THE LOSS OF GABAERGIC INHIBITORY INTERNEURONS CORRELATES WITH THE DEVELOPMENT OF THERMAL HYPERALGESIA AFTER SPINAL CORD INJURY

by

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ABSTRACT

Elimination of chronic pain is very important in improving quality of life for both paraplegics and quadriplegics alike. It is hypothesized that hyperexcitability found after injury may be attributed to a loss of GABA inhibitory control caused by the loss of GABAergic inhibitory interneurons. Transgenic mice with expression of green fluorescenct protein found in GABAergic inhibitory interneurons were used. SCI was performed at the 10^{th} thoracic spinal segment with a spinal cord impactor. Hyperalgesia was observed at 3 weeks post injury and continued on to week 6. 6 weeks post injury there was a loss of inhibitory interneurons in lamina I-III. Gabapentin and tiagabine were administered as analgesics. Gabapentin blocked hyperalgesia by 50% at 10mg/kg while tiagabine blocked hyperalgesia by 50% at 1mg/kg and completely blocked it at 10mg/kg. Enhancing GABAergic signalling decreases hyperalgesia and therefore GABAergic interneurons may play a key role in the development of hyperalgesia following SCI.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α2δ	Alpha-2 Delta
Aβ	A-Beta
Aδ	A-Delta
AMPA	α-Amino-3-Hydroxy-5-Methylisoxazole-4- Propionic Acid
ANOVA	Analysis of Variance
C	Cervical Segment
CCI	Chronic Constriction Injury
CNS	Central Nervous System
CGRP	Calcitonin Gene Related Peptide
DRG	Dorsal Root Ganglia
GABA	Gamma-Aminobutyric Acid
GAD	Glutamic Acid Decarboxylase
GBP	Gabapentin
GFP	Green Fluorescent Protein
IF	Injury Factor
L	Lumbar Segment
NeuN	Neuronal Nuclei
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
NO	Nitric Oxide
NT	Neurotensin

PBS	Phosphate Buffered Saline
PNS	Peripheral Nervous System
R	Receptor
RVM	Rostral Ventromedial Medulla
SCI	Spinal Cord Injury
SNI	Spared Nerve Injury
T	Thoracic Segment
TGB	Tiagabine
TBS-X	Triton X-100
TNF	Tumor Necrosis Factor
WDR	Wide Dynamic Range
zVAD	Benzyloxycarbonyl-Val-Ala-Asp(OMe)-Fluoromethylketone

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CHAPTER 1: INTRODUCTION

1.1 Spinal Cord Injury

Spinal cord injury (SCI) is a life changing event which may result from motor vehicle accidents, sports injuries, or other causes (Table 1). These injuries can be classified as one of 2 different injury types: 1) complete and 2) incomplete. In a complete injury, function is lost below the level of the injury. Voluntary movement is no longer possible and physical sensation is no longer present. Complete injuries are bilateral, that is, both sides of the body are equally affected. Incomplete injuries vary, and a person with an incomplete injury may be able to move one limb better than another, may be able to feel parts of the body which cannot be moved, or may be able to function on one side of the body more than the other. SCI can occur in a variety of ways. A contusion injury is an injury which causes bruising to the cord and is usually caused by direct impact to the spinal cord. A compression injury is an injury which causes pressure on the spinal cord and is usually caused by the vertebrae pressing in on the spinal cord. This occurs if the head, neck, and/or back are twisted abnormally during an accident or injury. This can also occur when bleeding, fluid accumulation, and swelling occur inside the spinal cord or outside the spinal cord but within the spinal canal. The accumulation of blood or fluid can compress the spinal cord, damaging it. Other types of SCI include lacerations (severing or tearing of nerve fibers) and central cord syndrome (specific damage to the cervical region of the spinal cord).

Table 1 Causes of SCI in Canada (Canadian Paraplegic Association 2007)

Car Collision	35%
Accidental Falls	16.6%
Medical	10%
Sports	6.7%
Other Motor Vehicle Collision	6.4%
Diving	5.5%
Industrial	5.4%
Other	14.4%

The timeframe for events following SCI can be divided into acute, sub-acute and chronic. Pathophysiological events that occur in the spinal cord within minutes, hours and days after the injury can be termed acute. Sub-acute events such as consolidation of injury and scar tissue formation will occur within the first 2 weeks of injury. A chronic injury will occur over 2 weeks post injury (Schwab and Bartholdi, 1996). Acute pain is common after a SCI. The pain may occur as a result of the damage to the spinal cord, spinal roots or from damage to other areas of the body at the time of injury. Chronic pain is defined as pain which persists past the time-point of natural healing and is common after prolonged injury. It can occur in areas where there is normal sensation, and it can also occur in parts of the body where there is little or no feeling after injury. Neuropathic pain is a form of chronic pain caused by injury causing functional changes in the nervous system. Neuropathic pain will be discussed later.

With approximately 40% of patients suffering from neuropathic pain, the development and persistence of chronic pain following spinal cord injury is a significant problem which is refractory to the majority of clinical interventions (Yezierski, 1996; Warms et al., 2002). Approximately 38,000 people in Canada are paralyzed by SCI with 1000 new injuries occurring every year. Spinal injury not only causes paralysis, but impacts tremendously on quality of life as sexual, bowel, bladder and cardiovascular functions are also disrupted (Lee et al., 1994, Mathias et al., 1999). For a significant percentage of patients, elimination of pain is more important than recovery of autonomic function as this represents the possibility of returning to work, improving social and family relationships as well as reducing pain medications

(Yezierski, 2000; Yezierski, 2004). Pain associated SCI can be divided into nociceptive which is defined as the sensation of pain (including musculoskeletal and visceral) and neuropathic (including at and below the level of injury) (Siddall et al., 2002) as discussed below.

1.2 Organization of the Spinothalamic Tract

The spinothalamic tract is the most important ascending nociceptive pathway in the spinal cord. It comprises the axons of nociceptive-specific and wide dynamic range neurons in lamina I, II, III and V-VII. Cutaneous pain, temperature, and non-discriminative touch are transmitted through this system. This pathway begins at free nerve endings throughout the skin, muscle, bone and connective tissue and travels to the dorsal root ganglia (DRG). The central processes of the DRG neurons enter the spinal cord through the dorsal roots. Primary afferents enter the Zone of Lissauer in the dorsal horn of the spinal cord. Once in the Zone of Lissauer, the pain and temperature (A-Delta (Aδ) and C fibres) and non-discriminative touch A-Beta (Aβ) fibers can descend or ascend one or two spinal segments prior to synapsing. Aδ fibers are fast conducting myelinated fibers which transmit nociceptive and non-nociceptive information. Aβ fibers are fast conducting myelinated fibers which transit non-nociceptive information exclusively. C-fibers are slow conducting unmyelinated fibers which transmit nocicptive information exclusively. These fibers then terminate in the posterior marginalis, the substantia gelatinosa, or the nucleus proprius (lamina I, II and III respectively) (Figure1). Aδ fibers enter the posterior marginalis and the nucleus proprius. The secondary afferents from these layers cross to the contralateral

side of the spinal cord via the ventral white commisure. The C fibers enter the substantia gelatinosa and synapse on interneurons which project to spinal neurons nearby. The interneurons carry the signal to the second order neurons (nociceptive specific and wide dynamic range) in either the posterior marginalis or the nucleus proprius. The axons of these neurons then cross over to the contralateral side (Basbaum and Jessell, 2000). Proceeding rostrally, the spinothalamic fibers continue into the medulla where these fibers constitute most of the spinal lemniscus. The spinal lemniscus continues through the ventrolateral region of the dorsal pons, and in the midbrain, it runs along the lateral edge of the medial lemniscus. Upon passing through the brain stem, axons give off collateral branches that terminate in the medullary and pontine reticular formation and in the periaqueductal gray matter of the midbrain. From here, most of the spinothalamic axons synapse in the ventral posterolateral division of the ventral posterior nucleus of the thalamus. The Axons of the third-order neurons then project to the primary somatosensory cortex located in the parietal lobe (Figure 2) (Kiernan, 2005).

1.3 Neuropathic Pain

Neuropathic pain is an unfortunate consequence of SCI. It is a traumatic experience impacting greatly on quality of life for the SCI patient. Neuropathic pain refers to pain that originates from pathology of the nervous system. Neuropathic pain, in contrast to nociceptive pain, is described as "burning", "electric", "tingling", and "shooting" in nature. It can be continuous or paroxysmal in presentation. While

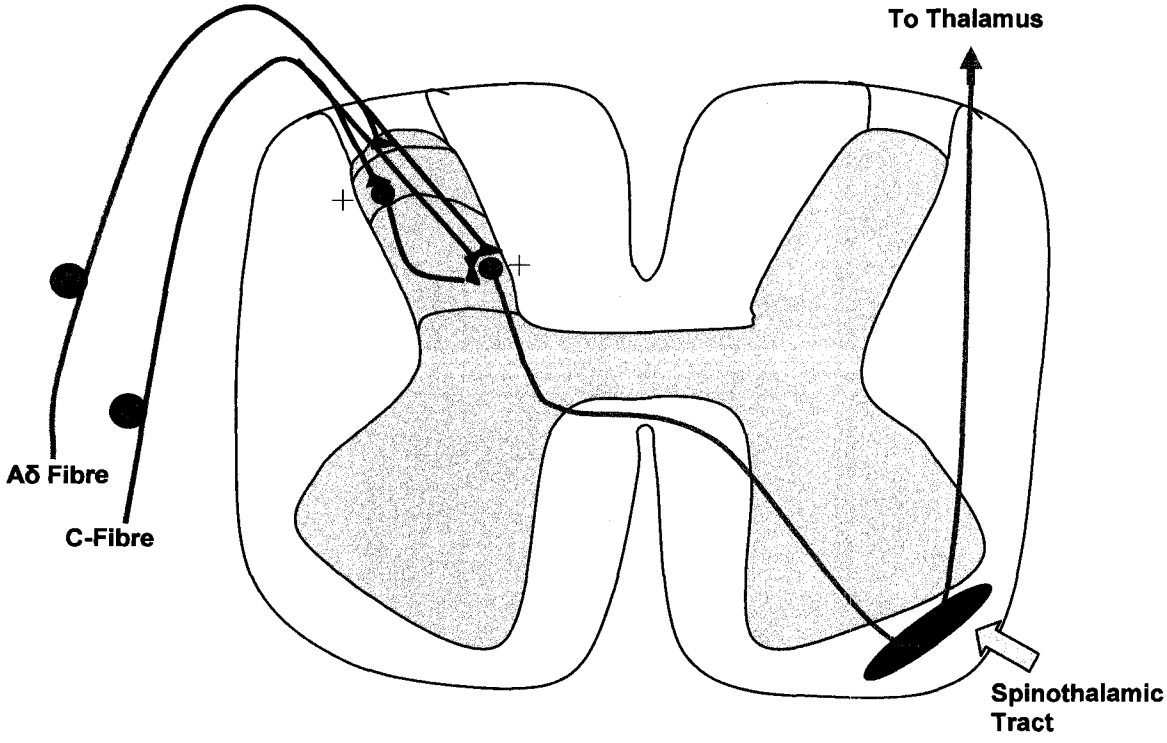


Figure 1 Nociceptive information being transmitted to spinothalamic tract via afferent neurons (Aδ and C fibers) and second order neurons. "+" denotes excitatory synapses. (Adapted from Kandel et al., 2000)

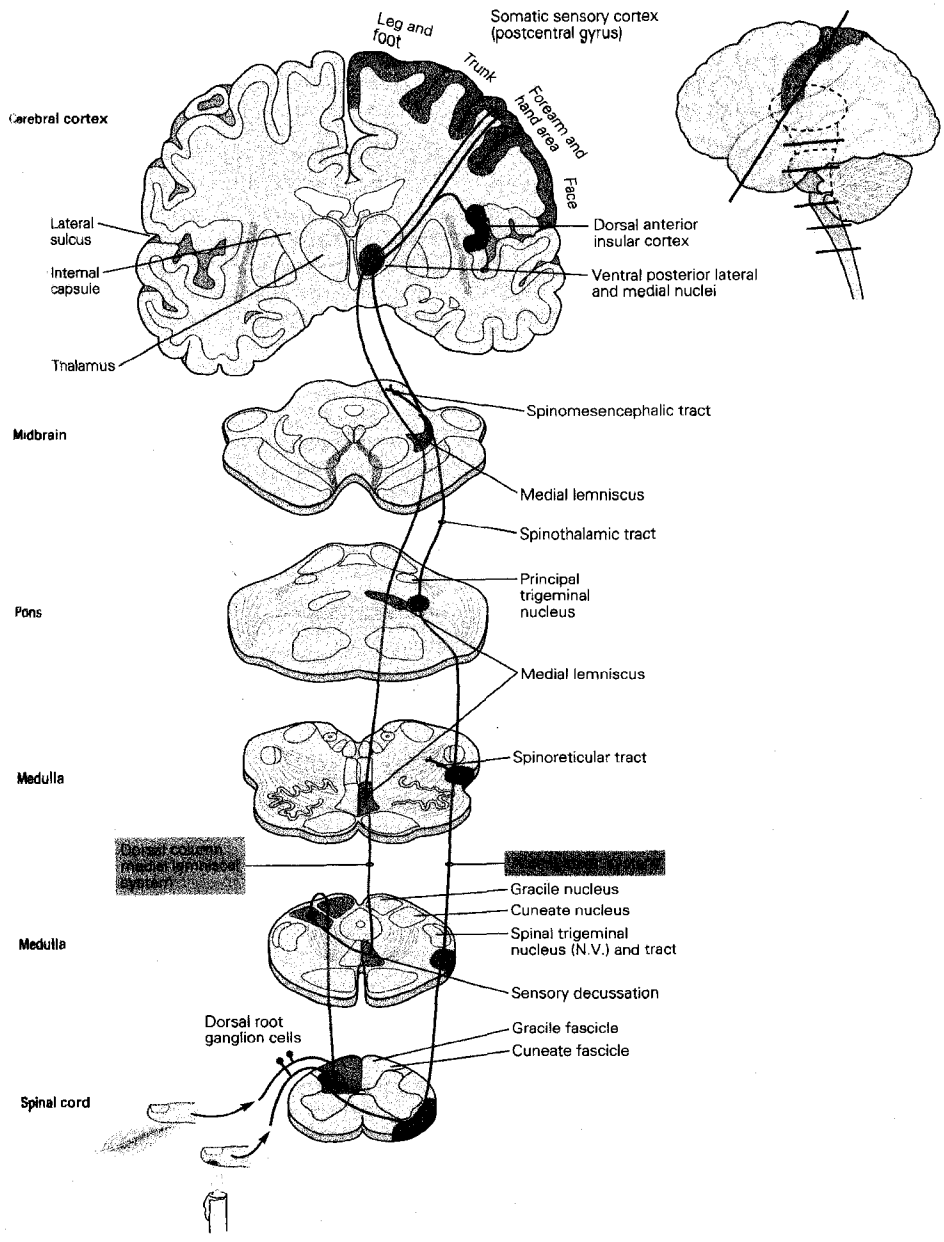


Figure 2 Tactile sensation and limb proprioception are transmitted to the thalamus by the dorsal column-medial lemniscal system (orange) and painful and thermal sensations are transmitted to the thalamus by the anterolateral (spinothalamic) system (brown). (Adapted from Basbaum and Jessell 2000)

nociceptive pain is caused by the stimulation of peripheral Aδ and C–polymodal pain receptors, neuropathic pain is produced by damage to, or pathological changes in the peripheral nervous system (PNS) or central nervous system (CNS) (Campbell and Meyer, 2006).

