THE ROLE OF FAS-MEDIATED APOPTOSIS IN THE PATHOPHYSIOLOGY OF ACUTE TRAUMATIC SPINAL CORD INJURY

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Abstract

The Role of Fas-Mediated Apoptosis in the Pathophysiology of Acute Traumatic Spinal Cord Injury

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Spinal cord injury (SCI) is a debilitating condition accompanied by motor and sensory deficits and a reduced quality of life. Current treatment options are limited and are associated with variable efficacy and a risk of adverse effects.

The pathophysiology of SCI is initiated by a primary mechanical insult to the spinal cord, followed by a complex series of deleterious events known as secondary injury. Secondary injury processes include free radical formation, glutamate excitotoxicity, inflammation and cell death. Apoptotic cell death in particular plays a key role in the secondary injury processes and exacerbates tissue degradation and loss of function.

The role of Fas-mediated apoptosis in SCI pathophysiology is poorly defined in the literature to date. Correlative evidence suggests that this form of cell death is delayed and occurs in white matter adjacent to sites of primary damage.

The cellular and temporal mechanisms of Fas-mediated apoptosis following experimental SCI were evaluated using a clinically relevant clip compression SCI model in the rat. Furthermore, therapeutic manipulation of Fas activation using a soluble form of the Fas receptor (sFasR) was carried out to

establish the efficacy and clinical relevance of targeting this aspect of secondary injury.

This work shows that Fas-mediated apoptosis is an important contributor to secondary SCI pathology. Oligodendrocytes are targeted by this form of cell death in a delayed fashion post-injury, providing an opportunity for therapeutic intervention. Intrathecal administration of sFasR following SCI reduced post-traumatic apoptosis, improved cell survival, enhanced tissue preservation and resulted in an improved motor recovery. Administration of sFasR was effectively delayed by up to 24 hours post-injury, however a shorter delay of 8 hours post-injury was most efficacious.

A surprising result emerged from this work. Delayed intrathecal administration of IgG following SCI showed significant efficacy in both cellular and tissue level outcomes, as well as at the functional level.

Fas-mediated apoptosis is an important aspect of secondary SCI pathophysiology and is an attractive therapeutic target. The beneficial outcomes of manipulating Fas activation using sFasR provide further evidence for this. Future work will refine this treatment strategy, bringing it into the SCI patient population.

Acknowledgements and Dedication

I would like to dedicate this thesis in loving memory of my mother, Sue Robins, who was taken from us tragically on the 22nd of February, 2007.

Mom, I know that you would be so excited and proud of me as I start this next chapter of my life. I cannot express how much I wish that you were here to celebrate this with us. You are greatly loved and missed with every day that passes, and your spirit and smile are never forgotten.

Sue Robins
May 19, 1951 – February 22, 2007

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AIF – Apoptosis Inducible Factor

Apaf-1 – Apoptotic peptidase activating factor 1

BSA – Bovine Serum Albumin

CIDP – Chronic Inflammatory Demyelinating Polyneuropathy

DD – Death Domain

DED – Death Effector Domain

DISC – Death Inducing Signalling Complex

Endo-G – Endonuclease G

FADD (Mort1) – Fas-Associated Death Domain

FAIM – Fas Apoptotic Inhibitory Molecule

Fas (CD95, Apo1) – Fas Receptor

FasL – Fas ligand

FG - FluoroGold

FLIP – Flice-Like Inhibitory Protein

HAMC – Hyaluronan Methylcellulose

H and E / LFB – Hematoxylin and Eosin / Luxol Fast Blue

IAP – Inhibitors of Apoptosis Proteins

IFNγ – Interferon gamma

IgG – Immunoglobulin G

IV-Ig – Intravenous Immunoglobulin

LFG – Lifeguard protein

MBP – Myelin Basic Protein

mGluR – metabotropic Glutamate Receptors

MMP – Mitochondrial Membrane Potential

MPO – Myeloperoxidase

MPSS – Methylprednisolone Sodium Succinate

MVA – Motor Vehicle Accidents

NASCIS – National Acute Spinal Cord Injury Study

NGF – Nerve Growth Factor

NMDA - N-methyl-D-aspartic acid

NOS – Nitric Oxide Synthase

iNOS – Nitric Oxide Synthase inhibitor

SCI – Spinal Cord Injury

sFas – soluble Fas receptor (endogenous)

sFasR – soluble Fas receptor human fusion protein

TGFβ – Transforming Growth Factor beta

TNFα – Tumor Necrosis Factor alpha

TNFR1 – Tumor Necrosis Factor Receptor 1

TRADD – TNFR Associated Death Domain

TRAF2 – TNFR Associated Factor 2

z-VAD-fmk – z-VAD-fluoromethylketone

Δψm - mitochondrial inner transmembrane potential

Chapter One: Introduction

Spinal cord injury (SCI) is a devastating condition that is accompanied with varying degrees of morbidity and mortality. Few, minimally effective treatment strategies are available to clinicians and patients, leaving them struggling to find adequate treatment for this condition (Bracken, Shepard et al. 1992; Geisler, Dorsey et al. 1992; Geisler, Dorsey et al. 1992; Fehlings, Sekhon et al. 2001; Sekhon and Fehlings 2001; Bracken and Holford 2002; Ackery, Tator et al. 2004). While there have been tremendous advancements in this field over the last decade, researchers are still searching for alternative, efficacious pharmacological treatments. An increase in quality of life is the modest wish of these patients, where pain, bladder and bowel function, sexual function and hand function are considered the highest determinants of quality of life (Anderson 2004). There is hope that science and medicine will come together to provide this patient population with hope and promise for a better future.

1.1 Incidence, Demographics and Impact of SCI

SCI is broadly defined as “the occurrence of an acute traumatic lesion involving neural elements in the spinal canal (spinal cord and cauda equina) resulting in neurological deficit” (Thruman DJ 1995). The reported prevalence of SCI throughout the world ranges from 14 to 80 people per million population, with this figure likely under-representative of the actual world wide incidence (Blumer and Quine 1995). There is a lack of consistent case documentation and differences in reporting protocol from country to country; in addition, significant

differences in reporting methods from the more developed world to the underdeveloped contribute to this discrepancy.

Studies focused primarily on the incidence and demographics of SCI in North America indicate that approximately 15,000 new cases are reported annually (Sekhon and Fehlings 2001). This translates into an average number of individuals suffering from acute or chronic SCI to be upwards of 500 per million population (Wyndaele and Wyndaele 2006). Motor vehicle accidents (MVAs) and falls are the top two causes of spinal cord trauma in developed countries. Over 50% of all reported cases result from MVAs, especially in the younger population (Ackery, Tator et al. 2004). The greatest incidence of falls occurs in the elderly population (Geisler 2002; Krassioukov, Furlan et al. 2003; Ackery, Tator et al. 2004). As well, the incidence of violence-related SCI is on the rise, with gunshot and stab wounds contributing to this increasing incidence (Nobunaga, Go et al. 1999).

It is well-accepted that the incidence of SCI is almost four times as high in males than females. Additionally, there has been a recent shift in the mean age of individuals suffering from SCI, due in part to an increased aging population. The current mean age of individuals suffering from a SCI is reported to be 44 years (Furlan, Bracken et al. 2008). In closer examination, two general age groups make up the total incidence of SCI, existing as a bimodal distribution containing an elderly and a young group.

There is a significant portion of the young patient population falling between the ages of 18 to 35 years (Bracken, Shepard et al. 1997; Nobunaga,

Go et al. 1999; Geisler 2002; Ackery, Tator et al. 2004; Dryden, Saunders et al. 2005). With such a young proportion of individuals suffering from SCI, a large socio-economics issue is introduced. As overall life expectancy continues to increase and younger individuals fall victim to injury, the personal and societal costs that each patient might incur over their lifetime begin to add up. It is estimated that a person with SCI will be responsible for over $1 million of health care and associated costs over a lifetime (McKinley, Jackson et al. 1999). These costs include such things as acute care and hospitalizations, medications, medical supplies and rehabilitation, personal assistance and home modifications. The Center for Disease Control in the United States estimates that an average of $9.7 billion (US) is spent annually on patients with SCI, making it the second most expensive medical condition to treat (McKinley, Jackson et al. 1999; Winslow, Bode et al. 2002; Ackery, Tator et al. 2004). Furthermore, the secondary medical complications arising in the SCI patient population are significant, contributing to several million dollars worth of cumulative healthcare costs over a lifetime (McKinley, Jackson et al. 1999).

1.2 Advancements in SCI Treatment and Clinical Trials

Despite recent advancements in the field of SCI research, there remains a lack of pharmacological treatment options available to both the acutely injured patient and to those suffering from chronic injuries. In the past several years there has been some success in the clinical translation of research findings, and in the establishment of world-wide SCI clinical trial networks

1.2.1 Methylprednisolone and NASCIS

To date, MPSS (methylprednisolone sodium succinate) is the only treatment option for SCI that has demonstrated neuroprotective efficacy in a phase III clinical trial. Its clinical use has stemmed from the NASCIS (National Acute Spinal Cord Injury Studies) trials which explored the glucocorticoid methylprednisolone as a potential therapy in the acute setting of SCI (Bracken 1990; Bracken, Shepard et al. 1997; Bracken, Shepard et al. 1998; Kwon, Tetzlaff et al. 2004; Baptiste and Fehlings 2008). The NASCIS trials were considered landmark for being the first randomized trial to show modest motor and sensory recovery in a SCI population. However, controversy and concern exist over the interpretations and conclusions ultimately drawn from these trials (Coleman, Benzel et al. 2000; Hurlbert 2000; Short, El Masry et al. 2000; Hurlbert 2001; Baptiste and Fehlings 2008). To highlight the most important criticisms, treatment of patients with MPSS was associated with a low ratio of therapeutic benefit to adverse risk. It was also felt that an insufficient improvement of primary outcome measures cautioned the recommendation for its use in all SCI patients (Hurlbert 2000; Geisler, Coleman et al. 2001). This being said, MPSS remains the most commonly administered pharmacologic therapy in the treatment of acute SCI. Clinicians are cautioned to weigh the small clinical benefit of the drug against the propensity for serious adverse systemic effects, such as increased wound infection, pulmonary embolism, severe pneumonia, sepsis and even death due to secondary respiratory complications (Kwon, Tetzlaff et al. 2004).

With the questionable success of the NASCIS trials, there has been a push to find better treatment options that can provide stronger and more consistent efficacy than MPSS. The NASCIS trials provided a the foundation for the development of future clinical trials. With MPSS as the only SCI-approved pharmacologic treatment, it became the "gold standard" treatment arm in subsequent clinical trials.

1.2.2 Sygen (GM-1) Clinical Trial

Sygen (GM-1) is a type of ganglioside, a group of glycolipids found in the plasma membranes of many nervous tissue cells. It was first discovered that therapeutic administration of GM-1 by exogenous application to the injured spinal cord was functionally beneficial (Bose, Osterholm et al. 1986). Several years later, GM-1 was evaluated in a phase I clinical trial that led to some success in primary outcome measures, and triggered the initiation of the largest prospective randomized clinical trial in acute SCI to date: the Sygen Multi-Center Acute Spinal Cord Injury Study (Hawryluk, Rowland et al. 2008). Several interesting points were introduced following this trial. First, while improvements in the primary outcome measure of functional recovery was not achieved, patients receiving GM-1 showed some improvements in neurological recovery and in the recovery of bladder and bowel function (Geisler, Coleman et al. 2001; Hawryluk, Rowland et al. 2008). Secondly, GM-1 was given after MPSS due to ethical consideration of the patients, creating problems associated with elucidating GM-1-specific effects (Hawryluk, Rowland et al. 2008). Much like the NASCIS trials,

the Sygen trial has continued to provide clinicians and scientists with great insights into the development and application of human clinical trials in SCI.

1.2.3 Other Therapeutic Compounds and Treatments

A number of potential molecules and compounds have been therapeutically beneficial following experimental models of SCI, but are not yet approved for use in human patients. Of note here are Riluzole which acts as a sodium channel blocker, the tetracycline antiobiotic Minocycline, the immune suppressants Cyclosporin and FK506 and the NMDA receptor antagonists MK801 and GK11 (Madsen, MacDonald et al. 1998; Buki, Okonkwo et al. 1999; Diaz-Ruiz, Rios et al. 1999; Scheff and Sullivan 1999; Wang and Gold 1999; Gaviria, Privat et al. 2000; Gaviria, Privat et al. 2000; Schwartz and Fehlings 2001; Tikka, Fiebich et al. 2001; Rossignol, Schwab et al. 2007). A handful of other molecules have made their way into SCI clinical trials over recent years, the most notable being Cethrin. Cethrin is a RhoA antagonist which passed through Phase I/IIa trials with positive outcomes and no serious adverse effects (Dergham, Ellezam et al. 2002; Baptiste and Fehlings 2008). In follow-up to the original Cethrin trials, a Canadian and US Phase IIb/III trial is in its early stages.

Additionally, several groups have shown that induction of mild to moderate hypothermia in the early stages of SCI can provide potential neuroprotective benefits. The type of injury, the extent of hypothermia and the duration of hypothermia after injury are all points of inconsistency in the literature, but there is still a general consensus that beneficial results are possible (Dietrich, Atkins et

al. 2009). Several groups have shown that reductions in body temperature to between 32 and 33 degrees Celsius (mild to moderate hypothermia) following experimental SCI in the rat allow for improved functional recovery as well as a preservation of both grey and white matter tissue (Yu, Jimenez et al. 2000; Ha and Kim 2008; Morochovic, Chuda et al. 2008; Dietrich, Atkins et al. 2009; Lo, Cho et al. 2009).

1.3 General SCI Pathobiology

Traumatic SCI pathophysiology consists of two distinct stages of trauma, beginning with an initial physical assault to the vertebral column and spinal cord known as the primary injury. Primary injury is followed by activation of a host of deleterious events and cellular cascades that are collectively known as secondary injury. These secondary injury processes are dynamic and complex and will be discussed in detail over the next several sections.

1.3.1 Primary Injury Mechanisms of SCI

Primary injury involve mechanical insults such as fracture and dislocation of the vertebrae, resulting in a compression, contusion and/or laceration of the spinal cord. It is a misconception that the spinal cord is actually severed completely during a traumatic event, as this happens rarely (Ackery, Tator et al. 2004). Compression or contusion most often leaves a rim of surviving white matter that persists throughout the injury pathology, creating potential for tissue repair and regeneration.

A greater physical impact to the vertebral column and spinal cord results in increased severity of damage to the tissues. As well, the location of the

primary injury along the vertebral column has a significant impact on the extent and severity of injury. Injuries at the cervical level have the highest incidence in the population today, with anywhere from 41% to 76% of all SCIs occurring in this region (Ackery, Tator et al. 2004). Trauma within the cervical spinal cord increases the severity of injury, resulting in a more debilitating functional deficit and a more significant loss in quality of life.

Following the initial traumatic insult to the cord, there are many adverse events and deleterious pathways that become activated within the spinal cord.

1.3.2 Secondary Injury Mechanisms Following SCI

Collectively known as secondary injury mechanisms, this dynamic and complex set of pathologies contribute to the tissue loss, cell death, demyelination and subsequent loss of function that is characteristic of human SCI. Immediately following the primary trauma, secondary injury events are initiated. In the hours, days and months following, the interplay between various aspects of secondary injury continues to exacerbate the loss of motor and sensory function and increase neurological. Because the cell death mechanisms in secondary SCI pathology are the main focus of this work, the background information and literature review of these elements will be discussed in separate sections to follow.

For a summary of primary and secondary injury mechanisms, refer to **Figure 1**.

1.3.3 Disruption of Spinal Cord Vasculature

Immediately following trauma to the spinal cord, several elements of secondary injury pathology become active. Disruption of spinal cord vasculature

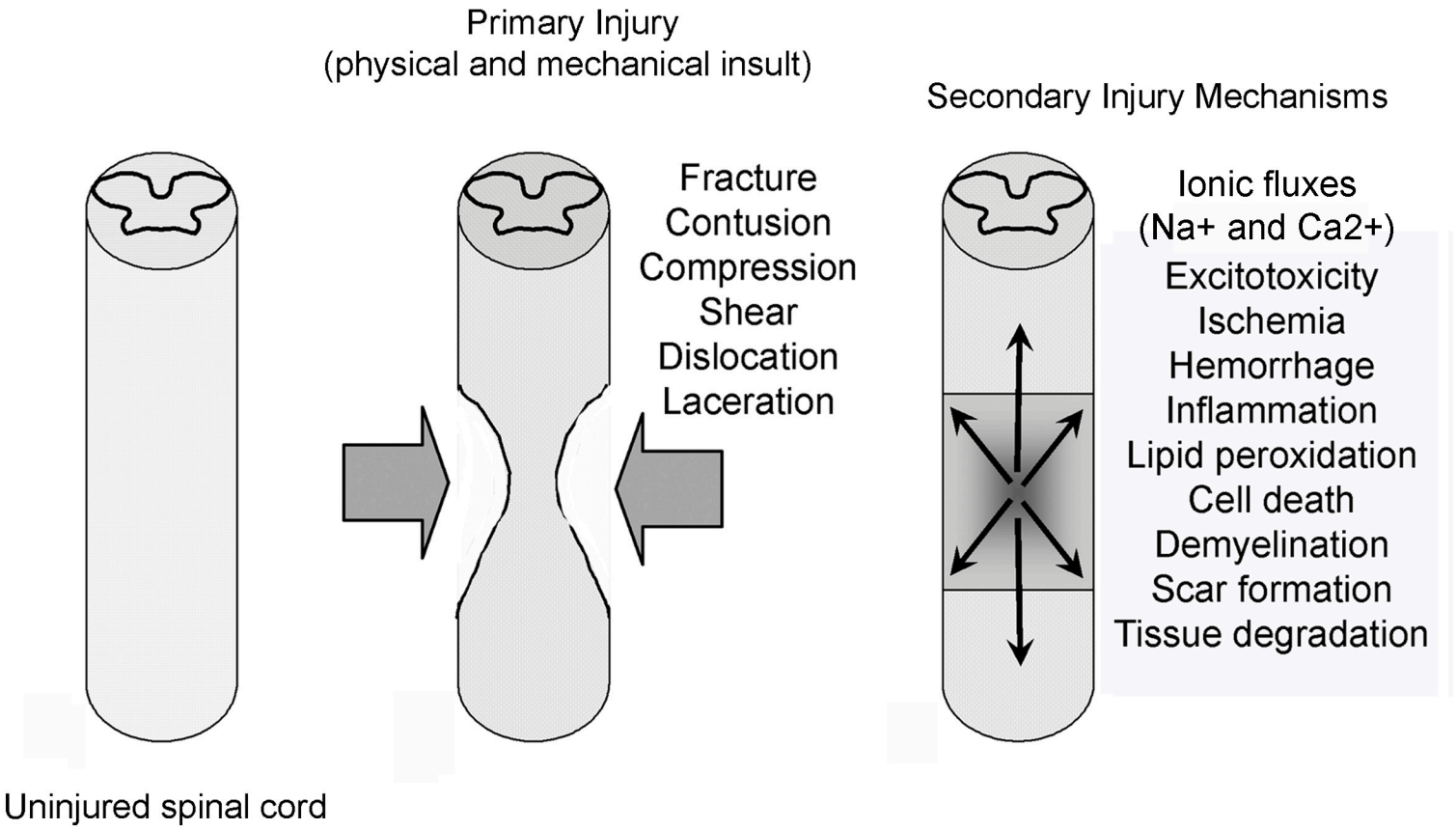


Figure 1: The Pathophysiology of SCI: Primary and Secondary Injury Mechanisms

Figure 1: The Pathophysiology of SCI: Primary and Secondary Injury Mechanisms

The pathophysiology of SCI is a complex and dynamic process that occurs in two distinct stages, so named primary and secondary injury events.

Primary injury occurs as a result of altered mechanical or physical forces acting to damage the delicate spinal cord tissues. This can occur through fracture or dislocation of the vertebral column, causing sheering, laceration, contusion and/or compression of the spinal cord.

Secondary injury mechanisms begin immediately following the primary trauma, and include a host of deleterious events such as hemorrhage and ischemia, loss of ionic homeostasis, inflammation and cell death. The elements of secondary injury pathophysiology are highly interrelated and often act on one another to perpetuate the tissue and cellular damage within the spinal cord. Secondary injury events can also spread in both rostral and caudal directions from the initial site of trauma, causing damage to neighbouring cells and tissues.

It is the impact of the physical primary insult that initiates and perpetuates the secondary injury mechanisms in the damaged spinal cord, contributing to the ultimate loss of function and paralysis in SCI patients.

by the mechanical forces of primary injury creates a setting of both hemorrhage and prolonged ischemia (Tator and Fehlings 1991). Ischemia is a well-known contributor to tissue injury and cell death; as a result of limited oxygen availability, death can be imminent. Neurons and oligodendrocytes can be highly sensitive to ischemic insult, and thus are severely affected by vascular disruptions occurring early in the secondary injury process. Subsequent to the initial disruption of vasculature is the development of thrombosis and vasospasm. Both of these mechanisms continue to exacerbate post-traumatic ischemia in areas adjacent to the initial site of damage (Tator and Fehlings 1991). There is a significant loss of integrity to the blood-spinal cord barrier as well, reducing the capacity for immune-privilege and increasing infiltration of systemic inflammatory cells.

1.3.4 Ionic Fluxes, Free Radical Formation and Excitotoxicity

Many of the secondary injury mechanisms after SCI are highly correlated. This interrelationship is noted in regards to ionic homeostasis, free radical formation and excitotoxic damage. Independent of one another, these processes are strong contributors to the early events of secondary injury. Ultimately, the cumulative effect of these deleterious events creates an environment resulting in progressive damage and limited repair.

1.3.4 (i) Na^+ and K^+ imbalances and dysfunction

Na^+, K^+ and Ca^{2+} play an important functional role in the physiology of the nervous system. Therefore the maintenance of ion gradients and appropriate intracellular and extracellular concentrations is crucial.

It has long been established that SCI leads to a significant disruption of intracellular Na^+, K^+ and Ca^{2+} concentrations (Happel, Smith et al. 1981; Young and Koreh 1986; Kwo, Young et al. 1989; Chesler, Young et al. 1994). The initial mechanical trauma results in widespread shearing and destruction of cellular membranes in the vicinity of the damage. Without the membranes acting as a restrictive border for ion fluxes, an immediate alteration in the normal physiology is observed. Further to this is the subsequent failure of Na^+/K^+ ATPase pumps that function to maintain crucial ion gradients, resulting in even greater physiological alterations (Faden, Chan et al. 1987; LoPachin, Gaughan et al. 1999). The net impact is dysfunction and inappropriate propagation of action potentials along damaged axons. Several groups have shown that with adequate blockage of Na^+ channels in the early stages of SCI, recovery of electrophysiological function and a reduced amount of tissue damage are possible (Agrawal and Fehlings 1996; Agrawal and Fehlings 1997; Teng and Wrathall 1997; Schwartz and Fehlings 2001). The limitations of this type of neuroprotective therapy are in the short time-window for therapeutic administration following SCI due to the early loss of ionic homeostasis.

1.3.4 (ii) Loss of Ca^{2+} homeostasis

Secondary to the disturbances noted in both Na^+ and K^+ ion gradients is that of Ca^{2+} at and adjacent to the site of primary injury. Calcium homeostasis is critical for healthy functioning of both neurons and glial cells. Hence, the disruption in calcium regulation following SCI contributes to white matter tissue loss and death of neurons. A major effect of calcium deregulation occurs at the

level of the mitochondria. Mitochondrial dysfunction as a result of excessive calcium influx causes apoptotic cell death of neurons following SCI (Lipton 1994). In white matter, calcium fluctuations result in the activation of ubiquitous proteases, specifically calpains, which contribute to neurodegeneration through cytoskeletal degradation and neurofilament damage (Banik, Matzelle et al. 1997; Banik, Shields et al. 1998; Schumacher, Eubanks et al. 1999; Schumacher, Siman et al. 2000). Collectively, the downstream effects of calpain activation result in compromised axonal integrity and loss of function post-SCI. Interestingly, this has been another site of extensive neuroprotective evaluation, through the use of calpain-specific blockers (Banik, Shields et al. 1998; Schumacher, Siman et al. 2000). Preservation of axonal filaments and improved motor function are observed following early blockage of these calcium-activated proteases. However, there is little clinical relevance to this therapeutic strategy due to the limited post-injury time window of efficacy and poor CNS penetration of the available inhibitors.

1.3.4 (iii) Free radicals and reactive oxygen species

Free radicals form as a result of inappropriate enzyme activity and with increases in the accumulation of reactive oxygen species. The impact of free radical formation and oxidative stress following SCI can exacerbate the sensitive ionic balances noted above. At the same time, loss of calcium homeostasis can trigger production of reactive oxygen species.

Concomitant with the deleterious effects of Na^+, K^+ and Ca^{2+} ion imbalance, the formation of free radicals and reactive oxygen species contribute

to oxidative stress and damage to the spinal cord tissue (Hamada, Ikata et al. 1996; Azbill, Mu et al. 1997). Increased production of nitric oxide (NO) due to over-activity of nitric oxide synthase (NOS) is a strong contributor to cell death following SCI Several groups have further investigated the impact of NOS on post-traumatic SCI using inhibitors of this enzyme (iNOS). It was observed that inhibition of nitric oxide synthesis reduces the amount of cell death occurring in the injured spinal cord (Sharma, Westman et al. 1996). Moreover, genetically altered mice lacking NOS showed a significant improvement in recovery following experimental SCI as compared to their wild-type littermates (Farooque, Isaksson et al. 2001).

Reactive oxygen species are known to cause lipid peroxidation, which disrupts the myelin sheath as well as cellular membranes (Chan, Persaud et al. 1984). Oxidative injury also involves cytoskeletal degradation, reduction in spinal cord blood flow, edema and inflammation (Sandler and Tator 1976; Hall, Yonkers et al. 1989; Banik, Shields et al. 1998). It is further postulated that reactive oxygen species may prevent astrocyte re-uptake of excess glutamate following SCI (Volterra 1994; Volterra, Trotti et al. 1994; Volterra, Trotti et al. 1994; Volterra, Trotti et al. 1994).

1.3.4 (iv) Excitotoxic damage

Glutamate is the primary excitatory amino acid responsible for the excitotoxicity observed in both grey and white matter following spinal cord trauma, and as such contributes to neuronal and glial damage.

Excitotoxicity refers to the injury process by which prolonged cellular exposure to excessive amounts of glutamate contribute to neuronal death and white matter damage (Doble 1999). Within minutes following SCI, it has been observed that extracellular levels of glutamate are significantly increased to toxic concentrations (Farooque, Hillered et al. 1996; Xu, McAdoo et al. 1998; Liu, Xu et al. 1999; McAdoo, Xu et al. 1999). Glutamate activates NMDA and non-NMDA (AMPA-kainate) receptors on the surface of neurons and glia. Prolonged activation of these receptors results in death of the receptor-bound cell (Choi 1988; Lipton 1994; Liu, Xu et al. 1999).

Further increase of the excessive amounts of glutamate in the injured spinal cord is due in part to the astrocyte. As indicated earlier, reactive oxygen species can promote the pathologic release of glutamate by astrocytes, contributing to excitotoxicity (Volterra 1994; Volterra, Trotti et al. 1994; Volterra, Trotti et al. 1994; Volterra, Trotti et al. 1994; Agrawal, Theriault et al. 1998; Mills, Xu et al. 2000; Mills, Xu et al. 2001). As well, perisynaptic astrocytes may actually release glutamate as a result of calcium-dependent damage (Araque, Li et al. 2000; Parpura and Haydon 2000). White matter damage by glutamate excitotoxicity is a less understood phenomenon, and it is believed that glutamate can be released from cells due to reverse operation of Na^+ / glutamate transporters in the membranes of white matter cells (McAdoo, Xu et al. 2000).

The early events of secondary SCI pathology are dynamic and complex, beginning immediately with alterations in normal ion flow and homeostatic gradients. Na+ and K+ imbalances lead to the initial deleterious changes in axon

function, perpetuating their own continued deregulation. Increased intracellular calcium levels exacerbate functional deficits and activate a variety of negative pathways. Multiple processes simultaneously intensifying the SCI pathology, including the formation of free radicals and the associated oxidative injury to cells and tissues. Inappropriate regulation of glutamate release is a strong contributor to the pathology, causing more cellular damage and neuronal death.

1.4 Post-SCI Inflammation

The inflammatory response following SCI is characterized by a complex and dynamic series of events initiated immediately following primary injury, and extending into the chronic phases of secondary pathology. There is a great deal of controversy in the literature surrounding the dual nature of negative impact and beneficial role that the inflammatory response ultimately carries following SCI. There are a wide range of inflammatory cell types involved in both of these actions, and the next several sections will detail the fundamental players involved.

1.4.1 Microglial Activation

The resident microglial cells of the spinal cord are activated early in secondary injury as a result of both the physical insult and the changes in vasculature and homeostatic imbalance (Tator and Fehlings 1991; Imperato-Kalmar, McKinney et al. 1997; Carlson, Parrish et al. 1998; Watanabe, Yamamoto et al. 1999; Jones, McDaniel et al. 2005).

The normal physiological role of microglia is to monitor and maintain the health of CNS tissue. Following a traumatic event, the activated microglia

release a cocktail of inflammatory cytokines and chemokines. These signal molecules continue to activate and recruit resident microglia from more distant areas of the cord and signal to the systemic circulation, alerting other immune cells that trauma has occurred (Popovich, Wei et al. 1997; Hausmann 2003). In this active state, microglia are known to secrete a toxic cocktail of IL-1, TNFα and IFNγ, which lead to damage of the myelin sheath and to the onset of oligodendrocyte apoptosis (Norris, Weston et al. 1977; Giulian, Corpuz et al. 1993; Selmaj, Walczak et al. 1998; Jones, McDaniel et al. 2005). Microglial release of IL-1 and TNFα facilitates the margination of immune cells such as leukocytes, neutrophils and systemic monocytes/macrophages by increasing the synthesis of key adhesion molecules on endothelial cells (Bartholdi and Schwab 1997; McTigue, Tani et al. 1998; Jones, McDaniel et al. 2005).

Active astrogliosis and the initiation of glial scar formation are triggered by the presence of IL-1 at the primary injury site (Giulian and Lachman 1985; Relton and Rothwell 1992; Blight, Cohen et al. 1995; Boutin, LeFeuvre et al. 2001). Much like inflammation in general, glial scar formation plays a dual role in SCI pathology. The scar acts as a boundary around the epicenter of tissue damage, thereby protecting the surrounding tissues from the spread of negative impacts. At the same time, the glial scar creates a physical barrier and limits regenerative capabilities following SCI.

It has been reported that active neutralization of IL-1 in the early stages of SCI can offer neuroprotective benefits (Giulian and Lachman 1985; Relton and Rothwell 1992; Blight, Cohen et al. 1995; Boutin, LeFeuvre et al. 2001). Of note

for later discussion is that microglial release of TNFα and IFNγ following SCI may play a role in the activation of Fas-mediated apoptosis of oligodendrocytes and microglia; it is known that these cytokines can upregulate the expression of Fas receptor and ligand (Spanaus, Schlapbach et al. 1998; Badie, Schartner et al. 2000; O'Connell, Bennett et al. 2000; Pouly, Becher et al. 2000).

1.4.2 Neutrophil / Leukocyte Infiltration

As a result of microglial activation, neutrophils are the first systemic cell to infiltrate the injured spinal cord. Neutrophils are part of a specialized group of white blood cells known as granulocytes. As noted above, after SCI there is an increased expression of adhesion proteins that facilitate margination of systemic immune cells into the spinal cord. Further, maintained disruption of the blood-spinal cord barrier in the early stages of injury aids in this recruitment. Neutrophil presence is accompanied by an increase in myeloperoxidase (MPO) activity, an enzyme limited to this cell population. MPO activity is significantly increased in spinal cord tissue by several hours after SCI, with maximum activity observed within three days (Means and Anderson 1983; Carlson, Parrish et al. 1998; Taoka and Okajima 1998). MPO activity contributes to the peroxidation of lipids, affecting important components of the myelin sheath and cell membrane. Neutrophils are also phagocytic, engulfing dead tissue and debris in an attempt to restore disrupted homeostasis. In the early setting of SCI, these actions contribute to the destruction of tissues and exacerbate secondary injury mechanisms. In addition, the infiltrating neutrophils release matrix metalloprotenases (MMPs) which further contribute to lipid peroxidation, tissue

damage and degradation (Taoka, Okajima et al. 1997; Gris, Marsh et al. 2004).

Several studies have shown a therapeutic benefit to reducing the infiltration of neutrophils into the acutely injured spinal cord by blocking adhesion molecules required for margination (Bao, Chen et al. 2004; Saville, Pospisil et al. 2004). The limitations to this approach include the lack of a clinically relevant time-window of opportunity. However, with maximum neutrophil infiltration occurring at three days post-injury, there is still potential for further refinement in these pharmacological techniques.

1.4.3 Role of the Macrophage / Microglial Population

The next type of inflammatory response post-SCI involves immature monocyte and blood-borne macrophage infiltration. Monocytes quickly mature into macrophages, at which point the systemic macrophage and resident microglia are indistinguishable from one another immunohistochemically. Interestingly, it is believed that the bulk of the negative inflammatory effects seen in the injured spinal cord are primarily a result of systemic macrophages, not the resident microglia (Blight 1994; Popovich, Guan et al. 1999; Popovich and Hickey 2001). There is debate among investigators regarding the potential negative and beneficial effects of hematologous macrophages in the injured spinal cord (Popovich, Guan et al. 1999; Mabon, Weaver et al. 2000; Popovich and Hickey 2001; Schwartz 2003). It is this cell type in particular that nicely illustrates the concept that neuroinflammation can be both deleterious and necessary, a so-called “double-edged sword”. The understanding is that the time period in which these cells are active following SCI is crucial to the type of effect had.

The peak occurrence of phagocytic macrophages in the injured cord is between two and three days post-injury, and this presence is maintained throughout the first week. Following this, activated and phagocytic cells remain in the spinal cord tissue for several weeks (Blight 1985; Blight 1992; Popovich, Wei et al. 1997; Sroga, Jones et al. 2003). It is believed that the early wave of macrophage infiltration negatively contributes to the secondary injury processes by adopting the same cytokine expression profiles as the resident microglia (Popovich, Wei et al. 1997). The attenuation or depletion of macrophage activity in the first week of injury results in reduced secondary demyelination and axon loss, as well as in improved functional recovery (Popovich, Guan et al. 1999; Mabon, Weaver et al. 2000). However, during the sub-acute injury phase in rat models of SCI, the presence of activated macrophages and microglia contributes to the beneficial processes of tissue repair and wound healing (Li, Carpio et al. 2001). The inherent switch to secretion of growth factors and neurotrophins in these later stages of injury make the macrophage and microglia crucial to a regenerative environment. Furthermore, active phagocytosis of dead tissue and debris are an integral part of the regeneration process (McTigue, Horner et al. 1998; Popovich and Hickey 2001; Jones, McDaniel et al. 2005).

The profile of cytokine secretion by the macrophage and microglial populations early in injury pathology is pro-inflammatory, as indicated earlier. The same cytokines are released in the sub-acute injury phase, however their overall impact is much more positive. For example, delayed release of IL-1 is important for the healing process, as it acts to aid in re-vascularization and new

blood vessel formation at the site of injury. As well, IL-1 stimulates glial synthesis of glucocorticoids and neurotrophic factors important for regeneration (Giulian, Baker et al. 1986; Giulian, Woodward et al. 1988; del Rey, Klusman et al. 1998; Herx, Rivest et al. 2000). Moreover, late release of TNFα can have beneficial effects, enhancing oligodendrocyte proliferation, stimulating remyelination and promoting axon growth (Shuman, Bresnahan et al. 1997; Selmaj, Walczak et al. 1998). This dual effect of macrophage and microglia activation on the injured spinal cord is a prime example of the need to more closely examine all aspects of the neuroinflammatory response. If it is possible to take advantage of the body's own inherent repair systems by optimizing the beneficial effects of inflammation while reducing the deleterious aspects, researchers and clinicians will be able to refine therapies for SCI patients.

1.4.4 T-lymphocytes

T-lymphocyte recruitment into the spinal cord is secondary to leukocyte and monocyte infiltration. These cells enter the spinal cord following receipt of cytokine and chemokine signals from activated microglia and macrophages. While T-lymphocytes are not the most abundant immune cell to enter the cord, they are observed in peak numbers between three and seven days post-injury with a lower but maintained presence for as long as ten weeks (Popovich, Wei et al. 1997; Popovich 2000; Sroga, Jones et al. 2003). Lymphocytes have the ability to secrete a variety of factors in addition to their role in immune surveillance and attack. The cytokines released by T-cells come from both pro- and anti-inflammatory families, and the cocktail released is dependent on the

presence of other cells, signals and soluble factors. For example, IFNγ and IL-10 can have a regulatory role in controlling macrophage and microglial activities (Lodge and Sriram 1996). It is also accepted that T-lymphocyte release of IL-10 prevents microglia, macrophages and other antigen-presenting cells from secreting TNFα and IL-6, while at the same time promoting the release of TGFβ, a powerful regenerative growth factor (Fiorentino, Zlotnik et al. 1991; Fiorentino, Zlotnik et al. 1991; Agnello, Villa et al. 2000; Cottrez and Groux 2001).

In addition to the sequestration of T-lymphocytes to the injured spinal cord through cytokine signalling, there is another theory of their activation and subsequent recruitment to the site of trauma. The concept is of autoimmunity and antigen-specificity. T-cells recognising self, CNS-specific antigen, for example to myelin-basic protein (MBP), accumulate at the site of injury and remain in the tissue (Hickey, Hsu et al. 1991; Wucherpfennig 1994; Hirschberg, Moalem et al. 1998; Moalem, Leibowitz-Amit et al. 1999; Kipnis, Mizrahi et al. 2002). In addition, presentation of CNS-specific proteins by activated microglia results in the activation of these autoimmune T-lymphocytes (Moalem, Leibowitz-Amit et al. 1999; Kipnis, Mizrahi et al. 2002; Kipnis, Avidan et al. 2004).

The overall function of CNS-reactive T cells in the injured spinal cord is not yet fully understood, however there is building evidence to suggest that a highly beneficial role exists for these cells following CNS trauma. The presence of activated, CNS-reactive T-lymphocytes at the injury site causes an increased infiltration of regulatory T-cells. The regulatory cells are then able to secrete various types of trophic factors important for regeneration and growth (Jones,

Mass et al. 1999; Moalem, Leibowitz-Amit et al. 1999; Moalem, Gdalyahu et al. 2000; Moalem, Yoles et al. 2000; Schwartz and Kipnis 2001).

Several studies have shown the post-SCI benefit of injecting autoimmune MBP-reactive T-lymphocytes. It was observed that MBP-reactive cells offered significant morphological and locomotor recovery effects as compared to ovalbumin-reactive control cells (Hauben, Nevo et al. 2000; Moalem, Yoles et al. 2000; Barouch and Schwartz 2002). Conceptually this is difficult to appreciate, as it this kind of autoimmunity that plays into the pathology of other CNS disorders such as encephalitis and multiple sclerosis. It should be noted that patients treated with this form of immune therapy would have to be extremely well-monitored to assess the balance between beneficial and potentially deleterious effects.

In regards to B-lymphocytes, the antibody producing immune cell, and its potential role in the secondary pathology of SCI, little is actually known. It has been observed that B cells do infiltrate into the injured spinal cord, likely as a result of blood-spinal cord barrier disruption, but the effect and duration of this has not been confirmed (Imperato-Kalmar, McKinney et al. 1997).

1.5 Cell Death Mechanisms

The secondary injury pathologies following spinal cord trauma are complex and highly interrelated. This concept has been highlighted already, with the interplay between ion imbalances, free radical formation and glutamate excitotoxicity. Similar relationships exist with cell death mechanisms that are activated post-injury, collectively contributing to cell loss and tissue degradation.

Importantly, loss of calcium homeostasis, excitotoxicity and the complex inflammatory response all have an impact on the health and viability of neurons and glia at and adjacent to the site of trauma.

There are three general ways that a cell can die: It can undergo a process known as apoptosis (also known as type-1 programmed cell death), a genetically programmed event leading to targeted cell death; it can undergo another process called oncosis which causes swelling, organelle disruption and lysis of the damaged cells (Kerr, Wyllie et al. 1972; Yasuhara, Asai et al. 2007). Both of these cell death mechanisms lead the dying cell to a state of necrosis, characterized by cell lysis. It is the characteristic stages of either apoptosis or oncosis in the preparation for necrosis that ultimately determines the impact of a cell's death on its surrounding tissues. Additionally, a more recent type of cell death that displays unique cellular characteristics has been defined as autophagy, also known as type-2 programmed cell death (Clarke 1990; Yasuhara, Asai et al. 2007).

There is continued debate of the proper terminology used to describe these various types of cell death. Strictly speaking, oncosis is considered to be unregulated, non-apoptotic cell death that leads to necrosis. However, the term necrosis has been widely accepted as the actual definition of this non-apoptotic mechanism of cell death, causing confusion and misinterpretation (Majno and Joris 1995; Yasuhara, Asai et al. 2007). For the purposes of this thesis, the more general definition of cell death mechanisms will be utilized to achieve consistency with the combination of literature from a range of fields. Apoptosis, necrosis and

autophagy will be the definitions used to identify the three distinct types of cell death possible.

It is important to note that cell death can also occur somewhere along a continuum from apoptosis to autophagy to necrosis, where cells can display a combination of characteristics. In addition, each type of cell death exerts unique consequence to the surrounding tissues (Ankarcrona, Dypbukt et al. 1995).

1.5.1 Apoptotic (type-1 programmed) Cell Death

Apoptosis is characterized as a highly ordered physiological mechanism that results in the controlled degradation of a single target cell (Cohen 1993; Nagata and Golstein 1995; Emery, Aldana et al. 1998). This type of cell "suicide" has been genetically conserved throughout evolution, from the nematode *C. elegans* all the way to mammals, including human beings (Horvitz, Shaham et al. 1994; Thornberry and Lazebnik 1998; Yin 2000). Apoptotic cell death is under strict genetic control and has an important physiological role in controlling developmental morphogenesis, regulating tissue homeostasis and in the proper functioning of the immune response and control of autoimmunity (Thornberry and Lazebnik 1998; Yin 2000; Penninger and Kroemer 2003). Apoptosis is important for normal development and immune system regulation. It can be initiated by a variety of physiological changes at the tissue and cellular level, such as pathogen invasion, reduced blood flow, and physical trauma (Kerr, Wyllie et al. 1972; Kerr, Winterford et al. 1994; Wyllie 1994; Majno and Joris 1995; Thornberry and Lazebnik 1998).

Initiation of apoptotic cell death can occur along two clearly defined pathways: the first involves death receptor activation at the target cell surface and the second encompasses mitochondria-specific events (Ashkenazi and Dixit 1998; Green and Reed 1998). Fas receptor and Fas ligand are key players in the death receptor pathway of apoptotic activation, and will be discussed in detail in subsequent sections. There is an additional means of apoptotic activation that occurs through the mitochondria but is independent of caspase activation (Susin, Lorenzo et al. 1999; Li, Luo et al. 2001; Arnoult, Parone et al. 2002; Cande, Cohen et al. 2002). These cell death pathways will be discussed in more detail in the following sections. **Figure 2** provides a generalized schematic detailing the most important aspects of the apoptotic pathways.

Apoptotic signalling is a biochemical process involving a series of events carried out by caspases, a family of cysteine proteases (Thornberry and Lazebnik 1998; Varghese, Khandre et al. 2003). An apoptotic cascade involves a predictable and logical sequence of cellular events that result in DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and eventual disassembly of the cell into membrane-bound vesicles known as apoptotic bodies (Thornberry and Lazebnik 1998). The inconspicuous tissue presence of an apoptotic cell is characteristic of this type of cell death, in that there is relatively no disruption of the surrounding environment. As a result, a slight inflammatory response is initiated, typified by engulfment of the apoptotic bodies by phagocytic cells (Weedon, Searle et al. 1979; Duvall, Wyllie et al. 1985; Savill, Fadok et al. 1993; Majno and Joris 1995).

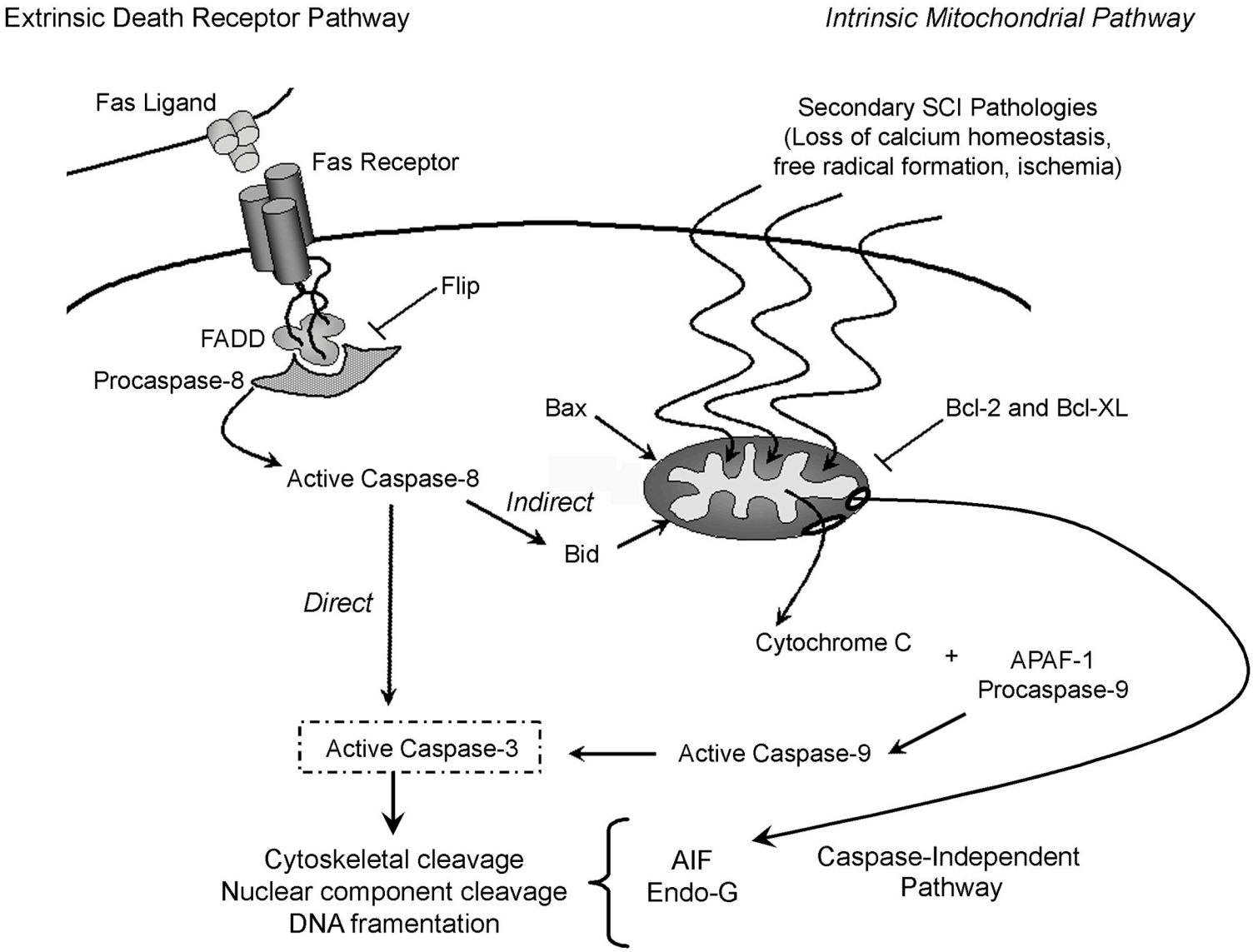


Figure 2: General Apoptotic Cell Death Mechanisms

Figure 2: General Apoptotic Cell Death Mechanisms

The cellular cascades responsible for apoptotic cell death are complex and there are some interactions that complicate the understanding even further. It is important to be able to recognise and understand the role of all key players in each of the three basic types of apoptotic cell death described throughout this work: the *Extrinsic Death Receptor* pathway, the *Intrinsic Mitochondrial Pathway*, and the *Caspase-Independent* pathway. In addition, it is necessary to note any interactions between the main players of each pathway. A fundamental understanding of the complexities of apoptosis is important for developing appropriate experimental outcomes and valid therapeutics designed to target this type of cell death.

The Extrinsic Death Receptor pathway is predominantly regulated by Fas receptor and Fas ligand. Here, activation of the Fas receptor initiates an intracellular cascade whereby recruitment of FADD and procaspase-8 triggers the autolytic activation of caspase-8. The **direct** pathway involves caspase-8 cleavage and subsequent activation of caspase-3, the final executioner caspase in the apoptotic cascade. The **indirect** pathway involves caspase-8 cleavage of Bid, a pro-apoptotic member of the Bcl-2 family. The cleaved fragment of Bid translocates to the mitochondria, triggering the release of cytochrome C into the cytoplasm. Cytochrome C joins together with APAF-1 and procaspase-9 to from the apoptosome complex, ultimately causing the cleavage and activation of caspase-9. Caspase-9 acts to cleave and activate caspase-3, hence the indirect nature of caspase-8 activation of caspase-3.

Recruitment of procaspase-8 to the death domains of FADD can be competitively inhibited by the presence of cellular Flip. Therefore, in the presence of competing amounts of both procaspase-8 and Flip, the extrinsic pathway can be reduced by a reduction in caspase-8 activation.

The Intrinsic Mitochondrial pathway takes place via the release of cytochrome C from the mitochondria, triggered by various signals from within the cell such as free radical formation, loss of calcium homeostasis or UV damage. Cytochrome C is released into the cytosol due to the permeabilization of the

(Figure 2 Continued)

mitochondrial membrane. Just as in the indirect pathway described above, caspase-3 is activated by caspase-9 following the formation of the apoptosome complex.

Mitochondrial release of cytochrome C can be regulated by the Bcl-2 family of apoptotic proteins. Both Bcl-2 and Bcl-XL are anti-apoptotic, acting to reduce the mitochondrial membrane permeability and release of cytochrome C, whereas both Bid and Bax are pro-apoptotic, promoting the release of cytochrome C. In the absence of any other apoptotic signals, it is the balance of both anti-apoptotic and pro-apoptotic members of the Bcl-2 family that regulate the apoptotic fate of a cell.

The mitochondria can also release Apoptosis-Inducible Factor (AIF) and Endonuclease G (Endo-G) in response to apoptotic signals from the cell. These factors are released into the cytosol where they are translocated to the nucleus and play a role in nuclear breakdown and DNA fragmentation. The apoptotic-inducing capabilities of these molecules are independent of caspase activation, and therefore create a novel Caspase-Independent apoptotic pathway.

1.5.2 Necrotic Cell Death (Oncosis)

Necrotic cell death is a passive process initiated by cellular and tissue damage, as a result of either physical or chemical insult. This type of cell death is not regulated genetically and does not normally occur in the absence of a pathophysiological interference. Necrosis is characterized by cellular edema, organelle swelling and ultimate rupture of the plasma membrane, at which time cell contents leak into the extracellular environment (Ankarcrona, Dypbukt et al. 1995). A significant inflammatory reaction is initiated following cell lysis. Inflammatory cells accumulate in the necrotic tissue, where they release pro-inflammatory cytokines and chemokines. Collectively, this response is deleterious to the surrounding tissues and can lead to further damage and exacerbate tissue and cellular loss.

