POST-SPINAL CORD INJURY BELOW-LESION NEUROPATHIC PAIN: MECHANISMS AND NOVEL THERAPEUTIC APPROACHES

By

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ABSTRACT

Neuropathic pain is a significant and frequent outcome of spinal cord injury (SCI), and is often refractory to treatment. A better understanding of the pathological processes following injury that contribute to the development of neuropathic pain will aid the search for novel therapeutics. In the second chapter of this thesis a murine model of post-SCI below-lesion neuropathic pain was utilized to investigate changes in GABAergic tone. The *gad1*:GFP transgenic mouse line allowed the study of a subpopulation of GFP-labeled GABAergic neurons under control of the GABA synthesizing glutamate decarboxylase enzyme. SCI was observed to result in a loss of GABAergic neurons, and secondary markers of GABAergic tone supported this observation. This finding suggests that GABAergic interneuron cell death accounts for the decreased GABAergic tone previously reported post-SCI.

In the third chapter of this thesis it was attempted to prevent the death of GABAergic neurons post-SCI using a transgenic mouse line expressing increased levels of the X-linked inhibitor of apoptosis (XIAP) under the ubiquitin C promoter. No differences were observed between ubXIAP and wildtype mice, indicating that increased expression of XIAP is not sufficient to prevent the development of neuropathic pain post-SCI.

The fourth chapter of this thesis attempted to prevent the development of neuropathic pain through a novel treatment schedule of the drug pregabalin. Pregabalin administered shortly after SCI prevented the development of neuropathic pain. Pregabalin initiated 1 week post-SCI had no effect. Early pregabalin treatment did not appear to dramatically alter glial activation, or expression of the pregabalin receptor, but we observed changes in markers associated with synaptic plasticity.

My findings build upon our knowledge of the mechanisms underlying post-SCI below-lesion neuropathic pain, and suggest new avenues of research, such as the uses of preemptive treatment with pregabalin, that offer promise for translation to clinical use.

List of Abbreviations and Symbols Used

5-HT – 5-hydroxytryptamine

AOI – area of interest

BDNF – brain-derived neurotrophic factor

CGRP – calcitonin gene-related peptide

CNS – central nervous system

COX2 – cyclooxygenase-2

dPGB – delayed pregabalin treatment

DRG – dorsal root ganglion

EAE – experimental autoimmune encephalitis

ECL – enhanced chemiluminiscence

eGFP – enhanced green fluorescent protein

GABA - γ-amino butyric acid

GAD – gluatamate decarboxylase

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GAT1 – GABA transporter 1

GLYT1 – glycine transporter 1

GTP – guanosine triphosphate

HRP – horse radish peroxidase

IAP – inhibitor of apoptosis

IB4 - isolectin B4

i.p. - intraperitoneal

iPGB – immediate pregabalin treatment

JGM – Jason George Meisner

kD –kiloDyne

LTP – long term potentiation

MAPK - mitogen activated protein kinase

NeuN – neuronal nuclei

NGS – normal goat serum

NMDA – N-methyl-D-aspartate receptor

nNOS – neuronal nitric oxide synthase

NSHRF – Nova Scotia Health Research Foundation

NYU-MASCIS – New York University – Multi-Centre Acute Spinal Cord Injury Study

OCT – optimal cutting temperature

PBS – phos-buffered saline

PGB - pregabalin

PGE2 – prostaglandin E2

PKCγ – protein kinase C isoform γ

PSD-95 – post-synaptic density protein 95

PVDF – poly vinyl difluoride

Rac-1 – ras-related C3 botulinum toxin substrate 1

SCI – spinal cord injury

SDH – superficial dorsal horn

SEM – standard error of the mean

SDS-PAGE – sulfur dodecyl sulfide polyacrylamide gel electrophoresis

SP- substance P

TBS-T – tris-buffered saline with tween

TBS-X – tris-buffered saline with triton-X

TNFα - tumor necrosis factor-α

TSP - thrombospondin

TRPV1- transient receptor potential cation channel, subfamily V, member 1

TTX – tetrodotoxin

VGAT – vesicular gaba transporter

VGLUT2 – vesicular glutamate transporter

WDR – wide dynamic range

XIAP – x-linked inhibitor of apoptosis

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CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION TO THE SPINAL CORD

The spinal cord, encapsulated within the vertebral column, is an important conduit for information transfer between the brain and the body, and independently possesses circuits important for reflexive locomotion and integration of sensory information. The spinal cord may be broadly described as containing white, and grey, matter; so named for the distinct coloration of the two regions when viewed in section using light microscopy. Both white, and grey, matter are susceptible to traumatic injury and subsequent inflammatory processes, damage from autoimmune attack, and neurodegenerative processes that alter normal physiological function. Endogenous repair and regeneration within the spinal cord is minimal, and current therapeutic interventions offer limited benefit to reduce degeneration, or restore function, following injury.

The white matter of the spinal cord is comprised of axonal tracts, with myelin sheaths accounting for the white colour. These axonal tracts may descend from distant supraspinal sites to their targets in the lower spinal cord, while others may ascend great distances from the body's periphery to targets in the brainstem. White matter encircles the outermost aspect of the spinal cord, and the axonal tracts therein conveying specific types of information are arranged in dorsal/ventral and medial/lateral pattern that is conserved across species. When viewed in transverse section the grey matter may be observed within the white matter of the spinal cord, in a pattern sometimes likened to a butterfly's wings, most notably in the cervical and lumbar region of the spinal cord. The grey matter of the cord is most prominent within the cervical and lumbar enlargements of the spinal cord, regions association with motor signals and sensory perception from the upper and lower limbs, respectively.

The grey matter enlargement within the cervical and lumbar cord is a result of the more complicated neural processing associated with these regions, such as mediation of voluntary movement, and fine sensory discrimination, and the associated neurons and synaptic connections required to support this processing. Neurons and glia are found within the grey matter. Processes extending from neurons comprise the axons contained in the tracts of the white matter. The neurons of the spinal cord are arranged within the grey matter according to their function. They may be broadly described as the dorsal region being important for the integration of sensory input from the periphery, and the ventral aspect providing innervation to the body's musculature.

1.2 SPINAL CORD INJURY

Spinal cord injury (SCI) can result in damage to both the grey and white matter of the spinal cord. Loss of motor function may be incurred through severing of motor axons, loss of primary motor neurons in the ventral horn of the spinal cord, or demyelinating events that occur secondary to injury. SCI can also impair the integration and conduction of sensory information through damage to ascending tracts conveying sensory information, descending tracts that modulate peripheral input, as well as loss of interneurons and/or damage to circuits which modulate sensory information.

1.2.1 Human SCI and Neuropathic Pain

Spinal cord injury (SCI) occurs with an incidence of 15-40 persons per million, striking approximately over 14 000 individuals each year in North America (Sekhon and Fehlings, 2001). SCI has historically impacted a young population, with an average age at time of injury of 28.7 in the years between 1973-1979. However, the increasing median age of the general population has led to a concurrent increase of the average age at time of injury to 39.5 years since 2005 (National Spinal Cord Injury Statistical Center, 2010). SCIs are often co-morbid with other traumatic injuries, as may occur during an automobile accident (ie. lacerations, fractures, dislocations, contusions, internal bleeds (Finn and MacDonald, 2010)); however, individuals who survive the first year post-injury have life expectancies only marginally shorter (2-4 years) than an uninjured age-matched population (National Spinal Cord Injury Statistical Center, 2010). While many of the comorbid musculoskeletal, internal, and hemorrhagic injuries associated with SCI may be effectively resolved, deficits arising from injury to the spinal cord itself will often afflict the individual for the remainder of their life.

The sequelae of SCI are diverse, and dependent on the location and severity of the injury. Injuries may be broadly classified as complete or incomplete tetraplegia, resulting from injury to C1-C8, and complete or incomplete paraplegia, resulting from injury to the thoracic, lumbar, and sacral spinal cord regions. While restoration of motor function remains the long sought goal of SCI research it's appreciated by researchers and patients that other symptoms of SCI require attention, "…the benefits that consumers [SCI patients] derive are directly related to the relevance of these developments to their lives. If any scientific and clinical discoveries are to translate into better care and quality of life for the patient, research efforts must consider these priorities" (Irene M Estores, 2002). Patient surveys have revealed other aspects of the SCI condition that may yield greater improvement to quality of life than motor restoration, and may be used by researchers to guide the priorities of their work. Paraplegics, for instance, most frequently rank regaining sexual function to be their highest priority; followed by: improvements in bladder, bowel and autonomic dysreflexia management; trunk stability; walking movement; chronic pain; normal sensation; and arm/hand function (Anderson, 2004). Individuals surveyed who do report the presence of neuropathic pain indicate that resolving pain is their foremost priority (Anderson, 2004), compelling researchers to find improved solutions to this problem.

1.2.2 Post-SCI Neuropathic Pain

Neuropathic pain is common following injury to the nervous system, and is a frequent outcome of SCI. Traumatic injury, metabolic disease, and viral infection are all capable of inducing changes in the nervous system that lead to a loss of sensory function, but also to maladaptive facilitation and spontaneous activity of the pain signaling system.

Neuropathic pain can manifest itself as hyperalgesia (previously noxious stimuli perceived as more intense), allodynia (previously innocuous stimuli perceived as noxious), spontaneous pain (noxious sensation in the absence of stimuli), dysesthesia (spontaneous unpleasant, abnormal sensations), and paresthesia (tingling or numb sensations), and is often refractory to conventional treatment. SCI neuropathic pain may be described as at-, below-, or above-level, where level refers to the dermatome associated with the site of SCI. At-level pain is believed to result from changes to both the peripheral and central nervous system, while below- and above-level pain is believed to reflect changes in the central nervous system alone (Siddall et al., 2000).

The gravity of neuropathic pain is evident in the language used by patients to describe their experience, such as "grueling, wrenching, lancinating, etc." (Melzack, 1987). Pain is the most common cause of disability among working age adults in Canada (Housing and Social, 2002), incurring substantial direct costs to Canadian health care ($6 billion) and indirect costs to the national economy ($16 billion) (Guerriere et al., 2010). Post-SCI pain causes a significant impairment to daily routines and quality of life to a greater degree than motor impairment (Rintala et al., 1998).

Past estimates of the prevalence of neuropathic pain after SCI have ranged from 18-63% (Mariano, 1992), with more recent studies showing a similar range from 18-67% of individuals developing at- or below-level neuropathic pain (Werhagen et al., 2004)(Finnerup et al., 2001). In one survey of individuals reporting pain post-SCI, 23% of individuals with high thoracic or cervical injury, and 37% of individuals with low thoracic or lumbosacral injuries, suggested they would be willing to trade sexual and bladder/bowel function, or the potential of locomotor recovery, for relief from pain symptoms (Nepomuceno et al., 1979).

Many drug classes have been investigated for post-SCI neuropathic pain, such as anticonvulsants, analgesics, antidepressants, antispastics, and cannabinoids. Often, the design of these studies makes it difficult to assess if the drugs improve neuropathic pain symptoms, or pain resulting from musculoskeletal, spastic, or affective origin (Teasell et al., 2010)(Attal et al., 2009). The strongest evidence for relief of neuropathic pain exists for the gabapentinoid class of anticonvulsants, comprised of gabapentin (Neurontin, Pfizer) and pregabalin (Lyrica, Pfizer) (Teasell et al., 2010), and these drugs are now considered first-line therapy for neuropathic pain (Gilron et al., 2006). Gabapentinoids still only seem to offer relief to 67-76% of individuals with post-SCI pain (To et al., 2002)(Putzke et al., 2002), incurring a need for the development of novel therapeutics, or improved treatment paradigms, to better serve this population.

1.3 MODELING NEUROPATHIC PAIN

For the past 30 years animal models have been utilized to investigate pathophysiological mechanisms underlying the development of neuropathic pain. The first animal models of neuropathic pain were developed beginning in the late 1980's to model symptoms experienced after peripheral nerve injury, and feature ligations (Bennett and Xie, 1988)(Seltzer et al., 1990)(Kim and Chung, 1992) or transections (Decosterd and Woolf, 2000) of the sciatic nerve and its branches. In these experimental models nerve fibres innervating the plantar surface of the hindpaw are damaged, permitting the application of thermal, mechanical, and chemical stimuli to the paw to evaluate altered sensory thresholds within the injured sensory field. The development of the first nerve injury model by Bennet and Xie (Bennett and Xie, 1988), and subsequent models, initiated a rapid period of progress in the understanding of neuropathic pain, and provided

a valuable tool for the pre-clinical assessment of novel therapeutics.

1.3.1 General Mechanisms of neuropathic pain

Peripheral nerve injury and the aforementioned injury models have generated the majority of our knowledge about the pathophysiology of neuropathic pain. The following section will provide a general description of mechanisms of pathological pain signaling emergent from peripheral nerve injury, and will be followed by a discussion of models of post-SCI neuropathic pain and resultant mechanisms.

Peripheral sensitization is the most acute form of neuropathic pain, and may be more reflective of prolonged exposure of sensory nerves to chronic inflammatory conditions. Sensitizing agents, such as the inflammatory mediators prostaglandin E2 (PGE_2), 5-hydroxytryptamine (5-HT), bradykinin, adrenaline, noradrenaline, and neurotrophic factors, are released following tissue damage and subsequently bind Gq or Gs protein-coupled receptors, or receptor tyrosine kinases, inducing the activation of phospholipase C, adenylyl cyclase, and protein kinases. This can lead to phosphorylation of tetrodotoxin(TTX)-insensitive sodium channels, transient receptor potential cation channel subfamily V member 1, multiple other ion channels, or to changes in gene regulation. This post-translational modulation acts to change the activation threshold and kinetics of specific ion channels (Woolf and Salter, 2000).

Transcriptional changes in DRG neurons can also result in the development of a neuropathic nociceptor phenotype. These changes are initiated downstream of cytokine and growth factor receptors which alter transcription via their respective signal transduction cascades (Scholz and Woolf, 2002). These transcriptional changes can result in a reduced depolarization threshold, such that previously non-noxious levels of

stimulation will induce pain signaling, or even spontaneous firing of the fibre. An example of this phenomenon is the awakening of silent, or sleeping, nociceptors. Primary afferent fibres which are sensitive to chemical stimulation, but insensitive to mechanical stimulation, can become mechano-sensitive following exposure to inflammatory mediators; thus contributing a novel source of nociceptive input to the spinal cord (Mizumura, 1998).

Changes in ion channel expression play a large role in phenotype switch following injury, in particular the sodium channels $Na_v1.1$, $Na_v1.3$, Nav1.6, $Na_v1.7$, $Na_v1.8$, $Na_v1.9$ and Na_X (Waxman et al., 1999)(Abe et al., 2002). TTX-insensitive channels, $Na_v1.8$ & $Na_v1.9$, are down-regulated following axotomy (Dib-Hajj et al., 1996); however, recent interest in the uninjured population of nerve fibres has demonstrated that $Na_v1.8$ (Gold et al., 2003) and the rapidly recovering $Na_v1.3$ (Abe et al., 2002) are upregulated after injury in adjacent fibre populations. A similar pattern of increased expression in uninjured fibres, and decreased expression in injured nerves, occurs with the TRPV1 receptor (Hudson et al., 2001), and novel expression of TRPV1 on heat/capsaicin insensitive Aδ fibres (Rashid et al., 2003) indicates that uninjured fibre populations are important mediators of heightened pain sensitivity.

Central sensitization may act to heighten pain sensitivity through a variety of mechanisms. Following injury, collateral fibres from Aβ nerves (mediating innocuous touch) which normally terminate in Rexed's laminae II and IV, have been observed to migrate to more superficial layers of the spinal dorsal horn previously occupied by nociceptive afferents (Scholz and Woolf, 2002). This migration allows excited Aβ fibres to form novel synapses on nociceptive second order neurons, creating a paradigm wherein

innocuous touch may result in activation of ascending pain tracts (Bridges et al., 2001)(Ueda and Rashid, 2003). Like peripheral sensitization, central sensitization may be mediated via activity-dependent or transcription-dependent mechanisms; both are mediated by some of the same processes, only transcription dependent sensitization is more delayed. Activity- dependent sensitization may occur following the activation of the G protein-coupled NK1 receptor by Substance P (SP), or activation of receptor tyrosine kinases such as TrkB by brain-derived neurotrophic factor (BDNF), leading to activation of intracellular signaling cascades that phosphorylate ion channels (Scholz and Woolf, 2002). Transcription-dependent sensitization may occur through the induction of a gene like cyclooxygenase-2 (COX2), and the subsequent production of PGE_2 which acts to sensitize pre- and post-synaptic terminals in the spinal dorsal horn (Ueda and Rashid, 2003).

Another aspect of central sensitization is wind-up, or long-term potentiation (LTP). This is a term given to a mechanism of neuropathic pain that involves both activity- and transcription-dependent changes, but with a prominent role of the neurotransmitter glutamate and the *N*-methyl-D-aspartate (NMDA) receptor. Wind up was first described as an increase in the excitability of second order nociceptive neurons resulting from, and outlasting, a short burst of activity from nociceptive afferents (Ji et al., 2003). An acute example occurs following intradermal capsaicin injection which activates peripheral TRPV1 receptors causing heightened sensitivity to pinprick and innocuous touch in a secondary zone outside the area of primary hyperalgesia (Willis, 2002). This can be problematic in cases of nerve injury, where increased Na channel activity can lead to the ephaptic firing patterns needed to initiate and maintain the sensitized state (Ji et al., 2003).

1.3.2 Models of post-SCI neuropathic pain

Models of SCI-induced central pain emerged following the development of models of peripheral neuropathic pain. The first models utilized photochemically induced ischemia to induce injury to the T10 spinal segment cord and assess alterations to below-lesion sensory thresholds (Hao et al., 1991)(Xu et al., 1992). These animals subsequently developed mechanical allodynia, and demonstrated the ability to model central neuropathic pain syndromes and assess potential therapeutic agents.

A variety of mechanical injuries were also developed to better recapitulate the injuries occurring in the clinical population, involving post-hemorrhagic ischemia and direct tissue damage. These models utilized a hemi-section (Christensen et al., 1996)(Christensen and Hulsebosch, 1997), cutting through one hemisphere of the spinal cord as may result from a stabbing or firearm injury, or compression injury to the dorsal surface of the spinal cord (Siddall et al., 1995)(Lindsey et al., 2000), as may result from a vertebral fracture and dislocation.

With the exception of injuries sustained during wartime, or as a result of violent crimes, the majority of SCIs are caused by motor vehicle accidents, or falls, and result in a contusive injury characterized by a core of necrotic tissue surrounded by a rim of demyelinated axons. These pathophysiological features can be recapitulated by experimental contusive injuries. The development of the NYU-MASCIS (Gruner, 1992) weight-drop contusive injury initiated progress into reproducing this more common injury. Modern techniques commonly used to induce SCI utilize tools to produce graded and reproducible contusion injuries to the dorsal surface of the spinal cord following a dorsal laminectomy, such as the Precision Systems Infinite Horizons impactor (Scheff et al., 2003), and the Ohio State University Electromagnetic Impactor (Jakeman et al.,

2000). These computer controlled devices allow the user to accurately assess the force of impact and tissue displacement of the time injury, important for producing consistent animal models (Ghasemlou et al., 2005).

1.4 MECHANISMS OF BELOW-LESION POST-SCI NEUROPATHIC PAIN

Spinal cord injury initiates a cascade of events that contribute to the facilitation of pain signaling. Below-lesion neuropathic pain may represent a purely central nervous system event, unlike at-level pain where there may be direct damage to terminals of the primary sensory afferent fibres and dorsal root ganglion, inducing changes in the transduction of environmental stimuli.

Afferent fibres from sensory neurons may synapse on three types of cells within the dorsal spinal cord: (1) inhibitory interneurons which modulate activity of second order projection neurons and primary nociceptive input; (2) excitatory interneurons which subsequently synapse on second order projection neurons; and (3) projection neurons that relay noxious pain signals to the brain. Classes and characteristics of sensory neurons are further described in Table 1.1. Classes and characteristics of dorsal interneurons are further described in Table 1.2. Thus, in the absence of overt damage to the peripheral sensory afferent fibres, the amplification of pain signaling must be mediated by changes in the neurochemical activity of sensory afferent fibres and/or in the properties of 2nd and 3rd order projection neurons. Spinal dorsal horn neurons may belong to one of three groups dependent upon their response properties. Neurons may be responsive to: low-threshold (LT) signaling, such as that mediated by Aβ fibres conveying innocuous sensations; high threshold (HT) signals, such as that mediated by Aδ and C fibres

conveying noxious stimuli; and wide dynamic range (WDR) neurons, responsive to a wide range of stimulus intensity and showing a graded response to increased stimulation.

1.4.1 Changes in GABAergic tone and integration

Second order wide-dynamic range (WDR) neurons mediate a reflexive modulation of pain signaling at the level of spinal integration of sensory input. WDR neurons may be excited by direct stimulation from Aδ and C fibres, or by excitatory interneurons. However, this activation is subject to simultaneous input from inhibitory interneurons activated by Aβ, and high sensitivity Aδ, fibres signaling low threshold mechanical stimulation; this forms the basis of the gate control theory of pain (Melzack and Wall, 1965). Inhibition of nociceptive input by inhibitory interneurons also provides a rationale for why gently rubbing (activating Aβ fibres) a sore area can reduce the perception of pain (involves C and Aδ fibres).

A loss of γ-amino butyric acid-(GABA)ergic inhibitory tone could increase the excitability of WDR neurons, lowering pain threshold and potentially increasing pain perception. This hypothesis was tested using the $GABA_A$ antagonist bicuculline in uninjured and allodynic animals post-SCI. In uninjured animals bicuculline increased the receptive field size, spontaneous firing rate, and response to skin brushes and pinching; whereas bicuculline had little to no effect in animals manifesting allodynia post-SCI (Drew et al., 2004). This observation suggests that the loss of effect of the $GABA_A$ antagonist bicuculline results from a loss of endogenous GABAergic tone post-SCI. This is supported by observations of reduced levels of the GABA synthesizing enzyme glutamate decarboxylase (GAD) post-SCI in the spinal dorsal horn (Gwak et al., 2008).

1.4.2 Pain facilitation by glial activation

Recent observations have highlighted the role of glia in the development and maintenance of neuropathic pain post-SCI. Glia is the umbrella term for the support cells of the nervous system: astrocytes (conventionally considered important in the regulation of extracellular homeostasis); oligodendrocytes (provide the myelin sheath to axons); and microglia (macrophage-like resident immune cells of the nervous system). Microglia have been most clearly linked to the development and maintenance of neuropathic pain post-SCI. Under normal physiological circumstances microglia exist in a quiescent state of surveillance during which there is rapid exploration by cellular filopodia of surrounding tissue for chemotactic cues indicative of injury or immune threat (Hanisch and Kettenmann, 2007). Upon detection of one of many stimulating cues microglia migrate along the chemotactic gradient and undergo a phenotype switch, changing their morphology from a ramified to amoeboid reactive state capable of responding to the site of altered homeostasis. The reactive state of microglia is recognized to be neurotoxic, releasing factors such as super oxide (Colton and Gilbert, 1987), nitric oxide (Moss and Bates, 2001), and tumor necrosis factor (TNF)-α (Sawada et al., 1989).

Following SCI microglia remote from the lesion site can also transition to the amoeboid reactive state and remain as such for months to years post-injury (Fleming et al., 2006). Chronic, remote activation has also been implicated as a factor in the maintenance of below-lesion neuropathic pain in animals models of SCI, leading to development of mechanical allodynia and hyperexcitability of projection neurons that may be reversed by treatment with microglial inhibitors (Hains and Waxman, 2006)(Gwak et al., 2008).

1.4.3 Synaptic adaptation post-SCI

The synaptic connections between sensory afferent neurons, interneurons, and projections neurons may undergo a period of plasticity in response to injury. This synaptic remodeling may permit the establishment of maladaptive, pain facilitatory synapses. The remodeling of synaptic connections in supra-spinal sites, such as the motor cortex, is recognized following spinal cord injury (Kim et al., 2006), but less attention has been paid to changes below the level of lesion. Recent investigations using an inhibitor of ras-related C3 botulinum toxin substrate 1 (Rac-1), a guanosine triphosphate(GTP)-binding protein involved in dendritic spine morphology, have supported the premise that inhibiting dendritic spine plasticity post-SCI can ameliorate reductions in behavioral withdrawal thresholds and reduce the excitability of wide dynamic range neurons (Tan et al., 2008).

1.5 GABAPENTINOID THERAPEUTICS FOR POST-SCI NEUROPATHIC PAIN

1.5.1 Development of gabapentinoids

Gabapentin and pregabalin, belonging to the gabapentinoid class of drugs, are increasingly prominent therapeutics for the treatment of neuropathic pain. Recognition of GABAergic dysfunction in convulsant disorders led to the synthesis of the gamma-amino butyric acid (GABA) analogs gabapentin, 2-[1-(aminomethyl)cyclohexyl]acetic acid (Neurontin, Pfizer Canada Inc), and subsequently pregabalin, (S)-(+)-3(aminomethyl)-5-methylhexanoic acid (Lyrica, Pfizer Canada Inc), comprising the gabapentinoid class of anti-convulsant drugs (Bryans and Wustrow, 1999). In the synthesis of gabapentin a cyclohexyl group was added to the carbon backbone of the GABA molecule with the intent of increasing lipophilicity and thus CNS pentrance, relative to GABA itself (Bryans and Wustrow, 1999). This addition did result in significant CNS penetrance of gabapentin, however not as a result of increased lipophilicity, but owing to conformational change in the molecule's shape. This change permitted gabapentin transport via the L system amino acid transporters (Su et al., 1995). The L system facilitates transport of amino acids such as leucine across the blood brain barrier, and is the unexpected primary transport mechanism for gabapentin entry to the central nervous system (CNS). Gabapentin was subsequently found to have a maximum 60% bioavailability, reaching peak brain levels 1-hour post oral administration (Vollmer et al., 1986). Saturable transport by system L amino acid transporters accounts for non-linearity between dose and plasma concentration (Stewart et al., 1993).