At-level pain is pain associated at the level of the spinal lesion. The primary characteristic of at-level pain is a hypersensitive band of skin in dermatomes associated with spinal segments adjacent to the site of injury. Injury-induced loss of intrinsic inhibitory control and changes in the functional state of neurons adjacent to the site of injury represent the current view of a mechanism responsible for this condition (Yezierski, 2001).

Below-level pain is characterized by pain and hypersensitivity to stimuli caudal to the lesion site. A dominating clinical feature of below-level SCI pain and other central pain conditions is an abnormal spinothalamic function with altered sensitivity to temperature and pinprick (Boivie et al., 1989; Vestergaard et al., 1995; Bowsher, 1996). Patients with neuropathic pain demonstrate distinct sensory symptoms that can coexist in various combinations. Responses can be graded as normal, decreased or increased to determine whether negative or positive sensory phenomena are involved. The stimulus-evoked (positive) pain types are classified as dysesthetic, hyperalgesic or allodynic, in accordance to the dynamic or static character of the stimulus (Rasmussen et al., 2004). Pain can be constant as in spontaneous pain (spontaneous firing of nociceptive neurons which become sensitized) or evoked from a stimulus.

1.4 Neuropathic Pain Models

A number of rodent peripheral neuropathic pain models have been developed to simulate human neuropathic pain conditions. These experimental models are described in this section.

1.4.1 Chronic Constriction Injury Model

A common peripheral nerve injury is the chronic constriction injury (CCI). In this model the sciatic nerve is loosely ligated with chromic gut sutures and animals develop spontaneous pain related behavior, hyperalgesia and allodynia to thermal and mechanical stimuli (Bennett and Xie, 1988). Allodynia can be defined as a painful response to an innocuous stimulus. Hyperalgesia can be defined as an extremely painful response to a noxious stimulus.

1.4.2 Spared Nerve Injury Model

The purpose of the spared nerve injury (SNI) is to enable a direct way of investigating changes in both injured primary sensory neurons and in neighboring intact sensory neurons so that their relative contribution to the pathophysiology of pain can be investigated. An example of this is to axotomize two of the three terminal distal branches of the sciatic nerve (tibial and common peroneal nerves) sparing one (sural nerve) (Zhao et al., 2007).

1.4.3 SCI Pain Model

This injury model involves direct injury of the spinal cord by way of clip compression, computerized spinal cord impactor, or weight drop contusion injury. A computerized spinal cord impactor was used in this study. This is an irreversible injury and is high maintenance with regards to surgery and post-operative care for the animal models used. For this reason, the other neuropathic pain models are more widely used. It was felt that in order to get an accurate depiction of chronic irreversible neuropathic pain this model would best replicate human SCI.

1.5 Neuropathic Pain Mechanisms

Neuropathic pain is a complex phenomenon whose mechanisms have been widely researched and discussed. There are many factors contributing to this pain and many different mechanisms have been proposed and studied.

1.5.1 Loss of Inhibition in the Dorsal Horn

Inhibitory mechanisms are present in the dorsal horn. This inhibition is mainly the result of the action of the neurotransmitters gamma-aminobutyric acid (GABA) and Glycine. GABA is the main inhibitory neurotransmitter in the CNS and will be discussed later. Glycine has less of a role in inhibition but is still able to produce an inhibitory postsynaptic potential when its ionotropic receptors are activated and allow the flow of Cl^- into the neuron. Inhibition may occur presynaptically by inhibiting the release of neurotransmitter or postsynaptically by binding to postsynaptic GABA receptors (discussed later).

One mechanism which has been proposed to contribute to neuropathic pain is peripheral nerve injury-induced loss of inhibition in the dorsal horn (Bennett et al., 1989; Dubner, 1991). It has been shown that in the superficial dorsal horn (lamina II) inhibition is decreased after partial nerve injury but this was not shown in complete nerve injury indicating that some input is still required for the loss of inhibition to occur (Moore et al 2002B).

Early changes in the dorsal horn after injury (e.g. reduced inhibition) and in primary afferent fibres (e.g. ectopic or abnormal discharges) may set the stage for neuron loss. It has been shown that after chronic constriction injury or spared nerve injury, $GABA_A$ receptors remain present, indicating that presynaptic, rather than postsynaptic changes in the dorsal horn are responsible for the lack of inhibition and that the amount of GABA being released into the superficial layers is being decreased (Moore et al. 2002B). It is hypothesized that this inhibition is due to the cell death of inhibitory interneurons in the dorsal horn. These cells may be dying for various reasons which will be discussed further.

1.5.2 Gate Control Hypothesis

The gate control hypothesis for pain is a four neuron model consisting of myelinated afferent fibers, unmyelinated afferent fibers, inhibitory interneurons and second order neurons. It was developed by Ronald Melzack and Patrick Wall in 1965. It is based on the theory that pain is not the result of direct activation of pain neurons but rather its perception is modulated by the interaction between different neurons. There are three possible scenarios for this model: 1) there is no stimulus, nothing is

activated and there is no pain. 2) There is a painful stimulus (ie burning hand) which activates unmyelinated C fibers. These fibers inhibit the inhibitory interneurons which therefore can no longer inhibit the second order neurons and a painful stimulus gets sent to the brain. 3) There is a non-painful stimulus (ie. light touch) which activates large myelinated A fibers. This in-turn activates inhibitory interneurons which inhibit the second order neurons and there is no pain. This theory explains why non-painful cutaneous receptor activation reduces perception of nociception by activation of the Aβ fibers. The Aβ fibers also have connections with second order neurons but is unclear as to their definite purpose. These cells are activated by low threshold stimuli so it is possible that these cells make direct connections with WDR neurons to transmit non painful information (Figure 1). If these Aβ fibers were inactive then the model would change to that of one having nociceptive specific second order neurons (Melzack 1991). The hypothesis of this study is that in this model, the inhibitory interneurons will undergo cell death resulting in a loss of inhibition. The lack of inhibition in this model will result in the inability to inhibit the large myelinated A fibers and therefore a normally innocuous stimulus will result in a painful response.

1.5.3 Primary Afferent Sprouting

The loss of inhibition of pain, which may be associated with interneuron death, may be attributed to the excitotoxic changes occurring in the dorsal horn after injury when axons develop spontaneous ectopic activity such as myelinated Aδ fibers sprouting from their normal terminations in lamina III into laminae I and II of the dorsal horn (Woolf et al., 1992). In these superficial laminae, they establish contacts

with neurons that normally receive C-fiber input. It has been shown that two weeks following a sciatic nerve lesion there was neuronal cell death in laminae I, II and III when myelinated fibers were stimulated (Coggeshall et al,. 2001). This indicates that the myelinated fibers had sprouted into laminae I and II and that upon stimulation, the glutamate released induces cell death. This has been shown to occur in other parts of the nervous system where glutamatergic fibre activation consistently resulted in postsynaptic neuron death (Sloviter et al., 1996; Kelsey et al., 2000). This can be attributed to excessive afferent activity or an increased number of activated fibers converging on a particular cell where the cell's capacity to handle the excessive glutamate load is exceeded (Coggeshall et al,. 2001). Primary afferent fiber sprouting can be found not only in the superficial laminae but also in deeper laminae and the central autonomic area (Weaver et al., 2001). The excitotoxicity of glutamate will be discussed further.

1.5.4 Central Sensitization and Wind-Up

When injury in the PNS or CNS occurs, dramatic secondary changes take place in the dorsal horn of the spinal cord. After injury, damaged cells and tissue release bradykinin, histamine, prostaglandins, leukotrienes, acetylcholine, serotonin and substance P (Kendel et al 2000). These all act to decrease the threshold for activation of nociceptors. There is an increased excitability of multi-receptive spinal neurons called wide dynamic range neurons. This hyperexcitability is manifested by increased neuronal activity in response to noxious stimuli, expansion of neuronal receptive fields and the spread of spinal hyperexcitability to other segments. This so-

called central sensitization is initiated and maintained by activity in sensitized C-fibers. These fibers sensitize (lower the threshold of activation) spinal cord dorsal horn neurons by releasing glutamate and the neuropeptide substance P, which act on post synaptic pain transmitting N-methyl-D-aspartate (NMDA) receptors (Baron 2006). NMDA is an NMDA receptor agonist and therefore mimics the action of glutamate at NMDA receptors. Before injury, moderate amounts of glutamate are released from first order neurons and can activate non-pain transmitting α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA) receptors but not Mg-blocked NMDA receptors without the aid of Substance P and constant release of glutamate. After injury, the constant activation of AMPA receptors by glutamate paired with substance P bound to Neurokinin-1 receptors releases the Mg block in the NMDA receptors. This allows glutamate to attach to these receptors and this results in an influx of calcium ions into the postsynaptic cell. These synaptically evoked voltage-gated currents contribute to a facilitation (windup) of dorsal horn neurons during repetitive C-fiber/nociceptive inputs by producing nonlinear cumulative depolarizations that last tens of seconds (Woolf and Salter, 2000). The consequence of this is a progressive increase in the amount of pain experienced per stimulus during a train of repetitive noxious stimuli, i.e., a change in the gain of the system during the course of its activation by repeated stimuli. If central sensitization is established, normally innocuous tactile stimuli become capable of activating spinal cord pain-signaling neurons via Aδ and Aβ low-threshold mechanoreceptors (Tal and Bennett 1994). This leads to an increase in responsiveness to noxious stimuli, the spread of

pain sensitivity to areas outside of an area of tissue injury, and the development of pain in response to low intensity stimuli (Woolf, 1983).

1.5.5 Role of Nerve Growth Factor in Neuropathic Pain

The neurotrophin, nerve growth factor (NGF) may be an important mediator in persistent inflammatory pain. Inflammatory pain is caused by damage to tissue which activates inflammatory processes and alters the function of the nervous system, causing pain to be perceived long after the initial injury. Transgenic animals lacking the NGF gene are born with virtually no small caliber primary sensory neurons and are hypoalgesic (Crowley et al. 1994). Also, transgenic animals lacking the gene for the NGF receptor trkA exhibit the same behaviour (Barbacid 1994). TrkA receptors are found selectively on nociceptive afferents and there is direct evidence that exogenous NGF can produce both mechanical and thermal hyperalgesia when administered systemically (Lewin et al., 1993, and 1994). After peripheral nerve injury, NGF has been shown to increase neuromodulators substance P and CGRP levels both in vivo (Kuraishi et al 1989, Donnerer et al 1992) and in vitro (Lindsay 1992) by increasing nociceptor activation. This increased production can be found in the central terminals of nociceptors. Many forms of central sensitization appear to depend upon the release of these neuropeptides (McMahon et al 1993) and one can therefore hypothesize that the increased levels of these peptides by NGF is an intermediate step in the generation of central sensitization and inflammatory pain.

1.6 Hyperalgesia

Traumatic and inflammatory processes cause, in addition to the pain of the primary lesion, an increase in pain sensitivity in areas of the body adjacent to or even remote from the site of injury. This pain sensitivity is termed hyperalgesia (Treede et al., 1992). In the case of neuropathic pain, hyperalgesia can occur in the absence of peripheral damage and can be maintained by ectopic activity from damaged nerves or from altered processing in the central nervous system (CNS) (Gracely et al., 1992). Hyperalgesia comprises both primary hyperalgesia and secondary hyperalgesia. Primary hyperalgesia is characterized as an increased sensitivity within the injured area predominantly due to peripheral nociceptor sensitization. Secondary hyperalgesia is characterized as an increased sensitivity in the surrounding uninjured area mediated by central changes (Treede et al., 1992; Treede and Magerl, 2000; Klede et al., 2003).

The mechanism involved in secondary hyperalgesia is unclear. What is known is that incoming nociceptive activity from an area of primary hyperalgesia is necessary for the development and maintenance of secondary hyperalgesia and that this process takes place inside the CNS (Dubner and Ruda, 1992). Incoming activity from low-threshold afferents from the secondary hyperalgesia area gains access to the nociceptive sensory pathway and therefore the activity that in normal circumstances will lead to a touch sensation now evokes pain (Cervero et al 2003).

1.7 Descending Pathways Involved with Hyperalgesia

Our understanding of descending modulatory influence is not well advanced; however, an increasing number of functional studies have implicated descending

modulation from the rostral ventromedial medulla (RVM) in hyperalgesia (Heinricher et al. 2003; Porreca et al. 2002; Ren and Dubner 2002). The RVM has long been known to suppress nociception through descending connections to the dorsal horn and to be an important substrate for opioid analgesia (Fields and Basbaum 1978; Mayer and Price 1976). Generally, low intensities of electrical stimulation or low concentrations of transmitter (e.g., glutamate, neurotensin) at the RVM facilitate spinal nociception, whereas greater intensities of stimulation or concentrations of transmitter at the same sites typically inhibit spinal nociception (Urban, and Gebhart, 1997; Urban,. and Smith, 1993; Zhuo, M.&Gebhart, 1992; Zhuo, and Gebhart, 1997). This dual influence appears to involve anatomically distinct spinal pathways and is mediated by different spinal receptors.

There is now strong evidence for two populations of nociceptive modulating neurons in the RVM: "ON" cells and "OFF" cells (Fields and Heinricher 1985; Fields et al. 2005). ON cells are characterized by a burst of activity associated with nociceptive withdrawal reflexes. Direct, selective activation of ON cells produces hyperalgesia, and a reduction in the threshold at which the ON cell is activated is associated with a decrease in reflex latency (Heinricher and Neubert 2004; McGaraughty et al. 2003; Neubert et al. 2004). Activation of OFF cells results in analgesia, and these neurons are generally thought to exert a net antinociceptive effect (Heinricher and Tortorici, 1994; Heinricher et al. 1994; Neubert et al. 2004). It is hypothesized that there exists a spinobulbospinal circuit that contributes significantly to central sensitization and secondary hyperalgesia. Both the RVM and adjacent areas receive direct afferent input from the superficial spinal dorsal horn and in turn send

descending projections through spinal funiculi that terminate in the superficial dorsal horn, completing a spinobulbospinal loop (Figure 3) (Almeida et al., 1993; Basbaum et al., 1978; Craig, 1995; Martin et al., 1985).

