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Reactive Oxygen Species and Lipid Peroxidation Products Contribute to Neuropathic Pain in Chronic Spinal Cord Injured Rats

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Reactive Oxygen Species and Lipid Peroxidation Products Contribute to Neuropathic Pain in Chronic Spinal Cord Injured Rats

by

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Dedication

To those who suffer from chronic pain, and to all those that try to end such suffering.

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Reactive Oxygen Species and Lipid Peroxidation Products Contribute to Neuropathic Pain in Chronic Spinal Cord Injured Rats

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Spinal cord injury (SCI) can result in loss of locomotion, sexual and bladder function, and chronic neuropathic pain. A rat model of spinal cord injury was used to study the mechanisms of chronic neuropathic pain. Chronically up-regulated reactive oxygen species (ROS) generated in neurons as a result of spinal cord injury contribute to the chronic neuropathic pain measured in rats. When ROS was reduced in chronic SCI by using PBN, a ROS scavenger, there was a reduction in neuropathic pain behaviors and a reduction in hyperexcitability of dorsal horn neurons in rats. Compounds that reduce ROS and lipid peroxidation are able to reduce measures of chronic neuropathic pain. Mechanical allodynia was reduced in chronic SCI rats with the intrathecal administration of Apocynin, 4-OXO-TEMPO, Tirilazad, and U-83836E. Apocynin was used to further examine the ability of ROS and lipid peroxidation products to generate neuropathic pain behaviors in chronic SCI rats. Apocynin was found to reduce expression of 4-HNE, a lipid peroxidation product, in chronic SCI rats when administered intraperitoneally 30 minutes before perfusion and fixation. Evoked and non-evoked measures of neuropathic

pain were reduced in chronic SCI rats when Apocynin was applied intrathecally and intraperitoneally. Apocynin was also able to reduce hyperexcitability of dorsal horn neurons in chronic SCI rats. These findings indicate that ROS is a major contributor to chronic neuropathic pain resulting from SCI. Further investigation of the relationship between reactive oxygen species and chronic neuropathic pain may lead to better understanding of the biological processes of chronic pain and more effective therapies for treating injury induced neuropathic pain.

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List of Abbreviations

UTMB	University of Texas Medical Branch
GSBS	Graduate School of Biomedical Science
ROS	Reactive Oxygen Species
LP	Lipid Peroxidation
SCI	Spinal Cord Injury
TBI	Traumatic Brain Injury
NP	Neuropathic Pain
CNS	Central Nervous System
4-HNE	4-Hydroxynonenal
WDR	Wide Dynamic Range
IP	Intraperitoneal
IT	Intrathecal

Chapter 1 Introduction

1.1 Chronic neuropathic pain resulting from spinal cord injury

Chronic pain affects 116 million people per year in the United States and costs the country over 635 billion dollars for treatment fees and lost productivity annually (Institute of Medicine, 2011). Pain is considered chronic if it persists for more than three months and is usually the result of an initial injury. Neuropathic pain is a chronic and difficult to diagnose and treat manifestation of pain which often does not respond to typical analgesic treatments (Murphy and Reid, 2001; Vissers, 2006). Patients experience the symptoms of neuropathic pain as sharp tingling, numbness, or burning sensations (Siddall and Loeser, 2001). The lack of effective treatments can leave the patients in constant pain, leading to increased episodes of depression and suicide (Cairns et al, 1996; Widerstrom-Noga et al. 2001; Blair et al, 2003). Chronic pain patients also experience disturbances to their sleep and often require medication to prevent insomnia (Rintala et al., 1998). Understanding the mechanisms behind chronic neuropathic pain will facilitate development of targeted therapies for people suffering from chronic neuropathic pain.

Neuropathic pain can manifest in two ways, either as an increase in perception of stimuli or as a spontaneous occurrence (i.e. not a result of a physical stimulus). Patients often complain of both aspects of chronic pain (Daviddoff and Roth, 1991; Willis and Coggeshall, 1991). The increase in pain evoked from normally non-noxious stimuli or an even more intense pain after application of noxious stimuli is easily measured and is the common means of studying pain. However, pharmacological compounds designed to treat chronic pain, that were tested only using evoked measures of pain, tend to be

unsuccessful when tested on human patients that are suffering from chronic pain. It is thought that this discrepancy is due to the spontaneous pain involved in chronic pain disorders being the major complaint. While novel pain medications may be excellent analgesics for traditional evoked pain, the mechanisms that underlie spontaneous pain may be so different from evoked pain that the compounds successful in treating evoked pain may not be effective for treating spontaneous pain (King et al., 2011; King and Porreca, 2014). For this reason many pain researchers are implementing behavioral assays that measure the behaviors associated with pain without using direct stimuli to elicit responses (Sufka, 1994; Sotocinal et al., 2011; Fuchs and McNabb, 2012).

1.2 CONTUSION MODEL OF SPINAL CORD INJURY

Major trauma to the back and neck often result in spinal cord injuries (SCI). When the spinal cord is injured, intracellular and extracellular signaling events contribute to enhanced maladaptive nociceptive transmission in the spinal dorsal horn via increased neuronal release of neurotransmitters and inflammatory cytokines (Detloff et al., 2008; Ferguson et al., 2008), overexpression of excitatory and inhibitory receptors and ion channels (Hains et al., 2003; Mills et al., 2001; Pabbidi et al., 2008), and activation of glial cells (Gwak et al., 2008; Gwak and Hulsebosch, 2009). The majority of injuries to the spinal cord involve blunt mechanical trauma to the vertebral column resulting in temporary or permanent paralysis below the level of injury and motor and bladder control deficits. A contusion mediated injury to the spinal cord is a highly translatable model to clinical settings since it parallels what a SCI patient population would encounter (Bung et al., 1993; Bunge, 1994). For these studies, a moderate (150kdyne) contusion injury was used to model SCI. The animals that underwent SCI had similar deficits to those seen in

clinical populations and recovered bladder control around 10 days after injury, and only had slight locomotor deficits 30 days after injury as seen in figure 1.1.

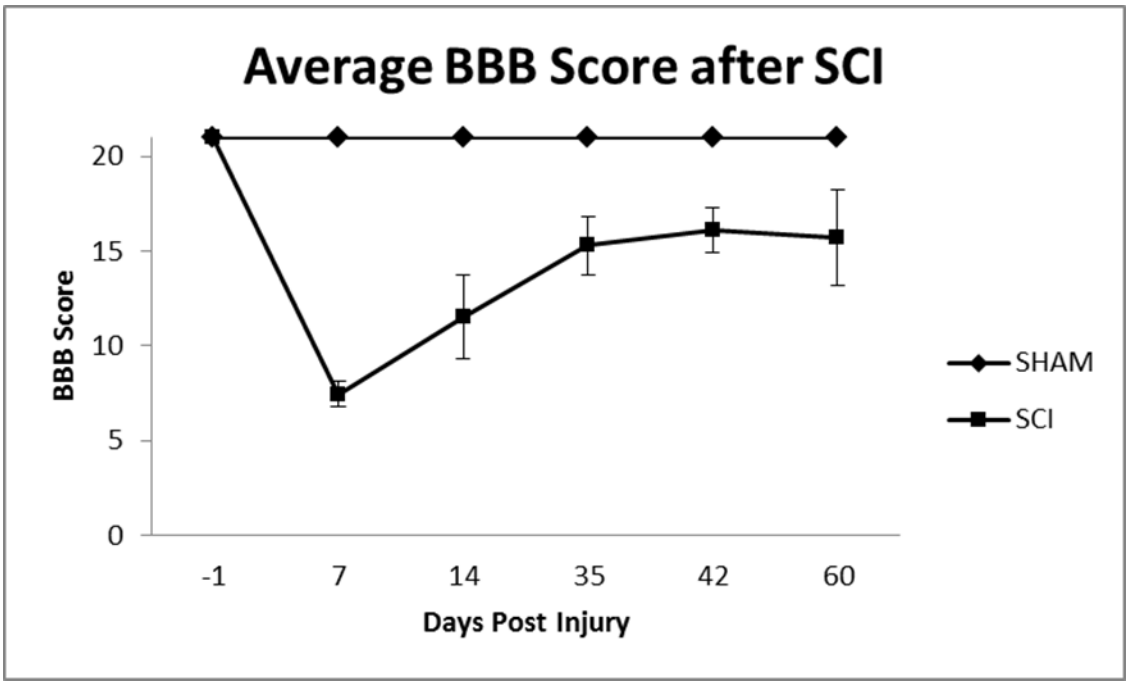

Figure 1.1 BBB scores from a representative group of SHAM and SCI rats.

Little or no motor deficits are seen with sham operated animals. SCI animals typically lose all function of hindlimbs (BBB Score ≈ 0) for the first few days after surgery and recover hindlimb function within 1 week of the surgery. 30 days after the surgery, the SCI animals will display only slight deficits in motor function.

A common complaint after a SCI is the development of persistent pain, often immediately after the injury or within a year of the injury (New et al., 1997; Stormer et al., 1997). Chronic pain is often the result of damage to nervous tissue, with up to 75% of SCI patients complaining of chronic pain (Anderson, 2004; Christensen and Hulsebosch, 1997). While most sensory systems will experience some measure of function loss after an injury, pain sensation is often times described as increased for noxious and non-noxious stimuli after an injury to nervous tissue. This maladaptive property of pain sensation is what is partly responsible for the difficulty in treating chronic pain disorders (Costigan et al., 2009). As a result of maladaptive changes in protein and lipid membrane structures in somatosensory neurons, especially in spinal cord neurons, neuropathic pain can develop and be maintained. This often results in the clinical symptoms of allodynia, where normally non-noxious stimuli become noxious, or hyperalgesia, where normal, noxious stimuli become even more noxious (Crown et al., 2006; Crown et al., 2012).

SCI-induced neuropathic pain is grouped into three categories based on the region of pain involvement (Slidell et al., 2002). First, above level pain, which is pain sensed in dermatome regions rostral to the spinal cord injury area. Above level pain is considered to be musculoskeletal in origin and not necessarily a result of neuronal pathophysiology, however, above level neuropathic pain has been observed in cervical and lower thoracic spinal cord injury models and may involve activation of glia cells (Apple, 2001; Carlton, et al., 2009). Second, at level pain, which is pain sensed in dermatome regions at or near the injury site. At level pain is considered to be a result of spinal cord cell loss, particularly local inhibitory neurons (Crown et al., 2006). Third, below level pain, which is pain sensed in dermatome regions caudal to the injury site. Below level pain, the most reported post SCI-induced pain, and is considered to be a result of loss of descending inhibition from the damage and degeneration of descending axons (Gwak and Hulsebosch, 2008). Typically at level pain is reported as proceeding below level pain,

given the indication that the mechanisms that develop neuropathic pain after SCI in these dermatome regions may be similar (Vierck et al., 2000).

The process by which neuropathic pain develops in the central nervous system after SCI is called central sensitization. Central sensitization is characterized by an increase in hyperexcitability in spinal dorsal horn neurons, which often results in the neurons becoming more responsive to noxious and non-noxious mechanical and thermal stimuli. Central sensitization can occur over weeks to months after an injury and ultimately leads to increased sensitivity to noxious stimuli, this process is observed in our SCI contusion model as seen in figure 1.2. Central sensitization is observed in many injury models involving major nervous tissue, with many hypotheses for the mechanisms which are involved in generating sensitization. Some of the proposed mechanisms are disinhibition of local or descending circuits (Basbaum and Wall, 1976; Sweet, 1991), increased sensitivity due to denervation (Nakata et al., 1979), activation of silent synapses (Devor and Wall, 1981), discharge of nociceptive C fibers (Willis, 1993), and increases in excitatory amino acids after injury (Faden and Simon, 1988). All of the proposed mechanisms for central sensitization involve a change in the central nervous system that is secondary to the injury or insult and always leads to a pathological pain state.

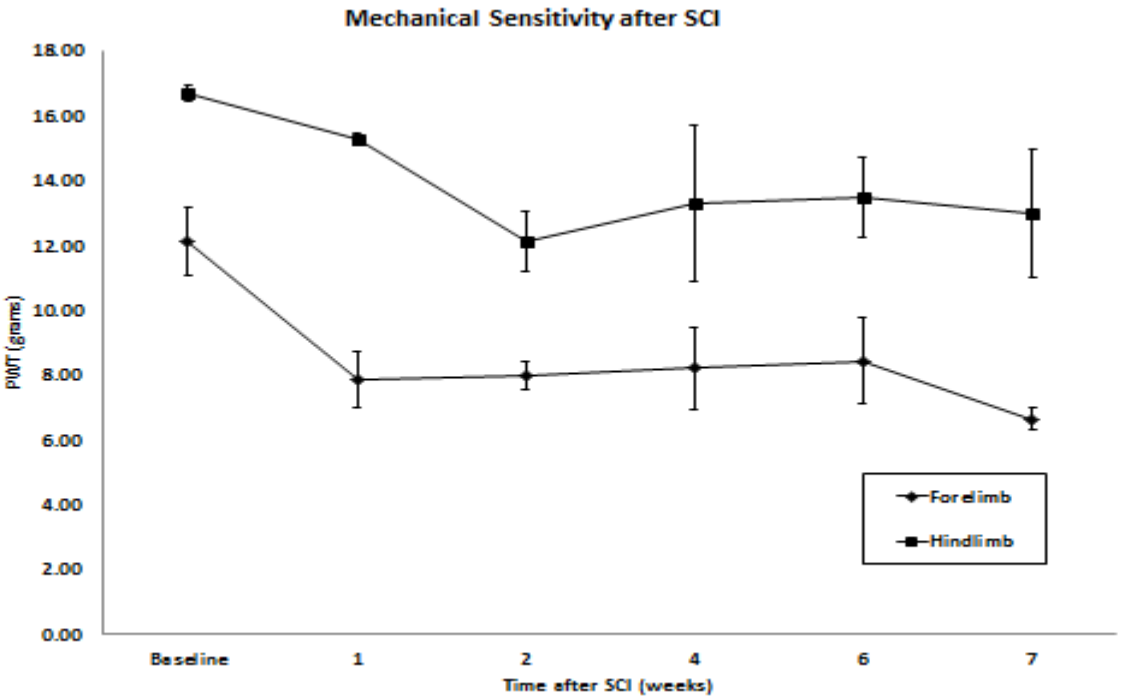

Figure 1.2 Mechanical Sensitivity after SCI in rat hindlimbs and forelimbs as measured by paw withdraw thresholds (PWT).

An increase in mechanical sensitivity is seen in animals starting one week after injury, with the increased mechanical sensitivity persisting for up to seven weeks. The animals are considered have increased sensitivity to mechanical stimuli since it takes less of a stimulus (force in grams) to initiate a response from the animals.

1.3 REACTIVE OXYGEN SPECIES AND LIPID PEROXIDATION AFTER SPINAL CORD INJURY

Reactive oxygen species (ROS) are a natural consequence of mitochondrial respiration. Cells take in and harvest an electron from oxygen to produce adenosine triphosphate (ATP), the energy currency used by biological organisms. Superoxide, the byproduct of this process is highly reactive with other molecules in the cell as it chemically seeks to restore its lost electron. Superoxide and similar molecules that make up ROS are normally kept in check and scavenged from the intracellular spaces by endogenous housekeeping mechanisms. However, in times of great stress or damage to

the cell, the housekeeping molecules are busy trying to repair the cell, leaving the superoxides to build up in the cell. When this occurs, built up ROS can oxidize important molecules required for cellular function leading to further damage to the cell. ROS is a particularly dangerous group of molecules for neurons since neurons require a great amount of ATP to function, and thus produce a great quantity of ROS. Reactive oxygen species are also highly involved in signal transduction mechanisms in the spinal cord (Kennedy et al., 2012). In the CNS, there are two types of ROS that have been identified; first there are radical groups, which have an unpaired electron in the valence shell, such as hydroxyl radicals, superoxide anions, and nitric oxides; and a second nonradical type of ROS composing of peroxynitrites and hydrogen peroxides (Kohen and Nyska, 2002). These two groups of ROS molecules are highly reactive and significantly contribute to cellular mechanism involved in signaling (Goldstone et al., 1997; Guedes et al., 2008). SCI and other forms of neurotrauma often result in increased production of ROS and a disruption of the neurons' production of oxidants and antioxidants. This disruption to the neuron is often followed by changes, both physiological and structural, and can initiate processes such as lipid peroxidation, protein S-thiolation, and DNA damage (Bains and Hall, 2012; Mikkelsen and Wardman, 2003; Rokutan et al., 1989; Sugawara et al., 2002). The oxidation of proteins, lipids, and DNA resulting from pathological concentrations of ROS are referred to as oxidative damage.

Researchers that study trauma to nervous tissue, such as spinal cord injury or traumatic brain injury (TBI) have to take into account the mechanisms of ROS when studying their diseases, since the secondary damage caused by oxidative damage can be just as significant as the initial injury. Limiting oxidative damage after nervous tissue injury is a major clinical challenge. ROS has recently been implicated in mediating chronic pain after spinal cord injury (Kim et al, 2004). At chronic time points after an injury ROS molecules were still up-regulated near the injury site (Gwak and Hulsebosch, 2008) and that application of ROS scavengers was able to reduce neuropathic pain like

behaviors (Gwak et al., 2013). ROS has been shown to alter calcium concentrations in neurons after an acute injury. This imbalance of calcium concentrations in neurons is a consequence of ROS stimulating mitochondrial signaling and has been shown to be involved in epilepsy and traumatic brain injury (TBI) (Martinc et al., 2012; Xiong et al., 1997). It is likely that ROS contributes to the hyperexcitability observed in dorsal horn neurons after SCI via stimulation of mitochondrial release of calcium, and that this hyperexcitability of dorsal horn neurons is the neuroanatomical correlate of neuropathic pain. However, it is not known to what the extent that ROS plays in the generation or maintenance of neuropathic pain.

Lipid peroxidation is a downstream consequence of ROS, where lipids are stripped of protons from their double bonds by hydroxide ions and further oxidized by molecular oxygen on the exposed single bond. This newly formed lipid peroxyl radical then propagates the lipid peroxidation cycle by stealing protons from other lipids. These alkoxyl radicals can be further broken down by iron dependent cleavage into the smaller toxic compounds such as 2-propenol (acrolein) and 4-hydroxynonenal (4-HNE). Increases in lipid peroxidation products have been observed in neurotrauma models, such as traumatic brain injury (TBI) and spinal cord injury (SCI). In fact, it is well established that the lipid peroxidation products play a functional role in the disease processes after a neurotraumatic injury (Gutteridge, 1995; Mark et al., 1997).