1.5.3 Autophagic (type-2 programmed) Cell Death

Another form of cell death is that of type-2 programmed cell death, also known as autophagy. Autophagic death is characteristically non-apoptotic and non-necrotic, is caspase-independent and involves the presence of autophagosomes within the cytoplasm of the affected cell (Clarke 1990; Yaginuma, Sato et al. 2001; Yasuhara, Asai et al. 2007). The mechanisms and contributing factors of this form of cell death are less understood than those of apoptosis and necrosis/oncosis, and therefore the impact of this specific cell death mechanism in SCI has is not yet fully understood. However, it has recently been shown that this novel form of cell death may play a role in cell loss following SCI in a mouse dorsal hemi-section model of injury (Kanno, Ozawa et al. 2009).

As noted previously, cells can exhibit signs of apoptosis, autophagy and necrosis along somewhat of a continuum of cell death processes. This is especially true in times of tissue damage and trauma. Certain cell types can be equally susceptible to apoptosis as they are to necrosis, even given the same type of initial insult (Ankarcrona, Dypbukt et al. 1995; Majno and Joris 1995). Given the subject of this scientific thesis, focus will be on apoptotic death mechanisms following SCI and where appropriate, important connections to necrotic cell death will be noted.

1.6 Apoptotic Cell Death

This thesis focuses on apoptotic cell death mechanisms in the pathophysiology of SCI, with specific emphasis on Fas-mediated apoptotic mechanisms. The following section serves to further introduce important background information regarding apoptotic cell death pathways and will provide relevant information to the understanding of apoptosis following traumatic SCI.

1.6.1 Apoptotic Caspases

The two main apoptotic pathways, death receptor-dependent and mitochondrial, share the existence of caspase proteins. Caspases are specific proteases that carry out proteolytic cleavage of target proteins at specific aspartic acid residues (Kerr, Wyllie et al. 1972; Wyllie, Kerr et al. 1980; Arends and Wyllie 1991; Ankarcrona, Dypbukt et al. 1995; Thornberry and Lazebnik 1998). Caspase activation is regulated through either self-cleavage or following proteolytic cleavage by another caspase (Nicholson and Thornberry 1997; Thornberry and Lazebnik 1998; Woo, Hakem et al. 1999). In this way, caspases

are found as either inactive precursors or following cleavage, as active proteolytic proteins (Nicholson and Thornberry 1997; Thornberry and Lazebnik 1998). In general, specific caspases have one of two specialized roles. The apical or regulator caspase is responsible for starting the activation cascade. Some examples of key initiator caspases are caspase-8 and caspase-9 (Ashkenazi and Dixit 1998; Emery, Aldana et al. 1998; Green and Reed 1998). Alternatively, caspases can be effectors, with the responsibility of carrying out targetrd cellular cleavage, resulting in the hallmark characteristics of apoptosis. The most notable effector caspase is caspase-3, whose activation marks the point of no return in the physiological events of apoptotic cell death (Varghese, Khandre et al. 2003).

The caspases involved in apoptotic cell death, namely caspase-8, caspase-9 and caspase-3, are responsible for the cellular and biochemical changes characteristic of this form of cell death (Thornberry and Lazebnik 1998). Active caspases work to cleave fundamental cell structure proteins, such as nuclear proteins and aspects of the cytoskeleton and in doing so, create a situation of cell degradation and disability (Orth, Chinnaiyan et al. 1996; Takahashi, Alnemri et al. 1996; Thornberry and Lazebnik 1998). For example, the nuclear lamina that underlies the nuclear envelope is necessary for proper chromatin organization. Activated caspase-3 will cleave this lamina and initiate the process of chromatin condensation, resulting in eventual DNA fragmentation. Caspases also work to inactivate many of the regulatory proteins required for DNA repair, mRNA splicing and DNA replication, setting the cell up for

inappropriate cell cycle control, eventual loss of homeostasis and further damage (Thornberry and Lazebnik 1998).

1.6.2 Death Receptors: The External Apoptotic Pathway

Fas (CD95 or Apo1) and TNF-R1 are cell surface receptors that belong to the Tumor Necrosis Factor (TNF) and Nerve Growth Factor (NGF) superfamily and have physiological ligands known as FasL and TNF, respectively (Suda and Nagata 1994; Nagata and Golstein 1995; Nagata 1997). The Fas and TNF signalling pathways are two of the most well-known “death receptor” pathways involved in the initiation of apoptotic cell death.

Fas and TNF-R1 are transmembrane receptors, consisting of an extracellular, an intracellular and a transmembrane domain. In both cases, upon receptor ligation by an appropriate ligand, homotrimerization of receptors forms a signalling unit. The bringing together of three identical receptors facilitates the intracellular grouping of their death domains (DD), recruiting the necessary machinery for apical caspase activation. In the case of Fas receptor, the amalgamated DD’s recruit the Fas-Associated Death Domain (FADD or Mort1) and procaspase-8 (also known as FLICE, Fadd-like Interleukin-1β converting enzyme) to the active intracellular site of the Fas receptor trimer. The amalgamation of Fas receptor, FADD and procaspase-8 is known as the Death-Inducing Signaling Complex (DISC). The formation of the DISC triggers procaspase-8 self-cleavage, producing active apical caspase-8 (Kischkel, Hellbardt et al. 1995; Boldin, Goncharov et al. 1996; Nagata 1997; Ashkenazi and Dixit 1998; Muzio, Stockwell et al. 1998; Thornberry and Lazebnik 1998). A

diagram of the components and formation of the DISC are illustrated in **Figure 3**.

The TNF-R1 pathway is very similar to that of Fas, whereby trimerization of the receptor triggers intracellular recruitment of TRADD (TNF Receptor-Associated Death Domain) and procaspase-8 autolytic activation. However, TNF-R1 activation can also cause TRADD to recruit other molecules such as TRAF2 (TNF Receptor Associated Factor 2), which can activate a protective pathway mediated by the NFκB transcription factor. This dual role of the TNF-R1 is mediated by the presence of certain transcription factors, making the decision between execution of a death signal or a protective signal dependent on cell type and intracellular conditions.

In the Fas-mediated apoptosis-inducing pathway, procaspase-8 is self-cleaved into its active form following formation of the DISC. Active caspase-8 acts downstream to cleave various substrates that contribute to the apoptotic signal. Specifically, caspase-8 will directly cleave the main executioner caspase, procaspase-3 into its active form. This direct activation of caspase-3 by caspase-8 is known as the **direct** Fas-mediated apoptotic pathway.

Active caspase-8 also acts indirectly by cleaving a member of the Bcl-2 family of proteins called Bid. Following cleavage of cytosolic Bid by caspase-8, an active fragment translocates to the mitochondrion (Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998; Gross, Yin et al. 1999; Yin 2000). Here, the action of truncated Bid is pro-apoptotic and triggers the mitochondrial release of cytochrome C, leading to further caspase activation and death. This **indirect** pathway for Fas-mediated apoptosis is most active in hepatocytes, where these

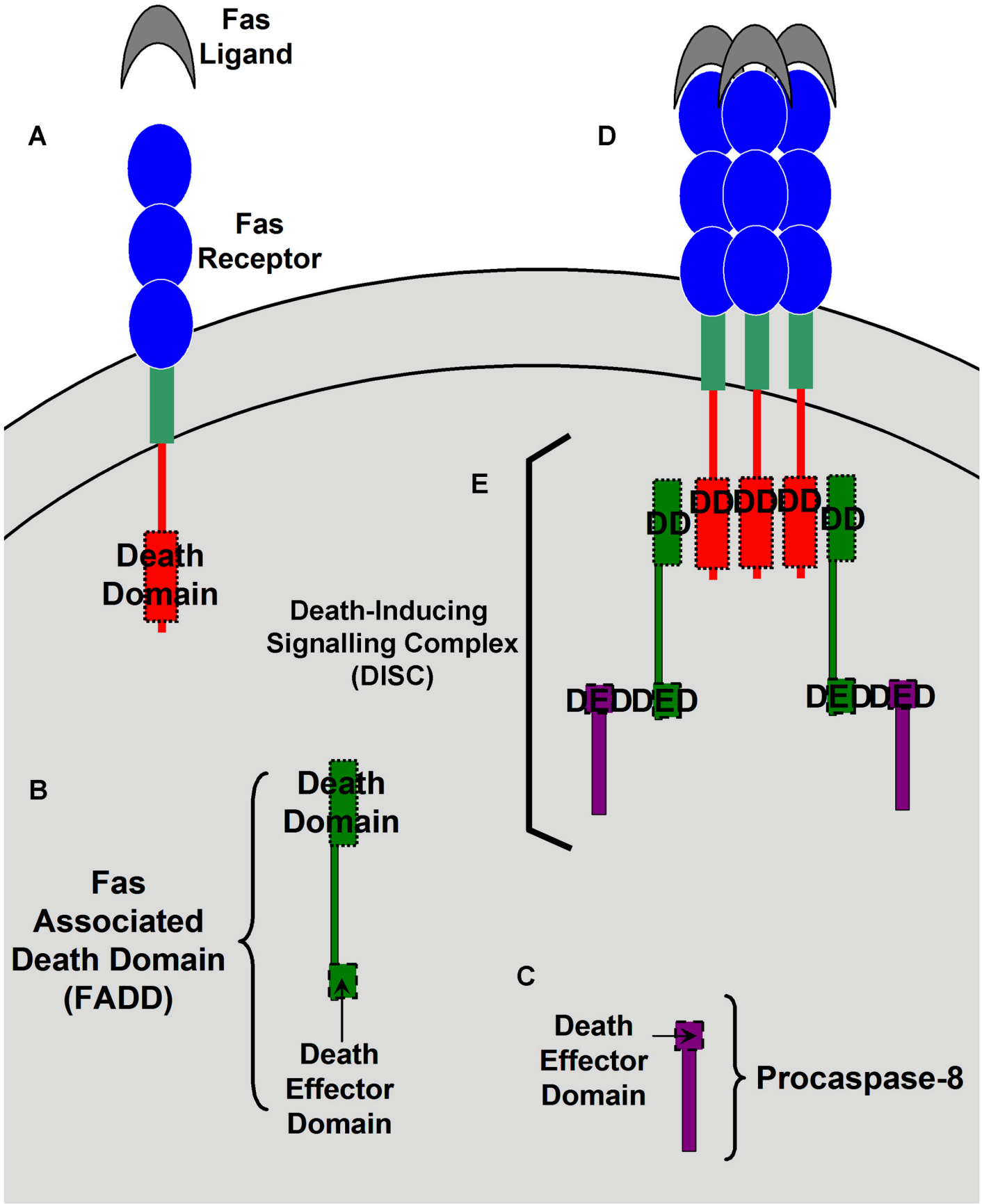


Figure 3: Fas receptor signalling through formation of the DISC

Figure 3: Fas receptor signalling through formation of the DISC

The Fas receptor, also known as CD95 or APO-1, is a member of the Tumor Necrosis Factor superfamily (A). The protein consists of 335 amino acids, which form six anti-parallel alpha-helices in its quaternary structure. Fas is a transmembrane protein, consisting on an extracellular domain, transmembrane domain and an intracellular domain, all of which are imperative for its ability to induce apoptosis (Nagata and Golstein 1995). The intracellular domain responsible for its death-inducing capabilities is termed the Death Domain (DD).

Fas-Associated Death Domain (FADD) is an adaptor protein recruited upon Fas activation to facilitate the apoptotic signal (B). FADD consists of two important domains, including its own DD and the Death Effector Domain (DED). The DD is able to interact with the DD on intracellular Fas, while the DED is able to interact with that found on procaspase-8.

Procaspase-8 is the third integral component of intracellular Fas apoptotic signalling (C). Procaspase-8 is an inactive protease that contains a DED to allow it to interact with that of FADD.

Upon activation of Fas receptor, the receptor trimerizes (D) and the intracellular DD's come together to recruit FADD by way of its DD's. FADD binds with intracellular Fas and further recruits procaspase-8 through interactions between the DED's. The Death-Inducing Signalling Complex (DISC) is formed by the amalgamation of these three molecules, intracellular Fas, FADD and procaspase-8 (E). Once the DISC is fully formed, procaspase-8 undergoes autolytic activation, and the Fas signalling pathway is active.

cells are highly sensitive to Bid-dependent Fas-mediated apoptosis and less so to the direct Fas pathway (Ogasawara, Watanabe-Fukunaga et al. 1993; Jones, Johnson et al. 1998; Yin, Wang et al. 1999).

1.6.2 (i) Type I and type II cells

More recently, it has been shown that a cell undergoing Fas-mediated apoptosis can be characterized into two categories, so named Type I and Type II cells. As explained above, ligation of Fas receptor with an appropriate ligand results in the formation of the DISC, and subsequent activation of caspase-8 is achieved (Kischkel, Hellbardt et al. 1995; Nagata 1997; Ashkenazi and Dixit 1998). The differentiation between Type I and Type II cells involves the speed at which the DISC is assembled following Fas activation, and the amount of active caspase-8 that is formed. In Type I cells, detection of the complete DISC is observed very soon after the Fas receptor is activated and large amounts of caspase-8 are quickly activated (Boldin, Goncharov et al. 1996; Muzio, Chinnaiyan et al. 1996; Kischkel, Lawrence et al. 2001; Sprick, Rieser et al. 2002; Aouad, Cohen et al. 2004). It is these Type I cells that primarily utilize the direct Fas pathway to carry out apoptosis, as there are sufficient amounts of active caspase-8 to cleave caspase-3 (Scaffidi, Fulda et al. 1998; Scaffidi, Krammer et al. 1999). On the other hand, Type II cells have an impaired ability to form the DISC upon Fas activation, and therefore do not have adequate amounts of active caspase-8. The caspase-8 that does become active cleaves Bid into tBid, thereby contributing to the indirect Fas-mediated pathway, functioning through the mitochondria (Li, Zhu et al. 1998; Scaffidi, Fulda et al.

1998; Scaffidi, Krammer et al. 1999). Type II cells are more likely to have an apoptotic signal inhibited by upregulation of Bcl-2 and Bcl-$_{XL}$ then would a Type I cell (Scaffidi, Fulda et al. 1998; Scaffidi, Krammer et al. 1999).

Quick and efficient recruitment of the machinery required to promote a Fas-mediated apoptotic signal is critical. Several groups have studied the regulatory role of membrane lipid rafts in the efficiency of Fas activation and DISC formation (Hueber, Bernard et al. 2002; Cahuzac, Baum et al. 2006). It is proposed that the presence of Fas receptor, Fadd and procaspase-8 in lipid raft microdomains allows for a more efficient and speedy formation of the DISC and greater propensity for caspase-8 activation. Type I cells more frequently show the majority of the DISC components being sequestered to membrane rafts as compared to Type II cells which lack key components. It follows that Type I cells would have a more efficient assembly of the DISC following Fas activation (Davis, Lotocki et al. 2007).

1.6.3 Mitochondria: The Intrinsic Apoptotic Pathway

Activation of the mitochondrial apoptotic pathway is usually a result of its receiving cellular stress signals and is most commonly triggered following trauma or cellular damage (Gross, McDonnell et al. 1999; Yin 2000). The basis for mitochondrial activation of apoptotic cascades involves mitochondrial membrane permeabilization (MMP). An increased membrane permeability results when the mitochondrial inner transmembrane potential (known as $\Delta\psi m$) is altered. A change in $\Delta\psi m$ triggers the opening of specialized channels in the mitochondrial membrane called mitochondrial PT pores (Petit, Susin et al. 1996; Qian,

Nieminen et al. 1997). There are several ways in which the Δψm can be disrupted, including rises in intracellular Ca^{2+}, pro-apoptotic effects of Bax and Bid, and by an apoptotic protein called ceramide (Green and Reed 1998). The outer mitochondrial membrane is permeable when the PT pores are opened, resulting in the release of cytochrome C into the cytosol. Cytochrome C is a protein normally associated inside the mitochondrial inner membrane where its primary responsibility lies in the proper functioning of the electron transport chain of cellular respiration (Wang, Yang et al. 2002; Penninger and Kroemer 2003).

The release of mitochondrial cytochrome C essentially commits the cell to death (Green and Reed 1998). The apoptotic events downstream of cytochrome C release involves the formation of the apoptosome complex. In the cytosol, cytochrome C combines with Apaf-1 and procaspase-9 to form an apoptosome, promoting the cleavage and activation of caspase-9 (Li, Nijhawan et al. 1997). Active caspase-9 moves forward to orchestrate the cleavage of procaspase-3 into its active form and finalizing the execution of the cell. As noted earlier, Fas-mediated signalling can also cause cytochrome C release from the mitochondria by way tBid (Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998; Gross, Yin et al. 1999; Yin 2000).

1.6.4 Caspase-Independent Apoptosis

Recently, a set of molecules have been discovered that are released from the mitochondria and trigger apoptotic cell death independently of caspase activation. Apoptosis Inducible Factor (AIF) and Endonuclease G (EndoG) are two such proteins that work together to elicite apoptotic signalling in the presence

of caspase inhibitors (Susin, Lorenzo et al. 1999; Li, Luo et al. 2001; Cande, Cohen et al. 2002; Wang, Yang et al. 2002). AIF is released from the mitochondria upon opening of the transition pores and subsequently translocates to the nucleus where it plays a role in causing chromatin condensation and DNA fragmentation, both hallmark signs of apoptosis (Susin, Lorenzo et al. 1999; Arnoult, Parone et al. 2002; Cande, Cohen et al. 2002; Penninger and Kroemer 2003). EndoG functions in much the same way, contributing to the fragmentation of DNA (Li, Luo et al. 2001; Penninger and Kroemer 2003).

1.6.5 Normal Physiological Roles for Apoptosis

1.6.5 (i) Developmental Regulation

Apoptotic cell death is well-recognised for its role in development, acting as a tool for structural formation and in the elimination of unnecessary and dysfunctional cells (Buss and Oppenheim 2004; Matalova, Setkova et al. 2006). Organ systems such as the lung, the kidney, the mammary and salivary glands, and the brain are dependent on apoptosis for proper morphogenesis (Humphreys, Krajewska et al. 1996; Piscione and Rosenblum 2002; Cecconi, Roth et al. 2004). For example, apoptosis has been recognised as a means for controlling neuronal development, as the developing cortical neuroepithelium is known to produce more neurons than are necessary for normal physiological functioning in the adult brain. It also acts to ensure that only those neurons with proper connections are present to form the mature brain. There must be a way for the extra and inappropriate neurons to be selectively removed, and the concept of neuronal attrition occurs as a result of neuronal apoptosis. It is

thought that this form of cell death is a consequence of limited target-derived growth factors (Oppenheim 1985). There has been suggestion that death-receptor-mediated neuronal regulation may play a role during development, however the impact of this is not clear (French, Hahne et al. 1996; Yeh, Pompa et al. 1998; Cheema, Wade et al. 1999).

1.6.5 (ii) Inflammatory Regulation

The Fas-mediated apoptotic pathway was first recognised in the immune system, followed by several non-neural tissues including the skin and retina (Watanabe-Fukunaga, Brannan et al. 1992; Oishi, Maeda et al. 1994; Esser, Heimann et al. 1995). While the extent and role of Fas-mediated apoptosis during development is still not entirely understood, its function in the immune system is well-known.

A fundamental understanding of Fas signalling in the immune system came from studies of mice deficient in either functional Fas receptor or Fas ligand (known as lpr and gld mice, respectively). The mutant mice display marked lymphadenopathy and have a high incidence of autoimmune disease (Rieux-Laucat, Le Deist et al. 1995; Watanabe, Suda et al. 1995). It is now understood that Fas and FasL interactions are critical for proper lymphocyte regulation, homeostasis and function. For instance, the ability of activated T lymphocytes and Natural Killer (NK) cells to kill target cells, either damaged or infected, is dependent on their expression of FasL (Kagi, Vignaux et al. 1994; Nagata 1997; Krammer 2000; Mabrouk, Buart et al. 2008; Strasser, Jost et al. 2009).

Fas and FasL signalling is important in lymphocyte development and maintenance of homeostasis within the lymphoid organs. Infectious insult activates T-cells leading to subsequent activation of additional lymphocytes. These cells are critical for attacking the source of insult and disabling the spread of infection. Once the body returns to a state of normalcy, the excess number of active lymphocytes can pose a threat to neighboring tissues and cells. Cytotoxic T-cells secrete a host of inflammatory cytokines that can cause a great deal of collateral damage if not removed from tissues. Fas-mediated cell death acts to downregulate the presence of active cells (Sprent and Tough 2001; Strasser 2005). Furthermore throughout development, lymphocytes can be inappropriately formed, either having incorrect TCR (T-cell Receptor) sequences or a high affinity for self-antigen. These cells must be removed before they can become an autoimmune risk. The clearance of these dysfunctional lymphocytes is achieved and regulated via Fas signalling (Strasser 2005; Strasser, Jost et al. 2009).

Fas-mediated apoptosis also plays a role in immune privilege. Certain organs or tissues of the body are protected from inflammatory cell infiltration because such a response could lead to serious consequences. The most notable of these is the immune privilege granted to the brain and spinal cord, where an unnecessary immune invasion could result in deleterious inflammation and tissue edema. Endothelial and non-hematopoietic cells such as microglia and astrocytes express FasL and activate the cell death of infiltrating T cells as

they attempt to cross the blood-brain-barrier (Bellgrau, Gold et al. 1995; Moalem, Monsonego et al. 1999).

The importance of apoptotic mechanisms in both developmental and immune system physiology is clear. However in many pathological situations, these necessary pathways become disrupted, and with inappropriate regulation can cause an exacerbation of cellular and tissue pathology.

1.6.6 Regulators of Apoptosis

There are naturally occurring agonists and antagonists of the Fas-mediated and mitochondrial apoptotic pathways that can complicate, exacerbate or minimize certain pathological conditions.

Under normal physiological conditions, cells contain a population of inactive proteins that work together to regulate the occurrence of programmed cell death, mainly by mitochondrial mechanisms (Gross, McDonnell et al. 1999; Yin 2000). These proteins belong to the Bcl-2 family of apoptotic regulators, and include the anti-apoptotic proteins Bcl-2 and Bcl-xl and the apoptosis agonists Bax and Bid (Gross, McDonnell et al. 1999). Upon initiation of apoptosis, initiator and effector caspases cleave and inactivate the antagonist members of the Bcl-2 family. The cleaved fragments then act to perpetuate the pro-apoptotic signal (Cheng, Kirsch et al. 1997; Xue and Horvitz 1997; Adams and Cory 1998). Bcl-2 and Bcl-XL over expression can inhibit the mitochondrial release of cytochrome C, and thereby prevent apoptosis (Seki, Hida et al. 2003). Bax and Bid on the other hand can exacerbate mitochondrial permeability, enhancing the apoptotic cascade.

There are several families of caspase-inhibitors known to exist. Inhibitor of Apoptosis Proteins (IAPs) are a family of caspase inhibitors that were discovered through the examination of viruses, and their inherent ability to prevent the normal host cell death response to viral invasion (Uren, Coulson et al. 1998). A very well-known caspase-inhibitor, z-VAD-fmk (fluoromethylketone) is a ketone peptide that acts to prevent caspases from actively cleaving their downstream targets. This molecule is not normally found physiologically, and therefore represents a potential therapeutic option for reducing caspase activation and apoptotic death initiation. There have been several studies showing the anti-apoptotic actions of z-VAD-fmk following traumatic injury in the spinal cord (Ozawa, Keane et al. 2002; Barut, Unlu et al. 2005).

Turning specifically to the Fas-mediated apoptotic pathway, there is one family of endogenous inhibitors that is well-known. FLIP, which stands for FLICE-like Inhibitory Protein, has a sequence homology to procaspase-8 and therefore has the ability to compete with procaspase-8 for binding with the DED of FADD. Via competitive inhibition, FLIP reduces Fas-mediated signalling because it lacks proteolytic functions (Irmler, Thome et al. 1997; Shu, Halpin et al. 1997). Several groups have investigated the impact of FLIP expression, reporting that increased expression reduces Fas-mediated apoptosis. In contrast, a downregulation of FLIP increases this form of cell death (Grassi, Piacentini et al. 2004; Wang, Azad et al. 2008).

Another means of regulating Fas-mediated cell death can be achieved by controlling the cellular expression of Fas receptor itself. The presence of Fas on

a cell dictates its vulnerability to death. It can be assumed that given control over the other regulatory factors discussed, increased expression of Fas would increase the probability of cell susceptibility to Fas-mediated cell death. There are several cytokines and chemokines that have the ability to alter Fas expression at the cellular level, and these may play an important role in Fas expression following SCI given the significant inflammatory response present. It has been widely shown that both TNFα and IFNγ expression can significantly increase cellular expression of Fas receptor (Moller, Koretz et al. 1994; Matsuyama, Hata et al. 1995; Choi, Park et al. 1999; Pouly, Becher et al. 2000; Fluhr, Krenzer et al. 2007). In the nervous system specifically, microglia normally express low levels of Fas, however *in vitro* application of TNFα and IFNγ can increase this expression, increasing the cellular sensitivity to Fas-mediated cell death. Interestingly, the presence of TGF-β in these cultures can reduce the increase in Fas expression and prevent apoptosis (Lee, Zhou et al. 2000). Another point to acknowledge is that TNFα and IFNγ can also upregulate the expression of FasL on astrocytes, giving some indication of a possible source for FasL expression in the traumatically injured spinal cord (Choi, Park et al. 1999).

1.7 Cell Death Mechanisms of Secondary SCI Pathobiology

The impact of both primary injury the cumulative events of secondary injury pathologies discussed previously play a role in cell death activation for both neurons and glia following traumatic SCI. Cell death contributes to the degradation and loss of tissue at the injury site, and exacerbates axonal

dysfunction, axonal loss and demyelination at sites proximal to the injury epicenter.

1.7.1 Necrosis After SCI

It is generally well-accepted that the most immediate form of cell death in the traumatically injured spinal cord is necrosis. The shear physical trauma to the tissue, associated calcium overload, glutamate excitotoxicity and ischemic insult cause the more sensitive cells located at the initial injury site to die by necrosis soon after the insult. These cells are mostly neurons, therefore the grey matter at the injury site is significantly necrotic early in the secondary injury phase (Hahn 1988; Manev, Favaron et al. 1989; Choi and Rothman 1990; Garthwaite and Garthwaite 1990; Siesjo 1992; Ankarcrona, Dypbukt et al. 1995; Schwab and Bartholdi 1996; Liu, Xu et al. 1997; McEwen and Springer 2005). As a result, there is a significant rostro-caudal spread of the lesion within the first of week of injury, creating a central cavity of necrosis and inflammation, surrounded by a rim of intact and potentially viable tissue (Siesjo 1992; Liu, Xu et al. 1997). It is in this spared tissue surrounding the necrotic lesion that exhibits the majority of apoptotic cells (McEwen and Springer 2005).

1.7.2 Apoptosis After SCI

Following the initial wave of necrotic cell death primarily affecting the neuron population, apoptosis of both neurons and glia is imminent (Rink, Fung et al. 1995; Katoh, Ikata et al. 1996; Li, Brodin et al. 1996; Springer, Azbill et al. 1999; Knoblach, Huang et al. 2005). Spared neurons surrounding the initial necrotic lesion are susceptible to a slightly delayed apoptotic cell death, due in

most part to the same kinds of insults that triggered the initial necrosis (Kure, Tominaga et al. 1991; Heron, Pollard et al. 1993; Linnik, Hatfield et al. 1993; Linnik, Zobrist et al. 1993; MacManus, Buchan et al. 1993; MacManus, Hill et al. 1994). Often the severity of the insult and the type of neuron affected dictates whether necrosis or apoptosis will result. The same neuronal cell type will undergo either apoptosis or necrosis, depending on the degree of insult (Dypbukt, Ankarcrona et al. 1994; Bonfoco, Krainc et al. 1995). DNA fragmentation, active caspase-3 activity and a TUNEL-positive phenotype are observed in neurons as early as one hour post-injury (Crowe, Bresnahan et al. 1997; Liu, Xu et al. 1997; Knoblach, Huang et al. 2005; McEwen and Springer 2005). Neuronal apoptosis is maintained for the first day following injury, with a maximal presence at eight hours post-SCI. Alternatively, the glial population, namely the oligodendrocytes, exhibit this phenotype in a more delayed and prolonged fashion following injury. Oligodendrocytes display apoptotic markers as early as six hours post-injury, with maximum expression at the injury site at 24 hours and in the adjacent, intact tissue at three to four days post-SCI (Crowe, Bresnahan et al. 1997; Liu, Xu et al. 1997; Shuman, Bresnahan et al. 1997; Springer, Azbill et al. 1999; Casha, Yu et al. 2001; McEwen and Springer 2005; Ackery, Robins et al. 2006). Interestingly, the oligodendrocyte population continues to show active apoptosis in the two weeks following injury, with another peak in expression around one week (Li, Field et al. 1999; McEwen and Springer 2005). The more delayed wave of oligodendrocyte apoptosis is observed in areas of white matter degeneration and along degenerating axonal tracts (Casha,

Yu et al. 2001). This observation was also noted in injured human spinal cord tissues (Bunge, Puckett et al. 1993).

It is important to note the relationship between apoptotic oligodendrocytes and demyelinating and degenerating white matter tracts. It is possible that the death of these important glial cells contributes to the loss of motor and sensory function and other neurological deficits experienced by patients suffering from SCI. The delayed nature of this form of cell loss provides an optimistic potential to therapeutically target apoptosis in the oligodendrocyte population, thereby reducing the extent of demyelination and axonal degradation.

The extent of apoptotic cell death following SCI in other glial cell types including astrocytes, macrophage and microglia is limited. Neither TUNEL-staining nor expression of active caspase-3 are commonly observed on these cell types (Springer, Azbill et al. 1999; McEwen and Springer 2005). The conclusion at this point is that both neurons and oligodendrocytes are susceptible to apoptosis following traumatic SCI. The neuronal population is affected in a more rapid and limited time line, and therefore is less therapeutically targetable than the oligodendrocyte population. Moreover, the number of neurons that succumb to necrotic cell death within minutes to hours following injury are not easily targeted.

Direct studies of human SCI are limited, due to the ethical restrictions in obtaining consistent tissue samples. It has been shown that following traumatic SCI in humans, there are a significant number of cells that show hallmark characteristics of apoptotic cell death, from specific DNA fragmentation and the

resultant TUNEL-positive phenotype to the formation of apoptotic bodies (Gavrieli, Sherman et al. 1992; Emery, Aldana et al. 1998; Harter, Keel et al. 2001; Hentze, Schwoebel et al. 2001). The occurrence of apoptotic cells in human tissue ranges from three hours to 2 months post-injury, however the exact timeline of apoptosis is not easily observed given the post-mortem tissue availability (Emery, Aldana et al. 1998). It is noted that a correlation of the number of apoptotic cells observed post-SCI is dependent in part of the severity of the initial trauma, and on the extent of ischemic insult to the spinal cord tissue.

Notably, the greatest occurrence of apoptotic cells in human spinal cord tissue was found in white matter areas of marked axonal degeneration. Furthermore, both Wallerian degeneration and apoptosis were observed to a greater extent in ascending tracts than in the descending tracts of the spinal cord. The cells expressing an apoptotic phenotype also expressed CNPase, indicating that the oligodendrocyte population was most severely affected by this form of cell death. It was also noted that phagocytic macrophages and microglia were abundant in the areas of white matter degeneration, and were observed engulfing apoptotic bodies (Emery, Aldana et al. 1998).

While it has been well-accepted that both neurons and oligodendrocytes are susceptible to apoptotic cell death, the mechanism of cell death might be different between the two cell populations. While a TUNEL-positive phenotype, the presence of DNA fragmentation and expression of cleaved caspase-3 are all strong indicators of apoptotic cell death, they do not give any indication as to the mechanism of activation. Recall that apoptosis can be initiated by two distinct

pathways, either by death receptor-ligand activation or through mitochondrial mechanisms. The following sections will delineate the current understanding of specific pathways of apoptosis that are active following SCI, the time line of this activation and the primary cell populations affected.

1.7.2 (i) Mitochondrial apoptosis following SCI

Mitochondria are the organelles responsible for cellular energy production and have the ability to activate apoptotic pathways, making them critical regulators of cell survival and functionality following CNS trauma (Fiskum 2000; Wieloch 2001; Friberg and Wieloch 2002; Friberg, Wieloch et al. 2002). Recall that the initial trauma to the spinal cord initiates a rapid secondary tissue response involving ion imbalance, glutamate excitotoxicity, free radical formation and lipid peroxidation (Choi 1988; Lipton 1994; Ankarcrona, Dypbukt et al. 1995; Choi 1995; Sullivan, Krishnamurthy et al. 2007). These secondary pathologies occur within the first several hours of SCI, and are most evident in the neuronal population, where specifically ionic imbalances and calcium homeostasis are critical for survival. It is well established that these deleterious alter MMP in damaged neurons (Faden, Demediuk et al. 1989; Braughler and Hall 1992; Bernardi 1996; Hamada, Ikata et al. 1996; Azbill, Mu et al. 1997; Sullivan, Keller et al. 1998; Sullivan, Thompson et al. 1999). The consequence is collapse of the mitochondrial membrane potential ($\Delta\psi m$) and the subsequent release of cytochrome C into the cytosol (Sullivan, Thompson et al. 1999; Sullivan, Geiger et al. 2000; Brustovetsky, Tropschug et al. 2002; Brustovetsky, Brustovetsky et al. 2003; Sullivan, Springer et al. 2004; Sullivan, Rabchevsky et al. 2005).

The release of cytochrome C into the cytosol of neurons following the various secondary insults of SCI is the primary trigger of apoptotic cell death in these cells. In as little as 30 minutes following SCI, there is a redistribution of cytochrome C from the mitochondria into the cytosol (Springer, Azbill et al. 1999; Lewen, Fujimura et al. 2001; Sullivan, Keller et al. 2002). Furthermore, caspase-9 activity has been correlated with cytochrome C release both temporally and spatially following SCI, leading to the conclusion that mitochondrial apoptotic signalling is present (Springer, Azbill et al. 1999; Knoblach, Huang et al. 2005). These mitochondrial-specific events are consistent with the observation of TUNEL-positive phenotypes and DNA fragmentation within the neuron population post-SCI (Katoh, Ikata et al. 1996; Liu, Xu et al. 1997; Knoblach, Huang et al. 2005; McEwen and Springer 2005). The presence of cytochrome C, activation of caspase-9 and subsequent activation of caspase-3 provide the evidence necessary to confirm that early neuronal apoptosis is most-likely due to mitochondrial mechanisms, triggered by the other secondary injury pathways.

Mitochondrial apoptotic mechanisms can also be triggered following activation of the Fas death receptor pathway, facilitated by the cleavage and activation of Bid. In order for this to happen, there is a need for cellular expression of Fas and the presence of active caspase-8. Furthermore, the more direct pathway of Fas-mediated apoptosis will contribute to the ultimate activation of cleaved caspase-3 in these cells. These aspects of Fas-mediated cell death following SCI will be discussed in the following section.

1.7.2 (ii) Fas-mediated apoptosis following SCI

In the normal, adult brain and spinal cord, there are generally low levels of Fas and FasL expression; in the pathological CNS, there is increased expression of both receptor and ligand on a variety of glial and neuronal cell populations (Martin-Villalba, Herr et al. 1999; Li, Farooque et al. 2000; Casha, Yu et al. 2001; Martin-Villalba, Hahne et al. 2001; Zurita, Vaquero et al. 2001; Choi and Benveniste 2004; Demjen, Klussmann et al. 2004; Zuliani, Kleber et al. 2006). There is no dispute over the deleterious impact of the Fas and FasL apoptotic pathway in many disorders and diseases of the central nervous system. Fas-mediated apoptosis of glia and neurons plays a role in the pathogenesis of multiple sclerosis, Parkinson's, Huntington's and Alzheimer's disease (Guo and Saxon 1995; Nishimura, Akiyama et al. 1995; Dowling, Shang et al. 1996; Mogi, Harada et al. 1996; Ciusani, Frigerio et al. 1998; Dzietko, Boos et al. 2008). It has also been widely accepted as a contributing factor to the apoptotic cell death observed after cerebral stroke and following traumatic brain injury (Martin-Villalba, Herr et al. 1999; Velier, Ellison et al. 1999; Beer, Franz et al. 2000; Martin-Villalba, Hahne et al. 2001; Grosjean, Lenzlinger et al. 2007).

The existence of Fas-mediated apoptosis during secondary injury SCI pathology is well-established (Crowe, Bresnahan et al. 1997; Shuman, Bresnahan et al. 1997; Springer, Azbill et al. 1999; Li, Farooque et al. 2000; Casha, Yu et al. 2001; Beattie, Hermann et al. 2002; Demjen, Klussmann et al. 2004; Casha, Yu et al. 2005; Knoblach, Huang et al. 2005; Ackery, Robins et al. 2006). There have been several studies to examine the impact of Fas-mediated

apoptosis following SCI using the *lpr* mutant mouse, a genetically altered mouse designed to express a non-functional Fas receptor. Collectively, this work provides evidence of Fas receptor activation post-SCI. Mutant mice consistently show a beneficial neuroprotective and enhanced locomotor response compared to their wildtype littermates. Fas-deficient mice show reductions in apoptosis, increased survival of oligodendrocyte and neurons, and a significant sparing of tissue post-injury (Yoshino, Matsuno et al. 2004; Casha, Yu et al. 2005; Yu, Baptiste et al. 2009). Furthermore, it has been shown that neutralization of the Fas signalling pathway, either by systemic injection of a neutralizing antibody, or by intrathecal administration of a soluble Fas receptor (sFasR) post-injury is therapeutically beneficial (Demjen, Klussmann et al. 2004; Ackery, Robins et al. 2006).

Fas receptor expression is significantly increased following traumatic injury. Increases in Fas receptor expression have been observed on the oligodendrocyte population, on certain sub-populations of neurons and on astrocytes (Li, Farooque et al. 2000; Casha, Yu et al. 2001; Zurita, Vaquero et al. 2001; Casha, Yu et al. 2005). The microglial population does not appear to express Fas receptor following SCI. In regards to Fas ligand expression, there is a general agreement that ligand expression is upregulated following SCI, and can be found on a variety of glial cells (Li, Farooque et al. 2000; Zurita, Vaquero et al. 2001).

The exact temporal and cellular profile of Fas expression in the injured spinal cord is not clear, however it has been shown that Fas expression begins to

increase by 24 hours post-SCI, peaking between three and seven days (Li, Farooque et al. 2000; Casha, Yu et al. 2001; Zurita, Vaquero et al. 2001; Casha, Yu et al. 2005). Moreover, there is a consistent observation that Fas-expressing cells concurrently express apoptotic markers, indicating that these cells are undergoing Fas-mediated apoptosis (Casha, Yu et al. 2001; Casha, Yu et al. 2005). Importantly, the appearance of these Fas-mediated apoptotic cells is delayed by approximately three days post-injury. Further support to this is the observation that post-SCI caspase-8 activation reaches peak levels starting at the day three and continuing through to day seven (Springer, Azbill et al. 1999; Casha, Yu et al. 2001).

In regards to the specific cell types expressing Fas and undergoing Fas-mediated cell death following SCI, there are some discrepancies within the literature, and this area of study needs to be given more focus. Currently, it has been shown that apoptotic nuclei, labelled by TUNEL, are found in greatest numbers in the degenerating white matter following SCI. In addition, the maximal appearance of these cells occurs in a delayed fashion, between three and seven days post-SCI (Casha, Yu et al. 2001; Casha, Yu et al. 2005; Ackery, Robins et al. 2006). It has been shown that TUNEL-positive cells found in white matter express Fas receptor, and this is consistent with the timing of caspase-8 activation (Shuman, Bresnahan et al. 1997; Springer, Azbill et al. 1999; Li, Farooque et al. 2000; Casha, Yu et al. 2001; Zurita, Vaquero et al. 2001). Moreover, several groups have shown that the oligodendrocyte population upregulates Fas expression in a similar delayed time course to the appearance

of apoptotic cells in the white matter following SCI (Casha, Yu et al. 2001; Zurita, Vaquero et al. 2001; Casha, Yu et al. 2005). Also of note is that oligodendrocytes express activated caspase-8 in a delayed time frame post-injury, with peaks in expression seen at three days (Knoblach, Huang et al. 2005).

The literature to date, summarized above, can be combined to suggest that Fas-mediated cell death is occurring in a delayed fashion, predominantly within the oligodendrocyte population. Furthermore, these dying oligodendrocytes are found in degenerating and demylinated white matter tracts in rostro-caudal cord segments distal to the injury site, implying that their death may be contributing to the degeneration and demyelination of distal white matter tracts following SCI.

Following SCI, neurons in areas adjacent to the injury epicenter are known to be susceptible to apoptosis (Katoh, Ikata et al. 1996; Li, Nijhawan et al. 1997; Liu, Xu et al. 1997; Springer, Azbill et al. 1999; Casha, Yu et al. 2005; McEwen and Springer 2005; Ackery, Robins et al. 2006). Neurons are also known to be highly susceptible to mitochondrial mechanisms of apoptosis, as discussed previously. Evidence of post-injury Fas expression on neurons has been limited and contradicting, with reports indicating either no or low levels (Matsushita, Wu et al. 2000; Casha, Yu et al. 2001; Zurita, Vaquero et al. 2001; Casha, Yu et al. 2005). It is noted that the extent and type of trauma to the spinal cord result in different neuronal patterns of Fas expression, giving validity to the inconsistent reports in the literature. Fas-mediated death of neurons in the CNS depends on

the sub-population of neuron. For example, motor neurons are the most susceptible, while other types including cerebellar granular, cortical and dorsal root ganglion neurons are unaffected by Fas (Raoul, Estevez et al. 2002). Also of note is that reports indicating neuronal expression of Fas receptor have been limited to *in vitro* work using primary and differentiated neuronal sub-populations. In addition, there is a lack of evidence showing apoptotic neurons co-expressing Fas receptor post-SCI, whereas there is an abundance of literature linking neuronal apoptosis to mitochondrial mechanisms.

Recent reports suggest that neurons have the ability to express endogenous Fas-inhibitory proteins, rendering them relatively resistant to Fas-mediated cell death (Fernandez, Segura et al. 2007; Segura, Sole et al. 2007). *In vitro*, neurons show a limited ability to undergo Fas-mediated cell death, even with concomitant expression of Fas receptor (Gerhardt, Kugler et al. 2001; Putcha, Harris et al. 2002). The protein known as Fas Apoptotic Inhibitory Molecule, long form ($FAIM_L$) has restricted expression in the CNS, and is only found in neurons. $FAIM_L$ acts as a competitive inhibitor of FADD binding to the active death domains of Fas receptor. It has been shown that overexpression of $FAIM_L$ invokes resistance to PC12 cells from Fas-induced apoptosis (Segura, Sole et al. 2007). In addition, a protein called LFG, or lifeguard (also known as the neuronal membrane protein 35) is upregulated in neurons during post-natal development and into adulthood. At these times points, LFG acts to protect cortical and differentiated cerebellar granular neurons from Fas-mediated apoptosis and subsequent caspase-8 activation. LFG can be found in the lipid

rafts of neuronal membranes, giving indication of its function in de-regulating the efficiency of Fas signalling in these cells (Davis, Lotocki et al. 2007; Fernandez, Segura et al. 2007).

1.7.2 (iii) Indirect Fas-Mediated Signalling after SCI

The impact of Fas activation on the mitochondrial pathway through tBid activation following SCI has only been recently addressed. As noted earlier, the main cell type that is seemingly affected by Fas-mediated apoptosis following SCI is the oligodendrocyte. Given this, the cellular mechanisms of oligodendrocyte apoptosis by Fas activation were evaluated using an oligodendrocyte cell line (Austin and Fehlings 2008). In this work, it was determined that Fas signalling in the oligodendrocyte causes caspase-8 activation and mitochondrial release of cytochrome C, followed by activation of caspase-9, providing evidence for the existence of indirect Fas signalling. In addition, Fas activation caused the mitochondrial release and nuclear localization of AIF, which occurred independently of caspase activation.

More recently using the *lpr* mutant mouse, the impact of indirect Fas signalling was evaluated post-SCI (Yu, Baptiste et al. 2009). Fas-deficient mice show a reduction in activated Bid following SCI as compared to their wildtype littermates. In addition, the *lpr* mice showed reduced caspase-9 activity at three days following experimental SCI. Taken together, the lack of Bid activation and a consistent reduction in caspase-9 activity observed in mice lacking a functional Fas receptor give merit to the concept that following SCI, Fas activation does play a role in mitochondrial apoptotic signalling.

By way of summary, Table 1 describes the major molecular components of Fas-mediated apoptotic signalling and its regulation.

1.8 Fas Receptor and Proliferation

The most notable accepted result of Fas-FasL signalling is that of apoptotic cell death. The role of Fas in the regulation of inflammatory cells and in the function of lymphocytes is well-known and there is evidence supporting the death-inducing capabilities of Fas activation following trauma to the CNS, specifically in SCI. However, recent reports have shown another, contrasting role for the Fas-FasL pathway. The evidence for a proliferative action of Fas activation comes from work in T-lymphocyte, B-lymphocyte and certain tumor cell lines (Alderson, Armitage et al. 1993; Owen-Schaub, Meterissian et al. 1993). Further reports show that stimulation of Fas receptor, either by ligand or an agonistic antibody, promoted cell proliferation in activated lymphocytes, fibroblasts, hepatocytes and in a variety of tumor cell types (Alderson, Tough et al. 1994; Aggarwal, Singh et al. 1995; Freiberg, Spencer et al. 1997; Husain, Chiocca et al. 1998; Jelaska and Korn 1998; Newton, Harris et al. 1998; Zhang, Cado et al. 1998; Desbarats, Wade et al. 1999; Desbarats and Newell 2000; Shinohara, Yagita et al. 2000). A broad range of molecular mechanisms are implicated in the proliferative actions observed, and were highly dependent on the cell type being evaluated. Currently there is no one specific pathway that has provided enough evidence to yield a unified model for Fas-mediated cell proliferation and growth.

Table One: Description of Key Molecular Components of Fas-Mediated Apoptosis

Name of Molecule	Description
Fas receptor (CD95, Apo-1)	The primary "death receptor" responsible for induction of Fas-mediated apoptosis; the receptor must be cell-bound to elicit death-inducing capabilities.
Fas ligand (CD95L)	The ligand capable of activating the Fas-mediated apoptotic pathway; there are both cell-bound and soluble forms of FasL.
Fas-Associated Death Domain (FADD)	The first component of the DISC that is recruited to the intracellular Death Domains (DD) of activated trimeric Fas receptor.
Caspase-8 (Flice)	The pro-form is recruited to complete the DISC after Fas activation; interaction between its Death Effector Domains (DED) and those on FADD trigger autolytic activation of caspase-8.
Death-Inducing Signalling Complex (DISC)	The complex formed following activation of Fas receptor that leads to activation of caspase-8; the components of the DISC are the intracellular DD of trimeric Fas, FADD and procaspase-8.
Caspase-9	The pro-form combines with cytochrome-C and APAF-1 to form the apoptosome complex, which triggers the cleavage and activation of caspase-9.
Caspase-3	The final executioner caspase in the apoptotic cascade; the pro-from is activated by either caspase-8 or caspase-9, marking the point of no-return for the cell. Active caspase-3 cleaves integral cytoskeletal elements, nuclear components and participates in DNA fragmentation.
Bcl-2	An anti-apoptotic member of the Bcl-2 family of apoptotic proteins; it works to prevent mitochondrial release of cytochrome C, AIF and EndoG.
Bcl-XL	A second anti-apoptotic member of the Bcl-2 family of apoptotic proteins; it works in the same fashion as Bcl-2 to prevent apoptosis.
Bid	A pro-apoptotic member of the Bcl-2 family of apoptotic proteins; cleavage of Bid by caspase-8 releases t-Bid (truncated-Bid) which acts on the mitochondria to induce the release of cytochrome C.

Bax	A second pro-apoptotic member of the Bcl-2 family of apoptotic proteins; it acts to trigger mitochondrial release of cytochrome C, AIF and EndoG.
Cytochrome C	A protein component of the mitochondrial inner membrane that is involved in electron transport during cellular respiration; upon receipt of an apoptotic signal, the mitochondrial membrane becomes permeable and cytochrome C is released into the cytosol, where it forms the apoptosome complex with APAF-1 and procaspase-9.
Apoptotic Peptidase Activating Factor-1 (APAF-1)	The protein that combines with cytosolic cytochrome C and procaspase-9 to form the apoptosome complex, triggering the activation of caspase-9.
Apoptosis-Inducible Factor (AIF)	The protein released from the mitochondria that triggers caspase-independent apoptosis by cleaving key DMA and nuclear elements.
Endonuclease-G (EndoG)	A similar protein to AIF also released from the mitochondria and where it is capable of triggering caspase-independent apoptosis.
Flice-Like Inhibitory Protein (Flip)	A family of proteins similar in structure to procaspase-8 (Flice) that can act to competitively inhibit procaspase-8 recruitment to FADD, thereby reducing caspase-8 activation and inhibiting Fas-mediated apoptotic signalling.
Inhibitors of Apoptosis Proteins (IAPs)	This is a family of proteins known for their abilities to inhibit caspases, thereby inhibiting the cascade of apoptotic cell death within a cell.
z-VAD-fmk	A well-known, pan caspase inhibitor; it functions by preventing active caspases from cleaving downstream targets.

In neurons, it has been shown that Fas activation induces neurite outgrowth *in vitro*, and promotes nerve regeneration following *in vivo* nerve injury (Desbarats, Birge et al. 2003; Pettmann and Henderson 2003). Interestingly, the observation of increased nerve outgrowth and associated improvement in functional recovery following nerve injury found by Desbarats *et al*. is in complete contradiction with the results showing that Fas neutralization stimulates regeneration and neurite outgrowth following transection SCI found by Demjen *et al*. in 2004. The mechanism of action in the SCI model is implied in the ability of Fas neutralization to reduce apoptotic cell death in the spinal cord tissue, while the mechanism proposed for neuronal proliferation is slightly more complex. It has been hypothesized that instead of signalling directly with caspase-8 activation, that Fas engagement can alternatively signal through the ERK pathway, causing growth benefits (Kataoka, Budd et al. 2000; Shinohara, Yagita et al. 2000; Desbarats, Birge et al. 2003). These authors believe that the increased expression of Fas receptor following trauma to the spinal cord is indicative of an endogenous defense and protective mechanism in place to enhance growth and regeneration (Lambert, Landau et al. 2003). With the impact of these findings remaining controversial in the context of CNS injury and trauma, the potential of proliferative signalling to be useful in therapeutic design for CNS trauma is an important area for future study.

1.9 Soluble Fas Receptor

Under normal physiological conditions, Fas is a 45 kDa, transmembrane receptor that functions upon extracellular activation by Fas ligand to induce

autonomous apoptotic cell death (Smith, Davis et al. 1990; Cascino, Fiucci et al. 1995; Nagata and Golstein 1995; Nagata 1997). There is accumulating evidence in the literature for the presence of a soluble form of this receptor naturally occurring in various tissues and physiological conditions. These soluble Fas receptor proteins (sFas) are thought to be either produced by proteolytic cleavage of the existing membrane-bound receptor or via alternative splicing of specific mRNA transcripts; to date there have been at least three fully-functional mRNA variants for human soluble Fas identified (Cascino, Fiucci et al. 1995).