1.8 Cell Death Following SCI

After SCI occurs, many changes occur within the spinal cord both in the lesion site and in spinal segments away from the lesion. One of these changes is the death of neurons, oligodendrocytes and astrocytes which can occur by way of several different mechanisms.

1.8.1 Necrosis and Inflammation Following SCI

The lack of inhibition in the dorsal horn may be due, in part, to the inflammatory response after injury. The initial or primary injury is followed by a cascade of biochemical events (secondary injury) that is thought to enlarge the area of cell death through necrosis and inflammation. It has been shown that when axons in the white matter are damaged, Wallerian degeneration can induce microglial activation and apoptotic cell death of oligodendrocytes long after the initial injury (Beattie MS. 2004) (Figure 4).

These events might also be exacerbated by the presence of inflammatory cells and proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) (Beattie et al., 2002). This is due to the infiltration of neutrophils and macrophages into the lesion site, ultimately resulting in secondary tissue necrosis and scar formation (Popovich et al., 1999 and Plunkett et al., 2001).

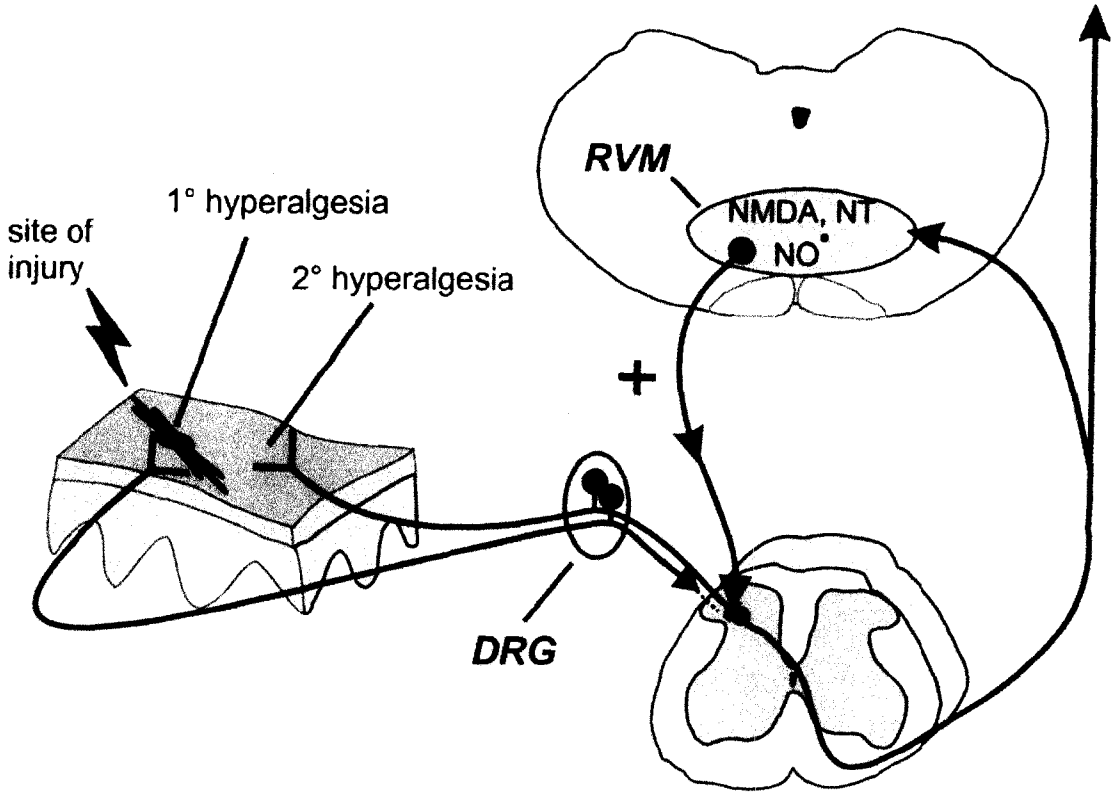


Figure 3 Diagram illustrating supraspinal contribution to secondary thermal hyperalgesia. It is proposed that stimulation of nociceptors activates a spinobulbospinal loop, engaging a centrifugal descending nociceptive facilitatory influence from the RVM. Facilitatory influences are activated by NMDA receptors, nitric oxide (NO), and neurotensin (NT) receptors in the RVM and descend to multiple spinal segments to contribute significantly to secondary hyperalgesia (Adapted from Urban and Gebhart, 1999).

Necrosis is the name given to the death of cells and living tissue which is not programmed. Necrosis is less orderly than apoptosis, which is part of programmed cell death. In contrast with apoptosis, necrosis results from acute traumatic injury and characteristically involves rapid lysis of cellular membranes without activation of the endogenous cell death program. The cleanup of cell debris by phagocytes of the immune system is more difficult, as the disorderly death generally does not send cell signals which tell nearby phagocytes to engulf the dying cell. This lack of signalling makes it harder for the immune system to locate and recycle dead cells. The release of intracellular content after cellular membrane damage is the cause of inflammation in necrosis (Kiernan, 2005). This inflammation will occur in the spinal cord and will lead to inflammatory pain.

1.8.2 Apoptosis Following SCI

Apoptosis is a programmed process which has specific cellular mechanisms. Apoptosis is characterized ultrastructurally by degradation of nuclear chromatin, condensation of the cytoplasm and nucleus, and ultimately fragmentation of the cell into apoptotic bodies (Collins et al., 1997 and Kerr et al., 1972). The major component driving apoptosis is a series of caspase proteases that target essential cellular functions. Caspases can be divided into two main groups: initiators (caspase-1, 2, 8 and 9) and effectors such as caspase-3 (Takagi et al., 2003). These enzymes participate in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell (Thornberry and Lazebnik, 1998). Apoptosis is initiated by a number of intracellular

and/or extracellular events. There appear to be three distinct cell death pathways (one extrinsic pathway and two intrinsic pathways) that lead to caspase activation and apoptosis. The extrinsic pathway will be the pathway that will be focused on here as this is the pathway associated with the release of glutamate and the subsequent activation of the death receptor. This extrinsic pathway or the "death receptor pathway" contains tumor necrosis factor family receptors (also known as death receptors). These include p75 and the Fas receptor. Fas receptor ligands oligomerize and cause oligomerization of the death receptor. The receptor then interacts with cytoplasmic FADD (Fas associated death domain) protein and converts pro-caspase 8 to active caspase 8. Caspase 8, in turn activates the effector caspase 3 from its inactive proform whose intracellular targets lead to cell disassembly and apoptotic cell death (Ashkenazi and Dixit, 1998). When apoptosis of inhibitory interneurons occurs, reduction of spinal cord inhibition is likely to occur (Moore et al., 2002B) as hypothesized in this study.

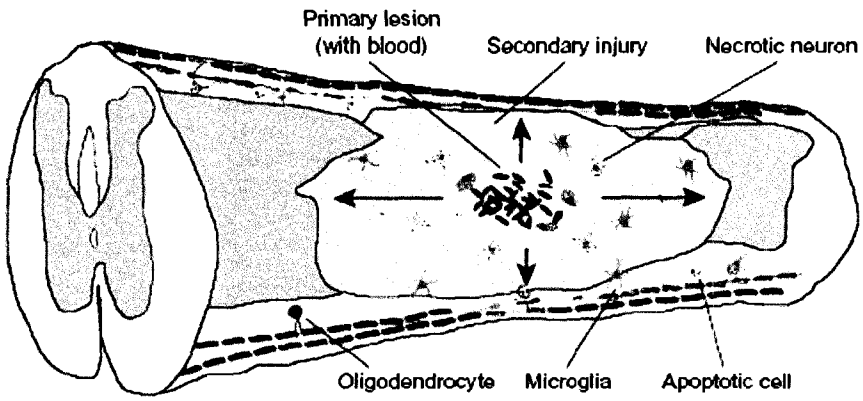


Figure 4 Secondary spinal cord injury. The initial injury is followed by a cascade of biochemical events (secondary injury) that is thought to enlarge the area of cell death through necrosis and apoptosis. (Adapted from Beattie 2004)

1.9 GABA and GABA Receptors

GABA is the main inhibitory neurotransmitter in the CNS. There is a particularly high concentration of GABA in the most superficial laminae (I, II and III) of the dorsal horn of the spinal cord. Normally, the dorsal horn GABA serves as a brake upon the excitatory action of glutamate. This produces a balanced state in the nervous system. In this state, GABA receptors on glutamatergic afferent terminals prevent excessive release of glutamate and substance P presynaptically. GABA clearly has an important role in regulation of nociceptive transmission, since local application of the $GABA_A$ receptor antagonist bicuculline (as well as the glycine receptor antagonist strychnine) to the spinal cord in the rat produces behavioural signs of tactile allodynia (Yaksh, 1989) and can cause low-threshold mechanical stimuli to produce a flexion withdrawal reflex (Sivilotti and Woolf, 1994). Ralston et al. (1997) found substantially reduced numbers of both GABA-immunoreactive neuronal cell bodies and axon terminals in the dorsal horn in a neuropathic model, that involved tight ligation of part of the sciatic nerve. This indicates that with a loss of GABA-immunoreactive cell bodies, results in an increase in the probability of neuropathic pain.

1.9.1 $GABA_A$ Receptors

$GABA_A$ receptors (R) mediate most of the inhibitory synaptic transmission in the CNS and serve as the target for many important neuroactive substances, including barbiturates, steroids, general anaesthetics and alcohol (Macdonald and Olsen, 1994).

The $GABA_AR$ is also the molecular target of the benzodiazepine class of tranquilizer drugs, and hence it is also often referred to as the benzodiazepine receptor. The receptor is a multimeric transmembrane receptor that consists of five subunits arranged around a central pore (Figure 5). Once bound to GABA, the protein receptor changes conformation within the membrane, opening the pore to allow chloride ions (Cl^-) to pass down an electrochemical gradient. Because the reversal potential for chloride in most neurons is close to or more negative than the resting membrane potential, activation of $GABA_ARs$ tends to stabilize the membrane potential, and can make it more difficult for excitatory neurotransmitters to depolarize the neuron and generate an action potential. The net effect is typically inhibitory, reducing the activity of the neuron (Siegel et al., 1999; Chen et al., 2005).

1.9.2 $GABA_B$ Receptors

The $GABA_BR$ differs from the $GABA_AR$ in many respects. It is now well accepted that $GABA_BRs$ assemble into heteromers composed of a $GABA_{B1}$ and a $GABA_{B2}$ subunit, which are both required for normal receptor functioning (Marshall et al.1999). Ligand binding and second messenger studies revealed that the $GABA_BR$ is metabotropic, distinguishing it from the ionotropic $GABA_A$ receptor. Thus, $GABA_BRs$ are coupled to G proteins, with activation causing a decrease in Ca^{2+} and an increase in K^+ membrane conductance (Bowery 1993). In the spinal cord, $GABA_BR$ activation decreases the duration of orthodromic action potentials and of excitatory neurotransmitter release from primary afferent fibers (Curtis et al., 1997). Both of these effects are explained on the basis of $GABA_BR$-mediated inhibition of

Ca^{2+} influx into primary afferent presynaptic terminals. Baclofen has been used for the treatment of spasticity, with its effectiveness believed to be related to $GABA_BR$ - mediated inhibition of excitatory amino acid release onto motoneurons in the ventral horn of the spinal cord. Side effects (in particular, sedation) limit its clinical usefulness (Penn and Mangieri, 1993). There are suggestions that the antinociceptive response to $GABA_B$ agonists may be mediated by effects in the brain. For example, studies with tiagabine, a GABA uptake inhibitor with high affinity binding sites in the brain (Suzdak et al., 1992), and therefore a nonselective activator of GABA receptors, have revealed that it displays antinociceptive properties in a variety of pain models (Ipponi et al., 1999).

1.9.3 $GABA_C$ Receptors

The $GABA_CR$ is an ionotropic receptor activated by cis-aminocrotonic acid, which is not recognized by either the $GABA_A$ or $GABA_BR$, suggesting that these receptors recognize the partially folded conformation of GABA. The $GABA_CR$, like the closely-related $GABA_AR$, is expressed in many areas of the brain, but in contrast to the $GABA_AR$, the $GABA_CR$ has especially high expression in the retina (Hull et al., 2006).

Table 2 Comparative Pharmacology of GABA Receptors (Martin and Dunn, 2002)

Compound	$GABA_A$	$GABA_B$	$GABA_C$
GABA	Agonist	Agonist	Agonist
Muscimol	Agonist	Inactive	Partial agonist
Isoguvacine	Agonist	Inactive	Antagonist
THIP	Agonist	Inactive	Antagonist
P4S	Agonist	Inactive	Antagonist
TACA	Agonist	Inactive	Agonist
CACA	Inactive	Inactive	Partial agonist
(*R*)-Baclofen	Inactive	Agonist	Inactive
Bicuculline	Antagonist	Inactive	Inactive
Picrotoxin	Antagonist	Inactive	Antagonist
CGP 35348	Inactive	Antagonist	Inactive
CGP 54626	Inactive	Antagonist	Inactive
CGP 64213	Inactive	Antagonist	Inactive
SCH 50911	Inactive	Antagonist	Inactive
TPMPA	Inactive	Inactive	Antagonist

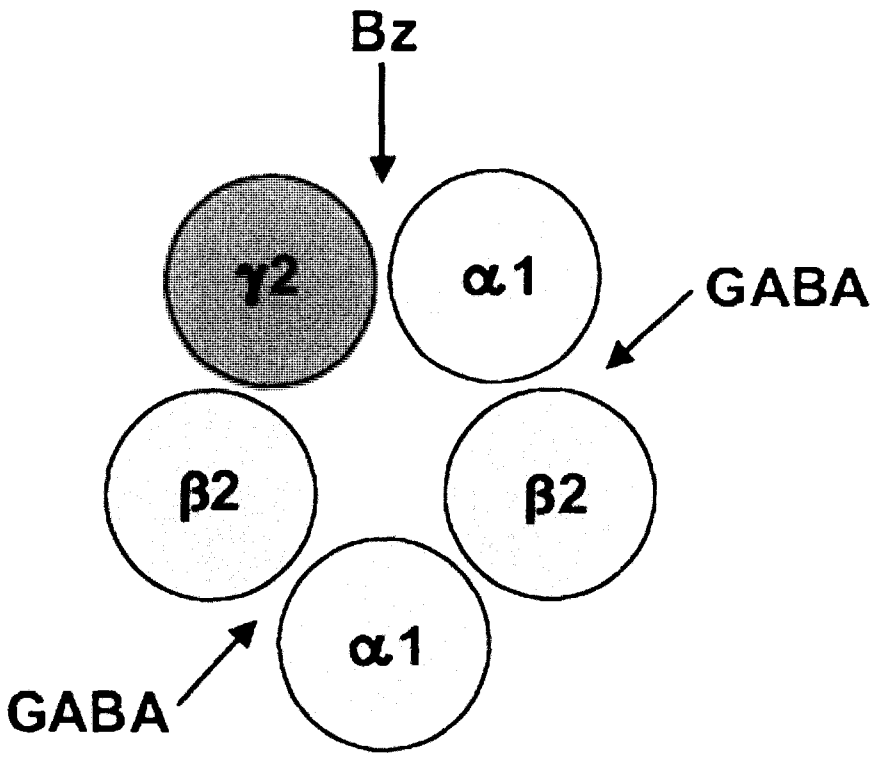


Figure 5 Viewing the $GABA_A$ receptor from the extracellular space, the orientation of the subunits within the pentamer together with the location of the benzodiazepine (Bz) and low affinity GABA sites shown is in accord with all the mutagenesis data that is available to date Adapted from Martin and Dunn (2002).

