackery and M.G. Fehlings. **P571. Neuroprotection after acute spinal cord injury by inhibition of the Fas apoptotic pathway.** *Journal of Neurotrauma*, 2002 Oct;19(10):1390. (Abstract)

S. Casha, A. Ackery, W. Yu and M.G. Fehlings. **Inhibition of FAS results in improved neurological outcome, reduced apoptotic cell death and enhanced axonal survival after spinal cord injury.** *Manuscript submitted to Journal of Neuroscience.*

Presentations

A. Ackery and M.G. Fehlings. **Neuroprotection After Acute Spinal Cord Injury by Inhibition of the Fas Apoptotic Pathway.** National Neurotrauma Society Symposium, Biloxi MS (2003/11). (Submitted abstract)

A. Ackery and M.G. Fehlings. **Neuroprotection After Acute Spinal Cord Injury by Inhibition of the Fas Apoptotic Pathway.** North American Spine Society, San Diego CA (2003/12). (Accepted Abstract)

A. Ackery and M.G. Fehlings. **Neuroprotection After Acute Spinal Cord Injury by Inhibition of the Fas Apoptotic Pathway.** Alan Wu Poster Competition, Institute of Medical Science, University of Toronto, Toronto ON (2003/06). (Poster Finalist)

A. Ackery and M.G. Fehlings. **Neuroprotection After Acute Spinal Cord Injury by Inhibition of the Fas Apoptotic Pathway.** Ontario Neurotrauma Foundation Conference, Toronto ON (2003/01). (Chosen for Oral Presentation)

A. Ackery and M.G. Fehlings. **Neuroprotection After Acute Spinal Cord Injury by Inhibition of the Fas Apoptotic Pathway.** Toronto Western Research Institute-Research Day, Toronto ON (2002/12). (Oral Presentation)

A. Ackery and M.G. Fehlings. **Neuroprotection After Acute Spinal Cord Injury by Inhibition of the Fas Apoptotic Pathway.** National/International Neurotrauma Society Symposium, Tampa FLA (2002/10). (Poster Presentation)

A. Ackery and M.G. Fehlings. **Neuroprotection of the Spinal Cord After Injury through Inhibition of the Fas Apoptotic Pathway.** University Health Network Research Day, University of Toronto (2002/11). (Poster Presentation)

A. Ackery and M.G. Fehlings. **Inhibition of Fas Apoptotic Pathway After Acute Spinal Cord Injury.** Canadian Institutes of Health Research- National Health Research Poster Competition, University of Manitoba (2002/06). (Poster Presentation)

A. Ackery and M.G. Fehlings. **Inhibition of Fas Apoptotic Pathway After Acute Spinal Cord Injury.** Southern Ontario Neuroscience Association/Program in Neuroscience, University of Toronto (2002/05). (Poster Presentation)

A. Ackery and M.G. Fehlings. **Inhibition of Fas Apoptotic Pathway After Acute - Spinal Cord Injury.** Gallie Batemen Day, Dept. of Surgery, University of Toronto (2002/05). (Poster Presentation)

A.Ackery and M.G. Fehlings. **Inhibition of Fas Apoptotic Pathway After Acute Spinal Cord Injury.** Institute of Medical Science Presentation, University of Toronto (2002/02). (Oral Presentation)

A.Ackery and M.G. Fehlings. **Inhibition of the Fas Apoptotic Pathway**. Trauma Research Day, University of Toronto (2002/04). (Oral Presentation)

Chapter 2- Materials & Methods

All experiments performed in the thesis are described in the following chapter. The methods are listed in the chronological order in which they were performed. These procedures are also separated into *in vitro* followed by *in vivo* techniques. Outcome measures will be described to help facilitate the interpretations given in the following results section (Chapter 3).

All facets of the methods described below have had their protocols approved by the animal care committee of the Toronto Western Research Institute & the University Health Network 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.

In vitro Experiments:

Spinal Cord Organotypic Slice Culturing

Spinal cord injury involves many distinct mechanisms that become activated simultaneously after trauma through primary and secondary injury. Our lab established a novel method for looking at these mechanisms *in vitro*. Krassioukov *et al.*[163] describes this method in its entirety for mice. Experiments completed involved rats as well as mice, and the procedures in culturing were identical (Figure 4).

Three to six week-old rodents (mice or rats) were anesthetized with pentobarbital (Somnotol, 65 mg/kg) and their thoracic spinal cords (T2-T10) were aseptically extracted and placed in ice-cold dissecting media (pH 7.15). 400μm-thick coronal spinal cord sections were cut, using a McIlwain mechanical tissue chopper (McIlwain, ON, Canada),

Timeline of Organotypic Experimental Procedures

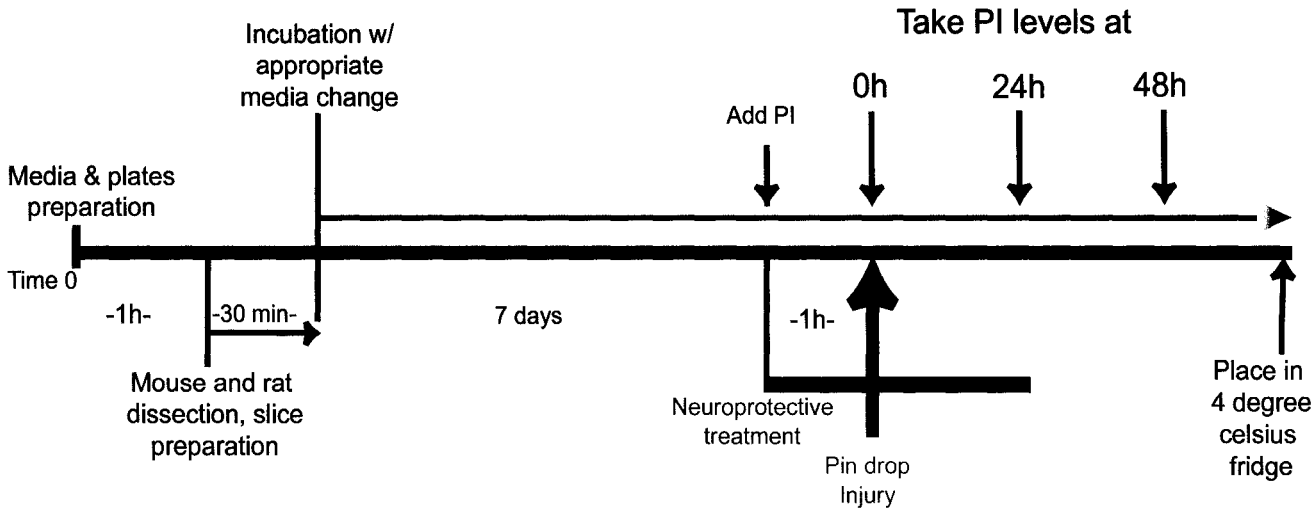


Figure 4. The time line for experiments undertaken in the organotypic slice model of spinal cord injury is shown. (PI= propidium iodide)

to generate the organotypic slices. Slices were placed into a Petri dish containing Earl's salt solution and separated, discarding slices of non-uniformity and with different cross-sectional size. The slices (approximately 8-12 slices per animal) were transferred to sterile porous membrane units (4 slices per membrane) (Millicell-CM, Millipore, MA, USA) which were submerged into wells of a six-well plate containing 1.4 ml of culture medium (50% MEM with Earl's salts and glutamine, 25% Hanks balanced salt solution and 25% horse serum supplemented with 20 mM of HEPES acid/salt and 6 mg/ml D-glucose, pH 7.2). Slices were placed in an incubator at 37°C in 6.5% CO_2 for seven days. Culture media were changed three times per week (Figure 5A).

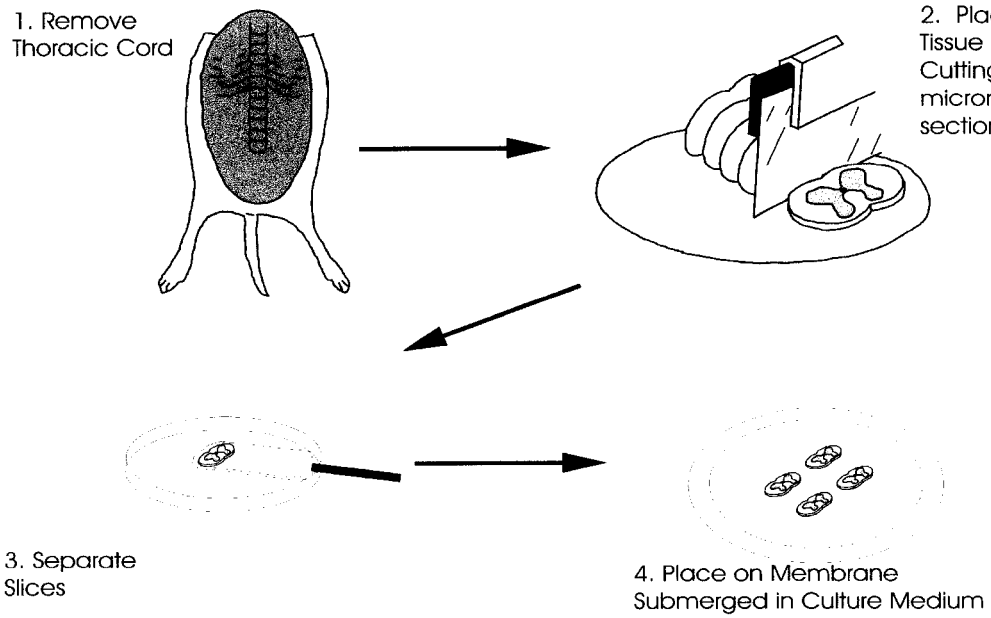
Organotypic slices were observed for viability on a daily basis, using an inverted bright field microscope (Nikon, Eclipse, TS 100) to observe the neurite outgrowth and cell viability within the gray and white matter (Figure 5B). Furthermore, a tetrazolium dye MTT was added to the medium of some wells to assess cell viability. MTT is a translucent yellow dye that is reduced by active mitochondria and other cellular enzymes to form a purple precipitate within the cytoplasm (Figure 5C).

In vitro Impact Injury Device

Mechanical trauma was elicited on the organotypic slices using a pin drop method. Seven days after culturing, an impactor (pin) with weight of 0.2 g was dropped from 1.7 cm onto the center of the culture slice (Figure 5D) in a sterile environment. Following the injury, the slices were placed back in the incubator at 37°C in 6.5% CO_2.

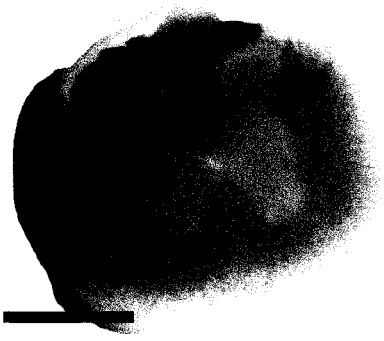
Preparation of Spinal Cord Organotypic Slice Cultures

A



* All Extraction Steps Were Done in Dissecting Medium

B



C

D

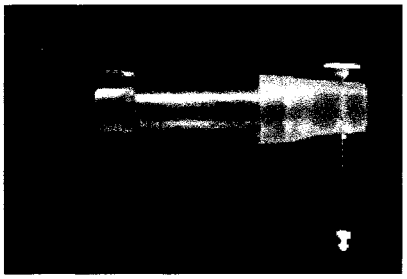


Figure 5. (A) Spinal cord slices were extracted in a manner similar to the schematic. (B) Whole spinal cord slice unstained. (C) MTT staining in organotypic slice. (D) Pin drop device used to injure slices. 0.2g piston was lifted to a height of 1.7 cm and dropped onto the center of the slice. Scale bar (B) = 500 μm, and (C) = 10 μm.

In vitro Administration of Neuroprotective Agents

Known neuroprotective agents were added to the medium to establish this model for testing new therapeutic interventions. CNQX, an AMPA/kainate receptor antagonist, and methylprednisolone (MPSS)(n = 3 slices per group), a clinically used anti-inflammatory agent, were applied at 10 μM, and were applied separately in the culture medium one hour before the injury. The drugs were left in the medium for 24 hours until slices were transferred into a new culture medium. These drugs were subsequently washed out to prevent potential neurotoxicity (Figure 4).

Assessment of cell death using PI in this model showed a progressive and significant increase within 24, 48 and 72 hours ($p<0.05$). The injured group showed a significant increase in cell death at each individual time point as compared to their uninjured counterparts ($p<0.05$). Pre-administration of MPSS showed a neuroprotective effect at 24 hours following injury ($p<0.05$), but this effect dissipated and was no longer significant at 48 and 72 hours. CNQX administration prior to injury resulted in neuroprotection at 24, 48 and 72 hours as compared to the untreated injured group ($p<0.05$) (Figure 6). We established the validity of our murine *in vitro* organotypic slice model of SCI and these results were published in Brain Research Protocols[163].