The study of various pathologies including cancer and autoimmune disease have provided insight into the function of soluble Fas. Specifically, it has been found that serum levels of sFas in cancer patients are positively correlated with greater malignancy and poor disease prognosis (Cascino, Papoff et al. 1996; Owen-Schaub 2001). In some cancers, the ability of sFas to reduce apoptotic cell death of cancerous cells allows the disease to become more aggressive. The incidence of T- and B- cell-related malignancies is increased significantly in the presence of elevated serum sFas levels (Knipping, Debatin et al. 1995). It has also been reported that patients suffering from autoimmune diseases show twice the levels of soluble Fas in their blood stream than did individuals without the disease (Cheng, Zhou et al. 1994). Here, it is believed that disease progression is at least in part initiated by the ability of sFas to alter lymphocyte development and proliferation by inhibiting regulatory apoptosis.

Several groups have taken to using soluble Fas-immunoglobulin fusion protein (sFasR) to examine the mechanisms behind the activation and death-

inducing capabilities of T-lymphocytes. The presence of sFasR causes competitive inhibition of the interaction between cell-bound receptor and ligand, thereby preventing the initiation of apoptosis (Brunner, Mogil et al. 1995; Ju, Panka et al. 1995). Examination of the therapeutic impact of this type of Fas and FasL disruption was evaluated in the clip compression model of SCI in the context of this thesis. **Figure 4** provides a schematic representation of molecular structure and potential therapeutic use of sFasR presented in Chapter 4 and 5 of this work.

1.10 Rationale, Hypothesis and Specific Aims

SCI is a devastating form of neurotrauma, often accompanied by secondary complications leading to morbidity and mortality. Individuals suffering from acute SCI have a limited number of efficacious treatment options available to them, leading to a reduced quality of life, loss of motor and sensory function and a significant economic burden.

Research aimed to understand the complexities and dynamic interactions between secondary injury pathologies following acute traumatic SCI will provide potential for the development of novel therapeutic strategies. Scientists are well on their way to understanding the temporal, spatial and cellular relationships of the various aspects of secondary injury, however there is still much more to be done.

In consideration of the apoptotic mechanisms after SCI, it is well-accepted that apoptosis contributes to a significant portion of the post-traumatic cell death observed. Experimental therapeutic targeting of apoptosis has been shown to be

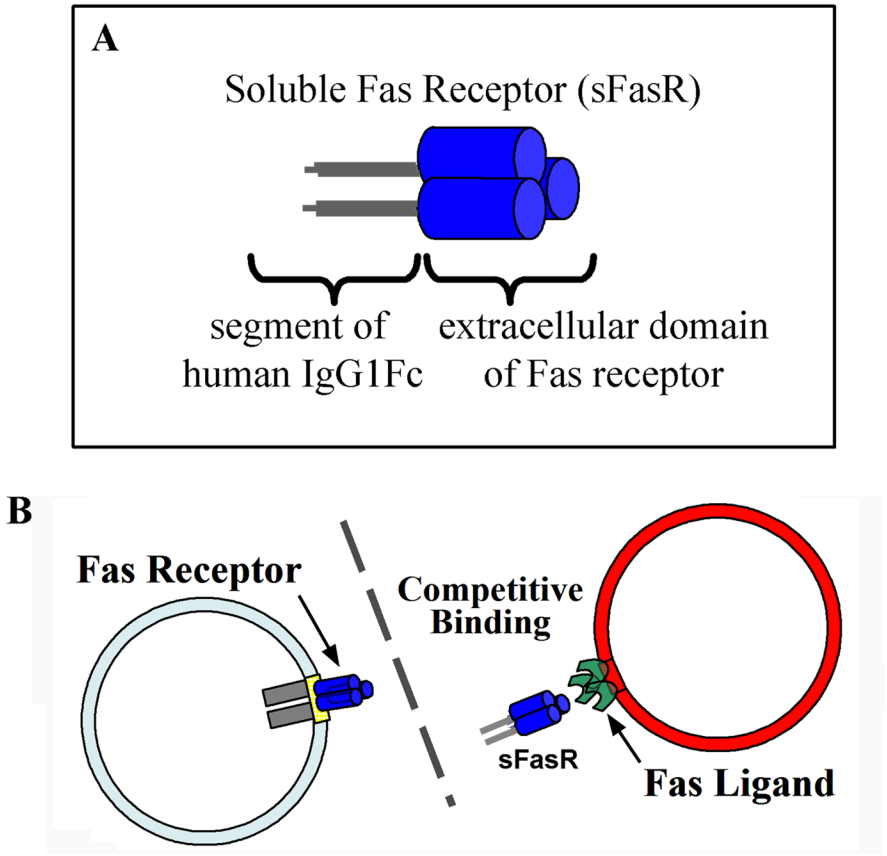


Figure 4: Soluble Fas Receptor (sFasR)

Soluble Fas receptor (sFasR) is a human fusion protein consisting of the extracellular, ligand-binding domain of human Fas receptor, stabilized by a 234 amino acid sequence of human IgG1 Fc (A).
sFasR acts to reduce Fas activation by competitively binding to Fas ligand, thereby reducing the probability of cell-bound Fas from binding to the ligand (B).

beneficial and neuroprotective by a number of research groups, however the clinical relevancy of this work is undefined.

First, the ability to reduce apoptotic cell death post-SCI is crucial, but more important is the ability to target this kind of cell death in a time window that is appropriate clinically. It is estimated that a patient with an acute SCI may not be treated medically by a physician for at least four to eight hours following the initial traumatic injury. This translates into the need for delayed therapeutic action to remain effective in more prolonged time windows.

Secondly, there needs to be a great deal of care given to the prevention and minimization of secondary complications in patients suffering from SCI. Systemic treatments with various anti-apoptotic or immune altering therapeutics run the risk of altering the patient's normal defence and reparative mechanisms, leading to a potentially worse outcome clinically.

The exploration of Fas-mediated apoptosis following SCI is an emerging field, and there is much lacking in the current literature regarding this secondary injury mechanism. The specific cellular incidence of Fas and FasL expression, and the specific target cells susceptible to Fas-mediated cell death following an experimental injury have not yet been directly shown. Furthermore, the exact time sequence of Fas and FasL expression and Fas-mediated apoptosis within the sub-acute setting following injury remain unclear. Prior studies lead to the hypothesis that Fas-mediated cell death is occurring in a significantly delayed time window following traumatic insult, and that the primary cell type being targeted in this delayed post-injury setting is the oligodendrocyte.

Given the above hypotheses, it was also proposed that manipulation of Fas activation following injury could be therapeutically beneficial and could offer neuroprotective effects, specifically to the oligodendrocyte population. It was hypothesized that disruption of Fas and FasL binding by competitive inhibition using a soluble form of the Fas receptor (sFasR) following trauma would be efficacious. Furthermore, if in fact Fas-mediated apoptosis was significantly delayed post-injury, treatment with sFasR could be delayed to offer a clinically valid time window of therapeutic administration.

1.10.1 Hypothesis

Following traumatic SCI, Fas-mediated apoptosis is an important contributor to the development of secondary pathophysiology. It is thus hypothesised that competitive inhibition of Fas and FasL signalling may confer a therapeutic benefit after spinal cord trauma.

1.10.2 Specific Aims of this Work

The goals and specific aims designed to investigate the hypothesis are three-fold and are as follows:

A) To determine the cellular and temporal relationships between Fas and FasL expression, and the specific profile for activation of Fas-mediated cell death following acute traumatic spinal cord injury.

This specific aim is key to resolving the interactions of Fas and FasL-expressing cells in relation to Fas-mediated apoptosis following SCI in order to fully understand this element of secondary injury pathology. Although it has been well-documented that Fas plays a major role in the secondary injury cascade

through its initiation of apoptotic cell death, it is not yet clear what cells play a central role in these events. The specific temporal dependence of Fas-mediated apoptosis on acute injury is unknown, as are the exact cell types affected. By answering these fundamental questions, novel therapeutic targeting will be possible and will ultimately provide a better opportunity for outcomes following SCI.

This specific aim was addressed in the following way:

a) The cellular and temporal expression profiles of Fas and FasL, and various key elements of the Fas-mediated apoptotic pathway were evaluated using immunohistochemical, Western blotting and real-time PCR techniques within the first week post-SCI.

b) Evaluation of the relationship between cellular Fas expression and apoptotic cell death in the acute pathology of SCI was carried out using triple labelling immunohistochemistry and cell quantification techniques.

B) To evaluate the therapeutic efficacy and neuroprotective outcomes through manipulation of Fas and FasL signalling by intrathecal administration of a soluble form of the Fas receptor (sFasR) following experimental SCI.

There is evidence to show that manipulation of the Fas-mediated apoptotic pathway could result in enhanced neurological recovery and cell survival following SCI. Mice expressing a non-functional Fas receptor reveal increased tissue preservation, enhanced cell survival and improvements in motor function as compared to wildtype littermates after experimental SCI (Yoshino, Matsuno et

al. 2004; Casha, Yu et al. 2005). In addition, neutralization of the Fas receptor following dorsal hemi-section injury in mice allowed for increased regenerative potential and promoted neurobehavioural recovery (Demjen, Klussmann et al. 2004).

The majority of the work encompassed by the following specific aims was published in the Journal of Neurotrauma in a co-first-authored paper: Ackery, A., S. Robins, and Fehlings, M.G. (2006). "Inhibition of Fas-mediated apoptosis through administration of soluble Fas receptor improves functional outcome and reduces posttraumatic axonal degeneration after acute spinal cord injury." J Neurotrauma **23**(5): 604-16.

a) The cells undergoing apoptotic cell death during the first week following traumatic SCI were evaluated using TUNEL and double-labelling immunohistochemical techniques.
b) The impact of prolonged intrathecal catheterization on spinal cord tissue were evaluated by uninjured animals given subarachnoid infusion of saline for one week using an intrathecal catheter. Cross-section and longitudinal tissue sections was evaluated for gross abnormalities, compression and damage using histological staining.
c) The anti-apoptotic effects of intrathecal sFasR administration was evaluated using quantification of TUNEL-positive cells at the injury site and in areas both rostral and caudal to the injury epicenter at five days following SCI.

d) The sub-acute cellular and molecular effects of sFasR administration were examined using Western blotting techniques for mature oligodendrocyte and axonal integrity markers.

C) To determine the presence and efficacy of a clinically relevant and therapeutic time window of opportunity for post-SCI intrathecal administration of sFasR.

In the development and evaluation of any potential therapeutic strategy, it is important to focus on the clinical applicability of the proposed treatment. Following demonstration of the strong therapeutic potential for sFasR administration at the time of injury, it was necessary to evaluate the potential for **delayed** administration of the molecule in the same injury model.

a) The ability to delay sFasR administration by eight hours post-SCI was compared with the established cellular benefit of zero hour administration of sFasR using Western blotting for a marker of mature oligodendrocytes.

b) The long-term behavioural impact of delaying sFasR administration by either eight hours or 24 hours post-SCI was carried out using weekly BBB hind-limb locomotor scores, for a period of six weeks following injury.

c) The potential for exacerbation of neuropathic pain responses with delayed sFasR administration post-injury was evaluated using VonFrey filament testing of mechanical allodynia responses bi-weekly for six weeks following SCI.

d) The impact of delayed sFasR administration on chronic cell survival was evaluated using immunohistochemical staining and cell-specific

quantification of tissue sections in rostral cord segments at eight weeks following injury.

e) The extent of tissue preservation achieved by delayed sFasR administration post-SCI was evaluated using quantitative histological analysis of tissue sections at the injury epicenter and in rostral and caudal cord segments at eight weeks post-SCI.

Chapter Two: Methodology

In the following chapter, the general and shared methodologies used to complete the previously detailed specific aims will be described in detail. Where appropriate, reference to more specific method descriptions will be made and these will be described in the subsequent thesis chapters accordingly.

2.1 Spinal Cord Injury Model

Adult female Wistar rats of approximately 280g weight were anaesthetized using 2% halothane, with a 2% oxygen and 1% nitrous mix. Body temperature was maintained throughout the procedure using an underlying thermal pad. After the area was shaved and prepared using iodine and alcohol washes, a dorsal incision was created along the midline at the approximate level of the 5th cervical vertebrae and the 3rd thoracic vertebrae, with careful attention to disrupt the underlying musculature as little as possible.

A three-level laminectomy was performed to remove the dorsal aspects of thoracic vertebrae two (T2) and one (T1), and cervical vertebrae seven (C7), allowing for full exposure of the dorsal spinal cord surface. For all sham-injured animals, this marked the end of the surgical procedure. For all spinal cord injured-animals, an extradural clip with a calibrated closing force of 35g was passed underneath the exposed spinal cord and was closed around the cord at the level of C7-T1 for exactly one minute. Refer to **Figure 5A** for a schematic diagram of the spinal cord clip compression injury. The next steps of the surgical procedure were dependent on the experimental design being carried out. There were three general categories of surgical procedure that an SCI animal was part

of. The post-operative treatment for all animals was generally the same, and will be highlighted in the following section.

The first surgical category of animals was one in which the animals did not receive any treatment surgically. Following a one minute clip compression of the spinal cord, the dorsal musculature was sutured back together and the incision was closed using Michel clips. The post-injury time line for these animals is shown in **Figure 6A**, where the animals were sacrificed at either one day, three days, five days or seven days following experimental SCI.

The second surgical category of animals were subject to the immediate post-SCI implantation of a mini-osmotic pump (Alzet mini-pump #2001) into a lateral skin pocket. This was followed by careful puncture of the dorsal dura using a 25-gauge needle and insertion of a catheter (Alzet rat subarachnoid catheter, special order) into the subarachnoid space where the tip was left to rest at the site of injury. The catheter was sutured to the surrounding musculature several times in order to stabilize it to the vertebral column. **Figure 5B** depicts a schematic of mini-pump and catheter implantation relative to the injury site. Following mini-pump and catheter implantation, this category of animals followed the same post-surgical procedures as those already described, with intramuscular suturing and closing of the dorsal incision using Michel clips. These animals followed the injury time-line depicted in **Figure 6B** where sacrifice was carried out at the fifth post-injury day.

The third and final surgical category of animals consisted of the delayed post-injury implantation of mini-pump and intrathecal catheter. Following the one

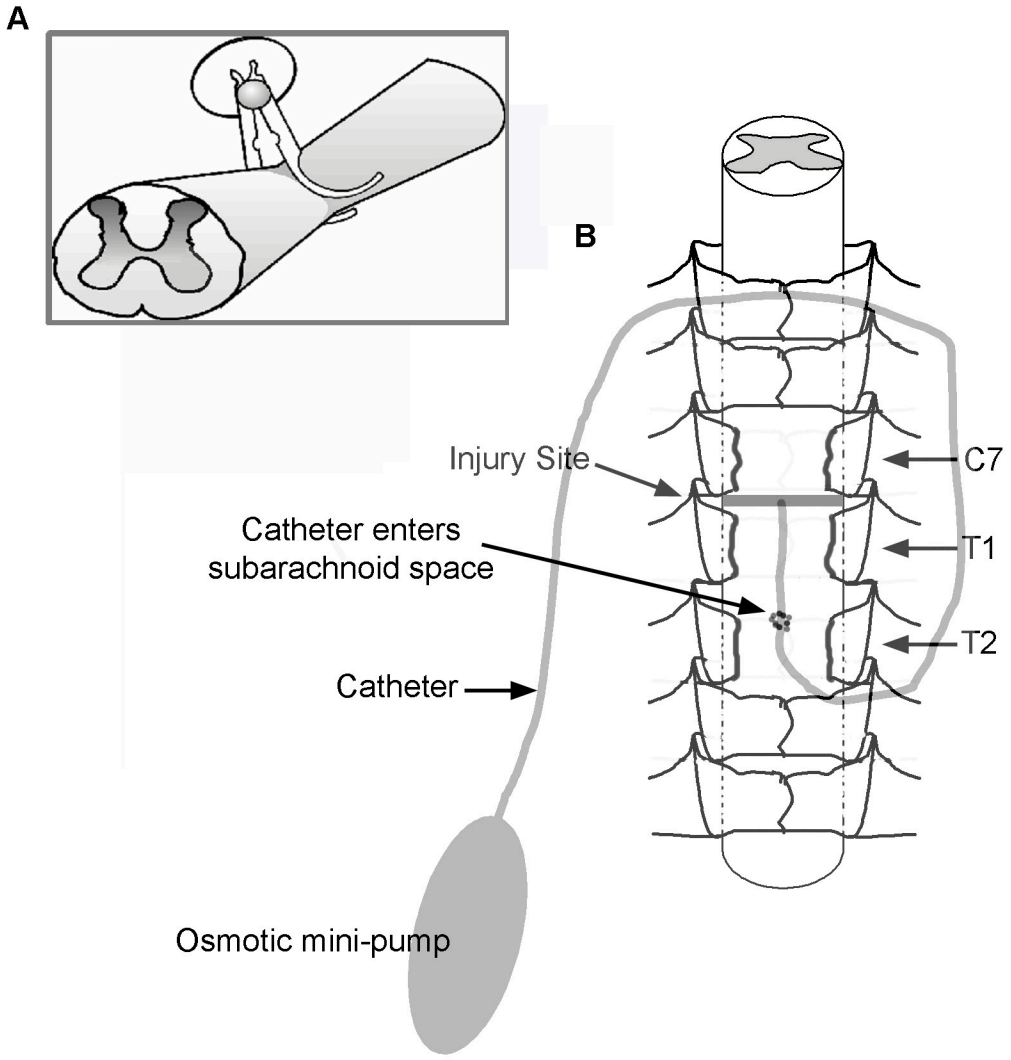


Figure 5: Experimental SCI Model and Therapeutic Design

Experimental compression SCI was induced using a calibrated aneurysm clip of 35g closing force applied around the spinal cord a the level of C7-T1. Schematic representation of this injury is shown in (A). The treatment delivery system adopted for these experiments is shown in (B), where an osmotic mini-pump and intrathecal catheter were employed. The mini-pump was implanted into the animals skin lateral to the injury site and the catheter was secured into place using sutures along the vertebral column. A small puncture in the dura caudal to the inury site allowed for passage of the catheter into the subarachnoid space. The catheter tip was left to rest immediatley dorsal to the site of injury at C7-T1.

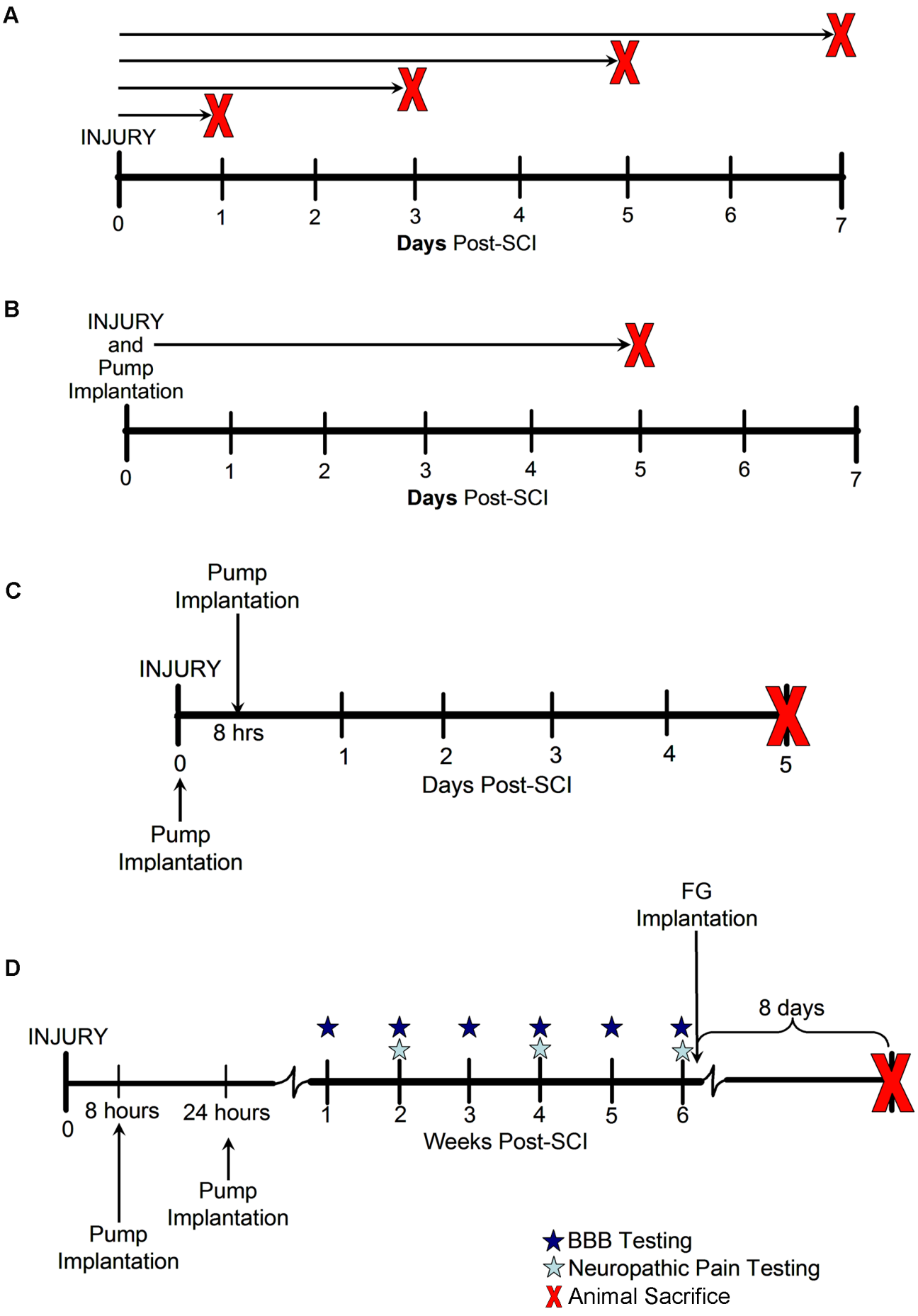


Figure 6: Experimental Timelines for Spinal Cord Injury Methods

Figure 6: Experimental Timelines for Spinal Cord Injury Methods

The clip compression model of spinal cord injury at the C7-T1 level was used throughout the experiments presented in this thesis. The experimental timeline followed was dependent on the particular analysis being carried out.

In evaluating the temporal and cellular expression profiles of apoptotic events post-injury, animals were sacrificed at either one, three, five or seven days post-SCI (A).

In evaluating the efficacy of treatment administration at the time of injury, animals were sacrificed at five days post-SCI for the evaluation of sub-acute outcomes (B).

Validation of a delayed administration time point was carried out by comparing a sub-acute outcome in animals implanted with pumps at the time of injury with animals implanted with pumps at eight hours post-injury. All animals in these experiments were sacrificed at five days following the time of injury (C).

The impact of eight and 24 hour delayed administration was carried out using the timeline depicted in (D). Animals were injured and then either eight or 24 hours post-injury were implanted with pump and catheter. These animals were followed for six weeks with weekly behavioural tests (BBB scoring) and bi-weekly neuropathic pain evaluation (see also **Figure 7A**). Following the conclusion of the neurobehavioural analysis, animals underwent a procedure intended to analyse the treatment effects on axonal sparing through the injury site. This FluoroGold implantation procedure is described in more detail in **Figure 7B**. Animals were left to recover for eight days and were then sacrificed for tissue processing.

minute clip compression of the spinal cord, animals had a single stitch put into the overlying musculature and had the dorsal incision weakly sealed by Michel clips. Animals were given 5cc. of saline and 0.4cc. of bupenorphine analgesic subcutaneously while they were left under heat lamps to recover. At either eight or 24 hours following the spinal cord injury, animals were re-anaesthetized with careful monitoring of breathing rate and a slightly increased percentage of oxygen, at 2.5%. The Michel clips and single suture were carefully removed and the area was gently cleaned using saline-moistened gauze pads. The site of spinal cord injury was re-opened to the extent where visualization of the bruised epicenter of injury was visible. The mini-osmotic pump was tucked into a lateral skin pocket, and the catheter was sutured several times to the surrounding musculature to stabilize it against the vertebral column. As described above, a small hole was made in the dorsal dura and the catheter was inserted into the subarachnoid space to the extent that its tip rested on the dorsal surface of the spinal cord at the injury epicenter. The same intramuscular suturing was carried out on these animals, and the dorsal incision was either closed tightly with Michel clips (for short-term animals) or was sutured tightly with a dissolving suture (for long-term animals). The delayed pump animals followed either one of the two post-injury time lines depicted in **Figure 6C** and **6D**, depending on whether they were short-term (sacrificed at five days post-SCI) or long-term (sacrificed at approximately eight weeks post-SCI) animals.

2.2 Animal Care

For two days prior to and five days following any surgical intervention, all animals received oral doses of the antibiotic Clavamox in their water to prevent infection. Following the surgical procedures detailed previously, animals were given 5cc. of saline subcutaneously along with 0.4cc of bupenorphine analgesic. The animals were left to fully recover from the anaesthetic under heat lamps and once stable, were taken to the animal for long-term care where room temperatures were maintained at 26 degrees Celsius.

Animals receiving the 35g clip compression injury were given an additional 5cc. subcutaneous dose of 5% dextrose in a lactated ringer's solution three times per day until their post-injury weight loss stabilized. In addition, any signs of bladder infection such as hematuria post-operatively was treated with oral doses of Clavamox and subcutaneous doses of 5cc. saline daily until the infection was cleared.

Animals were subject to manual bladder expression three times per day for the duration of the experiments. All animal use, handling and care were carried out in strict accordance with the guidelines set forth and approved by the Animal Care Council and the Toronto Western Research Institutes's Research Ethics Board.

2.3 Mini-Osmotic Pump Preparation and Treatment Dose

The particular Alzet mini-osmotic pump (model #2001) used in these experiments was designed to deliver its contents over a seven day time period, at an approximate rate of 1µl/hr. The pumps were filled with one of four possible

solutions, depending on the experimental plan being carried out. The primary treatment group received pumps containing a human fusion protein of extracellular Fas receptor and Fc-IgG domains for stability (Ancell). The primary and secondary control groups received pumps containing solutions of either the full form of human IgG (Sigma), BSA (Sigma) or plain saline. The proteins (sFasR, IgG and BSA) were diluted in saline at a concentration of 10µg protein per 200µl saline. This translated into a therapeutic dosage provided by the mini-osmotic pumps of ~0.6µg of the specific protein per hour, for up to seven days.

Preparation of the mini-osmotic pumps was done under sterile conditions approximately 12 hours prior to catheter implantation. This was done to ensure adequate priming time, and for the solution to reach the approximate position in the catheter to begin administration immediately upon insertion into the injured spinal cord.

In the experiments where animals were receiving different treatments, the implantation of pump and catheter was carried out in a blinded fashion, and the contents of each pump were not unblinded until after tissue analysis was complete.

2.4 Animal Sacrifice and Tissue Preparation

Animals were sacrificed at the various time points indicated in **Figure 6** in either one of two ways: Extraction or Perfusion. The method of sacrifice and tissue collection was dependent on what the tissue's main purpose was. For all protein and RNA work, the tissue was collected using the Extraction method, while for all histology, immunohistochemistry, cell quantification and tissue

preservation analyses, animals were sacrificed and tissue obtained following Cardiac Perfusion. The details of each of these methods are described in the following two sections.

2.4.1 Tissue Extraction

Animals were anaesthetized using a 2% halothane with 2% and 1% oxygen:nitrous mix, respectively. An incision was created along the dorsal midline to reveal the original laminectomy and/or injury site. Following the localization of the laminectomized region and spinal cord injury site, animals were quickly removed from the anaesthetic machine. Using a guillotine, the animal was quickly decapitated at approximately the 3rd cervical vertebral level and the section of vertebral column containing the injury site was removed and immediately placed into an icy Ringer's solution. Rapid microdissection to separate the spinal cord from the vertebral column and surrounding musculature was carried out under the surgical microscope, and it was ensured that the tissues were constantly bathed in icy Ringer's. Following careful removal of the dural membrane surrounding the spinal cord tissue, a 0.5cm segment of spinal cord, centered on the injury epicenter, was removed and placed immediately into liquid nitrogen to snap-freeze it. After 30 seconds in the liquid nitrogen, the tissue segment was place into a labelled 1.5ml Eppendorf tube and placed into the -80 degree Celsius freezer until all cords in the current study were extracted and snap frozen in this same fashion.

The tubes were removed from the freezer and kept on dry ice to prevent any melting of the tissue segment. A clay mortar and pestle were chilled using

dry ice, and filled with several millilitres of liquid nitrogen. The spinal cord tissue segment was removed from the tube and quickly crushed in a liquid nitrogen slush using the mortar and pestle. The resultant powder was then transferred into a new Eppendorf tube containing either homogenization buffer (for protein analysis) or Trizol (for RNA analysis), vortexed gently and kept on ice, or stored in the -80 degree Celsius freezer for future use.

2.4.2 Cardiac Perfusion

Animals were anaesthetized in an air-tight chamber containing a isoflourane-soaked piece of gauze. After ensuring the animal was completely anaesthetized using a toe-pinch, a quick incision was made in the ventral skin to expose the sternum and zyphoid process. The chest cavity was then opened completely and the left ventricle of the heart was grasped with forceps. Using a a pair of micro-scissors, a small hole was cut into the muscle of the left ventricle and the blunt end of an 18-gauge needle was carefully inserted through the hole and into the left ventricle. The needle was gently pushed through the aortic valve and into the proximal aorta, where it was secured in place using a curved forcep. A tiny incision was created in the right atrium using micro-scissors, and icy-cold PBS (Phosphate Buffered Saline) was slowly pushed from the syringe throughout the animal's vasculature. A suction was present at the right atrium to aid in removal of circulating blood and PBS. Following administration of 60cc. of PBS and after ensuring that all circulating blood had been removed, 120cc. of icy-cold 4% PFA (paraformaldehyde) was slowly pushed through the animals circulatory system using the same needle placement as with the PBS.

A large segment of the spinal cord containing the injury epicenter was carefully dissected away from the vertebral column and post-fixed in a 15ml conical tube containing a 10% sucrose in 4% PFA solution and stored at 4 degrees Celsius. After 1 day, the cord was transferred to a new tube containing a 20% sucrose in PBS solution and stored at 4 degrees for up to 3 days. Following the post-fixing procedure, the spinal cord segment was carefully trimmed to be 0.7cm long, with the length centered on the injury epicenter. The dura was carefully removed at this point as well. The small spinal cord tissue segments were embedded in tissue OTC medium and were stored in the -80 degree Celsius freezer until ready to be cryosectioned.

2.5 Cryosectioning and Injury Epicenter Determination

For tissues collected via cardiac perfusion and prepared for histological and immunohistochemical analysis, the 0.7cm segments of spinal cord tissue were carefully cryosectioned using a cryostat microtome machine. Cross-sections of spinal cord were cut at a thickness of 14µm and longitudinal sections were cut at a thickness of 10µm. The majority of the sections cut during these experiments were cross-sections, and they were serially collected on pre-coated glass slides to minimize tissue loss and maximize the staining potential of any given area of cord. In those experiments where brain stem neurons were quantified (described in detail in the FluoroGold Implantation and Analysis section), transverse sections of the brain were cut at 40µm total thickness.

The location of the injury site was determined for every SCI animal by staining every 3rd serial slide with a hematoxylin and eosin (H and E) and luxol

fast blue (LFB) histological staining procedure. Using a compound light microscope, tissue sections were inspected for the characteristic gross abnormalities, tissue loss, cavitation and demyelination associated with the injury model. By inspecting a series of sections and considering the section thickness and serial distribution on the slides, it was possible to determine the approximate slide numbers that would contain the tissue sections corresponding to the injury epicenter. It was important to be able to accurately select slides for specific staining purposes, for example to carry-out TUNEL-positive cell quantification in sections approximately 500µm rostral to the injury epicenter.

All slides containing spinal cord tissue sections were stored in slide boxes in a -80 degree Celsius freezer until ready to be stained.

2.6 Immunohistochemistry and Histology

Throughout the course of this work, several different adaptations of basic immunohistochemistry protocols were employed to evaluate specific events following SCI. The following section will detail each of these adaptations, and indication will be made as to where there methods were specifically employed throughout the course of this research.

2.6.1 Cell-Specific Labelling of Tissue Sections

Single-label immunohistochemical staining was carried out to allow for the identification and quantification of specific cell-type survival following spinal cord injury. This kind of experiment was conducted to evaluate the oligodendrocyte and neuronal survival in 700µm, rostral cord sections at eight weeks post-injury following sFasR, IgG and control treatments (Chapter 5 and Chapter 6).

Briefly, slides containing tissue sections obtained from specified regions at or surrounding the injury epicenter were brought to room temperature and tissue sections were covered with PBS to prevent dehydration. The PBS was aspirated from the tissue sections using a pipette, and an Immedge Pen was used to create a border around the tissue sections that would allow for minimal usage of antibody during the staining procedure. A blocking solution containing 5% milk in PBS with 1% BSA and 0.3% Triton-X was gently dropped onto the tissue sections and left to incubate at room temperature for one hour to prevent non-specific binding of antibody. Following the one hour incubation, the blocking solution was carefully aspirated from the sections using a pipette and a blocking solution containing the primary antibodies for either CC1/APC or NeuN were applied to the sections according to the specifications listed in **Table 2**. One section per slide was incubated in just blocking solution to act as a control for secondary antibody specificity. Following overnight primary antibody incubation at four degrees Celsius, the sections were washed three times with PBS with careful assurance not to disrupt the section's adherence to the slide. Secondary antibody incubation was carried out using a [1:400] of fluorescent-conjugated secondary anti-mouse antibody (Alexa 488) and blocking solution for one hour at room temperature. Another set of three washes in PBS was carried out and the slides were cover-slipped using DAPI and mowoil for nuclear localization.

2.6.2 TUNEL and Cell-Specific Labelling of Tissue Sections

Apoptotic cell death evaluation and quantification was carried out using TUNEL labelling, a procedure designed to exploit the specific and defined

Table Two: Immunohistochemistry Antibody Details

Primary Antibody Name	Main Molecular Target	Company	Concentration Used
Fas (A20)	Fas receptor	Santa Cruz Biotechnology, Inc.	[1:50]
FasL (N20)	Fas ligand	Santa Cruz Biotechnology, Inc.	[1:50]
CC1 / APC	mature oligodendrocytes	Chemicon	[1:40]
NeuN	neurons	Chemicon	[1:300]
CD11b / OX42	microglia / macrophages	Chemicon	[1:40]
GFAP	astrocytes	Chemicon	[1:200]
Cleaved Caspase-3 Alexa Fluor 488 conjugate	active caspase-3	Cell Signalling	[1:10]

cleavage of DNA found in apoptotic cells (Gavrieli, Sherman et al. 1992). TUNEL-positive cell quantification was carried out to establish the anti-apoptotic effects of sFasR treatment compared to a saline-treated control (Chapter 4). This technique was similar to that of single-label Immunohistochemistry, however the specific procedure followed was taken directly from the instructions provided in the TUNEL kit (from Chemicon).

Double-labelling of tissue sections with TUNEL and a cell-specific marker was carried out to determine the types of cells undergoing apoptotic cell death following clip compression spinal cord injury (Chapter 4). Here, selected tissue sections were prepared as in the TUNEL kit protocol, however the cell-specific staining was carried out prior to initiation of the TUNEL procedure. Cell-specific labelling followed the same procedure as outlined above, until the point of cover-slip application. Briefly, following secondary antibody incubation (with an Alexa 568 fluorescent-conjugated antibody), tissue sections were washed three times in PBS. At this point, the first steps in the TUNEL procedure were carried out according to the manufacturer's guidelines. Finally, the slides were cover-slipped with DAPI as discussed previously.

2.6.3 Triple Labelling of Tissue Sections

One, three, five and seven day post-injury tissue sections were selected and stained with cell-specific monoclonal antibodies CC1/APC, NeuN, OX42 and GFAP, for mature oligodendrocytes, neurons, microglia/macrophages and astrocytes, respectively. Please refer to **Table 2** for additional antibody information. Double and triple labelling with either Fas or FasL antibodies or

triple labelling with Fas or FasL and cleaved caspase-3 antibodies was carried out to evaluate the cellular and temporal relationships of Fas and FasL expression, and to determine the temporal relationship between Fas expression and apoptotic cell death (Chapter 3).

Selected slides were allowed to thaw at room temperature and were blocked with the same protocol as described above. The exception in the previous methods with these slides was the elimination of Triton-X from the primary and secondary antibody blocking solutions. The cell-specific antibodies were applied first, in the concentrations noted in **Table 2**. After an overnight primary antibody incubation at four degrees Celsius, slides were carefully washed with PBS three times. The anti-mouse Alexa 568 secondary antibody was applied in a concentration of [1:400] with blocking solution for one hour at room temperature. Following another three PBS washes, the sections were incubated overnight at four degrees Celsius in the second primary antibody solution, either polyclonal Fas or FasL in a [1:50] for both. Again, antibody details can be found in Table 1. Following incubation, three PBS washes were completed and the anti-rabbit Alexa 647 secondary antibody was applied to the sections in a [1:300] in blocking solution. This secondary antibody incubation was one hour in length, at room temperature. Three more washes in PBS followed and the double-labelled slides were cover-slipped with DAPI as described above. Following the washes, the slides receiving triple labelling had the final primary antibody solution applied. Cleaved caspase-3 fluorescent 488-conjugated antibody was mixed with blocking solution in a [1:10] and left on the

slides overnight at four degrees Celsius. After a final three PBS washes, the triple labelled slides were cover-slipped with DAPI and left to dry.

Normal cord and sham injured tissue sections were used, and antibody control tissue sections were made for each of the antibodies being used, in conjunction with the other antibodies used on the same slides to ensure the absence of secondary antibody non-specific binding and auto- fluorescence.

2.7 Cell Quantification and Tissue Preservation Analysis

Each of Chapter 3, Chapter 4 and Chapter 5 contain various analyses that required specific cell quantification. Chapter 5 also contains surface area analysis of tissue preservation. A LEICA fluorescent and light microscope combined with Stereo Investigator software were employed to image and count full tissue sections of interest. All cell counting and tissue preservation analysis was done in a blinded fashion, to treatment and/or time post-injury. Furthermore, cells were only considered part of the quantification if there was an obvious co-localization of all factors being examined with further co-localization of the nuclear marker DAPI.

In Chapter 3, quantification of either double or triple-labelled tissue sections was carried out in one, three, five and seven day post-SCI tissue sections to determine the post-injury temporal expression profile of Fas receptor on oligodendrocytes and neurons (n=3/timepoint post-SCI). The total number of oligodendrocytes and neurons were counted on each tissue section, and following that, the oligodendrocytes or neurons that expressed Fas receptor were also quantified. By combining the two sets of quantification together, the

percentage of oligodendrocytes and neurons expressing Fas receptor in a time-dependent manner post-injury was determined.

A second arm of this analysis involved the quantification and cellular identification of apoptotic cells at one, three, five and seven days post-injury. The total number of oligodendrocytes and neurons co-expressing cleaved caspase-3 were quantified. Again, the total number of oligodendrocytes and neurons on these sections was used to calculate a percentage of the total number of apoptotic cells (either oligodendrocytes or neurons) observed in a time-dependent manner following injury.

The last stage of this analysis involved quantification of the cells undergoing Fas-mediated apoptosis in a time-dependent manner post-SCI. This was done by quantifying the total number of oligodendrocytes and neurons expressing Fas receptor and expressing cleaved caspase-3. The percentage of cells undergoing Fas-mediated apoptosis was calculated using the total cell-type numbers determined in the first step.

Statistical analysis was carried out to first determine overall differences between oligodendrocyte expression and neuronal expression, and to evaluate the time-dependent differences in the expression profiles observed. The percentages of cell-specific Fas expression, apoptosis and Fas-mediated apoptosis were analyzed using a two-way ANOVA to evaluate cell-type and temporal differences, and post-hoc analysis employed the Holm-Sidak test, a post-hoc similar multiple comparison test.

For the quantification of TUNEL-positive cells as carried out in Chapter 4, sFasR-treated and saline-treated tissue sections were stained according to the procedures described above. Tissue sections from 1500µm, 1000µm and 500µm both rostral and caudal to the injury epicenter were examined. The average number of TUNEL-positive cells per section in each treatment group was calculated (n = 4 per group). Statistical analyses to determine differences in the degree of apoptotic cell death between treatments at each cord location were carried out using Student t-tests.

In Chapter 5, the impact of delayed sFasR and multiple controls (IgG, BSA and saline) following SCI was evaluated on multiple levels. Human IgG was selected as the primary protein control because of the fact that the sFasR molecule being evaluated was a fusion protein consisting of extracellular Fas and a portion of the human IgG Fc domain (refer to **Figure 4A**). BSA was selected as a secondary, non-specific protein control and saline was used to maintain consistency of controls in previous reports (Ackery, Robins et al. 2006).

One set of analyses involved the quantification of cell-specific survival at eight weeks post-injury. Here, tissue sections from ~800µm rostral to the injury epicenter were stained for either oligodendrocytes or neurons, as described above in the *Cell-specific Labelling section*. Quantification of the total number of oligodendrocytes and neurons on each slide was carried out, and after unblinding of treatments was done, an average number of surviving cells was determined for each treatment (n=8/treatment group). Treatments delayed by eight hours post-injury were compared using one-way ANOVA, followed by post-hoc analysis

with the Student Neumann Keul's (SNK) test. Treatments delayed by 24 hours post-injury were also statistically evaluated in this fashion.

A second type of analysis for the delayed treatment study involved determination of the area of various types of spared tissue. Preserved grey matter, preserved white matter, extent of cavity formation and extent of astrogliosis and inflammation were evaluated in H & E / LFB stained tissue sections from 1200µm and 700µm rostral and caudal to the injury epicenter, as well as sections taken directly from the injury EC. Following whole section imaging using the LEICA and StereoInvestigator software, ImageJ software allowed for the quantification of total section area, followed by the area of the specified regions above.

The percentage of total area for spared grey matter and cavity formation was calculated at each of the spinal cord locations, and the average percentages were determined for each treatment group. Two-way ANOVA for treatment and location effects was carried out for the eight hour delayed and 24 hour delayed groups, with post hoc analysis using the Holm Sidak test.

2.8 Western and Slot Blotting

Tissue homogenates obtained following the protocol detailed in the *Tissue extraction* section above were removed from the freezer and thawed on ice. Following several quick rounds of vortexing, the samples were spun down in a centrifuge at four degrees Celsius and 12,000 rpm for 15 minutes. The supernatant was collected and transferred to fresh tubes. A Lowry protein determination assay was performed on each of the samples to give an indication

as to the total protein concentration for each. The values obtained from the Lowry assay were used to determine the amount of sample required to give equal total protein amounts for all samples being run in the Western or Slot blot analysis.

Western and slot blot samples were prepared immediately following the Lowry assay and were then subsequently stored in the fridge or freezer for short-term or long-term storage, respectively. Depending on the protein of interest being examined, the amount of total protein in the samples varied, as did the concentration of the gel used. The specific specifications used for evaluation of different proteins by Western and slot blotting can be found in **Table 3**.

For each set of Western blot experiments, the prepared samples were run on a separating gel and transferred to a nitrocellulose membrane. The membrane was blocked for non-specific binding using a 5% milk blocking solution. For slot blot analysis, the prepared samples were drawn through a membrane within distinct wells using a vacuum-derived suction. For both Western and slot blot membranes, the primary antibodies were placed on the membrane for either one hour or overnight; please refer to Table 2 for specific details for each antibody used. Primary incubation was followed by an HRP-conjugated secondary antibody at [1:2000] in blocking solution for one hour at room temperature. The membrane was developed and exposed on x-ray film using an ECL chemilluminescence kit (PerkinElmer Lifesciences Inc., Boston, MA). Membranes were gently stripped using Re-Blot Plus Mild Solution (Chemicon) for 12 minutes in preparation for being re-probed with primary

Table Three: Western and Slot Blotting Antibody Details

Primary Antibody Name	Protein Target Weight (kDa)	Concentration Used	Total Protein Analysed and % Gel Used	Company
CNPase	47	[1:100]	30 µg and 12%	Chemicon
NF200	210 and 200	[1:1000]	10 µg and 7.5%	Sigma
Fas (A20)	48	[1:100]	50µg and 12%	Santa Cruz Biotechnology, Inc.
FasL (N20)	40	[1:100]	50µg and 12%	Santa Cruz Biotechnology, Inc.
Procaspase-8	50	[1:100]	50µg and 12%	Santa Cruz Biotechnology, Inc.
α-tubulin	48	[1:1000]	variable	Sigma
β-actin	46	[1:200]	variable	Sigma

antibody for β-Actin or α-tubulin to ensure proper and equal loading. In addition following transfer protocols, the gels were stained using Coomasie Blue to reveal any residual protein that had not transferred to the membrane. Examination of the Coomasie-stained gel also allowed for the evaluation of proper running and loading techniques.

Following film developing, Quantity One 4.2.1 software was used to determine the band density of the protein of interest and the actin or tubulin control. Each protein of interest was normalized to the actin or tubulin density in its corresponding lane to correct for any unequal loading; the proper linear range of detection was maintained throughout the analysis.

2.9 Real-Time PCR Analysis

Gene expression analysis was carried out to evaluate the temporal changes in expression of genes related to Fas-mediated apoptosis throughout the progression of SCI secondary pathology (Chapter 3). Cytokine expression profiles and elements of the Fas-mediated apoptotic pathway were assessed and gene expression changes in a time-dependent fashion following injury were correlated. Tissue collected at one, three, five and seven days following injury was prepared and evaluated using quantitative PCR (real-time PCR).

Primers were designed for the genes of interest, and ordered from Integrated DNA Technologies (IDT) and can be found in listed in **Table 4**. Primer sequences were designed using Primer Express software (PE, Applied Biosystems) where amplicons were between 100 and 150 base-pairs long. Primers were selected in sets intended to amplify the sequences as close as

Table Four: Forward and Reverse Primer Sequences

Gene Target	Forward Primer Sequence	Reverse Primer Sequence
Fas receptor	5' ACC ACT GTT ATC ACT GCA CCT CGT 3'	5' AGG ATC AGC AGC CAA AGG AGC TTA 3'
Fas ligand	5' AAG ACC ACA AGG TCC AAC AGG TCA 3'	5' TTC TCT TTG CCT CTG CAT TGC CAC 3'
Procaspase-3	5' AGA GTT GGA GCA CTG TAG CAC ACA 3'	5' TCA TGT CCA CTG AAG GAT GGT 3'
Procaspase-8	5' AAA GCA AGG ACC ACA AGG GCA AAG 3'	5' AGG GCA CTT TGA GCC AGT GAA GTA 3'
GAPDH	5' ATG CCA TCA CTG CCA CTC AGA AGA 3'	5' ACC AGT GGA TGC AGG GAT GAT GTT 3'

possible to the 3' coding region of the target genes. The amplicons chosen were specially located inside an exon to allow the use of genomic DNA to generate standard curves.

Tissue samples in Trizol, prepared as per directions in the *Tissue Extraction and Animal Sacrifice* section, were thawed on ice and then left at room temperature for five minutes. For each sample, the RNA was extracted, purified and quantified using a spectrophotometer. Equivalent amounts of RNA were used to synthesize the cDNA for each sample, yielding a final cDNA concentration of [1µg/20µl]. 1µl of each cDNA sample was used in the real-time PCR methods.

Qualitative PCR was performed on all samples using the ABI Prism 7,900 (PE, Applied Biosystems, Foster City, CA). Each SYBR Green reaction (10ul total volume) contained 4 ul of diluted cDNA (or genomic DNA standard) as the template. The final concentration of reagents was: 1× SYBR Green PCR Master Mix with 0.1 uM of each primer (50 uM stock) from Applied Biosystems. For all the primer sets, reactions were carried out with the standard SYBR Green protocol using either genomic DNA or cDNA as the template.

The reactions were incubated at 50 degrees Celsius for two minutes to activate the uracil N-glycosylase and then for 10 minutes at 95 degrees Celsius to inactivate this enzyme and activate the Amplitaq Gold polymerase. These steps were followed by 40 cycles at 95 degrees Celsius for 15 seconds (for denaturation) and another one minute at 60 degrees Celsius (for primer annealing and extension).

Because SYBR green indiscriminately binds to double-stranded DNA, other products in the PCR such as primer dimers may be detected along with the target gene. To verify that the SYBR green dye detected only one PCR product, the samples were subjected to a heat dissociation protocol after the final cycle of the PCR. Heat dissociation of the amplified DNA detects differences in melting temperature and will produce a single dissociation peak for each specific sequence (Schmittgen and Zakrajsek 2000). The PCR reactions were subjected to heat dissociation protocol present in the PE Biosystems 5,700 software. After the final cycle of the PCR, the reactions were heat denatured over a 35 degrees Celsius temperature gradient at 0.03 degrees per second from 60 to 95 degrees.

2.10 BBB Locomotor Recovery Scoring

In order to evaluate the behavioural recovery effects of delayed sFasR and control treatments following SCI (Chapter 5), animals were assessed weekly for six weeks following injury using an open field locomotor rating test of hind limb function known as the BBB score (Basso Beattie Bresnahan locomotor rating scale) (Basso, Beattie et al. 1996). The BBB scoring system is based on a 21-point, non-linear scale, the details for which are located in **Table 5**. Briefly, animals were observed for three minutes each week and were independently rated by two blinded observers. The average score for each hind limb was recorded and then averaged to provide one score per animal per week. At the end of the six weeks, the average weekly score for each treatment group was determined and the two main effects for treatment time post-injury were analyzed

Table Five: BBB Scoring Descriptions

BBB Score	Locomotor Function Displayed
0	No observable hindlimb (HL) movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or Extensive movement of one joint and slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints and extensive movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support or Plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e., when stationary) or Occasional, frequent, or consistent weight supported dorsal stepping and no plantar stepping
10	Occasional weight supported plantar steps, no forelimb (FL)-HL coordination
11	Frequent to consistent weight supported plantar steps and no FL-HL coordination
12	Frequent to consistent weight supported plantar steps and occasional FL-HL coordination
13	Frequent to consistent weight supported plantar steps and frequent FL-HL coordination
14	Consistent weight supported plantar steps, consistent FL-HL coordination; and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance or Frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent FL-HL coordination; and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement; Predominant paw position is parallel at initial contact and rotated at lift off
17	Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement; Predominant paw position is parallel at initial contact and lift off

18	Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs consistently during forward limb advancement; Predominant paw position is parallel at initial contact and rotated at lift off
19	Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs consistently during forward limb advancement; Predominant paw position is parallel at initial contact and lift off; and tail is down part or all of the time
20	Consistent plantar stepping and consistent coordinated gait; consistent toe clearance; Predominant paw position is parallel at initial contact and lift off; tail consistently up; and trunk instability
21	Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability, tail consistently up

Hind-limb joints observed include the **hip**, **knee** and **ankle** joints.

Slight movement of a joint implies that less than half the full-range of motion is achieved, whereas **Extensive** movement of a joint implies that more than half the full-range of motion of the joint is achieved.

Occasional observations imply that the task is achieved <50% of the time;
Slight observations imply that the task is achieved between 50 and 95% of the time;
Consistent observation implies that the task is achieved for 100% of the time.

using two-way ANOVA, with post-hoc analysis carried out using the Holm-Sidak test.

2.11 Mechanical Allodynia (Neuropathic Pain) Evaluation

It is important to establish whether or not a potential therapeutic strategy is contributing to the development of neuropathic pain mechanisms, if it is not altering the course of neuropathic pain development, or if in fact it is alleviating pain. Mechanical allodynia is a form of neuropathic pain common to SCI patients and involves a formerly non-noxious stimulus becoming noxious. The delayed administration of sFasR and control treatments was evaluated for the effect on neuropathic pain in Chapter 5.