Another product of oxidative stress is the production of reactive nitrogen species (RNS). RNS are produced when nitric oxide and superoxide react to form peroxynitrite (PN). Peroxynitrite is a highly reactive molecule that often deprotonates lipids causing the production of more lipid peroxidation products. Peroxynitrite also reacts with other molecules to form other types of RNS molecules. RNS has long been known to occur in models of neurotrauma (Beckman and Koppenol, 1996) and some of the mechanisms by which PN accomplishes this are well established (Bains and Hall, 2012).

1.4 Experimental Summary of Specific Aims

My central hypothesis is that ROS contributes to the maintenance of chronic neuropathic pain in SCI animals.

HYPOTHESIS 1: ROS and lipid peroxidation products are present and upregulated in SCI tissue at chronic time points.

Rationale: Lipid peroxidation products are an important downstream consequence of ROS production in the neuron before degradation or scavenging by protective enzymes occur, are found to be just as toxic as their ROS predecessors and have been shown to alter cell membrane properties and lead to increased calcium ion influx (Stark, 2005). Considering the above properties of lipid peroxidation it is likely that lipid peroxidation products are contributing to chronic neuropathic pain observed in SCI animals.

Specific Aim 1 To analyze spinal cord tissue of chronic SCI animals in order to determine concentrations ROS and specific species of lipid peroxidation products. Mass spectrometry and immunohistochemistry will be used to analyze spinal cord tissue for ROS concentrations, lipid peroxidation products, and concentrations of specific lipid peroxidation molecules at specific time points after SCI. Analysis of the tissue will give us insight into the expression and contribution of ROS and lipid peroxidation products during chronic SCI.

HYPOTHESIS 2: Molecules that inhibit ROS and lipid peroxidation will attenuate neuropathic pain behaviors in chronic SCI animals.

Rationale: ROS scavengers can attenuate neuropathic pain behavior and decrease membrane excitability during acute conditions. However, it is unknown if ROS scavengers affect cellular processes after SCI during chronic conditions.

Specific Aim 2.1 To test whether formation of superoxides and lipid peroxidation products are involved in generating neuropathic pain behaviors in chronic SCI animals. ROS and lipid peroxidation pathways will be inhibited using pharmacological compounds and monitored to see if they can attenuate measures of pain in chronic SCI animals.

Specific Aim 2.2 To test if application of a superoxide inhibitor can inhibit thermal and non-evoked measures of neuropathic pain behavior. Superoxide production will be inhibited using Apocynin and thermal and non-evoked measures of neuropathic pain will be monitored for signs of attenuation.

Specific Aim 2.3 To measure membrane hyperexcitability of dorsal horn neurons when an inhibitor of ROS is applied. Use whole cell recording to monitor the membrane excitability of superficial dorsal horn neurons, and observe the effects of different ROS pathway inhibiting substances on dorsal horn neuron membrane excitability.

Chapter 2 General Experimental Methods

2.1 SURGICAL PROCEDURES

2.1.1 Animals

Male Sprague-Dawley rats, 200-225g were used in all studies. The animals were housed in a reversed 12 hour day/night cycle while being fed ad libitum. The animals were obtained from Harlan Inc. (Houston), and all procedures were reviewed and approved by the UTMB Animal Care and Use Committee (IACUC).

2.1.2 Spinal Contusion Injury

Animals were anesthetized by i.p. injection of pentobarbital (40 mg/kg). Anesthesia was considered at the surgical plane when there was no withdrawal in response to noxious foot pinch. When the animal is fully anesthetized, its back was shaved, betadine applied, and a laminectomy was performed exposing spinal segment T10. A contusion spinal cord injury was produced using the Infinite Horizon impactor (150kdyne, 1 second dwell time). This device provides recordings of impact parameters (such as impact velocity, cord displacement, and impact force). Following the injury, the musculature was sutured, the skin autoclipped and the animals were allowed to recover from anesthesia. For sham controls, the same surgical procedure was followed, including

placement of the animal with the dorsal vertebral clips of the impacter except that the impactor was not dropped. The rats were eating and drinking within 3 hours of surgery. Antibiotic treatment began immediately after surgery with a subcutaneous injection of 0.3 cc of Baytril (22.7 mg/ml) followed by a second injection 7hrs later; from then on, Baytril injections were given twice daily for 7 days and once daily for 3 more days to prevent urinary tract infections. Bladders were manually expressed twice daily. Automatic bladder control was achieved in all spinally contused rats by 10 days post contusion. To ensure the general health of the rats, the animals were weighed daily and the animals demonstrated normal weight gains. Post injury animals were housed in UTMB Animal Care facilities. Records were kept on each animal as to when it was received, its weight at the time of injury, the computer record of the impact parameters, drug regimens that it received and the measurements on the animal and samples taken. These were entered into a spreadsheet and shared among all investigators.

2.1.3 Locomotor Assessment

The Basso, Beattie, and Bresnahan (BBB) open-field locomotor test (Basso et al., 1995) was performed to score locomotor deficits after the contusion SCI. BBB scoring is scaled from 21, normal locomotor function in limbs, to 0, no locomotor functions in limbs (paralysis). Each joint (ankle, knee, and hip) are scored, with each score depending on the amount of movement or use observed in each joint. BBB measures were taken prior to surgery, and on the 1, 14, 35 days after SCI.

2.1.4 Intrathecal Injections

Animals were given intrathecal lumbar injections at L3-L4 (Cauda Equina) of the spinal column to avoid damage to the spinal cord. The pH of each solution of inhibitors was set to 7.2-7.4 pH to match that of the animals' cerebrospinal fluid. The animals were anesthetized by inhalation anesthesia 4% isoflurane. Fifty microliters of vehicle or vehicle plus a test compound, was injected during a one minute period into the intrathecal space. Fifty microliters was chosen to ensure that the solution diffused up to cervical segments. The animals were alert and mobile moments after the inhalation anesthesia was removed, displaying no signs of distress or neural damage.

2.2 Pain Behavioral Assays

Measures from the mechanical and thermal sensitivity assays were taken before the SCI surgery for the purposes of a baseline measure, and starting every week 14 days after injury to monitor the animal's pain development. Animals that did not display an increase mechanical (at least 50%) or thermal (at least 25%) sensitivity were removed from the experiment as a non-pain behaving animal. Only about 5 percent of animals were removed from the experiments for failing to reach pain behaving criteria. All animals were habituated to the plexiglass cubicle testing containers for 2 hours for 2 days, and were acclimated to the testing boxes for 30 minutes before testing. All behavioral tests were performed by a blinded observer.

2.2.1 Mechanical Allodynia

Mechanical sensitivity of the forelimb and hindlimb of the animals were tested using a range of von Frey filament (0.2 to 25 gram force) using a modified Dixon up-down method with the paw withdrawal threshold being calculated. Von Frey filaments were applied through the mesh flooring of the testing boxes to the ventral glabrous skin of the animal's paws. During the von Frey assay, the animals were also monitored for supraspinal behaviors such as head turning, changes in body posture, avoidance, vocalizations, paw licks, and aggressive behavior toward the von Frey filament. These supraspinal responses were monitored in order to provide a quantified non-reflexive pain behavior.

2.2.2 Thermal Allodynia

Thermal sensitivity of the animal forelimbs and hindlimbs was tested using radiant heat stimulus of known temperatures delivered using a thermal delivery device (San Diego Instruments) and measuring the paw withdrawal latency; a modified version of the Hargreaves thermal sensitivity assay (Hargreaves et al., 1988). Each paw was tested at least twice during a session. Animals were placed in plexiglass containers that rested on an elevated glass plate. A radiant heat source was applied which directed a beam of light onto the ventral non-glabrous skin of the animals paws. The light beam is automatically turned off when the animals lifted their paws. The amount of time that occurs between the start of the beam and the limb-lift was defined as the paw withdraw latency. 5 minutes were lapsed between each trial and 2 trials were averaged for each limb.

2.2.3 Spontaneous Pain Assay

Animals were tested in a photobeam activity system (San Diego Instruments) during their nocturnal and diurnal cycles. The animals were monitored for total beam breaks, total locomotion, and rearing behaviors. Each animal was habituated to the activity boxes for two days before testing, for one hour each day. Animals were also monitored for stereotyped behaviors such as grooming or scratching.

2.3 ELECTROPHYSIOLOGY

Animals were anesthetized with pentobarbital sodium (50mg/kg, i.p.). A cannula was inserted into the trachea for artificial respiration and to measure end-tidal CO_2 levels; a catheter was placed in the jugular vein for continuous administration of anesthesia; the carotid artery was catheterized for blood pressure monitoring. Core body temperature was measured with a rectal thermometer and maintained at 37°C by means of a homoeothermic blanket system. Constant levels of anesthesia and paralysis of the musculature were maintained by i.v. infusion of a mixture of pentobarbital sodium (50 mg) and pancuronium (5 mg) in 30 ml NaCl (at ≈40 µl/min). The animal's spinal cord was supported in the stereotaxic frame by swan-neck clamps placed under the transverse processes of the exposed vertebrae. A laminectomy was made to expose segments T7-

T12. A pool was made with the use of skin flaps. The dura mater was opened and reflected; the pia mater was removed over the recording site to allow smooth insertion of the recording electrodes. For topical administration of an Apocynin 0.1mg/kg solution to the dorsal surface of the spinal cord, a small plastic trough (volume ≈30 µl) was built and tightly sealed with silicone gel to the surface of the spinal cord segments in which the recordings were performed. The trough was filled with artificial cerebrospinal fluid (ACSF), and agar was placed around the trough. ACSF contained (in mM): NaCL 117, KCl 4.7, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, and glucose 11. Extracellular recordings were made from single neurons in the dorsal horn with glass insulated carbon filament electrodes (2 MΩ) at depths of 100-1200 µm. The recorded signals were amplified and displayed on analog and digital storage oscilloscopes. Signals were also fed into a window discriminator, whose output was processed by an interface (CED 1401) connected to a Pentium III PC. Spike2 software (CED, version 3) was used to create peristimulus rate histograms on-line and to store and analyze digital records of single-unit activity off-line. Spike size and configuration are continuously monitored on the storage oscilloscopes and with the use of Spike-2 software. Neurons were identified by spike shape and height. Search stimuli included innocuous and noxious mechanical stimuli applied using a set of calibrated von Frey filaments and, periodically, brief pinch. Once an individual dorsal horn neuron was identified and its spike size optimized, the neuron's receptive field was carefully mapped and size and threshold of its total receptive field in the deep tissue and skin were determined. Following the end of the experiment, electrolytic lesions were performed to allow precise cellular localization by lamina. Apocynin treatment was delivered at the start of recording and monitored for two hours.

2.4 IMMUNOHISTOCHEMISTRY

Sixty days after injury, rats were given either vehicle alone (n=8) or Apocynin and vehicle (10 mg/kg, i.p. n=8) overdosed 30 minutes after injection with pentobarbital (100 mg/kg, i.p.) and perfused intracardially first with 300 ml of heparinized warm 0.9% saline followed by 500 ml of cold 4% paraformaldehyde. Spinal sections from T8-9 were removed and post fixed for 4 hours in 4% paraformaldehyde prior to protection for 2 days in 30% sucrose at 4^{O}C. The tissue was then embedded in OCT compound, frozen, mounted, and sectioned with a sliding microtome (model HM 400, Microm International, Waldorf Germany). Thirty micron sections from the T8-T12 tissue were blocked in 5% normal goat serum for 30 minutes and incubated overnight in primary antibody (Mouse Anti-4HNE, 1:500, Abcam; Rabbit Anti-MAP2, 1:1000, Abcam; Rabbit Anti-NeuN, 1:1000, Millipore). The sections were then rinsed in phosphate buffered saline (PBS) and incubated in anti-mouse antiserum conjugated to Alexa Fluor 568-Red (1:400, Molecular Probes) and anti-rabbit antiserum conjugated to Alexa Fluor 488-Green (1:400, Molecular Probes). After rinsing, the floating sections were mounted on gelatin-coated slides and coverslipped with non-fade media. Expression of primary antibody was quantified in the spinal cord gray and white matter rostral to the site of injury (T8/9) in sham and injured rats by measuring the density of the expression of primary antibody and comparing between sham and SCI rats using ImageJ Software (NIH). For immunofluorescent staining, data from three channels was collected by Sequential scan (BioRad Radiance 2100 Confocal Laser System coupled to a Nikon E800) to avoid bleeding through between channels. Localization of primary antibody and one of the cell marker images were collected with Krypton lasers of 568 nm excitation and 488 nm excitation. Red emission (a result of excitation of the conjugated AlexaFluor 568 to the 4-HNE primary antibody) demonstrated localization of 4-Hydroxynonenal; whereas, green

emission (a result of excitation of the conjugated AlexaFluor 488 to the NeuN or MAP2 antibodies) demonstrated localization of immunoreaction product in neurons or neuronal processes. Blue emission (DAPI) indicated nuclear localization. The yellow structures in merged images indicated the co-localization of the two antigens (Red+Green).

2.5 GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Spinal cord tissue was pulverized in liquid nitrogen with 0.6 M $HClO_4$ containing O-(pentafluorobenzyl) hydroxylamine hydrochloride and 2,2,6,6-d4-cyclohexanone (an internal standard) and incubated. Incubate was extracted with 10 volumes of methylene chloride, pooled and dried under gentle nitrogen stream. The extracted oxime-derivatives of hydroxy alkenals are subjected to trimethylsilylation of hydroxyl groups to trimethylsilyl (TMS) ethers. The derivatized products were analyzed by gas chromatograph-mass spectrometry (GC-MS) in electron impact (EI) mode. The criteria for identification of the peroxidation products of lipids in the samples were based upon the retention time and mass spectra (selective ion monitoring, SIM and corresponding mass fragmentation pattern) matching that of the corresponding standards. Analysis of oxidized proteins was done by measuring protein carbonyls using a spectrometric 2,4-dinitrophenylhydrazine (DNPH) assay. The oxidized proteins were identified essentially as described by Choi et al., 2004.

2.6 STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS software (ver. 14, SPSS Inc., Chicago, IL). The significant value for each test was $\alpha = 0.05$. A within group repeated measures ANOVA was used to analyze the data for the same group over time. A between groups ANOVA was used to analyze data when comparing two or more groups (i.e.

Sham, SCI, and SCI + Treatment). Data values are expressed and graphed as mean with standard error of the mean (mean $\pm$ SEM).

Chapter 3 Changes in Reactive Oxygen Species Expression and Lipid Peroxidation Expression at the Chronic Stage of Spinal Cord Injury

3.1 INTRODUCTION

It is well known that ROS is highly upregulated just after an injury and play an important role in secondary injury mechanisms, however, ROS has not been characterized in tissues at chronic time points and it wasn't until just recently known that ROS was upregulated during chronic time points after an neurotraumatic injury. As described in my first specific aim, I used behavioral, molecular, and electrophysiological methods to characterize the expression of ROS and LP products that are present in chronic SCI tissue (**Specific Aim 1.1**). Spinal cord injuries (SCI) produce direct and indirect spinal cord damage. Once the spinal cord is damaged, maladaptive intracellular and extracellular biochemical signaling events contribute to enhanced nociceptive transmission in the spinal dorsal horn via overexpression of receptors and ion channels (Hains et al., 2003; Mills et al., 2001; Pabbidi et al., 2008), increased release of neurotransmitters and proinflammatory cytokines (Detloff et al., 2008; Ferguson et al., 2008), and activation of glial cells (Gwak and Hulsebosch, 2009; Gwak et al., 2008).[1]

Subsequently, neuroanatomical and neurochemical changes following SCI produce persistent hyperexcitable states in spinal dorsal horn neurons, often called central sensitization which results in persistent central neuropathic pain (Gwak and Hulsebosch,

[1] Portions of Text and Figures from Gwak et al., 2013 are preprinted in this chapter with the permission of *Pain*

2011). However, the molecules that mediate the maladaptive nociceptive transmission in neuropathic pain after SCI are not well understood. Recent studies report that removal of ROS by spin trap ROS scavengers in several pathophysiological conditions, such as peripheral neuropathy (Kim et al., 2011; Kim et al., 2010), visceral pain (Wang et al., 2008), capsaicin-induced hyperalgesia (Schwartz et al., 2008), and inflammation (Keeble et al., 2009), significantly attenuated pain like behaviors. In addition, SCI studies have suggested that prevention of ROS production contributed to neuroprotection via reduction of tissue damage immediately after injury (Bao et al., 2005; Hachmeister et al., 2006). Taken together, the overproduction of ROS must be an important contributor to sensory and motor abnormalities in several pathophysiological conditions. However, the role of ROS in the induction and maintenance of central neuropathic pain and recovery of locomotion following SCI is not fully understood. More important, ROS-mediated intracellular signaling pathways following SCI are not clear. Although, some intracellular pathways that contribute to SCI-induced neuropathic pain behaviors have been suggested (Crown et al., 2012; Crown et al., 2008; Crown et al., 2006).

3.2 SPECIFIC METHODS

3.2.1 ROS immunohistochemistry

To test whether SCI influences the production of ROS double immunofluorescence staining was performed. We used 3 groups of rats (sham; SCI + vehicle; SCI + PBN). To test production of ROS, dihydroethidium (Dhet), an autoimmunoflorescent agent for detection of ROS (Invitrogen, Grand Island, NY), intrathecally administered 24 h prior to perfusion, and phenyl-N-tert-butylnitrone (PBN), a ROS scavenger (3mg/kg intrathecally, 100mg/kg intraperitoneally). Rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, ip) and perfused intracardially with heparinized physiological saline followed by 4% cold buffered paraformaldehyde/ 0.1 M

phosphate buffer (PB) solution. After perfusion, the lumbar spinal cord (L4/5) was removed immediately and postfixed overnight in 4% paraformaldehyde/0.1 M PB, followed by cryoprotection in 30% sucrose in 4% paraformaldehyde/0.1 M PB over several days. After postfixation, spinal cords were embedded in O.C.T. compound (VWR International, Radnor, PA) individually and then sectioned at 20 μm intervals. Antibodies for neurons (NeuN, 1:2000; Millipore, Billerica, MA) were incubated with a cocktail solution (0.1M PB, 0.15% Triton X-100 and 1% NGS) at room temperature, overnight. After 4 washes (each 10 m) with 0.1 M PB, sections were incubated with secondary antibodies (2 h, 1:600, Molecular Probes, Grand Island, NY). Sections were collected by free-floating methods and mounted on gelcoated slides with mounting media (DAPI, Vectashield; Vector Laboratories, Burlingame, CA). Images were captured by confocal microscopy (Radiance 2000, Bio-Rad, Hercules, CA) with Lasersharp 2000 imaging software (Sunnyvale, CA) and were evaluated by measuring intensity using a computer-assisted image analysis program (MetaMorph 6.1; Molecular Devices, Sunnyvale, CA). Imaging capture parameters, such as offset, gain and iris settings, were kept constant during image capture for valid comparisons of differential expression between-SCI and SCI + treatment groups.