Pregabalin is another structural analogue of GABA discovered in the late 1980's (Andruszkiewicz and Silverman, 1989). It was also found to have rapid absorption reaching peak plasma concentration within an hour of administration, and demonstrating 90% bioavailability (Kugler et al., 2002). These compounds were expected to act as agonists at the $GABA_{A/B}$ receptors and suppress hyperexcitability contributing to epileptogenic activity. Early preclinical testing demonstrated anti-convulsant efficacy, and later clinical testing revealed reductions in pain (Rosner et al., 1996) (Mellick and Mellick, 1995). Gabapentinoids are presently first-line therapy for post-SCI neuropathic pain (Teasell et al., 2010).

1.5.2 Ca_v $\alpha_2\delta$-1: High affinity binding target of gabapentinoids

The Ca_v $\alpha_2\delta$-1 subunit has been identified as the gabapentinoid receptor through site-directed mutagenesis studies (Bian et al., 2006). Ca_v $\alpha_2\delta$-1 is localized primarily to the pre-synaptic terminal of sensory afferent fibres within the dorsal horn (Bauer et al., 2009). At pre-synaptic sites this channel plays an important role in Ca^{2+} influx required for vesicle exocytosis. The ability of gabapentinoids to inhibit calcium influx remains controversial, and may be dependent on the level of $\alpha_2\delta$-1 expression relative to other subunits (reviewed in (Dooley et al., 2007)). Following peripheral nerve injury (Bauer et al., 2009) and SCI in the rat (Boroujerdi et al., 2011) Ca_v $\alpha_2\delta$-1 expression is upregulated in the dorsal spinal cord, and is believed to contribute to the development of neuropathic pain.

1.6 Aims for investigation into post-SCI below-lesion neuropathic pain

As previously described post-SCI neuropathic pain is often refractory to treatment, and it is hoped that a better understanding of the underlying pathobiology, and investigation of novel therapeutics, will alleviate unnecessary suffering. Until chronic neuropathic pain is reduced or eliminated, these SCI patients are unlikely to be able to participate in highly beneficial forms of physical rehabilitation and exercise. The focus of this thesis is to investigate some of the mechanisms implicated in post-SCI neuropathic pain, and investigate potential therapeutics, to better serve this patient population and promote advances in this field of research.

In Chapter 2 we investigate the fate of a population of GABAergic inhibitory interneurons post-SCI to determine if cell death may contribute to a loss of GABAergic tone, and assess a number of secondary measures to support this observation. This investigation lead to work discussed in Chapter 3, wherein transgenic mice resistant to apoptosis were studied to see if we could enhance neuroprotection and prevent the development of neuropathic pain. Chapter 4 presents an investigation into the gabapentinoid drug pregabalin as a pre-emptive agent to prevent the development of neuropathic pain-post SCI. We hope these observations will contribute to our understanding of the pathobiology of post-SCI neuropathic pain, and improve the development of treatment options for patients.

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Table 1.1 Characteristics of peripheral nervous system fibre types.

Fibre Type	Anatomical Traits	Conduction Velocity	Classes/ Specializations	Function	Termination
C	Unmyelinated Cell diameter ~20 μm	<1.4 ms^{-1}	Peptidergic (expressing Substance P, CGRP)	Responds to chemical, thermal and mechanical stimulus Expresses TRPV1, $P2X_3$	Laminae I, II_i Primarily interneurons
			Non-peptidergic IB4(+)	Responds to chemical, thermal and mechanical stimulus Expresses TRPV1, $P2X_3$	Laminae I, II_o Projection neurons
			Non-petidergic IB4(-)	Expresses TRPV1 Unresponsive to thermal, mechanical stimulus until sensitized by inflammation	Laminae I, II
Aδ	Lightly Myelinated Cell diameter ~30 μm	2.8-8 ms^{-1}	Type 1 Lightly myelinated	Mechanical sensitivity, thermal responsiveness at ~52°C	Laminae I, II, V
			Type 2 Lightly myelinated	Mechanical sensitivity, thermal responsiveness at ~43°C	
Aβ	Highly Myelinated Cell diameter ~30-50 μm	14-30 ms^{-1}	Meissner's corpuscles	Touch, Pressure (dynamic)	Laminae III-VI
			Pacinian corpuscles	Deep pressure, vibration (dynamic)	
			Merkel's disks	Touch, pressure (static)	
			Ruffini's corpuscles	Stretching of skin	
Aα	Highly myelinated Cell Diameter ~30-50 μm	30-55 ms^{-1}	Muscle spindles	Muscle length	Laminae III-VI
			Golgi tendon organs	Muscle tension	
			Joint receptors	Joint posistion	

Compiled from: Harper and Lawson, 1985*,* Basbaum, 1999, *&* Millan, 1999

Table 1.2 Interneurons of Laminae I-III

Post-synaptic Effect	Principle Neurotransmitter	Laminae	Markers	Morphology	Input
Inhibitory	GABA	25% of Laminae I cells 30% of LaminaeII cells 40% of Laminae III cells	VGAT GAD Galanin Neuropeptide Y	Islet cells Some Central cells	C fibres
	GABA/Glycine		nNOS		
	Glycine	Laminae III	GLYT2		
Excitatory	Glutamate	Laminae I-III	VGLUT2 Calbindin Calretinin PKCγ Somatostatin Neurotensin Substance P	Radial Cells Vertical Cells Some Central Cells	C and Aδ

Reviewed in: Todd, 2010

CHAPTER 2: LOSS OF GABAERGIC INTERNEURONS IN LAMINAE I-III OF THE SPINAL CORD DORSAL HORN CONTRIBUTES TO REDUCED GABAERGIC TONE AND NEUROPATHIC PAIN FOLLOWING SPINAL CORD INJURY

2.1 PREFACE AND SIGNIFICANCE TO THESIS

This chapter has been reproduced with permission from Mary Ann Liebert, Inc. (**Appendix A.1**) and was published as Meisner JG, Marsh A, and Marsh DR. Loss of GABAergic interneurons in Laminae I-III of the spinal cord dorsal horn contributes to reduced GABAergic tone and neuropathic pain following spinal cord injury. Journal of Neurotrauma 2010; 27:4, 729-737.

Results of this study have also been presented at the Canadian Association for Neuroscience Meeting in Vancouver, BC, May 2009.

I designed the experiments (with Dr. Marsh), conducted animals surgeries and post-operative care, conducted behavioural assessments of hindlimb locomotor function, mechanical allodynia, drug treatment studies, processed and prepared tissue for glutamate decarboxylase (GAD) 65/67 immunohistochemistry studies, GAD 65/67 and GAT1 Western blotting studies, and wrote the manuscript. Adam Marsh performed behavioural thermal hyperalgesia testing and conducted cell counts of the GIN neurons and caspase-3 co-localization.

GABAergic dysregulation in the spinal dorsal horn has been previously implicated following SCI, but the underlying cause of the loss of GABAergic tone was not understood. It was hypothesized that a loss of GABAergic inhibitory interneurons from the spinal dorsal horn may contribute to neural hyperexcitability, and resultant symptoms of neuropathic pain. A transgenic mouse line labelling a subpopulation of GABAergic neurons with enhanced green fluorescent protein (eGFP) under control of the GAD67

promoter (*Gad1)* was utilized to identify GABAergic interneurons and determine if below-lesion regions are susceptible to cell death following SCI. Our investigations observed reduced numbers of GIN neurons following SCI, and secondary markers of GABAergic neurons, the GABA synthesizing enzymes GAD65/67 and the GABA transporter (GAT)1, were also reduced following SCI. Application of the GABA transporter blocker tiagabine was able to restore behavioural withdrawal thresholds to pre-injury levels, suggesting that increasing GABAergic tone may be of utility to ameliorate symptoms of neuropathic pain post-SCI.

2.2 INTRODUCTION

Chronic neuropathic pain is a frequent outcome of spinal cord injury (SCI). Following SCI 40-80% of patients report clinically significant pain, and 40% of patients report neuropathic pain (Finnerup et al., 2001). Unfortunately, neuropathic pain (characterized by allodynia, hyperalgesia, and spontaneous pain) resulting from SCI is often refractory to treatment (Burchiel and Hsu, 2001; Hulsebosch, 2005), presenting a burden upon afflicted individuals.

The superficial laminae of the dorsal horn (SDH) of the spinal cord is the first level of modulation of primary afferent signalling by the central nervous system, and is of particular interest to studies of neuropathic pain. Modulation of primary afferent sensory fibres by excitatory or inhibitory interneurons in the dorsal horn may lead to enhanced, or reduced, signal transmission by nociceptive projection neurons (Willis and Westlund, 1997; Lu et al., 2008a; Daniele and MacDermott, 2009); a principle of the Gate Control Theory of pain (Melzack and Wall, 1965). In naïve rats, GABAergic interneurons comprise approximately 1/3 of neurons present in laminae I-III of the dorsal horn of the spinal cord (Todd and Sullivan, 1990; Todd and Spike, 1993; Todd et al., 1996; Heinke et al., 2004). These GABAergic interneurons are effectors of inhibitory modulation of primary afferent signalling. Under normal conditions, Aβ (Daniele and MacDermott, 2009) and low threshold C (Lu et al., 2008a) sensory afferent fibres drive the inhibitory tone arising from these GABAergic neurons, thus preventing the initiation of noxious pain signalling from innocuous stimulation. Mechanical hyperalgesia and allodynia, common manifestations of neuropathic pain, have been linked to aberrant processing of Aβ (Campbell et al., 1988) and some C (Lu et al., 2008a) fibre activity. This aberrant

signalling may be caused, in part, by reduced GABAergic tone as pharmacological antagonism of the $GABA_A$ receptor with bicuculline allows low threshold fibres to activate nociceptive projection neurons (Sivilotti and Woolf, 1994).

The development of animal models of post-SCI neuropathic pain has provided evidence for reduced GABAergic tone in the dorsal horn of the spinal cord. The lowered response thresholds to mechanical stimulation after bicuculline treatment is lost post-SCI (Drew et al., 2004), suggesting intrinsic GABAergic tone has been diminished. Additionally, after SCI spike activity of wide dynamic range projection neurons in the dorsal horn of the spinal cord is increased in response to non-noxious brush stimuli, and both spike activity and behavioural measures of mechanical hyperalgesia are restored by administration of GABA (Gwak et al., 2006; Gwak et al., 2008). Lu et al. (2008) have further demonstrated that polymodal C-fibres, likely mediators of thermal hyperagesia, synapse sequentially on both inhibitory and excitatory interneurons in the SDH. SCI results in a loss of inhibition on the excitatory interneuron, permitting this previously silenced input to the excitatory interneuron to fire, and leading to activation of spinal projection neurons (Lu et al., 2008b). These results suggest that SCI-induced reduction of nociceptive thresholds can be attributable to an attenuation of GABAergic tone, and that restoration of this tone may alleviate symptoms of neuropathic pain.

To investigate the role of spinal GABAergic tone in post-SCI pain, a transgenic mouse strain expressing enhanced green fluorescent protein (GFP) under control of the *gad1* promoter (Oliva et al., 2000) was utilized. This promoter is responsible for the 67 kDa isoform of the GABA synthesizing enzyme GAD67 (Bu and Tobin, 1994). The resulting GFP^+ cells constitute a third of the GABAergic neurons in Laminae II of the spinal dorsal horn; a distinct sub-population of GABAergic neurons that do not express

somatostatin (Heinke et al., 2004). Thermal and mechanical withdrawal thresholds were assessed prior to, and for 6 weeks following, SCI in *gad1*:GFP mice. These mice developed hyperalgesia and allodynia, that could subsequently be reversed by administration of the GABA transporter (GAT)1 antagonist tiagabine. A reduced number of GFP^+ neurons were present in the superficial dorsal horn of lumbar (L) segments 3-6 in 6 week post-SCI mice, and at two weeks post-SCI some of these cells appeared to be apoptotic; expressing active-caspase-3. In support of this observation, we also demonstrate decreased immunoreactivity for other markers indicative of GABAergic tone, such as: GAD 65, GAD 67; and the GABA transporter GAT1, that is predominately co-expressed with $GAD67^+$ neurons (Minelli et al., 1995; Yasumi et al., 1997).

2.3 METHODS

2.3.1 Animals

Adult (8-12 week) male FVB *gad1:*GFP mice (25-30g) (FVB-Tg(GadGFP) 45704Swn/J, #003718 Jackson Laboratory, Bar Harbour, ME) were used in this study. Experiments described herein were approved by the Dalhousie University Committee on Laboratory Animals and are in accordance with the Canadian Council on Animal Care guidelines. Animals were housed under a 12 h light/dark cycle at 22 ±2 °C with free access to water and food. Following injury animals were housed singly, and their cages kept over a heating pad at 37 °C for the duration of the experiment.

2.3.2 Spinal cord contusion injury

Mice were anesthetized under isoflurane (2%), placed on a heating pad at 37 °C, and given subcutaneous (s.c.) injections of 1 ml lactated Ringer's solution, 5 mg/kg Baytril, 0.05 mg/kg atropine and 5 mg/kg ketoprofen. A laminectomy was performed at spinal segment T11 and a moderate injury, 50 kiloDyne (kD) (Ghasemlou et al., 2005), was induced using the Infinite Horizon Impactor (Precision Systems, LLC). To ensure a consistent degree of injury only animals with impact force within ±5 kD of target force and a cord displacement between 400-600 μm were accepted for use in this study. Sham surgery consisted of exposing the vertebral column and snipping the vertical process of T11. Post-operative care included application of heat to maintain body temperature, and s.c. administration of saline, Baytril and ketoprofen as needed. Bladders were expressed twice daily until independent bladder expression returned (approximately 10-14 days).

2.3.3 Drug Administration

Tiagabine HCl (Sequoia Research Products Ltd) was prepared in saline and given via intraperitoneal (i.p.) injection to animals either 1 mg/kg or 10 mg/kg i.p. 30 minutes prior to behavioral testing (Takeuchi et al., 2007).

2.3.4 Hindlimb Locomotor Function

The right and left hindpaws of each mouse were scored one week prior to injury and 2, 3, 4, 5, and 6 weeks post-SCI using the Tarlov Scale. The mean score of right and left hindpaws was determined. The Tarlov score grades hindpaw locomotor function as follows: 0= Complete paralysis of the hindlimb, 1= Infrequent movement of hindlimb, 2= Frequent movement of the hindlimb, 3= Frequent movement, some weight bearing, 4= Minor deficits, 5= Normal.

2.3.5 Mechanical Allodynia

Two weeks prior to SCI surgery mice were habituated to a plexiglass box with a wire grid bottom. One week prior to SCI surgery baseline hindpaw withdrawal responses to a range of Von Frey filaments (Stoelting Co. Wood Dale, Illinois) were determined by applying the filaments to the plantar surface of the hindpaw using a modified version of the up-down method (Dixon, 1991; Chaplan et al., 1994). Hindpaw withdrawal responses after SCI were reassessed at 2, 3, 4, 5, and 6 weeks after surgery. The mean of right and left hindpaw withdrawal thresholds taken at each time point were used to calculate differences from respective sham or vehicle thresholds.

2.3.6 Thermal Hyperalgesia

A thermal hyperalgesia test apparatus (Department of Anesthesiology, University of California San Diego) was used. Two weeks prior to SCI, mice were placed in plexiglass observation chambers positioned on a glass surface maintained at 30°C and acclimatized until exploratory behaviour ceased. At 7 and 3 days prior to SCI radiant heat in the form of a focused light beam was directed through the glass at the plantar surface of the hind paw. Three baseline hindpaw withdrawal latencies were determined at 10-minute intervals for right and left hindpaws as baseline thermal latency. Mice were tested again at 2, 3, 4, 5, and 6 weeks post SCI to assess the development of thermal hyperalgesia. The mean of right and left hindpaw latency measurements taken at each time point were used to calculate differences from baseline. A cutoff value of 20 seconds was imposed on thermal stimuli to prevent tissue damage.

2.3.7 Immunohistochemistry

At six weeks post SCI animals were anesthetized with urethane (2.5mg/kg) and transcardially perfused with 10 ml of ice-cold PBS followed by 10 ml of room temperature 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline (PBS). Tissue was post-fixed in 4% PFA overnight at 4° C and then sequentially cryoprotected in 15% and 30% sucrose at 4° C prior to storage. The spinal cord segments L4-L5, representing sensory input from the plantar hindpaw, were identified and embedded in Tissue-Tek OCT (Sakura Finetik, USA) for cryostat sectioning. Free floating sections were collected for immunohistochemical staining with antibodies against GAD65/67 (Chemicon), CGRP (Peninsula Labs) and active-caspase-3 (R&D Systems). Sections

were blocked in Tris-buffered saline/triton-x 0.01% (TBS-X) w/5% normal goat serum (NGS) for one hour, incubated overnight with either 1:1000 GAD65/67, 1:2000 CGRP, or active caspase-3 1:5000 in 0.01% TBS-X w/1% NGS, washed 3x 10 minutes in TBS-X, incubated for 2 hours with 1:500 Alexa-fluor 546 Goat anti-rabbit (Abcam) in TBS-X w/1% NGS, washed 3x 10 minutes in TBS-X, and coverslipped. Image data was collected bilaterally at 10x magnification within the region of the spinal dorsal horn from equal size of field to ensure consistency between samples. Images were obtained using Zeiss Axioplan 2 imaging microscopes. Image Pro software (Media Cybernetics, USA) was used to determine level of staining intensity.

The number of GFP-expressing neurons was determined in 50 μm thick sections of lumbar spinal cord (L4-L5). The analysis was done "blind" with the researcher unaware to which group the numbered slide belonged. A Zeiss LSM 510 laser confocal scanning microscope was used to compile images in 2 μm increments through a 25x lens. CGRP-immunoreactivity in the superficial dorsal horn was used as an aide to help identify superficial laminae I-III and to set a consistent area of interest (AOI) to include these laminae. CGRP-immunoreactivity caudal to a T10 injury is similar between uninjured and SCI animals (Bruce et al., 2002). Within this established AOI, the nuclei of all GFP+ neurons were counted manually in at least 8, randomly selected sections of spinal segment L4-L5 per animal. The mean number of GFP-expressing neurons in the superficial dorsal horn was then tabulated for each animal.

2.3.8 Western Blots

At 6 weeks post-injury, SCI and sham animals from each group were anesthetized with urethane (0.3 ml of 0.25mg/ml) and perfused transcardially with 10 ml of ice-cold PBS. Animals were immersed in a solution of dry ice and methanol to flash freeze tissue and halt proteolytic degradation. The spinal cord segments L4-L5 were dissected out over dry ice. Frozen tissue was homogenized and extracted in 50 mM Tris buffer, pH 8.0, containing 0.5% Triton-X 100, 150 mM NACL, 1 mM EDTA and protease inhibitors. Protein concentration was determined using the Bicinchonic Acid Protein Assay kit (Pierce, Rockford, IL) and protein extracts separated using 8% stacking, 12% resolving SDS-Page gel electrophoresis. Samples from each treatment group were arranged on the same gel to allow for within gel comparison in semi-quantitative analysis of changes in levels of GAD65 and 67 (Chemicon) and GAT1 (Abcam) immunoreactivity. PVDF membranes were blocked for one hour in 5% skim milk powder in TBS-Tween (T) 0.01%, incubated overnight with GAD65/67 (1:10000), GAT1 (1:500), or GAPDH (1:1000) in TBS-T, washed 3x 10 minutes in TBS-T, and incubated with HRP-conjugated donkey anti-goat (Abcam) (1:15000) for two hours, followed by 3x wash in TBS-T and ECL chemiluminescence procdure (Bio-Rad, CA). Integrated densitometry values were determined as a ratio of GAPDH (Abcam) expression using Alpha Imager 2.1 (Alpha Innotech, USA). Comparisons between SCI and sham groups were performed after normalizing data to sham values.

2.3.9 Statistical analysis

Statistical analysis of results was conducted using one-tailed unpaired t-tests between groups. In behavioural tests, SHAM and SCI groups were compared at respective time points. Significance was set as $\alpha<0.05$. Analysis of variance (ANOVA) was used to test for significant differences among the GFP^+ neuron counts in spinal cords of uninjured sham, 2 week SCI, and 6 week SCI animals. Once significance was detected, Tukey's post-hoc comparison was used to identify differences among the groups.

2.4 RESULTS

2.4.1 Hindlimb locomotor function

All SCI mice demonstrated an immediate loss of hindlimb locomotor function following injury. All animals appeared incapable of hindlimb movement immediately following SCI (observed) but presented frequent hindlimb movements (Tarlov score = 2.1±0.1) one week post-SCI. Most animals gained some measure of weight bearing plantar stepping (Tarlov score =2.4±0.1) in one hindpaw by 6 weeks post-SCI. Sham surgery had no effect on Tarlov scores (5 at all time points).

2.4.2 Development of mechanical and thermal hyperalgesia

Mechanical hyperalgesia was assessed 7 and 3 days before surgery to establish baseline thresholds for noxious stimulation. The 50% withdrawal threshold in SCI mice reduced from 1.33±0.07 g and 1.42±0.09 g at 7 and 3 days before surgery, to 0.49±0.08 g at 6 weeks following surgery (Fig. 1A, $p<0.05$). Sensitivity of sham animals remained stable during testing periods (1.43±0.07 g and 1.49±0.07 g at 7 and 3 days prior to sham surgery, and 1.41±0.08 g six weeks following surgery) (Fig 1A).

Sham animals exhibited a baseline thermal hindpaw withdrawal latency of 14.10±0.81 s and 13.44±0.76 s at 7 and 3 days before surgery. SCI animals demonstrated a significant reduction in hindpaw withdrawal time at three weeks post-SCI (7.96±0.85 s) and this remained stable at 6 weeks post-SCI (6.50±0.344 s) (Fig 1C).

2.4.3 Reversal of hyperalgesia by the GAT1 antagonist tiagabine

The effect of i.p. administration of 10 mg/kg and 1 mg/kg tiagabine (Xu et al., 2008) was tested at week 5 and 6, respectively. Administration of 10 mg/kg tiagabine resulted in general sedation, such that animals met cut-off values for the behavioral tests (data not shown). Administration of 1 mg/kg tiagabine resulted in no obvious sedation (by assessment of ambulation and exploratory behavior), and increased the withdrawal threshold in SCI animals to mechanical (Fig 1B) (1.06±0.15 g and 0.50±0.10 g, respectively, $p<0.05$) and thermal stimuli (Fig 1D) (11.30±0.1 s and 6.70±0.4 s, $p<0.05$) of SCI mice. 1 mg/kg tiagabine was found to have no significant effect vs saline on the withdrawal threshold of uninjured mice (data not shown).

2.4.4 GABAergic interneuron loss in the dorsal horn after SCI

The dorsal horn of L4-L5 spinal cord was studied in *gad1*:GFP mice to determine if SCI resulted in a reduction of GFP^+ *gad1*-expressing cells (Fig 2A, 2B). GFP^+ cells were counted in sham mice and mice two and six weeks post-SCI. These SCI time points reflect the onset of neuropathic pain and fully developed neuropathic pain behaviour, respectively. A slight, but significant reduction in the number of GFP^+ neurons was observed in mice 2 weeks post-SCI (16%, $p<0.05$). The decrease in the number of GFP^+ neurons was more dramatic at 6 weeks, with a 43% loss occurring ($p<0.05$). This observation was further investigated with immunohistochemical staining of tissue from a mouse two weeks post-SCI for active-caspase-3, a marker of apoptosis-mediated cell death. This time point was chosen as pain symptoms developed between two and three weeks post-SCI. Active-caspase-3 immunoreactivity was observed co-localized in the

nucleus of GFP^+ cells, suggesting ongoing apoptotic cell death of GABAergic neurons (Fig 2D). Active-caspase-3 immunoreactivity was also observed in non-GFP^+ neurons in the grey matter, as well as numerous cells present in the white matter. In a separate group of animals (n=7), a more severe compression (75 kD) was used to injure the spinal cord. In this cohort of animals, a similar loss of GFP+ neurons (39%) was observed following the development of neuropathic pain symptoms (data not shown).

2.4.5 GAD 65 and 67 levels reduced after SCI

Reduced GAD 65 and 67 immunoreactivity was observed in the L4-L5 dorsal horn of 6 week post-SCI mice compared to sham mice. In transverse sections of the lumbar spinal cord GAD expression was most prominent in the dorsal horn. SCI reduced GAD immunohistochemical staining by approximately 30% (71.7±0.1 %, $P<0.01$) (Fig 3A&B). Western blotting techniques also demonstrated a ~17% reduction in GAD 65 approaching significance, and a 13% reduction of GAD 67 staining in 6 week post-SCI mice (83.29±0.04%, $p<0.06$ and 86.94±0.03%, $p<0.04$, respectively) (Fig 4A,B,C).