1.10 Analgesics

After hyperalgesia develops, it is necessary to find a way to alleviate this pain to improve quality of life. Many treatments are available to aid patients with SCI. The direct cost of SCI to the Canadian health care system is estimated at 450 million dollars annually (Spinal Cord Injury Treatment Center Society). The general effectiveness of these treatments is limited. Analgesic medications are very important in treating acute and chronic SCI to help in restoring quality of life for these patients. To help with the cost of treatments and equipment, it is important to find analgesics which are cost effective and potent enough to alleviate pain with a minimal dose and with minimal side effects. A commonly prescribed analgesic for SCI-induced neuropathic pain is gabapentin. In this study, gabapentin was tested on SCI mice and results were compared to that of tiagabine, a new anti-convulsant drug, with potential use as an analgesic.

1.10.1 Gabapentin

Gabapentin (2-[1-(aminomethyl)cyclohexyl]acetic acid) (GBP) is a structural analog to GABA whose mechanism of action is currently unknown. It lacks affinity for both $GABA_A$ and $GABA_B$ receptors and fails to inhibit GABA transaminase (Goldlust et al., 1995) or GABA uptake, (Su et al., 1995) but possibly binds to a subunit common to most voltage-sensitive calcium channels in the brain. Studies have shown that gabapentin is antinociceptive. It has been shown that GBP can reverse hyperalgesia and allodynia observed after peripheral nerve injury (Xiao et al, 2006).

In a pharmacological model of neuropathic pain (dynorphin-induced allodynia) GBP was shown to reverse behavioural signs of neuropathic pain in animal models (Xiao and Bennett, 1996; Hunter et al., 1997).

1.10.2 Tiagabine

Tiagabine [(R)-)N-(4,4-di(3-methylthien2-yl)but-3-enyl] (TGB) nipecotic acid hydrochloride] is a GABA re-uptake inhibitor which acts to increase synaptic GABA availability by blocking the reuptake of GABA into the presynaptic terminals and surrounding glial cells via selective inhibition of the GABA transporter protein (Borden LA et al., 1994). TGB has no affinity for catecholamine, serotonin, or glutamate uptake sites and lacks appreciable affinity for catecholamine, serotonin, acetylcholine, histamine, opiate, glycine, glutamate, GABA, and sigma receptors (Braestrup et al., 1990). It has been demonstrated that systemic administration of TGB produces antinociception in acute nociceptive tests in mice and nerve ligation-induced tactile allodynia in rats (Giardina et al., 1998). To date, TGB has not been tested for efficacy in reducing SCI-induced allodynia or hyperalgesia.

1.11 Purpose

The purpose of this project is to:

1) develop a mouse model of SCI induced thermal hyperalgesia,

2) determine if this hyperalgesia is associated with a loss of GABAergic inhibitory interneurons which would lead to a loss of inhibition in the dorsal horn, and

3) to attenuate hyperalgesia pharmacologically by blocking the reuptake of GABA.

1.12 Hypotheses

1) After SCI there will be a loss of GABAergic inhibitory interneurons in the dorsal horn.

2) This loss will lead to neuropathic pain in the form of thermal hyperalgesia.

3) Administration of a GABA reuptake inhibitor will decrease hyperalgesia.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Transgenic mice with the promoter glutamic acid decarboxylase (GAD)1 driven expression of green fluorescent protein (GFP) and non-GFP swiss FVB mice were used in these experiments. The generation of glutamic acid decarboxylase (GAD)67-GFP knock-in mice has been previously described (Tamamaki et al. 2003). Another isoform (GAD2) was used in the generation of GAD 65 GFP knock in mice. These mice have GFP concentrated in the membrane and projections of inhibitory interneurons and can be found in the dorsal horn, intermediate grey area and ventral horn of the spinal cord. In the dorsal horn, GAD 67 is the predominate isoform in a greater percentage of neurons than GAD 65 (Mackie et al., 2003). GFP expression in the GAD1 mice is restricted to a population of GABAergic interneurons prevalent in the cortex, hippocampus and dorsal horn of the spinal cord. GFP is concentrated in the soma and dendrites. The GFP positive cells account for 40% of the GABAergic neuron population in the spinal cord (Laing et al., 1994). All animal procedures were conducted following the Canadian Council on Animal Care (CCAC) "Guide to the Care and Use of Experimental Animals."

This study was divided into two parts. In one part, swiss FVB mice were used to develop an injury model using the Infinite Horizon Spinal Cord Impactor (PSI, Lexington, KY). 36 mice were injured at different dwell times (time of impactor in spinal cord) of 0, 10 and 60 seconds and with a force of 50 kilodynes at the tenth thoracic segment. Spontaneous pain behaviours were monitored and hindlimb

locomotion was assessed using the Tarlov scale scoring system. In the second part, GAD 1 transgenic mice were used to assess thermal hyperalgesia after chronic spinal cord injury. 53 mice were injured with the impactor at different dwell times (0 and 60 second) and with different forces (50 and 75 kilodynes) (Tables 3 & 4). Spontaneous pain behaviours were monitored and hindlimb locomotion was assessed using the Tarlov scale scoring system (discussed later).

2.2 Spinal Cord injury

All animals were anesthetized under isoflurane gas (2% with 1L/min oxygen) and given an ocular lubricant (Lacrilube; Allergan Inc.). All mice were given a 0.1 ml subcutaneous injection of buprenorphine (0.005 mg/ml), atropine (0.006 mg/ml) and baytril (1.25 mg/ml) prior to surgery. They were also given 1 ml of saline prior to surgery. Injections of baytril (antibiotic), buprenorphine (analgesic) and saline (to reduce dehydration) were continued for 3 days post injury or longer if needed to reduce pain and chance of infection.

A laminectomy was performed at T10 and the dorsal process of the vertebrae was removed. The mouse was then placed beneath the impactor which was programmed to injure the spinal cord by contusion with a force of either 50 kd or 75 kd for either 0, 10 or 60 seconds (depending on the experiment). After the inury, the incision was sutured with sterile 4-0 coated DEXION II braided absorbable sutures (CV-23; Syneture, Norwalk, CT). Mice were placed on heating pads and monitored after the injury. Mouse bladders were manually expressed for approximately 2 weeks

Table 3 Numbers of animals in four treatment groups in part one. Swiss FVB mice were injured at 50kd for 0, 10 and 60sec with the SC impactor. Hindlimb locomotion and spontaneous pain behaviours (biting and chewing of limbs) were monitored.

	Uninjured	**50 kd/0sec**	**50 kd/10sec**	**50 kd/60sec**
Swiss FVB Mice	10	7	9	10

Table 4 Numbers of animals in six treatment groups in part two. GAD1 GFP mice were injured at 50kd/60sec, 75kd/60sec and 50kd/0sec. They were put in one of two groups: 2wks and 6wks. Thermal hyperalgesia was tested and GAD-GFP interneurons were counted in laminae I-III.

GAD1 GFP Mice	**50 kd/60sec**	**75 kd/60sec**	**50 kd/0sec**
2wks	6	5	5
6wks	26	6	5

after injury or until bladder function returned. Mice were housed individually in microisolator cages in a temperature and light-controlled environment.

2.3 Tarlov Scale

Hindlimb locomotion was assessed weekly in an open field test according to the modified Tarlov 5 point scale. The scores are based on observations of the hindlimbs. Each hindlimb was scored independently then two scores were averaged to give the total score. 0 = no movement of the hindlimb, 1 = barely perceptible hindlimb movement with no weight bearing, 2 = frequent hindlimb movement with no weight bearing, 3 = occasional stepping with some weight bearing, 4 = mild deficits in hindlimb function, 5 = normal stepping and weight bearing.

2.4 Thermal Hyperalgesia Testing

Hindpaw thresholds were determined by using a thermal hyperalgesia device (developed by the Department of Anesthesiology, University of California, San Diego, La Jolla, CA). Mice were placed in plexiglass chambers which were 11cm by 8.5 cm in dimension. The chambers were placed on a clear glass surface. Mice were initially placed in the chambers for 30 min for acclimation. A radiant heat source evoked a hindpaw-withdrawal response to the thermal stimulus. The heat source was positioned to focus on the plantar surface of the hindpaw, which was in contact with the glass. Upon a flinch response, the heat source would be automatically turned off

by a motion sensor and the corresponding timer would be stopped. Both uninjured and injured mice were tested. The time from light activation to hindpaw withdrawal was determined electronically by an automated timing device. The stimulus was automatically terminated at 20 s to avoid tissue injury. Both paws were tested and then averaged for each mouse. After injury it was not possible in some cases to test both hindlimbs as there was usually only one limb with the plantar surface on the glass.

2.5 Drug Administration

Two drugs were used in this study: gabapentin and tiagabine. Drugs were administered by intraperitoneal injection. After injection, there was a 40 minute wait period to allow the drugs to take effect before testing began. Both drugs were diluted in saline to a dose of 1 mg/kg and 10 mg/kg based on concentrations in similar studies (Laughlin et al., 2002) and each concentration was tested. Tiagabine was also administered by transcutaneous intrathecal injection at a concentration of 0.4 mg/kg. Uninjured animals were injected with tiagabine intraperitoneally at a dose of 1 mg/kg. Animals were not injected with saline alone.

2.6 Spontaneous Pain Behaviours

Animals were assessed daily following surgery for the presence of excessive grooming behavior, i.e. self-directed biting and scratching resulting in damage to superficial and/or deeper layers of skin. Assessment of grooming behaviour was based on the following classification scheme: (A) Class I, hair removal over

contiguous portions of a dermatome; (B) Class II, extensive hair removal combined with signs of damage to the superficial layers of skin; (C) Class III, hair removal and damage to dermal layers of skin; and (D) Class IV, subcutaneous tissue damage (experiment terminated) (Abraham et al., 2004). To reduce spontaneous pain behaviours, a mixture of metronidazole and 8-hydroxyquinoline ("new skin") was applied to excessive grooming areas at Class I to prevent further self mutilation. The application of this mixture did not appear to affect behavioual testing.

2.7 Injury Factor

Based on Tarlov scores at 6 weeks post injury and their correlation with severity of injury, an equation was developed to describe the severity of an injury based on variables that can be controlled by the computerized spinal cord impactor. The equation was termed Injury Factor (IF). The following variables were used: F = Force (mm^4g), V = Velocity (mm/s), M = displacement (mm), D = Dwell Time (s). The equation is as follows: IF = [(F * V * M) / (1/D + 1)] / 1000. Each mouse was calculated an IF which was compared to their Tarlov score to assess injury severity. This formula was developed by me in hopes of being able to estimate SCI severity to ensure consistent injuries.

2.8 Spinal Cord Harvesting and Preparation

The animals were euthanized at 2 and 6 weeks post injury to examine neuron degeneration and loss. The mice were euthanized with an intraperitoneal injection of 25% urethane in saline (0.5 ml). The mice were then transcardially perfused with 10

ml of phosphate buffered saline (PBS) followed by 10 ml of 4% formaldehyde phosphate buffer (FPB). All thoracic and lumbar segments of the spinal cord were then removed and placed in a vial of 4% FPB for at least one night. The cords were then cryoprotected by equilibrating them with a 15% and 30% sucrose solution over at least 2 days.

2.9 Tissue Processing and Immuofluorescence

Spinal cord lumbar segments were frozen, mounted in Optimal Cutting Temperature (OCT;Tissue-Tek) and were cut using a microtome at 50 um transversely. The sections were collected in phosphate buffer and stored at 4°C. Sections were then placed in wells and washed free-floating in Tris-buffered saline containing 0.2% Triton X-100 detergent (TBS-X) 3 times for 10 minutes each time. The sections were then incubated in the wells with 5% normal goat serum (Biomeda) in TBS-X for at least 2 hours. The sections were then incubated with the primary antibodies (see below for dilutions) and 5% normal goat serum in TBS-X overnight. The sections were then washed with TBS-X 3 times for 10 minutes each time and then incubated with the secondary biotinylated antibodies (see below for dilutions) in TBS-X for 2 hours. The sections were then washed with TBS 3 times for 10 minutes each time and then incubated in streptavidin conjugated fluorescence (diluted appropriately) for 1 hour. The sections were then washed with TBS 3 times for 10 minutes each time. The sections were mounted on a slide and cover slipped with fluorescent mounting media (DakoCytomation). Sections were chosen randomly for cell count.

2.10 Antibodies

2.10.1 Primary Antibodies

Primary antibodies were applied as previously described on free floating sections. Sections were incubated in the following primary antibodies: Rabbit Anti-alpha Calcitonin Gene Related Peptide (CGRP) (T-4032; Peninsula Laboratories) at 1:2000 as a marker for primary afferent fibers (Zinck et al., 2007) and Rabbit Anti-human/mouse active caspase 3 (AF835; R&D Systems) at 1:5000 which is a marker for apoptosis (Joachim et al., 2005). Negative controls were used for all antibodies by omitting the primary antibody and continuing on with the immunohistochemistry with the secondary alone to observe background fluorescence in the tissue.

2.10.2 Secondary Antibodies

Secondary antibodies were applied as previously described on free floating sections. Sections were incubated in the following secondary antibodies: Biotin-SP-conjugated AffiniPure Goat Anti-Rabbit IgG (62880; Jackson Laboratories), Biotin-SP-conjugated AffiniPure Goat Anti-Mouse IgG (66984; Jackson Laboratories)

2.10.3 Immunofluorescence

Immunofluorescence was applied as previously described on free floating sections after incubation in the primary antibodies. Sections were incubated in the following: Alexa Fluor 568 goat anti-mouse IgG (A-11004; Molecular Probes) at 1:1000, Alexa Fluor 568 goat anti-rabbit IgG at 1:1000 (A11011; Molecular Probes),

streptavidin-Alexa Fluor 568 conjugate (S11226, Molecular Probes), streptavidin-Alexa Fluor 633 conjugate (S21375) at 1:1000.