Soluble Fas Receptor solution (0.5 mg/ml with 0.5 mg/ml Gentamycin Sulfate, Alexis Biochemicals, San Diego, CA) was administered to the culture medium at 1.0 μl/1.4ml of culture medium and was left in the medium for the duration of the quantification, which varied from 48 to 72 hours depending on the species being used.

Neuroprotection Following Organotypic Slice Injury

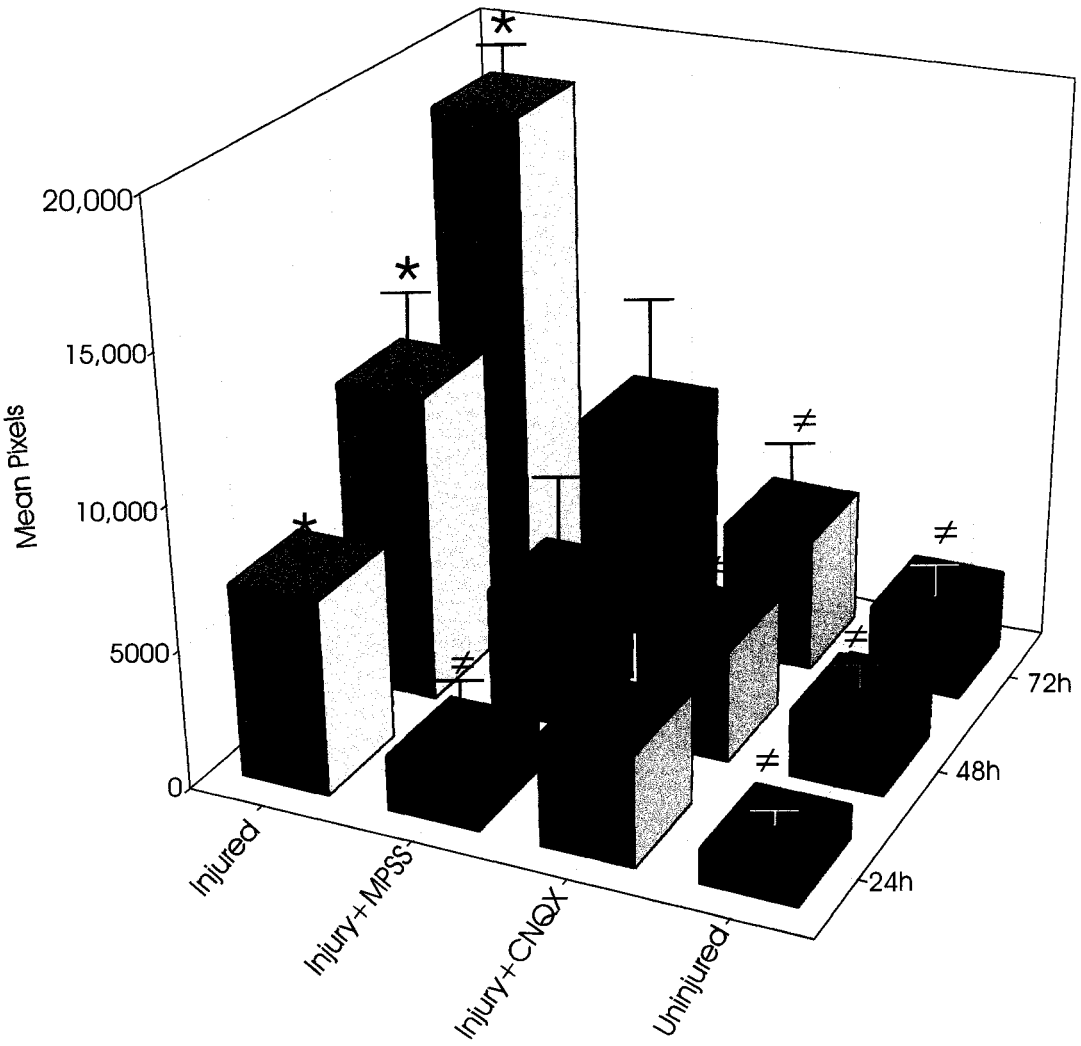


Figure 6. Average cell death of murine injured control spinal cord slices, injured treated with MPSS or CNQX and uninjured control slices, at 24, 48 and 72 hours after pin drop injury. (*) Statistical significance ($p<0.05$) among slices of injured control group times at 24, 48, 72 hours; (≠) statistical significance ($p<0.05$) among groups compared to the injured control slices and their coinciding times. Error bars = SEM. Published in Brain Research Protocols 2002.

Injured controls were not treated. (Total # slices: n =38 slices for murine, n =16 slices for rat).

Propidium Iodide and Quantification of Cell Death

Assessment of total cell death was done using propidium iodide (PI). PI is a nuclear marker that increases in fluorescence when bound to DNA. It has already been established as cell death marker in organotypic models and is quite proficient for quick observations of cell death[163, 164]. PI (10 μM) was added to each well one hour prior to injury. Confocal microscopy (BioRad) was used to capture fluorescence images using a 4X objective lens with a rhodamine filter (540-580/590 nm). The PI fluorescence emissions were immediately captured before, 24, 48, and 72 hours after injury, keeping standardized gains and black levels. Images were taken not on the surface of the slice, but at 150 μm below (approximately middle of 300 μm thick section) the surface as to avoid the glial scar layer on the surface of the slice.

Captured pixel intensities were analyzed with the help of image analysis software (Media Cybernetics, *Image Pro Plus 4.1*). Total cell death after injury was calculated as $F_1 - F_0$, where F_1 is the fluorescent levels (in pixels) at the specified time (24, 48 and 72 hours) intervals following trauma and F_0 is the fluorescent level (in pixel) prior to the injury (time 0). This number of mean pixels increase in response to injury was used as an index of cell death. Statistical Analysis was performed using t-tests, one-way or two-way ANOVA's with Student-Neuman-Keuls (SNK) analysis or Bonferroni corrections

depending on the experiment done. Significant statistical differences were established at $p<0.05$.

In Vivo Experiments:

In Vivo Clip Compression Model of Spinal Cord Injury

The rat *in vivo* clip compression model of SCI at C7-T1 was used for all *in vivo* experiments (Figure 7). Adult female Wistar rats (250 g; Charles River Laboratories, Wilmington, MA, USA) were anesthetized with halothane (1.5-2.0%) and N_20 (1 L/min), and a laminectomy was performed from C6 to T2 (extent of laminectomy will be explained in pump methods). The modified Kerr-Lougheed aneurysm clip (Figure 8A) was placed at C7 and released onto the spinal cord, compressing it for 1 minute at a calibrated force of 53 g (Figure 8B & C). This force of injury has been extensively characterized and denotes a severe spinal cord injury with initial complete paraplegia and with minimal delayed partial recovery of hind limb function weeks post-operatively[150, 165-167]. Acute post-operative care involved rehydration with 5 c.c. of saline, increasing body temperature through heat lamp and blankets, and the intra-peritoneal administration of buprenorphine hydrochloride (0.05 mg/kg) immediately following surgery. Chronic post-operative care included placement of animals in a room with an ambient temperature of 27-28°C, food and water given *ad libitum,* and manual expression of bladders three times daily. Animals were kept alive from one to seven weeks depending on the experiments being conducted.

Timeline of Behavioural Experiments

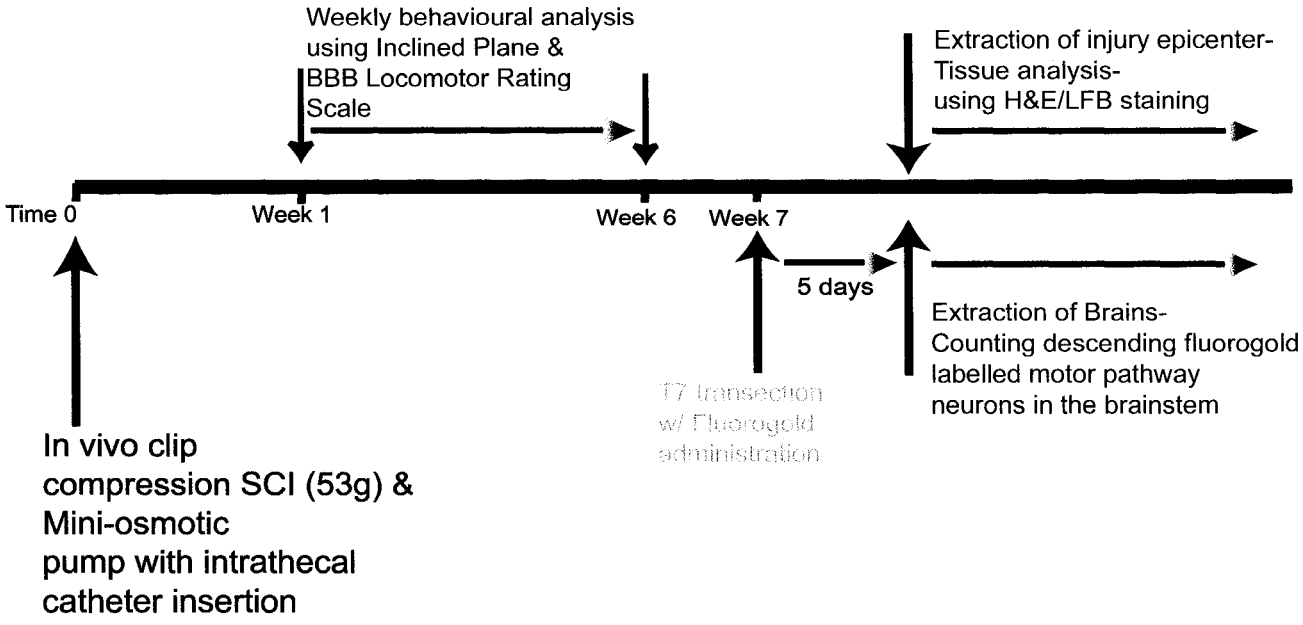


Figure 7. The time line for experiments undertaken in the *in vivo* clip compression model of spinal cord injury.

In Vivo Clip Compression Model of Spinal Cord Injury

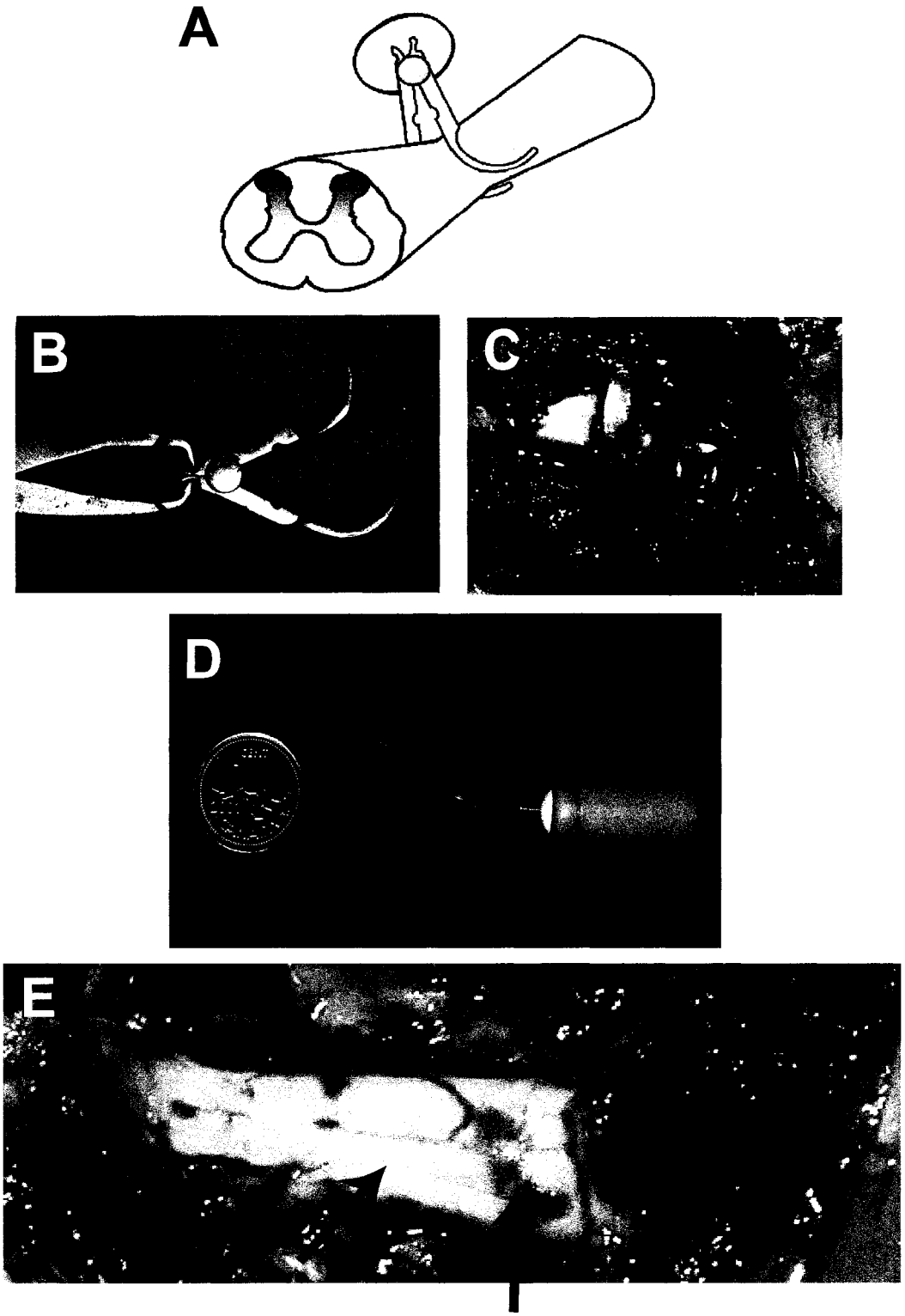


Figure 8. (A) A modified aneurysm clip calibrated at a closing force of 53g. (B,C) Operative photograph and schematic showing extradural clip compression of spinal cord at C7-T1. (D) Photograph of the 200 μl Alzet mini-osmotic pump delivering the solubleFas receptor at a rate of 1 μl/hr for seven days. (E) Photograph of intrathecal catheter delivering sFasR to the injury site.