2.4.6 Levels of GAT1 after SCI

Western blotting techniques revealed a decrease in the level of GAT1 in L4-6 spinal cord 6 weeks post-SCI. GAT1 expression in SCI mice was reduced by ~26% the expression of sham animals (73.7±0.02%, $p<0.01$) (Fig 5).

2.5 DISCUSSION

The proper integration and coding of noxious vs. innocuous sensory afferent input within the spinal cord is dependent upon inhibitory GABAergic and glycinergic tone. Superficial dorsal horn inhibitory neurons receive monosynaptic or polysynaptic input from Aδ and C-fibres (Lu and Perl, 2003; Hantman et al., 2004; Heinke et al., 2004), and loss of this inhibitory tone allows innocuous Aβ-fibre mediated activation of nociceptive specific projection neurons (Drew et al., 2004), in a pathway containing PKCγ interneurons (Miraucourt et al., 2007; Neumann et al., 2008), or facilitation of previously "silent" low-threshold C-fibre circuits (Lu et al., 2008b). Thus, loss of inhibitory neurons following SCI may create a permissive circuit for reduced nociceptive thresholds.

The present study of experimental spinal cord injury supports previous findings implicating reduced GABAergic tone in the development of neuropathic pain (Drew et al., 2004; Gwak et al., 2006; Gwak et al., 2008). The loss of *gad1*:GFP neurons observed in the dorsal horn following SCI likely contributes to reduced GABAergic tone. This postulate is supported by the observation of reduced levels of GAD 65/67, GAT1, and the ability of tiagabine to alleviate hyperalgesia and allodynia.

The death of neurons distal to the SCI lesion epicentre is variable. This variability in neuron survival may be attributable to the experimental model of SCI, or susceptibility of distinct neuronal populations to apoptotic signals (Yong et al., 1998; Beattie et al., 2002). Sigenthaler *et al*. (2007) demonstrated that contusion injury results in significantly more white matter apoptosis, demyelination, and macrophage infiltration away from the injury epicentre compared to a transection injury at the same level. Cytokine production, macrophage accumulation, and associated inflammation is known to have a deleterious

effect on cell survival (Fleming et al., 2006), likely to a similar extent in both white and grey matter. Using constitutive expression of GFP to identify a subset of GABAergic interneurons in the dorsal horn, we were able to demonstrate a 43% decrease in neuron number 6 weeks after SCI possibly attributable to caspase-3 mediated cell death. In addition, this loss of GABAergic interneurons coincided temporally with the development of SCI-induced neuropathic pain.

Differing susceptibility of neuronal populations to apoptotic signals may also be a contributing factor in the development of neuropathic pain after peripheral nerve injury. Although many studies report decreased inhibitory tone after peripheral nerve injury, it remains unresolved if loss of GABAergic neurons contributes to the development of neuropathic pain in this paradigm. Some studies report decreased GABAergic tone and a loss of inhibitory neurons (Moore et al., 2002; Scholz et al., 2005), while others observed no evidence of selective neuron loss (Polgar et al., 2005). Interestingly, Scholz *et al.* (2005) demonstrated that blocking caspase activity in the dorsal horn, prevented the loss of GABAergic interneurons, and diminished neuropathic pain after peripheral nerve injury. This suggests that a neuroprotective approach may be effective in preventing the development of pain symptoms following injury to peripheral nerves or the spinal cord.

In addition to loss of GABAergic neurons, a reduction in GABAergic tone in the dorsal horn after SCI could also be attributed to decreased activity of GAD65 and GAD67. GAD65 and 67 are the primary rate-limiting enzymes in the synthesis of GABA; each the product of the distinct genes *gad1* and *gad2*, respectively (Erlander et al., 1991). Some distinction has been made in the localization of each isoform, cortical GAD 65 being expressed in the cell body, and GAD 67 being expressed in axon boutons (Kaufman et al., 1991; Esclapez et al., 1994). Predominate expression of GAD 65 or

GAD 67 may vary among spinal inhibitory neurons, and the isoforms may not be equally co-expressed within neurons (Soghomonian and Martin, 1998; Martin and Tobin, 2000; Mackie et al., 2003). In the present study, use of an antibody recognizing both GABA synthesizing enzymes GAD 65 and 67, provides an accurate representation of total GABA synthesizing capacity of the spinal cord sections investigated. Reduced levels of GABA in the dorsal horn could be attributed to decreased enzyme activity of GAD65 and 67, decreased expression of *gad1* and *gad2* genes, or cell death of GABAergic neurons. The larger observed reduction in GAD65/67 intensity in immunohistochemistry relative to the analysis of Western blots may be supportive of a selective loss of GAD65/67 within Laminae I-III of the dorsal horn. Western blot experiments were conducted using a homogenate of whole spinal cord from the segment of interest, whereas immunohistochemistry studies were more specific to the region of interest within Laminae I-III of the dorsal horn. Thus, it is possible that the smaller relative reductions observed for GAD 65/67 in Western blots may be diluted by other GAD65/67 expressing cells in regions unaffected by injury. Our results suggest that death and subsequent decreased number of GABAergic interneurons in the superficial dorsal horn, plays a significant role in the diminished GABAergic tone and neuropathic pain after SCI.

GABAergic tone in the dorsal horn is also affected by reuptake of GABA from the synaptic cleft. The GABA membrane transporter GAT1 plays an important role in the synaptic regulation of GABAergic tone. Enhanced transgenic expression of GAT1 is associated with hyperalgesia (Hu et al., 2003), whereas transgenic knockout of the GAT1 gene results in hypoalgesia (Xu et al., 2008). Upregulation of spinal GAT1 expression has been associated with neuropathic pain following peripheral nerve injury, likely resulting in a decreased GABA concentration in the synapse (Daemen et al., 2008). A

reduction in GAT1 expression may also indicate a loss of $GAD67^+$ neurons, as GAT1 is expressed primarily, though not exclusively, in these neurons (Yasumi et al., 1997). The present results suggest that expression levels of the GAT1 may be of secondary importance to the GABAergic cells ability to survive the hostile environment in the spinal cord following injury. Previously, tiagabine, a GAT1 inhibitor, has been shown to alleviate neuropathic pain following peripheral nerve injury (Ipponi et al., 1999). We demonstrate for the first time that administration of tiagabine also diminishes SCI-induced neuropathic pain. This observation suggests that remaining GABAergic neurons at six weeks post-SCI are sufficient to mediate the reversal of hyperalgesia by tiagabine, and that GABA facilitatory treatments may be of therapeutic benefit in post-SCI neuropathic pain.

Decreased expression of the K^+/ Cl^- transporter KCC2 (causing increased $[Cl^-]_i$) in neurons of the dorsal horn of the spinal cord has also been implicated in neuropathic pain following peripheral nerve injury (Coull et al., 2003) and SCI (Cramer et al., 2008; Lu et al., 2008a). Reduced expression of KCC2 causes a shift in E_{GABA}, resulting in a net excitatory effect of $GABA_A$ receptor-mediated currents. Lu *et al.* (Lu et al., 2008a) demonstrate this reversal of anion gradient allows silent spinal circuits, normally inhibited by GABAergic inhibition, to activate excitatory spinal interneurons. Consistent with this observation persistent, remote activation of microglia has been observed following SCI (Hains and Waxman, 2006), and brain-derived neurotrophic factor released from microglia has been linked to decreased efficacy of KCC2 (Coull et al., 2005). Reduced microglial activation using the phosphodiesterase inhibitor propentofylline has been shown to ameliorate behavioral symptoms of hyperalgesia and loss of GABAergic tone (Gwak et al., 2008). Concordant with our observation of tiagabine alleviating mechanical

hyperalgesia, Gwak *et al.* (2008) also show application of GABA to the spinal cord after SCI reduces mechanical hyperalgesia and excitability of spinal projection neurons. This implies that a concurrent loss of GABAergic tone may be necessary to facilitate the "silent" circuits which contribute hyperalgesia following SCI. The net result is that augmenting GABAergic tone, or preventing its loss, may provide therapeutic benefit to individuals suffering symptoms of neuropathic pain.

Loss of GABAergic interneurons following SCI contributes to the reduction in GABAergic tone following SCI. Antagonism of GABA re-uptake by tiagabine alleviated symptoms of mechanical allodynia and thermal hyperalgesia. The apoptosis-mediated death of GABAergic interneurons distal to lesion after SCI likely contributes to disinhibition of dorsal horn neurons, and facilitation of pain signalling. These results suggest therapeutics aimed at restoring GABAergic tone in the dorsal horn, or neuroprotective treatments preventing the loss of GABAergic neurons, may be efficacious in reducing neuropathic pain following SCI. Future studies are needed to determine if inhibitory cell populations are selectively susceptible to death following, however, the results of the study support the hypothesis that loss of GABAergic tone contributes to neuropathic pain following injury. Furthermore, the partial loss of a population of inhibitory *gad1*$^{+}$ non-somatostatin expressing cells may contribute to this loss of inhibitory tone.

Competing interests

The authors declare no competing interests.

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Figure 2.1 Reversal of SCI induced hyperalgesia by tiagabine
Withdrawal thresholds to mechanical (A) and thermal (C) stimulation in SCI (n=7) and SHAM (n=6) mice, and reversal of mechanical (B) and thermal (D) hyperalgesia at 6 weeks post-SCI by tiagabine vs saline (SAL) (B, D). Points represent mean value and SEM of hindpaw withdrawal threshold. * indicates $P<0.05$ from SHAM or vehicle cont rol at equivalent time point.

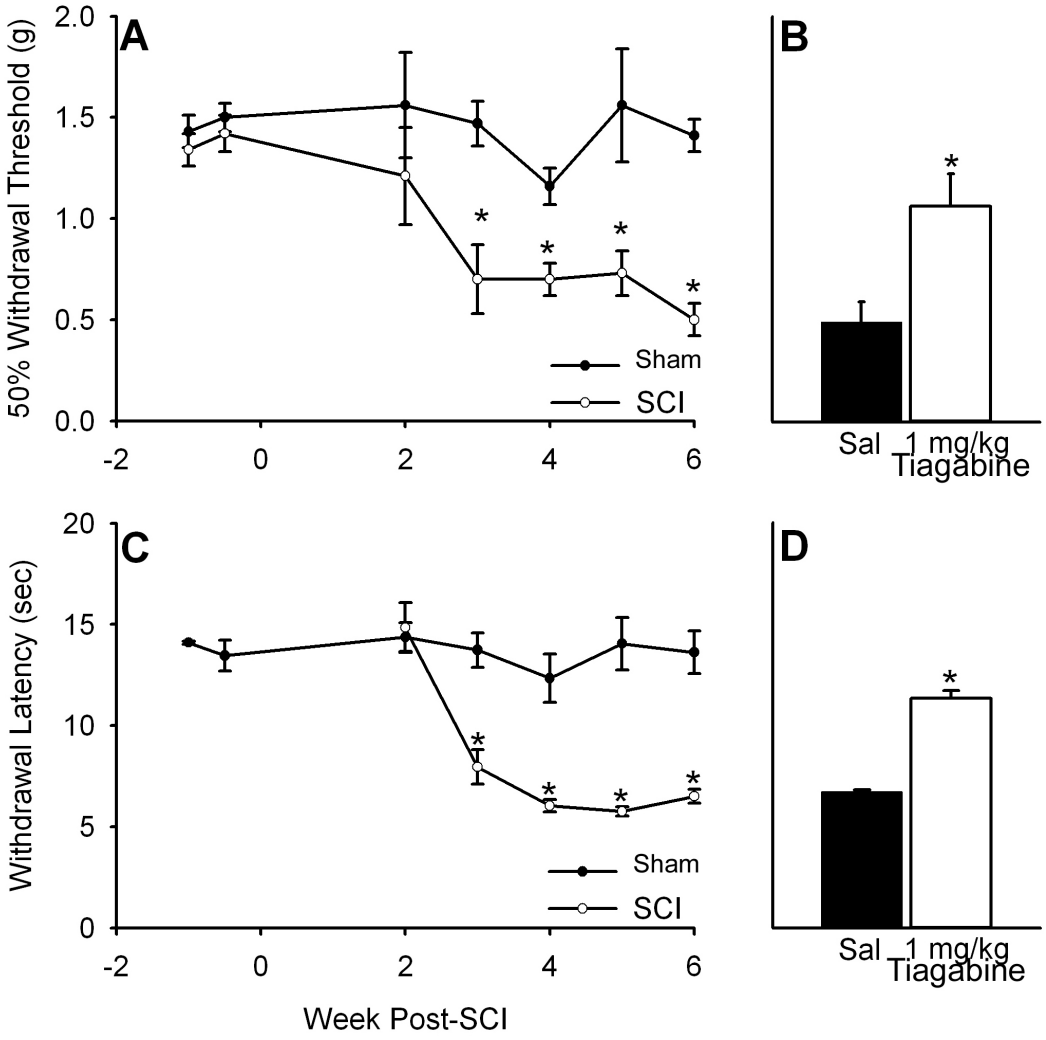

A
50% Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
-2
0
2
4
6
Sham
SCI
B
Sal
1 mg/kg Tiagabine
C
Withdrawal Latency (sec)
20
15
10
5
0
Week Post-SCI
D

Figure 2.2 SCI leads to loss of GABAergic inhibitory neurons
Representative images of *gad1*:GFP immunofluorecence in sham (A) and SCI (B) mice 6 weeks post-SCI. CGRP staining (red) was used to outline and define the Laminae I-III of the dorsal horn. Fewer GFP^+ neurons were observed in 2 (n=6) and 6 (n=8) week post-SCI mice compared to sham controls (n=4) (C) (143±3 and 96±4 vs. 167±3, $P<0.01$). Active-caspase-3 immunofluorescence demonstrates a GFP^+ neuron (arrow) and non-GFP^+ cell (arrowhead) undergoing cell death 2 weeks post-SCI (D).

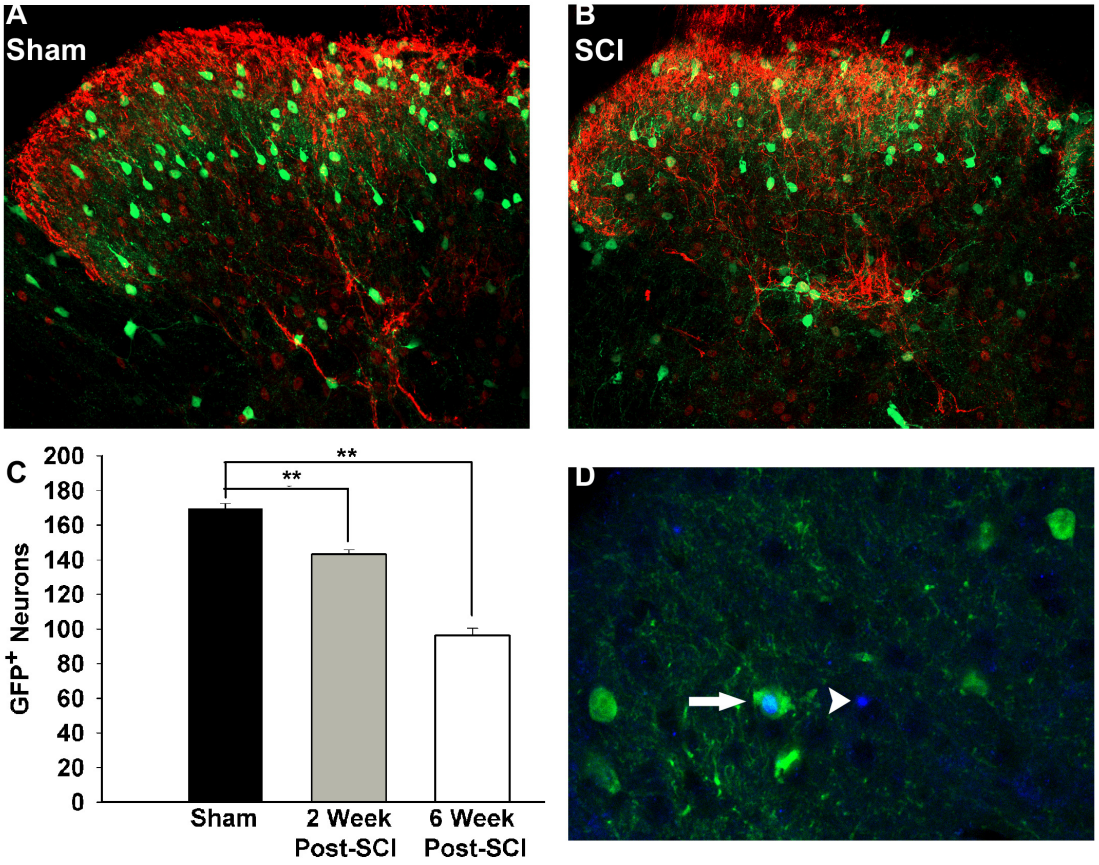
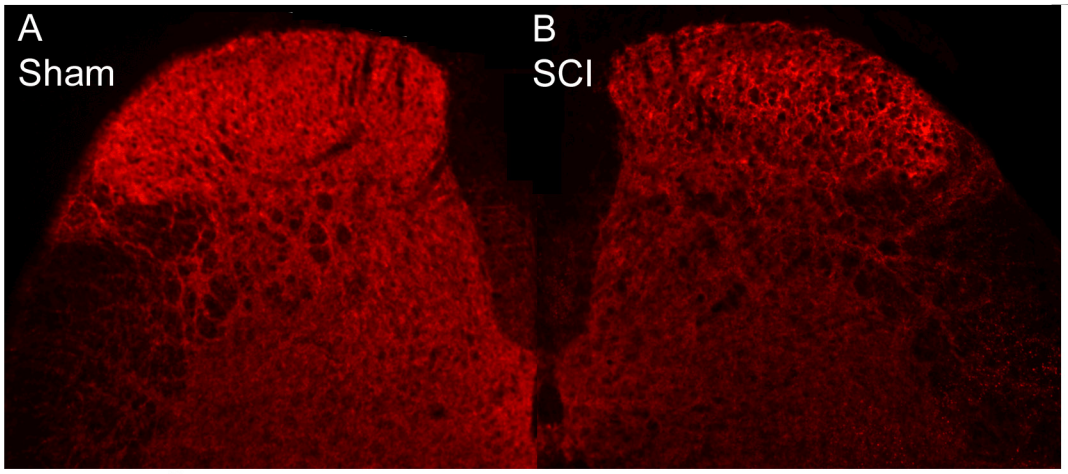

A
Sham
B
SCI
C
GFP+ Neurons
200
180
160
140
120
100
80
60
40
20
0
**
**
Sham
2 Week
Post-SCI
6 Week
Post-SCI
D

Figure 2.3. SCI reduces GAD65/67 immunoreactivity in the dorsal horn
Representative GAD 65/67 immunostaining in SHAM (n=6) (A) and SCI (n=7) (B) animals. In (C) GAD 65/67 immunoreactivity of SCI animals was observed to be 71.73±0.06% of SHAM (After normalization of SHAM animals) ($p<0.0.04$).


A
Sham
B
SCI

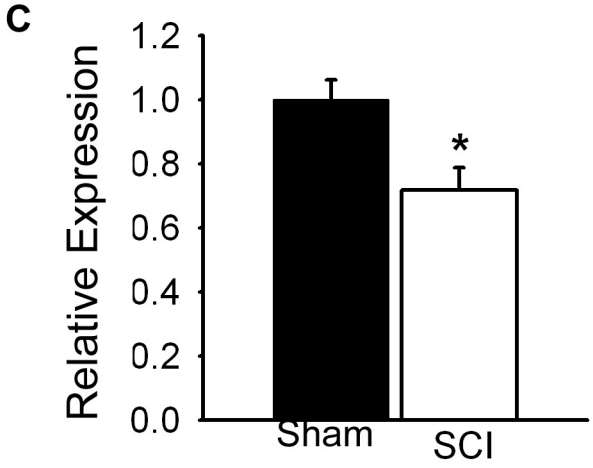

C
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Sham
SCI

Figure 2.4. SCI reduces levels of GAD65/67 by immunoblotting
Representative GAD 65/67 (A) immunoreactivity in SHAM (n=6) and SCI (n=7) mice six weeks post-SCI. SCI reduces immunoreactivity for both GAD 65 (B) (83.3%, NS, $P<0.06$) and GAD 67 (C) (86.9%, $P>0.04$) (After normalization of SHAM animals).

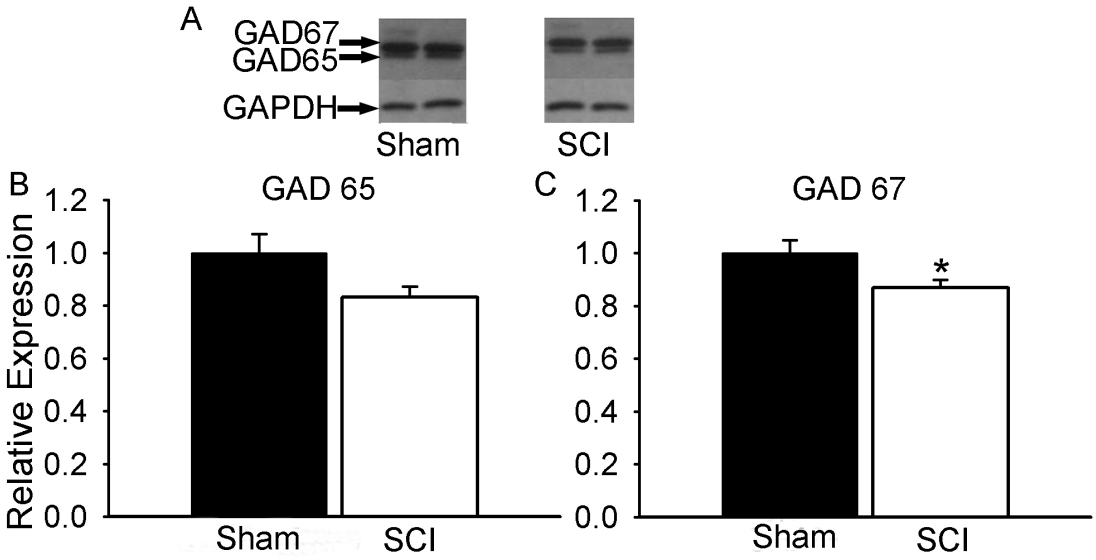
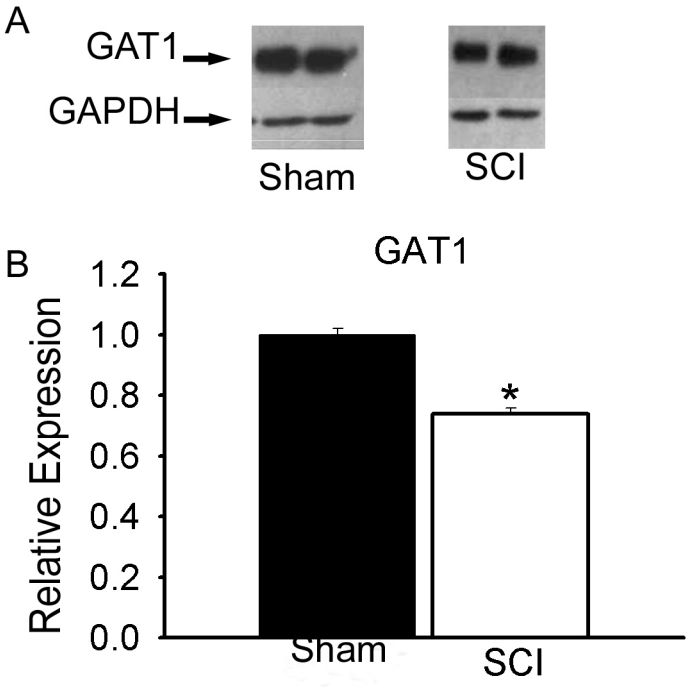

A
GAD67
GAD65
GAPDH
Sham
SCI
B
GAD 65
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
SCI
C
GAD 67
*
Sham
SCI

Figure 2.5. SCI reduces levels of the GABA transporter GAT1
Representative GAT1 immunoreactivity in SHAM (n=6) and SCI (n=7) mice six weeks post-SCI (A). GAT1 immunoreactivity is reduced following SCI (B) (83.3%, $P<0.01$) (After normalization to SHAM animals).


A
GAT1
GAPDH
Sham
SCI
B
GAT1
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Sham
SCI

CHAPTER 3: INCREASED EXPRESSION OF X-LINKED INHIBITOR OF APOPTOSIS PROTEIN (XIAP) DOES NOT PREVENT THE DEVELOPMENT OF BELOW-LESION NEUROPATHIC PAIN FOLLOWING SPINAL CORD INJURY

3.1 PREFACE AND SIGNIFICANCE TO THESIS

The results presented in this chapter have not been previously published as an abstract or manuscript.

I designed the experiments (with Dr. Marsh), performed animal husbandry, genotyping, conducted behavioral testing, animal surgeries and post-operative care, and collected tissue and performed Western Blot analysis.

In Chapter 2 we observed that the development of post-SCI below-lesion neuropathic pain was coincident with activation of caspase-3, induction of apoptosis and subsequent loss of GABAergic interneurons. This prompted the hypothesis that inhibiting cellular apoptosis following SCI may diminish the development of neuropathic pain. To investigate this hypothesis we utilized a transgenic mouse line overexpressing the X-linked inhibitor of apoptosis protein, driven by the ubiquitin C promoter (Moore et al. 2008). It was anticipated that over-expression of XIAP would reduce apoptosis and prevent SCI-induced loss of GABAergic neurons.