2.11 Confocal Microscopy and Neuron Count

A Zeiss LSM 510 laser scanning confocal microscope was used to analyze the mounted sections. Sections from L2-4 were analyzed. Wavelengths of 488, 568 and 633nm were used to excite fluorescence from the tissue. To get a count of the inhibitory interneurons, sections were scanned from top to bottom in two μm increments in a z-stack using a 25X objective lens. The stack was saved, and GFP positive GABAergic inhibitory interneurons were then counted manually. The neurons were counted in eight random sections from each slide, from each animal. Because the sections were not cut in serial sections, they were mounted randomly so it was not necessary to choose specific sections from each slide. A blind count was conducted for interneurons in lamina I-III in the dorsal horn as some interneurons in deeper lamina (lamina III) may still have some connections to the superficial layers. Lamina I-III were mapped out with a mouse spinal cord atlas and primary afferent immunoreactivity.

2.12 Cell Diameter Measurements

To investigate a possible change in size of GFP neurons that were counted, cell diameter was measured in the largest cells found in each segment. 48 GFP labeled cells from 6 animals were measured in each group. Cells were measured with Image Pro Discovery measurement software (MediaCybernetics).

2.13 Statistical Analysis

Analysis of variance (ANOVA) and paired T-tests were used to determine statistical differences which occurred at the $p \leq 0.05$ level using SPSS statistical software. Student-Neuman-Keuls post-hoc tests were used to assess significance. Standard error was calculated and is indicated by error bars.

CHAPTER 3: RESULTS

3.1 Thermal Hyperalgesia

Thermal hyperalgesia was induced by three different severities of SCI (50kd with a 60sec dwell time, 50kd with a 0sec dwell time and 75kd with a 60sec dwell time). Hindpaw withdrawal was measured in uninjured mice and injured mice for 6 weeks post injury. At 50kd-60sec, thermal hyperalgesia began to develop at week 3 and persisted to week 6 when the experiment was ended (Figure 6). Hindpaw withdrawal decreased from 16±1.2 to 6±0.7. Hyperalgesia was also measured by withdrawal time in the forepaw to see if the hyperalgesia was exclusive to areas caudal to the lesion. There was no significant change in withdrawal time for the forepaw when compared with ANOVA testing (Figure 6).

Hindpaw withdrawal was measured 6 weeks post injury for the 3 different injuries and the severity of hyperalgesia was compared. There was no significant difference in withdrawal time when the injuries were compared (Figure 7).

3.1.1 Sex Differences

Because both male and female mice were used in this study, it was deemed important to look at the withdrawal times for both as the female estrous cycle may have influence over pain threshold. It was found that there was no difference between male and female withdrawal times when tested with a paired t-test (Figure 8).

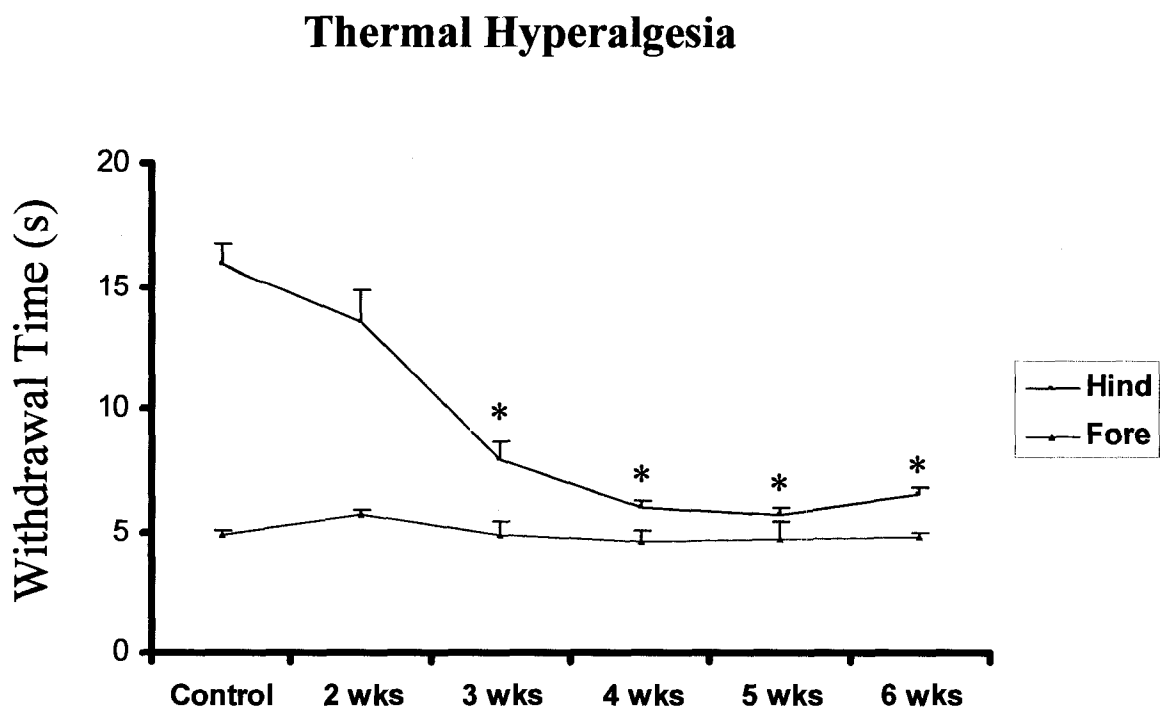


Figure 6 Thermal hyperalgesia testing for hindlimb and forelimb in uninjured and 50kd-60sec injuries. Thermal hyperalgesia was present from 3 weeks to 6 weeks in the hindlimb but did not manifest in the forelimb. * = significantly different from uninjured and 2 weeks post injury withdrawal times ($p<0.05$). Tested for significance with ANOVA. Error bars represent standard error of the mean. Fore n=8; Hind n=8.

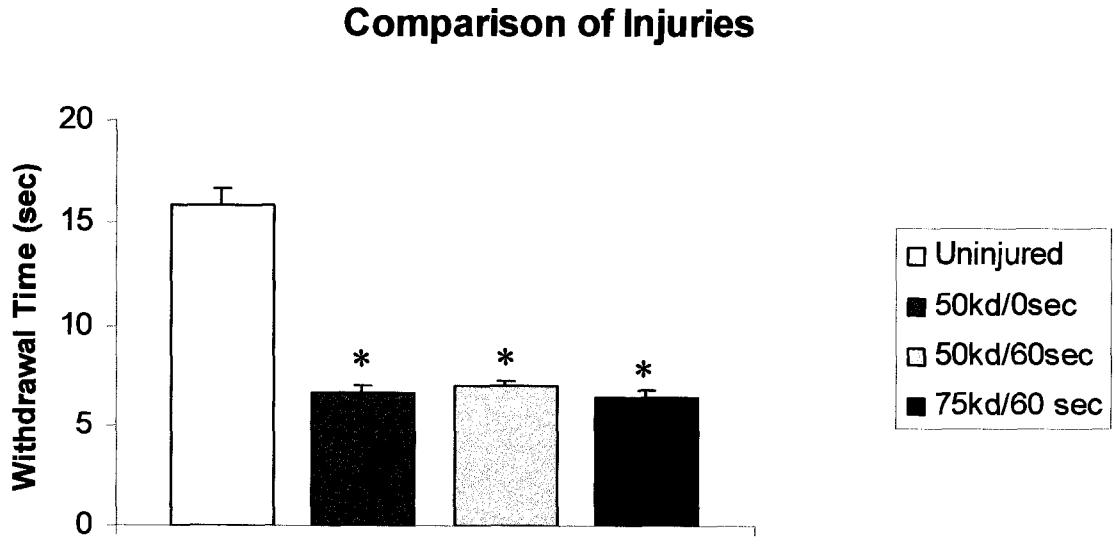


Figure 7 Withdrawal times for 50kd-60sec, 50kd-0sec and 75kd-60sec injuries at 6 weeks post injury. All injury groups are significantly different from uninjured controls (n=8) but not from each other. * = significantly different from from uninjured mice ($p<0.05$). 50kd-60sec n=26; 50kd-0sec n=6; 75kd/60sec n=6. Tested for significance with ANOVA.

3.2 Analgesia

Gabapentin and tiagabine were compared for efficacy in attenuation of thermal hyperalgesia. Tiagabine, which inhibits the reuptake of GABA, was administered to 50kd-60 sec injured mice in doses of 1mg/kg and 10 mg/kg by intraperitoneal injection and 0.4mg/kg by intrathecal injection. After injections, the mice were tested for thermal hyperalgesia. There was a significant increase in withdrawal time from 6±0.7 to 12±0.5 at 1mg/kg and 16±1 at 10mg/kg by intraperitoneal injection. Withdrawal time increased to 14±1.7 after intrathecal injection at 0.4 mg/kg (Figure 9).

Gabapentin at a dose of 10mg/kg showed an equal amount of analgesia when compared to tiagabine at a dose of 1mg/kg (Figure 10). From this it can be concluded that tiagabine is a more potent analgesic in attenuation of hyperalgesia in the mouse model of SCI.

To assess the effect of tiagabine on unininjured mice, thermal hyperalgesia was tested again after injections of 1mg/kg and 10mg/kg in uninjured animals. Withdrawal times for the respective drugs and doses were compared. There was no significant decrease in withdrawal time between each group (Figure 11). Uninjured mice were not tested for effects of injection of saline alone.

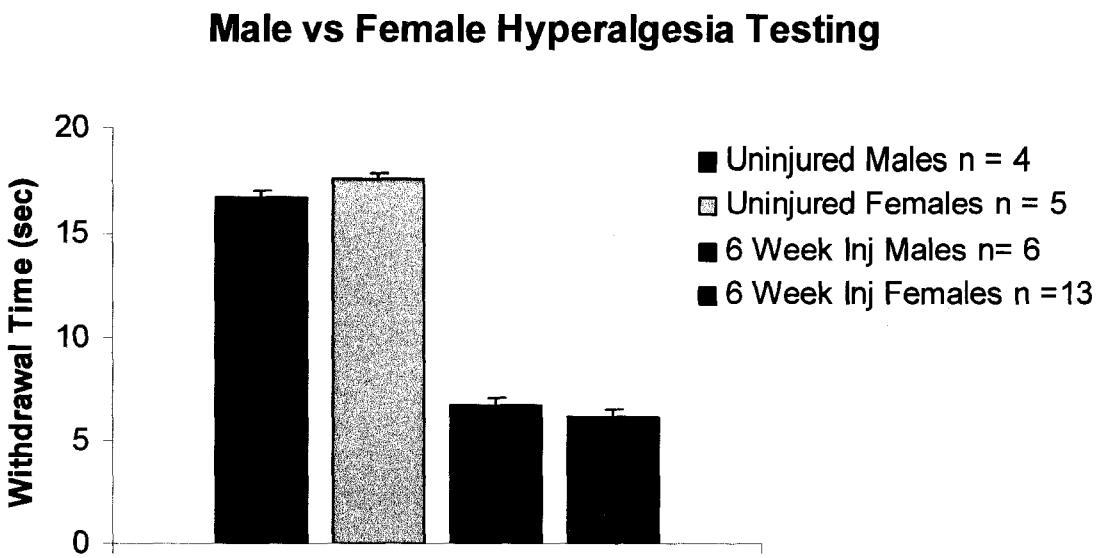


Figure 8 Thermal hyperalgesia testing comparing withdrawal times of male and female mice. There was no significant difference between withdrawal times of uninjured and injured male and female mice. All injuries were 50kd-60sec injuries. Tested for significance with paired t-test.

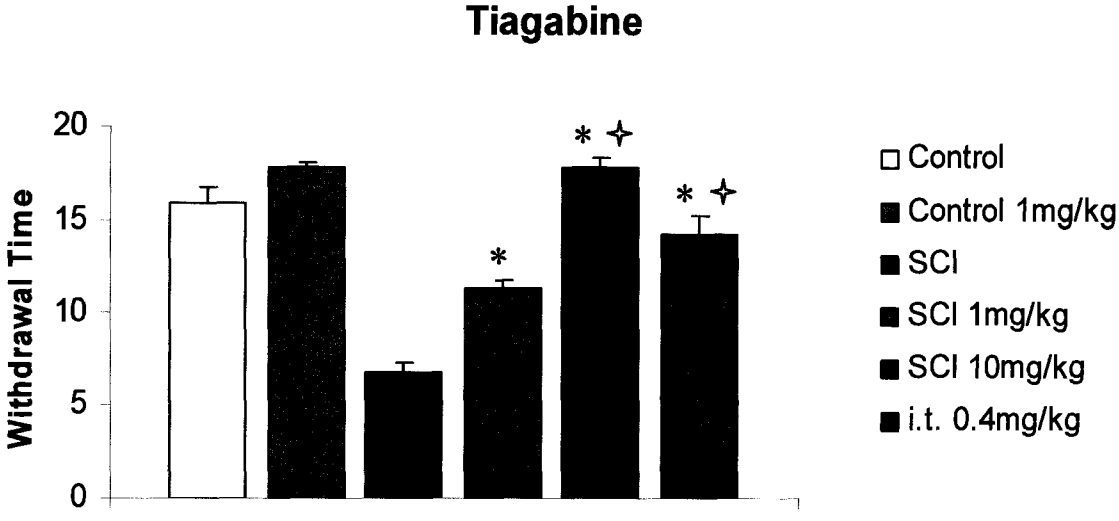


Figure 9 Thermal hyperalgesia testing at 6 weeks post injury with administration of tiagabine intraperitoneally (1mg/kg and 10mg/kg) and intrathecally (0.4mg/kg). * = Significant increase in withdrawal time from untreated SCI. ✧ = Significant increase in withdrawal time from 1mg/kg intraperitoneal SCI treatment ($p<0.05$). Uninjured n = 8; 50kd-60sec injury n = 8. Tested for significance with ANOVA

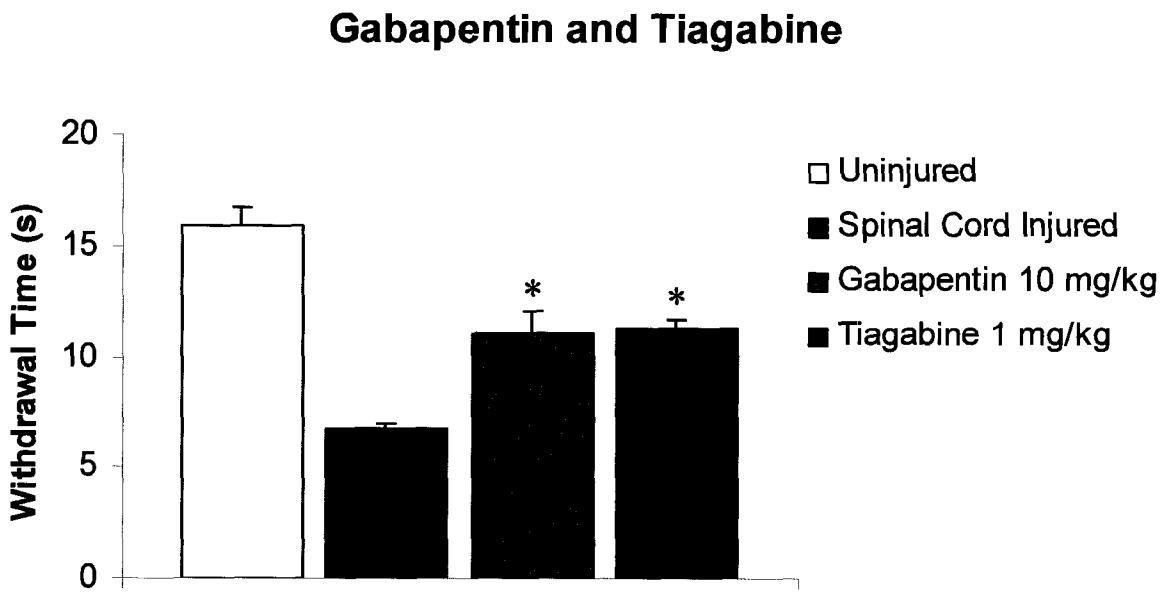


Figure 10 Thermal hyperalgesia testing at 6 weeks comparing gabapentin and tiagabine in attenuation of hyperalgesia when administered intraperitoneally. * Significant increase in withdrawal time from untreated SCI ($p<0.05$). These results indicate that tiagabine has the equivalent effect of gabapentin at 1/10 the dose. Tested for significance with ANOVA.