3.2.2 Electrophysiology

The electrophysiological responses of spinal lumbar 4/5 WDR dorsal horn neurons in response to mechanical stimuli were investigated using in vivo single extracellular recording techniques. Briefly, rats were anesthetized by sodium pentobarbital (60 mg/kg, ip) and then a laminectomy of vertebral segments T12 through L1 was performed to expose the lumbar enlargement (L4 through L5). Tracheal and jugular vein cannulae were inserted for breathing and infusion of sodium pentobarbital (5 mg/h/300 g) to maintain the physiological and anesthetic levels of the rat during the single unit recording, respectively. The rats were held in place by a stereotaxic apparatus,

and rectal temperature was maintained at 37°C. A single-unit WDR recording was performed by using a carbon-filament–filled single glass microelectrode (Kation Scientific, Minneapolis, MN) in the lumbar L4/5 dorsal horn. We sought WDR neurons in the lumbar dorsal horn that displayed graded activity patterns in response to increased intensities of mechanical stimuli given to the WDR receptive fields on the hind paw. After identification of single-unit activity, 3 graded mechanical stimuli were applied to that unit's peripheral receptive field to locate and characterize WDR neurons. These were: (1) brush stimulation of the skin with a fine tipped brush; (2) pressure stimulation by applying a large arterial clip (bulldog clamps; George Tiemann, Hauppauge, NY) with weak grip to a fold of the skin (firm pressure); and (3) pinch stimulation by applying a small arterial clip (Serrefines; Tiemann) with a strong grip to a fold of the skin (painful pressure). The 3 mechanical stimuli were applied successively for 10 s each, with an interstimulus interval of 20 s. The unit activity was amplified and filtered (DAM80; World Precision Instruments, Sarasota, FL), fed directly into either an oscilloscope (World Precision Instruments) or the data acquisition unit (CED-1401; Cambridge Electronic Design, Cambridge, UK), and stored on computer in order to construct the waveforms or to plot peristimulus time histograms (spikes/1 s bin width (Spike2 software, Cambridge Electronic Design). To test whether ROS influences on WDR neuronal activity, high doses of PBN (3 mg/kg/50μL by Hamilton syringe) and vehicles (for control) were delivered to test the changes in WDR activity. Responses were recorded for 10, 30, 60, and 120 m after baseline recording. As a control, to ensure that a single and the same WDR unit were held for the duration of the recording experiment, we used the Spike2 program to confirm the same action potential shape and amplitude.

3.3 RESULTS

Immunohistochemistry in conjunction with confocal microscopy to investigate the production of ROS in chronic SCI rats 35 days after injury. Figure 3.1 represents the increase in ROS seen in rat spinal cord tissue 35 days after injury. Immunohistochemistry was used to measure Dhet (shown as red), a marker of ROS, along with NeuN (shown as green), a neuronal marker, and DAPI (shown as blue), a cell nucleus marker. ROS is increased in the lumbar spinal cord tissue slices 35 days after injury and that the ROS is localized in the dorsal horn neurons. Spinal cord glial cells do participate in scavenging ROS, however, a GFAP marker was not used for this study.

Phenyl N-tert-butylnitrone, a free radical scavenger was used to test if ROS over production in chronic SCI rats could be reduced. Figure 3.2 represents the inhibitory effect of ROS scavenger PBN on ROS expression in lumbar spinal cord tissue 35 days after injury. To test whether SCI produces increased production of ROS in neurons we used immunohistochemistry in conjunction with confocal microscopy. PBN was able to reduce ROS expression after intrathecal and intraperitoneal administration. ROS intensity was significantly increased when compared to sham ROS measures and intrathecal and intraperitoneal administration of PBN significantly reduced ROS intensity when compared to vehicle only treatment measures. Dhet (a ROS indicator) intensity was measured using double immunofluorescence. Figure 3.2 represents the Dhet intensity in the lumbar 4/5 spinal dorsal horn neurons following T10 contusion injury. After normalization (100%) of sham control ($n = 5$), the mean intensity of Dhet in the SCI group (POD 35, no PBN group, $n = 5$) was 161.2 ± 12.9% and showed significant difference compared to sham controls ($p < 0.05$). By contrast, the Dhet intensity after early PBN treatment ($n = 4$, 100 mg/kg, ip, and $n = 4$, 3 mg/kg, it) was 118.8 ± 13.8% and 128.5 ± 10.2, respectively, and showed significant differences compared to SCI groups ($p < 0.05$).

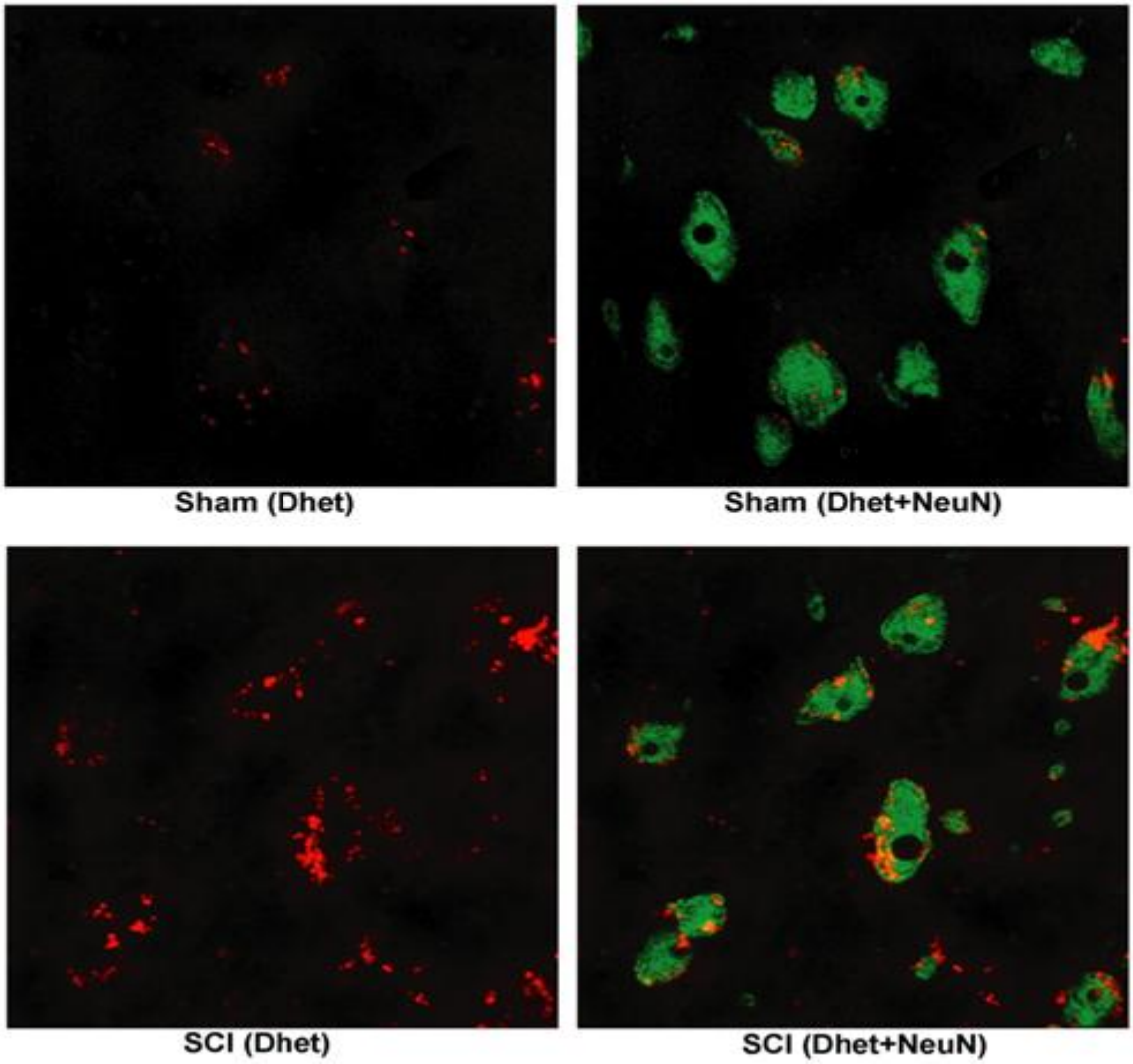

Figure 3.1 Dhet Expression in Sham and SCI Lumbar Spinal Cord Tissue 35 days after injury.

ROS expression is increased in SCI lumbar tissue when compared to Sham controls.

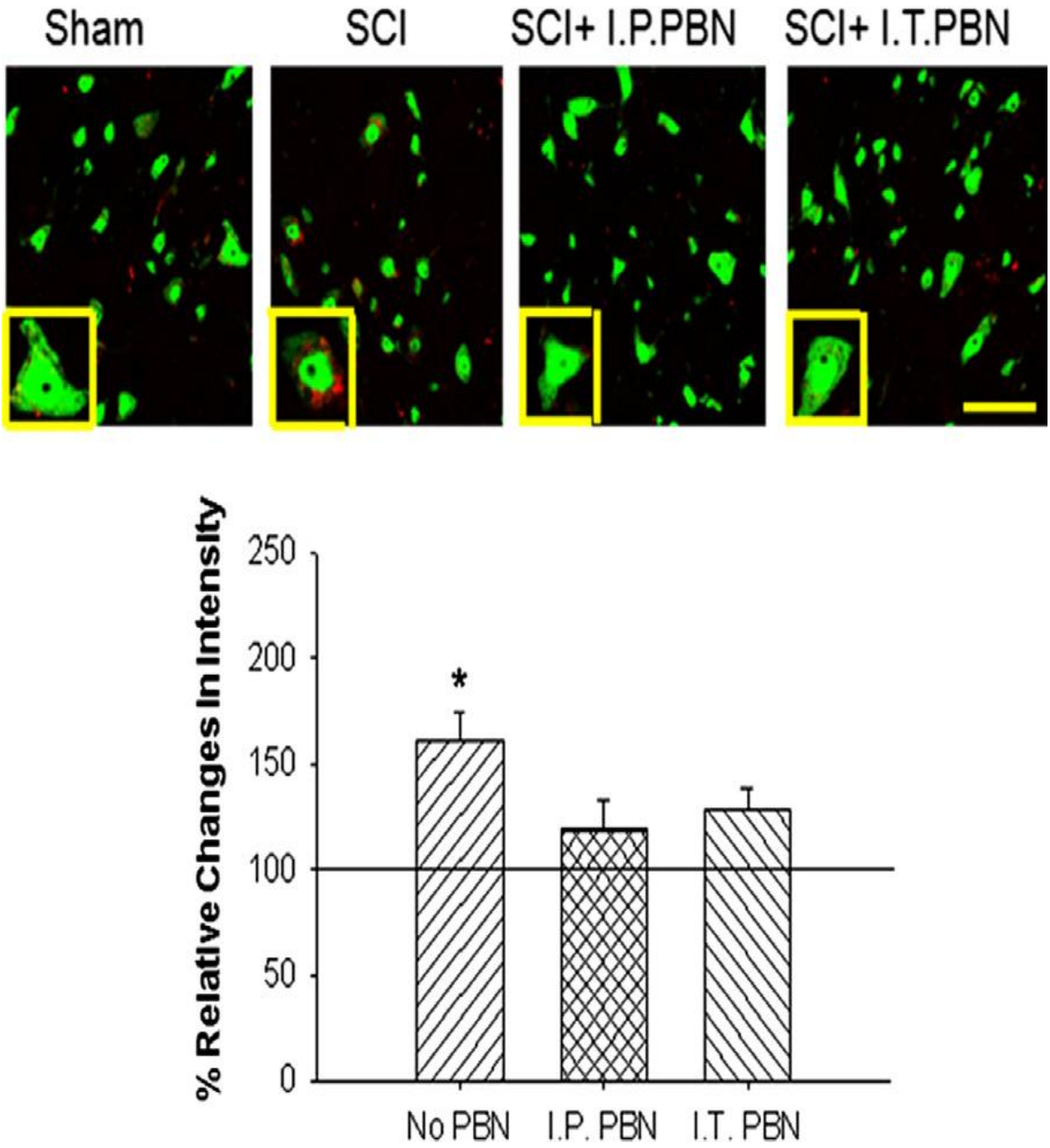

Figure 3.2 Dhet (ROS) Expression Intensity in Sham and SCI tissue 35 days after injury in response to application of PBN.

The ROS overproduction in the lumbar 4/5 spinal dorsal horn following T10 contusion injury. Double immnuofluorescence staining for neurons (NeuN, green) and Dhet (ROS marker, red) in the lumbar 4/5 spinal dorsal horn at 35 d following T10 contusion injury (top panel). Histogram of intensity measures of the 4 groups used in this study (bottom panel). Compared to sham controls, SCI groups showed increased intensity of Dhet that was attenuated by early PBN treatment, both by intraperitoneal (IP) and intrathecal (IT) administration (*$P < 0.05$). Small boxes are representative neurons from laminae III to V to demonstrate neuronal localization of Dhet at a higher magnification, not to demonstrate differences in soma size. Scale bar: 50 µm.

Whole cell electrophysiology was used in conjunction with application of PBN to test whether ROS overproduction in chronic SCI rats is responsible for the hyperexcitability found in dorsal lamina wide dynamic range (WDR) neurons. Figure 3.3 represents the decrease in WDR spinal cord dorsal horn neurons activity in response to early and delayed treatment of PBN. ROS is upregulated at chronic time points in a rat model of SCI, however, it was not known if this upregulation of ROS had any functional significance. To test whether ROS is involved in neuronal hyperexcitability following SCI, the changes in neuronal responsiveness to mechanical stimuli were measured. When ROS scavenger PBN is administered to SCI rats either daily for a week after their injury or as a single dose 35 days after injury that it reduces the excitability of WDR neurons to measures similar to those seen in sham animals. This is strong evidence that ROS plays a functional role in generating the transmission of maladaptive pain information in SCI animals. Fig. 3.3A and 3.3B are typical waveforms of activity patterns and histograms of WDR neurons in the lumbar 4/5 dorsal horn in response to brush (Br), pressure (Pr) and pinch (Pi) stimuli in sham, vehicle and PBN groups, respectively. In sham groups, the mean activity of WDR neurons (n = 23 from 6 rats) to brush, pressure and pinch stimuli was 8.6 ± 2.1, 15.9 ± 1.5 and 18.8 ± 1.8 spikes/s, respectively. In contusion groups (POD 35), the mean activity of WDR neurons (vehicle group, n = 55 from 14 rats) to brush, pressure and pinch stimuli was 16.8 ± 0.8, 29.4 ± 2.3 and 30.9 ± 2.1 spikes/s, respectively and showed significant differences compared to sham groups (*$p < 0.05$, Fig. 3C). However, the mean activity of WDR neurons after early PBN treatment (n = 45 from 14 rats, ip, 100 mg) to brush, pressure and pinch stimuli was 11.9 ± 0.9, 22.4 ± 1.6, and 24 ± 2 spikes/s and showed significant differences compared to SCI groups (#$p < 0.05$, Fig. 3.3C). In addition, delayed PBN treatment (n = 9 from 9 rats, intrathecal, 3 mg/kg) on POD 35 significantly attenuated the WDR hyperexcitability compared to SCI groups (*$p < 0.05$, Fig. 3.3D). The mean activity of WDR neurons 60 minutes after topical 3 mg PBN treatment, to brush, pressure and pinch stimuli, was 10.5 ± 2.1, 13.9 ± 2.6, and 15.7

± 2.9 spikes/s, respectively, and showed significant differences compared to vehicle (16.3 ± 1.2, 24 ± 3.5 and 26.3 ± 3.9 spikes/s, respectively, n = 4 from 4 rats) groups (*p < 0.05).

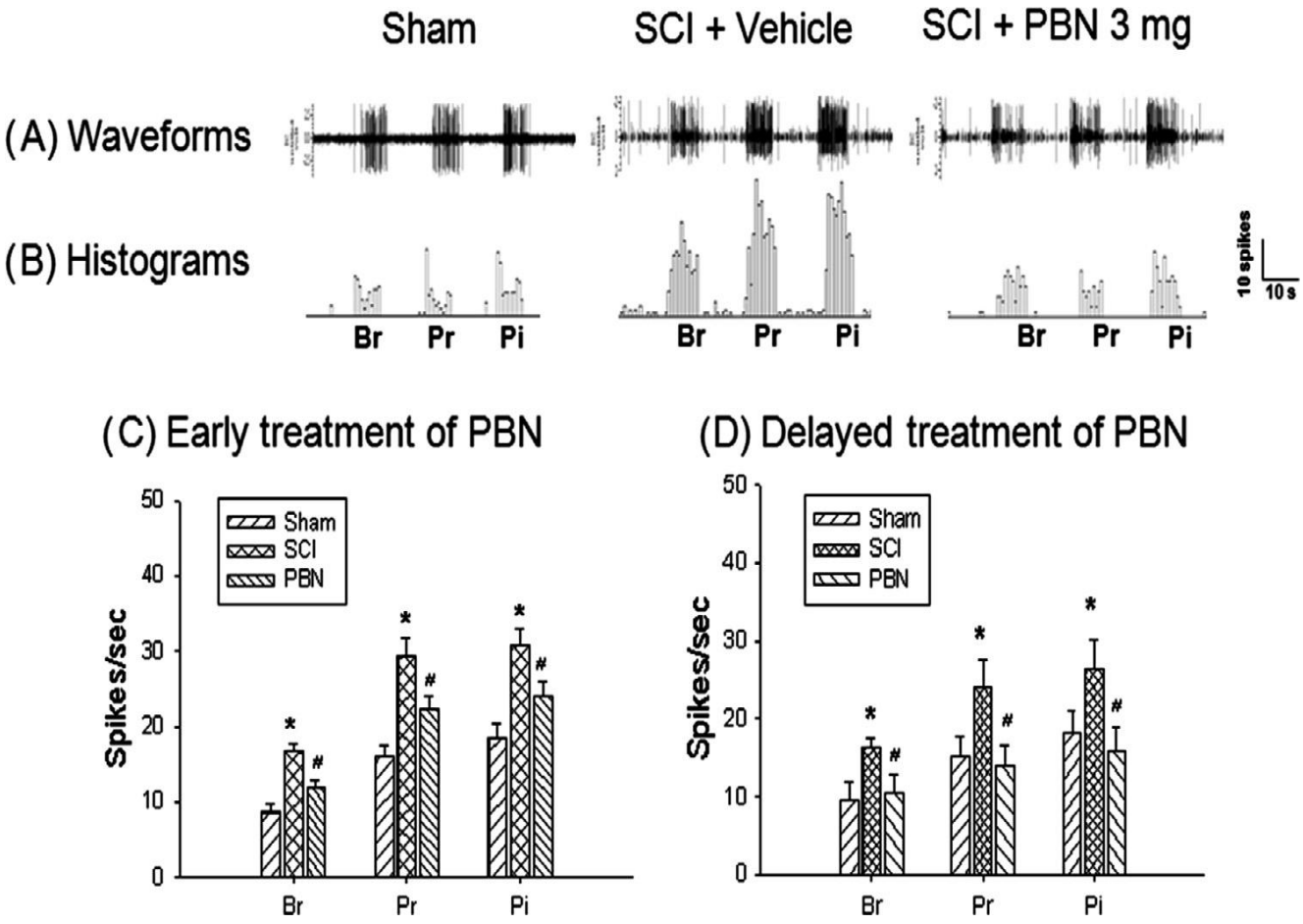

Figure 3.3 Electrophysiological recordings from dorsal lamina WDR neurons in Sham and SCI animals 35 days after injury.