If inhibition of apoptosis diminishes the development of below-lesion mechanical allodynia post-SCI it would provide strong evidence for further research into neuroprotective strategies as a means of preventing SCI-induced neuropathic pain post-SCI. Such therapies could then be used as adjunct's to current acute procedures post-SCI, such as spinal decompression surgeries.

The results of this study indicate that increased expression of X-linked inhibitor of apoptosis was not sufficient to prevent the development of neuropathic pain following

SCI. These findings do not rule out a role of GABAergic inhibitory interneuron apoptosis in the development of below-lesion pain post-SCI, as non-discriminatory increases in ubiquitin C promoter driven XIAP expression across all cell types, such as pro-inflammatory immune cells, may obscure a neuroprotective effect.

3.2 INTRODUCTION

The development of neuropathic pain is a frequent complication following spinal cord injury (SCI). A number of mechanisms have been suggested to contribute to the development of heightened pain signaling post-SCI (Christensen and Hulsebosch, 1997), including the loss of appropriate GABAergic tone within the dorsal horn of the spinal cord (Gwak and Hulsebosch, 2011). Reductions in GABAergic tone may permit otherwise innocuous sensory information to be encoded as a noxious stimulus, manifested in the experience of pain. As the loss of GABAergic tone (Gwak et al., 2008), and the loss of GABAergic neurons (Meisner et al., 2010) appears to be coincident with the development of neuropathic pain it may be possible to study the role of GABAergic cell death in the development of neuropathic pain through the inhibition of apoptosis following SCI.

Using transgenic animals expressing increased levels of the X-linked inhibitor of apoptosis (XIAP) protein, it may be possible to prevent apoptotic death of GABAergic interneurons. Previous characterization of this mouse has demonstrated high expression of XIAP in the central nervous system (Moore et al., 2008). Inhibitors of apoptosis (IAPs) are a family of anti-apoptotic proteins that suppress intrinsic and extrinsic mediated apoptosis (Hunter et al., 2007). XIAP is the most potent member of the IAP family, able to bind and inhibit caspases 3, 7, and 9 (Holcik et al., 2000), protecting cells from known apoptotic stimuli such as Fas ligand (Straszewski-Chavez et al., 2004), ultraviolet light (Duckett et al., 1998), and multiple cytotoxic drugs (LaCasse et al., 1998). Thus, increased XIAP expression may inhibit the actions of pro-apoptoic stimuli, such as Fas ligand, known to be increased following SCI (Marsh and Flemming, 2011).

It has also been suggested that a loss of GABAergic inhibition contributes to the development of neuropathic pain after peripheral nerve injury (Scholz et al., 2005). However, a loss of GABAergic inhibition may not be implicated in all models of nerve injury, and is possibly not necessary for the development of neuropathic pain (Polgar et al., 2005). Caspase inhibition has previously been shown to be successful in suppressing the development of nerve injury-induced mechanical allodynia through rescue of *GAD65* interneurons and restoration of inhibitory post-synaptic current levels observed in naïve animals (Scholz et al., 2005).

These studies will investigate if non-cell specific inhibition of apoptosis may prevent, or alleviate the development of neuropathic pain post-SCI.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Adult (8-12 week) male C57/B6 heterozygous *ubXIAP* and wildtype mice (25-30g) were used in this study (animals were a gift from Dr. G.S. Roberston, Dalhousie University). Experiments described herein were approved by the Dalhousie University Committee on Laboratory Animals and are in accordance with the Canadian Council on Animal Care guidelines. Animals were housed under a 12 h light/dark cycle at 22 ±2 °C with free access to water and food. Following injury animals were housed singly, and their cages kept over a heating pad at 37 °C for the duration of the experiment.

3.3.2 PCR Based genotyping of ubXIAP mice

Ear punches were collected from mice upon weaning and used to extract genomic DNA. *ubXIAP* transgene was detected using the following oligonucleotide primers:

FWD 5'5′dGGATCCTCTGATGCTGTGAGTTCTGATAG- GAATTTCCC-3′

REV 5′dGACTCGAGCTAAGTAGTTCTTACCAGA- CACTCCTCAAG-3′

DNA samples were amplified in a Thermal Cycler (Eppendorf, Canada) using the following conditions: denaturation temperature of 94 °C for 30 s, annealing temperature of 68 °C for 60 s (34 cycles), and an elongation temperature of 72 °C for 60 S. Samples were visualized on a 2% agarose gel using gel electrophoresis. *ubXIAP* transgene was detected at ~350 bp by visualization of ethidium bromide staining under ultraviolet light.

3.3.3 Spinal cord contusion injury

Mice were anesthetized under isoflurane (2%), placed on a heating pad at 37 °C, and given subcutaneous (s.c.) injections of 1 ml lactated Ringer's solution, 5 mg/kg Baytril, 0.05 mg/kg atropine and 5 mg/kg ketoprofen. A laminectomy was performed at spinal segment T11 and a moderate injury, 50 kiloDyne (kD) (Ghasemlou et al., 2005), was induced using the Infinite Horizon Impactor (Precision Systems, LLC). To ensure a consistent degree of injury only animals with impact force within ±5 kD of target force and a cord displacement between 400-600 μm were accepted for use in this study. Sham surgery consisted of exposing the vertebral column and snipping the vertical process of T11. Post-operative care included application of heat to maintain body temperature, and s.c. administration of saline, Baytril and ketoprofen as needed. Bladders were expressed twice daily until independent bladder expression returned (approximately 10-14 days).

3.3.4 Hindlimb Locomotor Function

The right and left hindpaws of each mouse were scored one week prior to injury and 2, 4, and 6 weeks post-SCI using the Tarlov Scale. The mean score of right and left hindpaws was determined. The Tarlov score grades hindpaw locomotor function as follows: 0= Complete paralysis of the hindlimb, 1= Infrequent movement of hindlimb, 2= Frequent movement of the hindlimb, 3= Frequent movement, some weight bearing, 4= Minor deficits, 5= Normal.

3.3.5 Mechanical Allodynia

Two weeks prior to SCI surgery mice were habituated to a plexiglass box with a wire grid bottom. One week prior to SCI surgery baseline hindpaw withdrawal responses to a range of Von Frey filaments (Stoelting Co. Wood Dale, Illinois) were determined by applying the filaments to the plantar surface of the hindpaw using a modified version of the up-down method (Dixon, 1991; Chaplan et al., 1994). Hindpaw withdrawal responses after SCI were reassessed at 2, 4, and 6 weeks after surgery. The mean of right and left hindpaw withdrawal thresholds and the standard error of the mean were taken at each time point were used to calculate differences from respective sham or vehicle thresholds.

3.3.6 Western Blots

At 1 week post-injury, wildtype and *ubXIAP* animals were anesthetized with urethane (0.3 ml of 0.25mg/ml) and perfused transcardially with 10 ml of ice-cold PBS. Animals were immersed in a solution of dry ice and methanol to flash freeze tissue and halt proteolytic degradation. The spinal cord segments L4-L5 were dissected out over dry ice. Frozen tissue was homogenized and extracted in 50 mM Tris buffer, pH 8.0, containing 0.5% Triton-X 100, 150 mM NACL, 1 mM EDTA and protease inhibitors. Protein concentration was determined using the Bicinchonic Acid Protein Assay kit (Pierce, Rockford, IL) and protein extracts separated using 8% stacking, 12% resolving SDS-Page gel electrophoresis. Samples from each treatment group were arranged on the same gel to allow for within gel comparison in semi-quantitative analysis of changes in levels of GAD65 and 67 (Chemicon) immunoreactivity. PVDF membranes were blocked for one hour in 5% skim milk powder in TBS-Tween (T) 0.01%, incubated overnight with GAD65/67 (1:10000), or GAPDH (1:1000) in TBS-T, washed 3x 10 minutes in TBS-T,

and incubated with HRP-conjugated donkey anti-goat (Abcam) (1:15000) for two hours, followed by 3x wash in TBS-T and ECL chemiluminescence procdure (Bio-Rad, CA). Integrated densitometry values were determined as a ratio of GAPDH (Abcam) expression using Alpha Imager 2.1 (Alpha Innotech, USA). Comparisons between SCI and sham groups were performed after normalizing data to sham values and calculating standard error of the mean.

3.3.7 Statistical analysis

Statistical analysis of results was conducted using one-tailed unpaired t-tests between groups and between respective time points. Significance was set as $\alpha<0.05$. Once significance was detected, Tukey's post-hoc comparison was used to identify differences among the groups.

3.4 RESULTS

3.4.1 Locomotor function post-SCI

Both wildtype and *ubXIAP* mice demonstrated an immediate loss of hindlimb locomotor function following injury (Figure 3.1). Immediate below-lesion paralysis was observed following SCI (observed). 2 weeks post-SCI both wildtype and *ubXIAP* animals presented frequent hindlimbs movements (Tarlov score 2.58±0.32 and 2.42±0.22, respectively). Wildtype and *ubXIAP* animals gained some measure of weight bearing plantar stepping in one hindpaw (Tarlov score 3.25±0.35 and 3.00±0.28, respectively) by 6 weeks post-SCI.

Baseline latency to fall in the rotorod task was similar in both wildtype and *ubXIAP* animals (180.72±17.34 s, and 208.47±21.43 s, respectively), and no significant differences were observed between wildtype and *ubXIAP* animals tested at week 2, 4, or 6 post-SCI. By week 6 both wildtype and *ubXIAP* animals were able to walk upon the rotorod for greater than one revolution of the bar (45.02±10.71 s and 26.11±5.85 s, respectively) (Fig 3.2).

3.4.2 Development of mechanical allodynia

Mechanical hyperalgesia was assessed 1 week before surgery to establish baseline thresholds for noxious stimulation. The baseline 50% withdrawal threshold was equivalent in wildtype and *ubXIAP* mice (2.07±0.19 g and 2.18±0.14 g, respectively). Development of mechanical allodynia was similar in wildtype and *ubXIAP* animals at week 2 (0.96±0.18 g and 0.77±0.22 g, respectively), 4 (0.80±0.16 g and 0.73±0.11 g, respectively) and 6 (0.72±0.19 g and 0.62±0.16 g, respectively) following surgery (Fig. 3.3).

3.4.3 GAD 65 and 67 levels 1 week post-SCI

Western blotting examination of GAD 65 and 67 immunoreactivity 1 week post-SCI indicated no significant differences in the relative expression levels of total and individual GAD isoforms between wildtype and *ubXIAP* animals in the respective lumbar spinal cord segments (Figure 3.4 A & B).

3.5 DISCUSSION

SCI results in below-lesion locomotor dysfunction and mechanical allodynia, deficits observed in both our wildtype and *ubXIAP* mice during weeks 2, 4 and 6 of behavioral testing. Increased XIAP expression did not result in obvious differences relative to wildtype animals in tests of locomotor function or mechanical withdrawal thresholds post-SCI.

No significant differences were observed between wildtype and *ubXIAP* mice one week post-SCI in the expression of GAD isoforms, suggesting increased XIAP expression may not have conferred a neuroprotective effect on GABAergic inhibitory interneurons.

Non-specific expression of the *ubXIAP* transgene may have reduced the potential for the intended neuroprotective effect. Expression of XIAP in this mouse has been reported in the heart, liver, kidney, testes, spleen, brain, spinal cord, and T cells, but the level of expression varies between tissue types (Moore et al., 2008). Factors contributing to transgene expression likely differ between cell types, and the level of expression in our cells of interest, the GABAergic interneurons of the dorsal spinal cord is unknown.

The ubiquitin C promoter of this transgene, resulting in ubiquitous expression of the XIAP, may also pose increased challenges to neuronal cell survival post-injury. Given the lack of significant differences in locomotor function and mechanical withdrawal thresholds after SCI it was not deemed necessary to investigate for potential pathological differences in the spinal cord. Previous evaluation of this mutant mouse following experimental autoimmune encephalopathy (EAE), a model of multiple sclerosis, indicated earlier onset, greater severity of EAE symptoms in *ubXIAP* mice (Moore et al., 2008). Heightened expression of XIAP in cells of the immune system, in particular T cells, was observed to confer greater resistance to culture- based apoptosis

assays, and furthermore, within the central nervous system of EAE treated animals *ubXIAP* mice were observed to have a greater number of cellular infiltrates, and increased demyelination (Moore et al., 2008)

Apoptosis is an important regulator of normal cell function. Moore et al. (2008) characterize an example of how inhibition of cell death may increase the severity of a disease state by conferring increased resistance to cell death upon immune cells. SCI also involves the recruitment of immune cells acutely following injury. In the context of SCI, the body's physiological response to tissue damage can exacerbate the injury, furthering damage beyond the initial traumatic insult. Migration of peripheral leukocytes by chemotaxis results in rapid infiltration of the injury site. The process of neutrophil extravasation into the lesion site begins within hours of injury, and by 1-3 days infiltration is diffuse within the spinal cord parenchyma (Fleming et al., 2006). 5-10 days post-SCI neutrophil nuclei begin to condense, indicative of apoptosis (Fleming et al., 2006). Inhibition of this apoptosis could prolong the activity of these cells, furthering exacerbating inflammation and damage secondary to the initial insult.

For this reason more targeted means of inhibiting neuronal apoptosis should be investigated to determine if a neuroprotective strategy offers clinical promise to guard against the development of neuropathic pain. Selective inhibition of inflammation was found to promote neuroprotection and was associated with decreased pain following SCI (Gris et al., 2004).

A transgenic mouse line overexpressing XIAP under control of the neuron specific *Thy-1* promoter has been created. These animals showed improved outcomes relative to wildtype animals following transient cerebral ischemia (Trapp et al., 2003).

Thus, neuron specific inhibition of apoptosis may still merit investigation as a therapeutic strategy post-SCI.

3.6 References

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Figure 3.1. Effect of ubXIAP on Tarlov Scores Post-SCI

Tarlov scores of hindlimb function were assessed prior to injury, and at weeks 2, 4 and 6 post-SCI. Both groups demonstrated paralysis immediately following injury, with gradual recovery of function until week 6. No significant difference was observed between wildtype and ubXIAP mice.

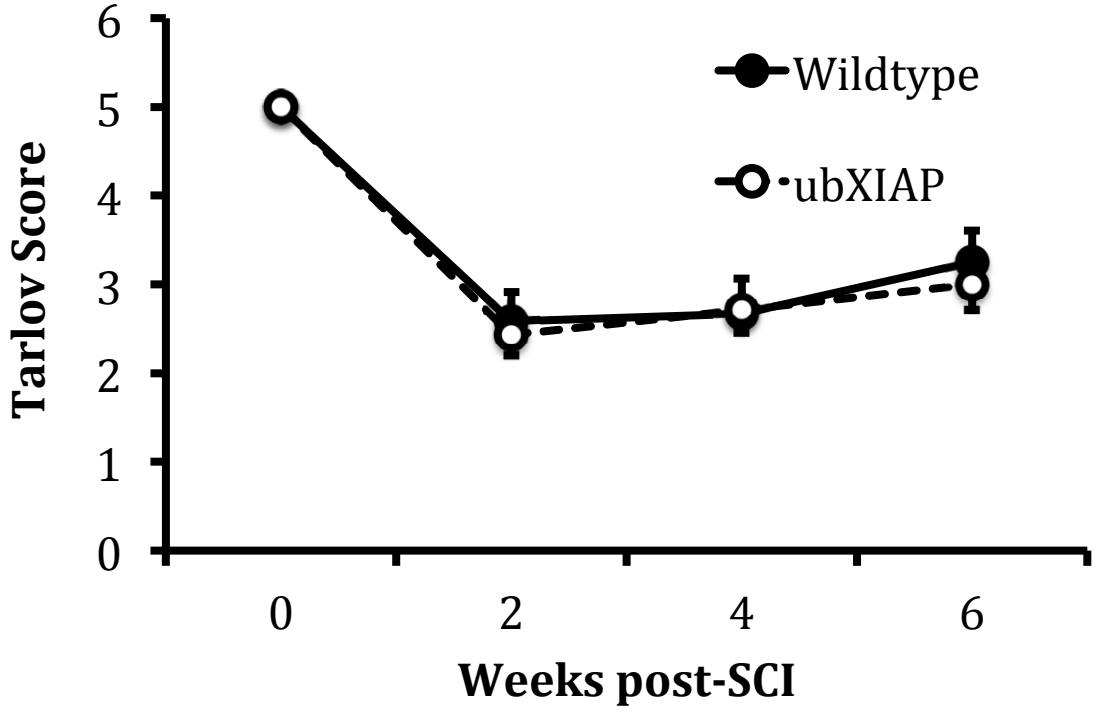

Tarlov Score
6
5
4
3
2
1
0
0
2
4
6
Weeks post-SCI
Wildtype
ubXIAP

Figure 3.2. Effect of ubXIAP on rotorod performance post-SCI

Rotorod performance was evaluated prior to injury and at weeks 2, 4, and 6 weeks post-SCI. Latency to fall dropped dramatically for both wildtype and ubXIAP mice, and showed minimal recovery at 6 weeks post-SCI. No differences were observed between wildtype and ubXIAP animals.

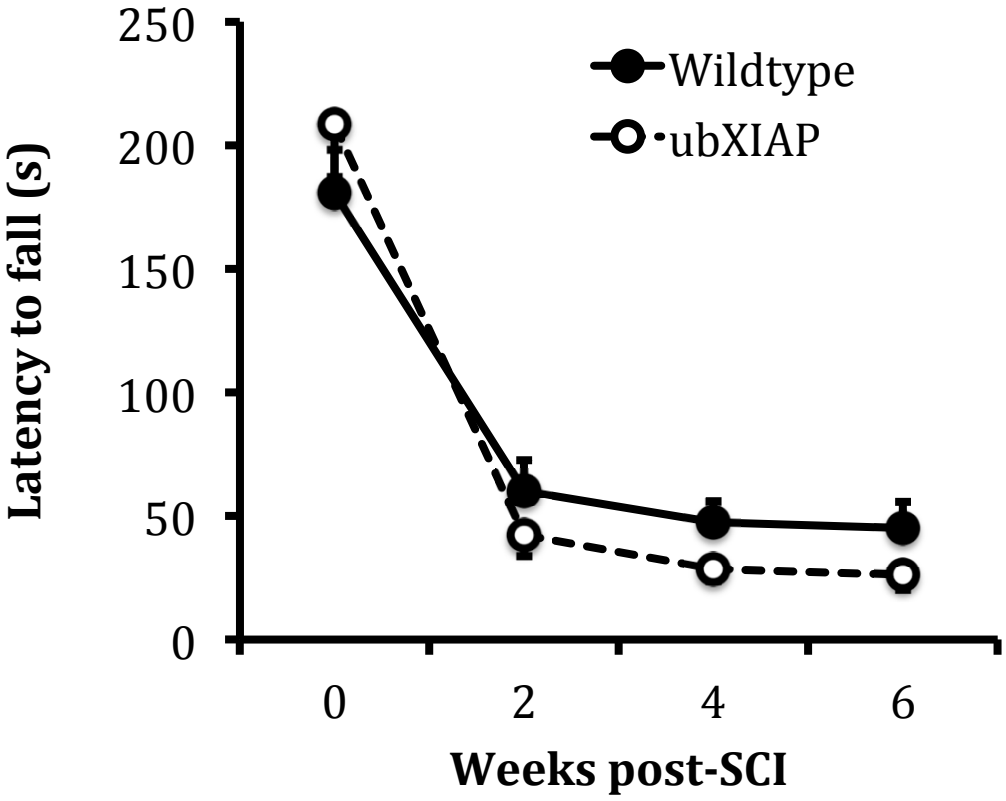

250
200
150
100
50
0
Latency to fall (s)
Wildtype
ubXIAP
0
2
4
6
Weeks post-SCI

Figure 3.3. Effect of ubXIAP on mechanical allodynia post-SCI

Baseline 50% withdrawal thresholds were obtained prior to injury and tested again at 2, 4, and 6 weeks post-SCI. SCI result in decreased withdrawal thresholds for both wildtype and ubXIAP mice, but no differences were observed between groups.

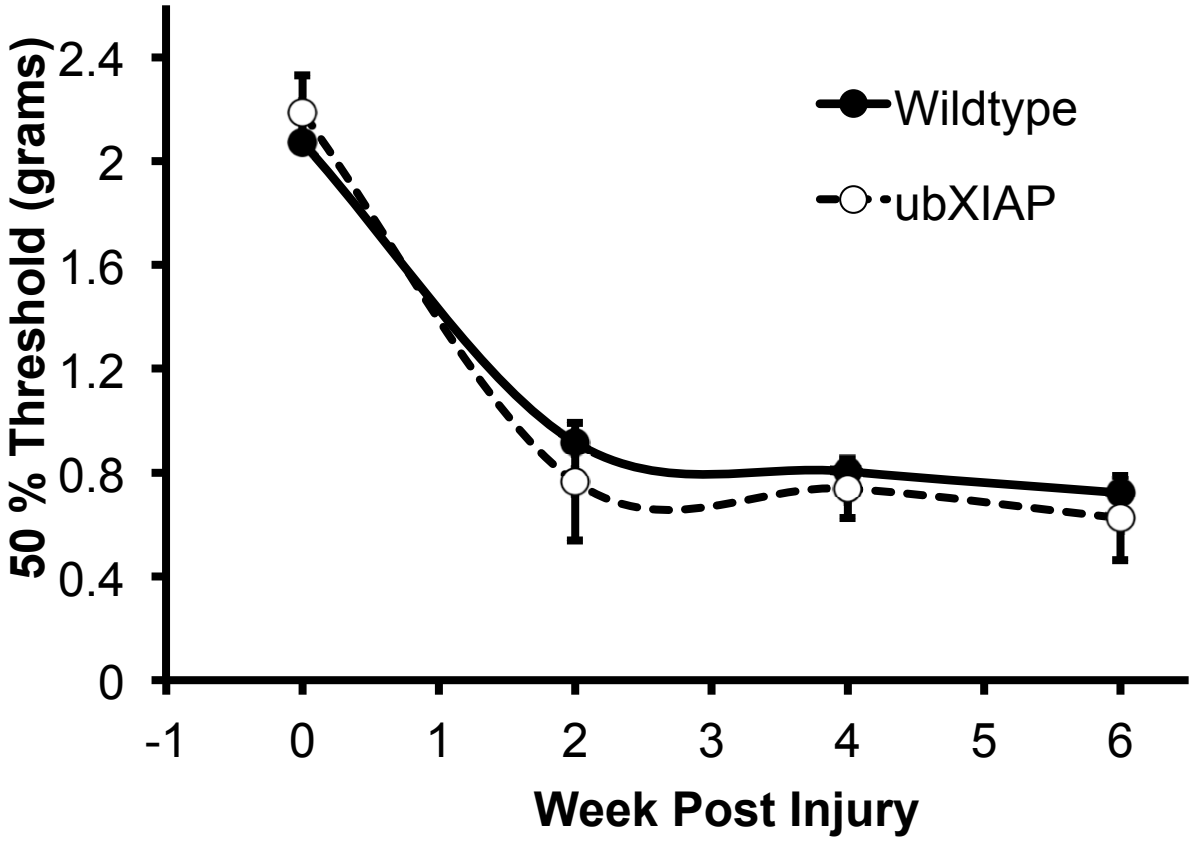
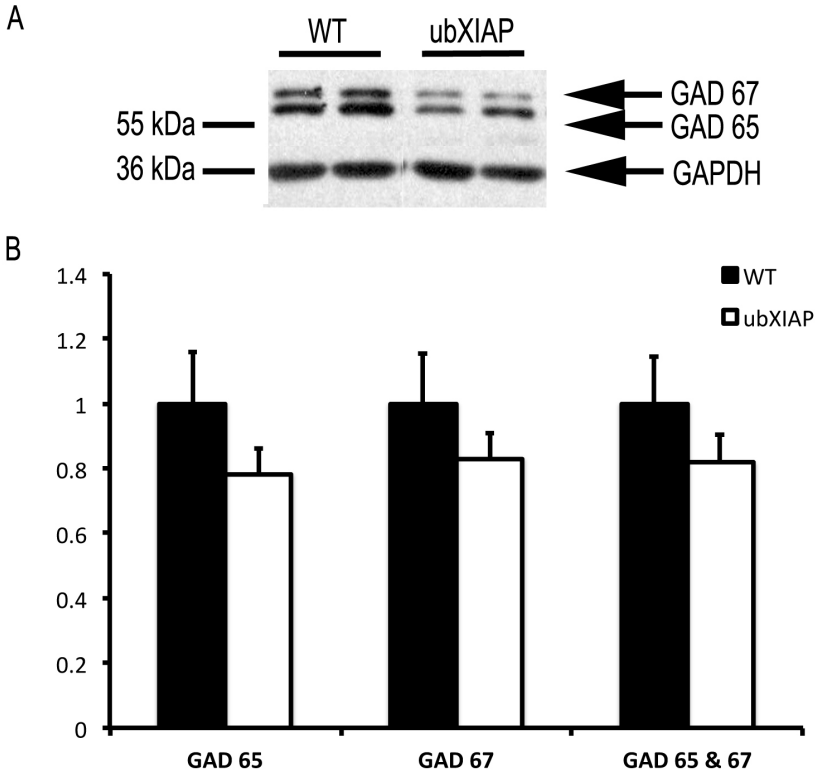

50 % Threshold (grams)
2.4
2
1.6
1.2
0.8
0.4
0
-1
0
1
2
3
4
5
6
Week Post Injury
Wildtype
ubXIAP

Figure 3.4. Effect of ubXIAP on GAD65/67 protein expression post-SCI

Representative GAD 65/67 (A) immunoreactivity in SHAM (n=6) and SCI (n=7) mice six weeks post-SCI. SCI reduces immunoreactivity for both GAD 65 (B) (83.3%, NS, $P<0.06$) and GAD 67 (C) (86.9%, $P>0.04$) (After normalization of SHAM animals).