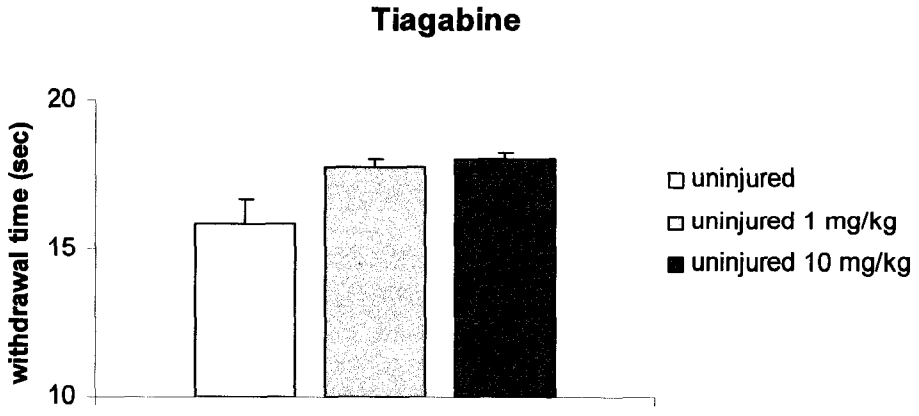


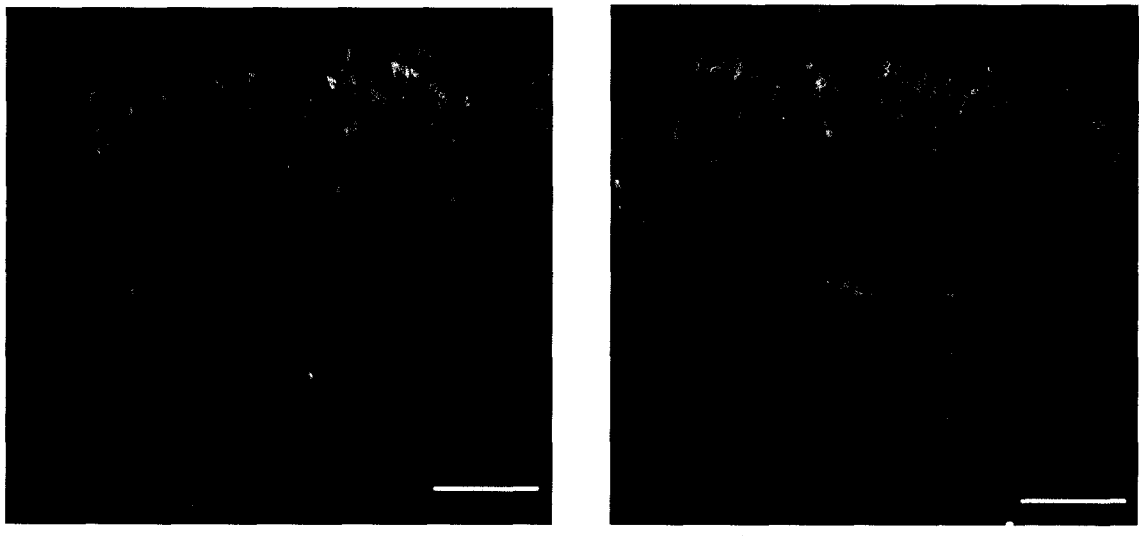
Figure 11 Tiagabine administered intraperitoneally on uninjured control mice at 1mg/kg and 10mg/kg. There was no significant difference in withdrawal time between the two treatments.

3.3 Interneuron Loss

GFP-positive GABAergic inhibitory interneurons were counted in the dorsal horn in order to assess their relationship to the development of thermal hyperalgesia. Immunofluorescence for CGRP which labels primary afferent fibers was used to determine the region of the dorsal horn to be counted (Lamina I-III) (Figure 12). Interneurons were counted for a 50kd/60sec injury at 6 weeks and a 75kd/60sec injury at 2 weeks and 6 weeks. Uninjured controls were also counted. There was no significant neuron loss between uninjured (n=8) and 2 weeks post injury (n=8). There was, however, a significant loss at 6 weeks post injury for the 50kd/60sec injury (n=8) and the 75kd/60sec injury (n=6) from the uninjured controls and the 2 week post injury group (Figure 13).

To ensure that there were no significant changes in size of these interneurons, the cell diameter of the soma was measured with Image Pro Discovery measurement software (MediaCybernetics). There was found to be no significant change in cell size between uninjured and injured mice (Figure 14).

Based on when hyperalgesia begins to develop it is hypothesized that the inhibitory interneurons begin to degenerate at 2 weeks post injury. To test this, immunofluorescence was performed with a caspase-3 antibody to label degenerating neurons. At 2 weeks post injury it was found that some GFP positive neurons were indeed degenerating although they were not quantified (Figure 15). Caspase-3 was also found to label other neurons but it is unclear as to what neurons these were.



UNINJURED **INJURED**

Figure 12 Images of left dorsal horn at L 2-4 taken on confocal microscope. CGRP Immunohistochemistry was used to identify primary afferent fibers (red). Interneurons in uninjured (left) and 6 weeks post injury (right) were counted and compared. 8 random sections were counted for each animal. Mice were injured with a 50kd-60sec or a 75kd-60sec injury. The 50kd injury is shown here. Laminas I-III were labeled with CGRP. Scale bars = 100um.

3.4 Tarlov Scale

To assess hindlimb locomotion after injury, the five point Tarlov scale was used (see methods). Hindlimb locomtion was assessed at 2, 4 and 6 weeks post injury. Both hindlimbs were observed and an average was taken (n=8 for each time point) There was a significant difference in withdrawal times at 6 weeks between treatment groups (Figure 16).

3.4.1 Injury Factor

A formula termed "injury factor" (IF) was developed to measure the severity of injury based on the variables available when using the spinal cord impactor (see methods). The injury factor was then compared to the Tarlov scores and it was found that as the injury factor increased, the Tarlov scores decreased indicating that this formula is able to grade SCI severity accurately (Fig 17). This formula was derived in hopes of being able to grade the severity of an injury based solely on hindlimb locomotion. This could prove to be important in maintaining a consistent injury when conducting SCI on mice. It would not have clinical relevance however as it is not possible to know the exact variables involved in a human SCI.

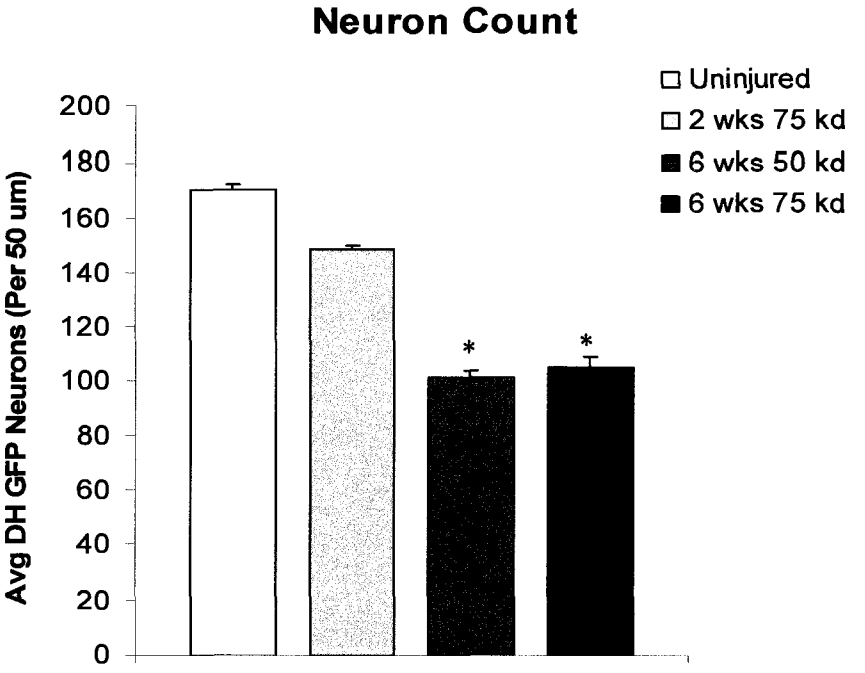


Figure 13 Interneurons were counted in lamina I-III of the dorsal horn in randomly selected 50 μm sections of L2-4 spinal cord sections at 2 and 6 weeks post injury and in uninjured mice. * = a significant loss of interneurons from uninjured and 2 weeks post injury were found at 6 weeks post injury in both the 75kd-60sec and 50kd-60sec injuries ($p<0.05$). There was no significant loss between the uninjured controls and the 75kd-60sec injury at 2 weeks. Uninjured n = 8; 2 weeks n= 5; 6 weeks 50kd n = 8; 6 weeks 75kd n= 6. Tested for significance with ANOVA.

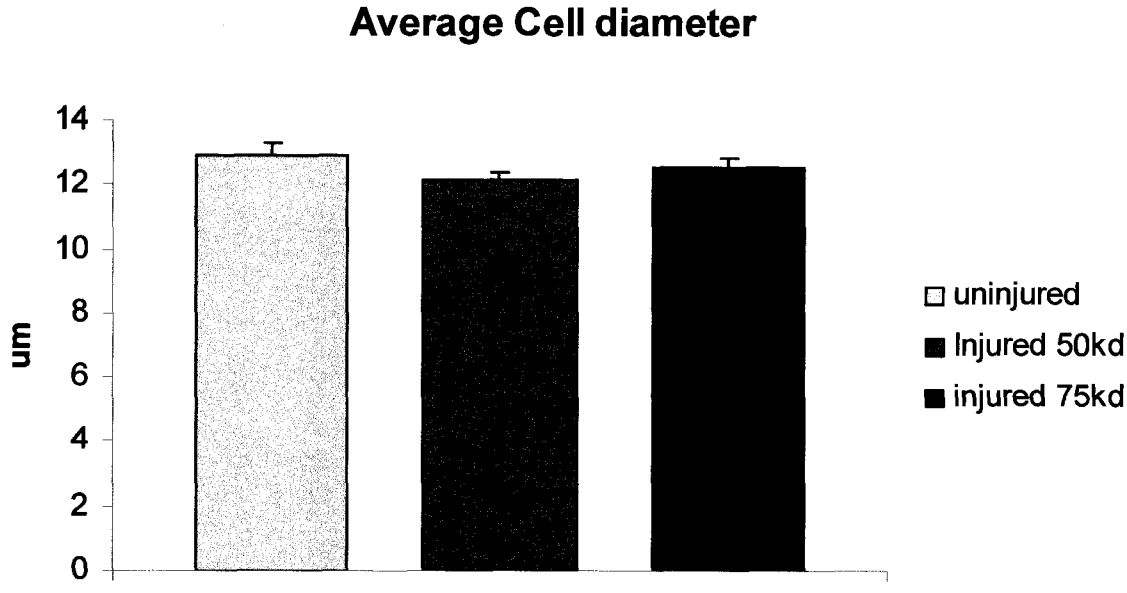


Figure 14 Cell diameter was measured and compared between injured and uninjured mice at 6 weeks post injury. There was no significant difference in cell size between the three treatment groups. Tested for significance with ANOVA. 48 GFP labeled cells from 6 animals were measured in each group.

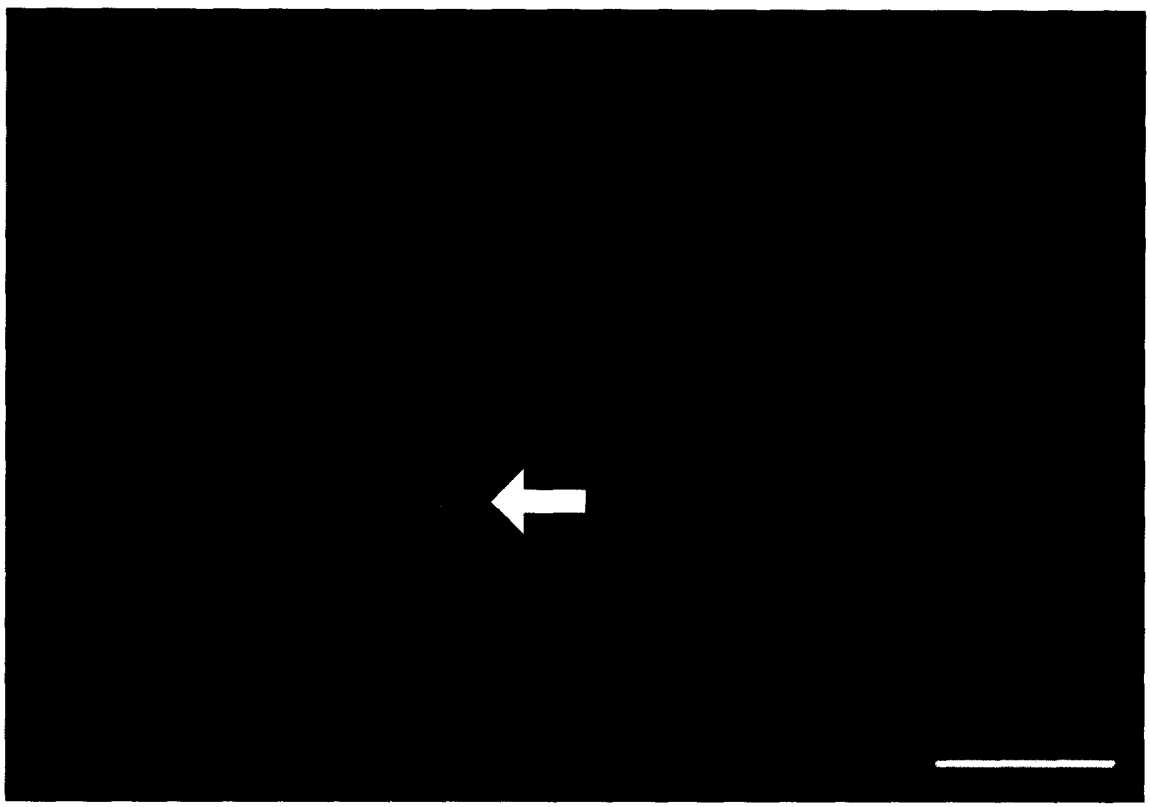


Figure 15 40X image taken on confocal microscope. Immunohistochemistry was used to identify degenerating interneurons 2 weeks post injury by using an antibody to the active form of caspase-3 (blue). Scale bar = 50um. Caspase-3 is a nuclear stain. Caspase-3 immunoreactivity was not tested on uninjured animals.