(A) **Representative** waveforms from Sham, SCI, and SCI with PBN treatment. (B) **Representative** stimulus histograms in response to brush (Br), pressure (Pr), and pinch (Pi) stimuli; which was applied to Sham, SCI, and SCI with PBN treatment. (C) Graph showing the attenuating effects of early PBN treatment on dorsal lamina neuron excitability. (D) Graph showing the attenuating effects of delayed PBN treatment on dorsal lamina neuron excitability. SCI measures were significantly increased when compared to Sham measures,*p < 0.05. PBN measures were significantly decreased when compared to SCI measures, #p < 0.05.

To test if ROS overproduction in chronic SCI animals leads to increased oxidation of lipids in dorsal spinal cord tissue, immunohistochemistry was used. Figure 3.4 represents the increase in lipid peroxidation molecule 4-HNE in chronic SCI tissue 42 days after injury. Shown is a representative image of a spinal cord dorsal horn that has been stained with 4-HNE (red, colocalized as yellow), MAP2 (neuronal marker, green), and DAPI (cell nucleus marker, blue). The below two images show only 4-HNE staining in SCI and Sham tissue. Tissue slices were taken from the T9-T8 region of the spinal cord, which was one to two spinal segments above the impact site. The recorded images were analyzed for the average intensity of 4-HNE staining in the dorsal and ventral aspects of the spinal cord and between Sham and SCI animals. Figure 3.5 shows the quantification of 4-HNE expression using mean intensity measures taken from the dorsal and ventral regions of Sham and chronic SCI spinal cord tissue. 4-HNE intensity was upregulated in SCI animals (1.62 ± 0.26, $n = 6$) when compared to Sham measures and that 4-HNE intensity is mainly localized to the dorsal horn region (1.29 ± 0.06, $n = 6$) of the spinal column. This finding confirmed that not only was ROS upregulated in SCI but that it also contributed to oxidation of lipids. It is likely that the lipid peroxidation products formed downstream of ROS are also involved in the generation of central sensitization in this chronic model of SCI ($^*p < 0.05$).

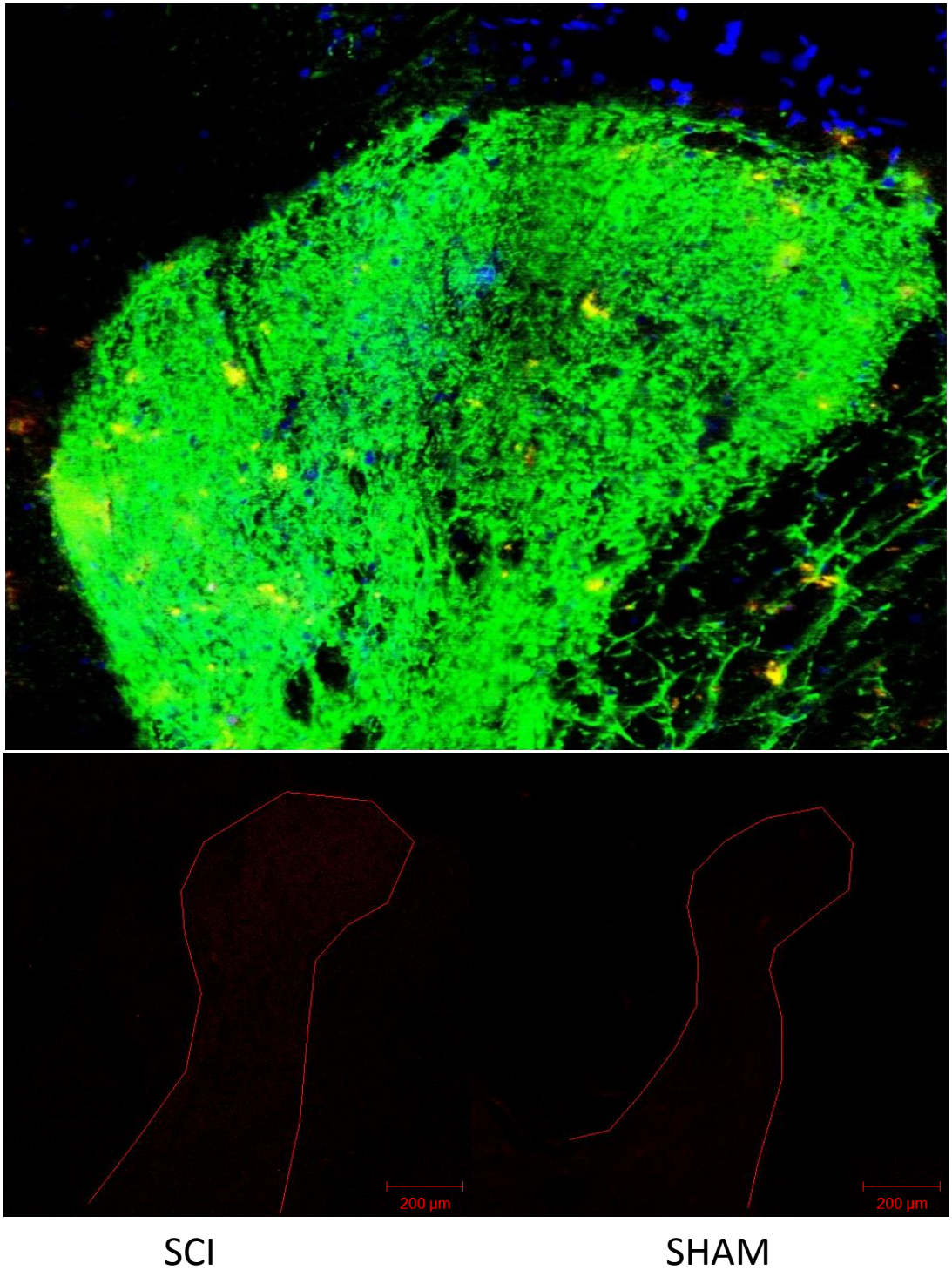

Figure 3.4 Representative confocal images showing expression of 4-HNE in Sham and chronic SCI spinal cord dorsal horns.

The above image is a composite of MAP2 (green), Cell Nuclei (Blue), and 4-HNE expression (Red). Some localization of 4-HNE expression with MAP2 is seen (Yellow). The below image shows two representative images from Sham and SCI dorsal horns (outlined) that have different expression of 4-HNE

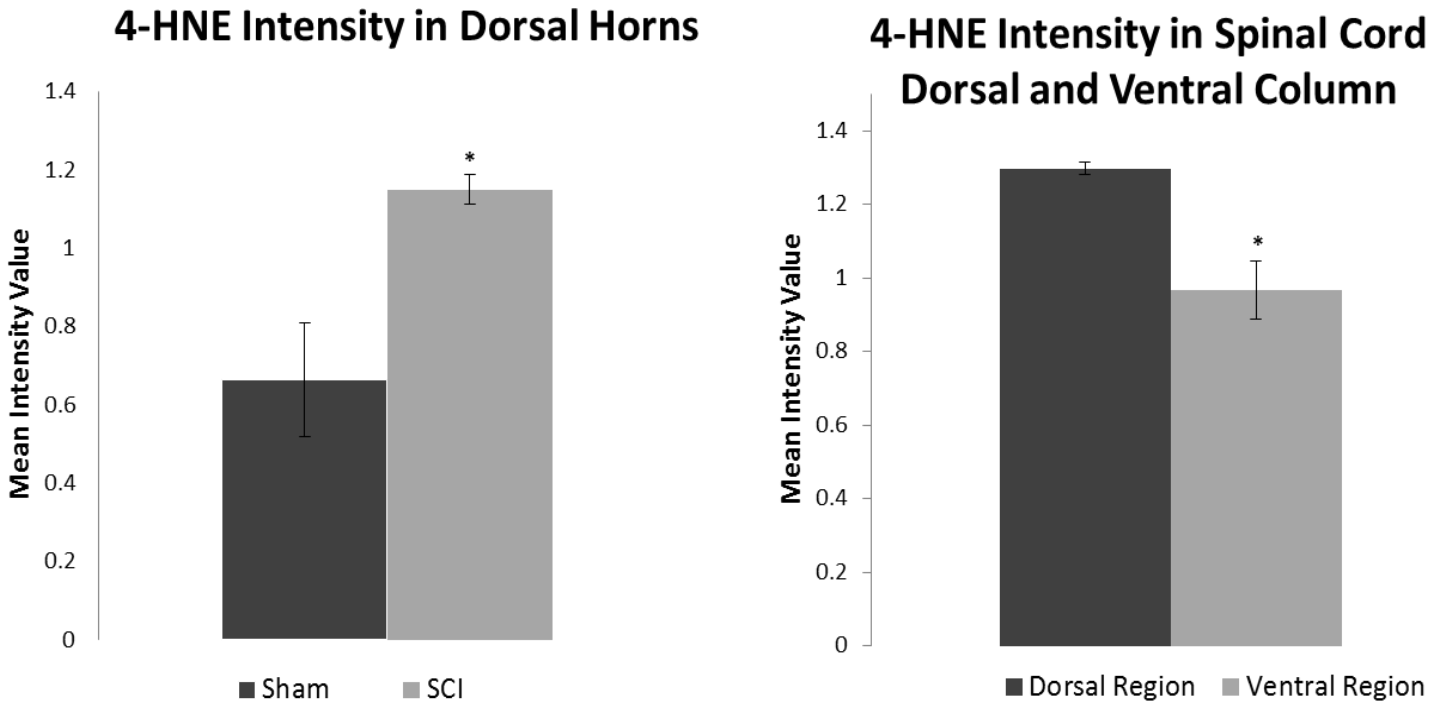

Figure 3.5 Mean intensity of 4-HNE expression in Sham and SCI spinal cord.

Gas chromatograph-mass spectrometry (GC-MS) was used to measure lipid aldehyde concentrations found in sham and chronic SCI spinal cord tissue. Figure 3.6 is a representative chromatograph from a gas chromatography-mass spectrometry experiment in which tissue from chronic SCI and Sham animals 42 days after injury. SCI animals had increased measures of lipid aldehydes (lipid peroxidation products). Lipid aldehyde molecules were upregulated in chronic SCI spinal tissue.

To look at expression of individual lipid peroxidation products in chronic SCI tissue GC-MS was used. Figure 3.7 represents the fold changes observed in lipid aldehydes between Sham and SCI animals. Note that 4-HNE is 2.34 fold increased in SCI animals when compared to Sham measures. The lipid aldehydes that were unknown were named by using their retention times.

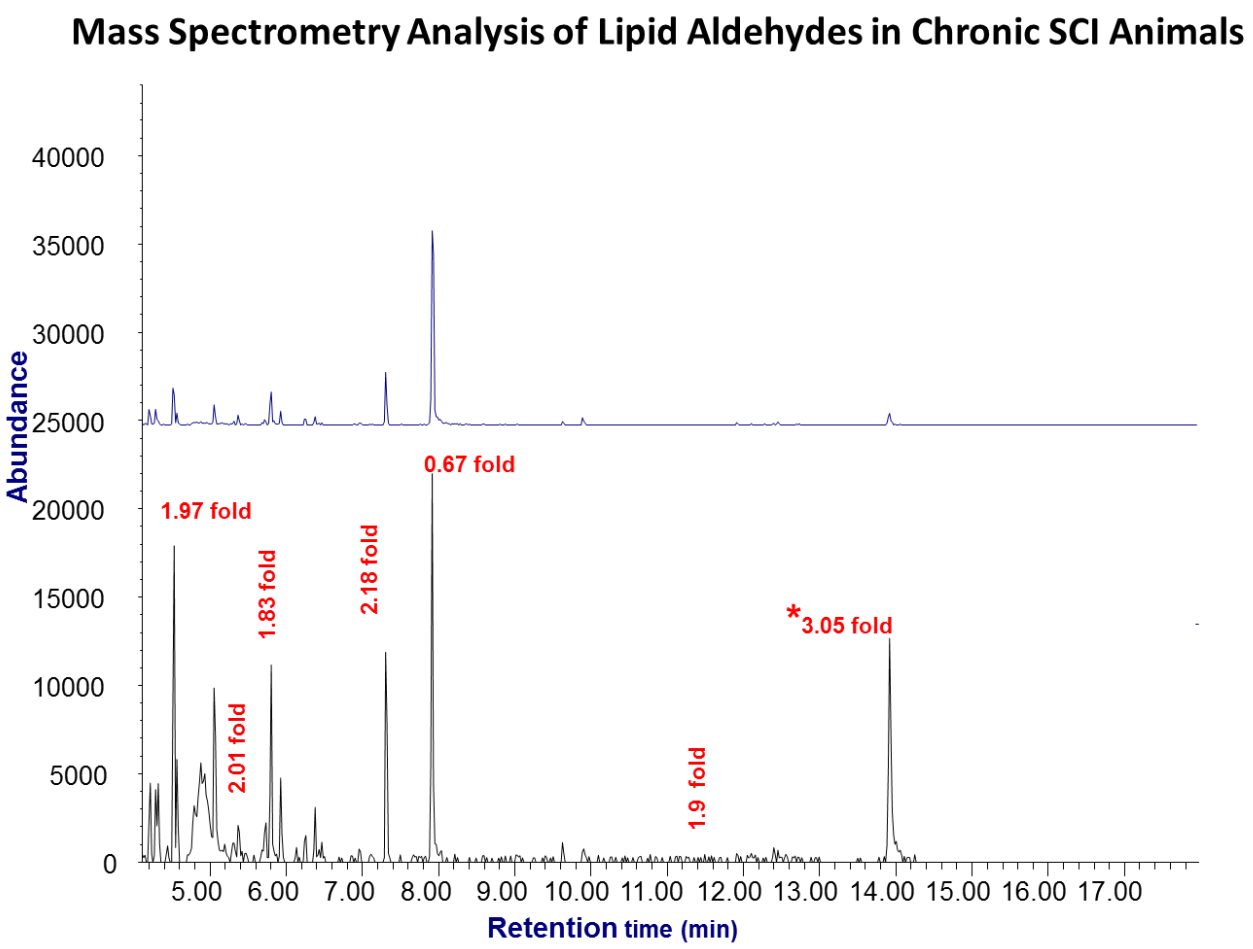

Figure 3.6 Gas Chromatographic Analysis of Lipid Aldehydes in Sham and SCI spinal cord tissue 42 days after injury

GC/MS (Negative Ion Chemical Ionization, NICI) chromatograms of extracted Single Ion Monitoring (SIM) of spinal cord tissue extract of naïve (top) and [SCI]

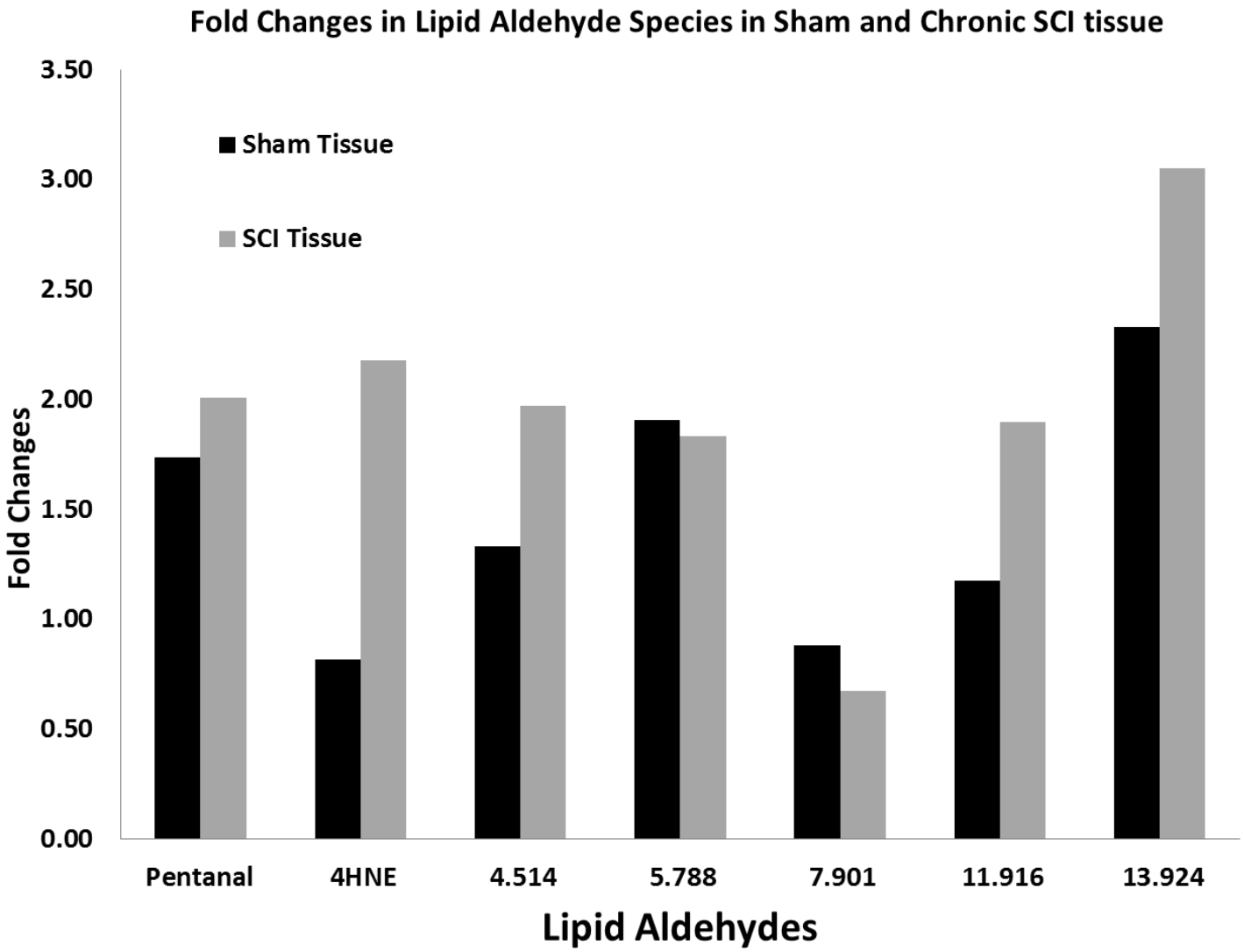

Figure 3.7 Fold Changes in Lipid Aldehyde Concentrations measured from Sham and SCI tissues harvested 42 days after injury

3.4 DISCUSSION

In the present studies, ROS scavenger, PBN, decreased the maintenance of ROS production and neuronal hyperexcitability in the wide-dynamic range lumbar dorsal horn neurons. The early systemic administration of PBN, given immediately after the injury is able to reduce ROS expression 35 days after injury, indicating that ROS overexpression can be attenuated by acute treatments of PBN. However, a single treatment of PBN at the chronic time point of 35 days post injury was able to reduce ROS expression as well. While it is interesting to find that ROS overexpression after an injury is involved in upregulating ROS at a later chronic time of 35 days post injury, the finding that administration of a ROS scavenger at a chronic time point is able to reduce ROS expression is more clinically relevant since immediate treatment will not always be available. The study also found that administration of PBN was able to reduce SCI mediated hyperexcitability of WDR dorsal horn neurons, which are responsible for relaying pain sensation information to the CNS. This result suggests that overproduction of ROS in chronic SCI animals contributes to below-level neuropathic pain following thoracic spinal contusion injury in rats, indicating that ROS are important to the maintenance of neuropathic pain-like behavior.