A
WT
ubXIAP
55 kDa
36 kDa
GAD 67
GAD 65
GAPDH
B
WT
ubXIAP
1.4
1.2
1
0.8
0.6
0.4
0.2
0
GAD 65
GAD 67
GAD 65 & 67

CHAPTER 4: CHRONIC PREGABALIN TREATMENT GIVEN IMMEDIATELY FOLLOWING INJURY PREVENTS DEVELOPMENT OF BELOW-LESION MECHANICAL ALLODYNIA FOLLOWING SPINAL CORD INJURY

4.1 PREFACE AND SIGNIFICANCE TO THESIS

The results of this chapter have been presented and published in part in abstract form at the meeting of the Society for Neuroscience in San Diego, California, November 2010 as Meisner JG, Short C, Christie S, and Marsh Dr. Preemptive pregabalin treatment prevents the development of below-lesion mechanical allodynia after spinal cord injury. 762.7/R6.

I designed the experiments (with Dr. Marsh), conducted animal surgeries and post-operative care, conducted behavioural assessments of hindlimb locomotor function, conducted behavioral assesments of mechanical allodynia, drug treatment studies, processed and prepared tissue for immunohistochemistry studies, and processed and prepared tissue for Western blotting studies (with assistance from Maggie Qi).

Neuropathic pain is a frequent outcome of SCI, and is often refractory to treatment. Chapter 2 of this thesis explored the phenomenon of loss of a population of interneurons associated with below lesion neuropathic pain, and in Chapter 3 we tried to prevent or diminish development of neuropathic pain using an apoptosis inhibitor to prevent cell death. In Chapter 4 we assess a pharmacological neuroprotective approach.

A therapeutic strategy that prevents the development of neuropathic pain following SCI would improve the quality of life for SCI patients, and reduce the health care costs associated with patient care. This study was undertaken to investigate the effect of early intervention with the drug PGB on the development of mechanical allodynia post-SCI. Evidence in the literature suggests pregabalin may have neuroprotective

effects, and may modulate descending monoaminergic tone, glial activity, trafficking of voltage-gated calcium channels, and the development of novel excitatory synapses; processes that may affect neuropathic pain. We observed that immediate, but not delayed, treatment with the drug PGB was able to prevent the development of mechanical allodynia following SCI. PGB treatment did not impair recovery of locomotor function as assessed by rotorod performance or Tarlov scores, suggesting that it may not prevent adaptive locomotor plasticity. Immediate PGB did not lead to reductions of astrocyte markers below the level of lesion. Expression of the microglial marker CD11b was altered 6 weeks post-SCI, suggesting that modulation of microglial activity may be implicated in the effect of PGB. A significant loss of GABAergic inhibitory neurons was observed in saline treated animals post-SCI, but not in PGB treat animals, while no effect of injury or treatment was observed on a population of excitatory interneurons. No effect on injury or PGB treatment was observed on the expression of the pregabalin receptor, $Ca_v\ \alpha_2\delta$. SCI resulted in a significant increase in expression of the pro-synaptogenic molecule thrombospondin, and PGB treatment induced increased expression of the marker of synaptic plasticity, PSD-95, suggesting that PGB treatment may impact synaptic plasticity occurring post-SCI.

4.2 INTRODUCTION

Below-lesion neuropathic pain is a common outcome following spinal cord injury (SCI). Approximately 80% of individuals with SCI self-report the presence of pain or unpleasant sensations, and 40-50% of individuals report characteristics of neuropathic pain (Finnerup et al., 2001)(Siddall et al., 2003). This pain is often refractory to treatment (Attal et al., 2009), dramatically impacting patients' global health ratings and ability to participate in daily activities (Siddall et al., 2003). Given the high incidence of pain following SCI, investigating interventions that preemptively suppress plastic processes contributing to the development of pain syndromes, without impairing recovery of autonomic and locomotor function, is an important focus of research in this area.

Many factors have been implicated in the development of post-SCI neuropathic pain (Hulsebosch et al., 2009). These include persistent, remote activation of microglia in the lumbar cord (Hains and Waxman, 2006) and ventroposterior lateral nucleus of the thalamus (Zhao et al., 2007), decreased GABAergic inhibition (Gwak et al., 2006)(Meisner et al., 2010), as well as hypersensitization sensitization (Bedi et al., 2010) and central sprouting (Ondarza et al., 2003) of nociceptive afferent fibres. The heterogenous nature of the clinical SCI population, and lack of understanding of the relative contributions of specific causative factors to post-SCI pain, have hampered the advancement of novel treatments to alleviate post-SCI neuropathic pain, or prevent its development.

The gabapentinoid class of anti-convulsant drugs, composed of gabapentin and pregabalin, have demonstrated efficacy in reducing established post-SCI central neuropathic pain in both animal models (Boroujerdi et al., 2011) and in human clinical settings (Teasell et al., 2010). The primary mechanistic action of gabapentinoid

antinociception has not been conclusively determined, and remains a current topic of investigation. Multiple modalities have been implicated in the pharmacodynamic action of gabapentinoids, such as modulation of descending noradrenergic (Takeuchi et al., 2007) and serotonergic tone (Rahman et al., 2009), inhibition of trafficking and membrane insertion of the voltage gated calcium channel (Ca_v) subunit $\alpha_2\delta$-1 (Bauer et al., 2009), and inhibition of excitatory synaptogenesis (Eroglu et al., 2009). These effects are mediated through high-affinity binding of PGB to Ca_v $\alpha_2\delta$-1. The pleiotropic nature of gabapentinoids may be useful in the treatment of SCI, wherein multiple processes contribute to the development of subsequent pain syndromes.

The study of experimental SCI animal models allows for the evaluation of novel therapeutic agents and identification of the ideal treatment windows for potential intervention. Using the gabapentinoid agent PGB we assessed the effect of preemptive treatment strategies on the development of post-SCI below-lesion mechanical allodynia. We evaluated changes in protein expression previously associated with SCI-induced neuropathic pain, such as Ca_v $\alpha_2\delta$-1, and markers for astrocytes and microglia (GFAP and CD11b, respectively), and changes in populations of excitatory and inhibitory interneurons associated with the integration of sensory information.

Recently, synaptic adaptation has been proposed to contribute to post-SCI neuropathic pain (Tan et al., 2008), and the gabapentinoid gabapentin has been shown to inhibit excitatory synaptogenesis (Eroglu et al., 2010), so we also examined if PGB treatment affected synaptic plasticity. Thrombospondin-1 (TSP1) is a molecule of interest for this investigation. TSP1 is a cell-cell and cell-extracellular matrix interaction protein secreted by astrocytes that has recently been shown to interact with the PGB receptor, Ca_v $\alpha_2\delta$-1. The interaction of TSP and $\alpha_2\delta$-1 has been shown to promote

synaptogenesis in retinal ganglion cell culture (Eroglu et al., 2009). Previously, increased TSP expression has been demonstrated at the spinal cord lesion site 12-24 hours following injury in the rat [34]. To investigate this observation in mice, we assessed the effect of SCI and PGB treatment on peri-lesional expression of TSP1 for the first week post-SCI, and on the expression of markers of synaptic plasticity such as PSD-95, synaptophysin, and cadherin.

4.3 MATERIALS AND METHODS

4.3.1 *Animals*

Adult 8- to 12-week-old male FVB mice (25–30 g) [Jackson Laboratories, Bar Harbor, ME] were used in this study. In experiments assessing survival of GABAergic inhibitory interneurons FVB *gad1:*GFP mice (25-30g) (FVB-Tg(GadGFP) 45704Swn/J, #003718 Jackson Laboratory, Bar Harbour, ME) were used. The experiments described herein were approved by the Dalhousie University Committee on Laboratory Animals, and are in accordance with the Canadian Council on Animal Care guidelines. The animals were housed under a 12 hour light/dark cycle at 22-28°C with free access to water and food. Following injury the animals were housed singly and cages were kept over a heating pad at 37°C for the duration of the experiment.

4.3.2 Spinal Cord Contusion Injury

The mice were anesthetized with isoflurane (2%), placed on a heating pad at 37°C, and given subcutaneous injections of 1.0 mL lactated Ringer's solution, 5 mg/kg enrofloxacin, 0.05 mg/kg atropine, and 5 mg/kg ketoprofen. A laminectomy was performed at spinal segment T11 and a moderate injury (50 k dyn, 60 s dwell time) [13] was induced using the Infinite Horizon Impactor (Precision Systems and Instrumentation, LLC, Lexington, KY). To ensure a consistent degree of injury, only animals with an impact force within 5 kdyn of the target force, and a cord displacement between 400 and 600 mm were accepted for use in this study. Postoperative care included application of heat to maintain body temperature, and subcutaneous administration of saline,

enrofloxacin, and ketoprofen as needed. Bladders were expressed twice daily until independent bladder expression returned (approximately 10–14 days).

4.3.3 Drug Administration

After SCI, mice were randomly assigned to saline groups, or pregabalin (PGB) treatment groups, for immediate (iPGB) or delayed (dPGB) treatment. PGB (Pfizer, Canada) was prepared fresh, as needed, in sterile saline and given via intraperitoneal (i.p.) injection to animals at 10 mg/kg BID (9 am and 5 pm) for two weeks beginning either two hours or one week following SCI for studies on chronic treatment. Saline vehicle was administered as control. For studies of acute effects, PGB (10 mg/kg), or saline, was injected i.p. 30 minutes prior to behavioral testing.

4.3.4 Hindlimb locomotor function

Rotarod testing was performed one week prior to injury and again at three, four, five, and six weeks following SCI with an Accurotor RotaRod apparatus (Accuscan Instruments Inc., Columbus, Ohio). Two initial training sessions were employed to acclimate animals to the testing apparatus, and baseline scores were recorded. Mice were allowed to stay on the unit until falling off. Three trials were performed, with at least two minutes between trials, and averaged to determine the latency (s) to fall for each animal. Rotorod was set to increase in speed at constant acceleration to 300 revolutions per minute over 300 seconds.

The right and left hindpaws of each mouse were scored 1 week prior to injury and 3, 4, 5, and 6 weeks post-SCI using the Tarlov Scale. The mean score of the right and left hindpaws was determined. The Tarlov score grades hindpaw locomotor function as

follows: 0 = complete paralysis of the hindlimb, 1 = infrequent movement of the hindlimb, 2 = frequent movement of the hindlimb, 3 = frequent movement with some weight bearing, 4 = minor deficits, and 5 = normal.

4.3.5 Mechanical Allodynia

Two weeks prior to SCI surgery mice were acclimated to an acrylic glass box with a wire grid bottom. One week prior to SCI surgery baseline hindpaw withdrawal responses to a range of Von Frey filaments (Stoelting Co., Wood Dale, IL) were determined by applying the filaments to the plantar surface of the hindpaw using the modified version of the up-down method [9; 11]. Hindpaw withdrawal responses after SCI were reassessed at 2, 3, 4, 5, and 6 weeks after surgery. The mean of right and left hindpaw withdrawal thresholds taken at each time point were used to calculate differences from respective uninjured or vehicle thresholds.

4.3.6 Immunohistochemistry

At 2 and 6 weeks post SCI animals were anesthetized with urethane (2.5mg/kg) and transcardially perfused with 10 ml of ice-cold PBS followed by 10 ml of room temperature 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline (PBS). Tissue was post-fixed in 4% PFA overnight at 4° C and then sequentially cryoprotected in 15% and 30% sucrose at 4° C prior to storage. The spinal cord segments L4-L5, representing sensory input from the plantar hindpaw, were identified and embedded in Tissue-Tek OCT (Sakura Finetik, USA) for 30 μm cryostat sectioning. Free floating sections were collected for immunohistochemical staining with antibodies against the

dihydropyridine receptor (Sigma, Cat.# D219, 1:200), PKCγ (1:1000), and NeuN (1:500) Sections were blocked in Tris-buffered saline/triton-x 0.01% (TBS-X) w/5% normal goat serum (NGS) for one hour, incubated overnight with either 1:1000 GAD65/67, 1:2000 CGRP, or active caspase-3 1:5000 in 0.01% TBS-X w/1% NGS, washed 3x 10 minutes in TBS-X, incubated for 2 hours with 1:500 Alexa-fluor 546 Goat anti-rabbit (Abcam) in TBS-X w/1% NGS, washed 3x 10 minutes in TBS-X, and coverslipped. Image data was collected bilaterally at 10x magnification within the region of the spinal dorsal horn from equal size of field to ensure consistency between samples. Images were obtained using Zeiss Axioplan 2 imaging microscopes. Image J (NIH, USA) was used to determine level of staining intensity.

The number of GFP^+, PKCγ, or NeuN positive cells was determined in sections of lumbar spinal cord (L4-L5) from animals 6 weeks post-SCI. The analysis was done blind with the researcher unaware to which group the numbered slide belonged. The superficial laminae I-III were outlined and all positive cells within this region were counted. A minimum of 6 sections of spinal segment L4-L5 were selected per animals and cells were counted manually within the establish region of interest. The mean number of GFP^+, PKCγ, and NeuN cells with visible nuclei in the superficial dorsal horn was then tabulated for each animal. The number of GFP^+ and $PKC\gamma^+$ positive neurons were expressed relative to the number of NeuN positive neurons in the same field.

To assess expression of Ca_v $\alpha_2\delta$ and PKCγ post-SCI lumbar spinal cord (L4-L5) sections from animals 2 and 6 weeks were stained and quantified using Image J software. A region of interest was defined, and placed from the edge of the dorsal horn grey matter into the deep dorsal horn to provide an average density plot within the region of interest. At least 6 sections per animal were compared and averaged before group comparison.

4.3.7 Western Blotting

At stated time points, uninjured or SCI animals from each group were anesthetized with urethane (0.3 mL of 0.25 mg/mL), and perfused transcardially with 10 mL of ice-cold PBS. The animals were immersed in a solution of dry ice and methanol to flash-freeze the tissues and halt proteolytic degradation. The spinal cord segments T9-T11 containing 1 mm on either side of the lesion at T10, and the lumbar segments L4–L6, were dissected out over dry ice. The frozen tissue was homogenized and extracted in 50 mM Tris buffer (pH 8.0) containing 0.5% Triton-X 100, 150 mM NaCl, 1.0 mM EDTA, and protease inhibitor cocktail (Amresco, M250). Protein concentration was determined using the Bicinchoninic Acid Protein Assay kit (Pierce Protein Research Products, Rockford, IL), and protein extracts were separated using 4% stacking, 10% resolving SDS-polyacrylamide gel electrophoresis (PAGE). Samples from each treatment group were arranged on the same gel to allow for within-group comparison and semiquantitative analysis of changes in levels of protein content. PVDF membranes were blocked for 1 hour in 5% skim milk powder in TBS-Tween (T) 0.01%, incubated overnight at 4° C with antibodies against glial fibrillary acidic protein (GFAP, Chemicon, Cat.# AB5804, 1:60,000), CD11b (abcam, Cat.# Ab75476, 1:500), dihydropyridine receptor (Sigma, Cat.# D219, 1:1000), thrombospondin (1:100), PSD-95 (1:1000), synaptophysin (1:5000), pan-Cadherin (1:1000) or glyceraldehyde phosphate dehydrogenase (GAPDH, 1:2000; Abcam Cat.# 9485) in TBS-T, washed three times for 10 minutes each in TBS-T, incubated with HRP-conjugated goat anti-rabbit (Sigma, Cat.# A4914, 1:5000) or HRP-congujated goat anti-mouse (Sigma, Cat.# A3682, 1:5000) for 2 hours, washed three times in TBS-T, followed by the ECL chemiluminescence procedure (Bio-Rad

Laboratories, Inc., Hercules, CA). Integrated densitometry values were determined as a ratio of GAPDH protein expression using Alpha Imager 2.1 (Alpha Innotech, San Eandro, CA). Comparisons between SCI and sham groups were performed after normalizing to sham values. An average obtained from at least 4 separate Western blots was used for analysis.

4.3.8 Statistical Analysis

Unless otherwise noted group size is n= 6 or 7. A two-tailed two-way analysis of variance (ANOVA) was used to determine whether there were any significant differences by TREATMENT and/or INJURY at the $P < 0.05$ level. If significant differences were observed then a one-way ANOVA was performed with the Tukey post-hoc test used to compare among the individual group means with significance set at $P < 0.05$.

4.4 RESULTS

4.4.1 iPGB treatment does not impair locomotor recovery post-SCI

Following a moderate contusion injury, paralysis is transiently observed below the lesion, similar to the period of spinal shock observed after human SCI. Animals gradually recover some measure of weight bearing ability by two weeks post-SCI but never recover normal locomotor function. Although animals receiving iPGB treatment consistently scored higher than animals receiving saline vehicle no statistically significant difference was observed between treatment groups. SCI resulted in a dramatic reduction in ability to maintain locomotion on the rotorod task (Figure 1A), such that animals were unable to balance on the bar for more than one revolution for the first 4 weeks following injury. By week 5 and 6 animals were able to walk upon the rotorod for multiple revolutions, but performance remained dramatically below baseline performance.

Recovery of locomotor function graded by Tarlov scores showed a similar pattern to that previously reported following a moderate contusion injury in the mouse [14]. Immediately after injury, the animals demonstrated complete hindlimb paralysis (observed), and a gradual return of some weight bearing support in one or both legs by six weeks post-SCI, irrespective of treatment group (Figure 1B).

4.4.2 Mechanical allodynia is blocked by early administration of PGB

To confirm the efficacy of acute PGB in reversal of established allodynia, baseline withdrawal thresholds were determined and tested again 6 weeks post-SCI, 30 minutes after i.p. injection of 10 mg/kg PGB or saline control. Acute treatment with PGB

resulted in reversal of mechanical withdrawal thresholds to levels not significantly different from baseline (Figure 2).

To assess the effect of iPGB or dPGB treatment on the development of below-lesion mechanical allodynia, we determined the 50% withdrawal threshold of the plantar surface of the hindpaw to stimulation with Von Frey filaments (Figure 3), compared to baseline testing prior to SCI. In saline treated animals a reduction in mechanical withdrawal thresholds was observed beginning 2 weeks following injury. The reduced threshold was persistant, and stable, to the end of the experimental period 6 weeks post-SCI. Animals receiving iPGB treatment did not develop mechanical allodnia. iPGB treated animals demonstrated significantly greater 50% withdrawal thresholds than saline controls at week 3, 4, 5, and 6; time points when PGB may be assumed to be cleared from the animals' system. When PGB treatment was delayed until 1 week post-SCI, no differences were observed between saline and dPGB treatments (Figure 3). This suggests the presence of a therapeutic window for preemptive PGB effect to suppress development of mechanical allodynia and neuropathic pain.

4.4.3 Expression of GFAP after SCI and PGB treatment

GFAP expression was investigated to assess astrocyte activation in spinal cord segments caudal to the SCI lesion. Previously, astrocyte activation has been demonstrated remote from the SCI lesion, whereas negligible changes have been reported in the lumbar spinal cord after peripheral nerve injury [24]. GFAP protein levels were not significantly altered by SCI as protein levels 2 or 6 weeks post-SCI were similar to uninjured spinal cord segments. A non-significant increase of 20% relative expression of GFAP at 6 weeks did not appear to be affected by iPGB treatment (Figure 4). GFAP

expression was also assessed at 12, 24, 48, 72 hours and 1 week post-SCI and no effect of injury or treatment was observed (Data not shown).

4.4.5 Expression of CD11b after SCI and PGB treatment

CD11b expression was used as a marker of microglia in the L4-6 spinal cord segments of uninjured and SCI mice. CD11b expression was significantly increased in the lumbar cord segments at two and 6 weeks post-SCI relative to uninjured animals. iPGB effectively diminished this increase in CD11b protein levels occurring 2 and 6 weeks post-SCI (Figure 5). CD11b expressed was also assessed at 12, 24, 48, 72 hours, and 1 week post-SCI and no effect of injury or treatment was observed (Data not shown).

4.4.6 Loss of GABAergic inhibitory interneurons post-SCI

The number of GFP, PKCγ, and NeuN positive cells were counted in Laminae I-III of the lumbar (L4-5) dorsal horn of uninjured mice, and mice 6 weeks post-SCI receiving either Saline or iPGB treatment (Fig 4.6 A). The number of GFP^+ GABAergic inhibitory interneurons relative to NeuN positive control cells was found to be decreased following post-SCI in saline treated mice (0.07±0.004 vs. 0.05±0.00, $P<0.05$), but no significant decrease was observed in iPGB treated mice (Fig 4.6 B, Table 4.1). However, the number of surviving GABAergic neurons was not different between saline- and iPGB-treated SCI mice.

A population of excitatory interneurons labeled by PKCγ staining was also counted (Figure 4.7 A). No significant differences were observed between uninjured, saline, and iPGB treated mice (Figure 4.7 B, Table 4.1).

4.4.7 Expression of Ca_v $\alpha_2\delta$-1 after SCI and PGB treatment

To investigate dynamic changes in the expression of the *$\alpha_2\delta$-1* subunit of voltage gated calcium channels, we assessed protein expression in spinal cord segments L4-6 caudal to the level of lesion. Analysis of L4-6 spinal cord homogenate by Western blot did not demonstrate significant differences in *$\alpha_2\delta$-1* protein levels between saline- and iPGB-treated animals, or between uninjured and injured animals at 2 and 6 weeks post-SCI (Figure 8).

Lumbar (L4-L5) spinal cord was sectioned and stained to investigate changes in the expression level, or expression profile throughout the dorsal horn, of Ca_v $\alpha_2\delta$ post-SCI. Immunoreactivity for PKCγ was also conducted as a reference standard for a protein that is expressed in excitatory interneurons of Laminae II inner. Tissue was collected from uninjured animals, and animals 2 weeks post-SCI and lumbar cord segments sectioned and stained (Figure 4.9 A) and 6 (Figure 4.10 A) weeks post-SCI. No difference in Ca_v $\alpha_2\delta$ staining intensity (assessed by area under the curve), or expression profile within the dorsal horn was observed between uninjured animals, and saline or iPGB treated animals at 2 (Figure 4.9 B) and 6 (Figure 4.10 B) weeks post-SCI.

Expression of PKCγ also showed a similar expression pattern between uninjured animals and saline, or iPGB treated animals at two (Figure 4.9 A) and six (Figure 4.10 B) weeks post-SCI. In saline treated animals 6 weeks post-SCI PKCγ staining was found to be significantly lower than uninjured animals at a 62.63 μm depth in the dorsal horn (43.50±3.91 vs. 72.14±3.05, $P<0.05$) (Figure 4.10 B). No other differences in staining intensity (assessed by area under the curve) or expression profile were observed (Figure 4.9 B and 4.10 B).

4.4.8 Expression of thrombospondin-1 is transiently increased at the lesion site following SCI

TSP1 expression was observed to rapidly increase following SCI (Figure 7). Compared to uninjured spinal cord, levels of TSP1 at the lesion site of SCI mice were increased at 12 and 24 hours, with peak expression levels occurring at 48 and 72 hours post-SCI. We observed this increase in TSP1 expression to be transient as levels were decreased significantly by 1 week post-SCI (Figure 7). iPGB was found to have no effect on expression of TSP1 protein expression levels at the lesion site.

4.4.9 Expression of PSD-95 is increased by iPGB animals post-SCI

PSD-95 expression was observed to be increased in iPGB treated animals at 24 (4.61 ± 1.99 vs 1.00 ± 0.34, $P<0.05$), 48 (2.65 ± 0.23 vs 1.00 ± 0.34, $P<0.05$), and 72 (3.27 ± 0.52 vs 1.00 ± 0.34, $P<0.05$) hours relative to levels in uninjured animals. No effect of injury or treatment was observed on levels of pan-Cadherin and synaptophysin immunoreactivity.

4.5 DISCUSSION

The observations reported in this study suggest that immediate intervention with pregabalin (PGB) following SCI may offer therapeutic benefit to preempt the development of below-lesion allodynia associated with neuropathic pain. Treatment with PGB for two weeks, initiated shortly after SCI (iPGB), prevented the development of mechanical allodynia. In contrast, mechanical allodynia was observed to develop in saline-treated SCI mice and mice receiving PGB treatment delayed till 1 week post-SCI (dPGB). These findings suggest that plasticity occurring within the spinal cord during the first week post-injury may be irrevocable and associated with detrimental neurological outcomes, such as the development of neuropathic pain. Furthermore, receiving appropriate early intervention may preempt this maladaptive plasticity and diminish the development of below-lesion neuropathic pain.

Increased activation of microglia has been associated with the maintenance of neuropathic pain (Hains and Waxman, 2006). Attenuation of microglial activity with propentofylline treatment reduces behavioral manifestations of neuropathic pain (Gwak et al., 2006). Chronic PGB treatment has also been associated with reduced microglia activity in a rat model of streptozotocin induced-diabetes (Wodarski et al., 2009), similar findings to those observed in our model of SCI. Suppression of mechanical allodynia by iPGB may be due in part to reduced microglial activity, as measured by the microglial marker CD11b.