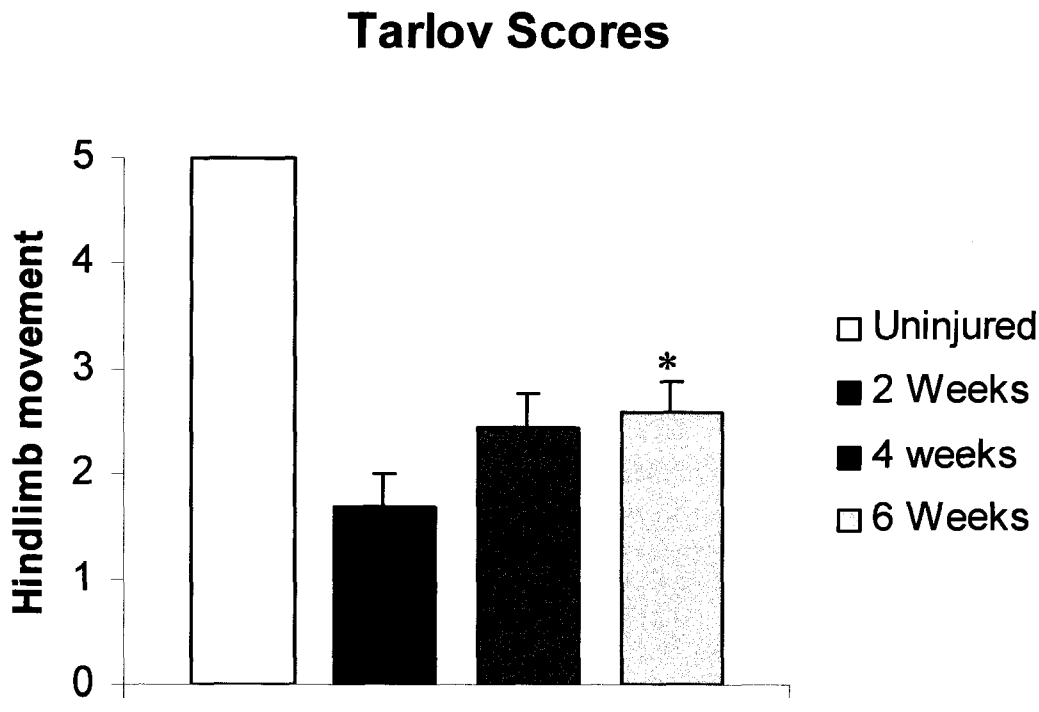


Figure 16 Hindlimb locomotion was assessed at 2, 4 and 6 weeks post injury at 50kd-60 sec. Both hindlimbs were observed and an average was taken. n=8 for each group. * = Significant increase in locomotion from 2 week timepoint ($p<0.05$). Tested for significance with ANOVA.

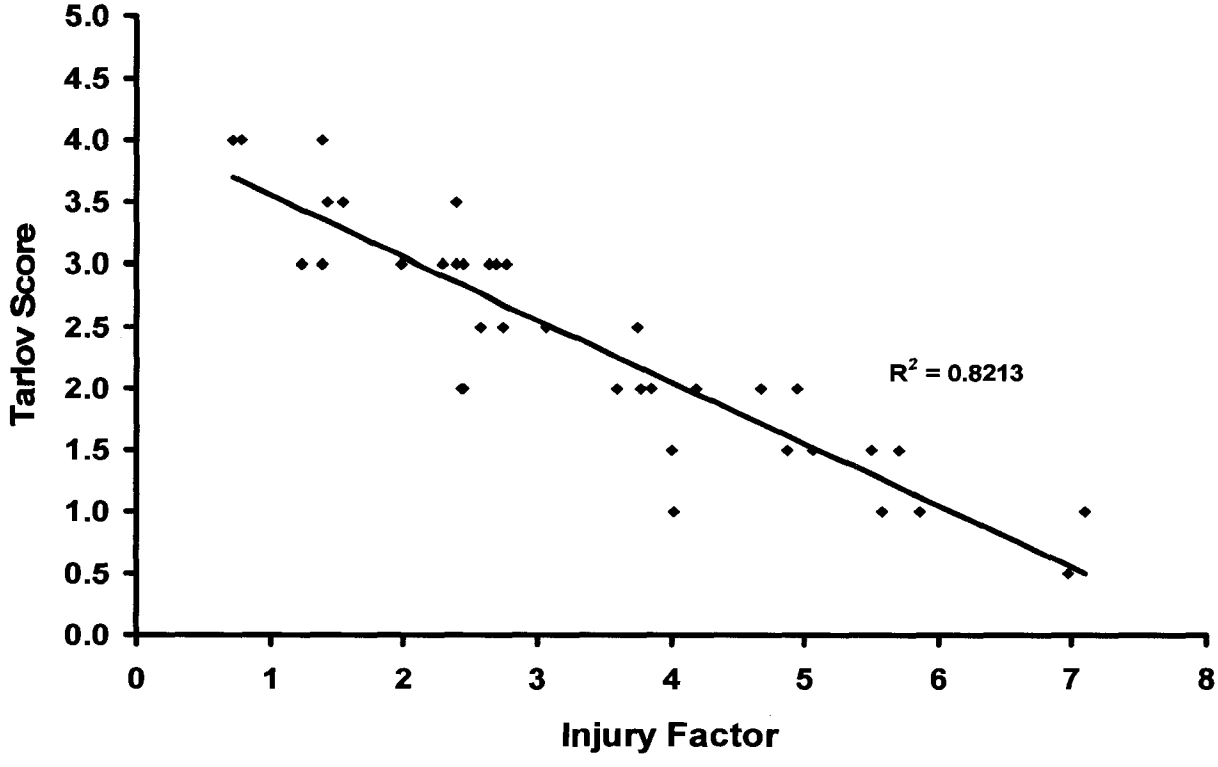


Figure 17 SCI was graded using the IF formula. As the injury factor increased, the Tarlov scores decreased indicating the formula is able to estimate severity accurately. n = 39. R^2 is the proportion of variability in this data set that is accounted for by this statistical model.

CHAPTER 4: DISCUSSION

4.1 Main Findings

In this study we have looked at thermal hyperalgesia associated with SCI and the factors contributing to it as well as methods of treating it pharmacologically. We were able to develop a mouse model of neuropathic pain in the form of thermal hyperalgesia via SCI. This hyperalgesia was found to begin at 3 weeks post injury and continued until testing ended at week 6. When injuries of different severities were compared for hyperalgesia there was no significant difference between the average withdrawal latencies, which indicates that the different injuries do not produce different amounts of hyperalgesia. We did not test whether the severity of injury influences the timeframe for development of hyperalgesia. It can be hypothesized, however, that the hyperalgesia will develop around the same time because of the fact that the severity of the hyperalgesia, in the form of hind paw withdrawal latency, is not significantly increased or decreased at 6 weeks post injury when the injuries are compared. This conclusion is further substantiated by the fact that the neuron counts between the different injuries were not significantly different, indicating that the same relative amount of sensitization and inflammation may be occurring. With respect to the neuron count, we found a significant decrease in a specific subpopulation of GABAergic inhibitory interneurons in the first 3 laminae of the dorsal horn at 6 weeks post injury but there was no loss at 2 weeks. Based on our hypothesis, it can be thought that there was a loss of inhibition in the dorsal horn in the form of

degenerating inhibitory neurons that we were able to show degenerating at the same time point when hyperalgesia begins to manifest (2 weeks).

Pharmacologically, we were able to investigate the role of GABA further by looking at pharmacological agents associated with GABA. GBP and TGB were administered at 6 weeks post injury and both were able to significantly attenuate hyperalgesia. TGB was able to completely block it at a higher dose, indicating that the role of GABA in neuropathic pain is an important one and also that there still remains some inhibitory interneurons for the TGB to act upon. Because of the motor in-coordination associated with a high dose of TGB, possibly a cocktail of TGB at a low dose coupled with GABAR agonists may give the same effect as a high dose of TGB but without the docility associated with it. More research is required to find the appropriate mix of pharmacological agents.

4.2 Thermal Hyperalgesia

The development and persistence of neuropathic pain and in particular hyperalgesia is a complex process which may be refractory to numerous events occurring simultaneously. These events include: central sensitization and windup (Woolf and Salter, 2000), loss of descending inhibition (Heinricher et al. 2003; Porreca et al. 2002; Ren and Dubner 2002), sprouting of primary afferent fibers (Ackery et al 2007) and loss of inhibition in the dorsal horn (Bennett et al., 1989; Dubner, 1991).

4.2.1 Development of Thermal Hyperalgesia

Thermal hyperalgesia was found to develop between 2 and 3 weeks after injury in a mouse model of spinal cord injury (50kd-60sec) and continued on to 6 weeks. This can be attributed to several changes within the CNS. After spinal cord transection, it has been shown that there is an increase in CGRP immunoreactivity in the dorsal horn at all laminas due to increased sensitization, beginning at 8 days and continuing in elevation to day 21 (Zinck et al 2007). Two weeks after spinal cord transection, there was a two fold increase in the area of CGRP immunoreactivity in the dorsal horn in the lumbar portion of the spinal cord (Krenz and Weaver 1998). The increase in sensory afferents causes an increase in c-fiber and Aδ fiber firing which can lead to an excess of glutamate at second order neurons leading to the release of the Mg blocks on the NMDA receptors of these neurons. This allows for phosphorylation of these receptors and an overall increase in the influx of calcium ions in the postsynaptic cell. Hypersensitivity of these neurons occurs and there is a facilitation or windup caused by repetitive firing of nociceptive inputs. At 8 days after injury, this sensitization may begin in accordance to sprouting and continue to increase as time after injury passes. At 2-3 weeks post injury, this sprouting may cause hypersensitivity of second order neurons and in turn contribute to hyperalgesia as observed in this study. This excess sprouting also leads to ectopic or abnormal activity in primary afferent fibres which may have an excitotoxic effect on dorsal horn inhibitory interneurons through glutamate "overload" which will be discussed later.

4.2.2 Forepaw and Hindpaw Hyperalgesia Testing

The lack of hypersensitivity found in the forepaw can be attributed to the fact that the injury is at T10 and sensitization may not reach that far rostral to the injury site. In addition, the forepaws still have supraspinal connections and therefore still have descending inhibitory input, unlike the hindpaw. A loss of dorsal horn inhibition due to apoptosis of inhibitory interneurons would be most prevalent in the segments closer to the injury site which could contribute to secondary hyperalgesia in the hindpaw which has connections to the L2-4 segments but not in the forepaw which has connections much further away in the C7-8 segments. Also, damage to ascending pathways will be more prevalent in areas caudal to the lesion which may lead to increased hyperalgesia.

4.2.3 Thermal Hyperalgesia and Injury Severity

Mice were injured at 50kd-60sec, 50kd-0sec and 75kd-60sec and tested for hyperalgesia. No significant difference in withdrawal times was found between the three groups. This can be attributed to the possibility that these injuries are similar in severity and there was not enough variability among the injuries to allow for a change in hyperalgesia. It has been shown in a human clinical study that the completeness of an SCI does not have a significant effect on the presence of pain overall (muscoloskeletal, neuropathic etc). Siddall et al (1999) found that there was no relationship between the presence of pain overall and the level or completeness of lesion, or the type of injury. Significant differences were found, however, when specific types of pain were examined. Musculoskeletal pain was more common in

people with thoracic level injuries. Neuropathic pain associated with allodynia was more common in people who had incomplete spinal cord lesions, cervical rather than thoracic spinal cord lesions, and central cord syndrome. It could be concluded from this that the mice in our study had an incomplete injury which allowed for the development of severe hyperalgesia. The injuries were, however, too close in severity to result in significant differences in hyperalgesia. After complete transection of the spinal cord, there would be no hyperalgesia as some input to the brainstem is required.

4.3 GFP Positive Inhibitory Interneuron Loss After SCI

We found a significant loss of dorsal horn GFP positive inhibitory interneurons (lamina I-III) at 6 weeks after the injuries (50kd-60sec and 75kd-60sec). This is the most important finding in this study as it supports the hypothesis that thermal hyperalgesia may be due in part to a loss of GABAergic inhibitory interneurons. This will lead to an overall loss of inhibition in the dorsal horn and therefore the loss of the ability to inhibit painful stimuli. There may be several reasons why this cell loss is occurring. It is important to note that not all inhibitory interneurons were counted, only those that were GAD 67:GFP positive. It is unknown as to whether the other inhibitory interneurons are degenerating. A further study is needed to examine if the remainder of the inhibitory interneurons are also undergoing cell death. It has been shown that there is no loss of GABAergic or glycinergic in the CCI model after 2 weeks post injury while hyperalgesia did occur (Polgar et al., 2003). Although the hyperalgesia did occur at an earlier point than our model, their

findings of no loss at 2 weeks post injury was the same result as our study shows. At 6 weeks post injury, it is possible that these GFP neurons are not going through apoptosis but are simply having their promoter, GAD67 turned off. It has been shown that after a thoracic spinal cord transaction, there was an increase in GAD67 expression in the dorsal horn (Tillakaratne et al., 2000) so it is unlikely that the promoter is being turned off. This, coupled with the fact that caspase-3 was found to localize in the GFP neurons, we can conclude that these neurons are going through cell death. Possible changes (ie. cell loss) in other types of neurons was not examined so selectivity remains to be investigated.