Soluble Fas Receptor Intrathecal Delivery

Due to the enormous costs of soluble Fas Receptor (sFasR, Alexis Biochemicals, San Diego, Ca, USA), an intrathecal dose of 5.0 μg of sFasR over seven days was determined for each 250 g animal, based on previous experiments[141]. This 5.0μg was diluted in 200μl of saline and placed in a mini-osmotic pump (Figure 8D)(Model 2001, 1μl/hour for seven days; Alzet Osmotic Pumps, Cupertino, CA, USA).

Delivery of the sFasR from the mini-osmotic pump was through an intrathecal catheter (Recathco, PA, USA), which was subdurally inserted below the level of injury through an incision (needle prick, 18 gauge) in the dura, resting on the dorsal surface of the spinal cord at the level of injury C7 (Figure 8E). This procedure was performed immediately following the clip compression injury. The catheters were anchored to the third thoracic (T3) lamina using cyanoacrylic glue (Krazy Glue, Elmer's Products Inc., Brampton, Ontario) and sutures. Lastly, the mini-osmotic pumps were placed underneath the skin on the dorsal side. Injured controls received saline infusions.

Western Blot Analysis for Caspase-3 Activation

One centimeter of spinal cord tissue was extracted centering at the injury site. Each extract sample was homogenized with 400 μl of homogenization buffer, placed on ice to minimize protein degradation, and centrifuged (10,000 rpm for 10 min). The Lowry protein quantification method was performed on each sample to assure equal loading when performing the immunoblot analysis.

40 μg of protein extract from each sample was run on a 12 % separating gel and transferred to a nitrocellulose membrane. The membrane was blocked for non-specific

binding using a 5 % milk-blotto blocking solution. The primary caspase-3 (Santa Cruz, pro-and activated caspase-3) monoclonal antibody (1:100) was placed on the membrane for 24 hours, followed by a one-hour incubation with a goat anti-rabbit secondary antibody (1:2000). The membrane was developed on X-ray film using an ECL chemilluminescence kit (PerkinElmer Lifesciences Inc., Boston, MA, USA) that was exposed for 30 minutes. The membranes were re-probed and re-developed for the actin monoclonal primary antibody (1:400) and an anti-mouse secondary antibody (1:4000) to ensure equal loading between lanes.

Immunoprecipitation of Fas Ligand, Fas Receptor and Caspase-8

Using one centimeter of spinal cord tissue centered at the injury epicenter, we extracted 300 μg of centrifuged protein and topped off to 1 ml using RIPA buffer. Next, we added 0.25 μg of IgG control protein (FasL uses normal mice IgG, and this helps lower non-specific binding) and 20 μl of the appropriate agarose conjugate. This solution was incubated & rotated for 30 minutes at 4° C. The pellet beads were then centrifuged (2,500 rpm) for 5 min at 4° C, followed by extraction of the lysate. The lysate was then incubated with 10 μl of FasL primary antibody for 2 hours at 4° C, followed by the addition of the protein A/G plus-agarose conjugate. Tubes were capped and incubated overnight on a rocker at 4° C.

Day two involved collection of the pellet by centrifugation (2,500 rpm for 5 minutes), carefully aspirating and discarding the supernatant. The pellet was washed and centrifuged (in similar fashion to the above step) using 1 ml of RIPA buffer, being

cautious to not suck up the pellet. The pellet was boiled (2-3 minutes, to separate the agarose from the antibody), and then re-suspended in 40 µl of 1X electrophoresis buffer with β-mercaptoethanol in preparation for PAGE.

20 µl of protein of each sample was loaded into the wells and Western blot analysis was performed using: 1) the primary polyclonal FasR (1:200) antibody with an anti-rabbit secondary antibody (1:2000), and 2) the primary polyclonal caspase-8 antibody (1:100) with an anti-goat secondary antibody (1:4000).

In situ (TUNEL) immunocytochemistry

Sham uninjured (n=3), injured with saline administration (n=3), and injured with sFasR administration (n=4) animals were trans-cardially perfused with 0.2M PBS (5min) and 4% paraformaldehyde (40 min) seven days following surgery. Spinal cords were extracted and post-fixed overnight in 4% paraformaldehyde and then transferred for 5 days into a 0.2 M PBS/30% sucrose solution.

One centimeter of spinal cord, centered at the injury epicenter, was snap frozen (spinal cords were frozen with Histoprep and dipped in 2-methylbutane cooled by dry ice) and cut on the cryostat (14µm thick coronal sections).

Hematoxylin & eosin, and luxol fast blue staining (described below) were performed to find the injury epicenter. Injury epicenter sections were taken to perform TUNEL immunocytochemistry.

The TUNEL protocol (Apoptag® Apoptosis Detection Kit, 2002 Serological Corporation, Norcross GA) was performed as follows: 1) Sections were rehydrated in dH_20, 2) equilibration buffer was aspirated onto the sections (fully covering them) and

left for 1-2 minutes, 3) working strength TdT enzyme (70% Reaction buffer and 30% TdT enzyme) was aspirated onto the sections and placed in an incubator at 4°C overnight. 4) On the following day, spinal cord sections were submerged in working stop solution for 10 minutes at room temperature. 5) Working strength anti-digoxenin working strength fluorescein was applied for 1 hour at room temperature, and cover-slipped with MOWIOL. TUNEL positive counts were performed at 10X magnification on Nikon E800 Eclipse epifluorescence microscope. Three non-overlapping pictures were taken to count whole crossections at the injury epicenter. Three whole spinal cord crossections were counted for each animal and averaged. Statistical significance was established at $p<0.05$ using students' t-test.

Behavioural Analysis

Inclined plane tests and the Basso Beattie Bresnahan (BBB) locomotor rating scale were used to assess hind limb motor function recovery. The inclined plane test looks at gross hind limb motor function recovery, using a rectangular padded board (Figure 9A) that rises in 5° increments relative to the horizontal. Scores are the maximum angle in which the animal can vertically maintain itself (minimum 5 seconds)[168]. BBB scores rank fine hind limb motor recovery using a scale that ranges from 0 to 21 (Table 3 & Figure 9B)[169].

Animals were injured using the clip compression model of spinal cord injury. Animals were blindly placed in either injured control (n=8) (Saline intrathecal administration) or injured sFasR treated (n=10) groups. Both groups started with 12 animals, but due to the level and severity of the injury some did not survive. Behavioural

Behavioural Tests Performed Following Spinal Cord Injury

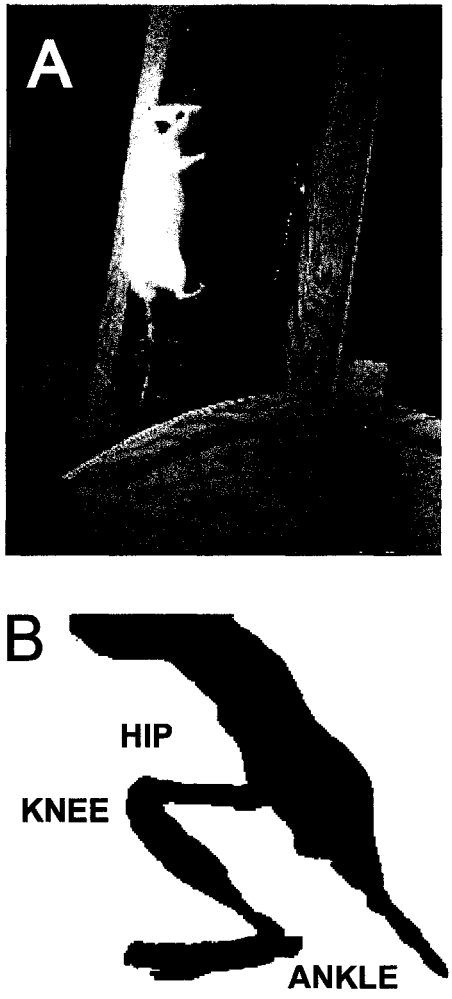


Figure 9. (A) Photograph of the incline plane test (Taken from RIvlin and Tator, J. Neurosurg., 1977). (B) Schematic illustrating the hindlimb joints used in scoring the Basso, Beattie, Bresnahan locomotor rating scale (Adapted from Basso et al. J. Neurotrauma, 1995)

Table 3. BBB fine hind limb locomotor rating scale

Score	*Function*
0	No hind limb movement
1	Slight[a] movement of 1-2 joints
2	Extensive[b] movement of 1 joint
3	Extensive movement of 2 joints
4	Slight movement of 3 joints
5	Slight movement of 2 joints, extensive movement of 1 joint
6	Extensive movement of 2 joints, slight movement of 1 joint
7	Extensive movement of 3 joints
8	Sweeping or plantar placement, no weight support
9	Plantar placement with weight support, or dorsal stepping with weight support
10	Occasional[c] weight supported plantar steps, no coordination[d]
11	Weight-supported plantar steps, no coordination
12	Weight-supported plantar steps, occasional coordination
13	Weight-supported plantar steps, frequent[e] coordination
14	Weight-supported plantar steps, consistent[f] coordination
15	Consistent plantar stepping and consistent coordination, no/occasional toe clearance
16	Consistent plantar stepping and consistent coordination, frequent toe clearance
17	Consistent plantar stepping and consistent coordination, frequent toe clearance, paw parallel throughout gait
18	Consistent plantar stepping and consistent coordination, consistent toe clearance
19	Consistent plantar stepping and consistent coordination, consistent toe clearance, tail down
20	Consistent plantar stepping and consistent coordination, consistent coordination toe clearance, tail up, trunk unstable
21	Consistent plantar stepping and consistent coordination, consistent toe clearance, tail up, trunk stable

[a] <50% range of motion.
[b] >50% range of motion.
[c] 0-50% of the time.
[d] Front limb-hind limb coordination.
[e] 50-94% of the time.
[f] 95-100% of the time.

Adapted from: Basso *et al.* J Neurotrauma. 1995 Feb; 12(1): 1-21.

recovery tests were performed weekly for six weeks. Three blinded observers were used to assign behavioural scores and all animals were videotaped (in case review of scoring was needed). Two-way ANOVA statistical analysis was performed to compare the two groups over time, with Student Neuman-Keuls post-hoc statistical analysis at the individual time points ($p<0.05$).

Hematoxylin & Eosin/ Luxol Fast Blue Staining and Injury Site Analysis

Animals from the behavioural experiments (Injured control n=5 and injured w/ sFasR n=8) were transcardially perfused (as described above) 7 weeks following injury. Their spinal cords were extracted, placed in 10% formalin, dehydrated through alcohols and chloroform and embedded in paraffin. Paraffin-embedded spinal cord tissue was cut into 12 μm-thick sections. Luxol fast blue (LFB) staining involves a series of rinses through xylene, 100% and 95% EtOH, which precedes submerging sections in LFB overnight at 37°C. After LFB incubation, the sections were placed in $LiCO_3$ with 70% EtOH until gray and white matter was properly differentiated. Next, sections were dipped in hematoxylin (20 minutes), H_20 (20 minutes), and quickly in eosin (10-20 seconds depending on strength of stain). Sections were dehydrated and cleared through alcohols & xylene, and coverslipped with Permount.

Bright field microscope images were taken in 500 μm increments both caudal and rostral to the injury epicenter until reaching a maximum distance of 2000 μm. Using Image Pro Analysis software, the residual tissue cross-sectional area was measured by analyzing the cavity area compared to the whole spinal cord cross-section. Statistical

analysis was performed using two-way ANOVA's with Student Neuman-Keuls post-hoc analysis ($p<0.05$).