ROS play an important role in synaptic transmission in the CNS (Forman et al., 2004; Kim et al., 2004), and has been found to induce hyperexcitability through the activation of TRPA1 and TRPV1 channels in dorsal horn neurons (Nishio et al. 2013). In physiological conditions ROS are tightly controlled by both antioxidant and redox reactions (Kohen et al., 2002). Unfortunately, upregulation of ROS, as a consequence of pathological conditions such as SCI, causes oxidative damages in neurons, such as lipid peroxidation and protein damage via oxidation processes (Goel et al., 1986; Raps et al., 1989; Teebor et al., 1988). The anatomical and chemical changes in neurons contribute to the maladaptive response of the cellular excitability which resulted from neural damage

and inappropriate nociceptive transmission (Goel et al., 1986). The early administration of PBN contributed to protective mechanisms in WDR neurons, whereas later intervention reformed intracellular signaling mechanisms that contribute to neuronal hyperexcitability. Decreases in ROS production in lumbar neurons and reduction of hyperexcitability in lumbar WDR neurons was observed after intrathecal and intraperitoneal administration of PBN, when treated early or delayed. The pharmacological properties of PBN are well known and PBN is considered the most effective ROS spin trap scavenging agent. PBN can readily penetrate all tissues and besides reducing endogenous concentrations of ROS, it also inhibits inducible nitric oxide synthesis (Ho et al., 2000; Miyajima and Kotake, 1997). The half-life of PBN is close to 2 hours and can easily penetrate the blood-brain barrier (Kotake, 1999). Intraperitoneal administration of PBN may produce its pharmacological effects by systemically effecting changes in the sensitivity of peripheral nociceptors, dorsal root ganglia and spinal cord circuits; whereas intrathecal administration presumably would produce effects only in the spinal cord circuits, dorsal root entry zone, and potentially dorsal root ganglia cells (Bedi et al., 2010). However, our behavioral data suggest that both intraperitoneal and intrathecal administration produce similar PBN effects on the attenuation of intracellular ROS.

It is reported that SCI produces immediate intracellular toxic levels of ROS through proinflammatory cytokines and activation of glutamate receptors (Barth et al., 2009; Mills et al., 2001). Consequently, the toxic intracellular levels of ROS lead to cell death, which includes apoptosis, necrosis and maladaptive synaptic circuits (Juurlink and Paterson, 1998) which adversely affect both sensory and motor function. Our immunohistochemistry results in increased Dhet intensity in the L4/5 spinal dorsal horn neurons following T10 thoracic contusion injury, and this increase in Dhet intensity was abolished by PBN treatment. Although the specific sources of ROS in these experiments were not investigated, it is known that mitochondrial dysfunction is a common source of

superoxide production that can contribute to neuropathic pain and motor dysfunction (Kim et al., 2011; Yune et al., 2008).

Lipid peroxidation products were found to be upregulated in our chronic model of SCI rats. Lipid peroxidation is a downstream consequence of ROS and is a common measure of oxidative damage (Gutteridge, 1995). Lipid peroxidation or lipid aldehyde products have been shown to have a toxic effect on neurons and can cause pathological conditions such as disturbances in ion homeostasis, trigger cell apoptosis, and impair neuronal mitochondrial function. (Mark et al., 1997; Vaishnav et al., 2010). 4-HNE, a highly toxic lipid aldehyde breakdown product and common marker for oxidative damage (McCraken et al. 2000), is upregulated in chronic SCI spinal cord tissue. The upregulation of lipid peroxidation products indicates that the overproduction of ROS observed in chronic contusion SCI model contributes to the oxidation of lipids in spinal cord neurons.

ROS and lipid peroxidation products are upregulated at chronic time points in SCI rats and that ROS can be reduced in these animals using the ROS scavenger PBN. ROS contributes to the dorsal lamina neuronal hyperexcitability seen in chronic SCI animals and that treatment with PBN is able to reduce hyperexcitability. The characterization of ROS in a chronic model of SCI provides strong evidence for a functional role for ROS in chronic neuropathic pain. However, whether ROS contributes to pain behaviors in SCI animals has not been tested.

Chapter 4 Reactive oxygen species and lipid peroxidation inhibitors reduce mechanical sensitivity in a chronic neuropathic pain model of spinal cord injury in rats

4.1 INTRODUCTION

It was previously reported that ROS is upregulated during chronic time points after SCI and found that certain lipid peroxidation products are upregulated and expressed in SCI rats as well. Now we will test if Reactive oxygen species (ROS) play an important role in chronic neuropathic pain (**Specific Aim 2.1**). ROS are highly oxidative molecules that naturally occur as a consequence of cellular energy production (Kallenborn-Gerhardt et al, 2013). Cellular stress or trauma results in higher than normal intracellular concentrations of ROS, which can overpower the homeostatic proteins and cause oxidative damage to the cell. Neurons are especially sensitive to ROS since neurons have greater energy demands to function as compared to glial and other cells in the central nervous system (Bell, 2013).[2]

It was previously reported that the downstream consequence of ROS, lipid peroxidation (LP) products, may also contribute to neuropathic pain in chronic SCI animals (Gwak et al, 2013). To investigate the role that oxidation damage plays in chronic neuropathic pain, we examined four compounds that are known to reduce ROS and lipid peroxidation (Khalil and Khodr, 2001; Hall, 1992; Mustafa et al, 2010;

[2] Portions of Text and Figures from Hassler et al., 2014 were used in this chapter with permission from *The Journal of Neurochemistry* and John Wiley and Sons.

Stefanska and Pawliczak, 2008; Wilcox, 2010). These four compounds are 1) Apocynin, a NADPH oxidase inhibitor, 2) 4-oxo-tempo (also known as TEMPONE), a spin trap nitroxyl radical, 3) U-83836E, a free radical scavenger that inhibits iron dependent lipid peroxidation, and 4) Tirilazad, a potent peroxyl scavenger and membrane stabilizer. Each of these compounds was tested based on different mechanisms of action involving ROS and lipid peroxidation reduction products. Intraspinal administration of Apocynin and 4-oxo-tempo, significantly attenuated the abnormal mechanical hypersensitivity that develops following SCI in rats.

The animals were given intrathecal lumbar injections with Apocynin (0.01mg/kg, 0.05mg/kg, 0.1mg/kg, Tocris Bioscience), Tirilazad mesylate (0.01mg/kg, 0.05mg/kg, 0.1mg/kg, Cayman Chemical), U-83836E (0.05mg/kg, 0.5mg/kg, 1.0mg/kg, Cayman Chemical), or 4-oxo-tempo (0.05mg/kg, 0.5mg/kg, 1.0mg/kg, Sigma-Aldrich) at L3-L4 of the spinal column to avoid damage to the spinal cord. The pH of each solution of inhibitors was set to 7.2-7.4 pH to match that of the animals' cerebrospinal fluid. The animals were anesthetized by inhalation anesthesia 4% isoflurane. Fifty microliters of vehicle (10% DMSO, in 0.9% Saline) or vehicle plus a test compound, was injected during a one minute period into the intrathecal space. Fifty microliters was chosen to ensure that the solution diffused to cervical segments. The animals were alert and mobile moments after the inhalation anesthesia were removed, displaying no signs of distress or neural damage.

Behavioral testing started 42 days post injury, during the animal's nocturnal cycle, which is when the animals are most active. The animals were first tested before injection, and then 30, 60, and 120 minutes after injection. This design was repeated for each compound trial with a minimum of one day in between trials to allow for drug washout, washout was determined by behavioral analysis and knowledge of the inhibitors half-lives. Animals were randomly assigned to either the vehicle or compound groups for each trial.

4.2 RESULTS

The Von Frey assay for mechanical sensitivity was used to assess the ability of Apocynin to reduce mechanical hypersensitivity in the forelimbs of chronic SCI rats. Figure 4.1 represents the changes in the forelimb paw withdraw thresholds (PWT, grams) before SCI, before intrathecal injection, 30, 60, and 120 minutes after intrathecal injection of Apocynin (0.01mg/kg, 0.05mg/kg, and 0.1mg/kg). The forelimb paw withdraw threshold values in the pre-injection groups were significantly different compared to baseline values. The 30 minute, 60 minute, and 120 minute values for the vehicle groups were not significantly different from the preinjection values. The Apocynin group values were significantly increased ($\alpha < 0.05$) from their pre-injection value at 30 minutes (14.35 ± 0.96), and 60 minutes (12.19 ± 0.81) after injection for the highest dosage of 0.1mg/kg.

The Von Frey assay for mechanical sensitivity was used to assess the ability of 4-OXO-TEMPO to reduce mechanical hypersensitivity in the forelimbs of chronic SCI rats. Figure 4.2 represents the changes in the forelimb paw withdraw thresholds (PWT, grams) before SCI, before intrathecal injection, 30, 60, and 120 minutes after intrathecal injection of 4-OXO-TEMPO (0.05mg/kg, 0.5mg/kg, and 1.0mg/kg). The forelimb withdraw thresholds values in the pre-injection groups were significantly increased compared to baseline values. The 30 minute, 60 minute, and 120 minute values for the vehicle groups were not significantly different from the preinjection values. The 1.0mg/kg 4-oxo-tempo values for 30 minutes (13.56 ± 0.92), and 60 minutes (10.26 ± 0.74) were significantly increased from the 4-oxo-tempo preinjection values.

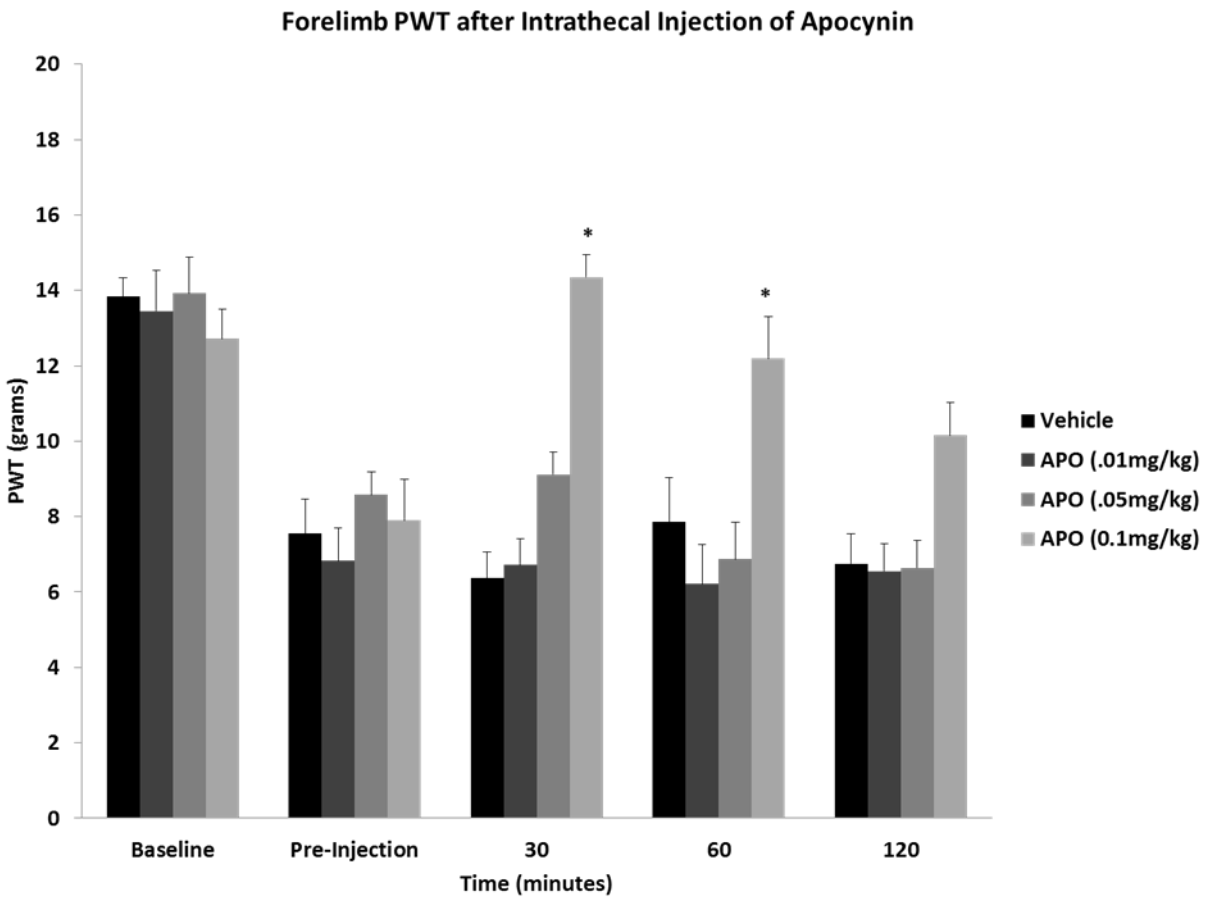

Figure 4.1 Forelimb paw withdraw thresholds (PWT) after intrathecal injection of Apocynin.

All baseline mean values are similar to each other with no significant difference from between groups. 0.1mg/kg Apocynin has the largest recovery of mechanical sensitivity with near baseline measurements 30 and 60 minutes after injection. All pre-injection mean values are significantly decreased compared to the baseline mean values. Data are plotted as mean $\pm$ SEM. *$p<0.05$ compared to vehicle treated group mean values. ($n = 6$)

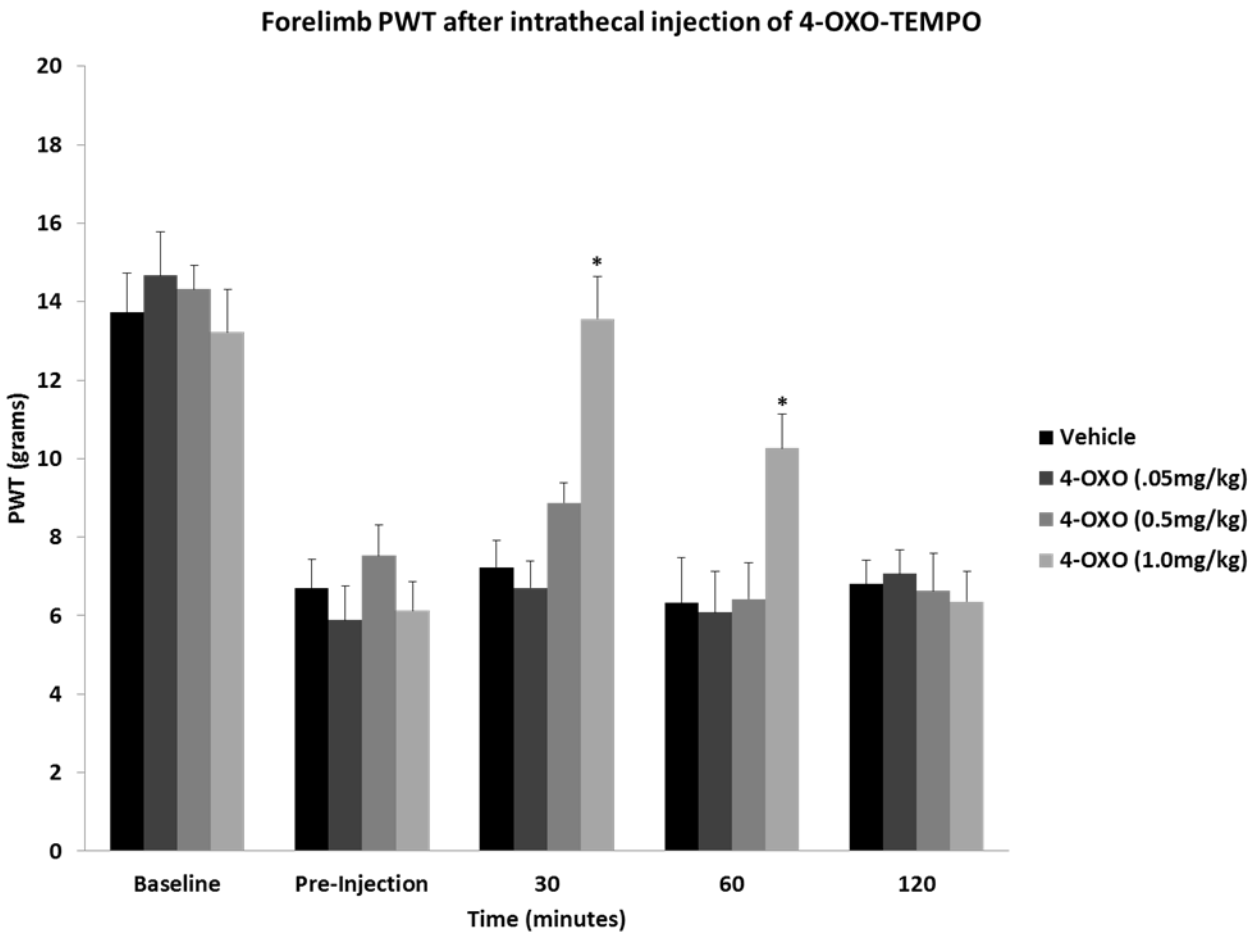

Figure 4.2 Forelimb paw withdraw thresholds (PWT) after intrathecal injection of 4-OXO-TEMPO.