4.3.1 Excitotoxicity of Glutamate

It has been suggested that the increased primary afferent activity in the neuropathic models discussed previously may result in excitotoxic death of some neurons in the superficial laminae of the dorsal horn (Sugimoto et al 1990), and this may therefore have contributed to the loss of GABAergic neurons reported in our study. Myelinated A-fibres terminate in lamina I and III-VI, while unmyelinated C-fibres terminate predominately in lamina II. Peripheral nerve injury triggers sprouting of A-fibre afferent terminals into lamina II which may be partly responsible for the sensory abnormalities that occur following nerve injury (Woolf et al 1992). The mechanism for this sprouting is not entirely clear although it is thought that two factors are involved: 1) C-fibre injury (through peripheral target deprivation which can cause C-fibre atrophy leading to vacant synaptic sites in the superficial dorsal horn and in particular lamina II) and 2) A-fibre injury (through induction of

regeneration mechanisms and conditioning of the sensory neurons by peripheral axotomy) (Woolf et al 1992). It has been shown by choleragenoid-horseradish peroxidase labeling that there were 15 times more synaptic terminals in lamina II, 2 weeks after axotomy than in the uninjured group (Woolf et al 1995). This sprouting is not permanent, however; in the peripheral nerve injury model at 6-8 months after injury, the A-fibre sprouting will withdraw from lamina II. If the nerve is resectioned, however, A-fibres will once again sprout into lamina II. On the basis of this it can be concluded that target deprivation induced C-fibre atrophy is not a necessary condition for A-fibre central sprouting (Qing-Pang Ma 2000). Once the A-fibres have sprouted into lamina II, they, along with the remaining C-fibres, will begin to develop spontaneous ectopic activity which is associated with central sensitization. Upon stimulation of these fibres and the consequent release of an excessive amount of glutamate, there will be cell death as the converging activated fibers on a particular cell exceed the cell's capacity to handle the excessive glutamate load. This has been shown to occur in a sciatic nerve injury model when the myelinated fibers in lamina II were stimulated (Coggeshall et al., 2001). This occurs because of the high calcium influx after activation of the NMDA glutamate receptor which is associated with excitotoxic apoptosis (Hardingham and Bading, 2003). After administration of glutamate via microdialysis at a concentration similar to that found after SCI, glutamate has been shown to damage neurons several hundred micrometers away from the fiber (Liu et al 1999). This supports the view that glutamate has an excitotoxic effect in a SCI model which may explain the cell death observed in the present study. Although most of these results were found in a peripheral nerve injury

model of neuropathic pain, these changes within the nervous system are similar to the sensitization of neurons within the dorsal horn as well as the pain behaviours found in SCI models and patients.

Caspases play a key role in mediating apoptosis when death receptors are activated. Apoptotic pathways initiated by these diverse events ultimately converge on the cleavage and activation of the "executioner" protease, caspase-3. Loss of neurons in the dorsal horn may be due in part to excessive glutamate released from primary afferent fibres which activates caspase-3. Glutamate induces caspase-3 mediated apoptosis by activating microglia in the spinal cord. Microglia activate the CD95 ligand which binds to the Fas receptor and initiate apoptosis (Beattie 2004). It has been shown in a SNI model of neuropathic pain that when the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD) is applied, it prevents the loss of GABAergic interneurons and increases inhibitory currents (Scholz et al 2005). zVAD, which has no analgesic properties when administered alone, was also able to attenuate neuropathic pain, giving strong evidence that the loss of inhibition in the dorsal horn may be due to cell death which does in fact lead to neuropathic pain. At 2 weeks post injury, we found the presence of caspase 3 in GFP positive neurons indicating apoptosis which leads to loss of inhibition at the time when hyperalgesia was found to develop. Second order neurons may also have an increase in primary afferent connections although it is not known as to whether these neurons are also degenerating or are only becoming sensitized.

4.3.2 Inflammation Following SCI

After injury, the area around the lesion contains damaged cells and excess blood from necrotic and apoptotic cell death. The surrounding white matter and grey matter rostral and caudal to the lesion will initially remain remarkably intact. However, over a time course of minutes to hours, the lesion is thought to spread centripetally, initially by the induction of necrotic cell death that is mediated, for example, by excitatory amino-acid-induced Ca^{2+} entry, nitric oxide (NO) production, oxidative stress and membrane breakdown (Casha et al 2001). The injury induces, first, a slowly spreading cell death that is characterized by apoptotic neurons at the lesion site and, later, microglial activation and apoptosis of oligodendrocytes in areas with degenerating axons that were injured at the original lesion site (Casha et al 2001 and Beattie MS et al 2000). This inflammatory response will lead to the release of peptides and cytokines which contribute to afferent fiber activation and sensitization.

4.4 Analgesia

Enhancement of GABAergic neurotransmission may provide a way to decrease the level of nociception in different pain states and in particular neuropathic pain. In an attempt to alleviate thermal hyperalgesia, two drugs were administered as analgesics. GBP, which was originally used as an anti-convulsant treatment as an add-on medication for partial seizures (Taylor et al 1998) and has been used in treatment of neuropathic pain (Wheeler 2002) was administered intraperitoneally at 6 weeks after injury in an effort to block thermal hyperalgesia. GBP has been shown clinically to improve neuropathic pain in patients (Nicholson 2000 and Backonja M 2003) as

well as experimentally in the rodent model (Moore et al 2002A). GBP is a proven analgesic for patients suffering from neuropathic pain. Because of this, we decided to compare a novel analgesic against GBP. Tiagabine is relatively new in comparison and has yet to be studied in SCI induced neuropathic pain and in particular thermal hyperalgesia. Tiagabine was also administered intraperitoneally at 6 weeks after injury in an effort to block thermal hyperalgesia.

4.4.1 Gabapentin

As expected, administration of GBP was found to increase withdrawal time and diminish thermal hyperalgesia. To understand the reason why GBP is effective, it is important to understand the mechanism of action of GBP. Despite its name, there is no clear relationship between GBP and GABAergic neurons. GBP shows little affinity to the $GABA_AR$ or the $GABA_BR$ (Suman-Chauhan et al 1993). The antinociceptive effects of intrathecal gabapentin in the formalin test (Yoon et al 2003) and L5/6 nerve ligation pain model (Hwang and Yaksh 1997) were unaffected by the $GABA_AR$ antagonist bicuculline. Therefore, it is generally believed that the $GABA_AR$ is not involved in the actions of GBP. It has been proposed that a heterodimeric subtype ($GABA_{B1a-B2}$) of the $GABA_BR$ predominates in the superficial laminae of the dorsal horn and is selectively activated by GBP (Bertrand et al 2001; Bertrand, Sullivan et al 2001). However, the $GABA_BR$ antagonist CGP 35348 has failed to diminish the effect of GBP in L5/6 nerve ligation (Hwang and Yaksh 1997, Hwang and Yaksh 1997) and partial sciatic nerve ligation (Patel et al 2001) despite being administered at doses that have effectively antagonized the antinociceptive effect of

baclofen. Therefore it can be concluded that $GABA_BR$ are not involved in gabapentin analgesia.

The α2δ subunit of voltage-dependent Ca2+ channels has been identified as a high affinity binding site of GBP (Suman-Chauhan et al 1993). By binding to the α2δ subunit, GBP may modulate Ca2+ currents to increase or decrease neurotransmitter release (Maneuf et al 2003) or neuronal excitability (Stefani et al 1998) presynaptically. GBP has the highest affinity for the α2δ-1 subunit when compared to the α2δ-2 and α2δ-3 subunits of Ca^{2+} channels (Maraise et al 2001). The α2δ-1 subunits of Ca^{2+} channels were found to be upregulated in the spinal cord and dorsal root ganglia (DRG) of the mechanical and diabetic neuropathic rat and mouse pain models (Luo et al 2001). Through this subunit, GBP is able to inhibit Ca2+ channels which would result in a decrease in neuronal excitability and synaptic transmission. GBP was found to inhibit glutamatergic synaptic transmission presynaptically in the superficial, but not deep, lamina of the spinal dorsal horn in normal rats (Shimoyama et al 2000). Since the primary neurotransmitter in afferent fibres is glutamate, this could explain GBP's method of analgesia if glutamate is inhibited in the dorsal horn. GBP has been shown to be able to inhibit C-fibre evoked response in a carrageenan induced inflammatory pain study but did not affect Aβ-fibre evoked response even when the dose was high enough to completely inhibit C-fibre response. This indicates that GBP is selective on noxious evoked responses of dorsal horn neurons (Stanfa et al 1997). GBP, when administered in uninjured mice, showed no increase or decrease in withdrawal times. This can be attributed to the fact that GBP has been shown to have no effect on normal afferent fiber activity but is able to inhibit the ectopic

discharge activity associated with peripheral nerve injury (Pan et al., 1999). This may be attributed to conformational changes of calcium channels after injury and in specific the α2δ-1 subunits. These channels may become phosphorylated and have a stronger affinity for GBP after injury. All of these factors may contribute to the analgesic effect of GBP which was observed in this study. Although GBP is useful as a clinically relevant "gold standard," the lack of a clearly defined mechanism of action makes it less attractive for basic research purposes. GBP is a well known drug which has well established efficacy. The purpose of its use in this thesis was to not test for its efficacy but to compare it to TGB, a drug with a clear selective mechanism of action.

4.4.2 Tiagabine

At 6 weeks post injury TGB was administered by intraperitoneal injection at 1 mg/kg and 10 mg/kg and by intrathecal injection at 0.4 mg/kg and thermal hyperalgesia was tested. We found a significant increase in withdrawal time after treatment. Unlike GBP the mechanism of action for TGB is known.

TGB is currently the only selective GABA reuptake inhibitor available in US markets; it exerts its action via GAT-1 (GABA transporter expressed in neurons, presynaptic terminals and glial cells around the synapse) blockade presynaptically, facilitating GABA neurotransmission (Schwartz and Nihalani 2006) and suppressing afferent input. This will indirectly activate $GABA_A$ and $GABA_BRs$. It was shown in this study that TGB was able to increase withdrawal latency in all doses. Enhancement of the neurotransmission of GABA may be an important factor in

diminishing the level of nociception in various pain models and in particular neuropathic pain. GABA is the main inhibitory neurotransmitter in the CNS and an increase in GABA will lead to an inhibitory response in post-synaptic neurons and in this case, nociceptive specific second order neurons.

At high doses (10mg/kg), TGB completely abolished thermal hyperalgesia. At this high dose, however, mice were observed to be in a docile state and the overall movement of the mice in the chambers was decreased. They were not observed to be in a catatonic state although signs of sedation and motor impairment were apparent. This can be attributed to the fact that TGB does not increase GABA levels in the dorsal horn alone but, with systemic administration, can increase GABA levels in the brain (Fink-Jensen et al., 1992). This in turn may be enhancing descending inhibition from the brain stem which may be decreasing the facilitation of movement. The globus pallidus of the basal ganglia contains binding sites with a very high affinity for TGB (Suzdak et al 1994). Such binding will lead to a decrease in the facilitation of movement. It has been shown that after systemic administration of TGB there was a significant increase in extracellular GABA levels in the globus pallidus (Fink-Jensen et al., 1992). This is why this is an important area in the study of Parkinson's disease and in treatments for antiepileptic drugs and why inhibition of movement can be observed after high doses of TGB.

After administration of TGB intraperitoneally and the subsequent behavioural testing, we wanted to look at the effects of a direct intrathecal injection on thermal hyperalgesia. TGB was administered via intrathecal injection at 0.4mg/kg and a significant increase in withdrawal time was observed. This can initially be attributed

to the fact that TGB is being injected centrally and is able to work directly at the spinal level. However, in previous studies it has been shown that TGB administered intrathecally was ineffective in the hot-plate test and the dynorphin-induced allodynia test (Laughlin et al., 2002). This would indicate that TGB evidently exerts its potent antinociceptive effect at a nonspinal site. Therefore, there may be several reasons why there is an increase in withdrawal latency after intrathecal injection in this experiment. Firstly, in this study SCI induced thermal hyperalgesia was used as opposed to the hot-plate test or the dynorphin-induced allodynia test which are not models of neuropathic pain. After this traumatic injury, there will be an inflammatory response which will eventually lead to scar tissue formation. This may make the diffusion of a drug rostrally up the spinal cord more difficult, leading to more of the TGB remaining caudal to the injury where the below level pain is found and where the effects of the drug can be concentrated. Secondly, there may have been a problem with the intrathecal injection. To administer the TGB, the drug was injected with a syringe directly intrathecally through the vertebrae as opposed to performing a surgical laminectomy and inserting an intrathecal catheter. Because of this, it may have been possible that the injection may have gone into the muscle adjacent to either sides of the vertebrae at the injection site or possibly into sub-cutaneous tissues. This may have had a similar effect as an intraperitoneal injection. Overall, TGB has been shown to be a promising pharmacological agent as an analgesic for neuropathic pain. In this study, TGB has been shown to be a more potent drug to GBP in the suppression of painful hyperalgesia after SCI.

4.5 Injury Factor

Based on the equation that was formulated, there is a significant correlation between the injury factor and Tarlov scores. The equation: IF = [(F * V * M) / (1/D + 1)] / 1000 was formulated by taking several different factors into account. The variables which were found to have the greatest impact on hindlimb locomotion were placed in the numerator (F, V, M) while the variable in the denominator (D) was placed there because of its impact on locomotion. The D is divided into 1 and then 1 is added to it to limit its ability to influence the IF significantly. The equation is divided by 1000 to keep the IF low so it can be easily compared to Tarlov scores.

CHAPTER 5: CONCLUSIONS

The results suggest that there is a potential loss of inhibition in the dorsal horn leading to an exacerbated response to thermal hindpaw stimulation. This loss of inhibition is suggested by the fact that there was a significant loss of dorsal horn inhibitory neurons which are a major source of inhibition for painful stimuli. It was beyond the scope of this project to determine the exact cause of this cell death. Excitotoxicity induced by excessive glutamate from sprouting is one hypothesized cause for this cell death.

Neuropathic pain is an extremely important field of study. A reduction of neuropathic pain can have a significant and immediate impact to enhance quality of life for SCI patients who already have many other obstacles to overcome. The goal of testing the analgesics gabapentin and tiagabine was to be able to find a therapeutic way to decrease hyperalgesia by using a drug whose method of action is based around the neurotransmitter GABA (tiagabine). Tiagabine was able to block hyperalgesia completely with a high dose, indicating that the role of GABA and in particular the GABAergic inhibitory interneurons is a very important one with respect to chronic neuropathic pain.

By using different degrees of injury severity, we originally hypothesized that there would be a change in the development and/or severity of hyperalgesia. Surprisingly there was no difference in severity of hyperalgesia which can be attributed to the fact that the injuries were relatively consistent even between injuries of different force. If there was a larger discrepancy between the severities of the

injury then the severity of hyperalgesia would change, although, if a small injury can still cause cell death of inhibitory interneurons then this may be enough to develop and maintain a constant state of hyperalgesia despite the difference in injury severity. The development of hyperalgesia was not tested between injury severities but based on the same manner of thinking, it would be thought that it would develop at relatively the same time as the 50kd-60sec injury tested.

In this study a neuropathic pain model was developed and used to study pharmacological attenuation of hyperalgesia. The results show that the reduced inhibition in the spinal cord may be due to the loss of inhibitory interneurons which is consistent with our hypothesis. These findings will hopefully shed some light onto the field of neuropathic pain after SCI and will hopefully lead to further research and findings which will eventually aid in improving quality of life to all SCI patients.

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