Gabapentin has been observed to act within the locus coeruleus to promote descending inhibitory tone, enhancing the release of noradrenaline within the spinal dorsal horn (Hayashida et al., 2008). In the dorsal spinal cord noradrenaline has an

antinociceptive action, and this may partially account for the acute effects of PGB treatment. Noradrenaline also acts directly upon microglia, reducing the phosphorylation of p38 MAPK (Morioka et al., 2009), an indicator of microglia which have transitioned from a quiescent to an activated state. Activation of p38 has been found to be necessary for the synthesis and release of BDNF from microglia (Trang et al., 2009), a factor known to contribute to shifts in the neuronal anion gradient associated with neuropathic pain after peripheral nerve (Coull et al., 2005) and spinal cord injury (Lu et al., 2008). Chronic facilitation of the descending noradrenergic system by PGB may have contributed to the noted reduction of SCI-induced CD11b expression reported in this study.

We have previously reported the loss of GABAergic inhibitory interneurons in the lumbar spinal cord post-SCI (Meisner et al., 2010). Here we investigated the potential for iPGB treatment to exert a neuroprotective effect on this population of neurons, and additionally, examined the population of $PKC\gamma^{+}$ excitatory interneurons that populate Laminae II inner (Polgar et al., 1999). We observed a significant loss of GFP positive GABAergic cells 6 weeks post-SCI in saline treated animals. This loss of GABAergic interneurons was attenuated in iPGB treated animals. The population of $PKC\gamma^{+}$ excitatory interneurons was not significantly affected by SCI or PGB treatment. Neuroprotective properties have previously been ascribed to gabapentionds, as pregabalin has been observed to reduce apoptosis of oligodendrocytes in a rat model of SCI (Ha et al., 2008).

It is of interest to note the rapid, and transient, upregulation of TSP immediately post-SCI. Our observations expand upon previous work demonstrating an increased expression of TSP in the rat at 12 and 24 hours post injury in the peri-lesion zone (Wang

et al., 2009). Purinergic stimulation promotes secretion of TSP from astrocytes (Cahoy et al., 2008)(Tran and Neary, 2006). Given the known release of ATP following spinal cord injury (Trang et al., 2009) the transient increase in TSP reported in our study may have been predicted. Recent observations have demonstrated a novel interaction of TSP family members with the $\alpha_2\delta$-*1* subunit of voltage gated calcium channels (Eroglu et al., 2009). This interaction promotes excitatory synaptogenesis, and it is intriguing to consider that *de novo* excitatory synaptogenesis may occur between sensory afferent neurons with high $\alpha_2\delta$-*1* expression (Bauer et al., 2009), and dorsal spinal cord projection neurons; thus, contributing to the development of neuropathic pain. The transient upregulation of TSP may be associated with the observed 1week therapeutic window for PGB treatment. Cell culture studies indicate that TSP1-induced synaptogenesis occurs rapidly, and that these synapses remain stable on susequent treatment with PGB (Eroglu et al., 2009). PGB treatment, begun within hours of SCI, may antagonize TSP binding of $\alpha_2\delta$-*1* and prevent the formation of *de novo* pain-augmenting synapses in the dorsal horn. In contrast, PGB treatment delayed by one week may miss this critical period of TSP1 exposure that facilitates the formation of pain-inducing spinal circuits.

Expression of PSD-95, a marker of synapse plasticity and post-synaptic excitatory synapses (Xu, 2011), is increased in iPGB treated animals, supporting the hypothesis that synapse formation may be implicated in its action. Our observation of an increase in PSD-95 expression in the iPGB group, but not saline treated animals, may seem counterintuitive to preemptive blockade of pain as other groups have reported that post-SCI neuropathic pain is associated with *de novo* synaptogenesis and increased expression of PSD-95 (Tan et al., 2008). Our observation is reported at early post-SCI time points, which may reflect dysregulation of normal synaptic modeling post-injury, and the

Western blotting study does not reveal the localization of the protein to inform whether it remains in the Golgi, the cytosol or has been inserted into a functional role at a synapse. Further investigations are needed to explore the effect of iPGB on synaptogenesis post-SCI.

Recent observations have reporting increased lumbar expression of the pregabalin receptor *$\alpha_2\delta$-1* post-SCI (Boroujerdi et al., 2011). In contrast, we observed no change in Ca_v $\alpha_2\delta$ protein levels post-SCI. Our analysis of Ca_v $\alpha_2\delta$ expression differed in that we utilized a spinal homogenate containing both dorsal and ventral spinal cord from mouse, while Boujerdi *et al.* (2011) utilized only the dorsal half of the spinal cord from a rat model of SCI. Inclusion of the ventral spinal cord in our assay may have diluted potential changes in *$\alpha_2\delta$-1* expression relative to total protein. Our observations are supported by immunohistochemical studies suggesting there is no obvious change in the intensity or expression profile of Ca_v $\alpha_2\delta$ due to SCI and saline or iPGB treatment. Differences in injury severity between our studies may also account for variation, or reflect strain- and species-dependent responses of rats (Popovich et al., 1997) and mice (Basso et al., 2006) to SCI. It had been postulated that the expression profile of *$\alpha_2\delta$-1* may expand following injury, providing greater co-localization with, and potential innervation of, excitatory $PKC\gamma^+$ interneurons, thus facilitating pain transmission, but no such pattern was observed.

Similarly, our analysis of GFAP expression in the lumbar cord was not found to significantly change after SCI. This is also in contrast to previous reports showing immunohistochemical evidence for below-lesion astrocyte hypertrophy post-SCI in rats (Gwak and Hulsebosch, 2009). An approximate 20% increase in expression of GFAP was noted in both saline- and PGB-treated animals 6 weeks post-SCI. This may be

indicative of an increase in lumbar astrocyte activation, but Western blot techniques utilized for analysis may not be sufficiently sensitive to detect a significant difference.

Gabapentinoids, such as PGB, may comprise an ideal early therapeutic intervention for SCI. A precedent for such early intervention following CNS trauma has been established by the use of the thrombolytic agent, tissue plasminogen activator, administered within 3 hours of acute ischemic stroke (Ahmed et al., 2010). Evidence presently exists to encourage the early (<24 hours) surgical decompression of spinal cord injury to reduce secondary damage (Cadotte et al., 2010), and there is no reason to believe PGB treatment may increase the risk associated with this, or other, procedures. In fact, as demonstrated in this study, PGB treatment may also be useful as a peri-surgical protective agent to reduce the risk of iatrogenic damage during decompression procedures that may contribute to neuropathic pain. Estimated incidence of persistent post-surgical pain is estimated to range from 10-30%, dependent upon the procedure (Hulsebosch et al., 2009). Patients undergoing spinal surgical procedures, such as scoliosis spinal realignment or tumor resection, may also risk damage to the spinal cord capable of inducing neuropathic pain.

In summary, we demonstrate that immediate treatment with pregabalin following SCI may be an effective and safe tool to block the development of neuropathic pain post-SCI. Although the mechanisms are not fully understood, they may involve suppression of microglia, and inhibition of *de novo* excitatory synaptogenesis among sensory afferents and pain transmission neurons. Use of drugs with established safety profiles as a preemptive agent against the development of neuropathic pain hold great promise and warrant further investigation as preemptive treatment strategies following SCI.

4.6 REFERENCES

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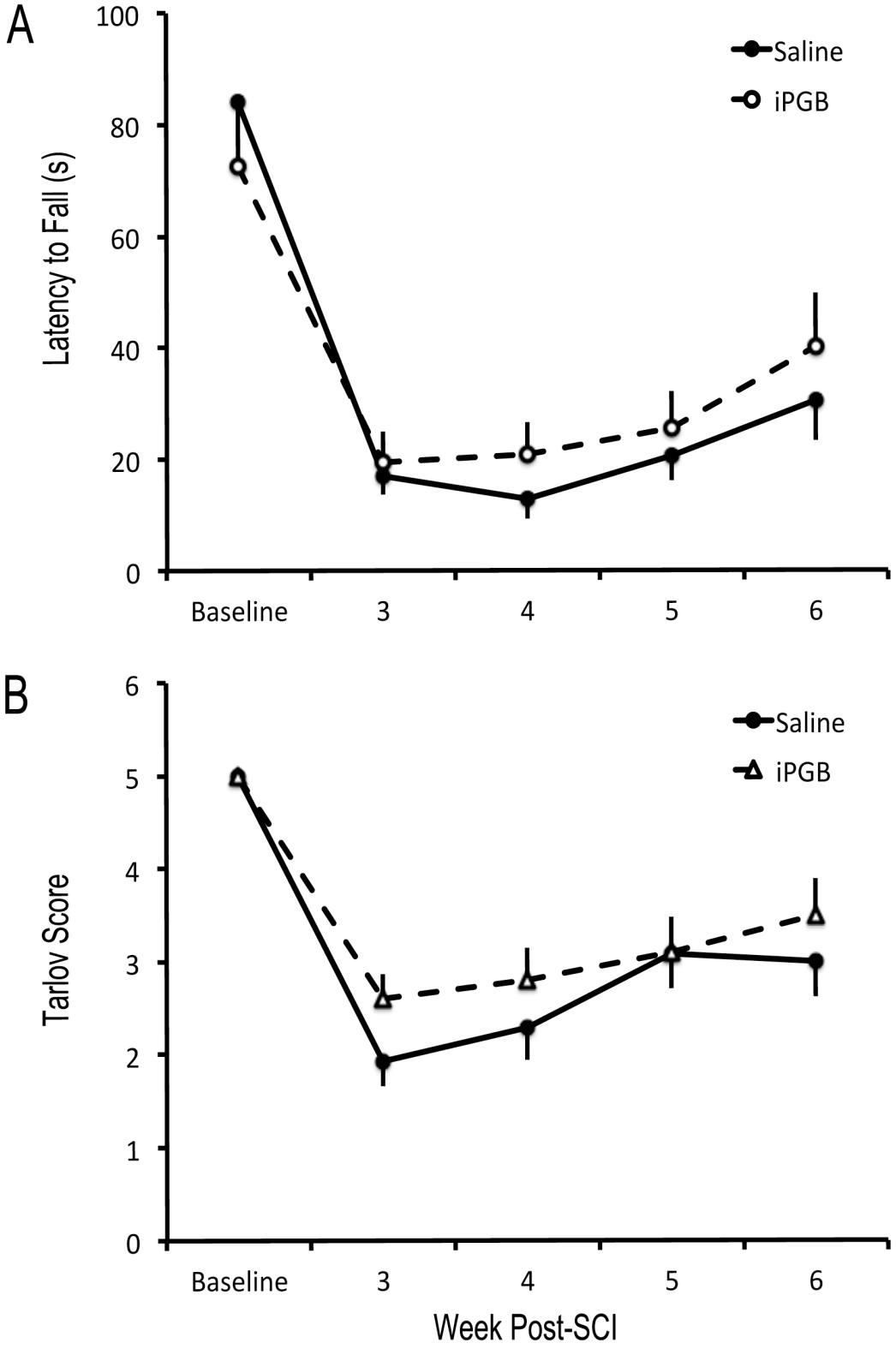
Figure 4.1. Effect of immediate PGB treatment on motor function post-SCI

Motor performance was evaluated following SCI to ensure that PGB did not impair motor recovery. (A) Rotorod performance was evaluated in saline and immediate PGB (iPGB) treated mice prior to injury and for 6 weeks post-SCI. Both saline and iPGB treated mice demonstrated significant loss of motor function post-SCI, with some recovery of function at week 6. No significant difference was observed between treatment groups. (B) Tarlov scores of hindlimb function were assessed for 6 weeks post-SCI. Both groups demonstrated paralysis immediately following injury, with gradual recovery of function until week 6. No significant difference was observed between saline and iPGB treated mice.


A
100
80
60
40
20
0
Latency to Fall (s)
Saline
iPGB
Baseline
3
4
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B
6
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4
3
2
1
0
Tarlov Score
Saline
iPGB
Baseline
3
4
5
6
Week Post-SCI

Figure 4.2. Acute PGB treatment is effective at reversing established post-SCI allodynia

50% withdrawal thresholds (g) were determined in uninjured mice and in SCI mice, 6 weeks post-SCI. SCI caused a reduction of 50% withdrawal thresholds observable at 6 weeks post-injury (1.87 ± 0.12 g vs 0.83 ± 0.12 g; $P < 0.05$). Thresholds are restored to levels similar to uninjured animals with PGB (10 mg/kg i.p) (2.41 ± 0.19 g).

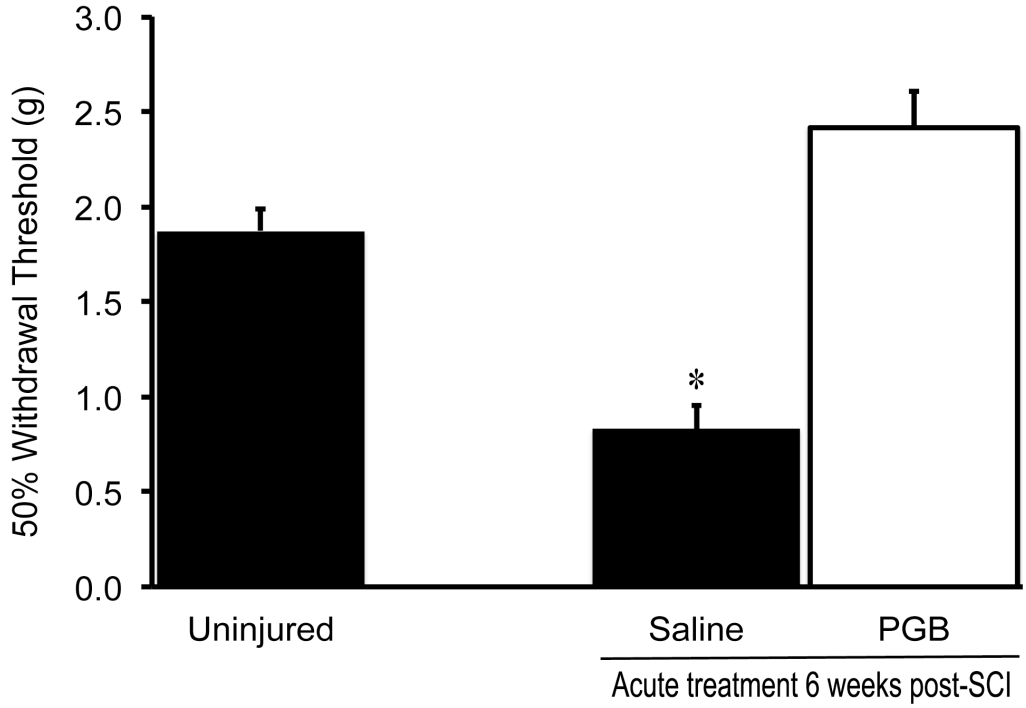

3.0
2.5
2.0
1.5
1.0
0.5
0.0
50% Withdrawal Threshold (g)
*
Uninjured
Saline
PGB
Acute treatment 6 weeks post-SCI

Figure 4.3. PGB stabilizes withdrawal thresholds when administered immediately (iPGB), but not after a 1 week delay (dPGB), following SCI.

After baseline 50% withdrawal thresholds were obtained, testing continued weekly to 6 weeks post-SCI in animals treated with saline vehicle (n=13), iPGB (n=11), or dPGB (n=12). iPGB alleviated the reduction in 50% withdrawal thresholds observed in saline- and dPGB-treated SCI mice, with significantly higher withdrawal thresholds observed in iPGB-treated mice at week 3, 4, 5, and 6 weeks post-SCI ($P < 0.05$).

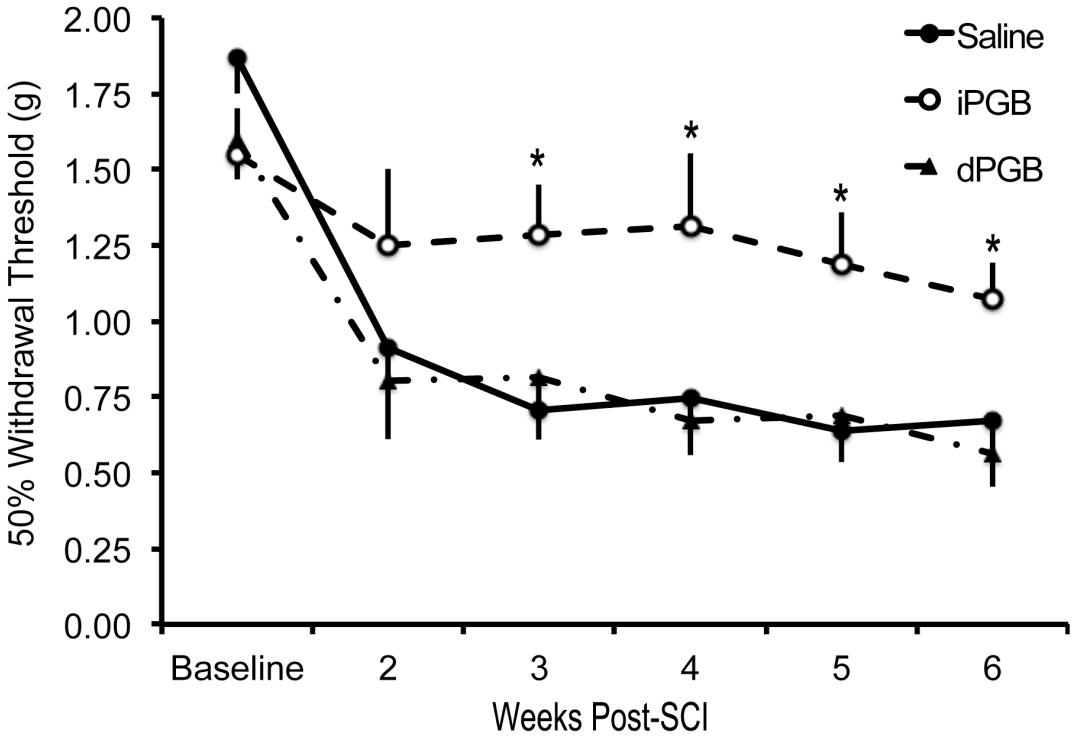

2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
50% Withdrawal Threshold (g)
Saline
iPGB
dPGB
*
*
*
*
Baseline
2
3
4
5
6
Weeks Post-SCI

Figure 4.4. Effect of iPGB on lumbar expression of GFAP after SCI.

Expression of GFAP protein in uninjured mice, and saline-, and iPGB-treated SCI mice at 2 and 6 weeks post-SCI. Panel (A) shows representative samples from L4-6 spinal cord homogenates processed by SDS-PAGE and Western blot analysis for GFAP protein. GAPDH protein expression was used as an internal loading control. Panel (B) shows quantification of relative GFAP protein levels by densitometry in L4-6 spinal cord in uninjured mice and after saline or iPGB treatment at 2 and 6 weeks post-SCI. Protein levels are expressed relative to uninjured as a ratio corrected for GAPDH levels. No significant change relative to uninjured mice was observed in GFAP protein levels in saline-, or iPGB-treated mice at 2 and 6 weeks following injury.

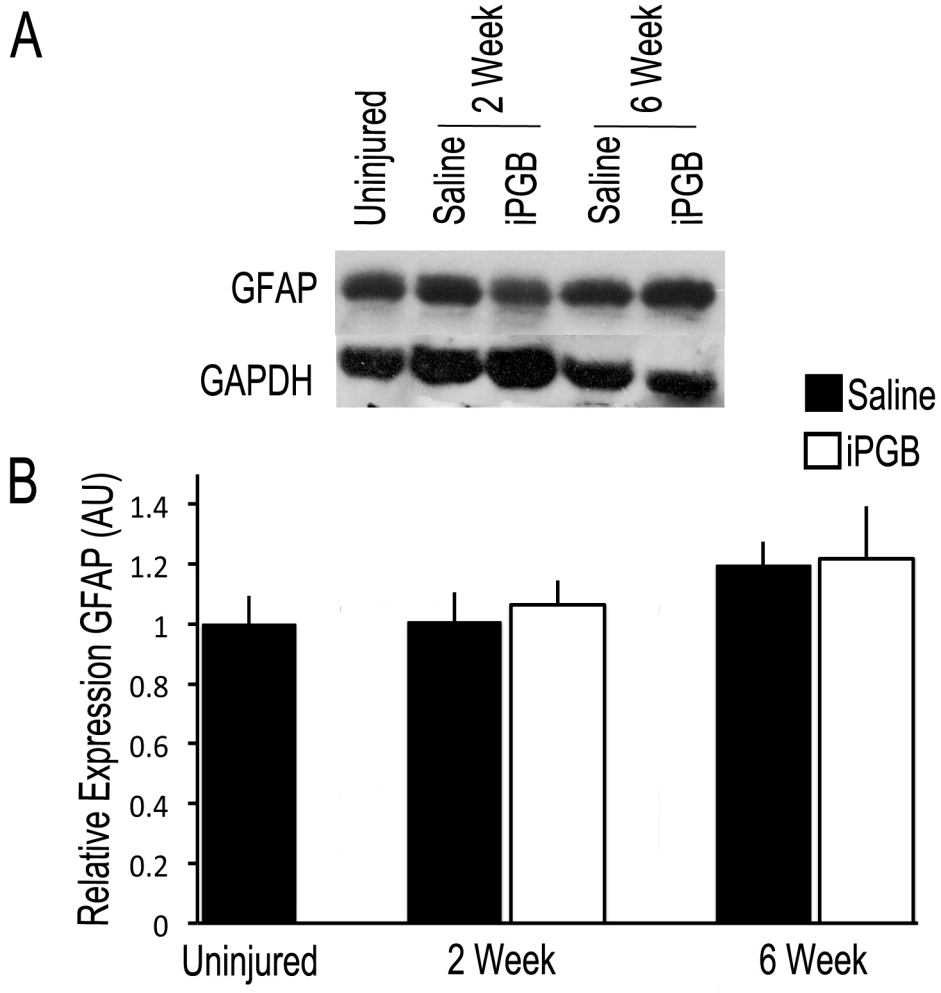

A
Uninjured
Saline
2 Week
iPGB
Saline
6 Week
iPGB
GFAP
GAPDH
B
Saline
iPGB
Relative Expression GFAP (AU)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Uninjured
2 Week
6 Week

Figure 4.5. Effect of iPGB treatment on lumbar expression of CD11b after SCI.

Expression of CD11b protein in uninjured mice and at 2 and 6 weeks post-SCI in saline-and iPGB-treated mice. Panel (A) shows representative samples from L4-6 spinal cord homogenates processed by SDS-PAGE and Western blot analysis for CD11b protein. GAPDH protein expression was used as an internal loading control. Panel (B) shows quantification of relative CD11b protein levels by densitometry in L4-6 spinal cord in uninjured mice and after saline or iPGB treatment at 2 and 6 weeks post-SCI. Protein levels are expressed relative to uninjured as a ratio corrected for GAPDH levels. Two-tailed two-way ANOVA showed no significant effect of time on expression of CD11b, but indicated a significant effect of iPGB treatment on CD11b protein levels. Subsequent analysis with a one-way ANOVA demonstrated CD11b levels were significantly elevated from uninjured animals in saline-treated mice 6 weeks post-SCI (*, $P<0.05$), and that expression in iPGB treated animals 6 weeks post-SCI approached a significant reduction ($P=0.062$).