Retrograde Axonal Labeling

Six weeks following SCI, the animals used for behavioural analysis were re-anesthesized (as described above) and a laminectomy was performed at T7-T8 followed by a T7 transection for retrograde axonal labeling (Figure 10). This was done in both injured control (n=5) and injured w/ sFasR (n=8). Some of the behavioural animals did not survive following the transection and could not be used for this experiment. A 4% fluorogold solution (Fluorochrome Inc., Engelwood, Colorado, USA) soaked foam pledget (size equaling the cross-sectional diameter of the T7 spinal cord, approximately 3x3mm) was placed in the transected gap and Vaseline was applied overtop of the cord to prevent non-specific fluorogold dissemination through the vasculature. The animals were transcardially perfused with 4% paraformaldehyde five days following pledget insertion and the rat brains were extracted, post-fixed in 4% paraformaldehyde overnight followed by 30% sucrose in PBS for seven days.

Brains were snap frozen and embedded in histoprep (as described previously), serially cut at 40 μm on the cryostat, and alternate sections were stained with cresyl violet to identify the red nucleus. Unstained sections were used to count fluorogold positive neurons in each individual nucleus. Fluorescent images were captured using the Nikon E800 Eclipse epi-fluorescence microscope and counted using Image Pro Analysis software. Groups were compared using student t-test ($p<0.05$).

Fluorogold Retrograde Tracing Following Spinal Cord Injury

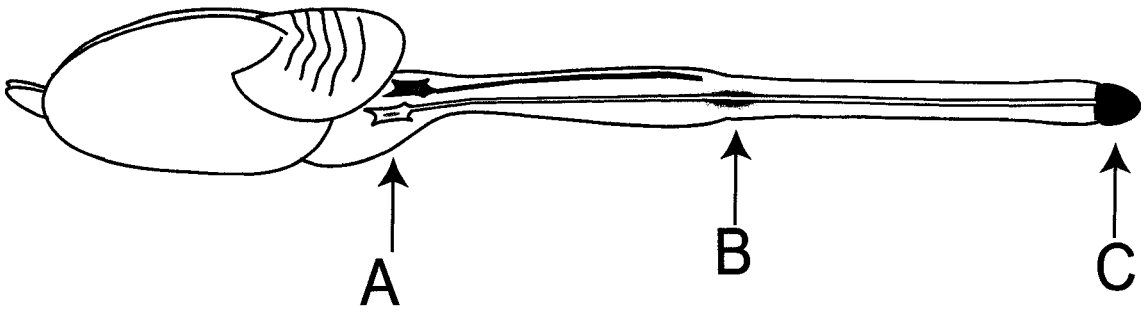


Figure 10. Schematic showing a sagittal view of the brain and spinal cord with (A) the area of the brainstem where the fluorogold positive red nuclei are found, (B) the injury epicenter at C7-T1 and (C) the transected T7 site where the fluorogold pledget was placed and left for 5 days (approximately 6 weeks following initial compression injury).

Chapter 3- Results

The following section will discuss the results that emanated from my time spent in the Fehlings laboratory. This section will describe all of the results from the above-described experiments. All results were obtained by the author, unless otherwise noted in the text.

Increased Fas Ligand/Fas Receptor Interaction Following Spinal Cord Injury

FasL binds FasR initiating the DISC to activate caspase-8. This triggers a caspase cascade that leads to apoptosis. We examined the interactions of these proteins following *in vivo* clip compression model of SCI. Using an immunoprecipitation technique we isolated FasL and performed immunoblots to analyze changes in the interactions between FasL, FasR and caspase-8 three and seven days following SCI.

We observed that three days following SCI there was an increased interaction between FasL (40 kDa) and FasR (48kDa) (Figure 11). Also, looking at the FasL/caspase-8 interaction, there was no observable difference in pro caspase-8 (55kDa) following injury. However, FasL developed an increased interaction with activated caspase-8 (20 kDa) three and seven days following SCI (Figure 11). Coomassie blue staining was performed on the immunogels because it was ascertained to be a suitable method to ensure equal loading in each of the immunoblots.

Increased Interactions of Fas Ligand, Fas Receptor and Caspase-8 After Spinal Cord Injury

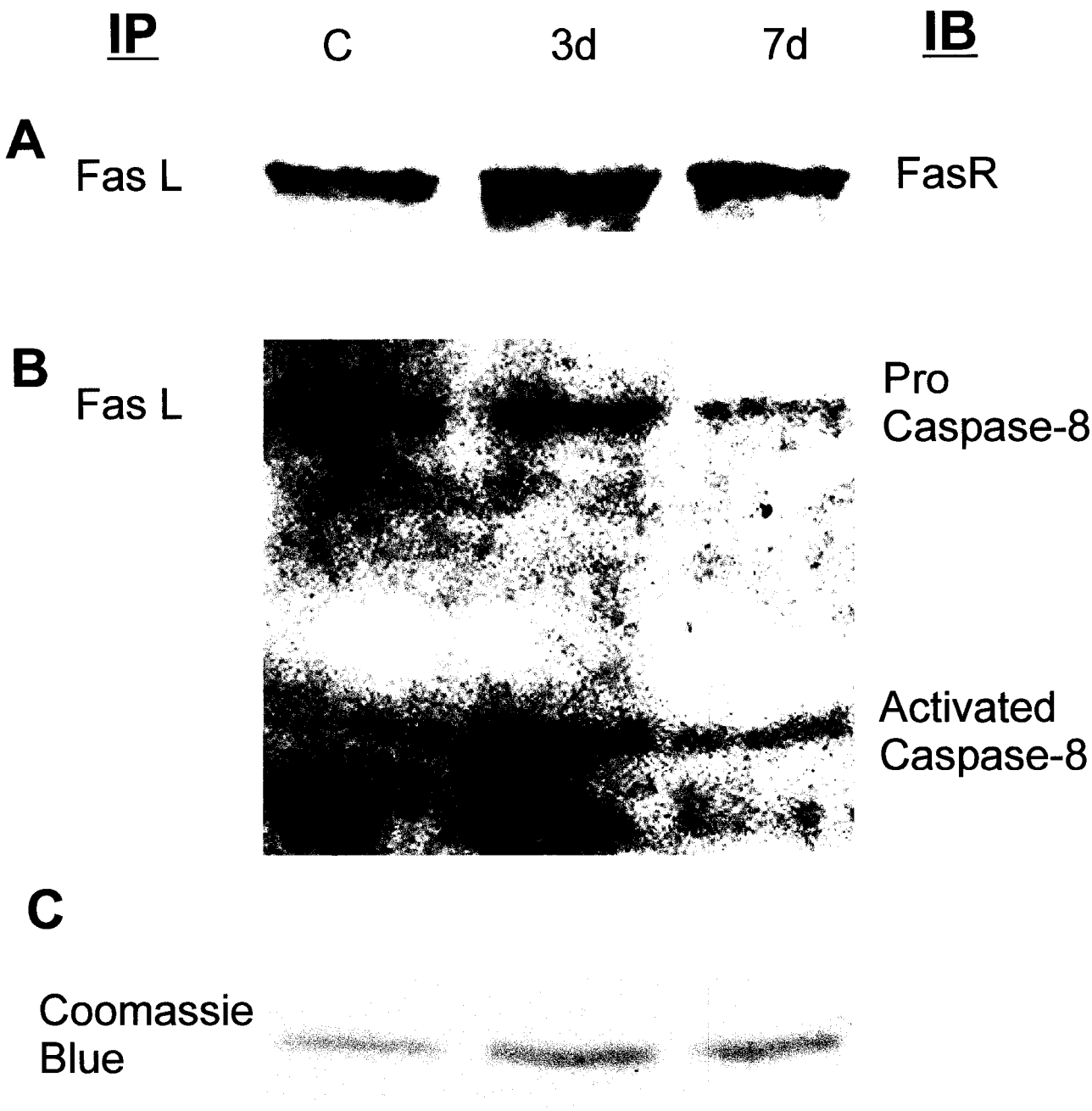


Figure 11. (A) Expression of Fas Ligand (FasL) and Fas receptor (FasR) after acute SCI over time. Increased interactions are seen at 3 days following injury. (B) Interaction of Fas Ligand (FasL) with the activated and pro-forms of caspase-8 after SCI over time. An increased interaction with activated caspase-8 is seen 3 and 7 days following SCI. (C) Coomassie Blue stain showing equal antibody loading throughout the immunoprecipitation. IP, immunoprecipitation antibody; IB, immunoblotting antibody. S=Sham, 3d= 3 day injury with 53g clip and 7d = 7day injury with 50g clip injury.

In Vitro Administration of Soluble Fas Receptor Results in Decreased Cell Death Following Injury

We administered sFasR to the medium of rat & murine organotypic slices one hour prior to the pin drop injury and left it in the medium for the duration of the experiment. In the rat model we observed an attenuation of PI uptake correlating to decreased cell death in the sFasR treated slices at 48 hours, as compared to the injured untreated group ($p<0.05$)(Figure12). In the murine model we extended the observations to 72 hours and found less post-traumatic cell death in sFasR treated groups compared to the injured untreated group (Two-way ANOVA: $p<0.001$ for both sFasR treatment and time; pair-wise comparisons with t-test and Bonferroni correction: $p<0.05$ at 48 and 72 hours) (Figure 13). The murine data has recently been submitted to the Journal of Neuroscience[121].

Soluble Fas Receptor Decreased Caspase-3 Activation Following Spinal Cord Injury

We examined the *in vivo* effect of 7-day mini-osmotic administration of sFasR following 50 g clip compression of the spinal cord. We qualitatively observed a decreased activated caspase-3 product (18 kDa) following in rat spinal cord homogenates as compared to injured saline administered control animals (Figure 14). Normal rat and sham operated animals showed no activated caspase-3 product present. β-Actin (42 kDa) was also run out on the Western blots to denote equal loading within each lane. Lastly to note, these results were difficult to reproduce consistently due to the high concentrations

Propidium Iodide Fluorograms of Rat Organotypic Slices Following Pin Drop Injury andSoluble Fas Receptor Administration

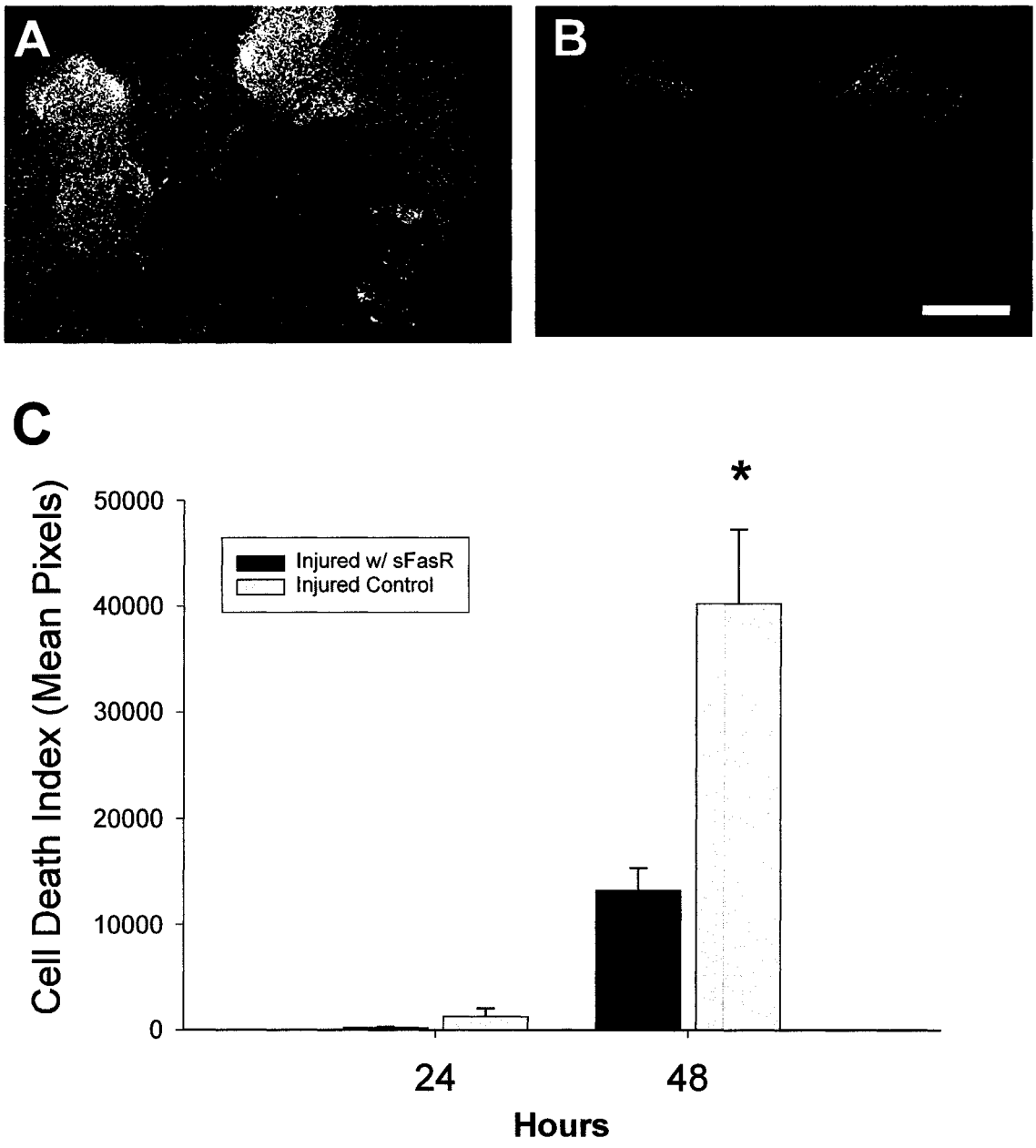


Figure 12. Propidium iodide fluorograms 48 hours after rat organotypic spinal cord weight drop injury with (A) injured control and (B) injured with soluble Fas receptor. (C) Soluble Fas receptor causes significant reduction in cell death (PI fluorescence) 48 hours after injury(*). Scale bar = 500 μm