The manifestation and progression of mechanical allodynia following SCI was assessed using von Frey filaments of 2g and 4g weights. At two, four and six weeks following SCI, each animal was acclimated to their surroundings for approximately 20 minutes prior to each test. Starting with the 2g filament, the animal was consistently touched ten times around the level of injury as shown in **Figure 7A**. The number of withdrawal responses out of ten stimulations was recorded, where a withdrawal response consisted of vocalization, adverse and direct movement away from stimulus, flinching, shivering, or scratching. The animal then was stimulated in the same way with the 4g filament. The average number of withdrawal responses out of ten was calculated for each treatment group and the 2g and 4g responses were each run through a one-way ANOVA at each week that pain analysis was carried out. The eight hour delayed treatments

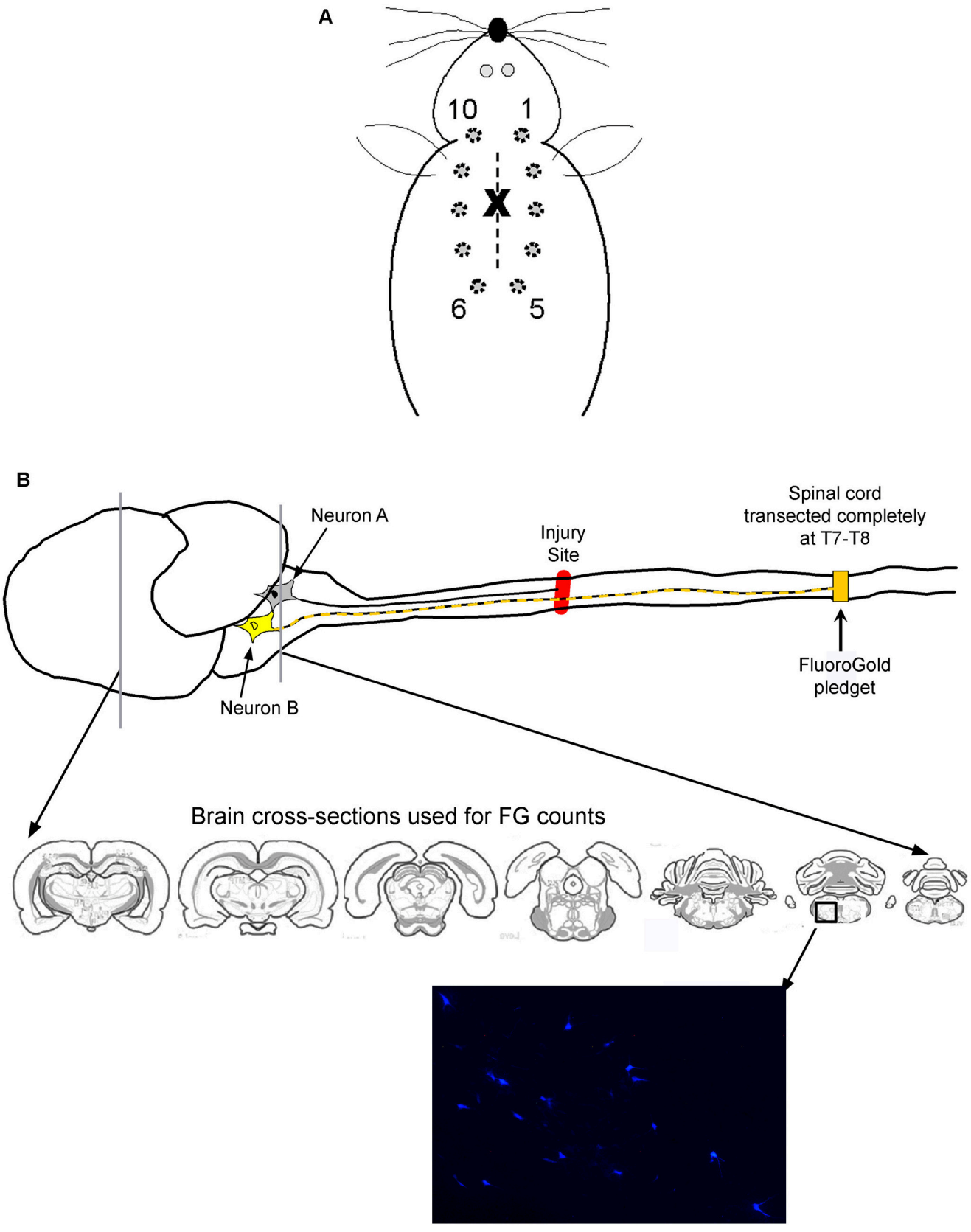


Figure 7: Neuropathic Pain and FluoroGold Methodology

Figure 7: Neuropathic Pain and FluoroGold Methodology

Mechanical allodynia was evaluated bi-weekly using 2g and 4g vonFrey filaments. The number of withdrawal responses elicited from mechanical stimulation around the injury site was recorded. The positioning of the mechanical stimuli around the injury level is illustrated in (A).

FluoroGold labelling of brain stem neurons and mid-brain neurons was performed to identify the approximate number of spared axons through the injury site. (B) depicts the important aspects of this type of analysis, including brain cross-sections and a representative image.

Brain section images were adapted from Neuropsychopharmacology (2005) 30, 1246-1256.

were analyzed separately from the 24 hour delayed treatments, with a sham and saline-treated control included for reference in each set of analysis.

2.12 FluoroGold Implantation and Analysis of Retrogradely Labelled Neurons

The effect of delayed treatment of sFasR, IgG and controls on axonal preservation and maintenance of integrity at and through the injury site was evaluated in Chapter 5 using FluoroGold (FG) implantation and brain nuclei quantification. The post-injury time line is depicted in **Figure 6D**.

Six weeks following SCI and pump implantation, animals were re-anaesthetized using the procedures described previously. An incision was made on the dorsal skin surface, slightly caudal to the original incision. The mid-thoracic vertebral column was exposed, and a two-level laminectomy was performed at the eighth thoracic (T8) and ninth thoracic (T9) vertebrae. The spinal cord was completely transected at this level, and after bleeding was brought under control, a foam pledget soaked in a 2% FG solution (Fluorochrome Inc., Engelwood, Colorado, USA). The FG pledget was inserted snugly against the caudal stump of spinal cord. The thin layer of overlying musculature was sutured together and the incision was closed using Michel clips. Following recovery from the anaesthetic, the animals were left for eight days before being sacrificed according to the cardiac perfusion protocol detailed above.

The brains from these animals were removed and post-fixed according to the same procedure used for the spinal cords. Brains were then embedded in OTC medium and frozen using dry ice. Coronal sections of the fixed brainstem and mid-brain were cut at 40µm thicknesses, and collected on glass microscope

slides, while care was taken to avoid excessive light exposure of the sections. Stereoinvestigator software on a LEICA microcope was used to capture full images of the brain sections, at ~120µm distances and the number of FG-positive cells were quantified within the Reticular, Vestibular, Raphe and Red Nuclei. Average numbers of nuclei for each brain region were calculated for the various treatment groups, and the data were analysed using a one-way ANOVA at each brain region, with SNK post-hoc analysis where appropriate. **Figure 7B** schematically illustrates this procedure.

In Appendix A, further analysis of IgG-treatment involved the establishment of 95% confidence intervals for the percent increase in axonal preservation of IgG-treated animals compared to the control BSA-treated animals.

2.13 Statistical Analysis

All continuous, parametric data were analysed for multiple comparisons using analysis of variance (ANOVA) methods followed by the appropriate post-hoc analysis. Any data failing normality assumptions was considered to be non-parametric are were analysed for multiple comparisons by the appropriate one or two-way ANOVA on ranks, followed by post-hoc analysis. The statistical significance between any pair-wise comparisons were evaluated using the t-test.

The various analyses of protein and gene expression changes in the first week following injury were carried out using one-way ANOVA on expression data of each protein or gene of interest, with post-hoc analysis by the Student Newman Keuls (SNK) test.

Quantification of the percent of either oligodendrocytes or neurons expressing Fas, caspase-3 and Fas + caspase-3 was carried out to evaluate the significance of Fas-mediated apoptosis in the first week post-SCI. These data were statistically analysed using two-way ANOVA to compare cell-specific and time post-injury differences; where appropriate, post-hoc analysis was carried out using the Holm-Sidak method.

The impact of sFasR versus saline treatment on TUNEL-positive cell counts was statistically analysed using Student t-tests at each location along the injured spinal cord. Additionally, the effect of sFasR versus IgG treatment on oligodendrocyte viability and neurofilament preservation was carried out using Western blot and was statistically analysed using Student t-tests.

Quantification of cell survival at 8 weeks post-injury following delayed treatments was carried out and the average number of oligodendrocytes and neurons observed were statistically compared across treatment groups using one-way ANOVA, followed by Student Newman Keuls post-hoc analysis.

Tissue preservation data evaluating the percent spared area at various locations along the cord length were statistically analysed for treatment effects and location effects using 2-way ANOVA and post-hoc analysis was carried out with the Holm-Sidak test.

FluoroGold analysis of select brain nuclei was used to evaluate the therapeutic impact of delayed IgG administration. One-way ANOVA was performed at each brain region to compare treatments and the Student Newman Keuls test was used for post-hoc analysis where appropriate.

Behavioural recovery throughout a six week period was evaluated using a weekly BBB score. The treatments and time post-injury were compared statistically using a two-way ANOVA and any necessary post-hoc analysis was carried out using the Holm-Sidak method.

The extent of neuropathic pain generated following treatment was evaluated bi-weekly, and the results were analysed at each time point to compare the treatment groups. A one-way ANOVA was performed, and only at the 2 week time point did normality fail. In this case, the Kruskal Wallis ANOVA on ranks was used. No post-hoc analysis was necessary for these data.

Chapter 3:

Elucidating and Understanding the Role of Fas-Mediated Apoptosis Following Acute Traumatic Spinal Cord Injury

Please note that the work presented in this chapter is currently being prepared as a manuscript for submission to the Journal of Neuropathology and Experimental Neuropathology and will constitute a first-authored paper for Sherri Robins.

3.1 Introduction

A review of the current literature related to Fas-mediated apoptosis following SCI reveals several gaps in knowledge. While it is known that both Fas and FasL show increased expression after trauma, the exact cellular expression and time-dependent changes in this expression are not fully understood (Li, Farooque et al. 2000; Casha, Yu et al. 2001; Zurita, Vaquero et al. 2001; Casha, Yu et al. 2005). In addition, there is only correlative data to suggest that Fas-mediated apoptosis does in fact target a specific cell population, and that the initiation of this form of cell death is delayed post-injury. It has been shown that Fas receptor expression is somewhat delayed following injury, however the exact temporal relationship is not clear (Casha, Yu et al. 2001; Zurita, Vaquero et al. 2001). Moreover, the apoptotic impact of delayed Fas expression on key cell populations like oligodendrocytes and neurons following traumatic SCI is not known. By gaining a better understanding of the key players involved in Fas-mediated apoptotic signalling after SCI, adequate treatment options will be one step closer.

3.2 Results

The work presented here gives a clear demonstration of the cellular and temporal profiles of Fas and FasL expression following SCI, the time dependent relationships of key players in the Fas pathway following injury, and confirms for the first time the specific cellular targets of Fas-mediated cell death in the injured spinal cord.

3.2.1 Fas receptor expression is increased post-SCI and follows a delayed temporal profile

Following a 35g clip compression SCI, the animals were sacrificed at one, three, five and seven days and the spinal cord tissue prepared for mRNA analysis using real-time PCR or for protein analysis using slot blotting. Another group of SCI animals had their spinal cords harvested at the same post-injury time points for immunohistochemical analysis of Fas receptor expression levels. A sham-injured group of animals was also used for evaluating pre-injury gene and protein expression levels of Fas receptor, providing a base-line for establishing injury-induced changes in expression.

In the first week following SCI, there was a significant increase in both message and protein expression as compared to sham-injured levels. Specifically, Fas gene expression was delayed until the third day following injury, at which point the largest increase in mRNA was observed. The increase in Fas mRNA expression was maintained in the fifth post-injury day, and by the end of the first week, levels had dropped slightly towards sham-injured expression (**Figure 8A**). In the first day post-injury, Fas protein expression was increased as compared to sham-injured controls. Fas receptor protein expression increased

consistently over three and five days where it reached its peak expression. By the end of the first week post-injury, consistent with the mRNA results, Fas protein expression had decreased to levels which were not significantly different from sham controls (**Figure 8B**).

Immunohistochemical analysis further confirmed the slot blot results. Sham-injured tissues revealed very little Fas expression (**Figure 8 C-E**), and following SCI significant increases were observed, specifically at five days post-injury (**Figure 8 F-H**).

These results confirm that Fas expression in spinal cord tissue is upregulated at the gene and protein level as a result of traumatic SCI. Importantly, Fas receptor expression is delayed following SCI and could therefore be a potential, clinically relevant therapeutic target.

3.2.2 Delayed post-SCI Fas expression is predominantly found on the oligodendrocyte population

The cellular localization of Fas receptor expression was examined using double-labelled tissue sections from animals at one, three, five and seven days post-SCI. An analysis of oligodendrocyte, neuron, microglia and astrocyte expression of Fas receptor in sham-injured tissues provided consistent findings as was shown above. There was little to no Fas expression found on any cell types in sham-injured animals. The increased Fas receptor expression noted following injury was interestingly limited to the oligodendrocyte population (**Figure 9A**).

Following quantification of Fas expression on oligodendrocytes and neurons, it was observed that the greatest increase in oligodendrocyte Fas

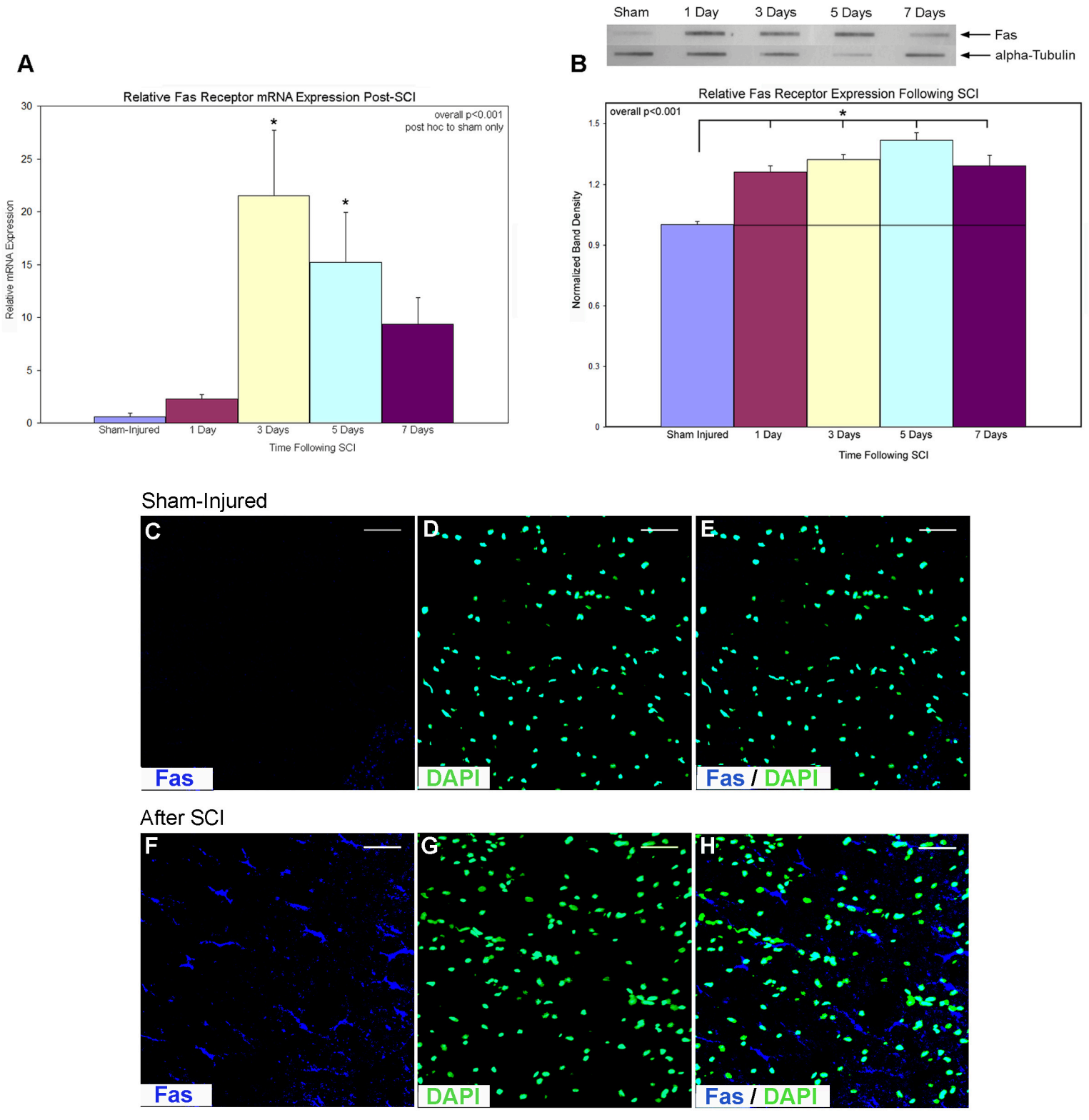


Figure 8: Fas Receptor Expression is Increased Following AcuteTraumatic SCI

Figure 8: Fas receptor expression is increased following acute traumatic SCI

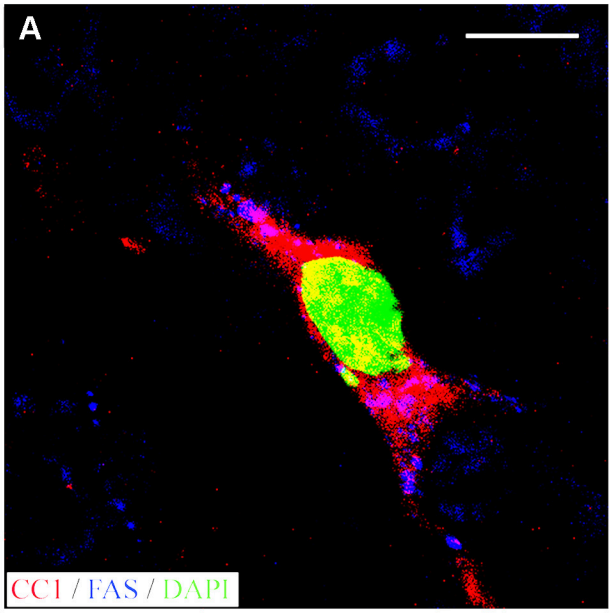
(A) Fas receptor mRNA expression was evaluated using real-time PCR in sham-injured and SCI rats at one, three, five and seven days post-injury. Messenger RNA expression levels are shown relative to sham-injured levels following normalization with GAPDH mRNA expression (error bars = SEM). Data were subjected to a variance stabilizing (square root) transformation. Kruskall-Wallis one-way ANOVA was performed and overall significance was achieved at a p-value of <0.001. Additionally, Dunn's post-hoc analysis revealed significant difference in expression levels from sham-injured at three and five days post-SCI, with p-values of <0.05 for each. The sham-injured control group had an $n = 6$ and the one, three, five and seven day post-SCI groups had an $n = 8$ in each. The seven day post-SCI group had an $n = 7$ due to the elimination of a data outlier given the criteria of ± 2 standard deviations from the mean.

(B) Fas receptor protein expression was evaluated using Slot Blot analysis in sham-injured and SCI rats at one, three, five and seven days post-injury. Expression levels are shown relative to sham-injured levels following individual normalization with α-tubulin expression. Fas receptor expression was significantly increased from sham-injured animals at one, three, five and seven days post-injury, where there was a peak expression observed at five days. Data were subjected to a one-way ANOVA and an overall significance of $p<0.001$ was achieved. Post-hoc analysis was carried out using the Student Neuman-Keuls analysis and revealed the significant difference between sham-injured and SCI animals at all time points with a $p<0.05$ for all. The sham-injured group had an $n = 4$ and the one, three, and five day groups had an $n = 6$ in each. The seven day SCI group had an $n = 5$.

Immunohistochemical analysis of Fas receptor expression was carried out in tissue sections taken approximately 800μm rostral to the injury epicenter (for sham-injured animals, tissue sections were taken from the C7-T1 vertebral level). Fas expression was very low in sham-injured tissues, shown in images C, D and E, with the scale bar = 50μm. At five days following SCI, Fas receptor expression was significantly elevated (F, G, H) compared to sham-injured

(Figure 8 Continued)

controls (scale bar = 50μm). This increase corresponded to the increased expression at five days observed using Slot Blot analysis above.



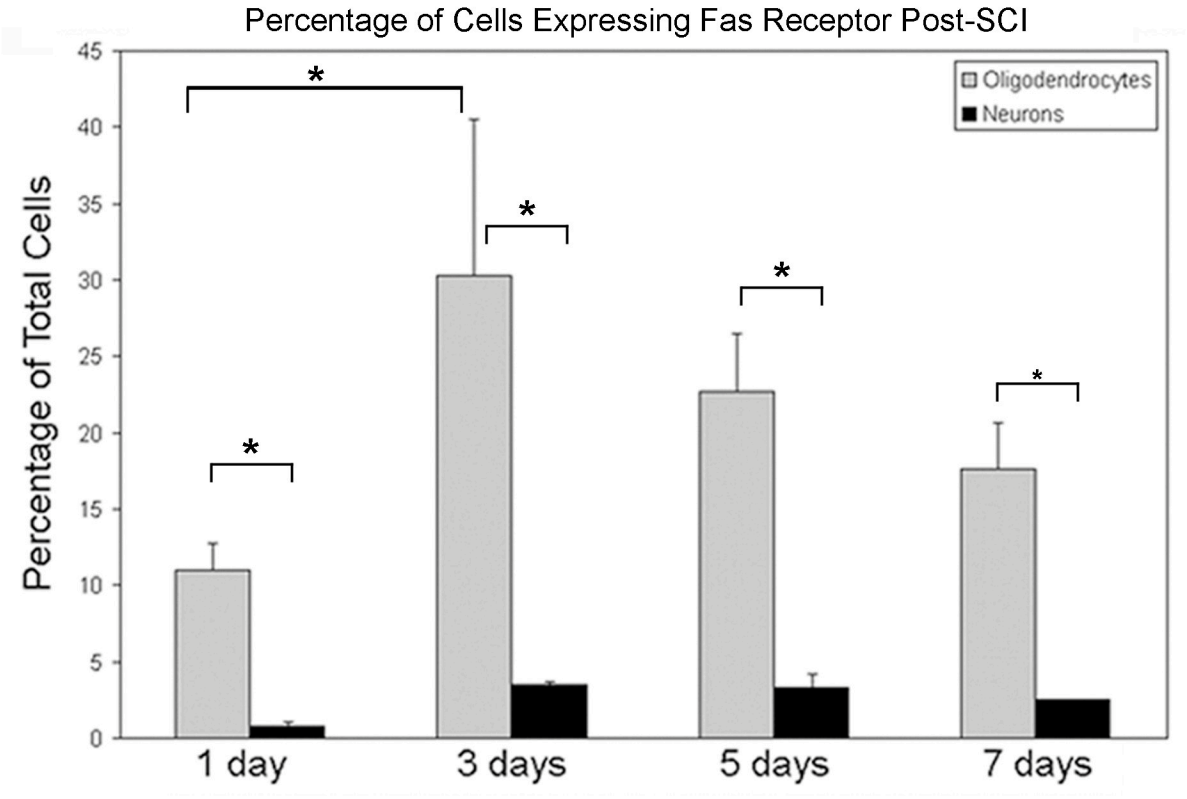


Figure 9: Oligodendrocytes predominantly express Fas receptor post-SCI

Figure 9: Oligodendrocytes predominantly express Fas receptor post-SCI

Immunohistochemical analysis of cell-specific Fas receptor expression was carried out in tissue sections approximately 650μm rostral to the injury epicenter at one, three, five and seven days post-SCI. Oligodendrocytes were labelled with CC1/APC and neurons were labelled with NeuN, with nuclear identification using DAPI.

The cellular and temporal profile of increased Fas receptor expression post-SCI showed a three to five day peak in Fas expression on the oligodendrocyte population. Image **A** shows a representative oligodendrocyte expressing Fas receptor at five days post-SCI. The tissue section was taken approximately 800μm rostral to the injury epicenter and shows co-localization of CC1/APC, Fas receptor and the nuclear marker DAPI, with the scale bar = 8μm.

The percentage of oligodendrocytes and neurons expressing Fas receptor was determined for each post-injury time point (**B**, error bars = SEM). A significant difference between the cell types was observed at three, five and seven days post-injury, where the percentage of oligodendrocytes expressing Fas receptor was higher than that of the neuronal population. Within the oligodendrocyte population, the percent of cells expression Fas at three days was significantly higher than that at one day post-SCI. There were no significant temporal differences in Fas expression within the neuronal population.

Statistical analysis of these data was carried out using a two-way ANOVA (for cell-type and time post-SCI), revealing a p-value of <0.001 for cell-type differences and a p-value of 0.092 for time post-SCI. Post-hoc analysis using the Holm-Sidak test (a variation of the Bonferroni correction) revealed statistical differences between oligodendrocytes and neurons at three, five and seven days post-SCI, with $p<0.05$ for each. Furthermore, a temporal difference within the oligodendrocyte population was determined between one and three days post-SCI, $p=0.004$. For this quantification analysis, there was an $n = 3$ tissue sections per time-point and per cell-type.

expression occurred between three and five days post-injury, remaining consistent with the findings of generalized Fas expression. Oligodendrocytes had a significantly higher percent of Fas expression than did the neurons at all post-injury time points examined. Furthermore, the greatest percent of Fas-positive oligodendrocytes was observed on days three and five, which shows consistency again with the overall temporal profile of Fas expression after SCI (**Figure 9B**).

3.2.3 Oligodendrocytes are highly susceptible to delayed Fas-mediated apoptosis following traumatic SCI

The above data do not reveal whether the increased expression of Fas receptor on the oligodendrocyte population is causing Fas-mediated apoptotic cell death of oligodendrocytes. It has been well-accepted in the literature that concurrent expression of both Fas receptor and apoptotic markers implies that a particular cell is undergoing apoptosis as a result of Fas activation (Casha, Yu et al. 2001; Yoshino, Matsuno et al. 2004; Dasari, Spomar et al. 2008). To establish the apoptotic actions of Fas expression on the oligodendrocyte population following SCI, tissue sections labelled for Fas, cleaved caspase-3 and either an oligodendrocyte or neuronal marker were evaluated. Quantification of these tissues sections at one, three, five and seven days following injury provided a more complete understanding of the temporal occurrence of Fas-mediated cell death following SCI.

First, the percentage of total neurons or oligodendrocytes that were undergoing apoptotic cell death was quantified using cleaved caspase-3 expression. These data further confirm what has been shown in the literature

regarding both neuronal and oligodendrocyte susceptibility following SCI. In addition, the temporal differences in apoptotic cell death between the two cell types was clear (**Figure 10A**). Neurons are more susceptible to apoptotic cell death at very early time points post-injury, as reflected by a greater percentage of neurons than oligodendrocytes expressing active caspase-3 at day one. However, oligodendrocytes were most affected by apoptotis at days three and five.

Next, the type of apoptosis in neurons and oligodendrocytes following SCI was evaluated. At five days post-injury, there were numerous oligodendrocytes showing concurrent expression of Fas and cleaved caspase-3, indicating that those cells were undergoing Fas-mediated apoptosis (**Figure 10B**). At one day post-injury, neurons were observed expressing cleaved caspase-3 in the absence of Fas (**Figure 10C**). This suggests that neurons are undergoing Fas-independent mechanisms of apoptosis in the first day following SCI. The idea that neurons are highly susceptible to mitochondrial-specific apoptosis has already been discussed, and the evidence shown here further confirms that concept.

The impact of Fas-mediated apoptosis on the neuron and oligodendrocyte population during the first week post-injury was evaluated. For each cell-type, the total number of apoptotic cells (shown as a percent of the total cell population in **Figure 10A**) was used to determine the percent of apoptotic cells that were concurrently expressing Fas receptor, and therefore were undergoing Fas-mediated apoptosis specifically. Apoptotic cell death in the neuron population is

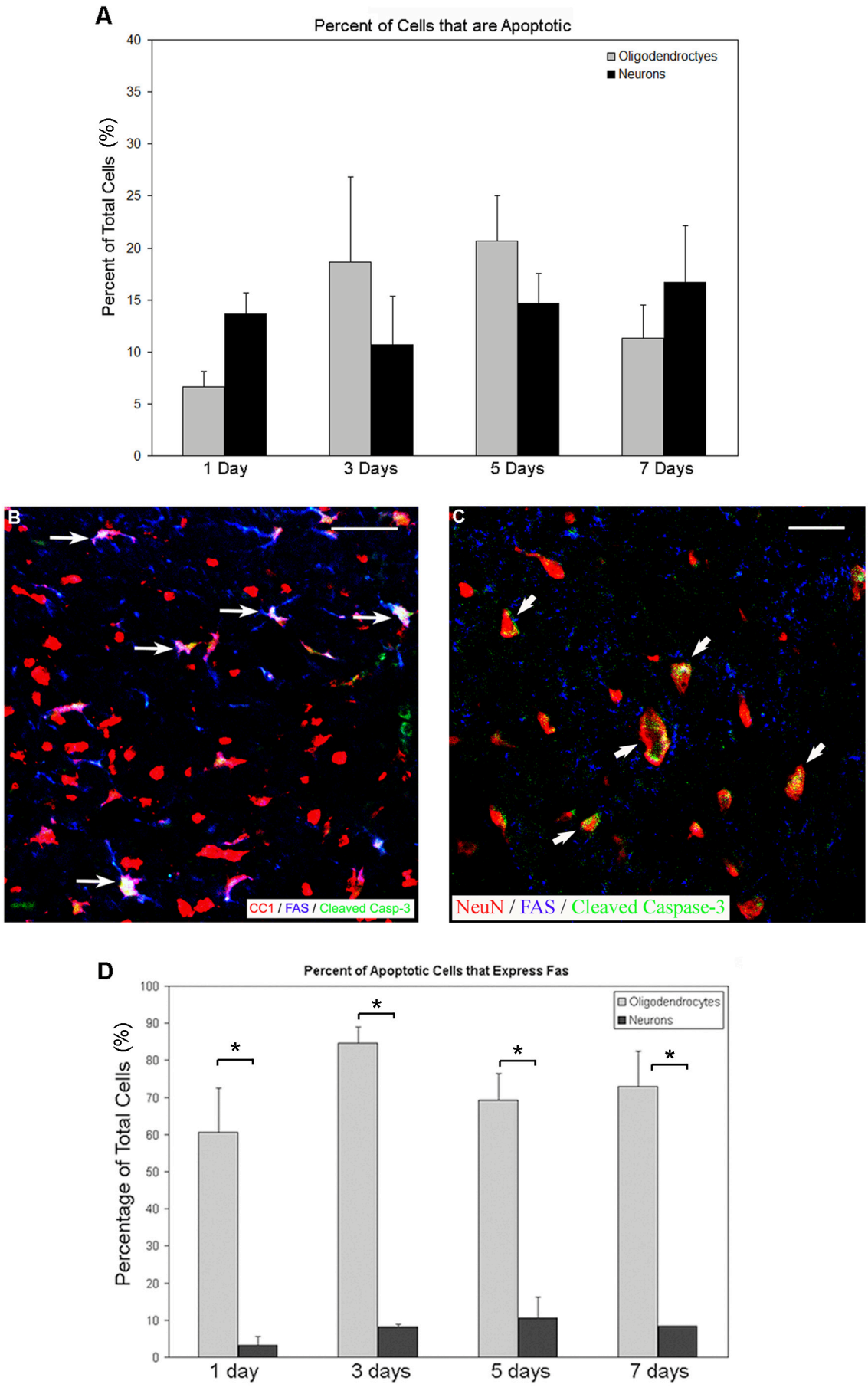


Figure 10: Oligodendrocytes are most susceptible to delayed Fas-mediated apoptosis post-SCI

Figure 10: Oligodendrocytes are most susceptible to delayed Fas-mediated apoptosis post-SCI

Immunohistochemical analysis of cell-specific apoptosis was carried out in tissue sections taken approximately 650μm rostral to the injury epicenter at one, three, five and seven days post-SCI. Oligodendrocytes were labelled with CC1/APC and neurons were labelled with NeuN, with nuclear identification using DAPI. Apoptotic cells were identified by the expression of cleaved caspase-3. Quantification of the total number of oligodendrocytes and neurons per tissue section was carried out, followed by quantification of apoptotic oligodendrocytes and apoptotic neurons. The percentage of oligodendrocytes and neurons that were found to be undergoing apoptosis was determined for each post-injury time point and are displayed graphically in A (error bars = SEM).

Statistical analysis was carried out using a two-way ANOVA for cell-type and time post-SCI, where there were no significant differences found between the cell-types and days post-SCI with $p = 0.266$ and $p = 0.446$, respectively.

Tissue sections taken approximately 800μm rostral to the injury epicenter were labelled with cell-specific markers (CC1/APC for oligodendrocytes and NeuN for neurons), Fas receptor and cleaved caspase-3. At five days post-injury, there were a number of oligodendrocytes showing both Fas and cleaved caspase-3 expression (shown by the arrows), indicating that these cells were likely undergoing Fas-mediated apoptosis (B). At one day post-injury neuronal expression of cleaved caspase-3 is present in the absence of Fas receptor expression (shown by the arrows), indicating apoptotic cell death is occurring in a Fas-independent manner (C). The scale bar for both B and C is equal to 50μm.

Immunohistochemical analysis of cell-specific Fas-mediated apoptosis was carried out in tissue sections taken approximately 650μm rostral to the injury epicenter at one, three, five and seven days post-SCI. Oligodendrocytes were labelled with CC1/APC and neurons were labelled with NeuN, with nuclear identification using DAPI. Cells undergoing Fas-mediated apoptosis were identified by the co-expression of Fas receptor and cleaved caspase-3. Quantification of the total number of oligodendrocytes and neurons per tissue

(Figure 10 Continued)

section was carried out, followed by quantification of Fas-mediated apoptotic oligodendrocytes and neurons. The percentage of oligodendrocytes and neurons that were observed undergoing Fas-mediated apoptosis was determined for each post-injury time point and are displayed graphically in D.

The oligodendrocyte population had significantly higher percentages of Fas-mediated apoptosis at all time points post-SCI, giving merit to the concept that this form of cell death is targeting the oligodendrocyte population over the neuron population. For example, at three days post-SCI, approximately 85% of apoptotic oligodendrocytes were experiencing Fas-mediated signalling whereas <10% of apoptotic neurons were experiencing Fas-dependent apoptosis.

These data were analyzed for statistical significance using a two-way ANOVA which revealed a p-value for cell-type differences of $p<0.001$, and a p-value for time post-SCI differences of $p = 0.234$. Post-hoc analysis of cell-type differences using the Holm-Sidak test gave p-values of $p<0.05$ for each time point post-SCI.

very unlikely occurring as a result of Fas activation, because less than 10% of the apoptotic neurons were found to express Fas. Contrasting this with the oligodendrocyte population, it becomes clear that Fas-mediated activation of apoptosis is playing a significant role in apoptosis of oligodendrocytes. Between 60% and 90% of apoptotic oligodendrocytes were also expressing the Fas receptor throughout the entire week post-injury (**Figure 10D**). The observations here act to further support the notion that Fas-mediated apoptotic cell death is likely a critical player in the delayed post-injury cell death of the oligodendrocyte population.

3.2.4 Fas ligand expression is slightly increased following SCI and is not limited to a specific glial cell type

Cellular expression of Fas can be considered more important than that of Fas ligand given the fact that a cell is not vulnerable to Fas-mediated apoptosis until it expresses the receptor. This is true regardless of the presence of Fas ligand in the system. However, the cellular source of Fas ligand activating Fas-mediated signalling post-SCI is of importance. Targeting of a particular cell type found to express FasL might very well have a valuable therapeutic role in a combinatorial approach to reducing apoptosis following trauma.

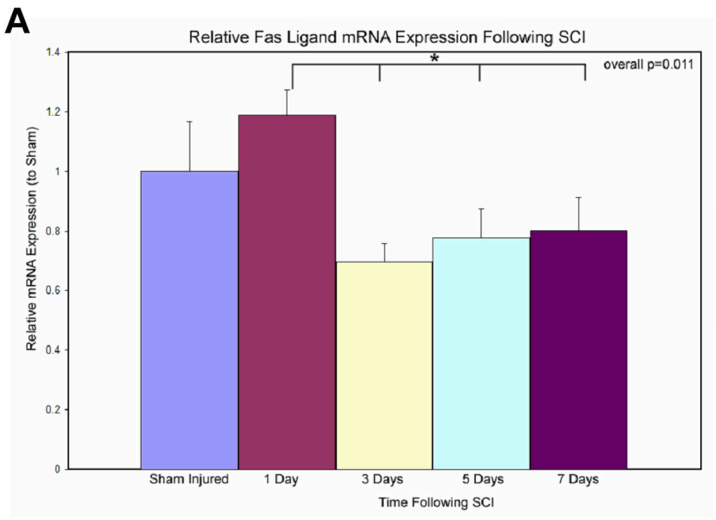
Following a 35g clip compression SCI, animals were sacrificed at one, three, five and seven days and the spinal cord tissue was prepared for mRNA analysis using real-time PCR and for protein analysis using slot blotting. As in the Fas receptor analysis, a second cohort of SCI animals had their spinal cords harvested at the same post-injury time points for immunohistochemical analysis of FasL expression. A sham-injured group of animals was used for evaluating

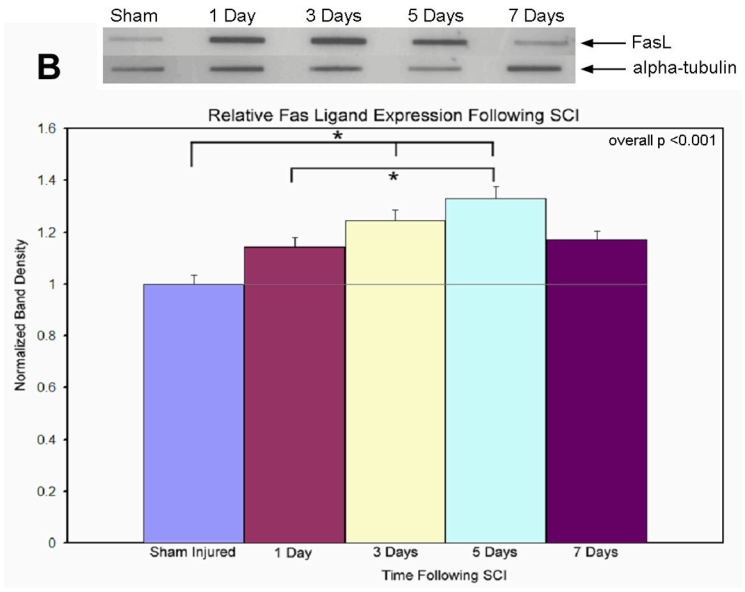
pre-injury gene and protein expression levels of FasL to establish a base-line of expression.

Injury-induced changes in expression of the FasL gene were evaluated using real-time PCR to determine relative mRNA expression as compared to sham-injured controls throughout the first week following injury. Fas ligand mRNA was increased as compared to controls by one day following SCI, at which point the expression levels dropped significantly below that found in sham-injured controls. Low levels of FasL mRNA expression were observed for the remainder of the first week post-injury (**Figure 11A**).

Changes in Fas ligand protein expression were evaluated using slot blotting and reveal a slightly different profile than what would have been predicted by the mRNA analysis. Levels of FasL were slightly increased from sham control following SCI, however this increase did not become significant until post-injury days three and five. Fas ligand expression returned to almost sham-injured levels by seven days post-SCI, and this is fitting with gene expression changes noted in days three and five (**Figure 11B**).

The cellular profile of FasL expression in sham-injured and in one, three five and seven day post-SCI animals was examined using immunohistochemical staining. It was noted that FasL expression is present in both sham-injured and SCI tissue sections, and there is a slightly greater staining intensity following SCI. It was not possible to determine a significant enough change in the ligand expression following injury to warrant further examination. However, with the greatest increase in FasL protein expression being found at 5 days post-SCI, the





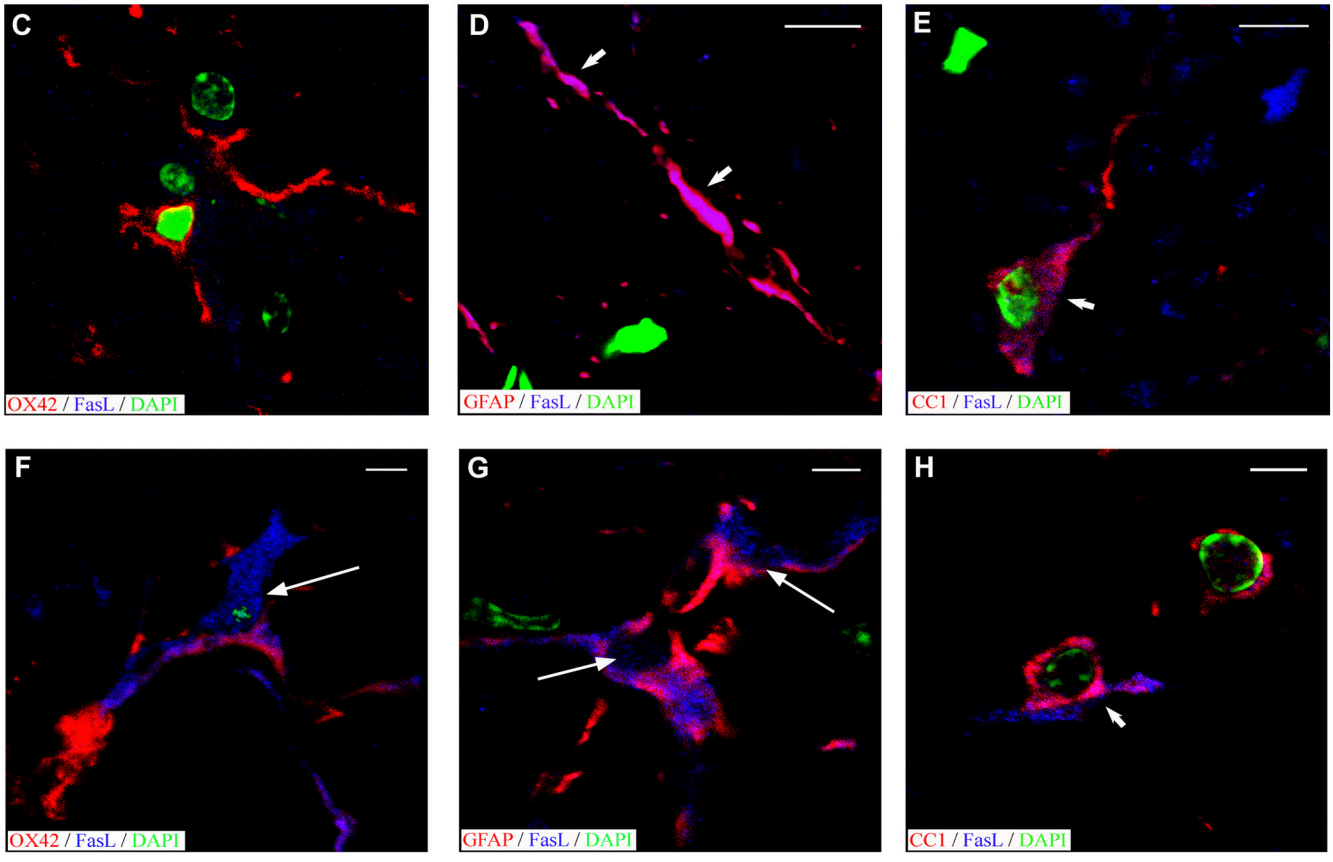


Figure 11: Fas ligand expression increases following SCI

Figure 11: Fas ligand expression increases following SCI

(A) Fas ligand mRNA expression was evaluated using real-time PCR in sham-injured and SCI rats at one, three, five and seven days post-injury. Messenger RNA expression levels are shown relative to sham-injured levels following normalization with GAPDH mRNA expression (error bars = SEM). Fas ligand expression is increased at one day post-SCI, but returns to levels similar to sham-injured animals at three, five and seven days post-SCI. Statistical analysis was carried out using a one-way ANOVA where the overall p-value was $p=0.001$. Post-hoc analysis using the Student Neuman-Keuls method found statistically significant differences in Fas ligand expression at one day post-injury, as compared to expression at three, five and seven days. The sham-injured control group had an $n = 6$ and the one, three, five and seven day post-SCI groups had an $n = 8$ in each. The seven day post-SCI group had an $n = 7$ due to the elimination of a data outlier given the criteria of ± 2 standard deviations from the mean.

(B) Fas ligand protein expression was evaluated using Slot Blot analysis in sham-injured and SCI rats at one, three, five and seven days post-injury. Expression levels are shown relative to sham-injured levels following individual normalization with α-tubulin expression (error bars = SEM). Fas ligand expression is slightly increased at three and five days post-SCI as compared to sham-injured controls. At seven days post-SCI expression levels returned to those found at one day post-SCI and in the sham-injured control. Data were subjected to a one-way ANOVA and an overall significance of $p<0.001$ was achieved. Post-hoc analysis was carried out using the Student Neuman-Keuls analysis and revealed the significant differences between sham-injured and SCI animals at three and five days post-injury with a $p<0.05$ for each. Furthermore, it was established that the Fas ligand expression level at five days post-injury was significantly higher than that at one day. The sham-injured group had an $n = 4$ and the one, three, and five day groups had an $n = 6$ in each. The seven day SCI group had an $n = 5$.

(Figure 11 Continued)

Immunohistochemical analysis of tissue sections taken from sham-injured animals and from approximately 800μm rostral to the injury epicenter in five day SCI animals was carried out to evaluate the cellular expression profile of Fas ligand. Tissue sections were stained with glial-specific markers (CC1/APC for oligodendrocytes, GFAP for astrocytes and OX42/CD11b for microglia and macrophages), Fas ligand and the nuclear marker DAPI. Images C, D, and E show sham-injured tissues expressing Fas ligand on all three glial cell types, except for the ramified microglia where very little Fas ligand expression was observed. Images F, G and H show tissue sections taken from animals at five days post-SCI and reveal a slightly more intense Fas ligand expression on all three glial cell types examined. The scale bars in all images represent 8μm.

immunohistochemical images corresponding to this time point provide the most realistic idea of which cell types are expressing FasL and therefore activate Fas. Interestingly, Fas ligand expression was not limited to any particular type of glial cell in either sham or injured tissue sections. Microglia, astrocytes and oligodendrocytes all expressed FasL, and the intensity of staining observed in the injured tissue sections was greater than in the sham-injured sections (**Figure 11 C-H**).

3.2.5 Activation of Fas by FasL-expressing astrocytes – a potential mechanism

Literature suggests that following SCI, the glial – axonal relationship becomes unhealthy, and perpetuates the axonal dysfunction, degradation and demyelination that is a well-accepted characteristic of SCI pathology (Fehlings and Tator 1995; Nashmi and Fehlings 2001). The contributing factors to this unhealthy interaction are dynamic and complex, however it is possible that Fas-mediated apoptosis plays a role in exacerbating this. Confocal microscopy was used to scan through the z-plane of tissue sections at five days post-injury, the peak of Fas-mediated apoptosis in the oligodendrocyte population. An interesting observation was made when examining z-stack images through a the white matter of a tissue section stained for GFAP (an astrocyte marker) and Fas receptor. It was noted that at the top of the stack of images, it appeared that an astrocytic process was co-labelled with Fas receptor (**Figure 12A**). However, upon further examination of the subsequent tissue sections in the stack, it became clear that the astrocyte process was most likely not itself expressing Fas, but instead was lying in close proximity to a different Fas-expressing cell,

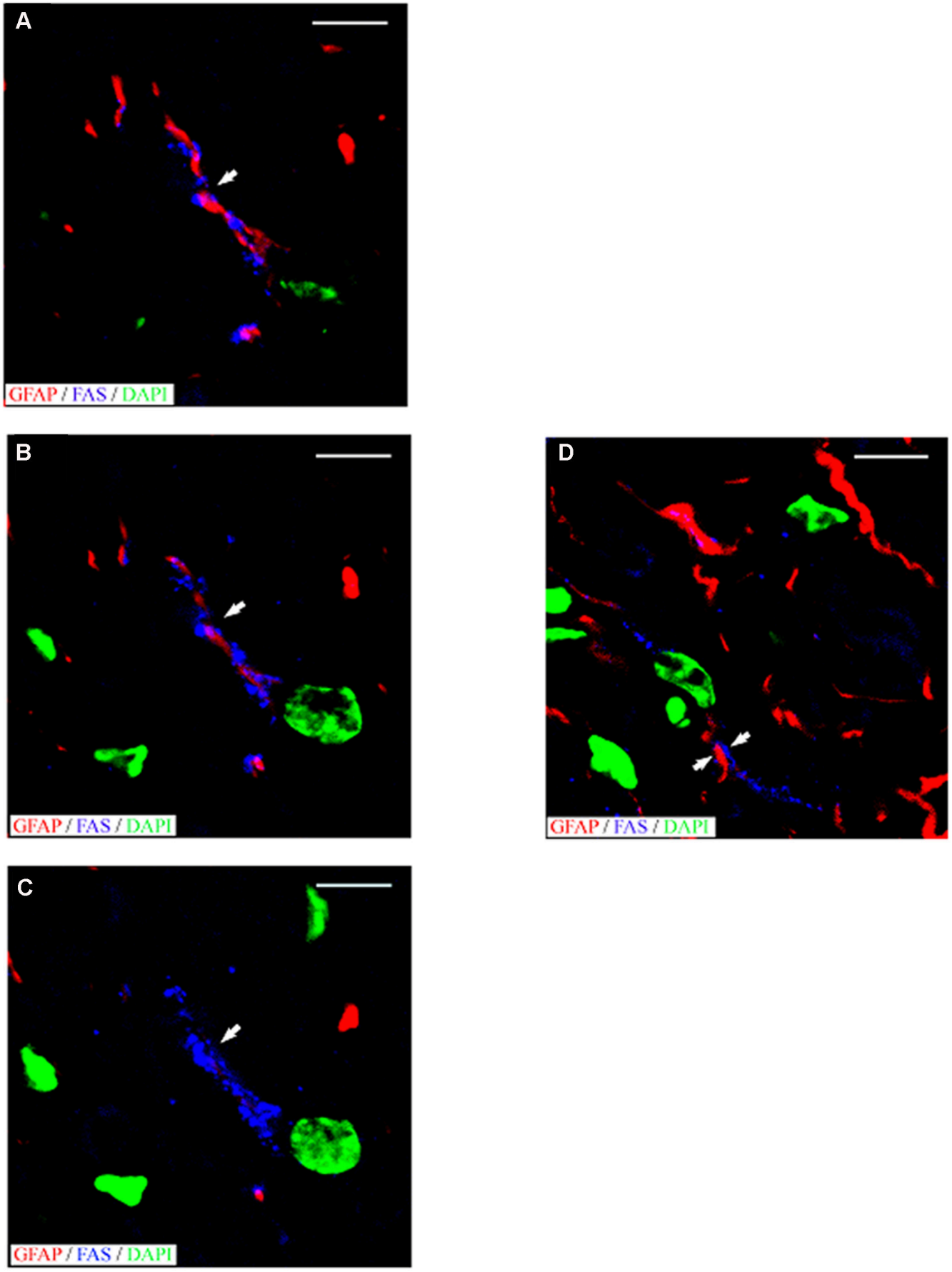


Figure 12: Close spatial relationship of astrocytes to Fas-positive cells

Figure 12: Close spatial relationship of astrocytes to Fas-positive cells

Tissue sections taken from an animal at five days post-SCI, approximately 800µm rostral to the injury epicenter were labelled for astrocytes (GFAP), Fas receptor and the nuclear marker DAPI. Confocal microscopy through the z-plane of the tissue was carried out starting with image A and moving through the tissue section to image C. Image A shows the co-localization of GFAP with Fas receptor, giving the indication that the astrocytic process is expressing Fas receptor. However, as one moves through the depth of the tissue section (from B to C), it becomes less clear as to whether the astrocytic process is in fact expressing Fas, or if it simply lying in very close proximity to another cell that is actually expressing Fas (indicated by the arrow). Further clarification of the fact that Fas expression is spatially distinct from GFAP expression is found in D, where the arrowheads point to close but not co-localized expression. The scale bars in all of these images represent 8µm.

identified by a separate DAPI-labelled nucleus (**Figures 12B and C**). Further confirmation is achieved by the close but not overlapping expression of Fas receptor and GFAP in a high magnified confocal section (**Figure 12D**).