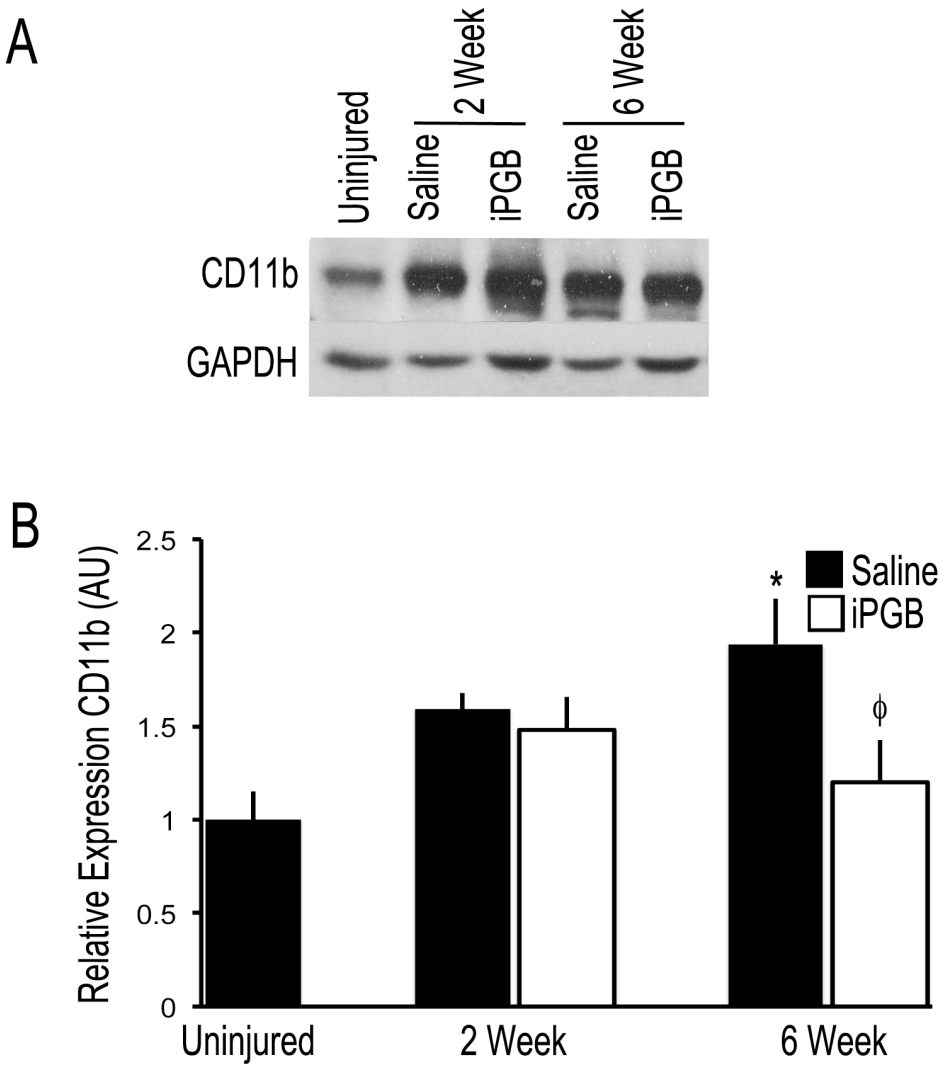
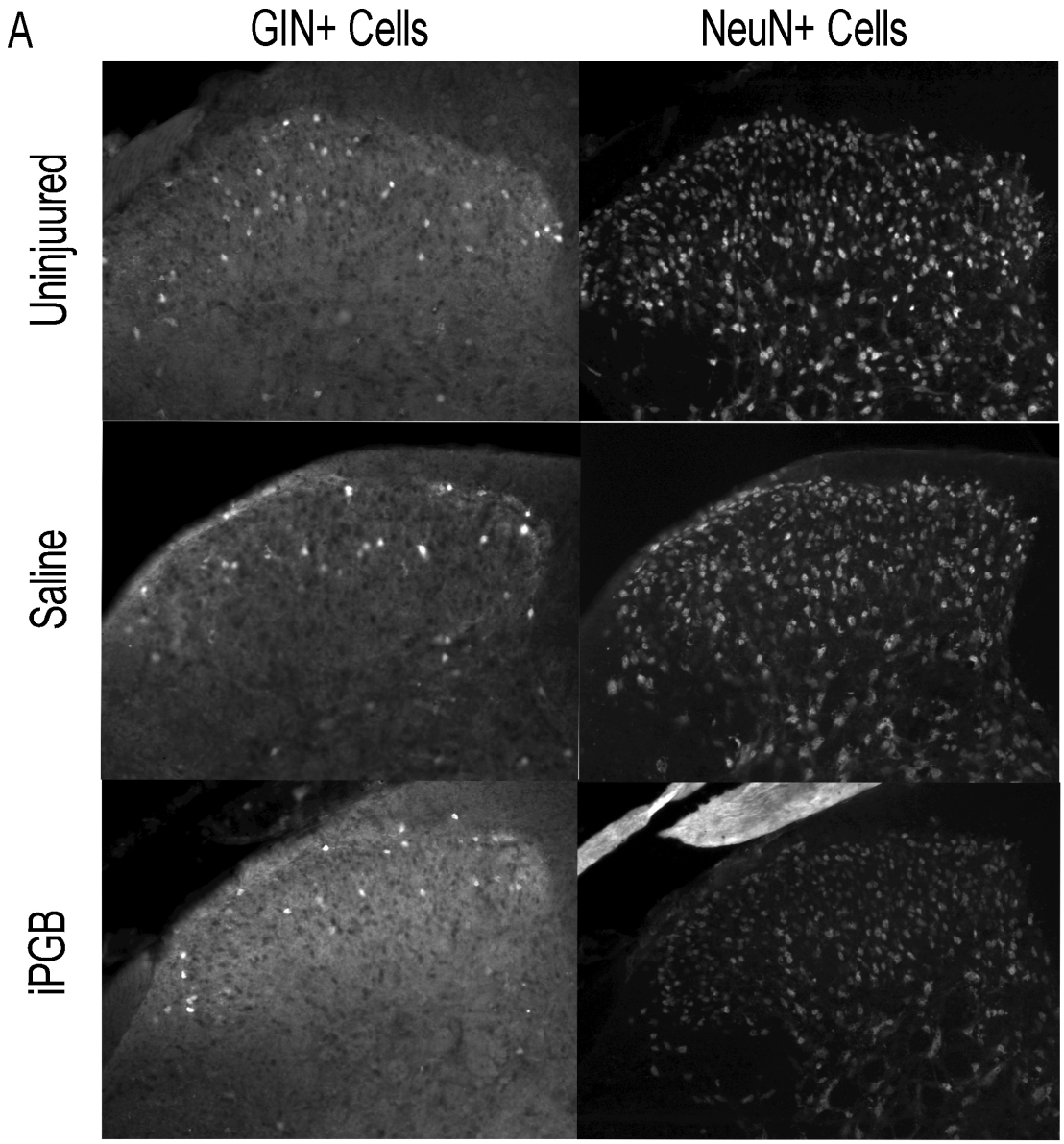

A
Uninjured
Saline
2 Week
iPGB
Saline
6 Week
iPGB
CD11b
GAPDH
B
Relative Expression CD11b (AU)
2.5
2
1.5
1
0.5
0
Saline
iPGB
*
ϕ
Uninjured
2 Week
6 Week

Figure 4.6 Relative number of inhibitory interneurons post-SCI

Representative images of GFP^+ GABAergic inhibitory interneurons and NeuN positive cells in uninjured and saline or iPGB treated mice 6 weeks post-SCI (A). Fewer GFP^+ GABAergic cells were counted in saline treated mice relative to uninjured animals at 6 weeks post-SCI (0.05±0.00 vs. 0.07±0.00, $P<0.05$) (B).


A
GIN+ Cells
NeuN+ Cells
Uninjuured
Saline
iPGB

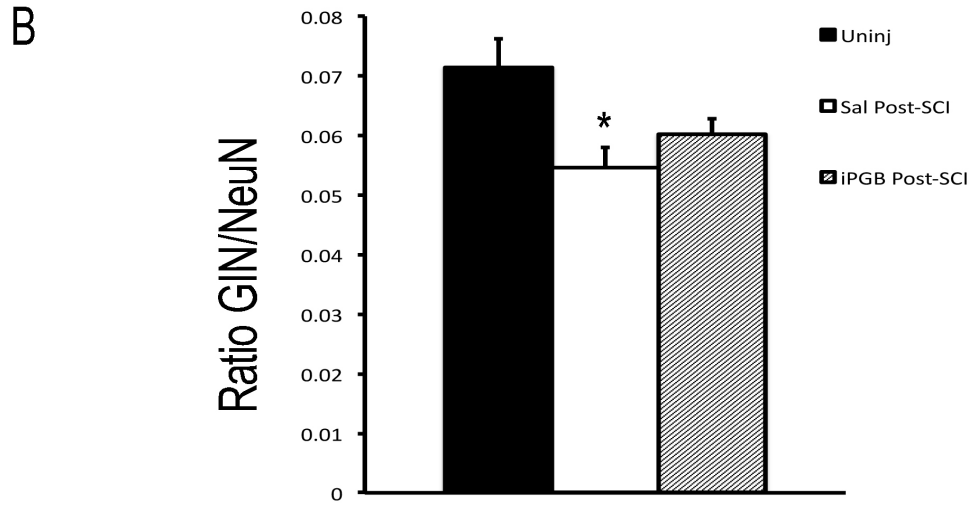

B
Ratio GIN/NeuN
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
*
Uninj
Sal Post-SCI
iPGB Post-SCI

Figure 4.7 Relative number of excitatory interneurons post-SCI

Representative images of $PKC\gamma^+$ interneurons and NeuN positive cells in uninjured and saline or iPGB treated mice 6 weeks post-SCI (A). No change in the number of $PKC\gamma^+$ cells was observed as an effect of injury or saline or iPGB treatment (B).

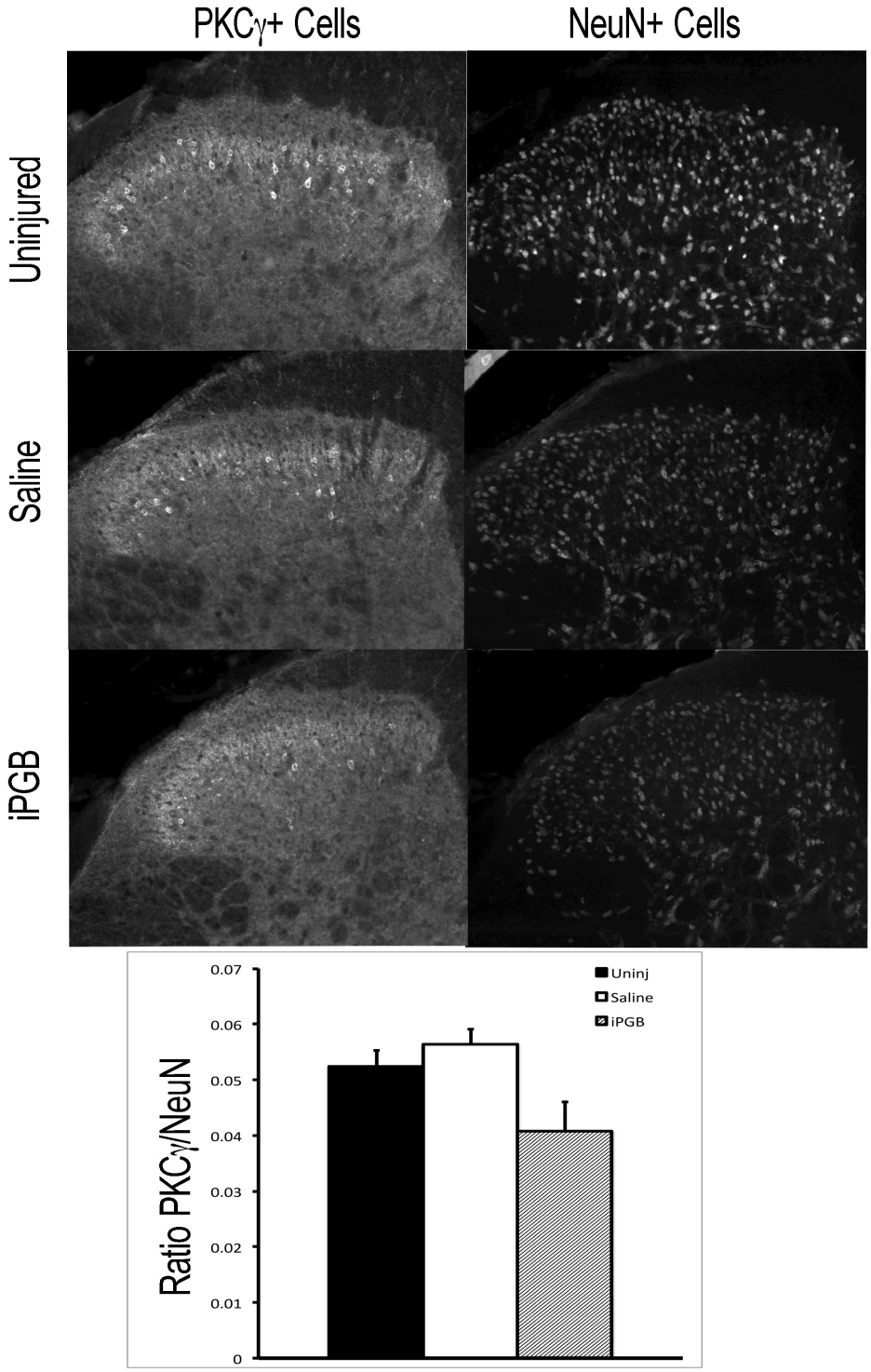

PKCγ+ Cells
NeuN+ Cells
Uninjured
Saline
iPGB
Ratio PKCγ/NeuN
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Uninj
Saline
iPGB

Figure 4.8. Effect of iPGB treatment on lumbar expression of Ca_v $\alpha_2\delta$-1 after SCI.

Expression of $\alpha_2\delta$-1 protein in uninjured mice, and saline- and iPGB-treated SCI mice at 2 and 6 weeks post-SCI. Panel (A) shows representative samples from L4-6 spinal cord homogenates processed by SDS-PAGE and subsequent Western blot analysis for $\alpha_2\delta$-1 protein. GAPDH protein expression was used as an internal loading control. Panel (B) shows quantification of $\alpha_2\delta$-1 protein levels by densitometry in L4-6 spinal cord in uninjured mice, saline-treated, or iPGB-treated mice 2 and 6 weeks post-SCI. Protein levels are expressed relative to uninjured as a ratio corrected for GAPDH levels. No significant change was observed in $\alpha_2\delta$-1 protein levels in saline- and iPGB-treated SCI mice at 2 and 6 weeks following injury.

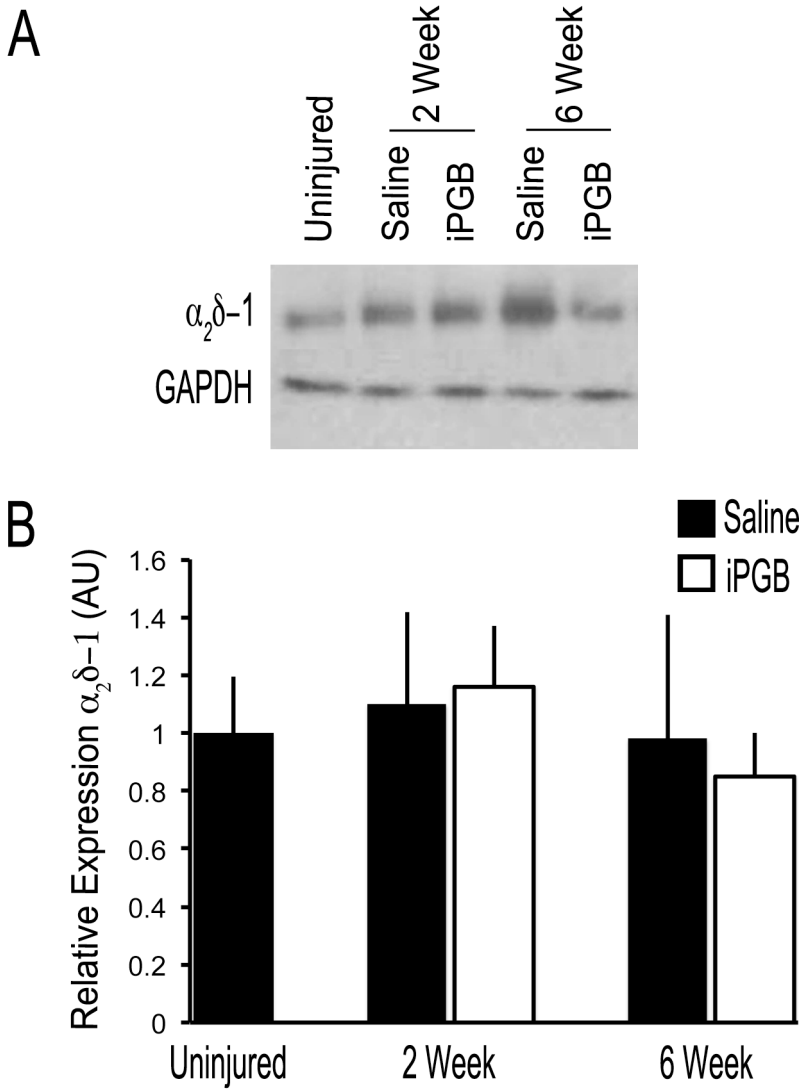

A
Uninjured
Saline
iPGB
2 Week
Saline
iPGB
6 Week
α2δ–1
GAPDH
B
Saline
iPGB
Relative Expression α2δ–1 (AU)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Uninjured
2 Week
6 Week

Figure 4.9 Intensity and expression profile of Ca_v $\alpha 2_2\delta$ and PKCγ in L4-5 dorsal horn 2 weeks post-SCI

Representative images of Ca_v $\alpha 2_2\delta$ and PKCγ staining in uninjured animals and saline or iPGB treated animals 2 weeks post-SCI (A). No effect of injury or treatment was observed in the area under the curve, or between expression intensities throughout the dorsal horn for both Ca_v $\alpha 2_2\delta$ and PKCγ staining (B).

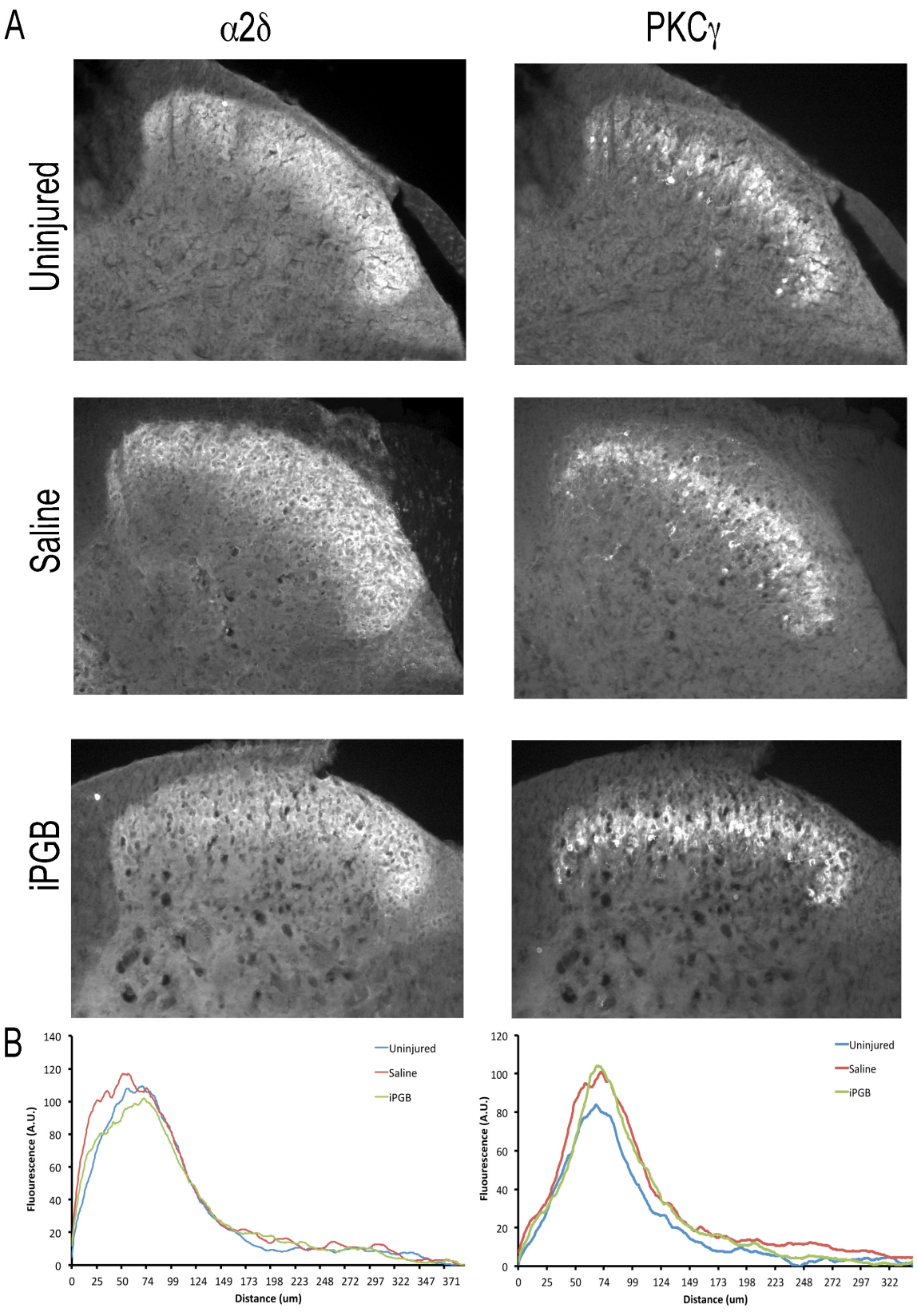

A
α2δ
PKCγ
Uninjured
Saline
iPGB
B
Uninjured
Saline
iPGB
Fluorescence (A.U.)
Distance (um)
0 25 50 74 99 124 149 173 198 223 248 272 297 322 347 371
0 20 40 60 80 100 120 140
0 25 50 74 99 124 149 173 198 223 248 272 297 322
0 20 40 60 80 100 120

Figure 4.10 Intensity and expression profile of Ca_v $\alpha 2_2\delta$ and PKCγ in L4-5 dorsal horn 6 weeks post-SCI

Representative images of Ca_v $\alpha 2_2\delta$ and PKCγ staining in uninjured animals and saline or iPGB treated animals 6 weeks post-SCI (A). No effect of injury or treatment was observed in the area under the curve for Ca_v $\alpha 2_2\delta$ and PKCγ staining (B). Saline treated animals were observed to express significantly less PKCγ immunoreactivity in the dorsal horn than uninjured animals at a depth of 62.63 μm in the dorsal horn (43.50±3.91 vs. 72.14±3.05, $P<0.05$), but at no other point or between expression intensities throughout the dorsal horn (B). No differences were observed in the staining profile of Ca_v $\alpha 2_2\delta$ (B).

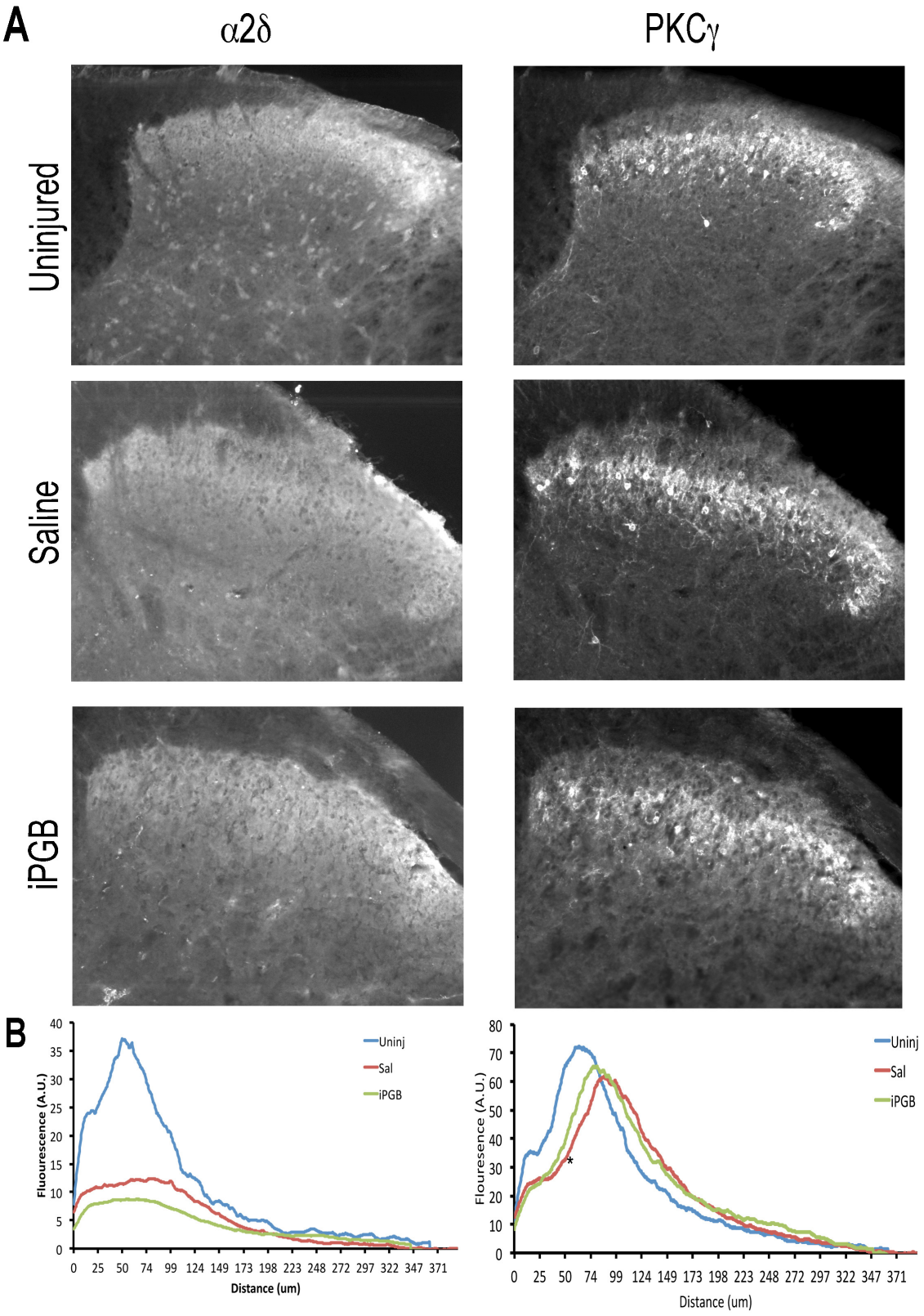

A
α2δ
PKCγ
Uninjured
Saline
iPGB
B
Uninj
Sal
iPGB
Fluouresence (A.U.)
Distance (um)
0 25 50 74 99 124 149 173 198 223 248 272 297 322 347 371
Flouresence (A.U.)
*

Figure 4.11. TSP-1 expression in mouse spinal cord after SCI.