Propidium Iodide Fluorograms of Murine Organotypic Slices Following Pin Drop Injury and Soluble FasReceptor Administration

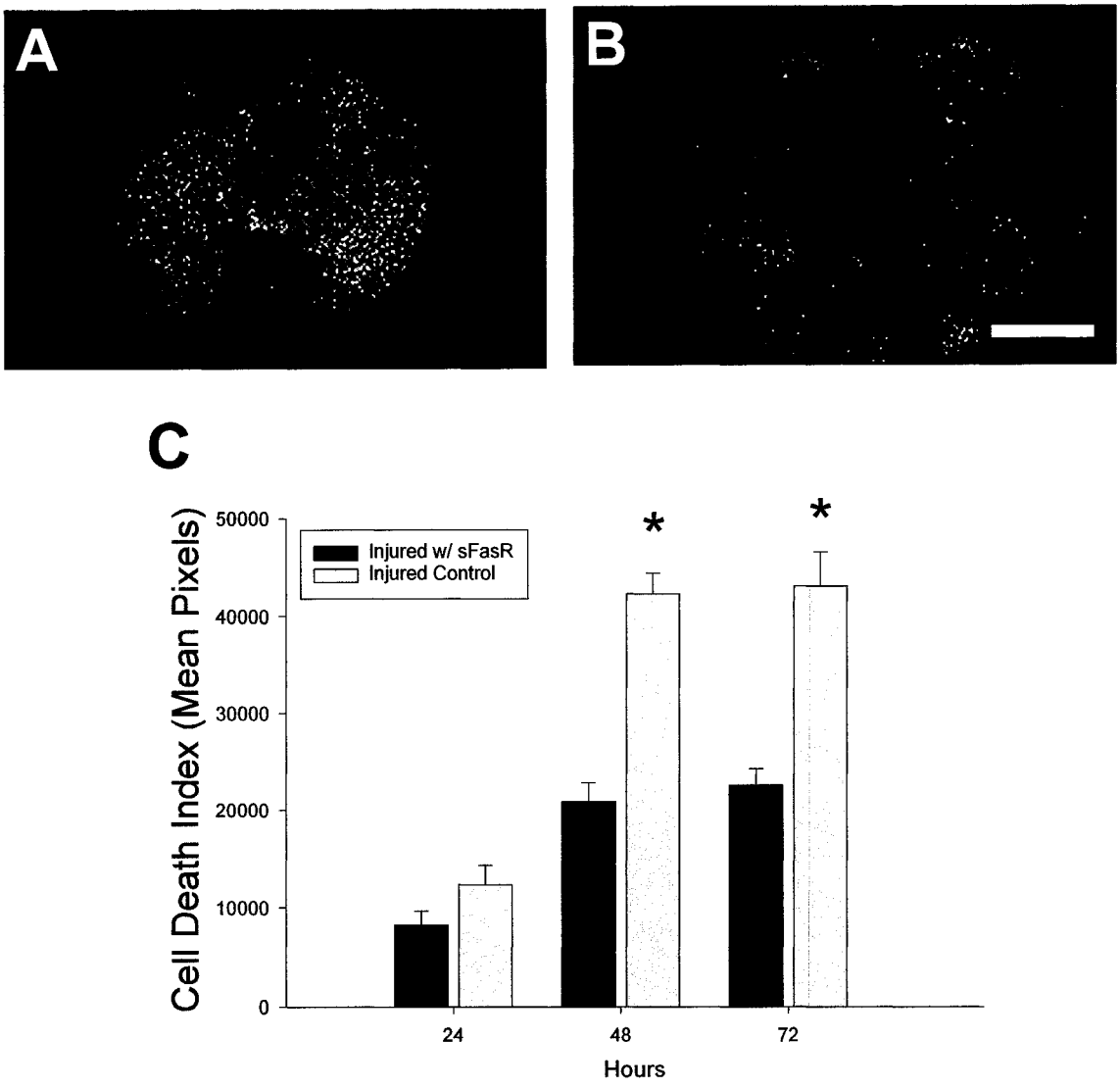


Figure 13. Propidium iodide fluorograms after murine organotypic spinal cord weight drop injury with (A) injured control and (B) injured treated with soluble Fas receptor slices. (C) Soluble Fas receptor causes significant reduction in cell death (PI fluorescence) 48 and 72 hours after injury (*). Scale bar = 500 μm

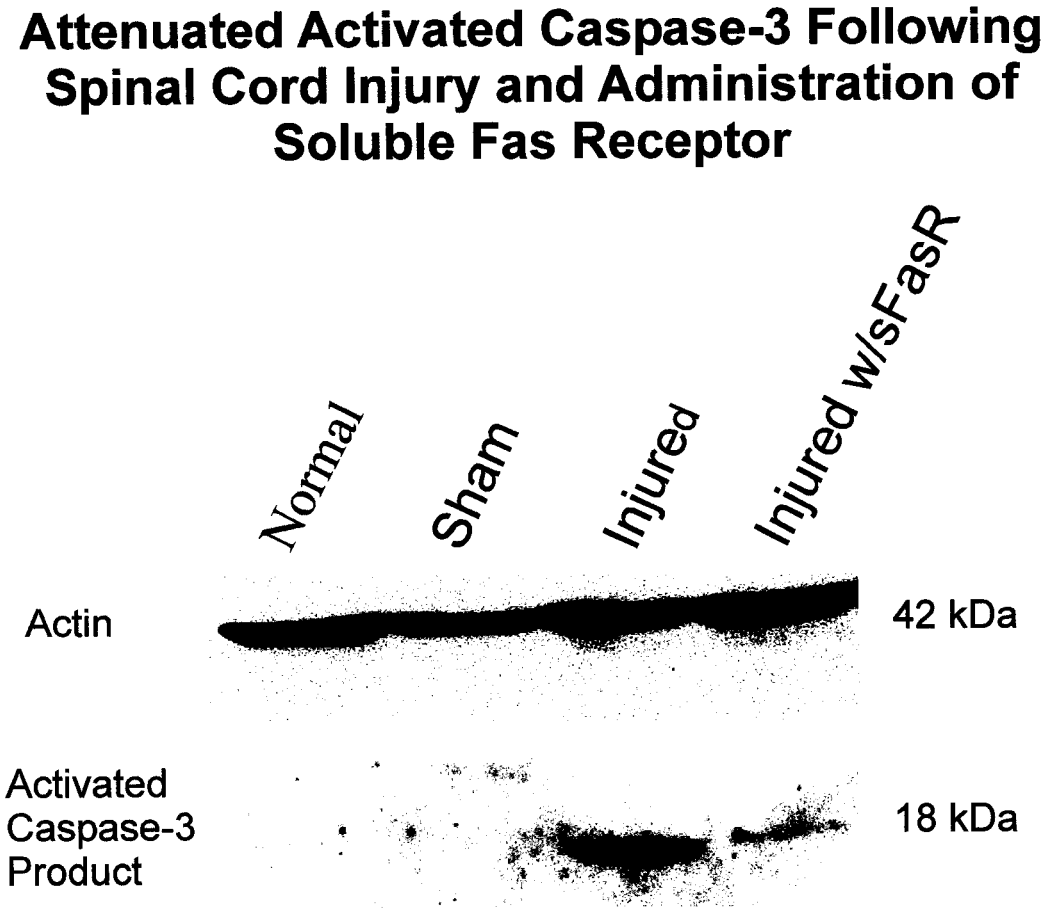


Figure 14. Western blot showing attenuated levels of caspase-3 activation with sFasR treatment 7 days following spinal cord injury.

of protein needed (>40 μg of protein per lane) and inconsistency of binding of the primary antibody.

TUNEL Labeling Following sFasR Administration

At day seven following SCI, we examined the extent of TUNEL labeling at the injury epicenter following administration of sFasR. Qualitatively we found that TUNEL labeling occurred both within the white and gray matter in all injured groups, while no observable TUNEL labeling was seen in uninjured shams. Quantitatively there appeared to be a trend toward decreased TUNEL labeling in the soluble Fas treated animals as compared to the injured saline treated animals (Figure 15). However, it did not reach the statistical significance ($p< 0.078$) due to insufficient numbers.

Soluble Fas Receptor Administration Improves Behavioural Recovery Following Spinal Cord Injury

We performed weekly behavioural examinations on animals for 6 weeks using the Basso Beattie Bresnahan (BBB) locomotor rating scale and the inclined plane test. Injured sFasR treated animals had significantly higher overall BBB scores as compared to the injured control saline treated animals (IC) (Two-way ANOVA: $P<0.001$ for both treatment and time). At three weeks student Neuman-Keuls post hoc analysis showed a statistically significant improvement in sFasR treated animals as compared to the injured controls ($P=0.009$; Mean ± SEM: sFasR 7.90 ± 0.94, IC 4.125 ± 1.058) (Figure 16).

Inclined plane scores showed overall improvement in the sFasR treated groups as compared to their injured controls (Two-way ANOVA: $P=0.002$ for treatment and $P<0.001$ for time). Post hoc student Neuman-Keuls analysis of the square root

TUNEL labeling Following Spinal Cord Injury and Soluble Fas Administration

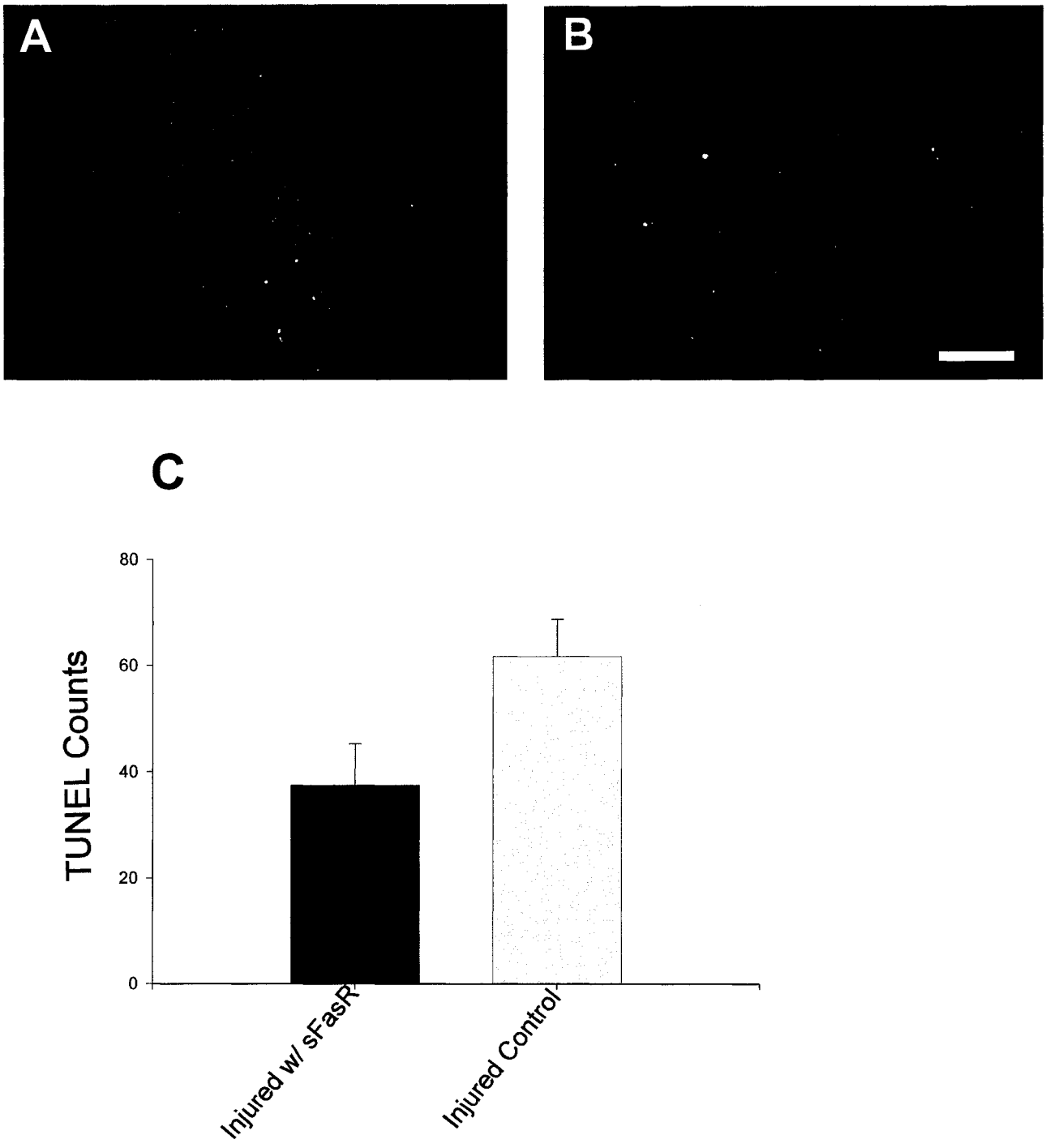


Figure 15. Low magnification of TUNEL positive cells of (A) Injured Control and (B) Injured with sFasR. (C) Soluble Fas receptor administration showed a trend towards decreasing apoptotic cell death with TUNEL labelling although it did not reach statistical significance ($p<0.078$). Scale bar = 150μm.