All baseline mean values are similar to each other with no significant difference from between groups. 1.0mg/kg 4-oxo-tempo shows the greatest recovery of mechanical sensitivity with near baseline measurements 30 and 60 minutes after injection. All pre-injection mean values are significantly decresed compared to the baseline mean values. Data are plotted as mean $\pm$ SEM. *p<0.05 compared to vehicle treated group mean values. (n = 6)

The Von Frey assay for mechanical sensitivity was used to assess the ability of Tirilazad to reduce mechanical hypersensitivity in the forelimbs of chronic SCI rats. Figure 4.3 represents the changes in the forelimb paw withdraw thresholds (PWT, grams) before SCI, before intrathecal injection, 30, 60, and 120 minutes after intrathecal injection of Tirilazad (0.01mg/kg, 0.05mg/kg, and 0.1mg/kg). The forelimb paw withdraw thresholds values in the pre-injection groups were significantly decreased compared to baseline values. The 30 minute, 60 minute, and 120 minute values for the vehicle groups were not significantly different from the preinjection values. The Tirilazad group was not significantly different from the preinjection value 30 minutes after injection, however, 60 minutes after injection (9.32 ± 0.85) was significantly increased for the 0.1mg/kg dosage as compared to the preinjection values.

The Von Frey assay for mechanical sensitivity was used to assess the ability of U-83836E to reduce mechanical hypersensitivity in the forelimbs of chronic SCI rats. igure 4.4 represents the changes in the forelimb paw withdraw thresholds (PWT, grams) before SCI, before intrathecal injection, 30, 60, and 120 minutes after intrathecal injection of U-83836E (0.05mg/kg, 0.5mg/kg, and 1.0mg/kg). The forelimb withdraw thresholds values in the pre-injection groups were significantly decreased compared to baseline values. The 30 minute, 60 minute, and 120 minute values for the vehicle groups were not significantly different from the preinjection values. The U-83836E group was not significantly different from the preinjection value 30, 60, or 120 minutes after injection.

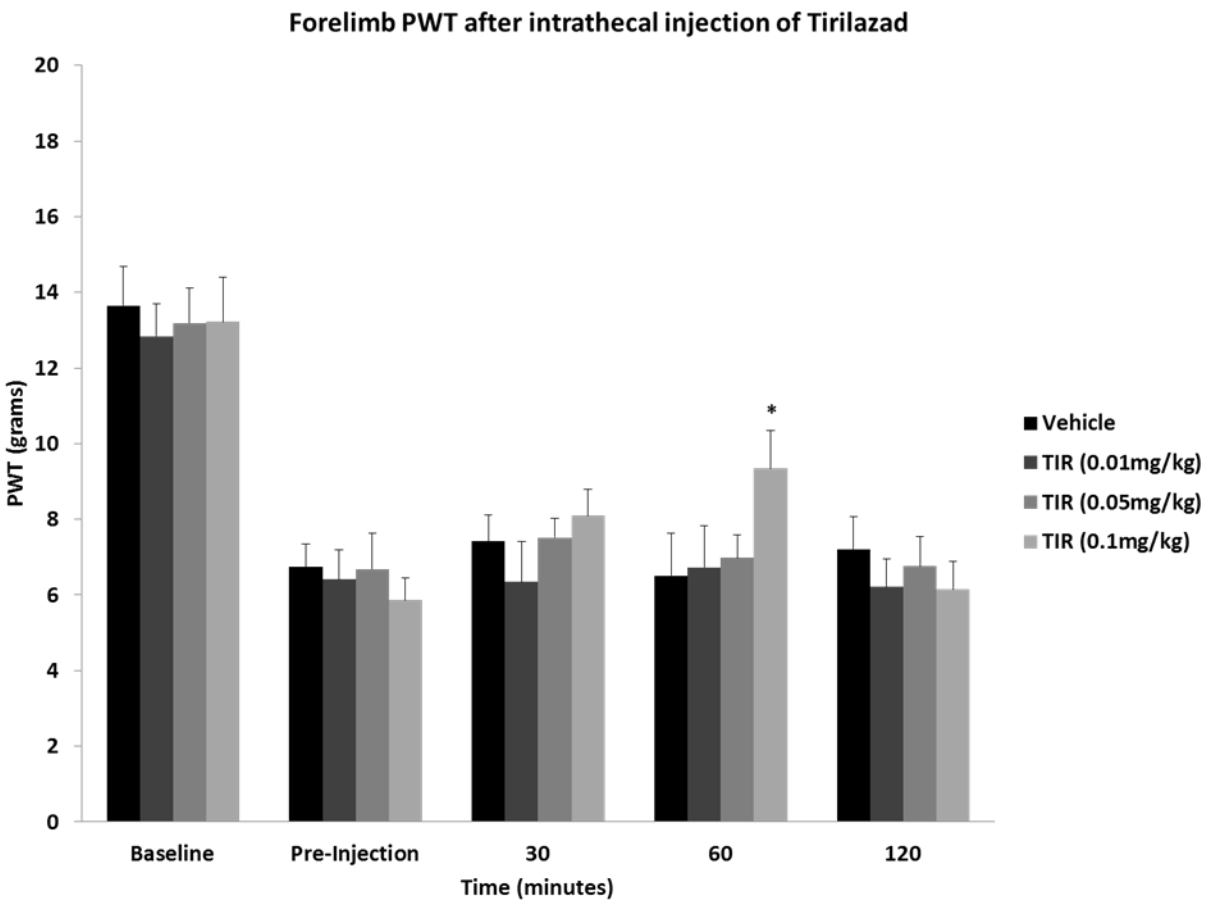

Figure 4.3 Forelimb paw withdraw thresholds (PWT) after intrathecal injection of Tirilazad.

All baseline mean values are similar to each other with no significant difference from between groups. 0.1mg/kg Tirilazad shows the greatest recovery of mechanical sensitivity with near baseline measurement 60 minutes after injection. All pre-injection mean values are significantly decreased compared to the baseline mean values. Data are plotted as mean ± SEM. *p<0.05 compared to vehicle treated group mean values. (n = 6)

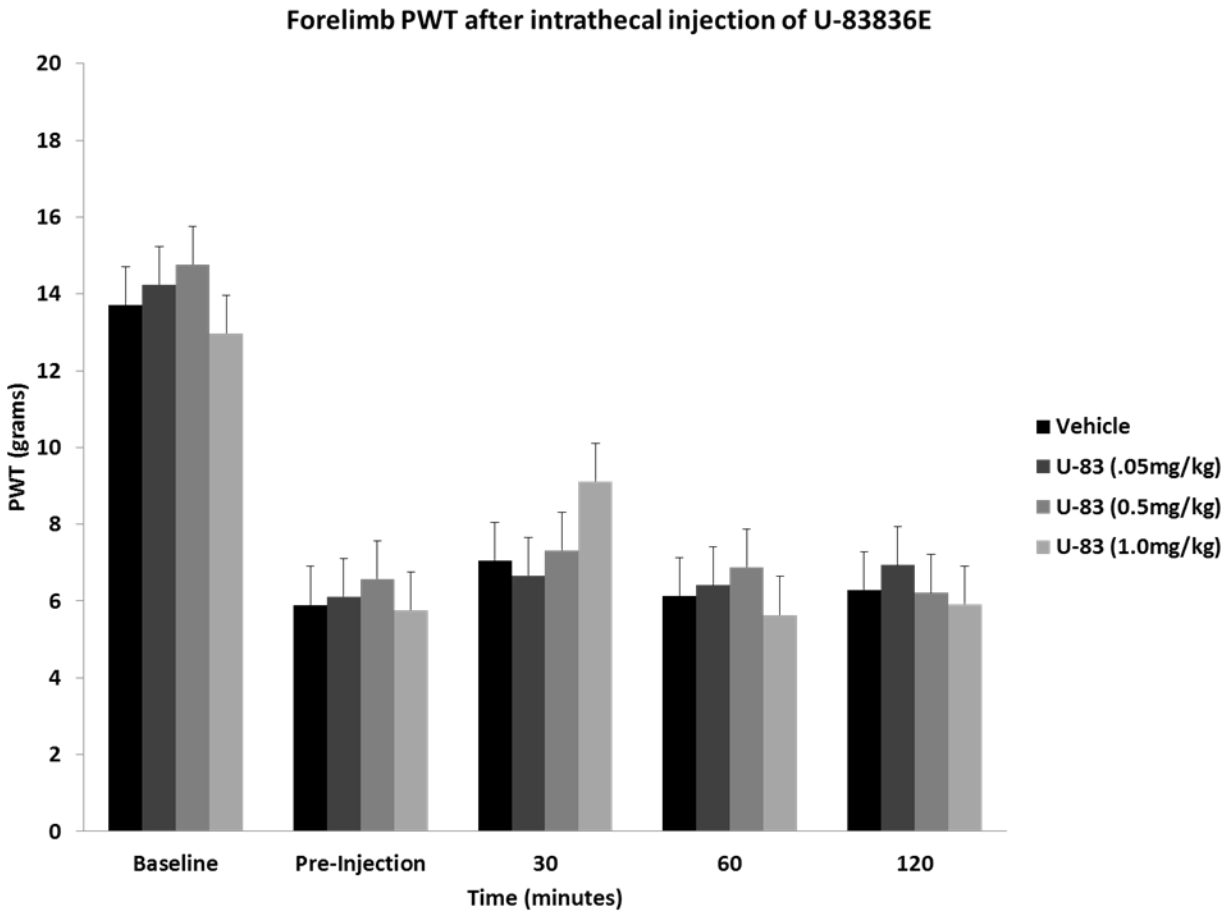

Figure 4.4 Forelimb paw withdraw thresholds (PWT) after intrathecal injection of U-83836E.

All baseline mean values are similar to each other with no significant difference from between groups. U-83836E was not significantly different when compared to SCI values. All pre-injection mean values are significantly decreased compared to the baseline mean values. Data are plotted as mean ± SEM. *p<0.05 compared to vehicle treated group mean values. (n = 6)

The Von Frey assay for mechanical sensitivity was used to assess the ability of Apocynin to reduce mechanical hypersensitivity in the hindlimbs of chronic SCI rats. Figure 4.5 represents the changes in the hindlimb paw withdraw thresholds (PWT, grams) before SCI, before intrathecal injection, 30, 60, and 120 minutes after intrathecal injection of Apocynin (0.01mg/kg, 0.05mg/kg, and 0.1mg/kg). All hindlimb paw withdraw thresholds values in the pre-injection groups were significantly decreased from baseline values. The 30 minute, 60 minute, and 120 minute values for the vehicle group were not significantly different from the preinjection values. The 0.1mg/kg Apocynin dosage group values of 30 minutes (17.14 ± 0.86), 60 minutes (17.11 ± 0.91), and 120 minutes (15.17 ± 0.87) were significantly increased from the preinjection values.

The Von Frey assay for mechanical sensitivity was used to assess the ability of 4-OXO-TEMPO to reduce mechanical hypersensitivity in the hindlimbs of chronic SCI rats. Figure 4.6 represents the changes in the hindlimb paw withdraw thresholds (PWT, grams) before SCI, before intrathecal injection, 30, 60, and 120 minutes after intrathecal injection of 4-OXO-TEMPO (0.05mg/kg, 0.5mg/kg, and 1.0mg/kg). All hindlimb paw withdraw thresholds values in the pre-injection groups were significantly decreased from baseline values. The 30 minute, 60 minute, and 120 minute values for the vehicle group were not significantly different from the preinjection values. The 1.0mg/kg 4-oxo-tempo dosage group values of 30 minutes (18.15 ± 0.67), 60 minutes (17.05 ± 1.07), and 120 minutes (15.07 ± 0.87) were all significantly increased from the 4-oxo-tempo preinjection values.

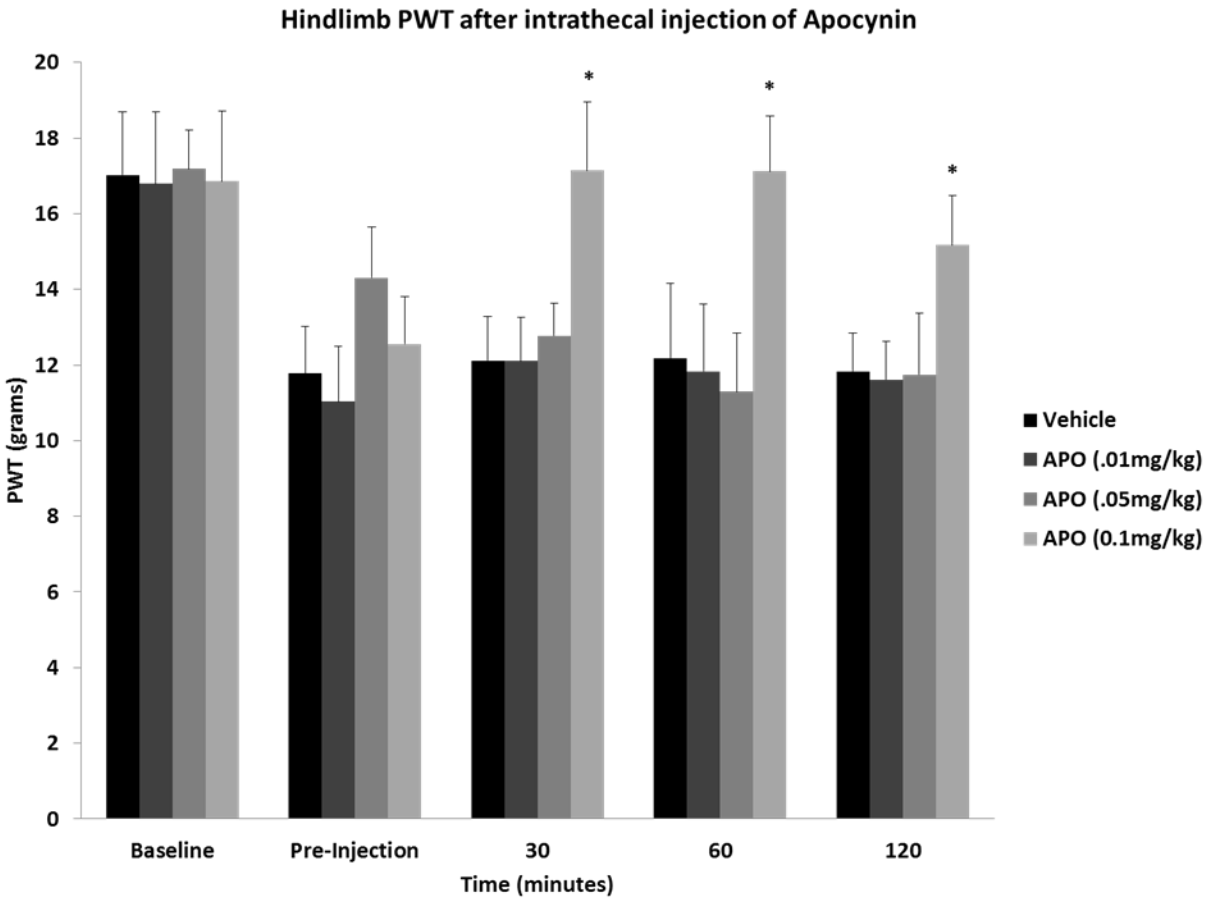

Figure 4.5 Hindlimb paw withdraw thresholds (PWT) after intrathecal injection of Apocynin.

All baseline mean values are similar to each other with no significant difference from between groups. All pre-injection mean values are significantly decreased compared to the baseline mean values. Apocynin shows the largest recovery of mechanical sensitivity, being significantly increased at 30 and 60 minutes after injection. Data are plotted as mean ± SEM. *$p<0.05$ compared to vehicle treated group mean values. (n = 6)

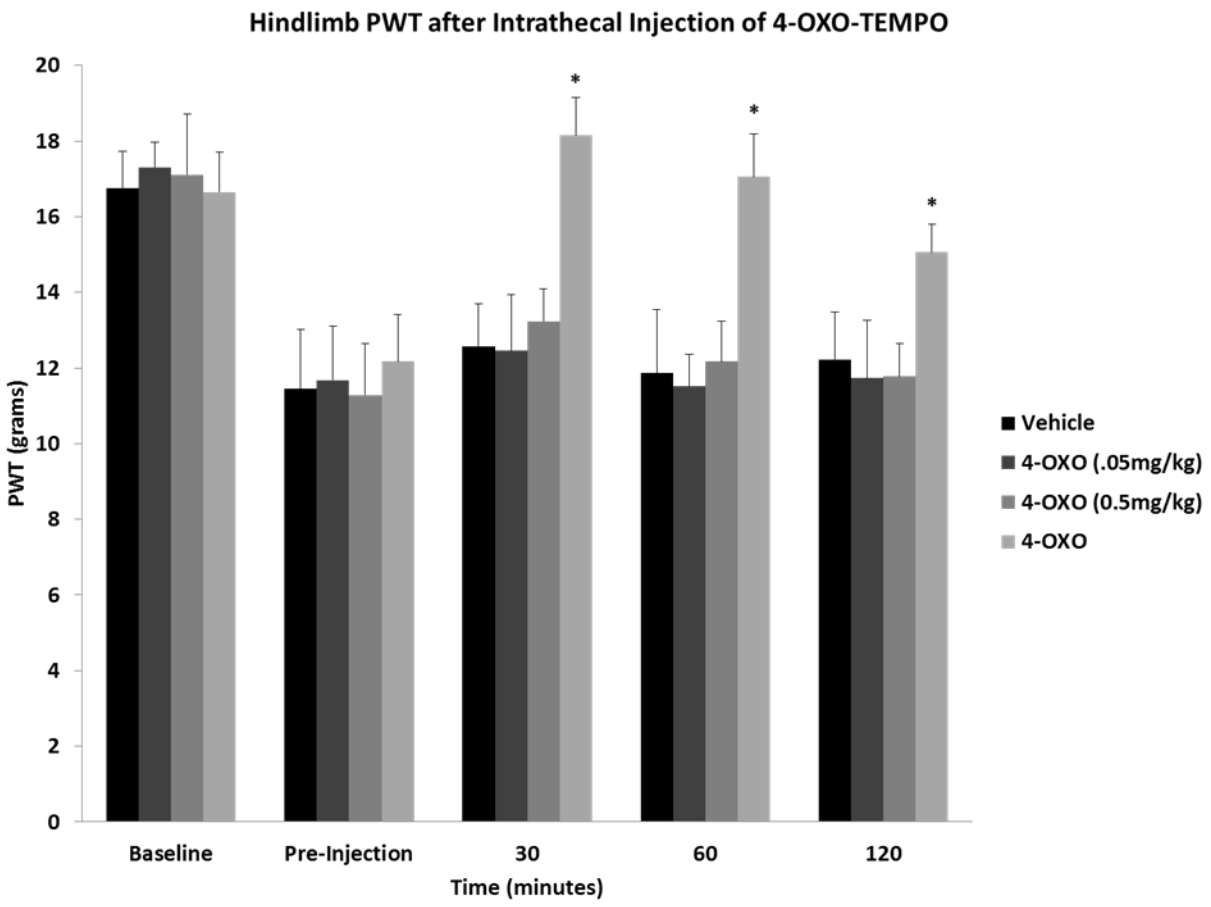

Figure 4.6 Hindlimb paw withdraw thresholds (PWT) after intrathecal injection of 4-OXO-TEMPO.