3.2.6 Caspase-8 and caspase-3 activation profiles support the theory of delayed Fas-mediated apoptosis following experimental SCI

The expression levels of mRNA for procaspase-3 and procaspase-8 were evaluated in injured spinal cord tissue from one, three, five and seven days post-trauma. These levels were made relative to the corresponding gene expression found in sham-injured tissues. Of important note here is that while the expression of the pro-forms of caspases do not directly imply changes in their activation, it has been accepted that increases in procaspase expression have strong implications on a similar increase in their activation (Beer, Franz et al. 2000). This has the potential to be extrapolated to the gene expression of the procaspases as well. The validity and acceptance of this type of interpretation will be examined more closely in the discussion section to follow.

Significant increase in procaspase-3 message expression was observed starting at one day post-SCI and continuing until its peak at seven days post-injury. There seem to be two-stages to the increased expression, where levels at one and three days are unchanged, followed by another significant increase at five and seven days (**Figure 13A**). This profile of procaspase-3 gene expression can be correlated with the known incidence apoptotic cell death during the first week following SCI.

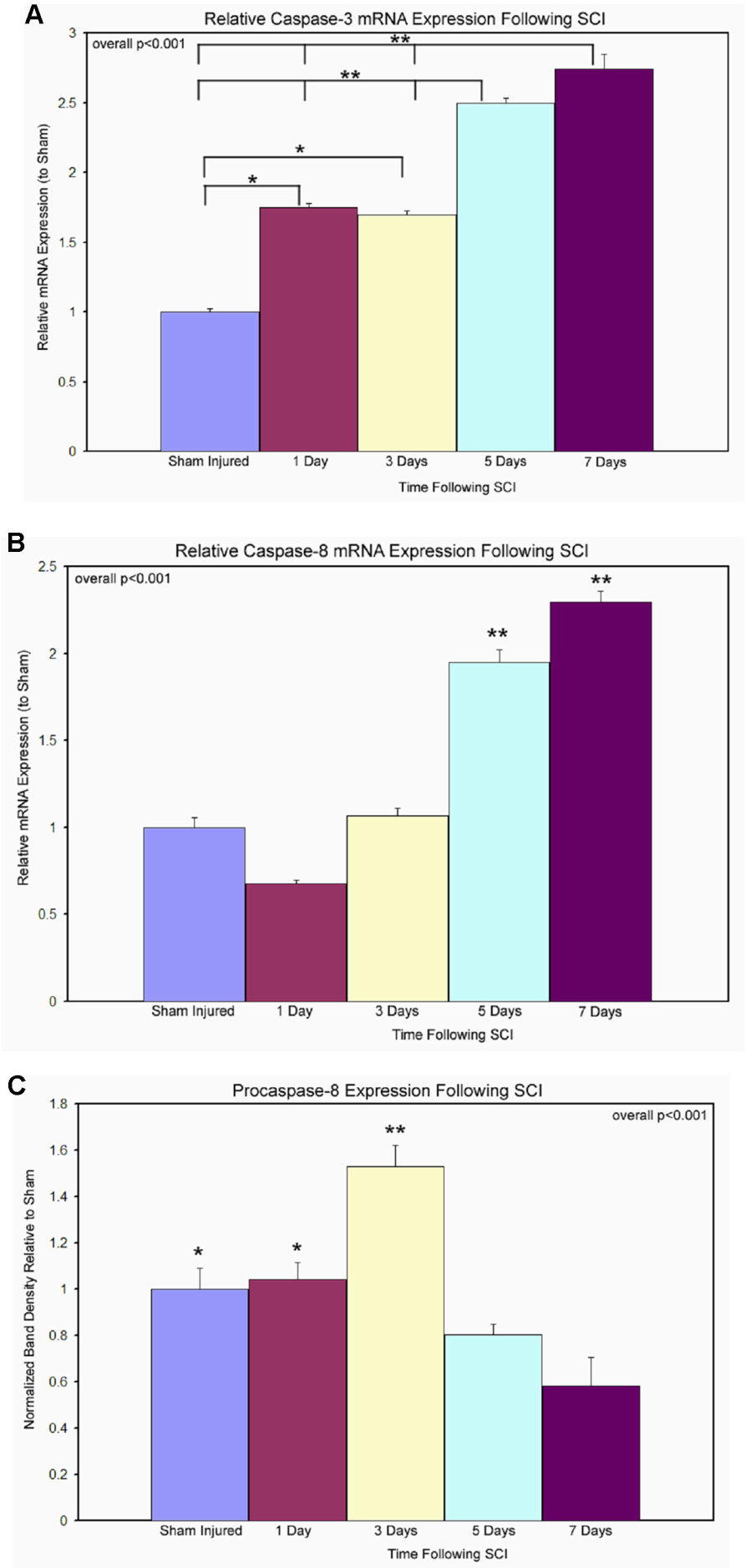


Figure 13: Procaspase expression profiles indicate delayed Fas-mediated apoptosis post-SCI

Figure 13: Procaspase expression profiles indicate the presence of delayed Fas-mediated apoptosis post-SCI

(A) Procaspase-3 mRNA expression levels were evaluated using real-time PCR in sham-injured and SCI rats at one, three, five and seven days post-injury. Messenger RNA expression levels are shown relative to sham-injured levels following normalization with GAPDH mRNA expression (error bars = SEM). It was found that procaspase-3 gene expression is increased following SCI, as soon as one day post-injury with another increase in expression found at five days post-injury, as compared to sham-injured controls. These data were analysed for statistical significance using a one-way ANOVA, which revealed an overall p-value of <0.001. Post-hoc analysis using the Student Neuman-Keuls method revealed significantly increased expression of procaspase-3 at one and three days post-injury, compared to sham-injured controls ($p<0.05$). Furthermore, the expression observed at five and seven days post-injury was also significantly higher than that in the sham-injured controls and in the one and three day post-injury tissue ($p<0.001$ for each comparison). The sham-injured group, one, three and five day SCI groups had an $n = 6$ and the seven day-injured group had an $n = 5$.

(B) Procaspase-8 mRNA expression levels were evaluated using real-time PCR in sham-injured and SCI rats at one, three, five and seven days post-injury. Messenger RNA expression levels are shown relative to sham-injured levels following normalization with GAPDH mRNA expression (error bars = SEM). Here it was observed that procaspase-8 gene expression does not significantly increase post-SCI until the fifth day post-injury, where the level is then maintained at seven days. These data were analysed for statistical significance with a one-way ANOVA, which revealed an overall p-value of <0.001. Post-hoc analysis using the Student Neuman-Keuls method revealed significantly increased expression of procaspase-8 mRNA at three and five days post-injury, compared to sham-injured controls and to one and three day post-SCI tissue ($p<0.001$ for all comparisons). The sham-injured group, one, three and five day SCI groups had an $n = 6$ and the seven day-injured group had an $n = 5$.

(Figure 13 Continued)

(C) Procaspase-8 protein expression was evaluated using Western Blot analysis in sham-injured and SCI rats at one, three, five and seven days post-injury. Protein expression levels are shown relative to sham-injured levels following individual normalization with β-actin expression (error bars = SEM). It was observed that procaspase-8 expression is significantly increased at three days post-injury, followed by a reduction in expression at five and seven days to below one day post-injury and sham-injured levels. These data were analyzed for statistical significance using a one-way ANOVA where the p-value was determined to be <0.001. Post-hoc analysis was performed using the Student Neuman-Keuls method and it was found that procaspase-8 expression is significantly increased at three days post-injury as compared to all other time points with a p-value of <0.001. In addition, it was found that expression at one day post-injury was greater than that at five and seven days, with a p-value of $p<0.05$. As well, the expression of procaspase-8 in sham-injured tissue was significantly higher than that found at seven day post-SCI ($p<0.05$). The sham-injured group, three day and seven day post-injury groups each had an $n = 4$ and the one day and five day post-injury groups each had an $n = 5$.

Expression changes for both procaspase-8 message and protein following SCI provide further confirmatory evidence in support of Fas-mediated apoptosis occurring in a delayed fashion post-injury.

Following SCI, the gene expression profile of procaspase-8 does not change significantly until five days post-injury at which point there is a dramatic increase (**Figure 13B**). The protein expression of procaspase-8 has a slightly different profile and at first glance seems contradicting to that of the message. Procaspase-8 protein presence is significantly increased only at three days post-injury, following which it is greatly reduced at days five and seven (**Figure 13C**). A possible explanation for this observation will be presented in the discussion section to follow.

3.3 Discussion

3.3.1 Summary of Findings

The work presented in this chapter highlights the importance of Fas-mediated apoptotic signalling in the secondary mechanisms of SCI. Fas expression is increased following injury in a delayed temporal profile, with predominant expression observed within the oligodendrocyte population. Furthermore, Fas receptor expression by oligodendrocytes simultaneously with cleaved caspase-3 indicates that the oligodendrocyte population is highly susceptible to delayed Fas-mediated apoptosis following SCI.

The cells responsible for Fas activation by expression of Fas ligand include the three main glial cell types: oligodendrocytes, astrocytes and microglia. The expression of Fas ligand on these cells is slightly increased

following SCI, and is likely influenced by infiltrating immune cells already expressing FasL prior to margination. In addition, a strong possibility exists for astrocytic expression of FasL to activate Fas-mediated apoptosis of oligodendrocytes along degenerating white matter tracts.

Procaspase expression profiles further contribute to the conclusion that Fas-mediated apoptosis has a major role in the cell death mechanisms in secondary SCI pathophysiology.

3.3.2 Rationale

Apoptotic cell death is a well-defined cellular phenomenon that plays a critical role in both the development, regulation and function of the immune system (Kerr, Wyllie et al. 1972; Kerr, Winterford et al. 1994; Wyllie 1994; Majno and Joris 1995; Thornberry and Lazebnik 1998; Yin 2000; Penninger and Kroemer 2003). However, the activation of both death receptor and mitochondrial apoptotic pathways following traumatic events and in several degenerative diseases of the CNS is known to cause marked cell loss, tissue destruction and loss of function. Specifically, the role of Fas-mediated apoptotic cell death is accepted as a key player in the secondary injury events following acute traumatic SCI. While the importance of Fas-mediated apoptosis in the injured spinal cord is not a new concept, a complete understanding of the cellular and temporal relationships of Fas and FasL expression and their contribution to apoptotic activation are not currently known. An understanding of the interrelationships involved in the secondary pathology after SCI is crucial for

furthering the development of and refining the therapeutic strategies designed to target this type of cell loss.

3.3.3 Fas Receptor Expression

In the clip compression SCI model, both gene and protein expression of Fas receptor were increased during the secondary injury phase. This suggests that cells are signalled to express Fas and in so doing, become susceptible to apoptotic cell death. The signal is likely in part due to the deleterious environment created by other secondary injury pathologies. The inflammatory response is a strong candidate for some of this signalling, as activated microglia and infiltrating macrophages are known to secrete pro-inflammatory cytokines such as TNFα and IFNγ following SCI (Norris, Weston et al. 1977; Giulian, Corpuz et al. 1993; Selmaj, Walczak et al. 1998; Jones, McDaniel et al. 2005). These cytokines have also been found to actively increase the expression of Fas receptor on a variety of cell types, contributing to the perpetuation of cell death signalling via Fas activation (Moller, Koretz et al. 1994; Matsuyama, Hata et al. 1995; Choi, Park et al. 1999; Pouly, Becher et al. 2000; Fluhr, Krenzer et al. 2007).

Of importance to the field of SCI is that the upregulation of Fas message and protein expression is delayed considerably following the initial traumatic insult. Fas receptor mRNA reaches a peak in expression at three days post-SCI and this is mirrored in the protein expression, which reaches peak presence at five days. Immunohistochemical analysis of Fas expression in adjacent tissue sections supports the concept of a delayed post-injury presence. This offers a

strong potential for therapeutic targeting of the Fas pathway by providing a significant window of opportunity post-SCI in which to disrupt Fas signalling.

Ultimately the specific cellular localization of Fas expression dictates what population of cells are most susceptible to Fas activation and subsequent cell death. Given this, the two most important cell types for proper support and function of the CNS, neurons and oligodendrocytes, were examined for increased expression of Fas receptor following SCI. With consistent significance, it was observed that the oligodendrocyte population contributed to the majority of Fas expression noted after SCI. While very few neurons were found to be Fas-positive (<5%), a great deal of the total oligodendrocyte population expressed Fas post-injury. Oligodendrocyte expression of Fas receptor reached peak levels at three and five days, with approximately 30% and 20% of the total cell population being Fas-positive, respectively.

3.3.4 Fas-Mediated Apoptosis Post-SCI

It is appreciated that Fas expression alone does not prove functional relevance. Following SCI, apoptotic pathways are activated and contribute to both neuronal and oligodendrocyte cell loss (Rink, Fung et al. 1995; Katoh, Ikata et al. 1996; Li, Brodin et al. 1996; Springer, Azbill et al. 1999; Knoblach, Huang et al. 2005). There are two main pathways for apoptotic cell death initiation, either by death receptor activation or through mitochondrial signalling. The impact of each of these pathways following SCI has yet to be confirmed in the literature.

By quantifying the temporal and cellular relationship of cleaved caspase-3 expression, the cell-specific dichotomy of the two apoptotic processes

was observed. Neurons showed an early rise in apoptotic cell death and had a somewhat consistent expression of cleaved caspase-3 throughout the first week post-injury. This correlated well with the idea that neurons are highly susceptible to mitochondrial mechanisms of apoptotic signalling, and are succumbing to cell death as a result of the ionic imbalances, inappropriate calcium homeostasis and free radical formation taking place in the early events of secondary injury pathology. Oligodendrocyte expression of cleaved caspase-3 was more delayed than that of the neuron population. There was a switch in the major cell type undergoing apoptosis at three and five days post-injury, where the oligodendrocytes became the major victim. Recall that Fas receptor expression on the oligodendrocyte population also reached peak levels at three and five days post-SCI. The correlative evidence suggests that these oligodendrocytes might be undergoing Fas-mediated apoptosis.

In examining the co-expression of Fas receptor and cleaved caspase-3, it was possible to more confidently state that a given cell was undergoing Fas-mediated apoptosis if both proteins were present. Immunohistochemical analysis of neuron and oligodendrocyte expression of Fas and cleaved caspase-3 was carried out to address this key issue. At five days following SCI, there was the largest amount of apoptotic cell death observed in the oligodendrocyte population. In closer examination of these dying cells, it was observed that approximately 70% of apoptotic oligodendrocytes were in fact co-expressing Fas receptor and cleaved caspase-3. This strongly implicates the activation of Fas-mediated apoptotic signalling in these cells. Contrasting this with the

observations from the neuronal population at one day post-SCI, the peak in neuronal apoptosis, it was found that very few apoptotic neurons were expressing Fas. This was not entirely surprising, given the neuronal predisposition to mitochondrial sensitivities. Furthermore, it has been shown that certain populations of neurons express regulatory proteins that render them unsusceptible to Fas-mediated apoptosis (Fernandez, Segura et al. 2007; Segura, Sole et al. 2007).

The conclusions to this point are quite specific given the evidence provided in the cervical clip compression model of SCI. While both neurons and oligodendrocytes are known to undergo apoptotic cell death following SCI, it is the oligodendrocytes population that is most severely affected by Fas-mediated apoptosis. Furthermore, this type of oligodendrocyte cell death is occurring in a delayed fashion following traumatic SCI, with peak occurrence between three and five days post-injury.

The idea that the oligodendrocytes are identified and targeted as necessary to die following injury is something that warrants further investigation. By manipulating the activation of Fas receptor following trauma to the spinal cord, it may be possible to protect these important, supportive glial cells. Enhancing the survival of the oligodendrocyte population, even in an injured, sub-optimal capacity, could hold promise for maintaining a supportive environment for neurons and axons following trauma to the spinal cord.

3.3.5 Fas Ligand Expression After SCI

The cellular and temporal expression of Fas ligand is important to the understanding of Fas-mediated apoptosis. To date, this line of investigation has not been fully appreciated in the literature. Fas receptor expression is ultimately the more critical factor to understand, given that it is the cellular expression of receptor that designates a cell for potential death. However, the death-inducing capability of FasL makes its expression profile following SCI an important aspect of secondary injury mechanisms, and provides further information for therapeutic development.

In the first week following SCI, FasL gene expression did not follow a predictable time course. An initial and significant increase in FasL mRNA was observed at one day post-injury, and could likely have been a result of the surge in microglial and astrocyte activation induced by primary trauma and early secondary pathological events. The drop in message expression from three days onward was slightly surprising, however it is possible that regulatory mechanisms exist to reduce the expression of FasL in order to keep Fas activation in check. When the protein expression of FasL was evaluated, it was observed that a steady and slight increase in receptor expression was noted starting at one day post-injury and peaking at five days. It is possible to link the message and protein expression profile in the following way: The infiltration of immune cells that may already express FasL is most likely to occur between three and five days post-injury, therefore the protein presence of FasL would increase with time. The message of FasL would them be reduced, to

accommodate and control for the increased presence of cells capable of activating Fas receptor.

A caveat to this work is in the inability to accurately distinguish between resident microglia and infiltrating macrophages in the injured spinal cord tissue. It is possible that systemic immune cells express constitutive levels of FasL, and upon margination into the injury cord, this presence is observed. There have been some attempts at understanding the systemic inflammatory cell impact on this particular issue, using mice whose bone marrow has been irradiated and then re-constituted with GFP-labelled cells (Letellier, Schreglmann et al. 2007).

In evaluating the cellular expression of both Fas receptor and ligand, a plausible explanation for Fas activation along degenerating white matter tracts was observed. Using confocal imaging through the z-plane of a tissue section, it was observed that an astrocyte can lie in very close proximity to a Fas expressing cell in the white matter. Due to problems in acquiring adequate molecular reagents, it was not possible to determine if in fact the Fas-expressing cell lying next to the astrocyte was an oligodendrocyte. However taking evidence from the work presented here, it has been shown that oligodendrocytes are the main cell type expressing Fas receptor post-SCI and that Fas ligand is expressed on reactive astrocytes. Furthermore, it is accepted in the literature that after SCI, astrocyte presence is observed along demyelinating and degenerating white matter tracts (Nashmi and Fehlings 2001). It therefore is a strong possibility that astrocytes expressing FasL are contributing to the Fas-mediated apoptosis of oligodendrocytes along damaged axons. While this theory has not been

confirmed due to a lack of necessary immunohistochemical reagents, it remains a potential explanation at least in part, for the initiation of Fas-mediated cell death signalling following SCI.

The work presented here identified the cell types that express FasL, before injury and following injury. It was surprising to see a range of glial cells showing FasL expression on their surface, with the most surprising cell type being the oligodendrocyte. Knowing that this cell population had the most significant Fas receptor expression, the fact that they also had the ability to express Fas ligand lends to an interesting theory. Cross-talk, or communication among the oligodendrocytes in close proximity to one another could be facilitated using Fas and FasL interactions. Note that recently, several groups have proposed that Fas and FasL ligation does not always infer an apoptotic signal (Alderson, Armitage et al. 1993; Owen-Schaub, Meterissian et al. 1993; Desbarats, Birge et al. 2003; Pettmann and Henderson 2003; Fernandez, Segura et al. 2007; Segura, Sole et al. 2007). This area of investigation merits future study.

FasL expression on microglia and astrocytes in sham-injured tissue was less intense than that following SCI. Of note in this context is the ability for microglial release of various pro-inflammatory cytokines to have an impact on FasL expression (Choi, Park et al. 1999). This might provide another way in which the various secondary injury pathologies interact with one another in perpetuating secondary cell and tissue damage.

3.3.6 Procaspase Expression Profiles

The expression profiles of procaspases involved in apoptotic signaling following SCI were evaluated. Ideally, the cleaved and activated forms of the caspases would have been examined to provide a unequivocal interpretation of apoptotic cascade signalling. However, there are currently a limited number of effective reagents available to address these concerns, making this exact line of investigation somewhat challenging (Letellier and Martin-Villalba 2007). It has been relatively well-accepted in the literature that changes in pro-form expression of specific caspases can correlate with their activation (Beer, Franz et al. 2000). Here, the increased activation of a caspase would cause the immediate yet short-term reduction in the amount of pro-form found within the cell. As a result, it is very likely that gene expression would increase to maintain the cell's supply of the pro-form. In an environment in which a great deal of apoptosis is occurring, gene expression would likely be quite high. Subsequently, there would be a steady rise of procaspase protein being translated to maintain the cellular supply.

Following cervical clip compression SCI, the gene expression of procaspase-3 rose steadily to a maximum level at seven days. This was consistent with the idea of an increased need for procaspase-3 in a setting of increased apoptosis would result in enhanced gene expression. This rise in procaspase-3 did not however explain the specific type of apoptosis that was occurring, as active caspase-3 is the final executioner caspase for all types of

apoptotic signalling. In examination of the Fas-specific caspase, caspase-8, more detailed information was obtained.

Procaspase-8 mRNA expression did not significantly increase until the fifth day post-injury. This was surprising given the burst in procaspase-8 protein expression noted at three days. However, it is possible to explain the drop in protein levels of procaspase-8 that were observed at five and seven days post-injury if one considers the increase in Fas-mediated apoptosis shown to occur at this time point. With a high level of Fas activation, procaspase-8 will be constantly cleaved into its active form following recruitment to the DISC. With this, an increase in procaspase-8 gene expression would attempt to maintain the cells' surplus of the pro-form. This effect was noted in examination of procaspase-8 mRNA expression, as levels rose significantly at five and seven days post-SCI. The protein expression of procaspase-8 at these times points did not mirror this, and for a very likely reason. There is only so much that increased gene expression can do to keep a surplus of procaspase-8 ready to be cleaved. It is possible that the extent of Fas activation at five days is so great that the gene expression cannot keep up with the cleavage of procaspase-8, resulting in the reduction in protein expression observed.

The obvious caveat to this line of investigation is in the inability to make solid conclusions based on the evidence provided. All that can be accomplished to this point is a correlative and estimated theory supported by the observations. Fas-mediated apoptosis, and therefore activation of caspase-8 and subsequently

caspase-3, is a predominant aspect of secondary injury pathologies following traumatic compression SCI.

3.4 Conclusions

For the first time, the specific cellular and temporal patterns of Fas and FasL expression have been accurately evaluated in a clinically relevant experimental model of spinal cord injury. The exact cellular target of Fas-mediated cell death has been established, and the delayed nature of this insult to the oligodendrocyte population provides a promising therapeutic potential for enhancing oligodendrocyte survival in the injured spinal cord. By maintaining the vitality of the oligodendrocyte in the white matter tracts of the spinal cord, the possible influence of survival and functional benefit to the neurons and their axons is proposed. Given this possibility, the following two chapters serve to examine a potential therapeutic strategy designed to disrupt the Fas-mediated apoptotic signalling in the acutely injured spinal cord.

Chapter Four:

Characterization of the neuroprotective efficacy of a soluble form of the Fas receptor (sFasR) via intrathecal infusion following acute SCI

Please note that the work presented in this chapter has been published:

> Ackery, A., S. Robins, et al. (2006). "Inhibition of Fas-mediated apoptosis through administration of soluble Fas receptor improves functional outcome and reduces posttraumatic axonal degeneration after acute spinal cord injury." J Neurotrauma **23**(5): 604-16

Ackery and Robins are co-first authors of this paper. Please note that only original data generated by S. Robins has been included in this chapter.

4.1 Introduction

Currently there is only one approved pharmacological treatment for acute SCI patients, Methylprednisolone. Unfortunately This option is accompanied by an inherent risk of side-effects and the potential for limited efficacy, making Methylprednisolone a less than desirable choice for most clinicians.

The activation of Fas-mediated apoptotic mechanisms following experimental SCI has been illustrated in the previous chapter. There is great promise for this aspect of secondary injury pathology to be targeted therapeutically with beneficial results. Given this, a human fusion protein consisting of the extracellular domain of human Fas receptor, attached to a 234 amino acid segment of the Fc region of human IgG was examined for its therapeutic efficacy following SCI. The extracellular domain of the soluble Fas receptor protein (sFasR) binds effectively to the physiological FasL in a variety of

in vitro settings (data provided by Ancell, the company that manufactures the sFasR molecule). However, due to the lack of transmembrane and intracellular domains, ligation of sFasR with FasL does not activate an apoptotic signal. By competitively binding with endogenous, cell-bound Fas receptor, the presence of sFasR is expected to reduce Fas activation, and thereby reduce Fas-mediated apoptosis.

The anti-apoptotic capabilities of sFasR administration following acute traumatic SCI were evaluated using the 35g, cervical, clip compression injury model. Intrathecal infusion of sFasR was initiated immediately following the primary injury, and administration was maintained for up to seven days through the use of an osmotic mini-pump. This work was originally started by Alun Ackery during his MSc. thesis, and as a result the manuscript detailing the complete story contains his work as well as that of Sherri Robins. The importance and contribution of Alun's findings will be more thoroughly addressed during the discussion section of the chapter.

Initially, saline was used as the primary control treatment for comparison against sFasR. Mid-way through the experiments, it was decided that a protein control would be a more appropriate choice of control treatment. Because of the fact that the sFasR molecule was stabilized by a segment of human IgG, the choice for the control protein was apparent. The full-length form of human IgG was introduced into the experiments as the primary control, with saline remaining a secondary control.

4.2 Results

The work presented in this chapter shows for the first time that soluble Fas receptor is an efficacious treatment in a clinically relevant model of traumatic cervical SCI. Furthermore, the targeted delivery system of a mini-osmotic pump and subarachnoid catheter is successfully employed for effective therapeutic dosing of sFasR at the time of SCI.

4.2.1 Subarachnoid catheterization does not adversely affect spinal cord tissues

Intrathecal or subarachnoid catheterization in the spinal cord is surgically challenging and unarguably invasive, however it does allow for localized delivery of a therapeutic to the site of injury. When compared with systemic administration of a therapeutic molecule, intrathecal administration reduces the dose requirement and more important, lessens the chance of adverse systemic effects. This being said, there has been a report in the literature suggesting that chronic intrathecal catheterization following SCI can cause associated tissue damage and potentially exacerbate the injury (Jones and Tuszynski 2001).

The subarachnoid space at the level of the cervical spinal cord is wider than that in more caudal regions. Given that the injury model used for these experiments is in the cervical region, there was less expectation for deleterious effects of catheter placement. In order to assess the impact of sub-acute catheterization at the level of C7 through T2 vertebral levels, non-injured animals underwent subarachnoid catheterization for one week and the histology of the spinal cord in the area of catheter implantation was assessed.

For a general point of reference, the impact of compression SCI on the normal histology of a spinal cord cross section is remarkable (**Figure 14A**). It is obvious that a severe disruption in tissue morphology and integrity results at one week following trauma. Following intrathecal catheterization, both cross and longitudinal sections of spinal cord tissue were examined for histological evidence of damage. There was no disruption of tissue morphology observed in these sections, as grey and white matter remain distinct and intact. Furthermore, there was no evidence of compression to the spinal cord caused by the catheter, and the occurrence of an inflammatory response was not observed (**Figure 14B and C**).

4.2.2 Neurons and oligodendrocytes are susceptible to post-traumatic apoptosis

In order to better understand the specific cell populations targeted by sFasR administration, TUNEL staining and cell-specific labelling was carried out at seven days following SCI. Note that this work preceded the more specific mechanistic evaluation that was presented in Chapter 3, and at the time was a valuable contribution to the literature. Both oligodendrocytes and neurons exhibit a TUNEL-positive phenotype following traumatic SCI, however at seven days post-injury there is a significantly higher proportion of apoptotic oligodendrocytes than neurons (**Figure 15**). This suggests that manipulation of Fas activation by sFasR would most likely target the oligodendrocyte population following experimental SCI.

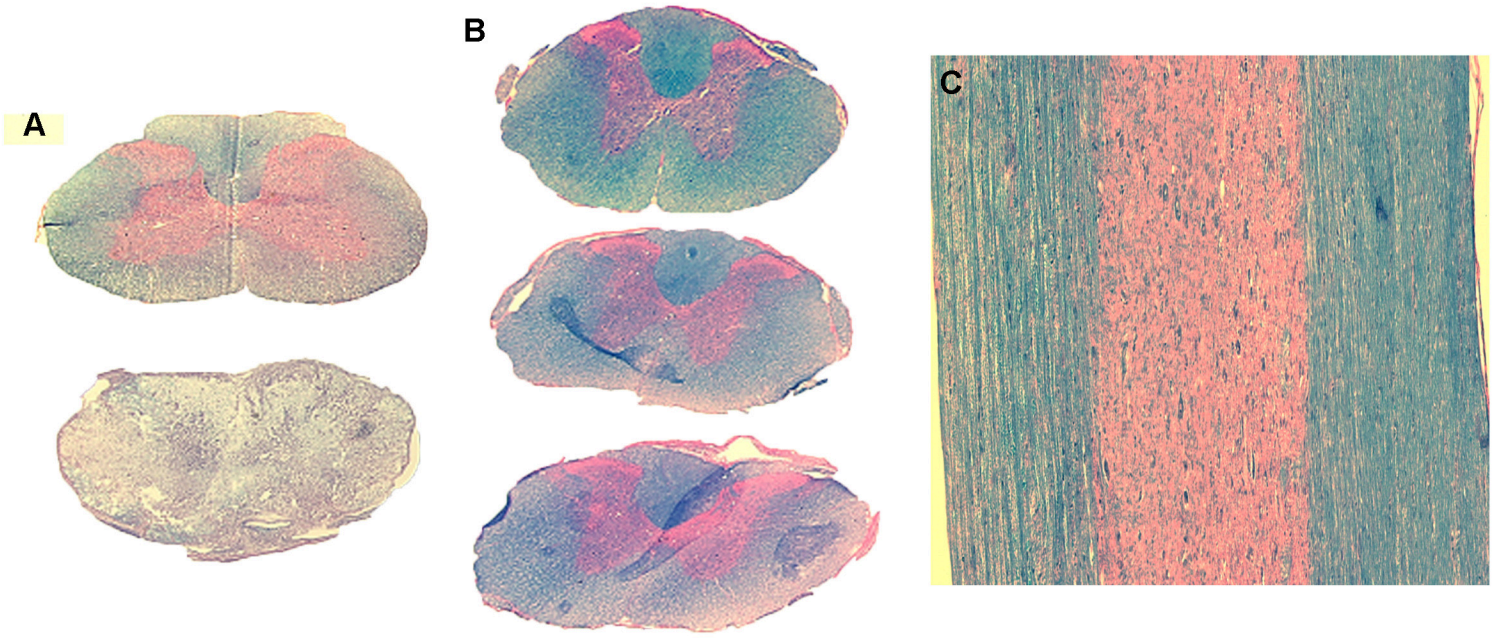


Figure 14: Cervical intrathecal catheterization does not damage tissue histology

Figure 14: Cervical intrathecal catheterization does not damage tissue histology

The effects of intrathecal catheterization at the cervical vertebral level were investigated using cross and longitudinal tissue sections and basic histological staining practices. The spinal cord tissue was evaluated for morphological disturbances, tissue damage and for signs of compression as a result of catheterization. A cross-section of a non-injured spinal cord is shown in the top panel of (A). Underneath the normal tissue section, the cross-section of an injured spinal cord is shown. There is a marked loss of tissue morphology and integrity as compared to the non-injured section.

Sequential cross-sections were taken along the length of spinal cord from an animal following seven days of intrathecal catheterization (B). It is noted that there is no sign of compression to the dorsal aspects of the sections, and there is no report of changes to tissue morphology or damage. A longitudinal section taken from the mid-plane of a spinal cord after seven days of catheterization reveals an intact and histologically normal tissue organization (C). It should be noted as well that the animals used for this analysis showed to signs of motor impairment due to catheterization in the subarachnoid space.

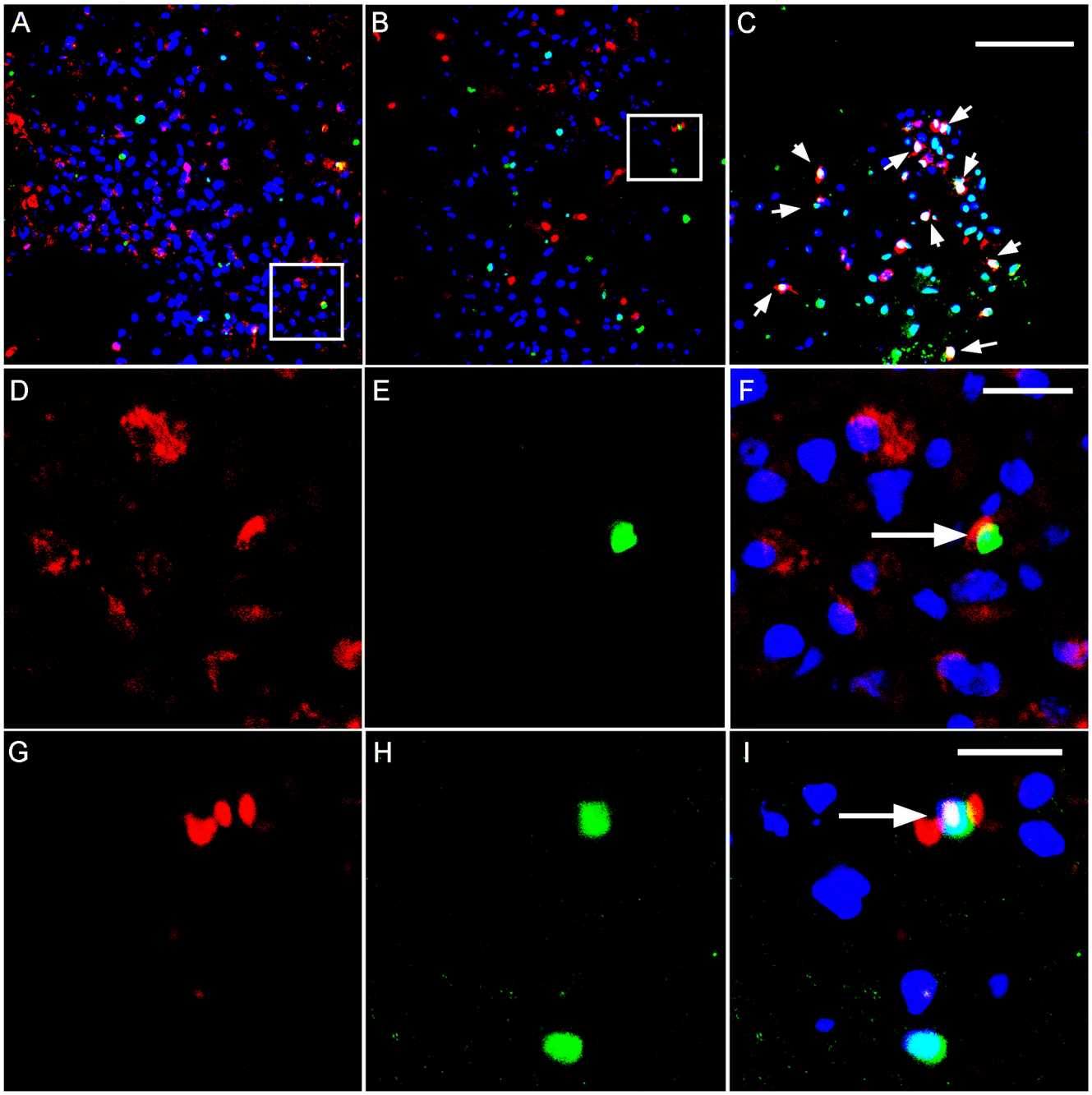


Figure 15: Neurons and oligodendrocytes are susceptible to post-traumatic apoptosis

Figure 15: Neurons and Oligodendrocytes are susceptible to post-traumatic apoptosis

This figure was published in the following manuscript:

Ackery, Robins and Fehlings (2006) Journal of Neurotrauma 23(5): 604-616

Immunohistochemical analysis of tissue sections taken approximately 1000μm rostral to the injury epicenter at seven days post-SCI was carried out determine the types of cells undergoing apoptotic cell death at that time-point following injury. Oligodendrocytes were labelled with CC1/APC and neurons were labelled with NeuN. Apoptotic cells were determined using TUNEL staining and nuclei were identified using DAPI.

A large number of oligodendrocyte were found to be TUNEL-positive at seven days post-SCI (A and C), while fewer neurons were found to be TUNEL-positive (B). Images D, E and F are higher magnifications of the area indicated in A, with the arrow pointing to a TUNEL-positive oligodendrocyte. Images G, H and I are higher magnifications of the area indicated in B, with the arrow pointing to a TUNEL-positive neuron.

The scale bar in image C is equivalent to 50μm, and the scale bars in images F and I are equivalent to 20μm.

4.2.3 Intrathecal administration of sFasR reduces post-traumatic apoptosis following SCI

The anti-apoptotic effects of sFasR administration were evaluated using TUNEL-positive cell quantification at various distances in both the rostral and caudal directions from the injury epicenter. It is clear that saline-treated animals have a greater proportion of TUNEL-positive cells than do the sFasR-treated animals (**Figure 16**). Following quantification of TUNEL-positive cells in full tissue sections at 500μm, 1000μm and 1500μm rostral and caudal to the injury site, it is clear that intrathecal administration of sFasR can significantly reduce apoptosis following experimental SCI as compared to saline control treatment (**Figure 16G**).

4.2.4 Oligodendrocyte viability and axonal preservation are improved with intrathecal sFasR administration post-SCI

In addition to the anti-apoptotic effects of intrathecal sFasR administration, the resultant cellular and molecular impact was evaluated. Analysis of oligodendrocyte vitality using a marker for mature oligodendrocytes (CNPase) was carried out at five days following SCI. Here, sFasR treatment significantly enhances oligodendrocyte survival when compared to IgG-treated controls (**Figure 17A**).

The degradation of axonal cytoskeletal elements like neurofilament 200 (NF200) following SCI is known to correlate with loss of axonal integrity and an associated loss of axonal function (Fehlings and Tator 1995). The ability of sFasR treatment to preserve the dephosphorylated band of NF200 was determined, as that neurofilament is particularly susceptible to early degradation

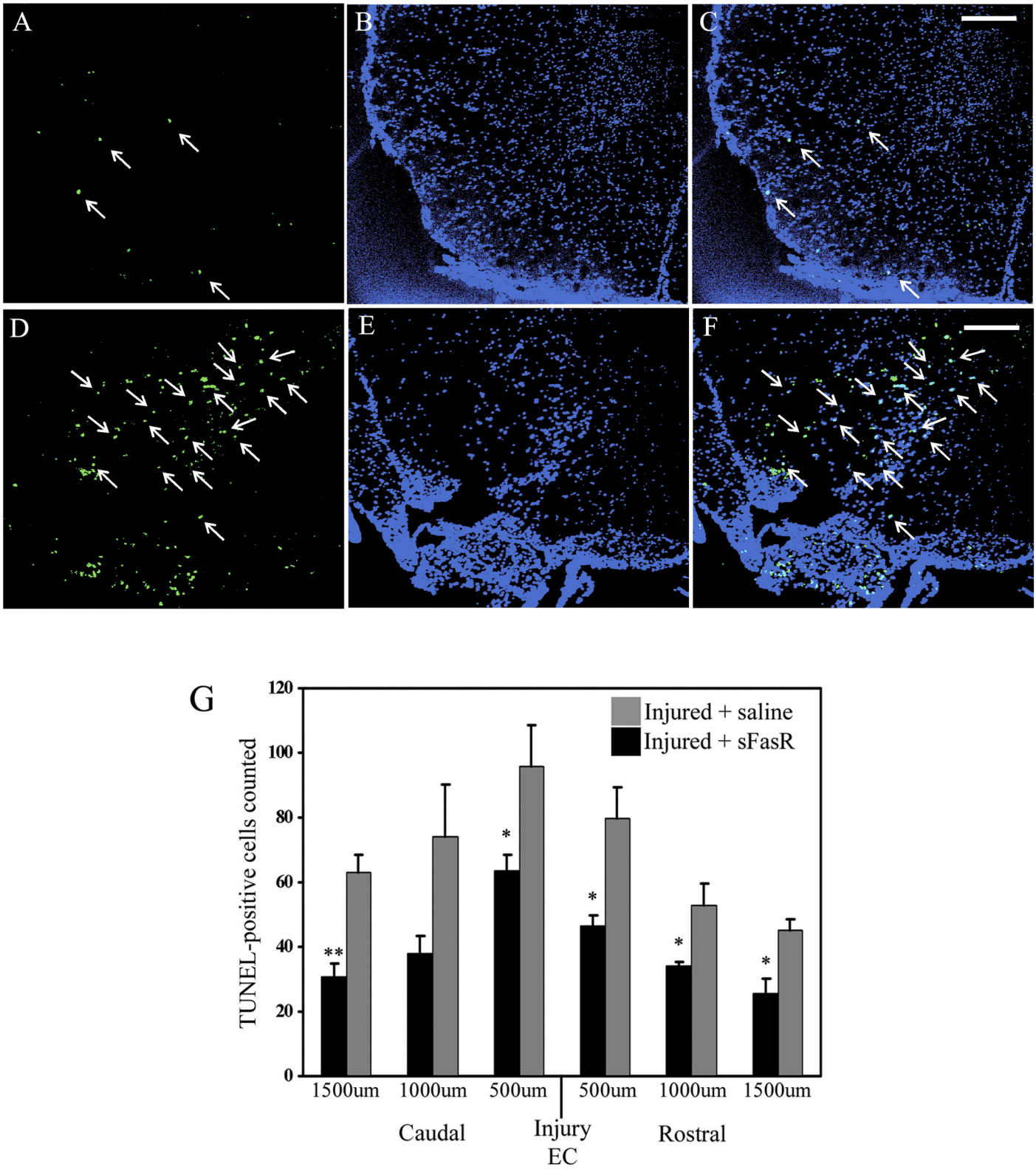


Figure 16: Administration of sFasR reduces post-traumatic apoptosis after SCI

Figure 16: Administration of sFasR reduces post-traumatic apoptosis following SCI

This figure was published in the following manuscript:

Ackery, Robins and Fehlings (2006) Journal of Neurotrauma 23(5): 604-616

The apoptotic effects of sFasR administration was evaluated using TUNEL labelling of tissue sections taken approximately 500μm, 1000μm and 1500μm both rostral and caudal to the injury epicenter at five days post-SCI. The nuclear marker DAPI was used for proper identification of cells during quantification of TUNEL-positive nuclei. Images A, B and C are representative images of tissue sections from an animal treated with sFasR. Images D, E and F are representative images of tissue sections from an animal treated with saline. Note the significant reduction in TUNEL-positive phenotypes in the sFasR-treated tissue.

Quantification of TUNEL-positive cells in full spinal cord cross-sections at the various locations surrounding the injury epicenter is graphically displayed in G. There was a significant anti-apoptotic ability of sFasR observed by the reduction in TUNEL-positive nuclei at almost all of the areas evaluated. These data were subjected to statistical analysis at each location using the Student t-test, where the p-values were determined to be $p<0.001$ at 1500μm caudal to the injury epicenter, and $p<0.05$ at 500μm caudal, 500μm, 1000μm, and 1500μm rostral to the injury epicenter. There was an $n = 4$ for each treatment group in this analysis.

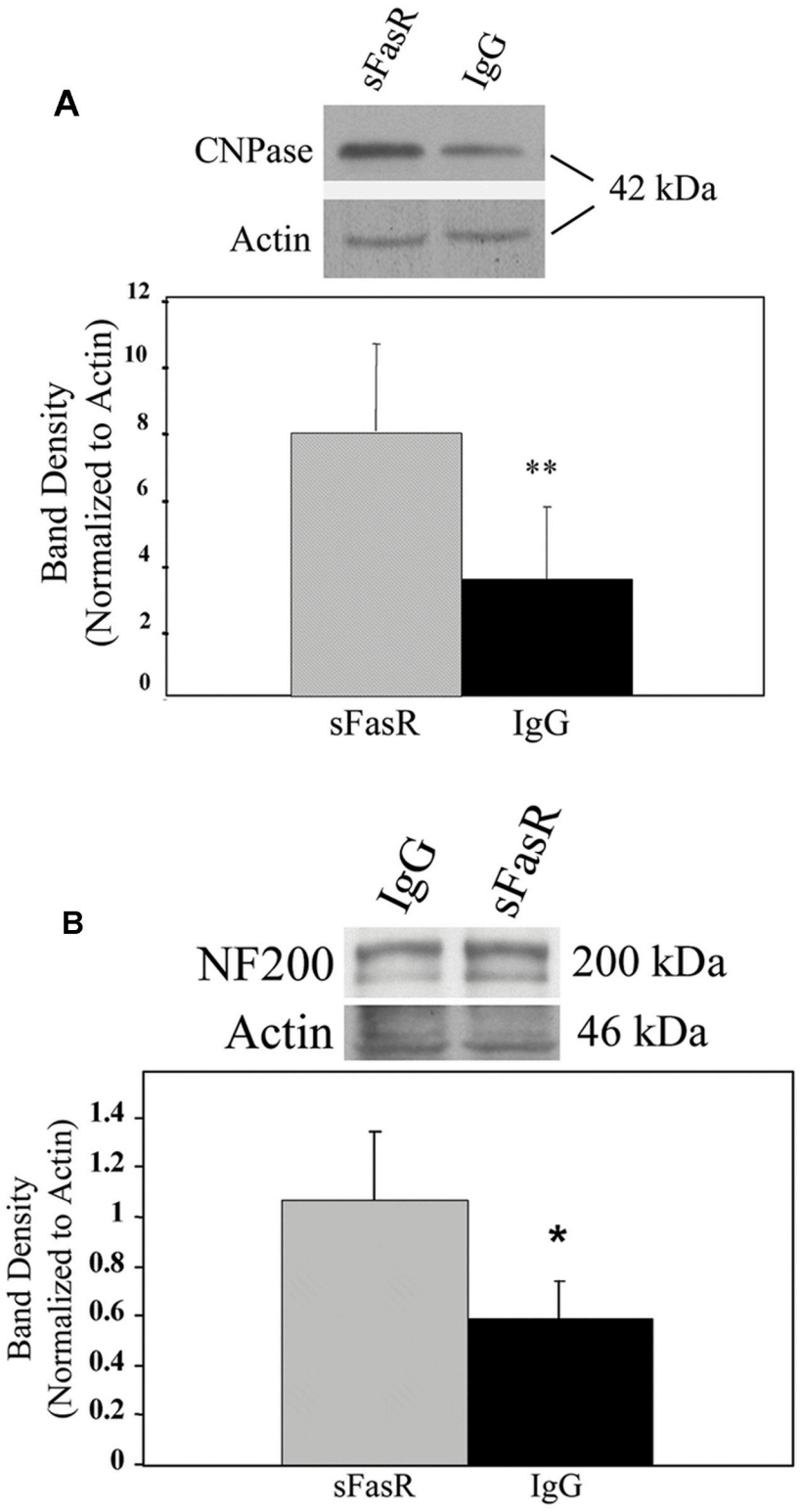


Figure 17: Administration of sFasR increases oligodendrocyte viability and reduces axonal degradation after SCI

Figure 17: Administration of sFasR increases oligodendrocyte viability and reduces axonal degradation after SCI

This figure was published in the following manuscript:

Ackery, Robins and Fehlings (2006) Journal of Neurotrauma 23(5): 604-616

The ability of sFasR to exert neuroprotective effects at the cellular level following SCI was evaluated using Western Blot analysis of tissue homogenates five days post-injury. The impact on oligodendrocyte viability was determined using the CNPase protein, a marker of mature oligodendrocytes (A). The band density of CNPase was significantly greater in those animals treated with sFasR, as compared to those treated with the IgG control.

The extent of axonal degradation was evaluated by measuring the degradation of neurofilament 200 (NF200), specially the de-phosphorylated band of the doublet (B). Soluble Fas receptor treated animals showed less breakdown of NF200, indicating that there was less axonal degradation in those tissues compared to IgG controls.

Both sets of data were subjected to statistical analysis using the Student t-test. The CNPase result achieved a p-value of $p<0.001$ and the NF200 result achieved a p-value of $p<0.05$. There were n's of $n = 6$ per treatment group in the CNPase analysis and $n = 5$ (sFasR) and 3 (IgG) in the NF200 analysis.

following SCI. It was found that sFasR-treatment significantly reduces the degradation of this neurofilament as compared to the IgG-treated controls (**Figure 17B**). The implications of these observation are that administration of sFasR limits axonal degradation following SCI, and therefore a strong potential exists for reduced axonal dysfunction with this treatment.

4.3 Discussion

4.3.1 Summary of Findings

This work illustrates the importance of Fas-mediated apoptosis in the development of secondary injury and cell loss following SCI. Complimentary analysis to that presented in chapter three further confirmed that while both neurons and oligodendrocytes undergo apoptotic cell death after SCI, the oligodendrocyte population is more greatly targeted by this type of cell death in a delayed time window after injury. Most importantly, the disruption of Fas and Fas ligand signalling using a competitive inhibitor, soluble Fas receptor, offers a significant anti-apoptotic mechanism, allowing for increased viability of oligodendrocytes and concomitant reduction in axonal damage after traumatic SCI. These observations provide further evidence that indeed Fas-mediated apoptosis plays a major role in secondary pathology and that this type of damage can be therapeutically targeted.

4.3.2 Anti-Apoptotic and Protective Abilities of sFasR

The anti-apoptotic ability of sFasR in the setting of the acutely injured spinal cord was illustrated in this work. A TUNEL-positive phenotype is indicative of the end stages of the apoptotic cascade, typified by specific DNA

fragmentation that is recognised using the TUNEL technique (Gavrieli, Sherman et al. 1992). Given what is now known about the main cell type affected by Fas-mediated apoptosis, the fact that post-SCI oligodendrocyte viability is maintained with sFasR treatment is exciting. Furthermore, the ability of the treatment to reduce axonal degradation is of great interest. The obvious question that comes to mind is in which capacity is the reduction in apoptosis functioning to maintain the health of axons. The answer to this is not trivial, however a possible explanation does exist.

Suppose that sFasR solely affects the survival of oligodendrocytes by preventing Fas activation. The resultant increase in oligodendrocytes health and presence along white matter tracts may provide the support necessary to decrease injury-induced degradation of axons. It is likely that although the oligodendrocyte population receives signals to undergo Fas-mediated apoptosis, these cells do not necessarily have to die. An injured oligodendrocyte might be able to provide beneficial support to the damaged spinal cord tissue, where if it were to simply undergo apoptosis as planned, it would offer no support at all.