Expression of TSP1 protein in uninjured mice and in mice 12, 24, 72 hours, and 1 week post-SCI with saline or iPGB treatment. Panel (A) shows representative samples from peri-lesion spinal cord homogenates processed by SDS-PAGE and Western blot analysis for TSP1 protein. GAPDH protein expression was used as an internal loading control. Panel (B) shows quantification of relative TSP1 protein levels by densitometry in L4-6 spinal cord in uninjured mice and after saline or iPGB treatment at 12, 24, 72 hours, and 1 week post-SCI. Protein levels are expressed relative to uninjured as a ratio corrected for GAPDH levels. Two-tailed two-way ANOVA showed no significant effect of iPGB treatment on TSP-1 expression. A significant effect of time ($P<0.001$, $F=5.568$) was observed. * denotes levels of TSP-1 were significantly increased from uninjured levels at 12, 24, 48, and 72 hours and one week post-SCI ($P<0.05$). φ denotes a significant decrease in protein compared to levels at 48 and 72 hours.

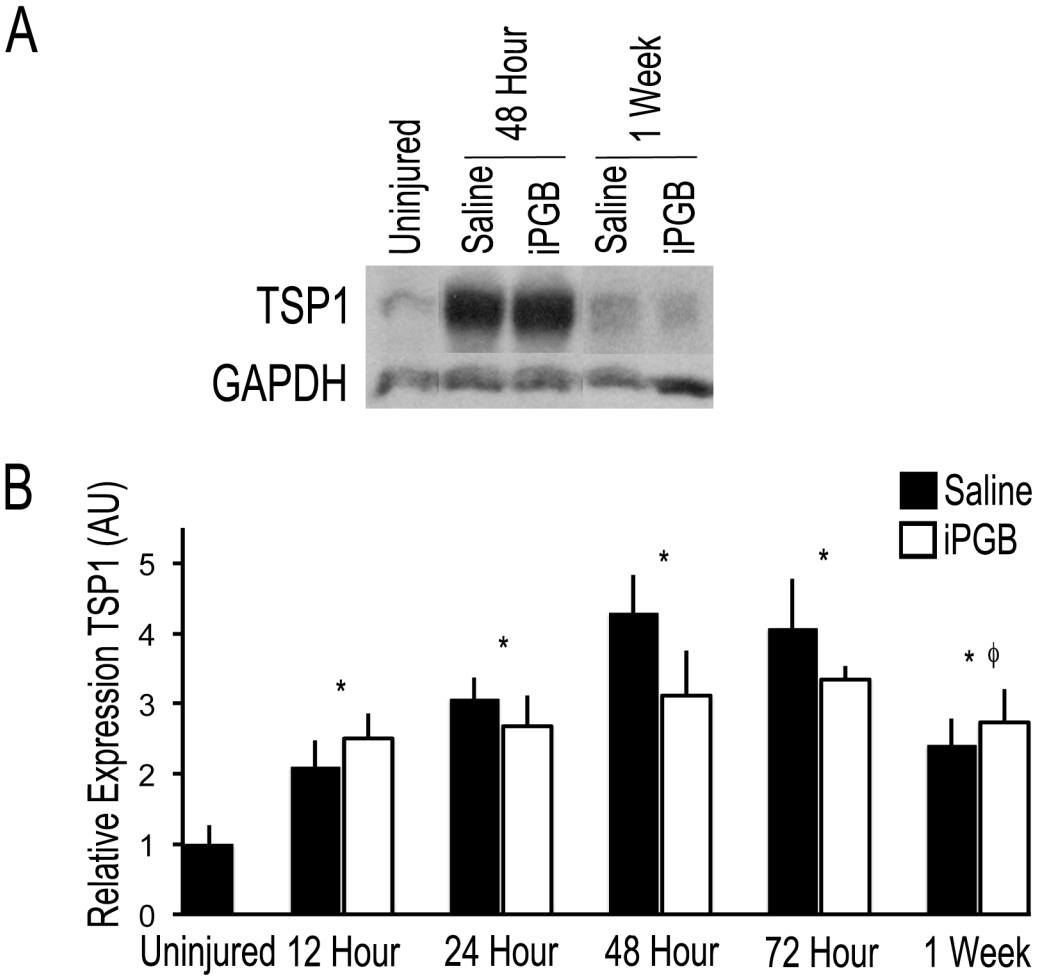
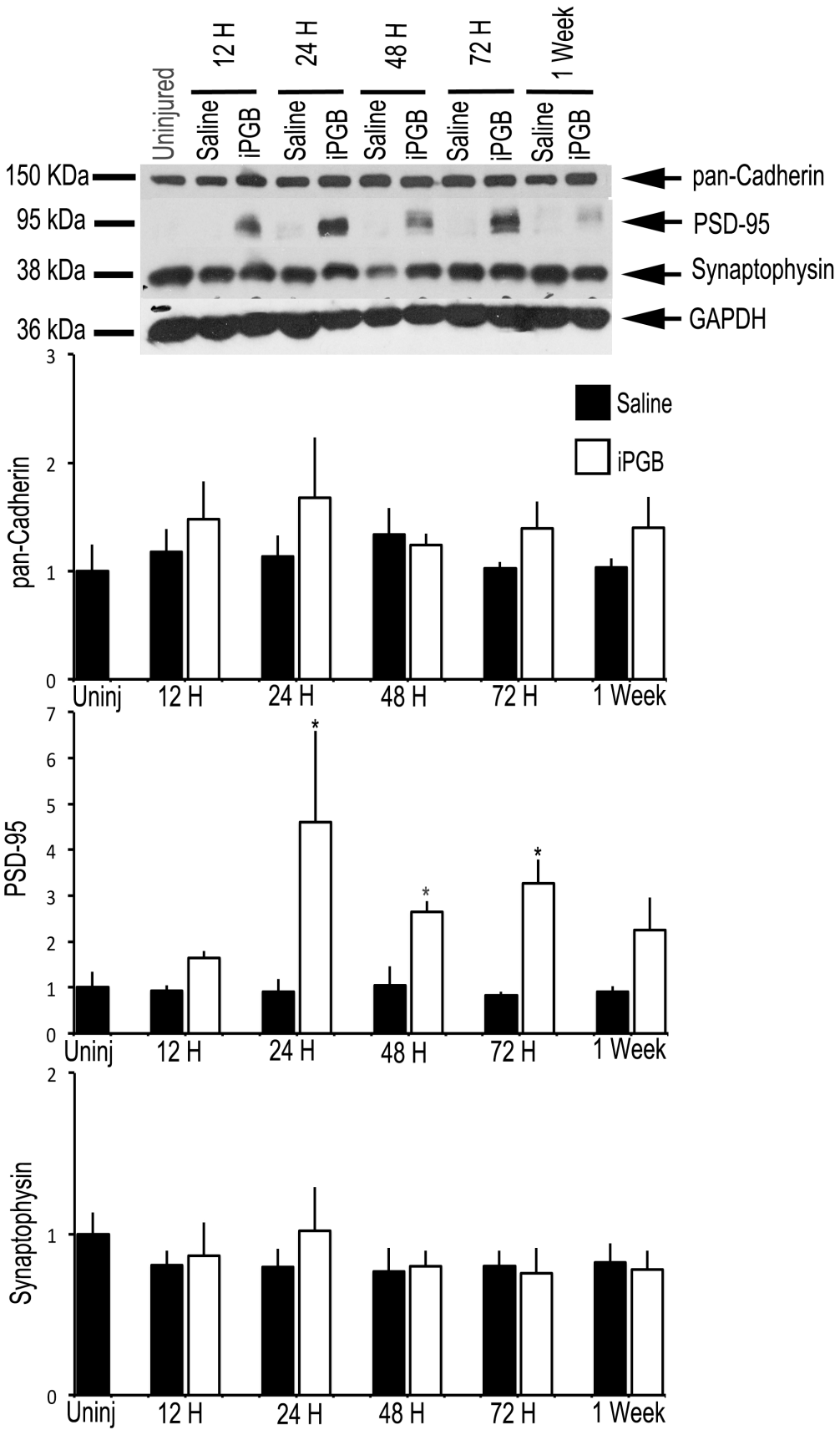

A
Uninjured
48 Hour
1 Week
Saline
iPGB
Saline
iPGB
TSP1
GAPDH
B
Saline
iPGB
Relative Expression TSP1 (AU)
0
1
2
3
4
5
*
*
*
*
* ϕ
Uninjured
12 Hour
24 Hour
48 Hour
72 Hour
1 Week

Figure 4.12. Effect of iPGB on expression of PSD-95, synaptophysin, and cadherin post-SCI

Representative images of blots for PSD-95, synaptophysin, cadherin, and GAPDH in uninjured animals and animals receiving saline or iPGB treatment post-SCI (A). PSD-95 expression was observed to be increased in iPGB treated animals at 24 (4.61±1.99 vs 1.00±0.34, $P<0.05$), 48 (2.65±0.23 vs 1.00±0.34, $P<0.05$), and 72 (3.27±0.52 vs 1.00±0.34, $P<0.05$) hours relative to levels in uninjured animals. Expression of synaptophysin and cadherin was not affected by injury or treatment.


Uninjured
12 H
24 H
48 H
72 H
1 Week
Saline
iPGB
150 KDa
95 kDa
38 kDa
36 kDa
pan-Cadherin
PSD-95
Synaptophysin
GAPDH
Saline
iPGB
pan-Cadherin
PSD-95
Synaptophysin
Uninj
12 H
24 H
48 H
72 H
1 Week
*

,

TABLE 4.1

GIN^+, $PKC\gamma^+$, and $NeuN^+$ cell counts in uninjured and saline or iPGB treated animals 6 weeks post-SCI

	GIN^+	$PKC\gamma^+$	$NeuN^+$
Uninjured	**26.31±1.82**	**18.48±0.49**	**371.32±20.54**
Saline	**21.34±1.56**	**22.05±0.99**	**395.76±15.65**
iPGB	**22.75±0.89**	**15.33±1.34**	**383.16±15.71**

CHAPTER 5: DISCUSSION

5.1 General Discussion

SCI patients surviving the first year post-injury survive to an age comparable to age-matched uninjured individuals (National Spinal Cord Injury Statistical Center, 2010), yet complications, such as neuropathic pain, arising from their injury may pose a burden throughout their life. Current therapeutics offer limited effectiveness to alleviate the burden of pain, compelling clinicians and researchers to better understand the underlying causes of post-SCI neuropathic pain and develop more targeted, effective therapeutics.

In Chapter 2 of this thesis we present a murine model of spinal cord injury that develops behavioral manifestations of below-lesion hyperalgesia and allodynia (Figure 2.1). This model system allowed for the study of neurobiological changes in pain signaling circuits that may occur as a result of SCI, and we focus on evaluating changes in a population of GABAergic inhibitory interneurons labeled by the *gad1*-GFP transgene. SCI reduced the number of GFP+ GABAergic cells at 2 and 6 weeks post-SCI, and some co-localization was observed with active caspase-3, indicative of ongoing apoptotic cell death (Figure 2.2). This observation suggested that previously reported reduced GABAergic tone in the spinal cord post-SCI (Gwak et al., 2006)(Gwak et al., 2008) may be attributable to death of GABAergic neurons. This observation was further supported by reductions in secondary markers of GABAergic tone, such as the GABA synthesizing enzyme isoforms GAD65 & 67, and the GABA transporter GAT1 (Figures 3, 4, 5).

Chapter three of this thesis utilizes another transgenic mouse line in which XIAP is expressed under the ubiquitin C promoter, increasing levels of XIAP is all tissue types

(Moore et al., 2008). It was hypothesized that increasing the resistance of cells to apoptotic signals may prevent the death of GABAergic inhibitory interneurons observed in Chapter 2, and thus prevent the development of neuropathic pain. Development of mechanical allodynia did not differ in animals expressing increased levels of XIAP relative to wildtype controls (Figure 3.3), nor did they differ in the recovery of locomotor function post-SCI (Figure 3.1 & 3.2). The lack of protection from development of mechanical allodynia suggested that this transgenic line may not be useful for further investigation into the role of GABAergic interneuron apoptosis in the development of neuropathic pain. Cursory investigation into possible altered GABAergic tone post-SCI indicated that animals expressing increased levels of XIAP did not differ from wildtype in animals in expression of GAD65/67 post-SCI (Figure 3.4).

Chapter 4 of this thesis investigated early chronic treatment with pregabalin, a medication with regulatory approval for treatment of central neuropathic pain. We administered pregabalin in a novel pre-emptive treatment schedule. The use of a drug with current regulatory approval may fast track the translation of any beneficial discoveries to clinical practice, offering great potential for the SCI patient population. Preemptive treatment strategies have the inherent risk of exposing individuals who would not go on to develop symptoms to potential adverse drug reactions. Given some reports of up to 67% of individuals developing at- or below-level neuropathic pain (Finnerup et al., 2001), the long-term imposition posed by neuropathic pain, and the difficulty to alleviate neuropathic pain, a preemptive treatment strategy may be justifiable.

To evaluate our treatment strategy we administered pregabalin 2 hours post-SCI (iPGB) to mimic the delay for human patients to arrive at a treatment centre, or 1 week post-SCI, to investigate the potential for a limited therapeutic window. The first priority

was to ensure that treatment did not inhibit adaptive plasticity, such as locomotor recovery, in tandem with the maladaptive plasticity underlying neuropathic pain. To this end we assessed rotorod performance, and determined Tarlov scores for animals and found no significant difference in performance between iPGB and saline treated animals (Figure 4.1). This observation suggested that our pre-emptive treatment schedule does not impair adaptive plasticity associated with recovery of locomotor function.

To determine if our dose of PGB was appropriate to have CNS penetrance and influence nociceptive signaling, we assessed an acute administration of our dose (10 mg/kg s.c.) in animals with established neuropathic pain and found it reversed thresholds (Figure 4.2), consistent with previously reported action of gabapentinoids on post-SCI neuropathic pain (Boroujerdi et al., 2011).

To investigate a possible pre-emptive effect on the development of neuropathic pain post-SCI we next administered saline, iPGB, or dPGB twice daily for 2 weeks and assessed behavioral withdrawal thresholds. Animals receiving saline and dPGB treatment demonstrated reduced 50% withdrawal threshold (Figure 4.3), while animals withdrawal thresholds of receiving iPGB treatment remained stble near baseline values, and were observed to be significantly greater than those of saline treated animals at weeks 4, 5, and 6 post-SCI (Figure 4.3). This is particularly noteworthy as it may be assumed that all pregabalin has been cleared from the treated animals at these later time points. This observation suggests that early intervention with pregabalin is able to have long-lasting effects, and that there is a therapeutic window post-SCI during which treatment must be received to exert an effect.

To investigate the mechanistic basis of this effect we chose to explore changes in 4 areas that may be relevant to the development, or lack thereof, of neuropathic pain: 1.

Glial activation, 2. $\alpha_2\delta$-1 receptor expression, 3. GABAergic inhibitory interneuron survival, and 4. Markers of synaptic plasticity.

Increased below lesion astrocytosis has been observed in tandem with the development of post-SCI neuropathic pain (Gwak et al., 2008), although it may not play a role in the initiation or maintenance of pain signaling. Below-lesion expression of GFAP, a marker for astrocytes, by western blot analysis was not altered by injury or treatment (Figure 4.4). Microglial hypertrophy and proliferation have also been associated with the development of neuropathic pain post-SCI (Hains and Waxman, 2006)(Gwak and Hulsebosch, 2009). Western blot analysis of CD11b expression, a marker of microglial cells, showed that levels of CD11b were significantly increased in saline treated animals 6 weeks post-SCI but not in iPGB treated animals (Figure 4.5). This observation indicated that iPGB treatment may suppress the hypertrophy and proliferation of microglia post-SCI. Supporting this hypothesis are reports that gabapentinoids enhance descending noradrenergic tone from the locus coeruleus (Hayashida et al., 2008), and that noradrenergic stimulation suppresses microglial activation (Morioka et al., 2009). This observation requires further exploration, and future studies investigating the effect of iPGB treatment on animals with chemically ablated noradrenergic tracts may help clarify if enhanced noradrenergic signaling contributes to iPGB's effect. Additionally, analysis of the expression level of phospho-p38 MAPK, a marker of reactive microglia, would clarify if iPGB suppress the transition of microglia from a surveillant to a reactive state.

Peripheral nerve injury (Bauer et al., 2010) and SCI (Boroujerdi et al., 2011) have been observed to result in increased expression of the $\alpha_2\delta$ subunit of voltage gated Ca^{2+} channels in the dorsal horn of the spinal cord. This may result in increased Ca^{2+} influx upon depolarization, and because these receptors are primarily located on pre-synaptic

terminals of senory afferents (Bauer et al., 2009) there may be a greater quantal release of neurotransmitter. Further, it has been reported that treatment with gabapentinoids restricts trafficking of Ca_v $\alpha_2\delta$ from the cell body to synaptic terminals (Bauer et al., 2009), and suppresses glutamate release from sensory afferents in the dorsal horn (Kumar et al. 2010). To investigate if increased expression of Ca_v $\alpha_2\delta$ is implicated in our model we examined the level of expression at two and 6 weeks post-SCI by western blot (Figure 4.6) and immunohistochemistry (Figures 4.7 and 4.8). No effect of injury or treatment was observed on the expression level or expression profile of Ca_v $\alpha_2\delta$. This result suggested that altered expression of Ca_v $\alpha_2\delta$ did not play a role in the effect of iPGB.

Changes in GABAergic tone have been observed post-SCI (Chapter 2) and we wished to investigate if iPGB treatment may have a neuroprotective effect on survival of GABAergic inhibitory interneurons. Cell counts performed in uninjured, saline, and iPGB treated animals 6 weeks post-SCI indicated reduced numbers of GABAergic inhibitory interneurons relative to uninjured animals, an effect not observed in iPGB treated animals (Figure 4.9). No change in the population or distribution within superficial laminae of an excitatory interneuron population labeled by PKCγ was observed as an effect of injury or treatment (Figure 4.10). This suggested that iPGB may have have reduced the loss of GABAergic interneurons post-SCI. Other reports have indicated a neuroprotective effect of gabapentionds post-SCI, showing reduced TUNEL staining and reduced demyelination (Ha et al., 2008).

A novel topic for exploration in post-SCI neuropathic pain is the concept of *de novo* synaptogenesis, or remodeling of existing synaptic connections. Evidence in support of this phenomenon has recently been published demonstrating a role of RAC-1 mediated dendritic spine remodeling in maintaining neuropathic pain memory post-SCI

(Tan et al., 2008). Additionally, gabapentinoid binding of Ca_v $\alpha_2\delta$ has been observed to inhibit the synaptogenic properties of the astrocyte secreted molecule thrombospondin (Eroglu et al., 2009). Thrombospondin expression was found to be dramatically upregulated by injury from 12-72 hours, and to return to uninjured levels by 1 week post-SCI (Figure 11). This transient upregulation of thrombospondin expression may be relevant to the therapeutic window observed for effect of pregabalin treatment. As thrombospondin expression peaked before dPGB treatment was initiated, sufficient time may have elapsed for the synaptogenic action of thrombospondin to occur. It has been reported that treatment with gabapentinoid has no effect on the stability of thombospondin induced synapses once established (Eroglu et al., 2009). To investigate if iPGB treatment impacts synaptic remodeling we assessed expression of PSD-95, a marker of synaptic plasticity and post-synaptic excitatory synapses (Xu, 2011). Unexpectedly, PSD-95 expression was observed to be increased in iPGB-treated animals post-SCI, but there were no changes in levels of the presynaptic marker synaptophysin, and synaptic adhesion protein cadherin (Figure 4.12). Altered expression of PSD-95 indicates iPGB is likely impacting some element of synaptic remodeling, but further evidence is required to elucidate the mechanism.

5.2 Implications from GABAergic inhibitory interneuron cell death for SCI treatment

The fundamental role of GABAergic signaling in synaptic physiology, and ubiquitous presence throughout the central nervous system presents a challenge for the use of GABA agonists as therapeutics for neuropathic pain. Existing GABAergic agonists, such as barbituates and benzodiazepines, have potential for abuse and adverse

effects with chronic use that may overshadow any relief from pain symptoms (Lader, 2011). At the dose used in our study, the GABA transporter antagonist tiagabine was able to reverse established neuropathic pain without obvious sedation (observed), and may represent a therapeutic approach preferable to the use of GABA agonists. Still, systemic administration of a GABA transporter agonist will be plagued by its non-specific actions through the central nervous system.

More promising is investigation into the use of neuroprotective drugs post-SCI. Treatment with neuroprotective agents beginning shortly following SCI may prevent the death of GABAergic interneurons and reductions in GABAergic tone that contribute to neuropathic pain post-SCI. Further research determining the cause of GABAergic cell death may allow for the use of specific antagonists to prevent the propagation of death signals. This research also lends support to the utility of cell replacement therapy in individuals with intractable neuropathic pain. The implantation of GABAergic predifferentiated embryonic stem cells to the spinal cord dorsal horn has been demonstrated to attenuate mechanical allodynia in rats following peripheral nerve injury (Mukhida, et al. 2007), and a similar therapy may also be effective following SCI.

5.3 Implications from preemptive inhibition of development of allodynia by iPGB

The promising findings presented in Chapter 4 describe the ability of a 2 week treatment with pregabalin to prevent the development of below-lesion mechanical allodynia in a mouse model of spinal cord injury. Given blockade of the development of neuropathic pain in our animal model, and low risks associated with pregabalin use, it is essential to further explore this treatment in a clinical setting.

Policy decisions surrounding pre-emptive therapeutics must be made with great caution to reduce exposing patients to unnecessary adverse drug effects. Given the dramatic impact of neuropathic pain on patient quality of life, and the safe and well tolerated profile of the gabapentinoids, their use may be well justified as a preemptive treatment.

The results of Chapter 4 describe the compelling behavioral effect of iPGB post-SCI, but provide only preliminary evidence toward a possible mechanism. Further understanding of the mechanism may reveal other conditions where iPGB treatment may also be of benefit, such as other surgical procedures where there is risk of injury to the nervous system and development of neuropathic pain.

5.4 Overall summary

SCI is a devastating injury, and the challenges imposed by the subsequent development of neuropathic pain are staggering. Many distinct processes appear to be implicated in the development and maintenance of neuropathic pain, each adding a layer of complexity to the understanding and development of treatments for this life altering condition. Nonetheless, multiple agents targeted at specific processes have demonstrated success in ameliorating behavioral symptoms of post-SCI neuropathic pain in animal models, offering hope that these techniques may be refined for clinical use.

The observations presented in this thesis contribute to our understanding of one of the processes underlying the development of neuropathic pain post-SCI, and provide exciting evidence for a novel treatment approach. Better treatment for post-SCI neuropathic pain will greatly aid its victims. Therapies incorporating physical rehabilitation, such as supported treadmill ambulation training, show good evidence for

improving locomotor function in SCI patients. (Protas, et al. 2001). However, the large number of SCI patients suffering from neuropathic pain face additional challenges to participating in such physically demanding forms of rehabilitation.

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Hello,

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I am preparing my PhD thesis at Dalhousie University in Halifax, NS, Canada, and am writing to request permission to include this article as a chapter in my thesis. Please let me know how I may direct this request, or confirm permission yourself.

Sincerely, Jason Meisner

Jason Meisner
Ph.D. Candidate, Dept. Anatomy & Neurobiology / Neuroscience
Faculty of Medicine, Dalhousie University
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Kind regards,
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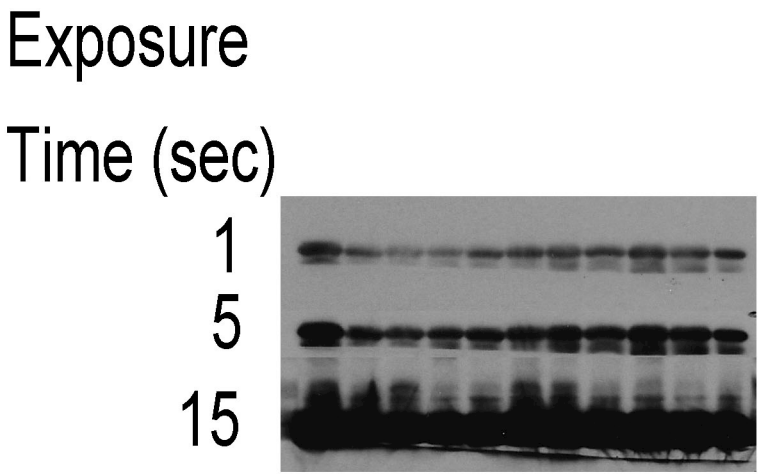
APPENDIX B: PRIMARY ANTIBODIES USED IN ASSAYS

Target	Host Species	Company	Catalogue Number
Active-caspase-3	Rabbit	R&D Systems	AF835
CD11b/c	Mouse	BD Pharmingen	554859
CGRP	Guinea Pig	Peninsula Labs	T-5027
Dihydropyridine Receptor	Mouse	Sigma	D219
GAD 65 & 67	Rabbit	Chemicon	AB1511
GAT1	Rabbit	Abcam	Ab426
GFAP	Rabbit	Immunostar	22522
NeuN	Mouse	Chemicon	MAB377
pan-Cadherin	Mouse	abcam	Ab6528
PKCy	Rabbit	Santa Cruz	sc-211
PSD-95	Mouse	Thermo	MA1-045
Synaptophysin	Rabbit	Zymed	18-0130
Thrombospondin	Mouse	abcam	AB1823

APPENDIX C: LINEAR WESTERN BLOT EXPOSURE



Western blot membranes were exposed to film for varying lengths of exposure times to determine the linear range of the photoluminescent response. In this example of a GFAP protein immunoblot, the 1 second exposure was chosen for analysis as saturation had not yet occurred and the response remained in the linear range. This was typical of all Western blot analyses.

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