All baseline mean values are similar to each other with no significant difference from between groups. All pre-injection mean values are significantly decreased compared to the baseline mean values. 4-OXO-TEMPO shows recovery of mechanical sensitivity, being significantly increased at 30 and 60 minutes after injection. Data are plotted as mean ± SEM. *$p<0.05$ compared to vehicle treated group mean values. (n = 6)

The Von Frey assay for mechanical sensitivity was used to assess the ability of Tirilazad to reduce mechanical hypersensitivity in the hindlimbs of chronic SCI rats. Figure 4.7 represents the changes in the hindlimb paw withdraw thresholds (PWT, grams) before SCI, before intrathecal injection, 30, 60, and 120 minutes after intrathecal injection of Tirilazad (0.01mg/kg, 0.05mg/kg, and 0.1mg/kg). All hindlimb paw withdraw thresholds values in the pre-injection groups were significantly decreased from baseline values. The 30 minute, 60 minute, and 120 minute values for the vehicle group were not significantly different from the preinjection values. The 0.1mg/kg Tirilazad group values for 30 minutes (14.38 ± 0.48), and 120 minutes (14.23 ± 0.95) after injection were all significantly increased from the preinjection values.

The Von Frey assay for mechanical sensitivity was used to assess the ability of U-83836E to reduce mechanical hypersensitivity in the hindlimbs of chronic SCI rats. Figure 4.8 represents the changes in the hindlimb paw withdraw thresholds (PWT, grams) before SCI, before intrathecal injection, 30, 60, and 120 minutes after intrathecal injection of U-83836E (0.05mg/kg, 0.5mg/kg, and 1.0mg/kg). All hindlimb paw withdraw thresholds values in the pre-injection groups were significantly decreased from baseline values. The 30 minute, 60 minute, and 120 minute values for the vehicle group were not significantly different from the preinjection values. The 1.0mg/kg U-83836E group value for 120 minutes after injection (14.57 ± 1.47) was the only value significantly increased from its preinjection values.

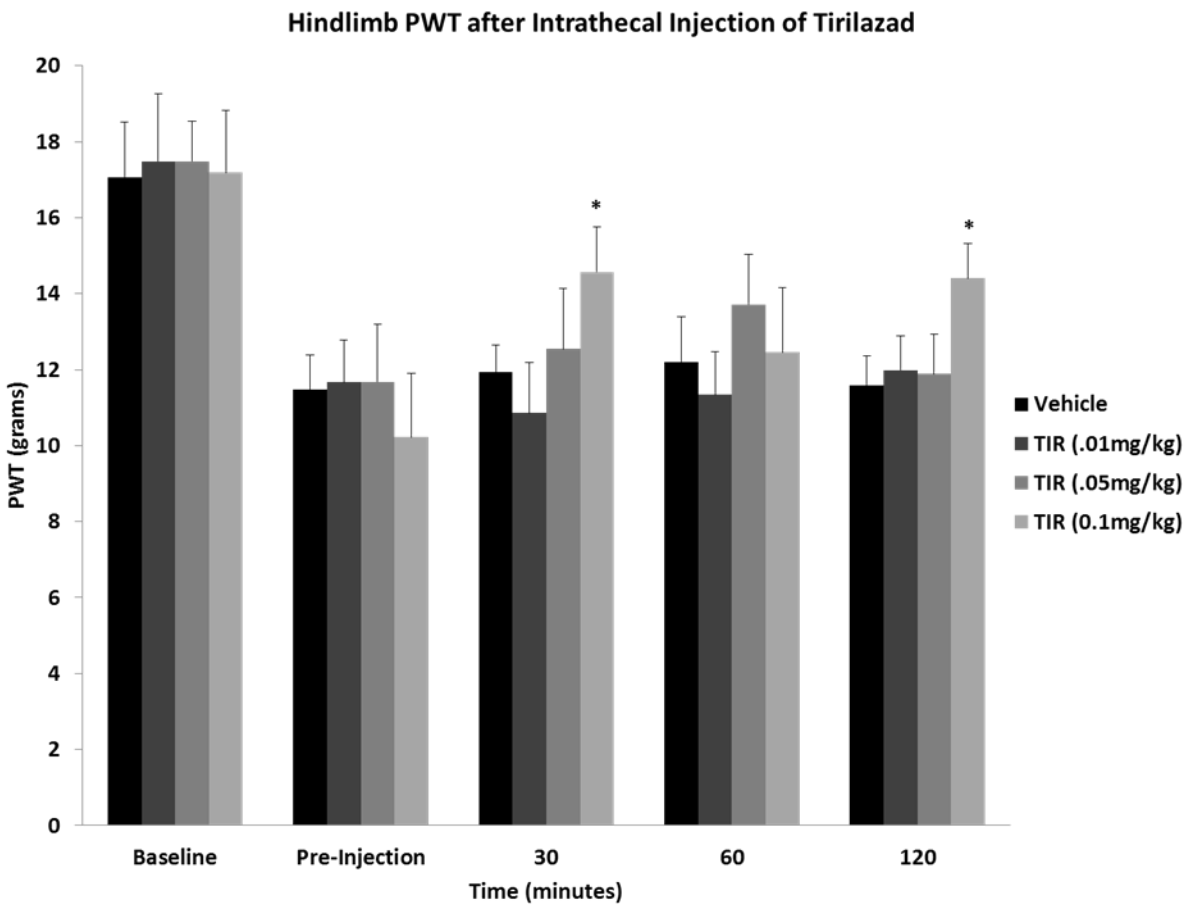

Figure 4.7 Hindlimb paw withdraw thresholds (PWT) after intrathecal injection of Tirilazad.

All baseline mean values are similar to each other with no significant difference from between groups. All pre-injection mean values are significantly decreased compared to the baseline mean values. Tirilazad shows recovery of mechanical sensitivity, being significantly increased at 30 and 120 minutes after injection. Data are plotted as mean ± SEM. *$p<0.05$ compared to vehicle treated group mean values. (n = 6)

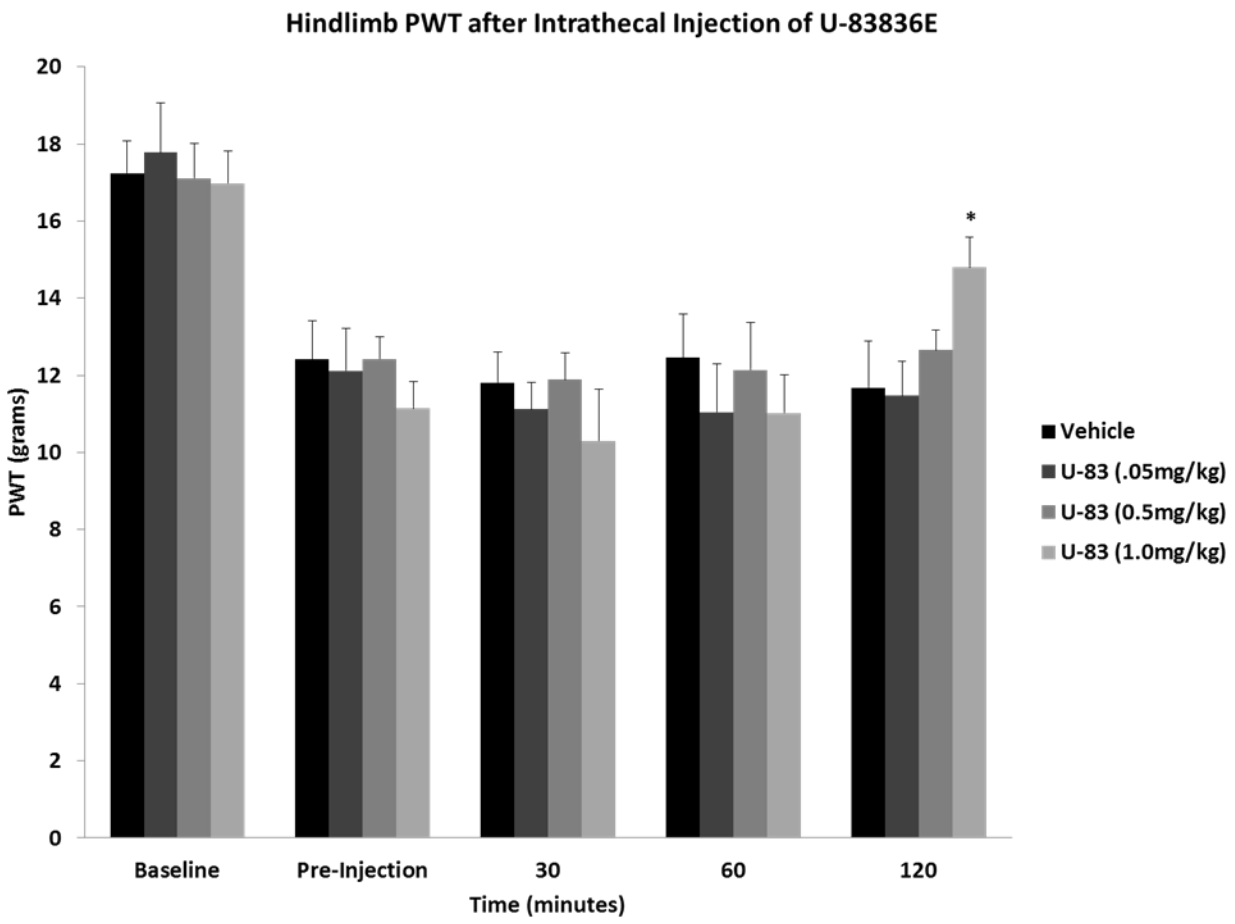

Figure 4.8 Hindlimb paw withdraw thresholds (PWT) after intrathecal injection of U-83836E.

All baseline mean values are similar to each other with no significant difference from between groups. All pre-injection mean values are significantly decreased compared to the baseline mean values. U-83836E shows recovery of mechanical sensitivity, being significantly increased at 120 minutes after injection. Data are plotted as mean ± SEM. *$p<0.05$ compared to vehicle treated group mean values. (n = 6)

4.3 DISCUSSION

Neuropathic pain is difficult to treat, because patients do not respond well to commonly prescribed analgesics (Murphy and Reid, 2001; Vissers, 2006). Underlying mechanisms of neuropathic pain are important to determine in order to create treatments that target these mechanisms and treat neuropathic pain. The present study tests four compounds that all reduce mechanical hypersensitivity in chronic SCI animals to different extents. However, the chemical mechanism of action is different for each compound and there may be off non-target effects on nervous tissue. By looking at the efficacy of each compound, insight into key processes can be gained in the ROS/LP pathway that affects chronic mechanical hypersensitivity. Apocynin and 4-oxo-tempo are effective and significantly reduced mechanical sensitivity in our model of chronic neuropathic pain. Apocynin, a NADPH oxidase inhibitor, reduces ROS by limiting the production of superoxides, which are the precursors of ROS. Apocynin has been shown to reduce inflammation in many other animal disease models as well as in animal models of nervous tissue damage (Valencia et al, 2012; Ghosh et al, 2012; Seo et al, 2012; Impellizzeri et al, 2011). The efficacy of Apocynin is likely due to its mechanism, since reducing superoxide would have a broad effect on the downstream ROS production. 4-oxo-tempo and other nitroxide scavengers, such as TEMPOL have been used in treating animal models of hypertension (Wilcox, 2010) and are potent scavengers of ROS radicals, particularly peroxynitrite (Carroll et al, 2000). The efficacy of 4-oxo-tempo is likely related to the broad effect that direct scavenging of oxidative radicals can have on

chronic SCI animals, and may implicate peroxynitrite being a major contributor to generating chronic pain in SCI animals.

There was limited efficacy of both tirilazad and U-83836E, both of which are in the lazariod family of compounds. The mechanisms of both tirilazad and U-83836E target downstream lipid peroxidation process, by either scavenging the peroxyl radicals or stabilizing the bilayer membrane. Tirilazad effects peaked at the 60 minute measurements; this is most likely because the lipid peroxidation scavenging and membrane stabilization process has a longer time course when compared to the mechanisms of the other compounds. All the compounds seemed to persist longer in the hindlimbs when compared to the forelimb time course; likely due to the close proximity of the injection site being near to the lumbar enlargement, which subserves the hindlimbs. The limited activity of the lipophilic compounds may also be due to the limitations of solubility of the compounds. The experiments were limited to dosages that would remain soluble in the vehicle and at the pH 7.2-7.4, which is the same as that of the animals' cerebrospinal fluid. It is possible that higher dosages of the lipophilic compounds may be more efficacious in reducing mechanical sensitivity in SCI animals; however, it is difficult to solubilize these compounds at higher dosages without altering the pH of the solution or using solvents that may adversely affect spinal tissue.

By investigating the analgesic properties of these compounds we are able to gain better insight into the significant roles that ROS and lipid peroxidation play in chronic neuropathic pain. A broad approach to reducing superoxides and other downstream effects of ROS via scavenging is more likely to produce an analgesic effect then that of inhibiting lipid peroxidation products which could also reduce mechanical

hypersensitivity, but it requires longer intervals to be effective. This study demonstrates the novel finding that compounds that inhibit or reduce reactive oxygen species or lipid peroxidation products reduce mechanical sensitivity in chronic spinal cord injury animals and should be considered when developing treatments for chronic neuropathic pain

Chapter 5 Apocynin Inhibits Measures of Neuropathic Pain on Chronic Spinal Cord Injured Rats

5.1 INTRODUCTION

SCI-induced chronic neuropathic pain behavior are reduced by using ROS and LP inhibitors. For the current study we tested and further characterized the inhibitory effect that superoxide (ROS progenitor) inhibitor Apocynin has on mechanical, thermal, and non-evoked measures of neuropathic pain behaviors in chronic SCI rats (**Specific Aim 2.2**). We also investigated the attenuating effects of Apocynin on membrane hyperexcitability in dorsal lamina WDR neurons (**Specific Aim 2.3**). Neuropathic pain is chronic, difficult to diagnose and treat and, often does not respond well to commonly prescribed analgesic treatments (Murphy and Reid, 2001; Vissers, 2006). Chronic pain affects 116 million people per year in the United States at a cost of over 635 billion dollars for treatment fees and lost productivity annually (Institute of Medicine, 2011). The lack of effective treatments can leave patients in constant pain, leading to increased episodes of depression and suicide (Blair et al, 2003; Cairns et al, 1996; Widerstrom-Noga et al. 2001). Patients commonly develop chronic neuropathic pain by trauma to nervous tissue, either peripherally or centrally. Specifically, up to two-thirds of all spinal cord injured (SCI) people develop neuropathic pain syndromes (Finnerup and Jensen, 2004; Werhagen et al, 2004). Our lab has developed a SCI animal model that produces chronic neuropathic pain (Hulsebosch et al., 2000; Hulsebosch, 2003), parallels the pathophysiology described in people with SCI (Bunge et al., 1993; Bunge, 1994), and

allows the rigorous study of cellular and molecular mechanisms of neuropathic pain after SCI in a controlled environment. The contusion model of SCI was chosen to test the role of reactive oxygen species (ROS) in chronic neuropathic pain.

Reactive oxygen species (ROS) are highly oxidative molecules that naturally occur as a consequence of cellular energy production. Cellular stress or trauma results in higher than normal intracellular concentrations of ROS, which can overpower the homeostatic proteins and cause oxidative damage to the cell. Neurons are especially sensitive to ROS since neurons have greater energy demands to function as compared to glial and other cells in the central nervous system (Bell, 2013). Besides being implicated in ischemia and neurodegenerative diseases, ROS are also found to be increased in chronic neuropathic pain animal models (Kim et al,, 2004; Kallenborn-Gerhardt et al, 2013).

It was previously reported that downstream consequence of ROS, LP products, may also contribute to neuropathic pain in chronic SCI animals and that compounds that inhibit ROS, also reduce measures of neuropathic pain. (Hassler et al, 2014; Gwak et al, 2013). To better investigate the role that oxidation damage plays in chronic neuropathic pain we used Apocynin, a NADPH inhibitor that reduces the endogenous concentration of superoxides, the progenitor of ROS.

The current study looks at the ability of Apocynin to reduce measures of chronic neuropathic pain in SCI rats. We used behavioral assays that examined evoked and spontaneous measures of chronic pain, in-vivo whole cell electrophysiology that examined the membrane excitability of dorsal horn neurons in the spinal cord, and immunohistochemistry in conjunction with confocal microscopy. Apocynin is capable of producing an analgesic effect on chronic SCI rats thus, it is likely that ROS is a major contributing factor to the maintenance of chronic neuropathic pain.

5.2 RESULTS

To examine if Apocynin is able to reduce overexpression of ROS in chronic SCI rats, immunohistochemistry in conjunction with confocal microscopy was used to measure expression of 4-HNE in chronic SCI spinal cord tissue. Figure 5.1 represents the expression of 4-HNE (red), a lipid peroxidation product, NeuN (green), a neuronal marker, and DAPI (blue), a cell nuclei marker, in spinal cord (T8-9) tissue taken from Sham, SCI, and SCI + APO (1.0mg/kg) animals. SCI animals were administered either vehicle or vehicle with 1.0mg/kg Apocynin intraperitoneally 30 minutes before tissue fixation and harvest. Figure 5.7 are representative confocal images of the spinal cord.

The expression of 4-HNE in chronic SCI spinal cord tissue was quantified to provide an objective measure of 4-HNE expression, optical settings during image capture were standardized so that comparison between images would remain appropriate. Figure 5.2 is the quantification of the 4-HNE expression in the spinal cord tissue samples as mean intensity of the dorsal horn region. The intensity value measurements from the SCI group (416.32 ± 49.83, $n = 8$) are significantly increased when compared to the SHAM group (32.74 ± 19.58, $n = 8$) measures ($*p < .05$), and the measurements from the SCI + APO group (193.07 ± 74.05, $n = 8$) are significantly decreased when compared to the SCI group measures ($\#p < .05$).