BBB Locomotor Test Following Spinal Cord Injury and Administration of Soluble Fas Receptor

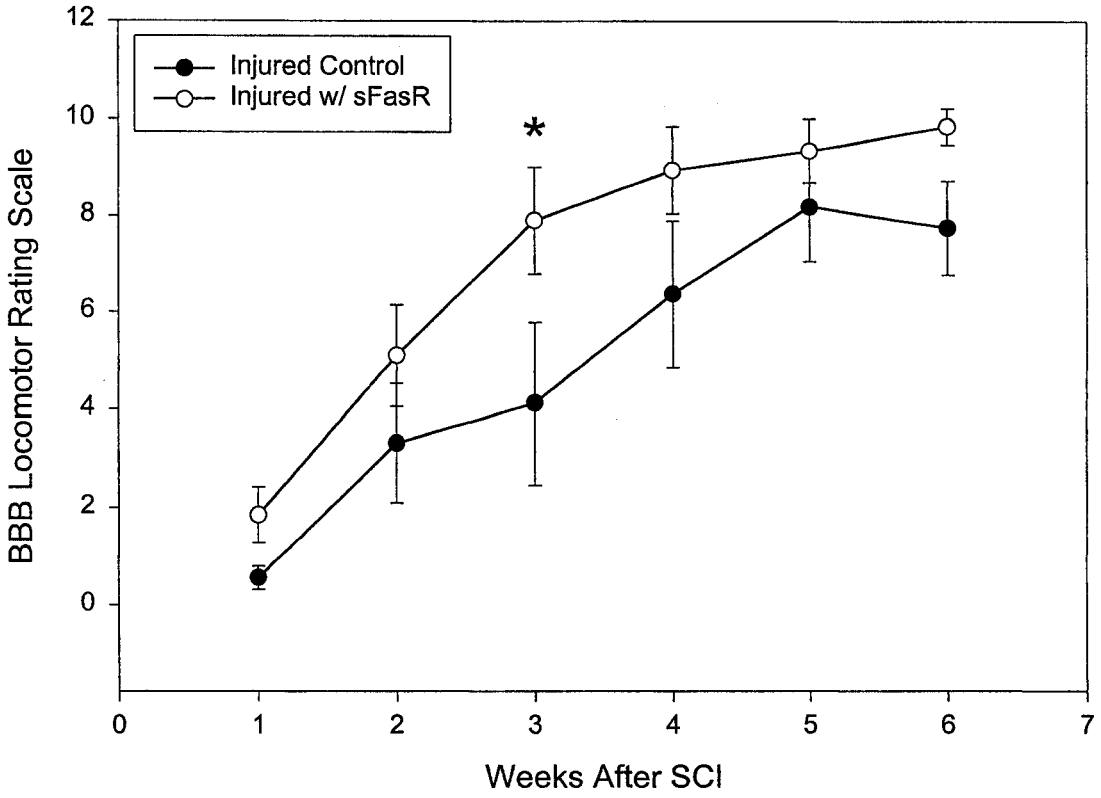


Figure 16. The Basso, Beattie, Bresnahan (BBB) locomotor rating scale (0 to 21) showed an overall increase in fine hind limb motor function recovery in animals that received sFasR treatment, as compared to their control saline treated group (Two way ANOVA: $p<0.001$). Post hoc student Neuman-Keuls analysis showed a statistically significant improvement in sFasR treated animals as compared to injured control 3 weeks following injury (*). Error bars = SEM

transformed data showed at two weeks a statistically significant improvement in sFasR treated animals as compared to the injured controls (P=0.04; Mean ± SEM: sFasR 30.5 ± 3.23, IC 23.1 ± 3.61) (Figure 17).

Increased Tissue Preservation with Soluble Fas Administration Following Spinal Cord Injury

Using the animals from the behavioural experiments, we analyzed the residual tissue using hematoxylin & eosin and luxol fast blue staining. We found that animals that received sFasR following injury had an overall significant increase in residual tissue collected 2000 μm caudal to 2000 μm rostral to the injury site (Figure 18)(Two way ANOVA: $P<0.001$ for both treatment and distance). Furthermore, post-hoc analysis showed that at the injury epicenter and at both 500 μm caudal & rostral to the injury epicenter the normalized residual tissue was increased with sFasR (Student Neuman-Keuls: $P<0.001$ for 500, 0, -500 μm).

Increased Neuronal Survival in the Red Nucleus with Soluble Fas Administration after SCI

Using the animals from the behavioural experiments, we looked at the difference in fluorogold positive red nucleus neurons (both magnocellular & parvocellular) to see if there was an increased preservation of rubrospinal tract axons following SCI with sFasR administration as compared to saline administered injured controls. Performing student t-tests on square root transformed data, we found a statistically significant increase of fluorogold positive neurons in the sFasR-administered animals as compared to the injured

Inclined Plane Test Following Spinal Cord Injury and Administration of Soluble Fas Receptor

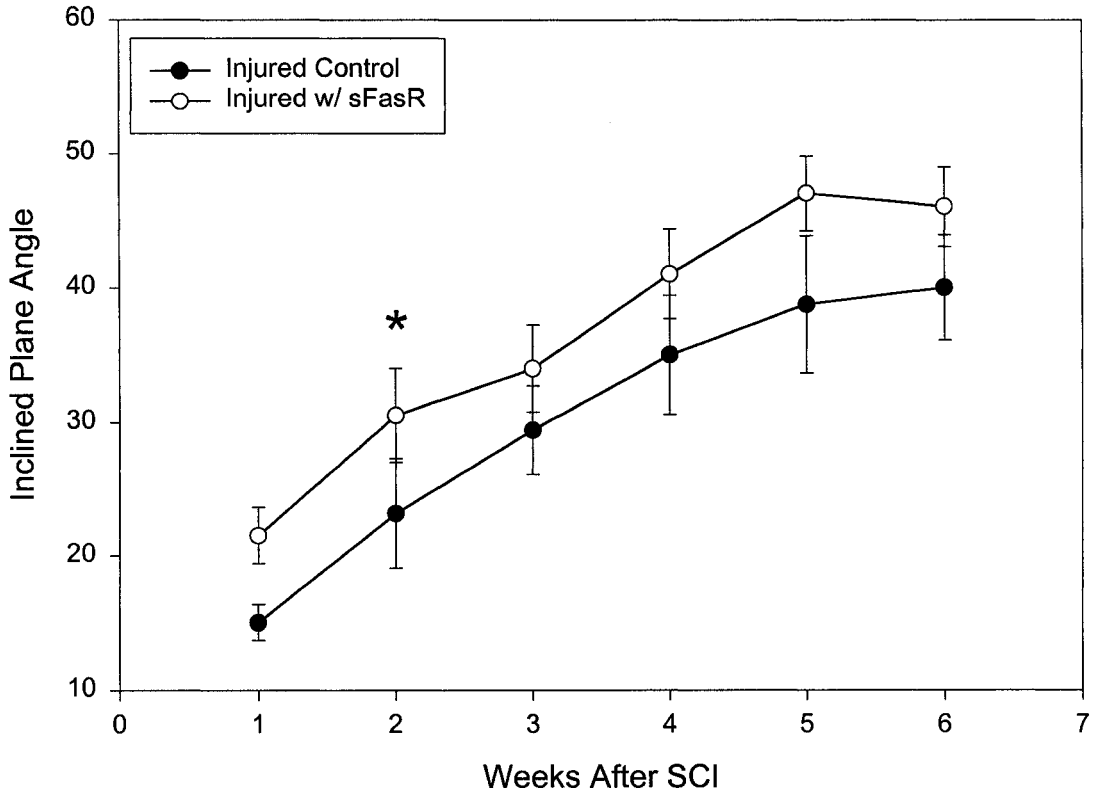


Figure 17. Inclined plane test showed an increased overall gross motor function recovery of animals that received sFasR treatment for seven days following SCI, as compared to control saline treated groups (Two way ANOVA: p=0.009). Square root transformations of the data and student Neuman-Keuls post hoc analysis showed significant improvement in the sFasR group (*). The inclined plane test measures the maximal angle that an animal can maintain itself vertically for five seconds.

Morphometric Analysis Following Spinal Cord Injury and Administration of Soluble Fas Receptor

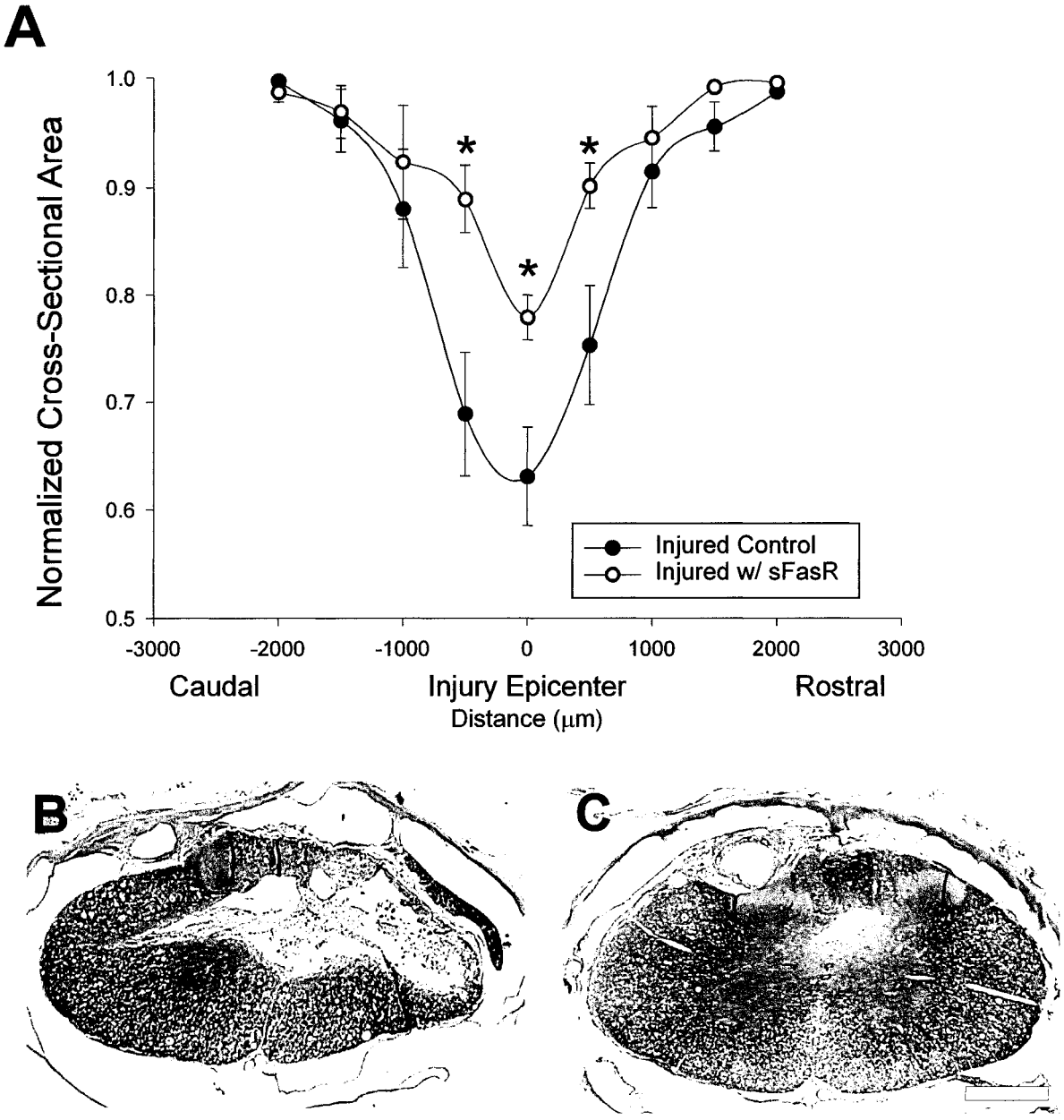
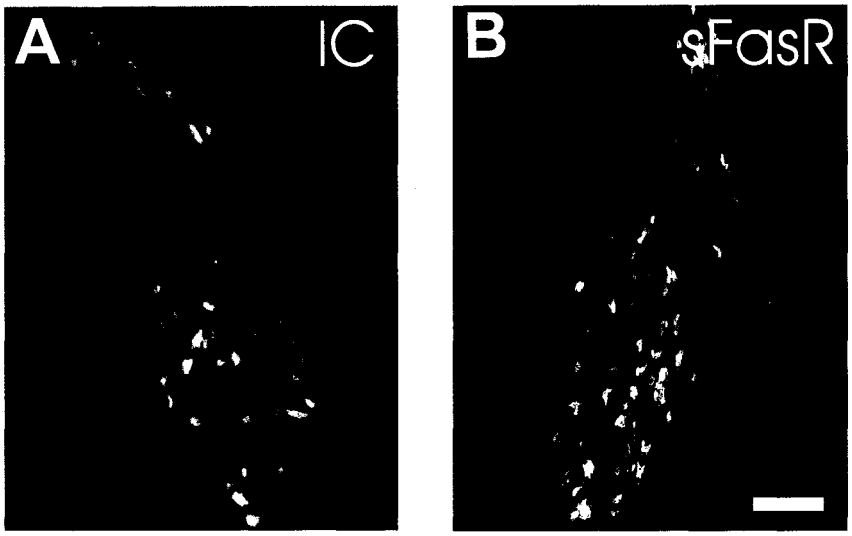