4.3.4 Additional Supportive Evidence

As previously mentioned, the work presented in this thesis chapter was carried out to complement the work done by Alun Ackery during his MSc. thesis in the laboratory of Dr. Michael Fehlings from 2001 through 2003. Using a similar experimental design to what was presented here, Alun showed that intrathecal administration of sFasR enhances long-term neuron survival following injury as compared to saline controls. As well, behavioural analysis of animals

treated with sFasR and saline over six weeks post-injury reveal that sFasR-treated animals have a significantly improved recovery of locomotor function than do the control animals. Furthermore, retrograde labelling of brain stem neurons shows that sFasR treatment increases the number of axons extending through and beyond the injury site as compared to saline-treated animals.

4.4 Conclusions

The work completed by Alun Ackery and the subsequent work presented in this thesis by Sherri Robins were combined to create a unified and complete picture of the neuroprotective abilities of intrathecal sFasR administration following cervical SCI. The logical next step was to evaluate the clinical relevancy of this therapeutic in the same injury model. The experiments designed, carried out and evaluated in Chapter Five of this PhD thesis embody the steps required to examine the full clinical potential of intrathecal sFasR administration following traumatic SCI.

Chapter Five:

Evaluation of the therapeutic efficacy and clinical relevance of delayed soluble Fas receptor administration following acute traumatic SCI

Please note that the work presented in this chapter is currently being prepared for submission to the Journal of Neurotrauma and will constitute a first-authored paper for Sherri Robins.

5.1 Introduction

Highlighted throughout this work has been the fact that Fas-mediated cell death following acute traumatic SCI offers a solid therapeutic opportunity for clinical translation. The fact that Fas expression was delayed between three and five days post-injury, and that this coincided with a delayed activation of Fas-mediated apoptosis is complimentary to this idea. The three day delay presents a potentially wide window for post-SCI therapeutic intervention, making Fas-mediated cell death a target with great clinical relevance. Patients acquiring a traumatic SCI are most often not seen by a physician qualified or capable of adequately treating the injury for several hours after the initial trauma. This means that therapies showing efficacy in experimental models of injury given right at the time of insult are not clinically valuable. The delayed nature of Fas-mediated apoptosis gives promise of being able to successfully overcome this clinical issue.

The neuroprotective benefit of intrathecal administration of soluble Fas receptor shown in chapter four and in the publication by Ackery, Robins et al.

(2006) provides the foundation for expanding sFasR evaluation as a novel and clinically relevant treatment option for patients suffering from acute SCI.

5.2 Results

Intrathecal administration of sFasR immediately following SCI offered protection to the mature oligodendrocyte population at five days post-injury, as noted by the increased presence of the CNPase protein expression in treated tissue homogenates. This finding formed the basis for the sub-acute evaluation of delayed sFasR treatment efficacy.

5.2.1 Eight-hour delayed sFasR administration maintains ability to enhance oligodendrocyte viability following SCI

An eight hour post-injury delay was selected for its strong clinically relevancy, and was the largest treatment delay to be noted in the current literature. Animals received either sFasR or the control IgG protein via subarachnoid catheterization and mini-pump implantation immediately following SCI or at an eight hour delayed time point. The expression levels of CNPase were then evaluated by Western blot analysis and the treatment averages were compared. The beneficial effect of sFasR treatment on promoting oligodendrocyte survival was observed for a second time in animals treated at the time of injury (t=0 hours), providing further validation to the results presented in chapter four (**Figure 18A**). Examination of the same outcome measure in animals given the eight-hour delayed treatment of either sFasR or IgG showed similar results (**Figure 18B**). An eight hour delay of sFasR treatment did not take away from its ability to improve oligodendrocyte survival at five days post-injury (**Figure 18C**). With these preliminary data in place, the proceeding work was

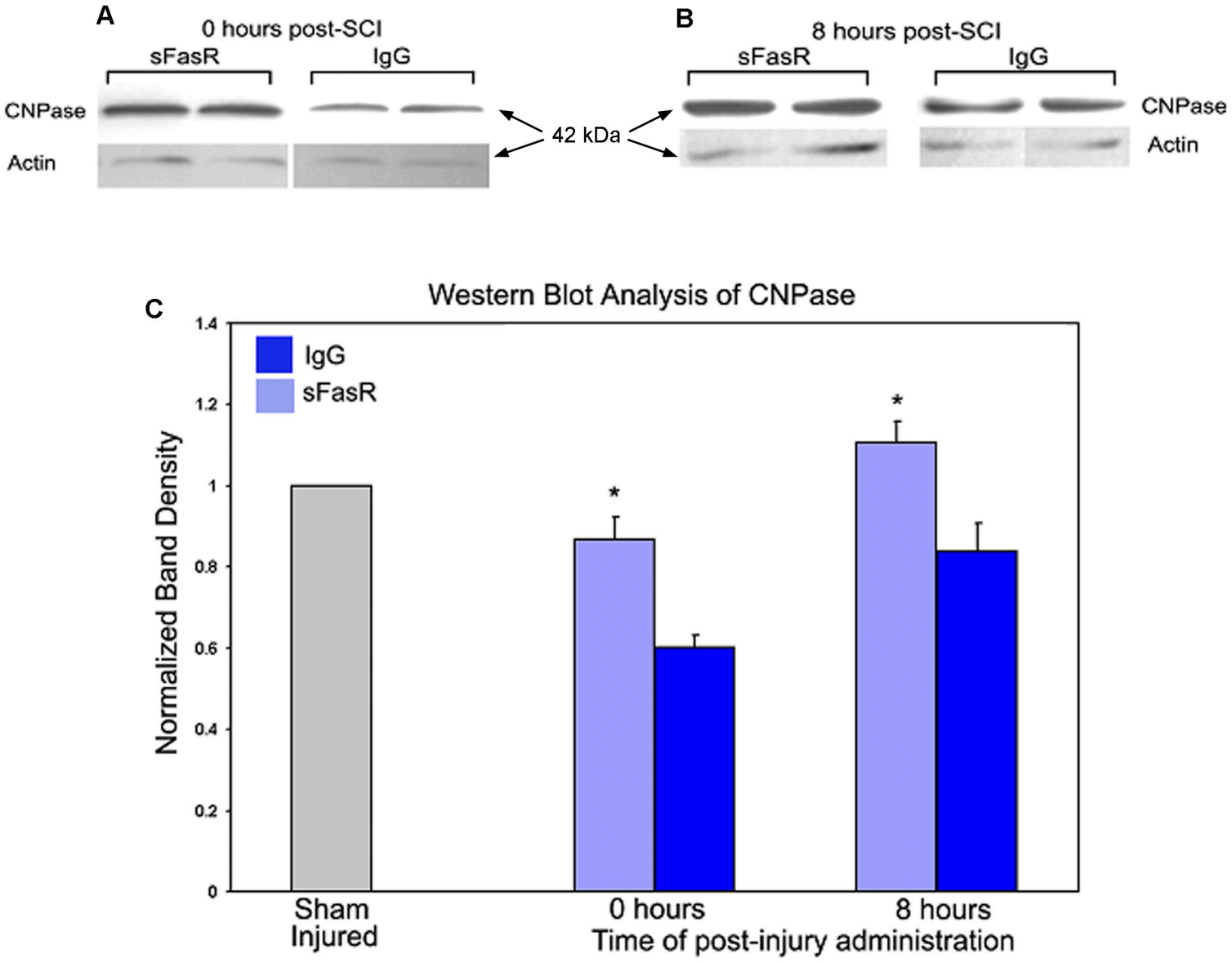


Figure 18: Soluble Fas receptor administration can be delayed by eight hours post-SCI with maintained efficacy

Figure 18: Soluble Fas receptor administration can be delayed by eight hours post-SCI with maintained efficacy

Verification of the effects of post-SCI sFasR administration on oligodendrocyte viability were carried out using Western Blot analysis, as in Figure 17. The impact of delaying the treatment by eight hours post-injury was also carried out in this way. The ability of sFasR administration at the time of injury (t = 0 hours) was verified as previously shown, with an increased presence of CNPase at five days post-injury (A).

The ability to delay the initiation of sFasR administration by eight hours after SCI (t = 8 hours) was determined to have a maintained efficacy as compared to delayed IgG administration (B). The quantification of these results are depicted graphically in C, showing the ability of either t = 0 hour or t = 8 hour sFasR administration to improve oligodendrocyte viability, as compared to IgG controls (error bars = SEM).

These data were statistically analysed separately with regards to post-injury administration using the Student t-test. Statistical significance was achieved in both cases, in which sFasR treatment showed improved CNPase expression, with p-values of $p<0.05$ for each post-injury delay. The values of n for these experiments were n = 3 for the t = 0 hour post-injury delayed administration, n = 5 (sFasR) and 7 (IgG) for the t = 8 hour post-injury delayed administration, and n = 2 for the sham-injured group.

carried out to establish the potential for long-term benefit of delayed sFasR administration post-SCI.

In the development of the chronic experiments, several additional treatment-control groups were added due to some interesting and unexpected findings. Animals were grouped into three basic treatment groups: the main treatment (sFasR), primary treatment-control (IgG), and an additional secondary control (saline). The sFasR and IgG treatment groups were thought to be the most important animals for this analysis, and therefore each of these treatments was further broken down into both an eight hour and a 24 hour post-injury delayed group. The saline-treated animals acted as a secondary control, with saline administration initiated at the time of injury.

All animals were followed for six weeks following injury and pump implantation as was previously described (**Figure 6D**). At the end of the study following spinal cord and brain extractions, the six weeks of behavioural analysis using BBB scoring was analysed. It was at this point that unexpected results were observed.

5.2.2 IgG administration exerts surprising, beneficial effects following SCI

In the eight hour delayed groups, differences in improvement of hind-limb motor function were not apparent between the sFasR-treated animals and the IgG control-treated animals, however there was a marked reduction in the behavioural recovery observed between the saline-treated animals and the sFasR and IgG groups (**Figure 19A**). Following examination of the 24 hour delayed treatment groups, a similar observation was had: Both the sFasR- and

IgG-treated animals had a significantly improved behavioural recovery when compared to the secondary saline-control animals. In fact, while not statistically significant, the 24 hour delayed IgG treatment group had a stronger recovery profile than did the sFasR group (**Figure 19B**).

These results were surprising and introduced the need to revaluate the experimental design for the evaluation of delayed sFasR administration. It was decided that another protein control group would be added to these experiments to distinguish between a non-specific protein effect and an effect which was specific to IgG. BSA (bovine serum albumin) was selected as the secondary protein control for these experiments due to its benign and non-reactive properties. BSA treatment groups were also divided, with one group receiving intrathecal BSA at the eight hour time-point post-injury and the other group with administration starting at 24 hours post-SCI. The complete summary of treatment groups can be found in **Table 6**.

Due to the unknown beneficial effects observed with delayed IgG treatment, it was thought best to remove these animals from the presentation of the results for sFasR efficacy to avoid confusion and to more clearly present the findings as they relate to the main purposes of this thesis. The next thesis chapter (Chapter 6) will serve to present the data showing IgG's protective effects. More insight and information regarding this topic will be addressed in Chapter 7.

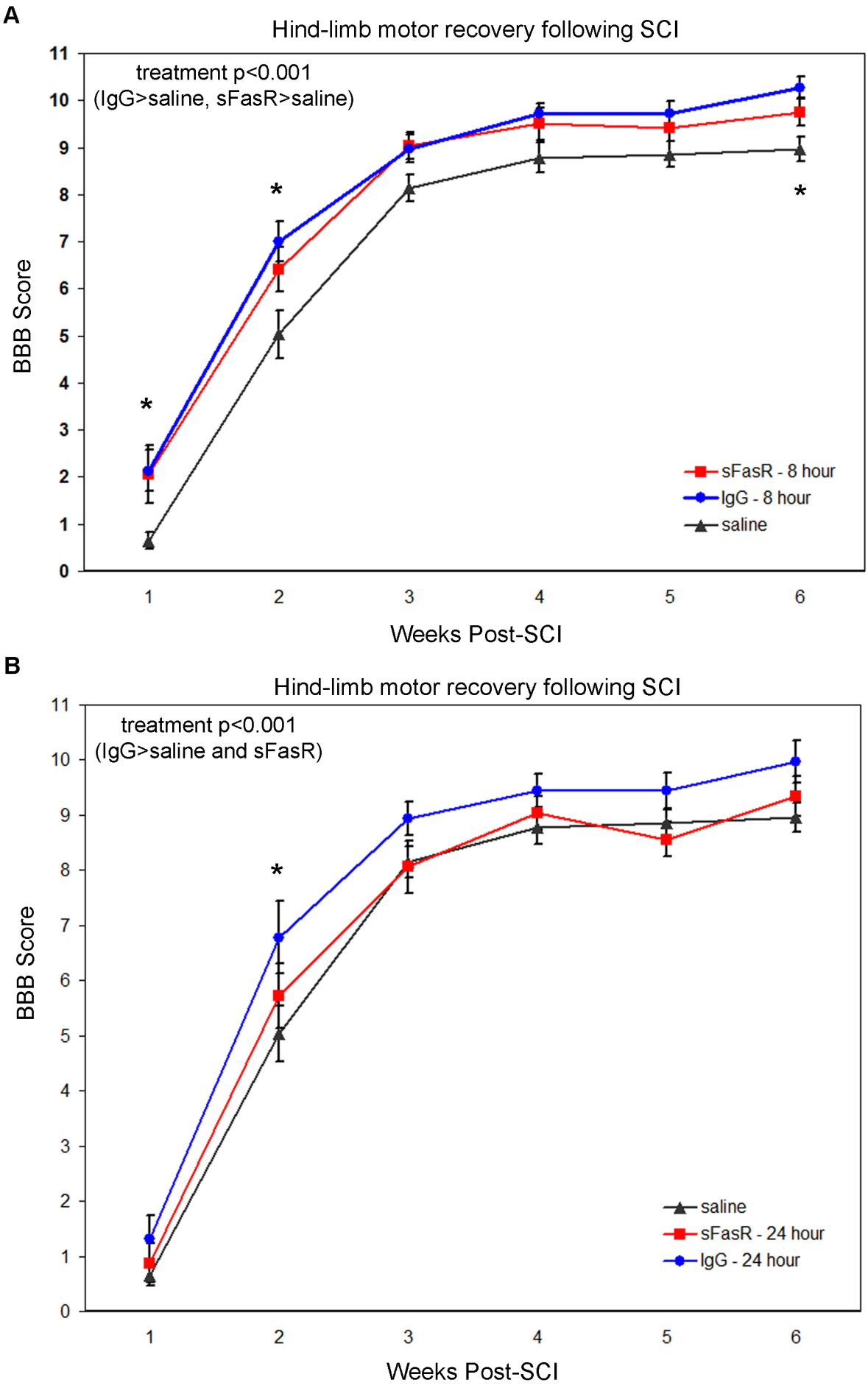


Figure 19: IgG administration exerts surprising, beneficial effects following SCI

Figure 19: IgG administration exerts surprising, beneficial effects following SCI

Hind-limb motor recovery was evaluated weekly using the BBB scoring system in animals treated with either delayed sFasR, delayed IgG or saline administration following experimental spinal cord injury. The ability of eight-hour or 24-hour delayed post-injury administration was being evaluated, when it became apparent that the IgG treatment control was offering the same, or improved, efficacy as sFasR, when both were compared to saline controls.

Graph A shows the recovery curves associated with an eight-hour treatment delay in sFasR and IgG administration, compared to the saline control (error bars ± SEM). It was found that there were no significant differences between sFasR and IgG animals, however both were significantly better than the saline-treated animals. Statistical analysis of these data was carried out using a two-way ANOVA, where the overall impact of treatment achieved a p-value of $p<0.001$. Post-hoc analysis using the Holm-Sidak method confirmed that both sFasR and IgG-treated animals showed better recovery than saline-treated controls at a p-value of $p<0.001$. There was no statistical difference between sFasR and IgG treated animals. Furthermore, the differences between sFasR and saline and IgG and saline were noted specifically at one, two and six weeks post-injury with p-values of $p<0.05$ for each time-point.

Graph B shows the recovery curves associated with a 24-hour treatment delay in sFasR and IgG administration, compared to the saline control (error bars ± SEM). It was determined that sFasR was less effective at with this delay in administration than was IgG, when compared to saline. Statistical analysis was performed as noted above, there was an overall treatment effect giving a p-value of $p<0.001$, where post-hoc analysis revealed that IgG was significantly better than both saline and sFasR overall ($p<0.001$). In addition, the improved effects had by IgG treatment were noted specifically at two weeks post-SCI ($p<0.05$).

The values of n for these experiments were as follows: 8-hour sFasR had an $n = 16$, 8-hour IgG had an $n = 18$, 24-hour sFasR had an $n = 18$ and saline-treated group had an $n = 14$.

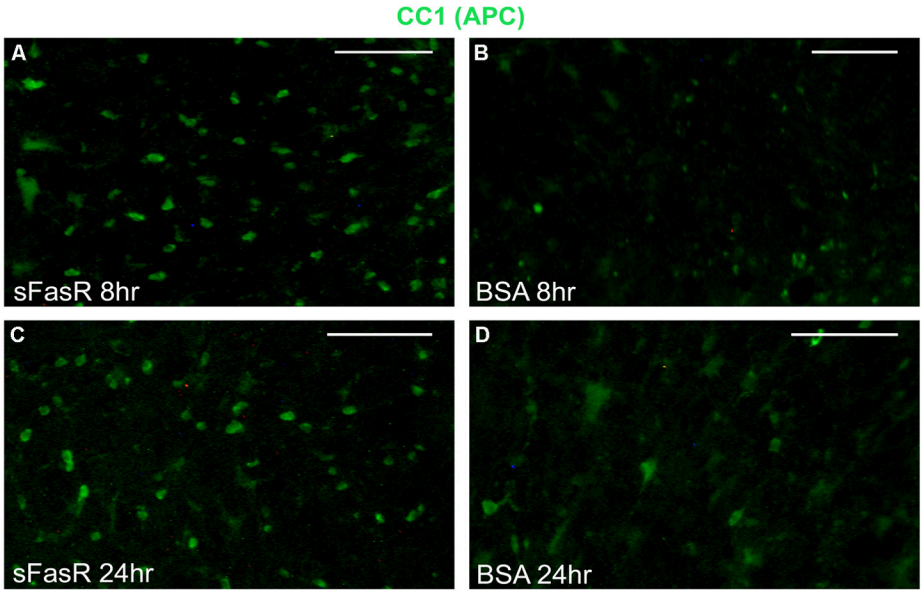
Table Six: Treatment Groups for Delayed Treatment Administration

Treatment Group	Molecule Administered	Time of Post-SCI Delay	Final n
8 hour sFasR	sFasR	8 hours	16
24 hour sFasR	sFasR	24 hours	18
8 hour IgG	IgG	8 hours	18
24 hour IgG	IgG	24 hours	18
8 hour BSA	BSA	8 hours	8
24 hour BSA	BSA	24 hours	10
Saline Control	Saline	0 hours	14

5.2.3 Delayed sFasR administration improves long-term oligodendrocyte survival following SCI

To evaluate the impact of delayed sFasR administration on cell survival, quantification of the total number of oligodendrocytes in rostral cord sections was carried out at seven weeks post-injury. Tissue sections from approximately 700μm rostral to the injury site were selected because of the high level of apoptotic cell death known to occur in the rostral cord segments. With the knowledge that sFasR has anti-apoptotic effects, it was decided that this area might offer the best depiction of therapeutic benefit.

The eight hour treatment groups of sFasR and BSA and the 24 hour treatment groups of sFasR and BSA were analyzed independently, with the secondary saline control as a common comparison. In evaluating the ability of sFasR administration to be delayed by eight hours post-injury, it was revealed that increased oligodendrocyte survival was possible given the treatment delay. sFasR-treated animals had significantly more oligodendrocytes at seven weeks post-injury than did the BSA and saline-treated controls (**Figure 20 A, B and E)**. The results of oligodendrocytes survival in the 24 hour delayed groups were also positive. Treatment with sFasR was able to increase the survival of oligodendrocytes when delayed by 24 hours post-injury, and this impact was significantly greater than both BSA and saline controls (**Figure 20 C, D and E**). This analysis provides evidence to suggest that delayed administration of sFasR is able to effectively maintain oligodendrocyte viability, providing a large window for therapeutic intervention post-SCI.



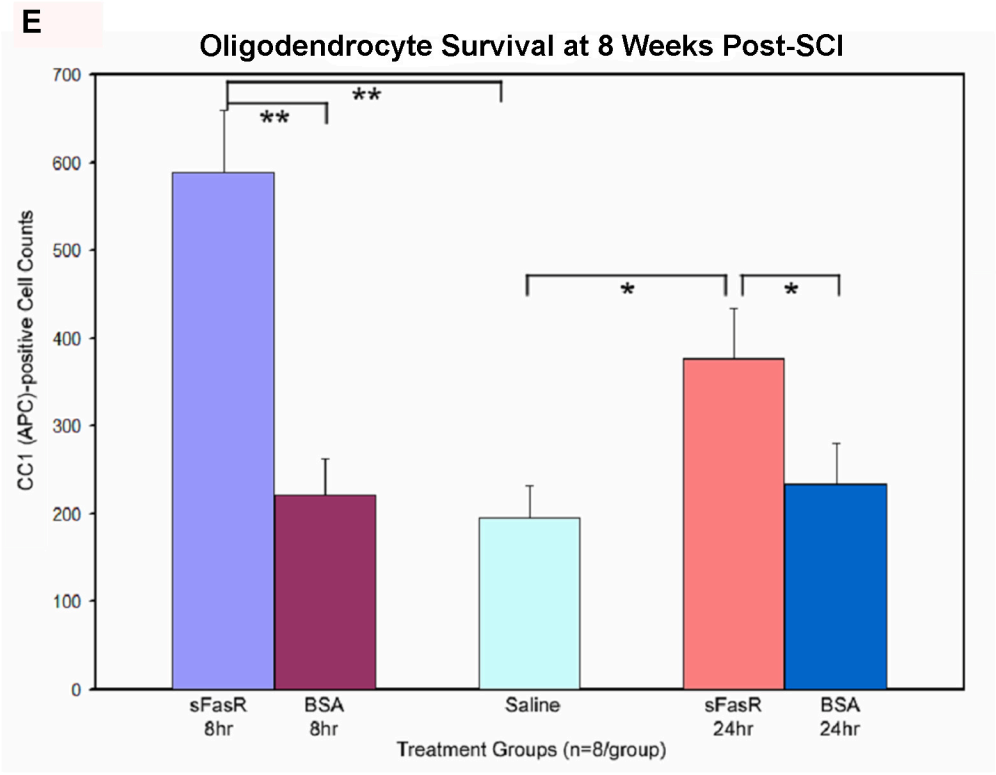


Figure 20: Delayed sFasR administration enhances long-term oligodendrocyte survival post-SCI

Figure 20: Delayed sFasR administration enhances long-term oligodendrocyte survival post-SCI

The impact of delayed sFasR administration following SCI was evaluated using tissue sections stained for oligodendrocytes (using CC1/APC) taken from approximately 700µm rostral to the injury epicenter at eight weeks post-injury. The number of CC1-positive cells on each tissue cross-section was quantified and an average was calculated for each treatment group. Images A and B are representative images of tissue sections from sFasR and BSA-treated animals with an eight-hour post-SCI delay in administration, respectively (scale = 75µm). Images C and D are representative images of tissue sections from sFasR and BSA-treated animals with a 24-hour post-SCI delay in administration, respectively (scale = 75µm). It was observed in both treatment delay groups that there were a greater number of oligodendrocytes in the sFasR-treated animals as compared to the BSA-treated animals.

Quantification of the total number of oligodendrocytes in each group are displayed graphically in E (error bars = SEM). Saline-treated animals were used as a secondary control for both eight-hour and 24-hour delayed groups. Statistical analysis of the data was carried out using a one-way ANOVA on the eight-hour treatments and then on the 24-hour treatment groups. It was confirmed that sFasR administration delayed by eight-hours post-SCI was significantly better than both eight-hour delayed BSA and saline ($p<0.001$). In addition, 24-hour delayed sFasR administration was significantly better than both 24-hour delayed BSA and saline ($p<0.05$). Furthermore, the difference in oligodendrocyte survival between the eight-hour delayed sFasR group and the 24-hour delayed sFasR group was significant at a level of $p=0.034$. Post-hoc analysis to determine the specific treatment effects was carried out using the Student Neuman-Keuls method. In all treatment groups, there was an $n = 8$.

5.2.4 Delayed sFasR administration improves long-term neuronal survival following SCI

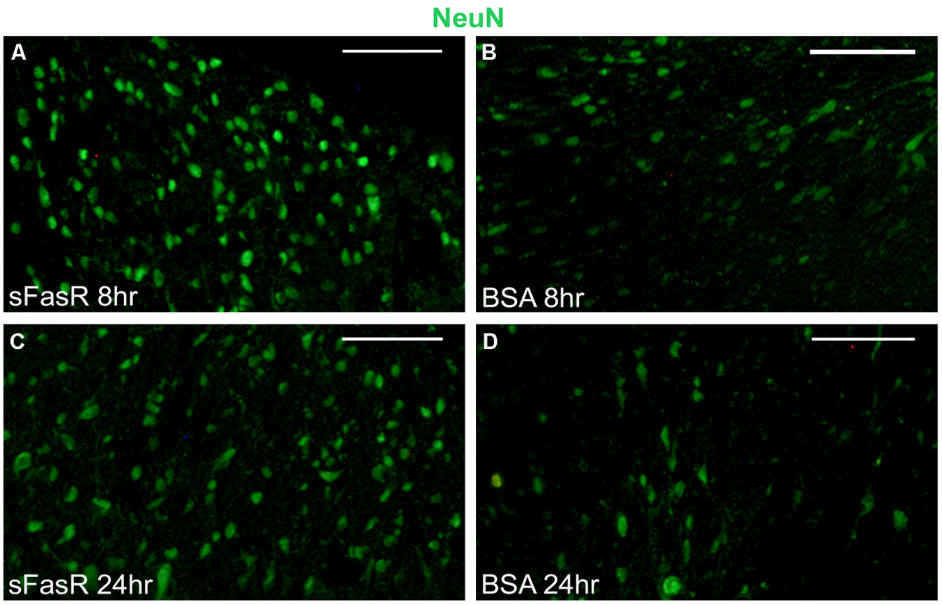
The impact of delayed sFasR administration on the viability of neurons at seven weeks post-SCI was also evaluated. In the same methods used to evaluate oligodendrocyte survival, serial tissue sections were collected and stained with the neuron-specific marker NeuN and were quantified based on co-expression with DAPI.

The effect of eight hour delayed sFasR administration on chronic neuron survival was positive. Tissue sections from sFasR-treated animals showed a significantly higher number of NeuN-positive neurons than did either of the control groups (**Figure 21 A and B**). Moreover, the ability to delay sFasR treatment by 24 hours post-SCI with maintained efficacy was also observed. The numbers of neurons found in animals treated with 24 hour delayed sFasR were significantly higher than in those animals treated with 24 hour delayed BSA or saline (**Figure 21 C, D and E**).

In summary, the ability to positively influence cell survival with delayed administration of sFasR by eight hours and 24 hours is strong. In both cases, sFasR-treated animals had substantial improvements in both oligodendrocyte and neuron survival when compared to BSA- and saline-treated controls.

* * *

Traumatic compressive SCI causes an histologically apparent reduction in tissue morphology and integrity. There is a marked demyelination of white matter following this type of injury, and a heavily necrotic lesion early on ultimately leads to a high level of inflammatory infiltrate and formation of a cystic



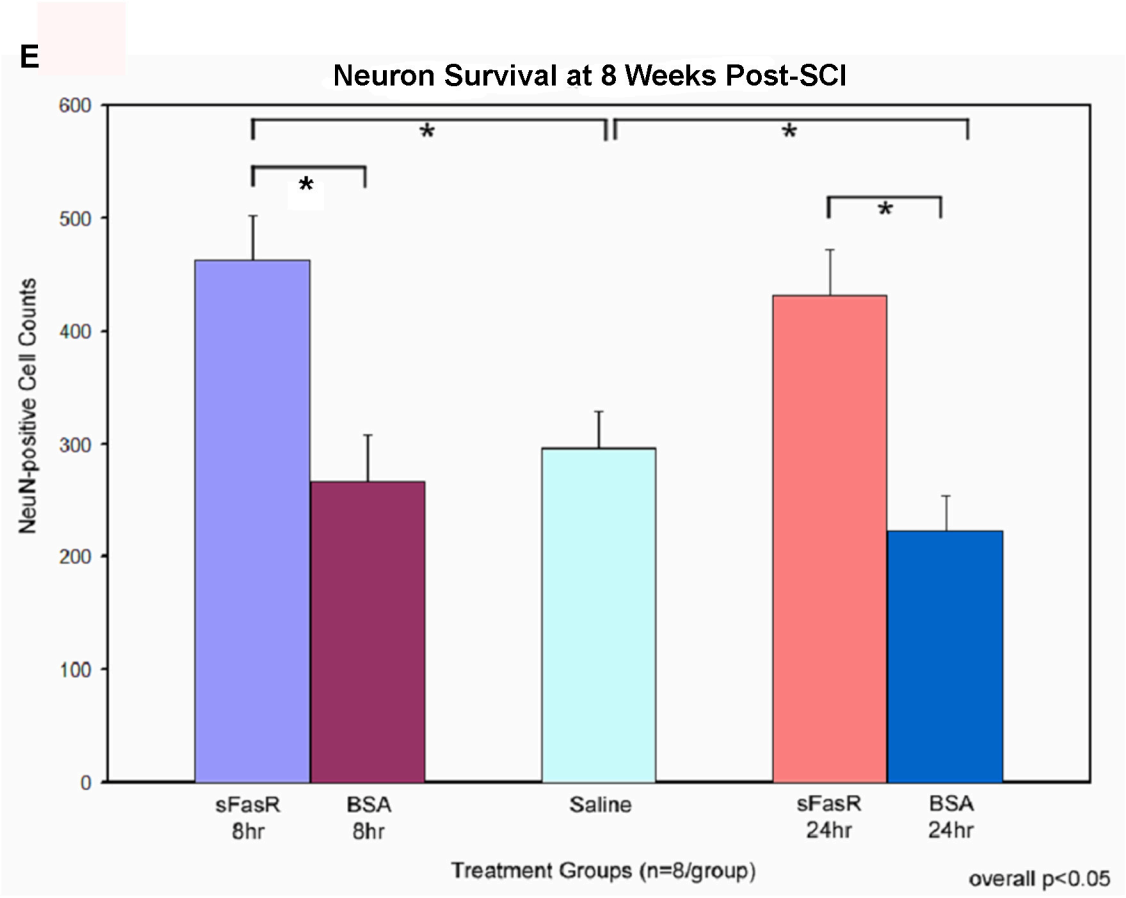


Figure 21: Delayed sFasR administration improves long-term neuron survival following traumatic SCI

Figure 21: Delayed sFasR administration improves long-term neuron survival following traumatic spinal cord injury

The impact of delayed sFasR administration following SCI was evaluated using tissue sections stained for neurons (labelled with NeuN) taken from approximately 700μm rostral to the injury epicenter at eight weeks post-injury. The number of NeuN-positive cells on each tissue cross-section was quantified and an average was calculated for each treatment group. Images A and B are representative images of tissue sections from sFasR and BSA-treated animals with an eight-hour post-SCI delay in administration, respectively (scale = 75μm). Images C and D are representative images of tissue sections from sFasR and BSA-treated animals with a 24-hour post-SCI delay in administration, respectively (scale = 75μm). It was observed in both treatment delay groups that there were a greater number of neurons in the sFasR-treated animals as compared to the BSA-treated animals.

Quantification of the total number of neurons in each group are displayed graphically in E (error bars = SEM). Saline-treated animals were used as a secondary control for both eight-hour and 24-hour delayed groups. Statistical analysis of the data was carried out using a one-way ANOVA on the eight-hour treatments and then on the 24-hour treatment groups separately. It was confirmed that sFasR administration delayed by eight-hours post-SCI was significantly improved neuron survival compare to both eight-hour delayed BSA and saline ($p<0.05$). In addition, 24-hour delayed sFasR administration was significantly better than both 24-hour delayed BSA and saline ($p<0.05$). Post-hoc analysis to determine the specific treatment effects was carried out using the Student Neuman-Keuls method. In all treatment groups, there was an $n = 8$.

cavity. The ability of potential therapies to reduce the degradation of tissues, and to prevent the growth of the lesion cavity are worthwhile outcome measures to assess. Reductions in tissue loss may ultimately contribute to a more functional and permissive environment.

5.2.5 Delayed sFasR administration improves grey matter preservation and reduces cavity formation following SCI

Spared grey matter area, as a function of the total section area, was analysed in both rostral and caudal tissue sections and at the injury epicenter itself at approximately seven weeks post-injury. In addition, the size of cystic cavity present in the injured spinal cords was measured in this way.

In evaluating the effects of eight hour delayed sFasR treatment, it was found that both grey matter tissue preservation and cavity-size showed beneficial outcomes. There was an overall significant increase in area of intact, healthy-looking grey matter, characterized by a lack of necrotic and inflammatory infiltrate and an appropriately consistent eosinophilic staining pattern within tissues treated with sFasR as compared to BSA and saline controls. Additionally, the extent of cavity formation was reduced in the eight hour delayed sFasR-treated animals (**Figure 22**).

Tissue preservation and cavity formation were also evaluated in the 24 hour delayed treatment groups. Here, grey matter tissue integrity at seven weeks post-SCI was improved by delayed sFasR treatment, with overall significant increases in the intact tissue area, and specifically at areas in the rostral cord segments. BSA-treated and saline-treated control tissue sections showed a reduction in preserved grey matter areas compared with the sFasR-

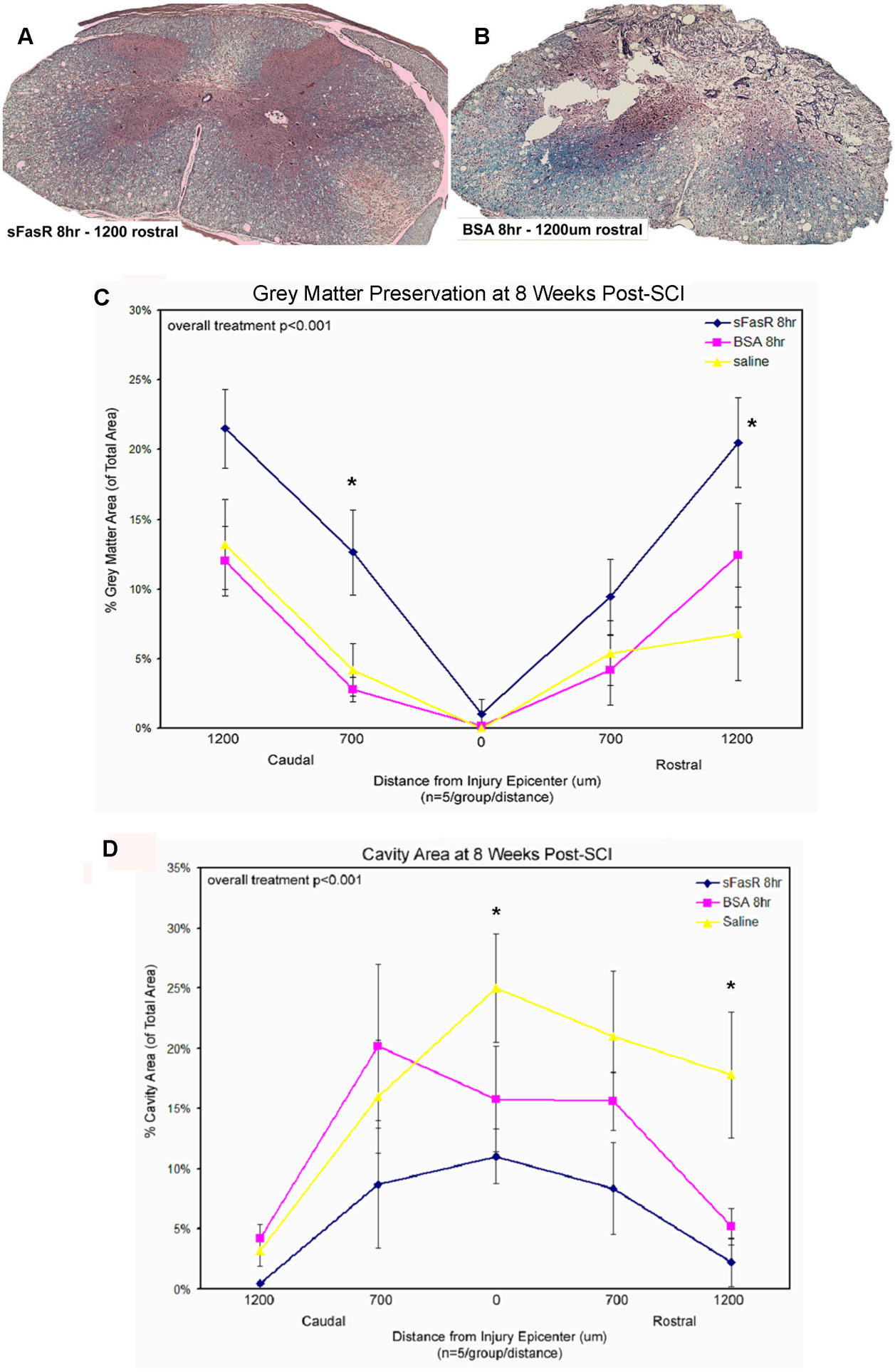


Figure 22: Eight hour delayed sFasR enhances tissue preservation following SCI

Figure 22: Eight-hour delayed sFasR enhances tissue preservation following SCI

The impact of eight-hour delayed sFasR administration post-SCI was evaluated using histologically stained tissue sections taken from approximately 1200μm and 700μm rostral and caudal to the injury epicenter, and from the injury epicenter itself at eight weeks post-injury. The tissue morphology and neuroanatomical structure of the tissue sections was evaluated using H and E with LFB staining.

Representative tissue sections taken from approximately 1200μm rostral to the injury epicenter from sFasR and BSA-treated animals are shown in A and B, respectively. Quantification of the percent grey matter preservation (of the total section area) was carried out and the results are shown graphically in C (error bars ± SEM). As compared to both controls, eight-hour delayed sFasR administration provided an improvement in grey matter preservation, especially at 700μm caudal and 1200μm rostral to the injury epicenter. Quantification of the percent cavity formation (of the total section area) was carried out and the results are shown graphically in D (error bars ± SEM). Again when compared to both controls, eight-hour delayed sFasR administration reduced the cavity size, specifically at the epicenter and at approximately 1200μm rostral to the injury epicenter.

Each set of data were subjected to a two-way ANOVA where eight-hour delayed sFasR administration effects on grey matter preservation and cavity size produced p-values of $p<0.001$ in each case. Post-hoc analysis using the Holm-Sidak method revealed the specific time-points of significance, each at a p-value level of $p<0.05$. There were n's of 5 used for each treatment group and set of analysis.

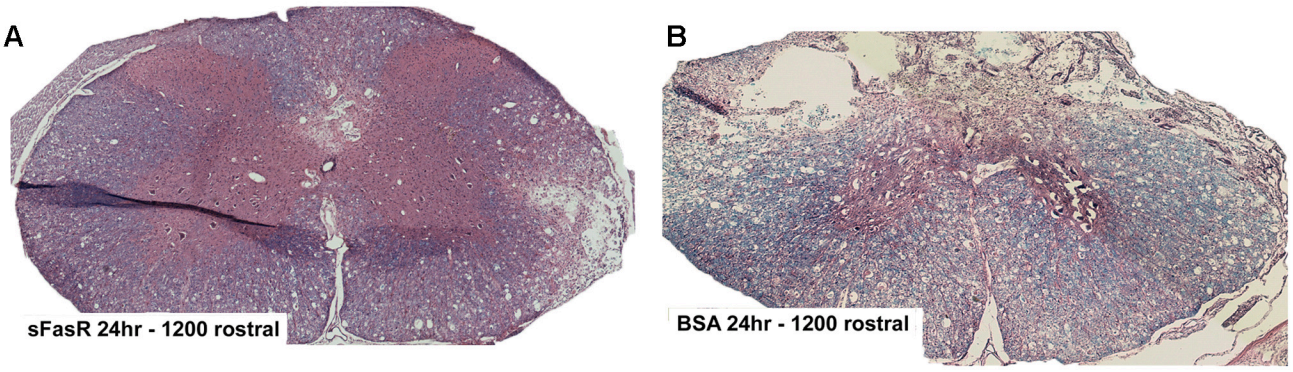
treated group. In terms of cavity formation, 24 hour delayed sFasR reduced cavity size overall, however significance was not reached during post-hoc analysis of the spatial relationship along the injured cord (**Figure 23**).

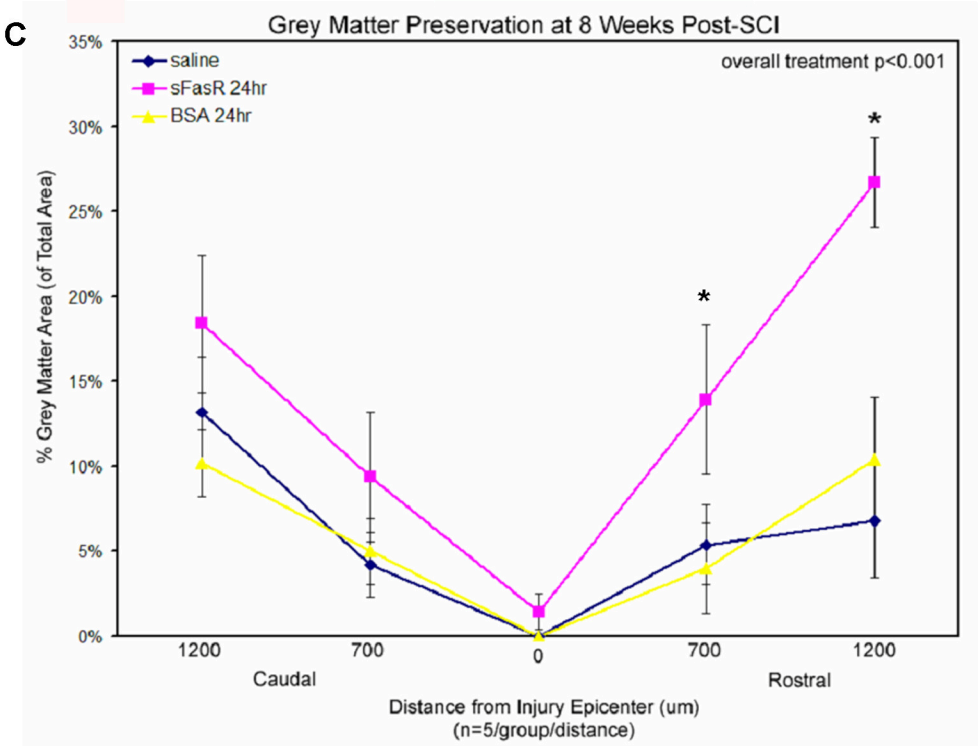
5.2.6 Eight-hour delayed sFasR administration effectively improves hind-limb motor function after SCI

Functional motor recovery was evaluated in all treatment groups for six weeks following injury and pump implantation. Examination of delayed sFasR impact on hind-limb function was carried out using the BBB (Basso, Beattie, Bresnahan) open field locomotor rating scale.

An eight hour post-injury delay in the initiation of sFasR treatment did not prevent its ability to infer positive effects. When the overall BBB scores obtained by animals treated with delayed sFasR were compared to those in the BSA and saline control groups, there was a significant improvement in motor scores in those animals treated with sFasR (**Figure 24 A**). Furthermore, post-hoc analysis of treatment effects at each weekly time points revealed that animals treated with eight hour delayed sFasR showed a statistically significant improvement at one week post-SCI, as compared to both BSA and saline groups.

Behavioural recovery was also evaluated in the animals receiving 24 hour delayed treatments using the BBB scoring system. Twenty-four hour delayed treatment of sFasR improved functional recovery in comparison to those animals treated with BSA, however there was little difference between it and the saline-treated group (**Figure 24B**). The lost impact on behavioural function observed with 24 hour delayed sFasR was disappointing however there was a modest beneficial impact maintained with such a wide post-injury timeline.





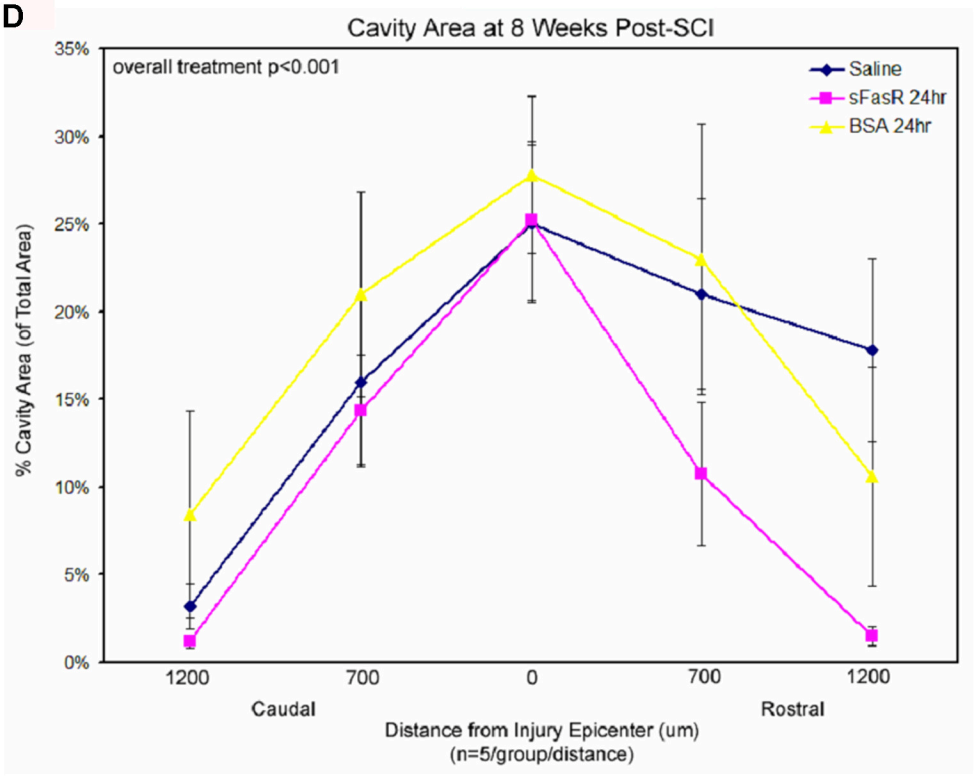


Figure 23: Twenty-four hour delayed sFasR administration offers a modest improvement in tissue preservation post-SCI

Figure 23: Twenty-four hour delayed sFasR administration offers a modest improvement in tissue preservation post-SCI

The impact of 24-hour delayed sFasR administration post-SCI was evaluated using histologically stained tissue sections taken from approximately 1200μm and 700μm rostral and caudal to the injury epicenter, and from the injury epicenter itself at eight weeks post-injury. The tissue morphology and neuroanatomical structure of the tissue sections was evaluated using H and E with LFB staining.

Representative tissue sections taken from approximately 1200μm rostral to the injury epicenter from sFasR and BSA-treated animals are shown in A and B, respectively. Quantification of the percent grey matter preservation (of the total section area) was carried out and the results are shown graphically in C (error bars ± SEM). As compared to both controls, 24-hour delayed sFasR administration provided an improvement in grey matter preservation, especially at 700μm and 1200μm rostral to the injury epicenter. Quantification of the percent cavity formation (of the total section area) was carried out and the results are shown graphically in D (error bars ± SEM). Again when compared to both controls, there was an overall treatment effect but no specific significant time points where 24-hour delayed sFasR administration reduced the cavity size.

Each set of data were subjected to a two-way ANOVA where 24-hour delayed sFasR administration effects on grey matter preservation and cavity size produced overall p-values of $p<0.001$ in each case. Post-hoc analysis using the Holm-Sidak method revealed the specific time-points of significance for grey matter tissue preservation, each at a p-value level of $p<0.05$. There was no statistical result found in post-hoc analysis of cavity formation. There were n's of 5 used for each treatment group and set of analysis.

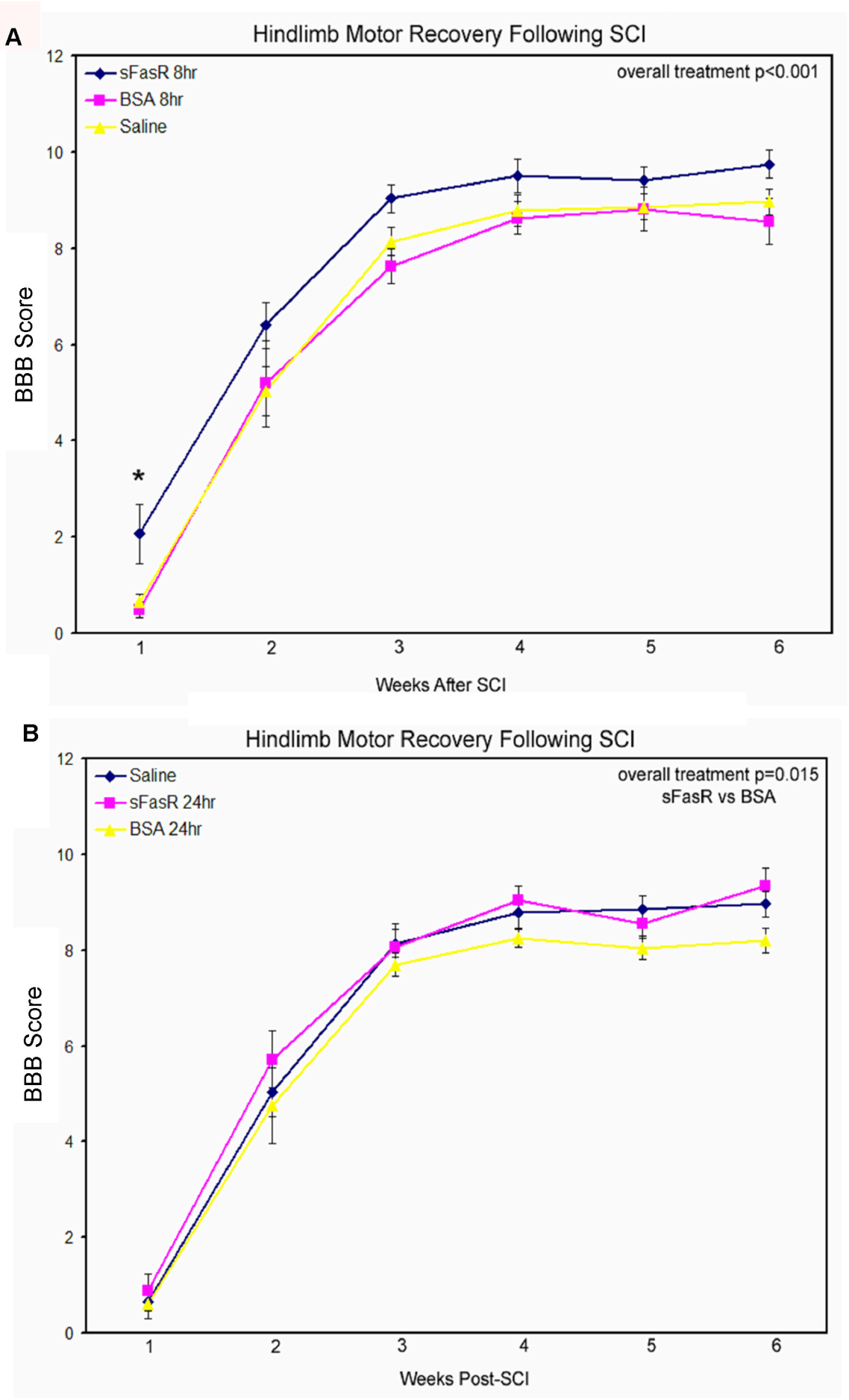


Figure 24: Eight-hour delayed sFasR offers significant behavioural recovery effects, while 24-hour delayed sFasR administration offers a modest effect

Figure 24: Eight-hour delayed sFasR offers significant behavioural recovery effects, while 24-hour delayed sFasR administration offers a modest effect

The effect of delayed post-injury sFasR administration was evaluated using weekly hind-limb motor recovery observation and BBB scoring. The graphical representation of the behavioural recovery data for the eight-hour delayed groups is shown in A (error bars ± SEM). Here, eight-hour delayed sFasR treatment provided improved motor recovery when compared to BSA and saline controls. A slightly reduced effect was seen in the 24-hour delayed groups, shown graphically in B (error bars ± SEM). Animals given 24-hour delayed sFasR administration showed only a modest improvement in locomotor recovery as compared to the BSA-treated controls.