Figure 18. (A) Morphometric analysis of the injury epicenter and adjacent rostral and caudal sites showed increased tissue preservation following sFasR administration. Soluble Fas receptor administration improved tissue preservation with post-hoc univariate analysis demonstrating significant improvement at the injury epicenter and 500 μm both rostral and caudal (*)(Two way ANOVA: $p<0.001$ for both treatment and distance; Student Neuman-Keuls: $p<0.001$ for 500, 0, -500 μm). Staining with H&E/LFB 500 μm caudal to the injury epicentre: (B) Injured control, and (C) Injured with sFasR. Error bars = SEM. Scale bar = 500 μm.

controls (P=0.05 on square rooted data, Raw Mean ± SEM: Injured control 333 ± 59.4, injured w/ sFasR 582 ± 87.7) (Figure 19).

Retrograde Fluorogold Tracing Following Spinal Cord Injuryand Soluble Fas Receptor Administration



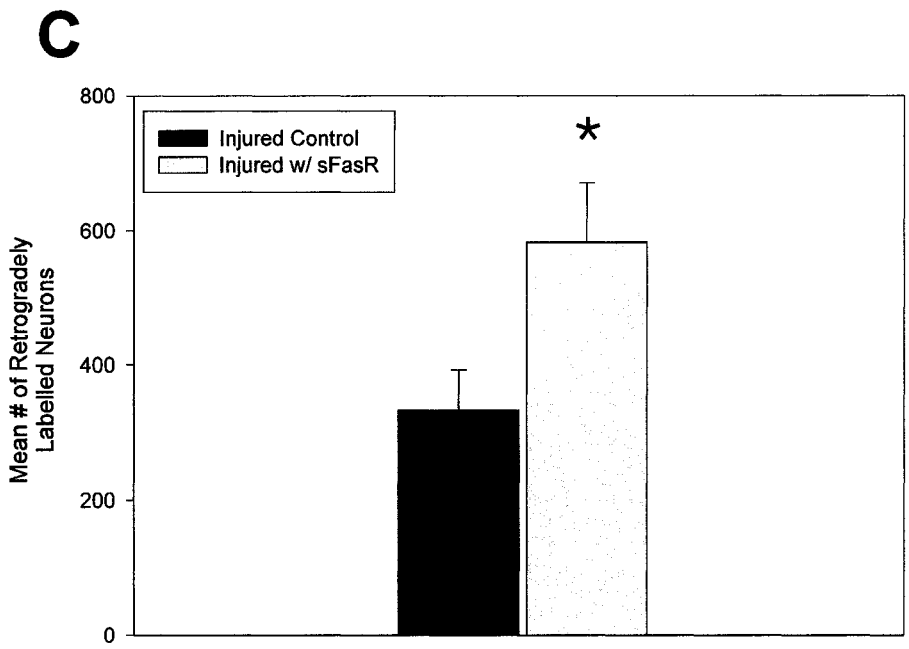


Figure 19. Counts of fluorogold positive red nucleus neurons demonstrated an increased amount of axons crossing the injury epicenter with (B) sFasR administration as compared to (A) Injured Controls. (C) Square root transformation of the data showed statistically significant improvement with sFasR administration ($p = 0.05$)(*). Error Bars = SEM. Scale Bar = 150 μm.

Chapter 4- Discussion

There is strong evidence supporting the role of apoptosis following SCI [1, 24, 68-70, 72, 73, 76, 81, 82, 89, 148, 162]. Apoptosis has been shown to evolve maximally over some days following injury. This offers the potential for a broad therapeutic time window for neuroprotective strategies. Our laboratory has previously shown that the apoptotic Fas death receptor plays an integral role in the pathophysiology of spinal cord injury, with increased upregulation on glial cells (especially oligodendrocytes) and neurons[68, 121]. Moreover, our laboratory has shown that genetic deletion of the FasR results in an improvement of tissue preservation and behavioural recovery following injury [121].

In this study we undertook the task of identifying potential clinically relevant approaches to inhibit the Fas apoptotic pathway following acute spinal cord injury. We used *in vitro & in vivo* models of SCI and administered sFasR to inhibit the FasL/FasR interactions. Furthermore, we looked at sFasR administration following experimental compressive SCI, and found that sFasR administration decreased apoptosis, increased tissue & axonal preservation, and improved behavioural recovery.

Increased Protein-Protein Interactions with Fas Ligand after SCI

Immunoprecipitation (IP) experiments after SCI showed increased interactions of FasL with FasR and activated caspase-8. We observed increased binding of FasL to FasR three days following SCI and increased interactions of FasL and activated caspase-8, three and seven days following SCI. Qiu *et al.* [123]showed increased interactions

between FasL & FasR and increased interactions between FasR & pro-caspase 8 after traumatic brain injury. However, they looked at early time points (3, 6, and 12 hours) after the traumatic injury. Furthermore, Matsuhita.*et al.* [143]used a spinal cord ischemia model and demonstrated increased interactions between FasR & pro-caspase 8 both 3 and 12 hours following spinal cord ischemia.

Our IP results expand and re-confirm these already published results on increased FasL/FasR interactions after traumatic injury in the CNS. Firstly, looking at later time points showed the delayed persistence of activation of the Fas receptor following SCI. The second and more intriguing observation was the increased interaction of FasL with activated caspase 8 at these later time points. We initially expected to only see changes in pro-caspase 8 levels because it is believed that following FasR activation pro-caspase 8 is cleaved and departs from the DISC into the cytosol triggering the proteolytic cleavage of downstream effector caspases[109]. These results conflicted with our group's initial idea, that we would see changes in pro caspase-8 instead of activated caspase-8; however, we found literature supporting the possibility to have cleaved caspase-8 products in the DISC[170].

Further experiments should be performed to see if the FasL interaction with activated caspase-8 is a phenomenon that has an earlier onset than three days, while analysis should be performed to examine the temporal progression.

Rationale for using Soluble Fas Receptor

The idea to use sFasR came from Dr. Steven Casha whose PhD thesis in Dr. Fehlings laboratory also looked at the role of the FasR following SCI[68, 121]. Further

reading found that the sFasR was an endogenous negative feedback mechanism that had been elevated in humans following traumatic brain injury[142]. Also, Cheng *et al.* demonstrated that *in vivo* administration of sFasR could protect against FasR-mediated apoptosis [141].

The sFasR is a soluble fusion protein consisting of an extracellular (175 amino acids) domain of human FasR fused to Human IgG Fc (234 amino acids). Nagata *et al.*[145] noted that human and murine FasL have similar identity (76.9% identity at the amino acid level) and do not demonstrate species specificity.

It was elected to use intrathecal administration of sFasR to minimize systemic complications, and enhance delivery to the injured spinal cord. We decided to use approximately a 1000 fold decrease of an intraperitoneal dose that Cheng *et al.* [141]used to block FasR-mediated apoptosis. Our decision to decrease the amount intrathecally was based on the studies of drugs like baclofen, which showed improved bioavailability with intrathecal administration[171, 172].

Soluble Fas Receptor Decreases Cell Death in Organotypic SCI

The work we initially performed on the organotypic model of SCI assessed the viability of the slices to see whether it could be a valid model to observe new neuroprotective treatments. We found that it was a valid method to assess neuroprotection by testing already proven neuroprotective drugs (MPSS and CNQX). This work was published and discussed in more detail in a manuscript in Brain Research Protocols [163].

Soluble Fas receptor was administered in the organotypic model of SCI and showed a significant reduction of cell death in the rat slices 48 hours following pin drop injury ($p<0.05$). The sFasR administration on murine slices was performed following the observations already seen in the rat organotypic slices. The reason for repeating the experiments was to confirm whether human sFasR was able to inhibit FasL interactions in both murine and rat models, and to compare the relative reduction of organotypic cell death as compared to the murine FasR knockouts.

An overall difference between control and sFasR treated groups was found with a statistically significant reduction in cell death 48 and 72 hours following injury. (Two way ANOVA: $p<0.001$ for both sFasR treatment and time; pair-wise comparisons with t-test and Bonferroni correction: $p<0.05$ at 48 and 72 hours). Extending the length of observation was done to correlate our results with that of the organotypic murine FasR knockouts already completed. Similarly, methods of statistical analysis were changed to match the murine FasR knockouts analysis. These data were combined with the work done by Dr. Casha and submitted to the Journal of Neuroscience [121].

An important note is that propidium iodide quantified all types of cell death. We attempted to quantify apoptotic TUNEL labeled cells in the organotypic slices, but it proved to be inconsistent. The main problem was that the 250 μm thick slices were very difficult to cut consistently on the cryostat. Furthermore, it is important to note that neurons did survive in organotypic slices (it was observed that they continued to grow out of the ventral horns.).

Literature on this model of spinal cord injury is restricted to the work performed and published from our laboratory[163]. A similar hippocampal organotypic slice model has been used with a pin drop injury to assess neuroprotection treatments[164].

These results helped give initial indications that sFasR had the potential to be neuroprotective *in vivo*. Its role was not to provide definitive answers in regards to a neuroprotective treatment, but simply to provide efficient indications on whether our neuroprotective treatment ideas (like sFasR) were viable.

Reduction in Apoptosis with Soluble Fas Receptor Administration Following *In Vivo* Spinal Cord Injury

Previous work performed in our laboratory has shown an increase in apoptosis following clip compression SCI. This apoptosis appeared to maximize at seven days and has been shown through TUNEL counts and double labeling with cell specific markers. These studies indicated that oligodendrocytes and neurons are both susceptible to this delayed apoptotic cell death[68, 121]. Liu *et al.* [76] have also demonstrated neuronal and glial apoptosis following spinal cord injury and showed that cyclohexamide, a protein synthesis inhibitor, helped prevent this cell death and improve behavioural recovery.

We examined whether sFasR would decrease apoptosis after SCI. Immunoblot analysis showed an attenuation of activated caspase-3 with administration of sFasR for seven days following injury. It is important to note that the immunoblots were difficult to reproduce due to the sensitivity of the caspase-3 antibody. Varying laboratory conditions appeared to have an effect on antibody efficiency. Also, large doses of protein (40 μg) were needed see the appearance of the activated caspase-3 band. Controlled experiments

increasing the amount of injured tissue protein were performed to decipher the optimal amount of protein needed to be loaded within the wells. Positive (purified caspase-3 products) and negative controls were performed to assure specific binding of the antibody.

Looking at seven days following injury, quantified TUNEL results showed a trend ($p<0.078$) towards decreased apoptosis at the injury epicenter with the addition of sFasR following injury. Emery *et al.* [70] noted that apoptosis is a more rapid process and its debris is removed swiftly following injury. Any given cross-section could potentially be underestimating the amount of apoptotic cell bodies. This suggests that the sFasR treated animals delayed the apoptotic cell death and that cells that had early apoptotic cell death would be cleared away in the injured untreated group before being able to be counted. Looking at sFasR treated groups at earlier time points (i.e. 3 days) would help answer this question.