The eight-hour and 24-hour data were subjected to separate two-way ANOVA, with the saline-treated group acting as a secondary control in both analyses. The overall treatment effect in the eight-hour delayed group achieved a p-value of $p<0.001$, where sFasR showed improvement over both BSA and saline-treated animals. Post-hoc analysis using the Holm-Sidak method showed further significance for sFasR benefit at one week post-SCI ($p<0.05$). The overall treatment effect in the 24-hour delayed group had a p-value of $p=0.015$, where sFasR treatment was better than BSA only. Post-hoc analysis revealed no specific post-injury time points of statistical interest. The values of n for these experiments were as follows: 8-hour sFasR had an n = 16, 8-hour BSA had an n = 8, 24-hour sFasR had an n = 18, 24-hour BSA had an n = 10 and the saline-treated group had an n = 14.

5.2.7 Neuropathic pain development is not significantly altered with delayed sFasR administration post-SCI

The value of any experimental therapeutic in a clinical setting is dependent on its ability to infer therapeutic benefit. In addition, it is important to ensure that adverse reactions are not part of the process. The development of neuropathic pain is a serious problem experienced by patients with SCI. The pain experience by these individuals can be extremely debilitating and can significantly reduce quality of life. Research is ongoing to figure out the complex processes involved in the development of neuropathic pain following SCI, and there is a strong hope that treatment options specifically targeting pain mechanisms will be developed. In terms of the research presented here, it is important to evaluate whether or not treatment with intrathecal sFasR administration exacerbates the existing occurrence of neuropathic pain.

A common form of neuropathic pain following SCI is the development of mechanical allodynia. The manifestation of this type of pain comes from changes in the perceived pain response from a stimulus. Following SCI, a normally benign stimulus like a gentle touch on the leg, can elicit a tremendously painful response. In effect, a stimulus that was once tolerable becomes extremely noxious. This type of pain response is evaluated following experimental SCI through the use of vonFrey filaments, calibrated fibres of various thickness and strength. The details of this procedure were discussed more specifically in chapter two of this thesis.

Following bi-weekly assessment using vonFrey filaments, it was observed that none of the treatment groups showed a significant alteration, either

beneficial or deleterious, to the normal manifestations of pain responses in the animals (**Figure 25**).

5.3 Discussion

5.3.1 Summary of Findings

The data presented in this thesis chapter provide for the first time a clinically relevant and therapeutically efficacious treatment strategy for targeting Fas-mediated cell death following spinal cord injury. It has been shown that sFasR treatment can be delayed by as much as 24 hours post-injury and still have the maintenance of modest therapeutic effects, while an eight hour delay is capable of offering an even greater overall benefit. Both neuroanatomical and functional improvements were observed in animals treated with sFasR delayed by eight hours post-SCI, and this was mirrored with long-term increases in both oligodendrocyte and neuron survival.

5.3.2 Rationale

Given the lack of clinically available treatment options for patients suffering from SCI, there is a strong need for novel therapeutic strategies. The ability to delay the initiation of treatment administration in a valid post-injury time window is crucial and presents a challenge in the development of these types of therapies. Currently, the only one acute SCI therapy in wide clinical use is the corticosteroid methylprednisolone. There is an accepted amount of caution considered with the use of methylprednisolone, as there has been a number of controversial issues raised in relation to its limited efficacy and the high risk of potential adverse effects in the critical care of SCI patients. With this, the need

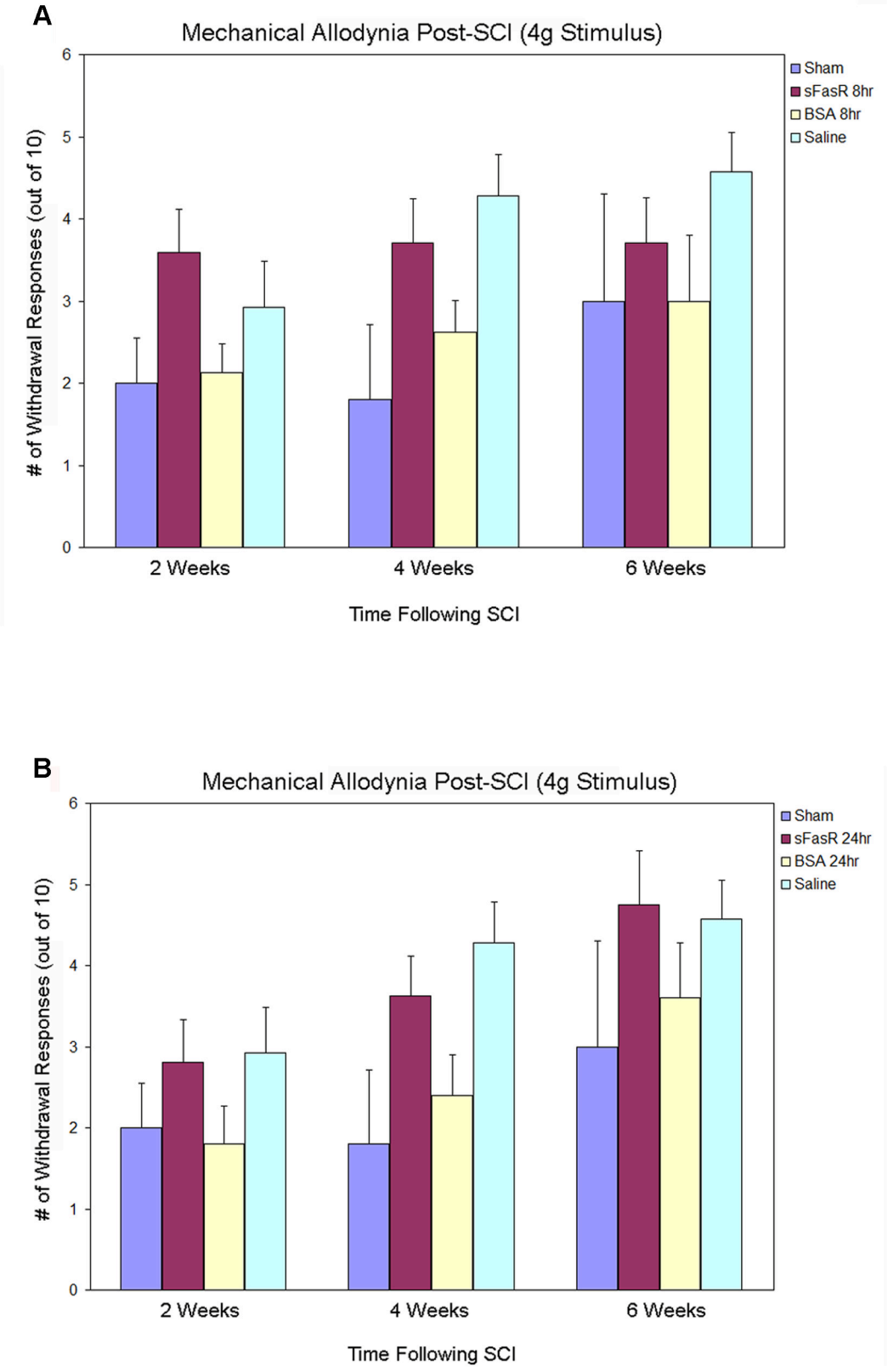


Figure 25: Delayed sFasR administration does not alter the development of neuropathic pain following SCI

Figure 25: Delayed sFasR administration does not alter the development of neuropathic pain following SCI

The impact of delayed post-injury sFasR administration on the development of neuropathic pain, specifically mechanical allodynia, following SCI was evaluated bi-weekly using vonFrey filaments. The number of withdrawal responses upon stimulation by the 4g vonFrey filament around the level of injury was reported and averaged for each treatment group. There was no significant change in the development of mechanical allodynia after SCI in any of the treated animals, in either of the delayed administration groups. These results are displayed graphically in A (eight-hour delay) and B (24-hour delay), error bars = SEM.

The data were analyzed for statistical differences using a one-way ANOVA at each bi-weekly time point after injury and gave the following p-values:
At two weeks post-injury, the eight-hour delayed group p-value was p=0.208 and

the 24-hour delayed group p-value was 0.502 (note that normality failed in the 2 week, 24-hour delayed group, so a Kruskall Wallis ANOVA on ranks was performed).

At four weeks post-injury, the eight-hour delayed group p-value was p = 0.081 (note that normality failed in the 4 week, eight-hour delayed group, so a Kruskall Wallis ANOVA on ranks was performed) and the 24-hour delayed group p-value was p = 0.029 (post-hoc analysis revealed no significant differences between groups).

At six weeks post-injury, the eight-hour delayed group p-value was p = 0.328 and the 24-hour delayed group p-value was p = 0.339.

The values of n for these experiments were as follows: 8-hour sFasR had an n = 16, 8-hour BSA had an n = 8, 24-hour sFasR had an n = 18, 24-hour BSA had an n = 10, the saline-treated group had an n = 14 and the sham-injured group had an n = 5.

for developing better and more reliable treatment options for patients suffering from SCI is obvious.

To date, several groups have been successful in targeting Fas-mediated apoptosis following experimental SCI. Studies using the *lpr* mutant mouse have shown that absence of Fas signalling post-injury has an important beneficial effect. The absence of a functional Fas receptor reduces post-traumatic apoptotic cell death, increases survival of oligodendrocytes and neurons and significantly improves locomotor recovery as compared to wildtype littermates (Yoshino, Matsuno et al. 2004; Casha, Yu et al. 2005).

Neutralization of Fas ligand with systemic injection of an antagonistic antibody following dorsal hemi-section injury in a mouse model of SCI proved to be beneficial in several respects. Mice receiving the neutralizing antibody show enhanced behavioural recovery and cell survival, and display an improved regenerative capacity than that observed in the control-treated mice (Demjen, Klussmann et al. 2004). Published work presented in the third chapter of this thesis show that intrathecal administration of a soluble Fas receptor therapeutically targets SCI-induced apoptosis, resulting in improved survival of oligodendrocytes and neurons, enhancing tissue preservation and improving locomotor recovery in a highly relevant cervical compression model of SCI (Ackery, Robins et al. 2006).

The relevancy of Fas-mediated apoptosis as a therapeutic target after acute SCI is appreciated. However, the clinical applicability of the previously

mentioned treatment options is lacking, greatly reducing the potential for success in clinical translation of these findings into the SCI patient population.

5.3.3 Impact of Delayed sFasR Administration on Cell Survival Post-SCI

The significance of enhanced cell survival following traumatic SCI is important. Not only does an increase in the number of surviving cells indicate a reduction in cell death, it also alludes to the possibility that a more supportive post-injury environment was established and maintained throughout the secondary injury process. In the work presented here, delayed intrathecal soluble Fas receptor administration increased the chronic survival of both oligodendrocytes and neurons. In the evaluation of cell viability at such a late time point, it is a challenge to propose to what extent the inhibition of apoptosis influenced the results. It is possible that an early anti-apoptotic effect provided the means for a more supportive environment to be had, thereby compounding the cell survival potential. With the previous work highlighted in chapter four showing the anti-apoptotic actions of sFasR treatment post-SCI, there is some credibility to this concept. This can be more readily understood in the evaluation of oligodendrocyte survival, given that it has been shown in chapter three that the oligodendrocyte population predominantly expressed Fas and underwent Fas-mediated apoptosis in the first week following injury.

The observed improvement in neuronal viability with delayed sFasR treatment is not as easily understood as that of the oligodendrocyte population. Soluble Fas receptor must exert its main anti-apoptotic effect by competitively inhibiting Fas and FasL binding, preventing subsequent activation of the Fas

pathway in the receptor-bound cells. With the knowledge that neurons do not predominantly express Fas receptor following SCI and in addition, that they have the inherent ability to express inhibitory proteins against Fas-mediated apoptosis, the therapeutic actions of sFasR on neurons is intriguing. Enhancement of supportive glial survival, specifically the oligodendrocyte population, could very well maintain a supportive environment following trauma that may contribute to neuron and axonal health and viability. This theory will be expanded upon in Chapter 7.

Delaying sFasR administration by either eight hours or 24 hours post-injury did not alter the therapeutic capability to enhance long-term survival of both oligodendrocytes and neurons, as compared to BSA and saline controls. It should be noted that while the beneficial impact of 24 hour delayed sFasR treatment on oligodendrocyte cell counts was significant compared to controls, the eight hour delay showed a more significant efficacious effect. The same trend was not apparent in the evaluation of neuron survival, where the strength of efficacy was similar between the eight hour and 24 hour groups. This raises an interesting point in that the earlier initiation of sFasR treatment had an apparently stronger impact on oligodendrocyte survival than it did on neuronal survival. Considering the Fas-mediated anti-apoptotic effects of sFasR, one might offer the following explanation for this observation: an early attenuation of Fas-mediated apoptosis in oligodendrocytes may allow for a greater preservation in numbers than if the prevention of apoptosis was delayed even slightly. This could explain why the eight hour delayed treatment resulted in a greater sparing

of oligodendrocytes than the 24 hour delayed treatment – there was simply more time to prevent oligodendrocyte cell death and therefore more cells survived in the end.

The next point addresses why this effect was not observed in the neuron population. Here, one might propose the theory that given a particular increase in the viability of oligodendrocytes, a resultant amount of supportive benefit is achieved by the neurons. There may be a threshold where any further increase in oligodendrocyte survival would not have much impact on the viability of neurons. It is possible that both the eight hour and 24 hour delayed treatment with sFasR achieved this threshold of oligodendrocyte survival, and hence little difference between the treatment delays was observed in the neuron counts.

5.3.4 Tissue Preservation by sFasR Administration

The beneficial effect of eight hour delayed sFasR treatment was further supported by an increase in grey matter preservation and a reduction in cavity formation. More specifically, it was observed that in both outcome measures, rostral cord sections showed the most significant therapeutic benefit from eight hour delayed sFasR administration. This is fitting with the observations that the most consistent inhibition of apoptotic cell death by sFasR was rostral to the injury epicenter [Chapter Four and (Ackery, Robins et al. 2006)]. In addition, the findings of increased health and integrity of grey matter tissues in the rostral cord are highly correlated with the enhanced neuron survival previously discussed. This is not the first time that a stronger therapeutic effect has been observed in rostral cord segments specifically (McTigue, Tripathi et al. 2007). The reason for

these observations can only be speculative in nature. It is possible that maintenance of a supportive environment at the injury epicenter has a positive effect on retrograde degeneration. Reduced axon degradation would result in less inflammatory activity, potentially less cell death and improved tissue preservation.

Overall, the effects of 24 hour delayed sFasR treatment on grey matter preservation and cavity reduction were more modest than those of the eight hour treatment group, when compared to the respective controls. The enhanced rostral impact on grey matter tissue was also noted in the 24 hour treatment group, and remains consistent the findings of increased neuronal survival in this region. This finding was not carried into the cavity size evaluation however. While overall statistical significance was observed with cavity reduction by 24 hour delayed sFasR, post-hoc analysis of treatment effects at various cord locations did not reveal any further findings.

5.3.5 Functional Recovery Improvements and Considerations

The results of the behavioural analysis on open-field hind-limb assessment provided relevant validation for the cellular and tissue benefits observed with delayed sFasR treatment. Evaluation of the eight hour delayed treatment groups revealed that animals treated with either BSA or saline scored between one and two point differences from animals treated with sFasR. In addition, the significant improvement in sFasR-treated animals at the one week time point is interesting. It was noted in chapter four that at five and seven days post-injury, sFasR treatment had shown a reduction in TUNEL-positive cells, an

increased presence of oligodendrocytes and a reduction in axonal degradation (Ackery, Robins et al. 2006). It is possible that the anti-apoptotic effects of sFasR within the first week of SCI could manifest in such a way as to provide this burst in behavioural recovery.

Differences in BBB scores are an important area to explore in the context of biological significance. The scale is non-linear, ranging from a score of zero where an animal has no hind-limb movement in any joint in either limb, to a score of 21 which would be achieved by a fully functioning, uninjured animal (Basso, Beattie et al. 1996). The non-linear nature of the scale is especially note-worthy in the range from seven to ten, where the animals in this study are typically found by approximately four weeks post-injury. Here, the motor difference from one point to the next indicates a strong biological effect. For instance, an animal achieving a score of eight displays full movement of all three hind-limb joints but lacks the ability to bear any weight on the hind-limbs and does not make any attempt to take steps. In contrast, an animal achieving a score of ten shows the ability to step occasionally and can maintain weight support while standing and occasionally during stepping. When taken into the context of a human patient suffering from SCI, the difference between these points could translate into large differences in quality of life and would greatly reduce the possibility of developing co-morbidities often associated with injury severity.

The behavioural efficacy of 24 hour delayed sFasR treatment was certainly not as significant as the eight hour delay. The fact that cell survival and certain aspects of tissue preservation were positively influenced with 24 hour

delayed treatment gives merit to future the potential for this therapy. The correlation between neuron and oligodendrocytes survival and behavioural recovery following SCI is not entirely clear. At a certain point in the recovery curve, increases in cell survival and axon preservation do not correspond to increases in behavioural outcome (Fehlings and Tator 1995). This relationship is non-linear and the correlation is not always obvious.

In terms of other neurobehavioural tests commonly used to evaluate motor function following experimental SCI, the level and severity of injury model used here does not lend well to recovery evaluation using other well-known tests, such as grid-walking or foot-print analysis. Although the cervical 35g clip compression model is highly relevant clinically, there exists a strong degree of functional deficit accompanied by spasticity in the first four to six weeks following injury. These manifestations render the use of test other than BBB scoring challenging.

Evaluation of neuropathic pain manifestation, specifically that of mechanical allodynia, revealed that delayed administration of sFasR using an intrathecal catheter and mini-pump did not adversely affect the onset of pain. At the same time, the treatments did not reduce the acquisition or severity of neuropathic pain. This was not surprising because the anti-apoptotic action of sFasR was not intended to target the development or exacerbation of pain mechanisms. It was important to determine that the treatment did not cause a greater pain response in the animals, and the conclusions to this end are promising.

5.3.6 Intrathecal (Subarachnoid) Catheterization

The invasiveness of therapeutic delivery using an intrathecal catheter and mini-pump system is appreciated, and certainly one might argue that IV treatment would reduce the associated risks. It is believed that intrathecal catheterization offers a positive option for direct drug delivery following SCI. The proper and effective placement of the catheter into the cervical dural space can be achieved by a qualified and practiced spine surgeon, and the use of intrathecal catheterization can be found in the clinical setting already. Furthermore, the ability to target the injured area of the spinal cord with this type of system is of incredible value when compared to systemic injections. The dose required to achieve efficacy can be much smaller and cost-effective, and the concern over a drug's ability to cross the blood – spinal cord barrier is not necessary. More importantly, there is a significant reduction in the possibility of adverse systemic effects being attributed to the administration of the compound if locally delivered. Unlike the systemic administration of methylprednisolone and other corticosteroids that can create dangerous adverse effects, this type of targeted delivery system confines the molecule to the area of need. There are several novel drug delivery option that warrant consideration for this purpose, and these will be discussed in Chapter 7.

5.4 Conclusions

The ability of an experimental therapy to be considered for translation into a clinical setting is dependent in part on its ability to provide a significant functional impact. Improvements to the quality of life in individuals suffering from

SCI needs is a priority in assessing potential therapeutic strategies. The ability of delayed sFasR treatment to improve cell survival of both oligodendrocytes and neurons, to promote the maintenance of tissue integrity and to reduce the lesion cavity size are all important factors to be evaluated. Combining these beneficial cellular events with a significant improvement in behavioural recovery firmly validates the efficacy of the treatment. Here, eight hour delayed sFasR administration showed both of these outcomes with significant cellular and tissue effects, and a positive, biologically significant locomotor recovery. While the 24 hour delayed sFasR treatment showed significant cellular and tissue benefit, the modest behavioural effects make it less applicable for clinical translation.

In order for researchers and clinicians to bring potential therapies from the laboratory into a clinical setting, another hurdle must be crossed. There must be an effective to translate the therapeutic strategy into the SCI patient population. In order to accomplish this, suitable modes of administration and appropriate treatment paradigms must be demonstrated in the laboratory before being introduced into a clinical trial. This work has demonstrated that intrathecal administration of sFasR can be effectively delayed by up to eight hours post-injury while maintaining strong neuroprotective and behavioural effects. Furthermore, the treatment can be delayed by up to 24 hours post-SCI with substantial cellular and tissue benefits observed.

This work has begun to set the stage for the translation of SCI research into the clinical setting. Future studies on acute traumatic SCI should have the

ultimate goal of providing strong efficacy with treatment initiation beginning as long as possible after the initial trauma takes place.

Chapter Six:

Evaluation of the unexpected neuroprotective benefits of delayed IgG administration following acute traumatic SCI

6.1 Introduction

In an attempt to effectively evaluate the therapeutic efficacy of sFasR administration following SCI, it was determined that an appropriate protein control was needed. The sFasR molecule being evaluated consisted of the extracellular aspects of human Fas receptor, stabilized by a 234 amino acid segment of human IgG Fc domain. As a result, it was decided to use human IgG as the protein control. Initial experiments with outcomes within the first week following SCI, namely apoptotic cell death, oligodendrocyte survival and axon degradation, showed significant differences between sFasR and IgG. IgG administration did not show any neuroprotective characteristics at these early post-SCI time points.

Moving ahead, experiments were designed to evaluate the delayed effects of sFasR administration following SCI. Five initial experimental groups were used, including two lengths of post-injury delay (eight and 24 hours) and three treatments (sFasR, IgG and saline). Because the saline-treated group was considered secondary, this group received treatment at the time of injury. The outcome measures included six weeks of motor recovery analysis, seven weeks post-injury cell survival, seven weeks post-injury tissue preservation and seven weeks post-injury axon preservation at the site of injury.

Following initial data analysis, it was observed that no difference in behaviour recovery existed between the sFasR and IgG treated animals, specifically within the eight hour delayed groups. In addition, there existed statistically significant differences between the saline-treated group and both sFasR and IgG-treated animals. These surprising observations led to the question of whether or not sFasR and IgG had independent effects, or if the benefit seen in both treatments was simply due to non-specific protein interactions. A second protein control was added to the experiments, and the rest of the data analysis was carried out using bovine serum albumin (BSA) as a secondary protein control. Saline-treated animals were also used as another control group like before.

Once a full analysis of all treatment groups and outcomes measures was complete, the results were quite interesting. Across almost all outcome measures, the sFasR and IgG treatment groups showed significantly beneficial outcomes as compared to both the BSA and saline-treated groups. Moreover, there was very little difference between the sFasR and IgG animals, specifically in the eight hour delayed groups. This allowed for the conclusion that the observed efficacy of sFasR and IgG was not due to non-specific protein interactions, but more likely a result of the molecule itself.

In order to more clearly delineate the therapeutic efficacy of sFasR for the purposes of this thesis, it was decided to remove the IgG data and present each in separate chapters. Note that the BSA and saline controls are used in each chapter.

6.2 Results

6.2.1 Eight hour delayed IgG administration improves long-term oligodendrocyte survival following traumatic SCI

In evaluating the impact of IgG administration on long-term oligodendrocyte survival, quantification of the total number of surviving oligodendrocytes in rostral cord sections was carried out at seven weeks post-SCI. The tissue sections were selected from approximately 1200μm rostral to the injury epicenter and stained with an antibody for CC1/APC to label oligodendrocytes. DAPI co-labelling was used for nuclear identification.

Quantification of oligodendrocytes in tissue sections from the eight hour delayed post-SCI group revealed that IgG administration significantly increased the long-term survival of oligodendrocytes, as compared to both BSA and saline control groups (**Figure 26**). The same significance of treatment effect was not observed in the 24 hour delayed group.

6.2.2 Eight hour delayed IgG administration improves long-term neuron survival following traumatic SCI

Long-term neuron viability following IgG administration post-SCI was evaluated in tissue sections taken from approximately 1200μm rostral to the injury epicenter, at seven weeks post-injury. Tissue sections were labelled with NeuN to identify neurons, and DAPI co-labelling was carried out for nuclear localization.

Following full section quantification of NeuN-DAPI-positive cells, it was observed that IgG administration delayed by eight hours post-SCI showed the ability to increase long-term neuron survival, as compared to both BSA and

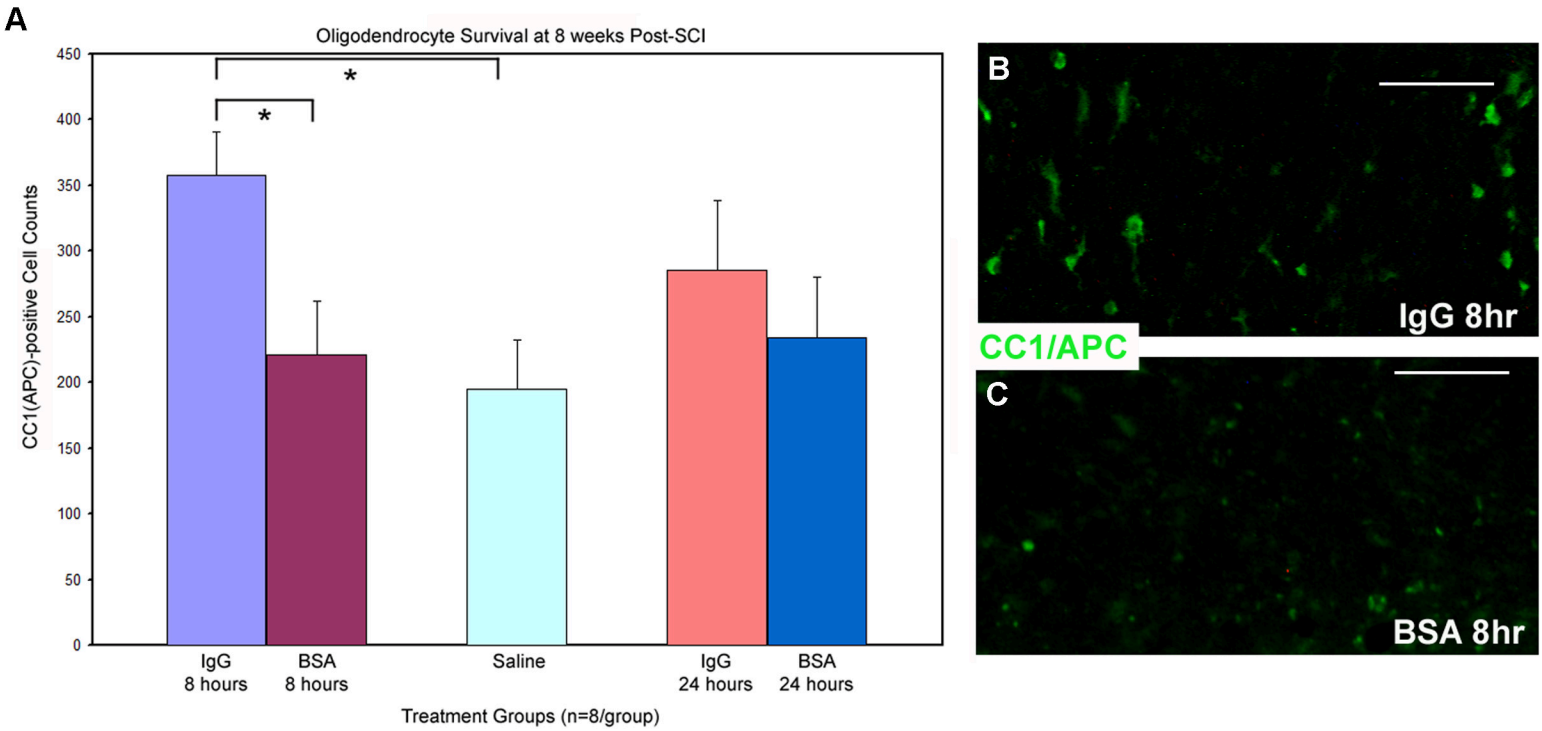


Figure 26: Delayed IgG administration improves long-term oligodendrocyte survival following SCI

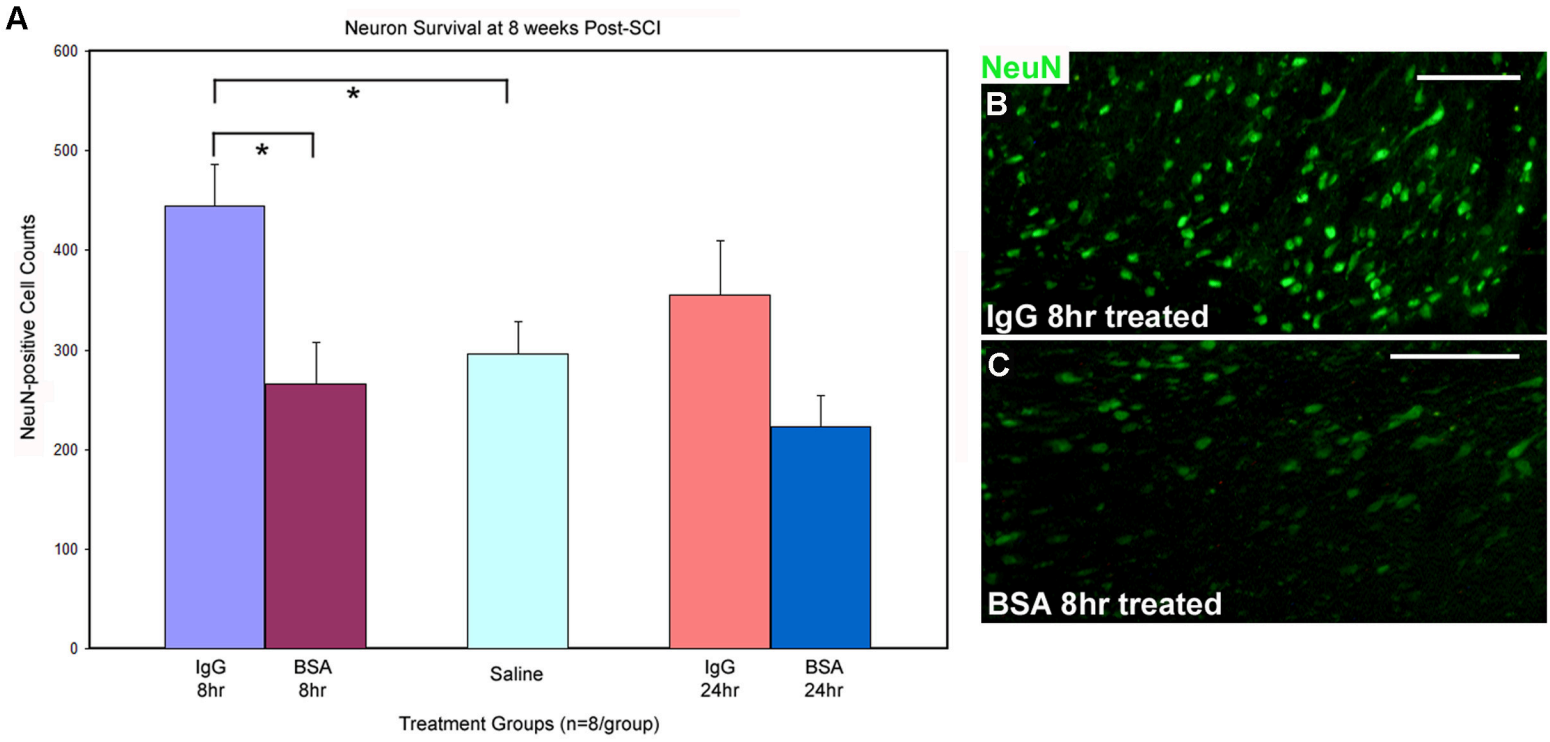


Figure 27: Delayed IgG administration improves long-term neuron survival following SCI

Figure 26: Delayed post-injury IgG administration improves long-term oligodendrocyte survival

The impact of delayed IgG administration following SCI was evaluated using tissue sections stained for oligodendrocytes (using CC1/APC) taken from approximately 700µm rostral to the injury epicenter at eight weeks post-injury. The number of CC1-positive cells on each tissue cross-section was quantified and an average was calculated for each treatment group.

Quantification of the total number of oligodendrocytes in each group is displayed graphically in A (error bars = SEM). Saline-treated animals were used as a secondary control for both eight-hour and 24-hour delayed groups. Images B and C are representative images of tissue sections from IgG and BSA-treated animals with an eight-hour post-SCI delay in administration, respectively (scale = 75µm). It can be observed that there was a greater number of oligodendrocytes in the IgG-treated animals as compared to the BSA-treated animals.

Statistical analysis of the data was carried out using a one-way ANOVA on the eight-hour treatments and then on the 24-hour treatment groups. It was confirmed that IgG administration delayed by eight-hours post-SCI was significantly better than both eight-hour delayed BSA and saline ($p<0.001$ overall, with post-hoc analysis yielding a $p<0.05$ for each comparison). The 24-hour delayed IgG administration was not significantly different than either 24-hour delayed BSA or saline. Post-hoc analysis to determine the specific treatment effects was carried out using the Student Neuman-Keuls method. In all treatment groups, there was an $n = 8$.

Figure 27: Delayed post-injury IgG administration improves long-term neuron survival

The impact of delayed IgG administration following SCI was evaluated using tissue sections stained for neurons (labelled with NeuN) taken from approximately 700µm rostral to the injury epicenter at eight weeks post-injury. The number of NeuN-positive cells on each tissue cross-section was quantified and an average was calculated for each treatment group.

Quantification of the total number of neurons in each group is displayed graphically in A (error bars = SEM). Saline-treated animals were used as a secondary control for both eight-hour and 24-hour delayed groups. Images B and C are representative images of tissue sections from IgG and BSA-treated animals with an eight-hour post-SCI delay in administration, respectively (scale = 75µm). It can be observed that there was a greater number of neurons in the IgG-treated animals as compared to the BSA-treated animals.

Statistical analysis of the data was carried out using a one-way ANOVA on the eight-hour treatments and then on the 24-hour treatment groups. It was confirmed that IgG administration delayed by eight-hours post-SCI was significantly better than both eight-hour delayed BSA and saline ($p<0.001$ overall, with post-hoc analysis yielding a $p<0.05$ for each comparison). The 24-hour delayed IgG administration was not significantly different than either 24-hour delayed BSA or saline. Post-hoc analysis to determine the specific treatment effects was carried out using the Student Neuman-Keuls method. In all treatment groups, there was an $n = 8$.

saline-treated controls (**Figure 27**). Similar to the evaluation of oligodendrocyte survival, the same increase in neuron survival was not observed in the 24 hour delayed treatment groups.

6.2.3 Delayed IgG administration effectively increases grey matter tissue preservation in areas adjacent to the injury epicenter following SCI

The ability of a potential therapeutic to reduce the extent of tissue degradation at and around the injury site following trauma is important. The nature of SCI pathophysiology is such that functional deficits result in part due to an overall loss of tissue integrity, particularly at the epicenter of injury and reaching out into rostral and caudal cord sections. Reductions in this spread of tissue loss are important and correlate well with functional improvements (Joshi and Fehlings 2002).

Spared grey matter area, as a function of total section area, was evaluated in stereologically from caudal through rostral cord sections around the injury epicenter. Tissue sections were taken from animals sacrificed at seven weeks post-SCI and were histologically stained using H and E / LFB.

Preserved grey matter was characterized by the presence of intact, healthy-looking tissue, showing a lack of necrotic and inflammatory infiltrate and an appropriately consistent eosinophilic staining pattern. In animals treated with eight hour delayed IgG, there was a significant increase in the amount of spared grey matter in areas adjacent to the injury epicenter, specifically around the 1200μm rostral location, as compared to both BSA and saline controls (**Figure 28**). Similarly, animals treated with IgG delayed by 24 hours post-SCI showed increased preservation of grey matter in areas adjacent to the injury epicenter as

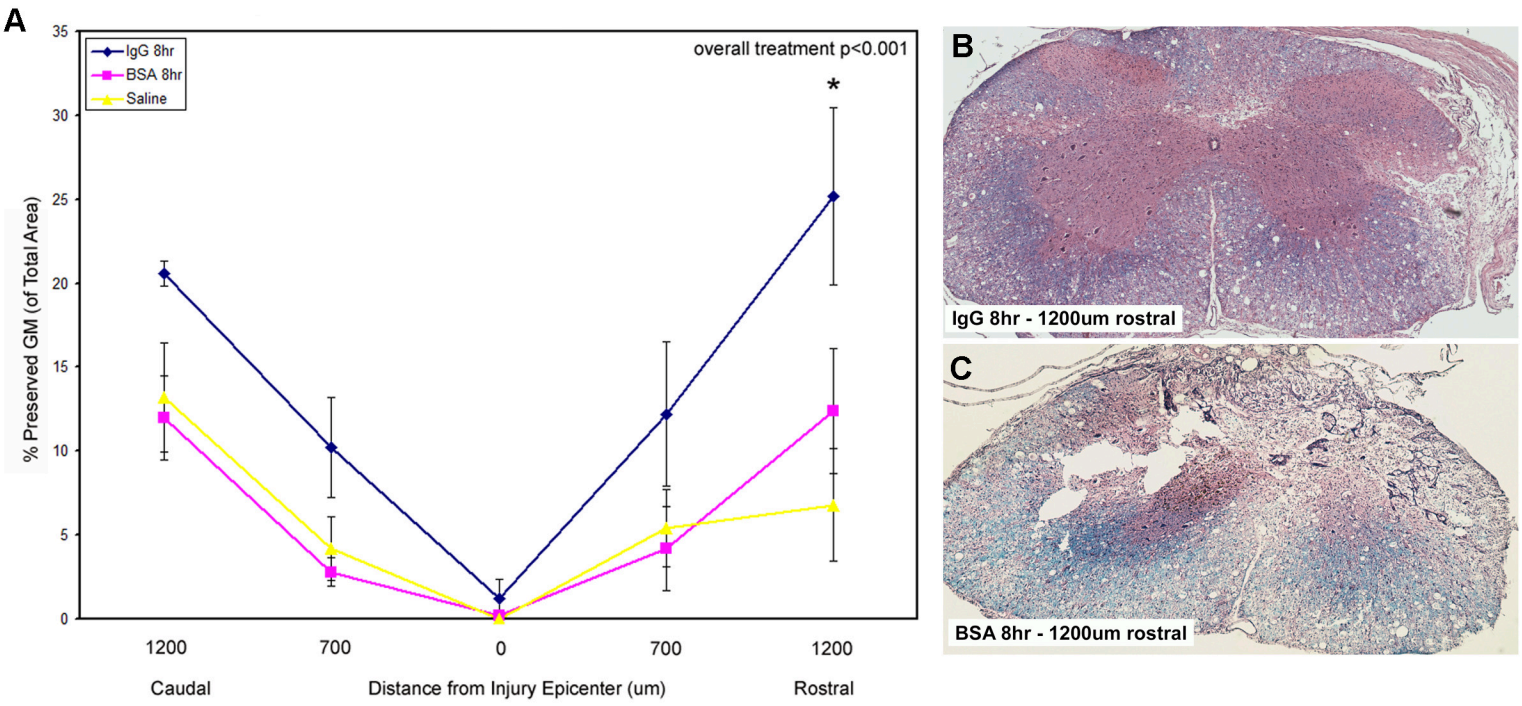


Figure 28: Eight hour delayed IgG administration improves grey matter preservation post-SCI

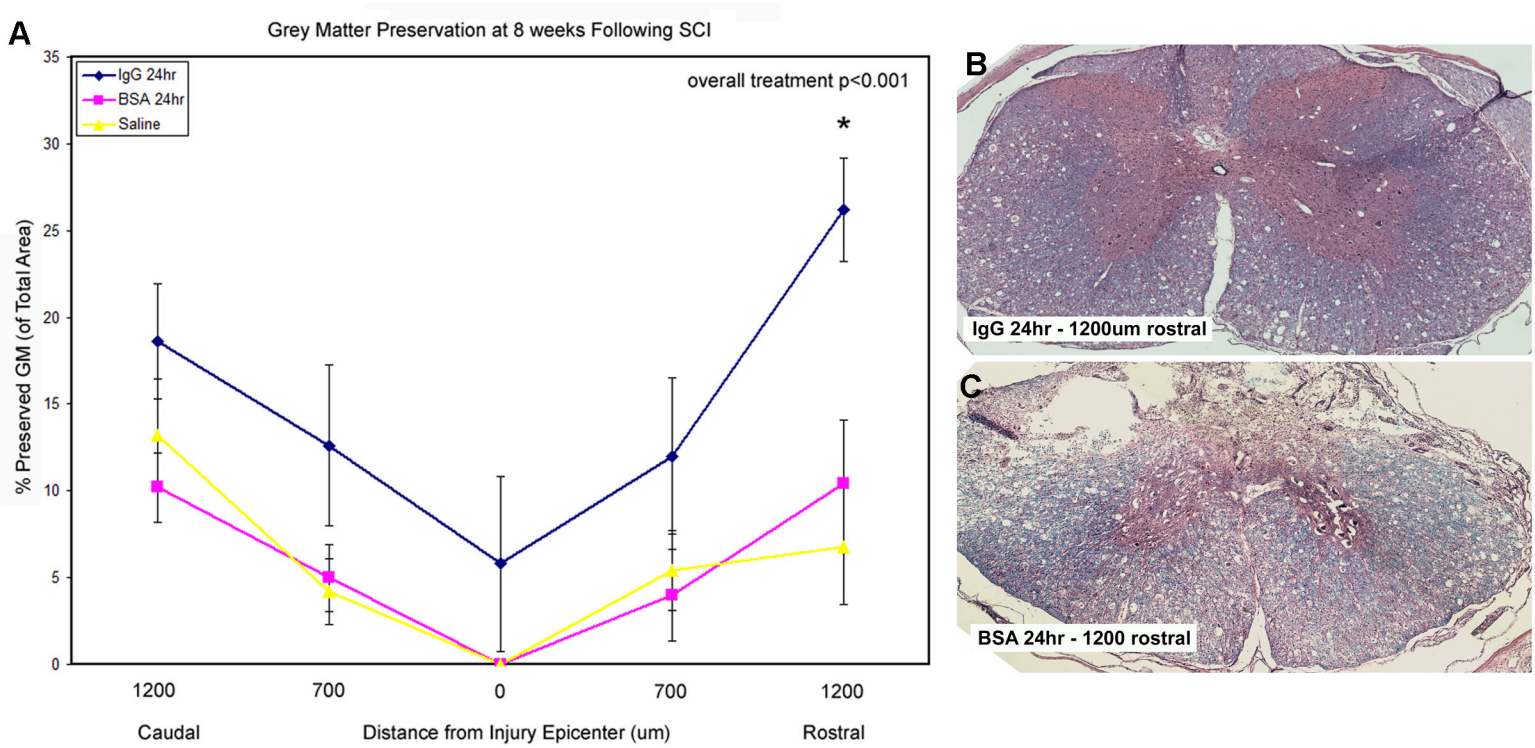


Figure 29: Twenty-four hour delayed IgG administration improves grey matter preservation post-SCI

Figure 28: Eight-hour delayed IgG administration improves grey matter preservation post-SCI

The impact of eight-hour delayed IgG administration post-SCI was evaluated using histologically stained tissue sections taken from approximately 1200µm and 700µm rostral and caudal to the injury epicenter, and from the injury epicenter itself at eight weeks post-injury. The tissue morphology and neuroanatomical structure of the tissue sections was evaluated using H and E with LFB staining.

Quantification of the percent grey matter preservation (of the total section area) was carried out and the results are shown graphically in A, error bars ± SEM. Representative tissue sections taken from approximately 1200µm rostral to the injury epicenter from IgG and BSA-treated animals are shown in B and C respectively. As compared to both controls, eight-hour delayed IgG administration provided an improvement in grey matter preservation, especially at 1200µm rostral to the injury epicenter.

The data were subjected to a two-way ANOVA where eight-hour delayed IgG administration effects on grey matter preservation produced p-values of $p<0.001$. Post-hoc analysis using the Holm-Sidak method revealed the specific location of significance, at a p-value level of $p<0.05$. There were n's of 5 used for each treatment group and set of analysis.

Figure 29: Twenty-four hour delayed IgG administration improves grey matter preservation post-SCI

The impact of 24-hour delayed IgG administration post-SCI was evaluated using histologically stained tissue sections taken from approximately 1200μm and 700μm rostral and caudal to the injury epicenter, and from the injury epicenter itself at eight weeks post-injury. The tissue morphology and neuroanatomical structure of the tissue sections was evaluated using H and E with LFB staining.

Quantification of the percent grey matter preservation (of the total section area) was carried out and the results are shown graphically in A (error bars ± SEM). Representative tissue sections taken from approximately 1200μm rostral to the injury epicenter from IgG and BSA-treated animals are shown in B and C respectively. As compared to both controls, 24-hour delayed IgG administration provided an improvement in grey matter preservation, especially at 1200μm rostral to the injury epicenter.

The data were subjected to a two-way ANOVA where eight-hour delayed IgG administration effects on grey matter preservation produced overall p-values of $p<0.001$. Post-hoc analysis using the Holm-Sidak method revealed the specific location of significance, at a p-value level of $p<0.05$. There were n's of 5 used for each treatment group and set of analysis.

compared to BSA and saline controls. This difference was observed most significantly in the 1200µm rostral cord sections (**Figure 29**).

6.2.4 Delayed IgG administration maintains axon integrity at the injury site following SCI

An important aspect of neurological function is the ability of projecting axons to reach their target. SCI causes disruption to these axons, often leading to their degradation and ultimate loss. Axonal destruction within the spinal cord contributes to the functional deficits observed in these patients. By therapeutically preventing the loss of descending axons, an improvement in functional outcome is possible.

By retrogradely labelling mid-brain and brain stem neurons using FluroGold (FG), a fluorescent molecule transported by the axon, it is possible to establish the extent of axon preservation achieved at the injury site. If an axon has maintained integrity and function as it descends past the injury epicenter, it will successfully reach the source of FG and retrograde transport will localize the molecule in the neuron cell body. Quantification of labelled neurons in the brain stem and mid-brain will correlate with the extent of axon preservation at the injury site.

Four specific brain stem and mid-brain nuclei were selected for analysis, including the reticular formation, the vestibular formation, the raphe nuclei and the red nuclei. A fifth assessment included the sum of FG-positive cells quantified in all four specific brain regions Stereological brain sections were selected at even intervals through out each of these brain regions and the total

number of FG-positive nuclei were quantified (representative image is shown in **Figure 30A**). IgG and BSA-treated groups were used for this analysis.

The ability of delayed IgG administration to improve axon integrity and survival at the injury site was only apparent in those axons projecting from the raphe nuclei, as compared to BSA-treated controls (**Figure 30 B and C**). Interestingly, achievement of only a 10% increase in axon preservation is accepted as providing biological significance (Fehlings and Tator 1995). With this, the data were analysed to examine the percent increase in axon preservation achieved by IgG administration as compared to BSA. It was found that eight hour delayed IgG improved axon preservation at the injury site by 189% and 115% for reticular fibres and raphe fibres, respectively. The 24 hour delayed IgG administration improved raphe axon preservation by 152% over that of the BSA treatment (**Figure 30D**).

6.2.5 Delayed IgG administration offers significant functional improvement following traumatic SCI

The impact of delayed IgG administration on hind-limb motor recovery following SCI was evaluated using weekly open-field motor assessment and the BBB rating scale as described in the methods chapter (Basso, Beattie et al. 1996).

Animals treated with eight hour delayed IgG showed highly significant improvements in motor recovery as compared to both BSA and saline-treated animals. Post-hoc analysis revealed further significance in function between the IgG treated and control animals at one, two, five and six weeks post-SCI (**Figure 31A**).

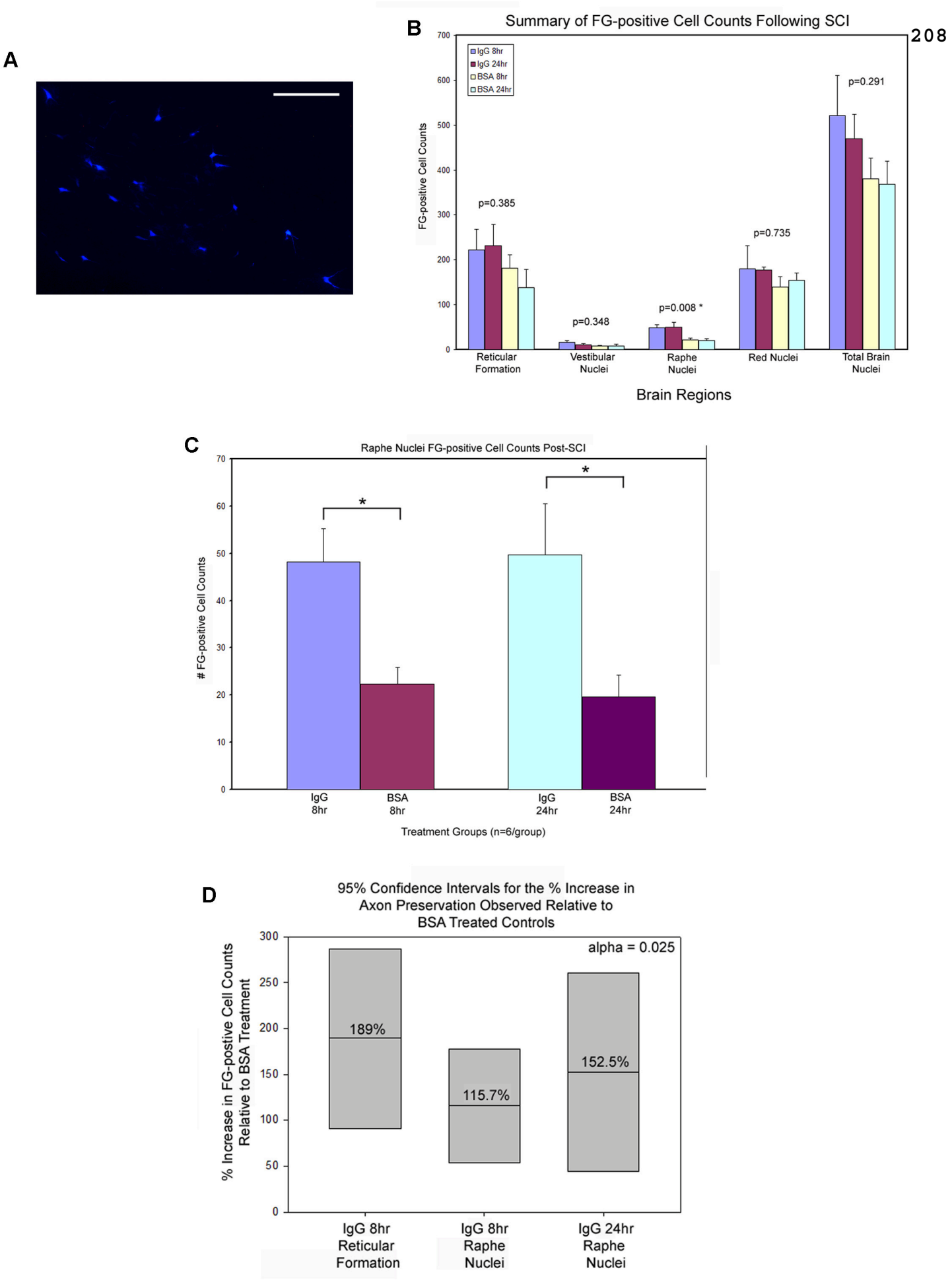


Figure 30: Delayed IgG administration reduces axonal degradation at the injury site following traumatic SCI

Figure 30: Delayed IgG administration reduces axonal degradation at the injury site following traumatic SC

The ability of delayed post-injury IgG administration to reduce axonal degradation through the site of injury was evaluated through quantification of brain stem nuclei using FluoroGold (FG) retrograde labelling; a representative FG-labelled image is shown in A (scale = 50μm). The number of FG-positive nuclei in each of four specific brain regions was determined, and the results are shown graphically in B (error bars = SEM). The data were subjected to statistical analysis using a one-way ANOVA for each of the brain regions: reticular formation, vestibular formation, raphe nuclei, red nuclei and the sum of all nuclei counted. The only region that showed a significant treatment effect was the raphe nuclei, with a p-value of $p=0.008$ overall.