Soluble Fas Receptor Increases Tissue & Axonal Preservation, and Improves Behavioural Recovery

We used a variety of methods to assess the functional recovery of rats that received sFasR. The mini-osmotic pumps and intrathecal catheters delivered the sFasR for a duration of seven days, but they were left in for the duration of the experiment (7 weeks), the glue anchoring of the catheter made them difficult to extract without creating further damage.

Statistically significant improvements in neurological function with sFasR treatment versus saline controls were seen by two-way ANOVA of scores on the BBB locomotor rating scale and inclined plane tests. At six weeks there appeared to be a

plateau in both behavioural scores. Post-hoc student Neuman-Keuls univariate analysis showed significant improvements with sFasR at three weeks for the BBB locomotor rating scale and two weeks in the inclined plane test. Examination of the behavioural data suggests that sFasR administration increased early restoration of neurological function, followed by parallel recoveries at later time points. Increasing the length of sFasR administration (i.e. to 2 or 3 week) may help improve the later time point neurological scores and may further separate the scores between the treated and control groups.

Morphometric analysis showed a highly significant overall improvement in tissue preservation with sFasR administration ($p<0.001$). Univariate post hoc analysis showed improvements at the injury epicenter and at both 500 μm rostral and caudal to the epicenter ($p<0.05$). The literature has established that oligodendrocytes are susceptible to apoptotic cell death following trauma[68, 69, 147, 148]. This oligodendrocyte cell death coincides with an upregulation of the Fas receptor and is associated with Wallerian degeneration of the white matter tracts, a process thought to be a main contributor in the rostral and caudal spread of injury. Casha *et al.* suggest that the rostral and caudal preservation that we see with sFasR can be attributed to preservation of oligodendrocytes, which myelinate multiple axons and provide trophic support to the white matter.

The injury epicenter we believe has a different hypothesized mechanism. The amount of tissue preservation at the injury epicenter with sFasR administration was quite pronounced, a result not always found in treatments successful in neuroprotection [161]. Neuron cell death has also been linked to the FasR activation and trauma to the CNS[124, 125]. Moreover, the immune system has been shown to play a role in the secondary

injury response that follows SCI [48-50]. T cells have long been shown to regulate immune responses and are highly susceptible to FasR-mediated cell death[126, 132, 173]. Hauben *et al.* [47, 50] have recently demonstrated that autoimmune T cells are neuroprotective to the spinal cord following injury. These mobile cells regulate other immune cells and provide neurotrophic factors and cytokines that help with maintenance and repair of damaged areas. Lastly, Prewitt *et al.* [51] demonstrated that activated microglia/macrophages can have beneficial roles in regenerative and repair responses following spinal cord injury. Our lab demonstrated that microglia up-regulate the FasR following SCI [68] and hence, the administration of sFasR could potentially attenuate their cell death, allowing them to exert their beneficial mechanisms. Therefore, we believe that our tissue preservation at the epicenter is the result of multipotent affects of the sFasR inhibiting apoptosis in a variety of immune cells and neurons.

We quantified fluorogold positive red nucleus neurons following sFasR administration versus saline injured control because it has been shown to be an objective and accurate way to assess recovery following experimental spinal cord injury[166, 174]. Following *in vivo* clip compression experiments, these brainstem red nuclei have been reported to be most susceptible to neuroprotection treatments and have a direct correlation with hindlimb motor function [57, 59, 150, 161]. Red nucleus neurons have axons that run exclusively in the dorsal course down the rubrospinal tract (located in the dorsal part of the lateral funiculus). Counts of fluorogold-labeled neurons found in the red nucleus revealed statistically significant improvement with sFasR treatment in axonal survival of rubrospinal neurons ($p<0.05$).

The improvement in the survival of red nucleus axons crossing the injury epicenter is relatively modest in comparison to the robust tissue preservation found at, and adjacent to the injury epicenter. An explanation for this is that sFasR locally affects a variety of cells, whether they are the glial or immune cells that provide maintenance, nourishment and repair to the damaged environment. Even though this occurs it does not robustly preserve the rubrospinal tract axons.

Mechanism of Action

It is hypothesized that sFasR (Figure 20) inhibits the interactions between FasL & FasR. This decreased interaction likely increases preservation of neurons, oligodendrocytes, T cells & microglia; all cell types that have upregulation of FasR following SCI and have been shown to be directly or indirectly beneficial to improving functional recovery[49, 50, 68, 72, 76, 121, 148].

Limitations

The following section is a description of the limitations of the present study that should be performed to strengthen the results that were found.

Administration of a control protein of similar size (i.e. un-conjugated IgG) could have been added as another control (to supplement with injured saline treated controls) to confirm that it was not just the addition of any small protein that would give similar sFasR treated results.

Effect of Soluble Fas Receptor Following Spinal cord Injury

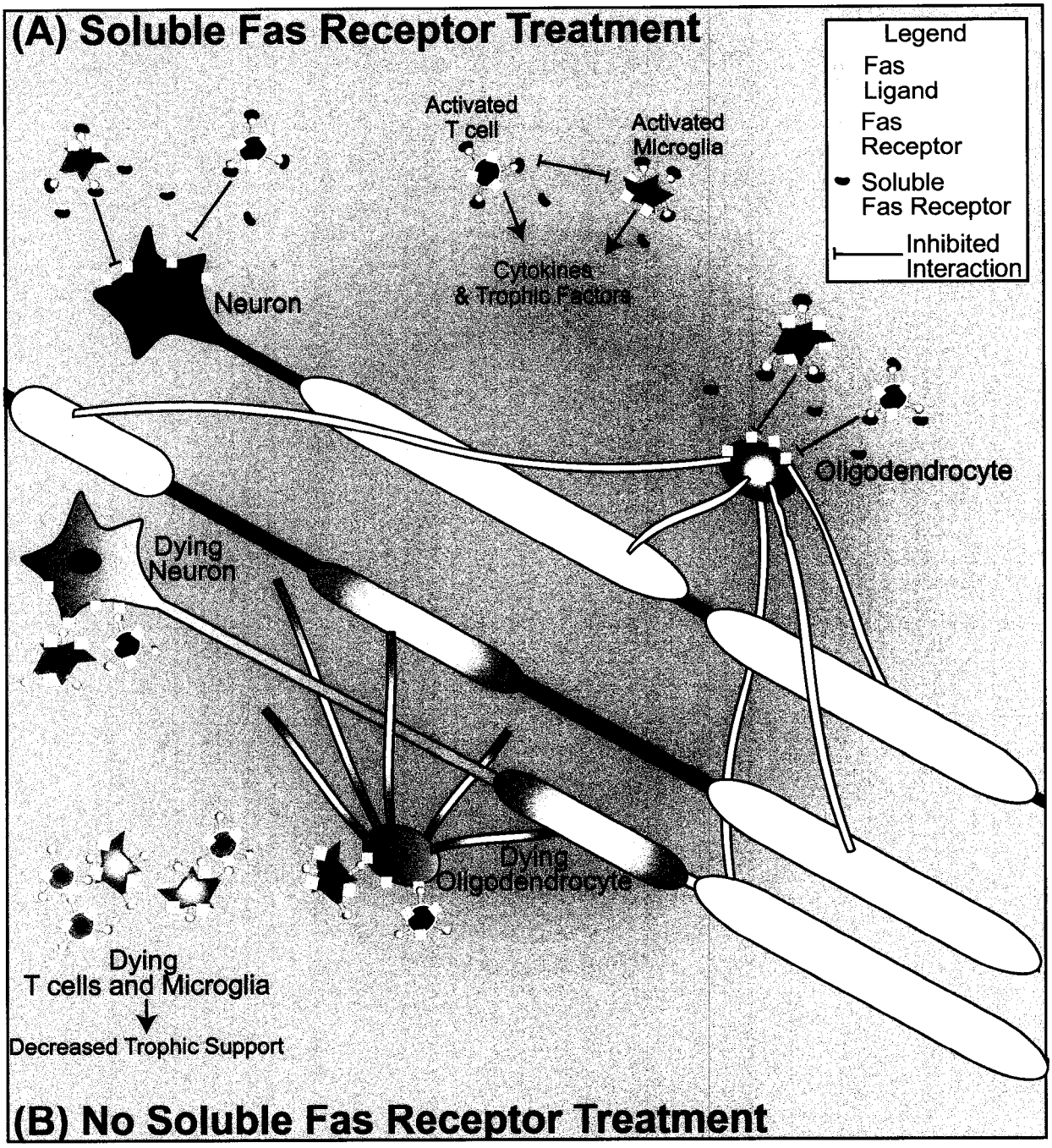


Figure 20. Schematic showing the interactions of neurons, microglia, T cells and oligodendrocytes following spinal cord injury with (A) soluble Fas receptor administration and (B) without treatment.

Additionally, increasing the number of animals used to count TUNEL positive cells could potentially bring our p-value ($p<0.078$) closer to our established value of significance of $p<0.05$.

Future Directions

The results of this thesis have led to a number of areas for future study:

1. Soluble Fas receptor therapeutic time windows and concentrations
2. The role of sFasR on the cell populations involved in SCI
3. Neuroprotection through alteration of adaptor proteins linked to the Fas receptor

Soluble Fas Receptor Therapeutic Time Windows and Concentration

In our studies we delivered sFasR immediately following injury and delivered the 5 μg of the protein in 200 μl of saline over a seven-day period. The potential of this experiment to have clinical implications requires that we better understand the timelines of administration. Further experiments will have to be conducted to see what happens when we delay the administration to 3, 8, or 24 hours; times of administration that would be more clinically relevant. Also, it would be much more clinically practical to administer a bolus dose, whether it be intrathecally or intraperitoneally. These are all strategies that must be discussed to confer the most cost efficient and neuroprotective results and to offer a patient the most positive outcome following injury.

The Role of Soluble Fas Receptor on Certain Cell Populations

Our study has delineated the utility of soluble Fas receptor administration following spinal cord injury. However, there still remains questions of which cells sFasR is keeping alive following injury. We believe that cell sparing adjacent to the injury epicenter is predominantly a glial cell story due to the work showing oligodendroglial cell death following injury. However, due to the predominant tissue preservation at the injury epicenter, we must further investigate to see the amount of T cell and microglia present and decipher whether they are truly beneficial following injury. Immunocytochemical techniques using antibodies specific for immune cells will enable us to determine whether sFasR preserves the amount of immune cells present at the injury site.

Neuroprotection Through Alteration of Adaptor Proteins Linked to the Fas receptor

Our study has confirmed that Fas receptor/Fas ligand interactions help activate the apoptotic pathophysiology that arises following spinal cord injury. Accordingly, below is a potential experiment that would enable us to look at Fas-related downstream intracellular proteins and to understand their role following SCI.

FLICE Inhibitory Protein (FLIP) is an endogenous inhibitor of the FAS receptor pathway. FLIP resembles caspase-8 except it does not possess any of the caspase-8 proteolytic properties[175, 176]. Moreover, increased FLIP has been shown to divert the FAS receptor apoptotic pathway and recently, in certain cells, has been shown to divert the FAS apoptotic signal into a cell replication signal[135].

One idea is to insert the FLIP peptide into cells following SCI using a novel HIV-1Tat protein method of transduction. The Tat-mediated protein method of transduction renders cells permeable to any protein that is attached to its transduction domain and has been successful in a variety of experiments to demonstrate neuroprotection[99, 177-179]. The Tat-mediated protein method of transduction offers the potential to insert a variety of dominant negative proteins (like FLIP) delivering maximal concentrations rapidly (less than five minutes) regardless of size (the largest protein transduced to date is 120 kDa)[180].

Impact

The findings arising from this thesis demonstrate that sFasR has neuroprotective effects following SCI. To our knowledge, it is the first time that the administration of a protein (sFasR) has been used to extracellularly attenuate the interactions of a death receptor and its activating ligand resulting in a functional recovery following SCI. It suggests that sFasR has a role in decreasing apoptotic cell death in oligodendrocytes, neurons, microglia and infiltrating T cells, although further experiments are needed to decipher which cells, if any, are predominantly affected.

These observations also offer the potential to translate these findings into a clinically relevant way to treat spinal cord injury. We used human sFasR to inhibit the interactions between FasL and FasR, making it conceivable that this method of neuroprotection could be translated to a clinical setting. Furthermore, an increased endogenous level of sFasR has already been shown following CNS trauma, and therefore we could just be enhancing an already active neuroprotective mechanism.

Concluding Remarks

In summary, apoptosis has been strongly implicated in the pathophysiology of spinal cord injury and attempts to inhibit it contribute to neurological and functional recovery. The sFasR appears to be a novel method of inhibition of the extrinsic apoptotic pathway, targeting the FasR induced delayed cell death. Further experiments are needed to decipher whether it is possible to move this method of treatment to a clinical situation and to decipher what cell populations it is predominantly affecting.

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