Further investigation into the significance within the raphe nuclei is shown graphically in C, where the eight-hour delayed IgG and BSA were compared and the 24-hour delayed IgG and BSA were compared using post-hoc analysis. In both cases, the ability of IgG treatment to significantly improve the integrity of axons travelling descending through the injury site was determined at a $p<0.05$ level.

The impact of axonal preservation at the injury site is known to be biologically significant with only a 10% increase in surviving axons (Fehlings and Tator 1995). With this in mind, the ability of IgG administration to increase the number of axons at the injury site was compared directly to the BSA control. A 95% confidence interval of the percent increase in FG-positive counts of IgG-treated animals relative to BSA-treated was generated with an alpha of 0.025; these data are presented graphically in D. It was established that eight-hour delayed IgG administration significantly increased the number of preserved axons at the injury site by 189% (reticular formation) and 115% (raphe nuclei) of that found in eight-hour delayed BSA. In addition, 24-hour delayed IgG increased the axonal presence at the injury site by 152% of the BSA-treated controls in the raphe nuclei.

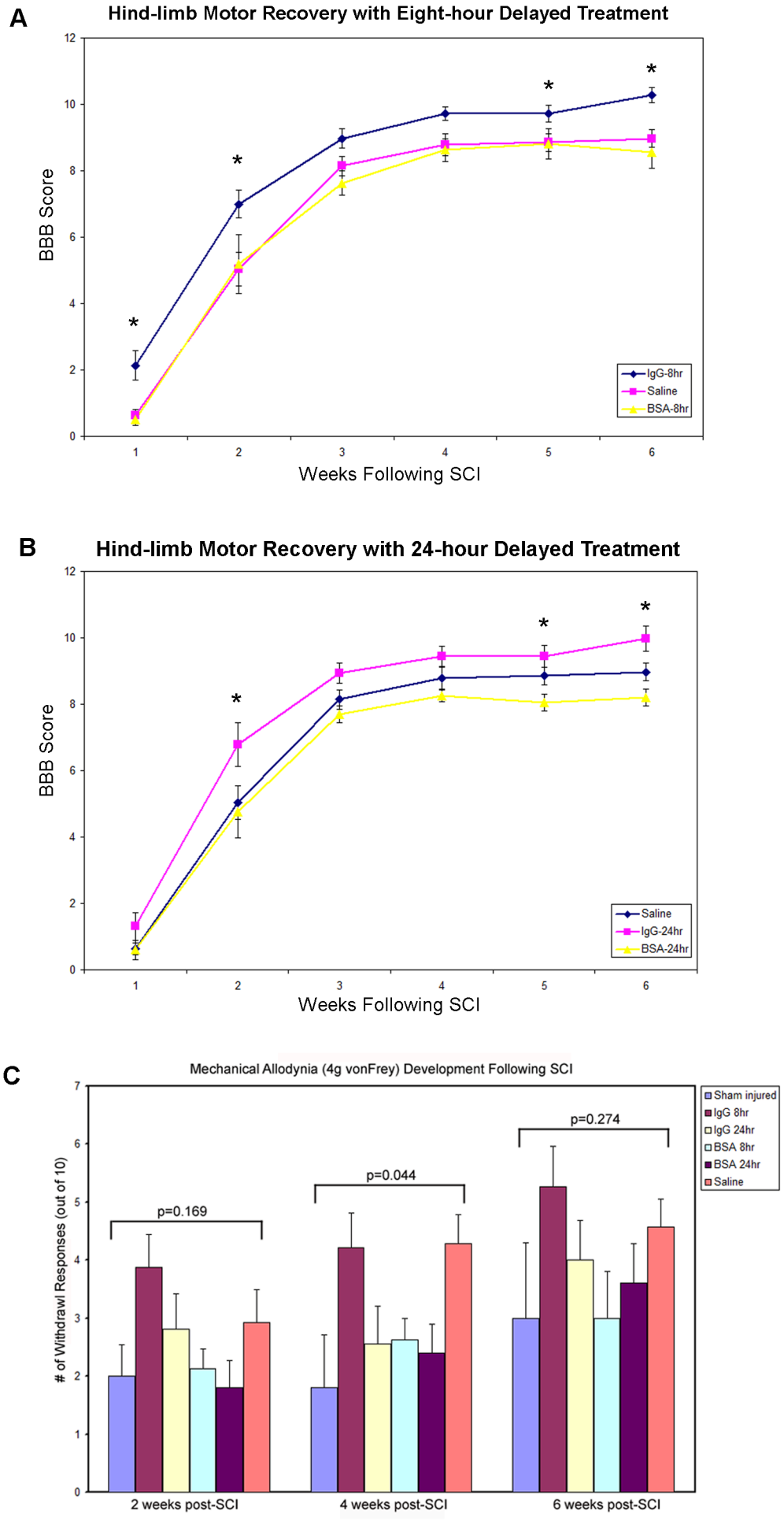


Figure 31: Delayed administration of IgG improves neurobehavioral outcome

Figure 31: Delayed IgG administration improves neurobehavioral outcome post-SCI

The impact of delayed IgG administration on hind-limb motor recovery was evaluated using BBB scoring weekly, for six weeks following SCI. Eight-hour delayed IgG administration offered a significantly improved motor recovery as compared to both BSA and saline-treated control animals. Furthermore, this difference was statistically significant at one, two, five and six weeks post-injury using post-hoc analysis. These data are displayed graphically in A (error bars ± SEM). Twenty-four hour delayed IgG administration also offered an improvement in motor recovery as compared to 24-hour delayed BSA and saline controls. These differences were further found to be significant at two, five and six weeks post-injury using post-hoc analysis.

Statistical analysis was performed using a two-way ANOVA on the eight-hour and 24-hour delayed groups independently, with saline used as a common secondary control. Post-hoc analysis was carried out using the Holm-Sidak method.

The influence of delayed IgG administration on the development of neuropathic pain following SCI was evaluated using bi-weekly von Frey hair stimulation, with the number of withdrawal responses recorded out of ten. The data are displayed graphically in C, where there were no significant difference in neuropathic pain between any of the treatment groups using one-way ANOVA at each time point post-injury (error bars = SEM).

The values of n for these experiments are as follows: Sham-injured n = 5, IgG 8 hour n = 18, BSA 8 hour n = 8, IgG 24 hour n = 18, BSA 24 hour n = 10, and saline n = 14.

Administration of IgG delayed by 24 hours post-SCI improved hind-limb motor function significantly compared to both BSA and saline-treated controls. Although the effects were more modest than that shown with the eight hour delayed IgG treatment, there were still significant differences between IgG and both controls at two and six weeks post-SCI (**Figure 31B**).

Finally, establishment of the effect had by delayed IgG administration on neuropathic pain development following SCI was evaluated as previously described. Although the eight hour delayed IgG animals appeared to have slightly elevated pain responses when compared to the other treatment groups, these observations were not statistically significant (**Figure 31C**). Therefore, IgG administration had little effect on reducing or exacerbating pain mechanisms following SCI.

6.3 Discussion

6.3.1 Summary of Findings

An unexpected line of investigation was presented in this chapter. The therapeutic effects of delayed IgG administration following SCI were surprising, but offer a novel therapeutic option for future exploration. It was found that eight hour delayed post-injury administration of IgG had a beneficial impact on the long-term survival of both oligodendrocytes and neurons, but that a 24 hour delayed administration was not able to maintain this efficacy. Furthermore, both eight and 24 hour delayed IgG administration showed the ability to reduce grey matter tissue preservation in spinal cord sections adjacent to the injury site, with particular significance around the 1200μm rostral area. The number of preserved

raphe nuclei axons descending through the injury site were significantly increased with delayed IgG administration, and it was noted that a tremendous biological significance in axon preservation was achieved by IgG treatment when compared to BSA controls. Finally, the ability of delayed post-injury IgG administration to facilitate an improvement in motor recovery throughout the six weeks following injury was remarkable. With little alteration to the existing development of neuropathic pain following SCI, delayed administration of IgG has shown to be a strong therapeutic option.

6.3.2 Early outcome measures did not reveal a therapeutic effect with IgG

Studies in the early stages of the sFasR work (presented in Chapter 4), used IgG as a control protein, for the reasons described above. These experiments were designed to evaluate the therapeutic efficacy of sFasR administration after SCI by examining early molecular outcomes. Comparisons between sFasR and IgG treatment on the viability of oligodendrocytes at five days post-SCI were evaluated using western blot. Administration of sFasR significantly improves the viability of oligodendrocytes after SCI at this time point when compared to IgG (Ackery, Robins et al. 2006). In addition, the same significant difference in effect is observed when axon preservation is assessed. Soluble Fas receptor-treated animals have significantly less degradation of neurofilament 200 than do animals treated with IgG, indicating a significant preservation of axons at the injury site (Ackery, Robins et al. 2006).

The observations presented in this thesis chapter seem to contradict the early outcome results. The therapeutic ability of IgG was clear in long-term

evaluation of cellular and neuroanatomical outcomes, and early improvements in functional recovery were noted compared with BSA and saline-treated controls. Of note is that within the eight hour delayed groups, there was very little difference in effect between sFasR and IgG. In examining all of the outcome measures, it was observed that 24 hour delayed IgG showed a slightly more therapeutic ability than did 24 hour delayed sFasR.

A potential explanation for the inconsistent results might be that sFasR and IgG are exerting their effects in different mechanistic ways. With this, the type of outcome measure and the time post-injury that the outcome is evaluated would provide variable results. The potential for mechanistic differences between sFasR and IgG is not difficult to appreciate. First, it is known that sFasR has an anti-apoptotic effect following SCI and that a likely mode of action is in the disruption of Fas-mediated apoptotic signalling (Ackery, Robins et al. 2006). Although the exact mechanisms carried out by IgG are not clear, the anti-inflammatory nature of its impact clinically is well-accepted (Kaneko, Nimmerjahn et al. 2006; Nimmerjahn and Ravetch 2007).

The early post-SCI outcomes evaluated in Chapter 4 focused on the ability of sFasR to reduce cell death, and as a result may not have been the most appropriate choice to show the effects had by IgG. Further to that, the more long-term outcomes described in this and the previous chapter evaluate a broader therapeutic effect. The specific mechanisms of action for both sFasR and IgG are likely taking place within the first week of injury, as the course of administration for both molecules is limited to seven days. Future work that will

ultimately extend from these findings should focus on determining the cellular and molecular changes occurring during and shortly after therapeutic administration of IgG. Evaluation of inflammatory cell infiltrate and cytokine expression between one and two weeks post-SCI could provide insights into the specific mechanisms of action carried out by intrathecal IgG administration. These findings could then be correlated with the long-term observations presented here in an attempt to make sense of these findings.

6.3.3 Effects of IgG administration on long-term cell survival

It is a challenge to explain the observations of IgG's therapeutic effect due to the fact that these experiments were not planned according to a previous understanding of IgG mechanisms. Furthermore, little is known regarding the specific anti-inflammatory mechanisms of action by IgG.

Current clinical use of IgG is carried out in various autoimmune neuropathies such as Guillain Barré Syndrome and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) (Nimmerjahn and Ravetch 2007). Very recently, the first report of IgG use following SCI attempted to delineate the possible mechanisms of therapeutic action (Gok, Sciubba et al. 2009). Here, IgG administration following experimental SCI appears to reduce the infiltration of neutrophils into the spinal cord tissue, as determined by relative levels of myeloperoxidase activity. Critical evaluation of this work reveals that the outcomes were measured at premature time points to the accepted time of neutrophil activity post-SCI. However, the notion that IgG might work to alter the

presence and/or activity of inflammatory cells in the injured spinal cord is an interesting concept to explore in future studies.

Given the minimal understanding of IgG anti-inflammatory actions, the way in which delayed intrathecal administration post-SCI increased the long-term survival of both oligodendrocytes and neurons is open for discussion. It is possible that by minimizing the deleterious aspects of the inflammatory response within the first after injury, the health of both oligodendrocytes and neurons was enhanced. Preservation of cellular health in the sub-acute setting of experimental SCI could easily translate into an improved long-term survival.

The observation that 24 hour delayed IgG lost the ability to significantly improve both oligodendrocyte and neuron survival is worth noting. The target of IgG action could be occurring early enough in the secondary injury process that by delaying the administration past eight hours, the benefit is reduced. Interestingly, the other outcome measures examined did not reveal this loss of effect to the same extent between eight and 24 hour delays. The relationships between cellular, neuroanatomical and behavioural outcomes following SCI are not strictly linear, therefore there exists the possibility for improvements to be significant in one and not in others (Fehlings and Tator 1995; Joshi and Fehlings 2002).

6.3.4 Functional and neuroanatomical benefits of delayed IgG administration

The most significant therapeutic findings coming from this work are the powerful ability of IgG administration to improve functional recovery post-SCI. The BBB locomotor rating scale was used weekly to evaluate the progression

and degree of functional improvement between the treatment groups. Eight hour delayed IgG administration showed significant efficacy throughout the course of the six week study, with multiple time points of statistical significance achieved in post hoc analysis. The observed difference in BBB scores between IgG and controls at six weeks deserves note. The discussion surrounding biological significance and non-linearity of the BBB score in Chapter 5 can be useful here as well. Briefly, animals scoring 10 on the BBB scale are able to occasionally bear weight and are successful in stepping attempts. On the other hand, animals receiving a score of 8 do not bear weight at all and make sweeping motions with their paws in the place of steps. The functional difference is obvious, while two point separation can be perceptively misleading.

The groups with 24 hour delayed treatment administration also showed the ability of IgG to significantly improve functional recovery after SCI. The observations from these groups are more modest than those of the eight hour delayed group, however the presence of a treatment effect is still obvious.

Tissue preservation analysis revealed that delayed IgG administration had a beneficial impact on grey matter, specifically in rostral cord sections. A similar observation was noted in Chapter 5, and potential reasons for this effect were carried out.

6.4 Conclusions

Observations stemming from previous experiments have provided evidence that delayed IgG administration following traumatic SCI was therapeutically beneficial. The efficacy of IgG was established in a variety of

outcomes measures including long-term cell survival, functional recovery and tissue preservation. The significant impact of this novel molecule in experimental SCI is shown for the first time in these experiments. Only one other report in the literature has used this type of treatment post-SCI, and the extent of outcome measures is limited (Gok, Sciubba et al. 2009).

The surprising set of observations that led to this chapter has served to open up a new line of investigation in the quest to refine therapeutic strategies for SCI. The fact that IgG is currently accepted for therapeutic use for other neurological indications gives promise for its translation into the SCI patient population. Although the exact mechanisms of IgG's effect are still being evaluated, the anti-inflammatory nature of the molecule itself provides a foundation for which to begin the analysis. Future work that will delineate the exact molecular and cellular targets of IgG administration post-SCI will aid in the establishment of an appropriate treatment regime. Furthermore, the potential exists to give IgG intravenously instead of intrathecally post-SCI, as this is the common mode of administration in current clinical use. The significant reduction in invasiveness and the ease of delivery could mean that patients would not necessarily need to wait for a physician to being the treatment.

Chapter Seven: General Discussion and Future Directions

Traumatic SCI is a devastating condition accompanied by varying degrees of morbidity and mortality. A significant loss in quality of life is experienced by those persons suffering from SCI, and there are currently few efficacious treatment options available.

The pathology of SCI is dynamic and complex, in part due to the host of deleterious processes working in tandem to exacerbate tissue and cell loss, ultimately reducing motor and sensory functions. Researchers have spent a great number of years attempting to delineate the various elements of the secondary injury process and to date have made a great deal of progress. One of the areas that still requires focus is the impact, regulation and mechanisms of cell death post-SCI.

Apoptotic cell death is known for causing both neuron and oligodendrocyte cell death in the injured spinal cord (Rink, Fung et al. 1995; Katoh, Ikata et al. 1996; Li, Brodin et al. 1996; Springer, Azbill et al. 1999; Knoblach, Huang et al. 2005; Ackery, Robins et al. 2006). Specifically, Fas-mediated apoptosis has shown an emerging role in the activation of cell death cascades following SCI (Casha, Yu et al. 2001; Demjen, Klussmann et al. 2004; Yoshino, Matsuno et al. 2004; Casha, Yu et al. 2005; Ackery, Robins et al. 2006). The work presented in this thesis was designed to evaluate the specific cellular and temporal profiles of Fas and Fas ligand expression, while elucidating the impact of Fas-mediated apoptosis in the acutely injured spinal cord.

7.1 Fas receptor expression post-SCI

Following SCI, the temporal correlation of Fas receptor expression in the oligodendrocyte population and the presence of oligodendrocyte apoptosis were strong. In addition, the presence of delayed Fas-mediated apoptosis of oligodendrocytes post-SCI was observed, leading to the conclusion that this type of cell death is activated in a delayed time-course after traumatic injury, and predominantly targets the oligodendrocyte population. A more thorough appreciation for the correlation of these observations can be achieved with graphical representation of the cellular and temporal expression profile of Fas after SCI (**Figure 32**).

The importance of cellular Fas expression to the ultimate activation of cell death is clear. A cell must express Fas in order to be vulnerable to Fas-mediated apoptosis. Following SCI, there was a significant increase in Fas expression observed on the oligodendrocyte population. Ultimately, the oligodendrocyte is signalled to increase Fas expression in order to facilitate its own death following SCI. The possible source of these “death signals” lie in the preceding secondary injury mechanisms within the injured spinal cord tissue. These include, and are necessarily not limited to, cytokine release from activated microglia and inflammatory cells. It is known that both pro-inflammatory cytokines TNFα and IFNγ can trigger *in vitro* Fas receptor expression (Spanaus, Schlapbach et al. 1998; Badie, Schartner et al. 2000; O'Connell, Bennett et al. 2000; Pouly, Becher et al. 2000). The impact of TNFα and IFNγ release on oligodendrocyte Fas expression after SCI is not known. In order to elucidate these interactions, it

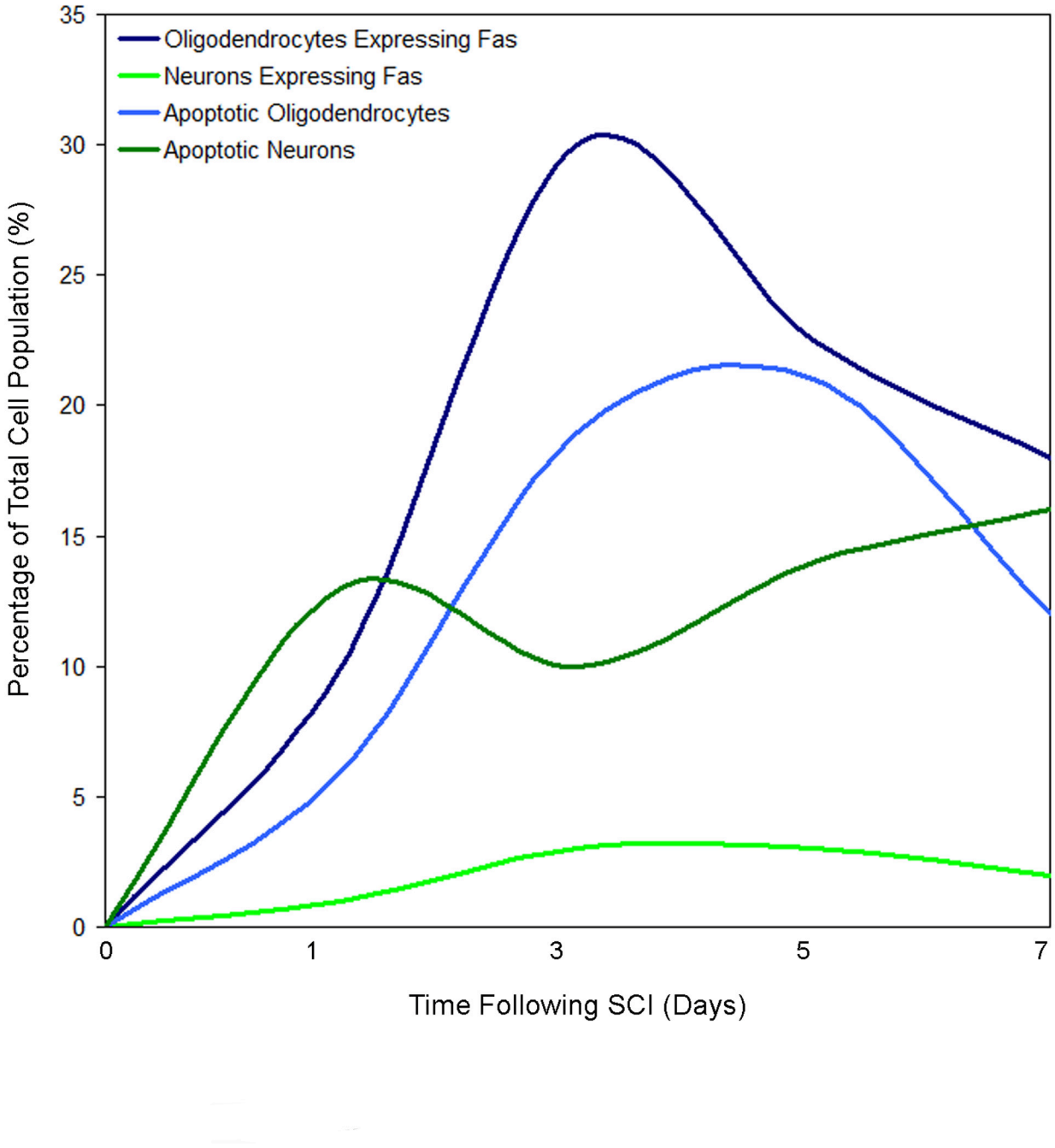


Figure 32: Time Course of Fas Expression and Apoptosis After SCI

Figure 32: Time Course of Fas Expression and Apoptosis After SCI

The cellular and temporal profile of Fas expression and apoptotic cell death following SCI contributes to the idea that Fas-mediated apoptosis targets the oligodendrocyte population over the neuron population. Furthermore, this type of cell death is delayed by three or more days following traumatic SCI.

Quantitative expression data within the first week of SCI from chapter three were fit to curves and are shown graphically. There is a positive correlation of increased oligodendrocyte apoptosis with increased oligodendrocyte expression of Fas starting at three days following SCI (shown by the light and dark blue lines, respectively). This combined with the observation that Fas-mediated apoptosis of oligodendrocytes is also occurring at these time points provides strong evidence to suggest that this form of cell death does target the oligodendrocytes in a delayed fashion post-SCI.

In addition, neuronal expression of Fas is very minimal (light green line), and the slight increase in expression that is observed does not correlate with the incidence of neuronal apoptosis seen at one day following SCI (dark green line). This provides correlative evidence that neuronal apoptosis is Fas-independent, and likely a result of activation of the mitochondrial pathways post-SCI.

would be interesting to run *in vivo* experiments designed to manipulate the presence of these cytokines in the injured spinal cord and evaluate the impact had on oligodendrocyte Fas expression. This could be achieved in several ways, including the use of genetically modified mice, lacking TNFα and/or IFNγ. Following experimental SCI in these mice, the extent of oligodendrocyte Fas expression could be compared between knockout mice and their wild-type littermates. Another question that could be addressed in this way is to what extent either TNFα or IFNγ each contribute to the increased Fas expression. It is possible that one cytokine may have a more important role than the other. Comparison of Fas expression levels in animals lacking each cytokine, and in those animals lacking both cytokines would provide preliminary information to this end. Furthermore, this type of investigation could be carried out in a rat model of experimental SCI using viral-induced gene expression of TNFα and IFNγ or by administering the cytokines to the site of injury. With presence of TNFα or IFNγ artificially increased post-SCI, any resultant changes in oligodendrocyte Fas expression could be attributed to the cytokines. If a particular cytokine could be shown as having a significant impact on post-SCI Fas expression, therapeutic targeting of that molecule could provide further anti-apoptotic actions against Fas-mediated apoptosis.

Because the inflammatory response in the injured spinal cord is so dynamic, it becomes difficult to pin-point the source for cytokines and chemokines. One theory would be that infiltrating immune cells are contributing to the increased expression of Fas receptor following injury by releasing pro-

inflammatory cytokines. By preventing the infiltration of systemic immune cells using key integrin blockers, it would be possible to being to evaluate their impact on Fas expression following SCI. This kind of manipulation has been successful in preventing immune cell margination following SCI (Mabon, Weaver et al. 2000; Bao, Chen et al. 2004; Gris, Marsh et al. 2004; Saville, Pospisil et al. 2004).

7.2 Fas ligand expression following SCI

Another important factor in the activation of Fas-mediated apoptosis is the presence of FasL. A cell can express receptor and will not fall victim to the cell death pathway unless the receptor is activated by its appropriate ligand. The cells that may be contributing to the activation of Fas-mediated cell death through their expression of FasL include astrocytes, reactive microglia and oligodendrocytes. The limitation in the results presented here is that the increased FasL protein expression following SCI was not completely evaluated. The extent of impact had by systemic immune cell infiltration on the presence of FasL in the injured spinal cord was not determined.

While it was observed that FasL expression increases throughout the first week following SCI, FasL mRNA increased significantly after one day, but was reduced in the days that followed. A potential explanation for this dichotomy is that systemic cells already expressing FasL infiltrate the spinal cord throughout the first week of injury, contributing to the increases presence of protein. It is possible that neutrophils and/or systemic macrophages known to infiltrate the spinal cord after injury are introducing additional FasL to the injury site, ultimately

contributing to the activation of Fas-mediated apoptosis in the oligodendrocyte population.

This line of investigation is limited by the challenge of discriminating between systemic macrophages and endogenous microglia using traditional immunohistochemical approaches. In addition, there are few effective antibodies that specifically label neutrophils in tissue sections, making the determination of their role in Fas-mediated apoptosis difficult. There has been a suggestion in the literature of the use of a chimera mouse model to elucidate these cellular mechanisms related to FasL expression (Letellier, Schreglmann et al. 2007). Here, a mouse would undergo bone marrow irradiation followed by reconstitution with GFP-labelled hematopoietic stem cells. The systemic immune cells originating from the newly grafted bone marrow would display the identifying characteristic of GFP-fluorescence. Following experimental SCI in these mice, identification of FasL-expressing cells in the spinal cord would be carried out. Cells double-labelled with GFP and FasL would be easily characterized as systemic and the contribution of these cells to the overall FasL presence following injury could be evaluated.

7.3 The role of astrocyte FasL expression on Fas-mediated apoptosis post-SCI

It was observed that following SCI, reactive astrocytes expressed FasL. In addition, it was observed that astrocytic processes may be closely associated with Fas-expressing cells in white matter areas surrounding the injury epicenter. Because it has been shown that the oligodendrocyte population predominantly expressed Fas receptor following SCI, there is a strong possibility that the

unidentified Fas-expressing cell would be an oligodendrocyte. These observations taken together suggest that reactive astrocytes might contribute the Fas-mediated apoptotic activation in oligodendrocytes. An unfortunate caveat to this work is that limited immunohistological reagents prevented the specific illustration of this hypothesis.

An experiment intended to address this issue is possible. A cre-lox mouse designed such that FasL expression in the astrocyte population could be manipulated after SCI would be a valuable tool. Following conditional knockout of the FasL gene in astrocytes, the extent of Fas-mediated apoptotic death of oligodendrocytes after SCI could be evaluated. If the removal of FasL from astrocytes resulted in a significant reduction in oligodendrocyte cell death, it would be more easily concluded that astrocytic expression of FasL after SCI was in fact contributing to Fas activation on oligodendrocytes.

A summary figure illustrating the proposed mechanisms of Fas-mediated apoptosis following SCI is helpful to conceptualize this secondary injury mechanism (**Figure 33**).

7.4 Therapeutic targeting of Fas-mediated apoptosis following SCI using sFasR

The delayed nature of Fas-mediated oligodendrocyte apoptosis presents itself as an advantageous mechanism to target in the reduction of injury-induced cell loss. Manipulation of this secondary injury process has a strong degree of clinical relevancy because treatment may be appropriately delayed post-SCI.

Oligodendrocytes are important supportive cells within the spinal cord, and the maintained presence of these cells following a traumatic insult is critical for

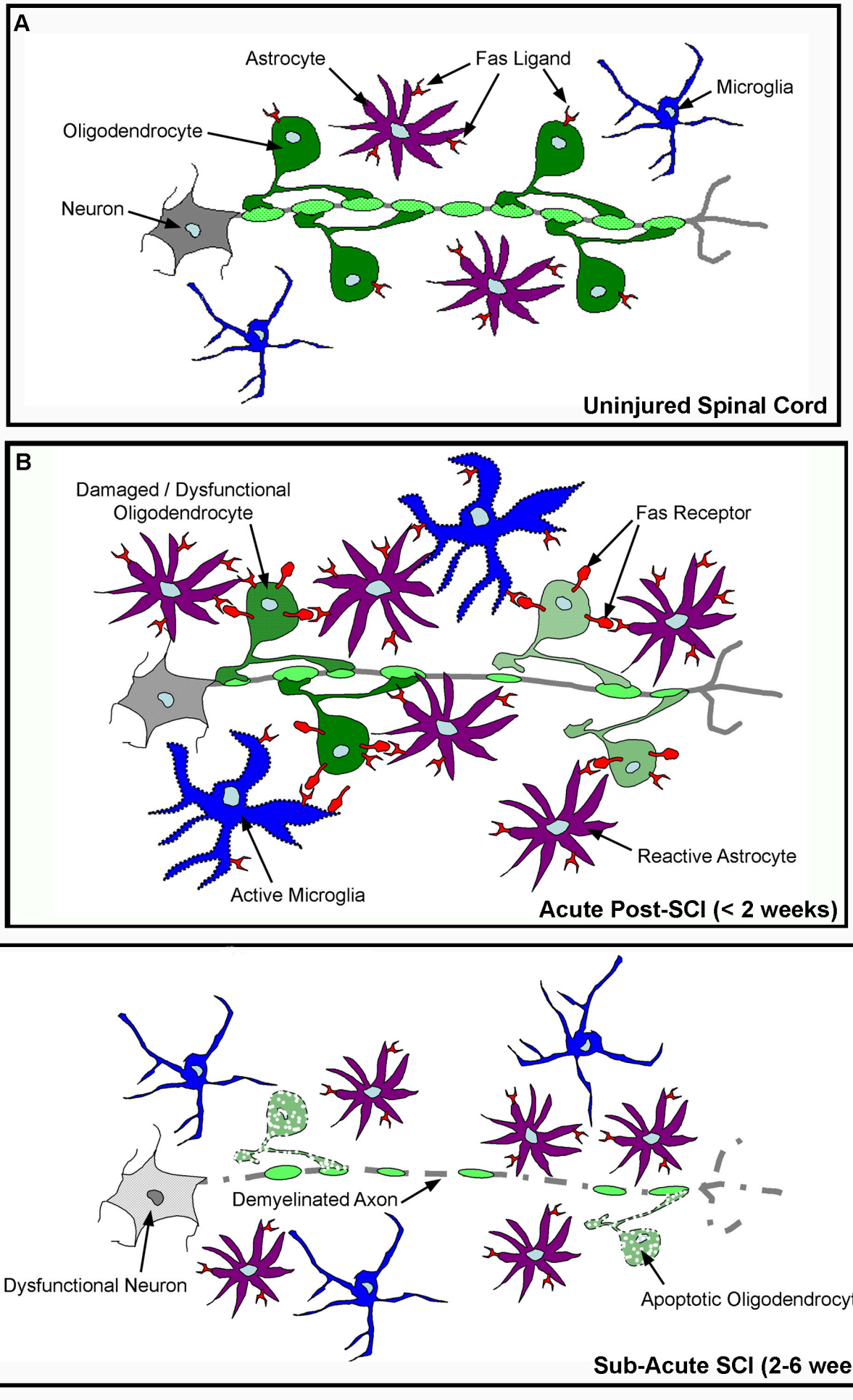


Figure 33: Proposed Mechanism of Fas-Mediated Oligodendrocyte Apoptosis After SCI

Figure 33: Proposed Mechanism of Fas-Mediated Oligodendrocyte Apoptosis After SCI

In the normal physiology of the spinal cord, the glial cells provide a healthy and supportive environment to the neurons and their axons. This relationship is depicted in an uninjured spinal cord (A), where oligodendrocytes are found to myelinate proximal axons, astrocytes provide support and maintain neuronal contact with capillaries, and the resting microglia survey the tissue for inappropriate cells, debris or infection. In this state, Fas receptor expression is non-existent and Fas ligand is expressed in low levels on oligodendrocytes and astrocytes.

In the acute post-injury setting of traumatic SCI, the physiology becomes pathologic, depicted in (B). Damaged and dysfunctional oligodendrocytes express Fas receptor, and there is an increased expression of Fas ligand on reactive astrocytes and microglia. A disruption in the glial-axonal relationship by Fas-expressing astrocytes perpetuate the activation of Fas-mediated apoptosis in the oligodendrocytes. There is likely some Fas activation by FasL-expressing activated microglia as well.

The sub-acute manifestation of this altered physiology is depicted in (C). Fas-mediated apoptosis targets many of the oligodendrocytes normally found myelinating white matter tracts. This results in a loss of support to axons, demyelination and a compromised, dysfunctional neuron. Axonal degradation and possibly neuronal cell death are likely consequences to the loss of oligodendrocyte support. Taken together, this cellular damage results in impaired motor and sensory function and paralysis.

overall tissue preservation and functional recovery. The role of oligodendrocytes in the health and maintenance of the spinal cord tissue is complex, involving an intimate and mutually-dependent axonal-glial relationship contributing to myelin formation (Rosenberg, Ng et al. 2006). Additionally, the oligodendrocyte population offers a significant trophic support to neurons and axons in their vicinity through the release of neurotrophins such as NT-3, BDNF and IGF-1 (Dai, Qu et al. 2001; Wilkins, Chandran et al. 2001; Du and Dreyfus 2002).

This work evaluated a potential therapeutic agent intended to target the Fas-mediated apoptotic pathway. Soluble Fas receptor administration at the time of SCI displays a significant anti-apoptotic ability, and enhances the viability of oligodendrocytes in the first week post-injury. In addition, a significant reduction in axonal degradation at the injury site is observed in sFasR-treated animals. It is possible to imagine that maintenance of the supportive oligodendrocyte population had an impact on the health of the surrounding axons, minimizing their degradation. The potential for sFasR treatment to be effective in a clinical setting was also established in this way. Post-injury treatment delay of eight hours resulted in significant oligodendrocyte viability in the early stages of injury, and contributed to enhanced long-term oligodendrocyte and neuron survival. The question remaining is seemingly straightforward: Does sFasR reduce post-traumatic apoptosis of oligodendrocytes, thereby maintaining a supportive environment that contributes to enhanced neuron and axon viability? Or is there an alternative explanation for the beneficial effects of sFasR on neurons?

There are several lines of additional investigation that might provide further insight. The first experiment could be designed to assess the impact of oligodendrocyte protection by sFasR on the survival of the neuron population. The development of another cre-lox mouse model where oligodendrocyte-specific expression of the Fas gene could be deleted after SCI would be necessary. If the oligodendrocytes do not express Fas, they would not be susceptible to Fas-mediated cell death. Additionally, the proposed impact of sFasR would not be possible. Following experimental SCI and the administration of sFasR, survival of neurons in the cre-lox mice and their wildtype littermates could be compared. If no significant difference in neuron survival is observed between the wildtype and knockout mice, it would be possible to conclude that sFasR effects are independent of oligodendrocyte survival and not related to the Fas-mediated apoptosis of oligodendrocytes.

Another experiment could be carried out to confirm that sFasR administration affects the oligodendrocyte population and its ability to maintain a supportive environment post-SCI. Oligodendrocytes produce myelin and the extent of myelin production can be correlated with the presence of myelin basic protein (MBP). If sFasR administration were to prevent the reduction of MBP associated with the demyelination of axons, it could be inferred that sFasR treatment maintains the health and viability of oligodendrocytes, in turn maintaining the integrity of the myelin sheath surrounding axons.

7.5 Post-injury Fas expression and critical therapeutic time window

The temporal expression pattern of Fas receptor following SCI (evaluated in chapter 3) revealed that both gene and protein expression show significant increases from uninjured tissue at 3 days post-injury. Only a slight increase in Fas expression was noted at 1 day, however there was a strong proportion of oligodendrocytes exhibiting markers of Fas-mediated apoptosis as early as 1 day following injury. In light of these observations, it would be plausible to suggest that therapeutic targeting of Fas-mediated cell death should be able to be delayed by as much as 3 days post-injury while maintaining a beneficial effect. Additionally it would be understandable that an earlier administration might show a slightly stronger efficacious effect.

Comparison of the overall cellular and behavioural impacts of delayed sFasR administration of 8 hours following SCI and 24 hours following SCI reveal interesting observations. Based on the aforementioned expression patterns, it would be expected that sFasR administration should be equally beneficial if delayed by 8 or 24 hours post-injury. It was observed that an 8 hour delay contributed to a stronger and more consistent benefit overall than did the 24 hour delay. This was puzzling, as both delayed time points fall well before the 3 day post-injury onset of increased Fas expression, making the pursuit of an adequate explanation challenging.

Intracellular signalling cascades such as in Fas-mediated cell death are inherently complex. The extracellular and intracellular machinery required to initiate and propagate the pathway are regulated by a host of environmental and cellular aspects, such as inflammatory cytokine secretion, cellular interactions,

and a myriad of gene and protein expression. It is possible that the cellular preparation for Fas-mediated signalling is initiated early in the injury process, and does not fully manifest as a functionally active pathway until 3 days post-injury. A gene and/or protein expression analysis of regulatory components of the Fas pathway from the time of injury throughout the first 3 days would provide an interesting perspective to this end.

Administration of sFasR is thought to have a strong anti-apoptotic ability due to the competitive nature of the molecule with cell-bound FasL. However, the possibility exists that sFasR has additional effects on the early regulatory aspects of Fas-mediated signalling. Moreover, the consideration that sFasR might have non-specific or non-apoptotic effects is worthwhile. In this event, the administration of sFasR could have unknown beneficial impacts when initiated at earlier post-SCI time points than expected. To further analyze this possibility, the regulatory elements dissected from the earlier proposed analysis of gene and protein expression would be evaluated in sFasR-treated and control-treated animals following SCI. This line of investigation would begin to de-lineate the non-specific and non-apoptotic impacts of sFasR that lend to its increased efficacy at early post-injury time points of administration.

7.6 Alternative strategies for sFasR administration post-SCI

In general, the therapeutic use of sFasR in the acutely injured spinal cord warrants further attention. In an attempt to bring this therapy closer to human SCI patients, it will be important to explore the possibility of novel modes of administration, in an attempt to reduce the invasiveness of subarachnoid

catheterization. There are several important considerations that must be maintained with any novel approaches. The ability to locally deliver sFasR to the site of injury within the spinal cord is critical to prevent adverse systemic effects. If this kind of treatment were given systemically, the potential for severe immunological complications would be high. In addition, the ability for a systemically administered therapeutic to easily penetrate the blood-spinal cord barrier is a concern. The large dose requirement often needed for efficacy in this instance would surely cause unnecessary side effects. Moreover, the cost of treatment could be significantly higher if such large doses were required to achieve beneficial results. With this in mind, the evaluation of novel, bioengineered drug delivery system designed for use in SCI is an important next step.

A synthetic hydrogel of hyaluronan (HA) and methylcellulose (MC), abbreviated HAMC, has shown promise for effectively delivering a therapeutic molecule into the intrathecal space following SCI (Gupta, Tator et al. 2006; Kang, Poon et al. 2009). This bioengineered polymer reduces the risk of post-traumatic infection and reduces the invasiveness created by chronic intrathecal catheterization. A therapeutic molecule, like sFasR, can be mixed into the HAMC *in vitro* and then the mixture can be carefully injected into the subarachnoid space at the site of SCI. The biochemical properties of HAMC allow it to be a liquid at room temperature and quickly become gel-like when exposed to body temperature. The fast-gelling capabilities of HAMC allow for its maintained presence at the site of injury. While sitting in the subarachnoid

space, the therapeutic molecule is effectively released over a restricted period of time. The caveat to date with this delivery system is the inability of HAMC to prolong the release of the molecule for longer than 24 hours. Due to the fact that Fas-mediated apoptosis peaks around five days post-injury, HAMC would need to show release kinetics in the length of days in order to be effective at administering an efficacious dose of sFasR. The ability of HAMC to provide a prolonged release past 24 hours is currently being evaluated (Kang, Poon et al. 2009).

Finally, a common concept in the field of SCI is the need for combinatorial approaches to therapy. There is no dispute that the secondary SCI pathologies are complex and that many deleterious events occur simultaneously, collectively contributing to the cellular and tissue destruction and functional deficits. It will be important to effectively target several of these injurious pathways in the acute setting of SCI to achieve optimal therapeutic effects. In addition, therapeutic manipulation of the chronic spinal cord to enhance its regenerative capacity and promote remyelination will be necessary. The field of stem cell therapy has exploded in recent years and will no doubt provide tremendous opportunity for the repair and regeneration of chronic SCI.

7.7 Novel therapeutic actions of IgG following SCI

In the course of the investigation looking into Fas-mediated apoptosis and the associated therapeutic effects of sFasR after traumatic SCI, it was observed that delayed intrathecal administration of human IgG was also therapeutically beneficial. This was a surprising result due to the fact that IgG was initially

chosen for the purpose of providing a non-reactive protein control in the study of sFasR. Following a preliminary literature review, it was determined that IgG may have a strong potential to become a clinically valid approach to neuroprotection following SCI.

Current clinical use of intravenous (IV) immunoglobulin (Ig) is widespread in the field of autoimmune neuropathies including Guillain Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) and multifocal motor neuropathy (Cats, van der Pol et al. 2008; Hughes 2008; Hughes 2008).

IV-Ig therapy involves administration of purified immunoglobulin fractions derived from extensive pools of plasma donors (Koski and Patterson 2006). Interestingly, it is believe that the most important and therapeutically active component of IV-Ig is IgG (Kaneko, Nimmerjahn et al. 2006; Nimmerjahn and Ravetch 2007; Anthony, Nimmerjahn et al. 2008; Kaveri, Lacroix-Desmazes et al. 2008). Surprisingly there is little conclusive evidence regarding the mechanism of action by IV-Ig in inferring its beneficial effects. The general consensus is that the effect must be immune-modulating in nature, and likely involves protection against autoantibody-mediated pathology (Kaneko, Nimmerjahn et al. 2006; Nimmerjahn and Ravetch 2007).

Recently, it has been suggested that the anti-inflammatory ability of IV-Ig, mediated by IgG molecules specifically, has to do with alterations in the glycosylation of the Fc domains of a sub-population of IgG molecules (Kaneko, Nimmerjahn et al. 2006; Nimmerjahn, Anthony et al. 2007; Nimmerjahn and

Ravetch 2007). It is believed that alteration of the Fc domain in this way heightens its ability to increase the expression of inhibitory Fcγ receptors, thereby reducing the impact of autoantibody activity (Nimmerjahn and Ravetch 2007).

There have been several studies published recently showing the potential for IV-Ig and IgG administration to offer therapeutic benefit following traumatic brain injury, experimental cerebral stroke and SCI (Knoblach and Faden 2002; Lapointe, Herx et al. 2004; Arumugam, Tang et al. 2007; Arumugam, Woodruff et al. 2009; Gok, Sciubba et al. 2009). In particular, its use in experimental SCI shows a reduction in neutrophil activity and resultant lipid peroxidation, giving merit to a potential mechanism of action SCI (Gok, Sciubba et al. 2009).

There is still much to be learned at this time regarding the therapeutic mechanisms taking place with IgG administration post-SCI. In addition, it will be important to establish an optimal treatment regime for the clinical use of IgG in the SCI patient population. Extrapolation from existing human studies will no doubt provide valuable insight into furthering its use in SCI. Because the work presented here involved intrathecal administration, it will be interesting to evaluate the potential of IV administration of IgG in the same rat model of SCI. This will require alterations in dosing, and a dose-response curve will be an important first step in evaluating the efficacy of IV IgG after SCI. Finally, the possibility exists that an even greater therapeutic effect might be observed if the Fc domains of IgG were subjected to sialylation.

This unexpected development provides an exciting opportunity for furthering the field of SCI therapeutics. The clinical effectiveness of IgG is well-established in other neuro-immune disorders and the preliminary work presented here provides a strong foundation for further work in this field. Moreover, the fact that IV-Ig is already approved for clinical use in other disorders gives its exploration in SCI an advantage in overcoming regulatory issues on the way to human clinical trials.

7.8 Evaluating the impact of sFasR and IgG therapeutic effects following experimental SCI

The molecular composition of the sFasR human fusion protein used in these experiments consists of a 234 amino acid sequence of human IgG Fc attached to the extracellular domain of human Fas receptor. Following inquiry to the manufacturing company, it was determined that the piece of IgG was necessary for molecular stability of the fusion protein. In response to this, it was determined that a valuable control in the experiments evaluating sFasR efficacy following SCI would be human IgG. It was unexpected that such a significant treatment effect was observed in the IgG-treated animals.

The first task after the observation that sFasR and IgG were more efficacious than the secondary saline control was to determine if the observed treatment effects were a result of non-specific protein interactions. An additional treatment group using a known benign protein was needed, and BSA was selected as such a protein. If the treatment effects observed with sFasR and IgG administration were in fact a result of non-specific protein interactions, then the same effects should be observed following BSA treatment. This was not the

case. Both sFasR and IgG showed significantly improved outcome measures following SCI when compared to both BSA and saline controls. There was also no reported difference in any of the outcomes between BSA and saline-treated animals. These findings helped to establish the theory that a specific functional effect was being carried out in the injured spinal cord by sFasR and IgG.

The challenge here is in the interpretation of the observed effects and the proposal of mechanism of action. Without the IgG findings, it would have been assumed that sFasR was beneficial solely as a result of its anti-apoptotic mechanisms. This idea has already been demonstrated in the ability of sFasR administration to reduce the number of TUNEL-positive cells following SCI (Ackery, Robins et al. 2006). However, the important question to address now is whether or not the beneficial impact of post-SCI administration of sFasR is also due in part to the stabilizing segment of IgG Fc.

It has been shown in the literature that the anti-inflammatory activity of IgG is predominantly a result of its Fc domain (Kaneko, Nimmerjahn et al. 2006; Nimmerjahn, Anthony et al. 2007; Nimmerjahn and Ravetch 2007). It therefore remains a distinct possibility that the neuroprotective effects of both sFasR and IgG are, at least in part, a result of the Fc domain found on both molecules.

In order to establish whether or not sFasR is acting independently of the Fc domain, it would be necessary to engineer a soluble Fas receptor that does not possess any element of the functional IgG molecule. The implications that this might have on the new protein's stability would have to be addressed accordingly. If the engineered soluble Fas and the original sFasR were

administered in separate treatment groups following experimental SCI, any differences in the outcomes measures between the two groups would provide valuable insight into the actual mechanisms at work.

Along the same lines, an experiment designed to compare the treatment effects of the full human IgG molecule against a mutated form, lacking the Fc domains, would provide insights into the mechanism of action. As unlikely as this might be given the background literature reviewed earlier, if IgG is exerting its beneficial effects independently of the Fc domain, this would lend to the idea that sFasR was acting independently of its Fc portion as well.

Another interesting experiment would be to investigate the therapeutic impact of Fc sialylation following SCI. If indeed the Fc domain of IgG was responsible for the therapeutic benefit, it would be expected that sialylation would further increase the beneficial outcomes observed when compared to regular human IgG. Results like this would establish the importance of glycosylation on the functional impact of IgG, and would serve to further the development of this novel therapeutic for use in SCI.

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7.9 Conclusions

The pathophysiology of SCI is complex, as are the active mechanisms of cell death throughout the course of secondary injury. Fas-mediated apoptosis has been known to occur following SCI, however the impact and mechanisms of its involvement were not clear. This work has been successful in answering some of the important questions in this field. It has been identified that the

oligodendrocyte is a major target for post-SCI Fas-mediated apoptosis. Secondly, this form of cell death can be therapeutically targeted using delayed sFasR administration. Aside from the impact of Fas-mediated apoptosis, this work has opened a new line of investigation into the therapeutic impact of IgG administration following SCI.

The work presented in the first part of this thesis established much of the cellular and temporal relationships involved in the activation of Fas-mediated apoptosis following SCI. The principle cellular target of this type of cell death was determined to be the oligodendrocyte, and it was confirmed that Fas-mediated apoptosis of oligodendrocytes occurs in a delayed fashion after injury. Furthermore, the expression of FasL following SCI contributes to the activation of Fas, and perpetuates the apoptotic signal in the oligodendrocytes. In all, the delayed nature of Fas-mediated oligodendrocyte cell death following SCI demonstrates the importance of this type of cell death mechanism.

This work has also provided an exciting contribution to the literature by illustrating the promising therapeutic potential of targeting Fas-mediated apoptosis after SCI. Intrathecal administration of sFasR following injury was effective at multiple outcome levels, and a large window of therapeutic opportunity existed in its use. The combination of the work presented here provides key information regarding secondary SCI pathophysiology. Fas-mediated cell death plays a deleterious role, however it is also targetable in a clinically relevant post-injury setting.

A surprising conclusion to this work is that intrathecal administration of human IgG offers significant therapeutic benefit following experimental SCI. As with most scientific discovery, many more questions are introduced with each new observation. The results presented here will serve as the foundation for future studies to investigate and refine the use of IgG as a treatment strategy following SCI.

There is great hope for the future of SCI research, with promise for the discovery and establishment of efficacious therapeutic options for patients. Subtle changes in functional ability for individuals suffering from SCI translate into a significantly improved perception of quality of life. The ability to potentially offer this kind of improvement to such a deserving patient population is incredibly rewarding and there is great confidence that scientists and clinicians will be successful in these endeavours.

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