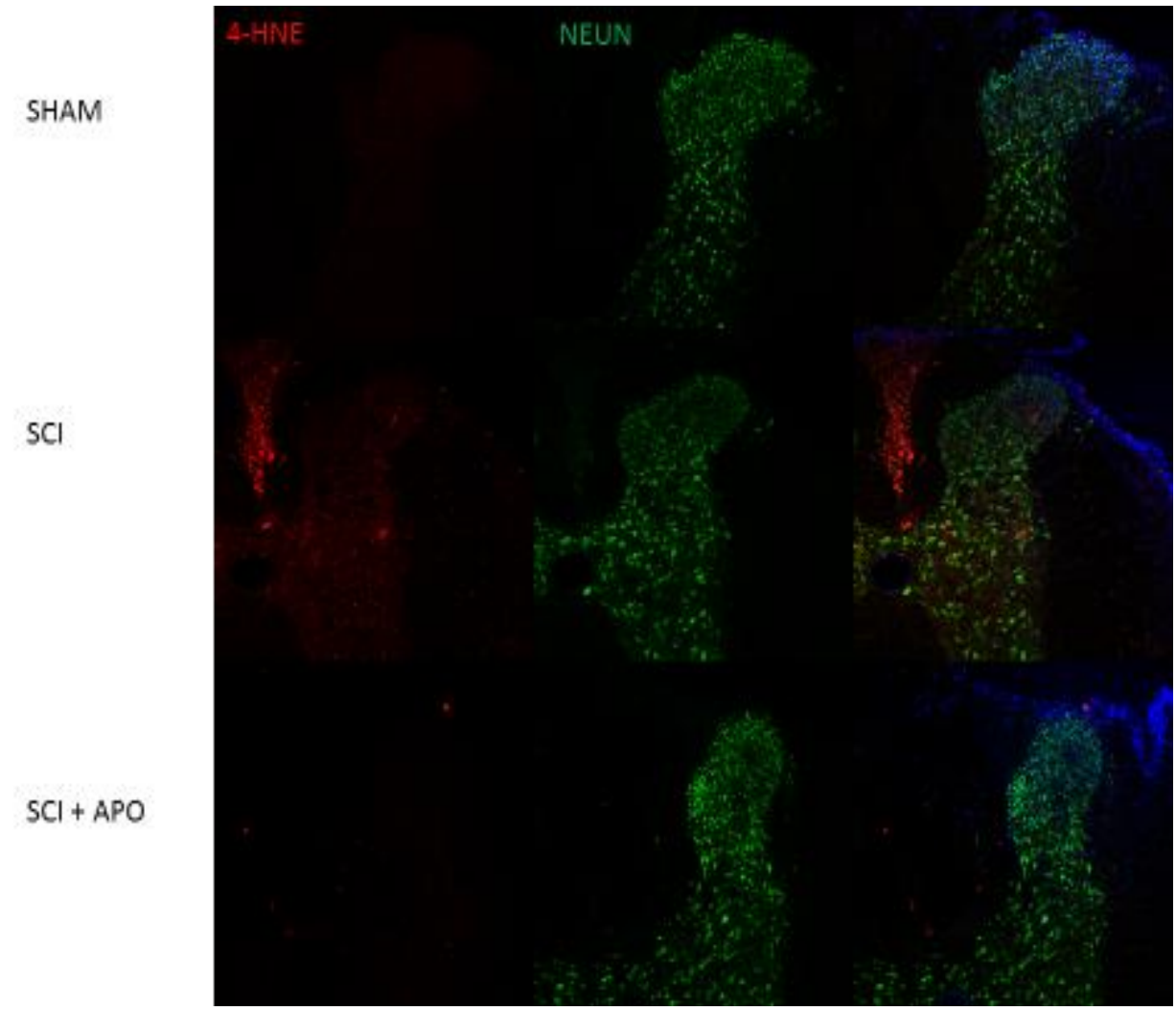

Figure 5.1 Confocal imaging showing expression of 4-HNE, NeuN, and DAPI in Sham, SCI, and Apocynin treated SCI tissue.

There is a robust increase in 4-HNE expression in spinal cord tissue 60 days after SCI. This increase in 4-HNE is attenuated with intraperitoneal administration of Apocynin (1.0mg/kg) 30 minutes before tissue fixation and harvest.

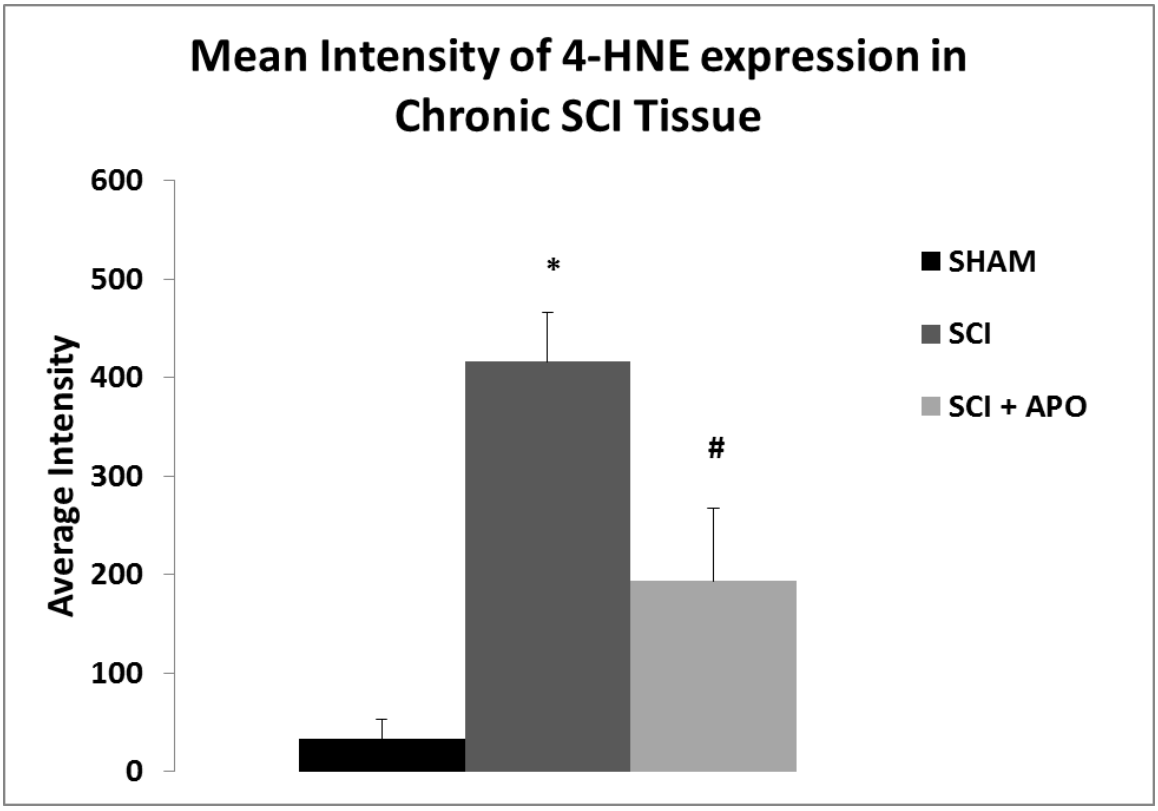

Figure 5.2 Quantification of 4-HNE intensity found in Sham, SCI, and Apocynin treated spinal cord tissue.

There is a robust increase in 4-HNE expression in spinal cord tissue 60 days after SCI. This increase in 4-HNE is attenuated with intraperitoneal administration of Apocynin (1.0mg/kg) 30 minutes before tissue fixation and harvest.

A Von Frey mechanical sensitivity assay was used to characterize the ability of Apocynin to reduce measures of mechanical allodynia in the forelimbs and hindlimbs of chronic SCI rats. Figure 5.3 represents changes in the forelimb and hindlimb paw withdraw threshold (PWT, grams) to mechanical stimuli measured in Sham, SCI, and SCI + Apocynin (0.01mg/kg, 0.05mg/kg, and 0.1mg/kg, i.t.) treated animals. All withdraw threshold values from the SCI groups were significantly decreased when compared to the Sham group measures ($\alpha = .05$). For figure 5.3a, at 30 (14.35 ± 0.66), 60 (12.19 ± 1.41), and 120 (10.16 ± 1.16) minutes after injection, the 0.1mg/kg Apocynin group measures were significantly increased when compared to the vehicle only group measures. For figure 5.3b, at 30 (17.14 ± 1.66), 60 (17.11 ± 1.81), and 120 (15.17 ± 1.62) minutes after injection, the 0.1mg/kg Apocynin group measures were also significantly increased when compared to the vehicle only group measures.

A Hargreaves-like thermal sensitivity assay was used to characterize the ability of Apocynin to reduce measures of thermal allodynia in the forelimbs and hindlimbs of chronic SCI rats. Figure 5.3 represents changes in the forelimb and hindlimb paw withdraw latency (PWL, seconds) to thermal stimuli measured in Sham, SCI, and SCI + Apocynin (1.0mg/kg, i.p.) treated animals. All withdraw latency values from the SCI groups were significantly decreased when compared to the Sham group measures ($\alpha = .05$). For figure 5.3c, at 30 (10.12 ± 0.44), and 60 (9.32 ± 0.64) minutes after injection, the 1.0mg/kg Apocynin group measures were significantly increased when compared to the vehicle only group measures. For figure 5.3d, 60 (14.71 ± 1.17) minutes after injection, the 1.0mg/kg Apocynin group measures were significantly increased when compared to the vehicle only group measures.

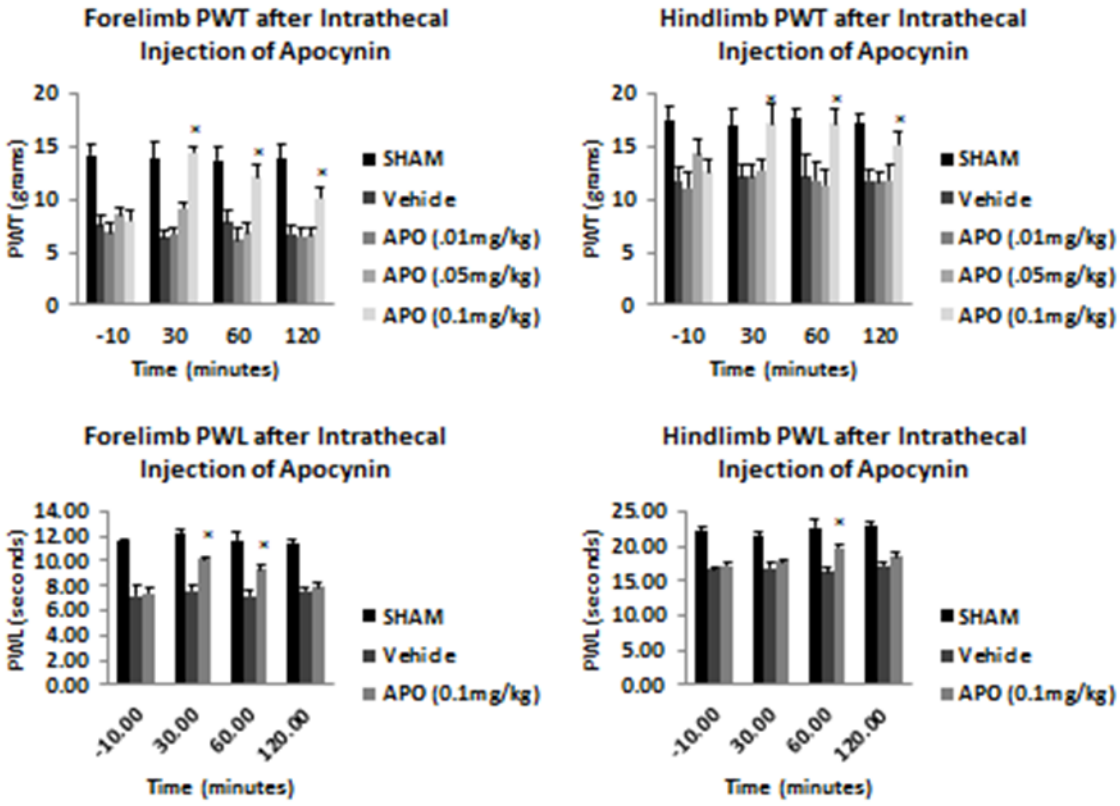

Figure 5.3 Thermal and mechanical sensitivity in Sham and SCI animals after intrathecal injection of Apocynin.

Intrathecal administration of Apocynin (0.1mg/kg) attenuated SCI-induced mechanical hypersensitivity in forelimb and hindlimb paws 30, 60, and 120 minutes after injection. Intrathecal administration of Apocynin (0.1mg/kg) also attenuated SCI-induced thermal hypersensitivity in forelimb and hindlimb paws 30 and 60 minutes after injection. All Sham group mean values are significantly different compared to the SCI group mean values. Data are plotted as mean ± SEM. *$p<0.05$ compared to vehicle treated group mean values.

To assess the ability of Apocynin to reduce non-evoked measures of neuropathic pain in chronic SCI rats we used a novel assay that measures differences in activity in SCI animals during diurnal and nocturnal periods. Figure 5.4 represents changes in animal activity during awake and sleep cycles in response to Sham, SCI, and Apocynin (1.0mg/kg). The total beam breaks during the animals sleep cycle (Diurnal) activity for the SCI + Vehicle (1124 ± 65) group was significantly increased when compared to the Sham group measures, and the SCI + APO (742 ± 128) group measures were significantly decreased when compared to the SCI + Vehicle group. The total beam breaks during the animals awake cycle (Nocturnal) activity for the SCI + Vehicle (1023 ± 72) and SCI + APO (977 ± 84) groups were significantly decreased when compared to the Sham group measures, however, the SCI + Vehicle group and the SCI + APO group were not significantly different when compared to each other. The total distance traveled during the animals sleep cycle (Diurnal) activity for the SCI + Vehicle (1036 ± 53) group was significantly increased when compared to the Sham group measures, and the SCI + APO (774 ± 89) group measures were significantly decreased when compared to the SCI + Vehicle group. The total distance traveled during the animals awake cycle (Nocturnal) activity for the SCI + Vehicle (978 ± 43) and SCI + APO (1023 ± 91) groups were significantly decreased when compared to the Sham group measures, however, the SCI + Vehicle group and the SCI + APO group were not significantly different when compared to each other.

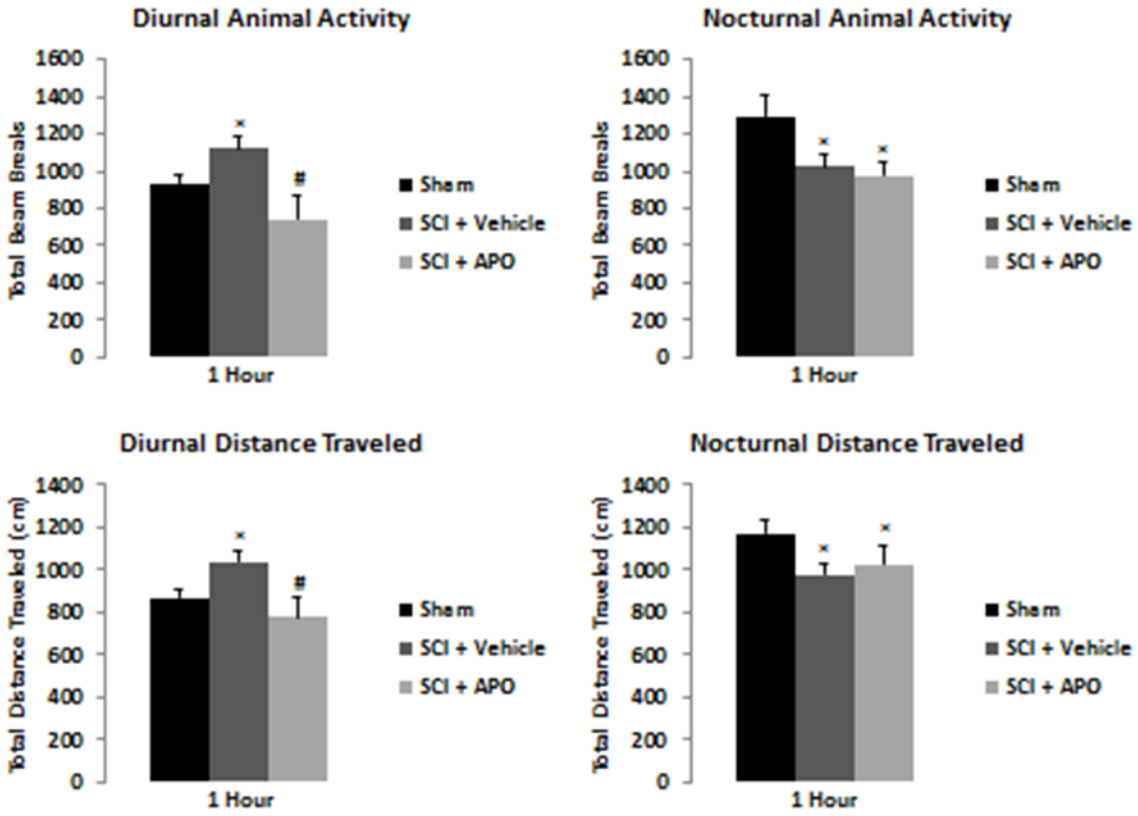

Figure 5.4 Diurnal and Nocturnal activity and distance traveled in Sham and SCI animals after intrathecal injection of Apocynin.

Intraperitoneal administration of Apocynin (1.0mg/kg) attenuated SCI-induced hyperactivity during the animal's diurnal (sleep) cycle when given 30 minutes prior to testing. Intraperitoneal administration of Apocynin (1.0mg/kg) was not able to restore the SCI-induced hypoactivity during the animals nocturnal (awake) cycle. All Sham group mean values are significantly different compared to the SCI group mean values. Data are plotted as mean ± SEM. *$p<0.05$ compared to Sham group mean values. #$p<0.05$ compared to vehicle treated SCI group mean values.

To examine the ability of Apocynin to reduce hyperexcitability in dorsal lamina WDR neurons found in chronic SCI rats' electrophysiology was used. Activity of dorsal lamina WDR neurons was measured during stimulation of the ipsalateral hind paw. Stimulation consisted of brush, pressure, pinch, and Von Frey filaments, which were applied to the hind paw in 10 second intervals. Figure 5.5 represents changes to wide dynamic range (WDR) neurons in the spinal cord dorsal lamina in animals with Sham, SCI, and SCI + APO (0.1mg/kg). Stimulus histograms were recorded from dorsal lamina WDR neuron activity from Sham, SCI, and SCI + Apo animals in response to brush (light touch), pressure (firm touch), pinch (noxious stimuli), and Von Frey filament stimuli to the hind ipsalateral paw. All neuron activity was recorded in this fashion and used in the analysis to investigate differences between the experimental groups.

Figure 5.6 represents the average spike activity during stimulation of the hind ipsalateral paw with brush, pressure, and pinch stimuli. All average spike activity values from the SCI groups were significantly increased when compared to the Sham group measures ($\alpha = .05$). The average spike activity measures for the SCI + APO group during the 30 (16.18 ± 0.42), and 60 (19.72 ± 0.35) minute time points for brush stimuli were significantly decreased when compared to the SCI group measures. The average spike activity measures for the SCI + APO group during the 30 (25.17 ± 0.92) minute time point for pressure stimuli were significantly decreased when compared to the SCI group measures. The average spike activity measures for the SCI + APO group during the 30 (27.23 ± 1.31), and 60 (33.84 ± 1.01) minute time points for pinch stimuli were significantly decreased when compared to the SCI group measures.

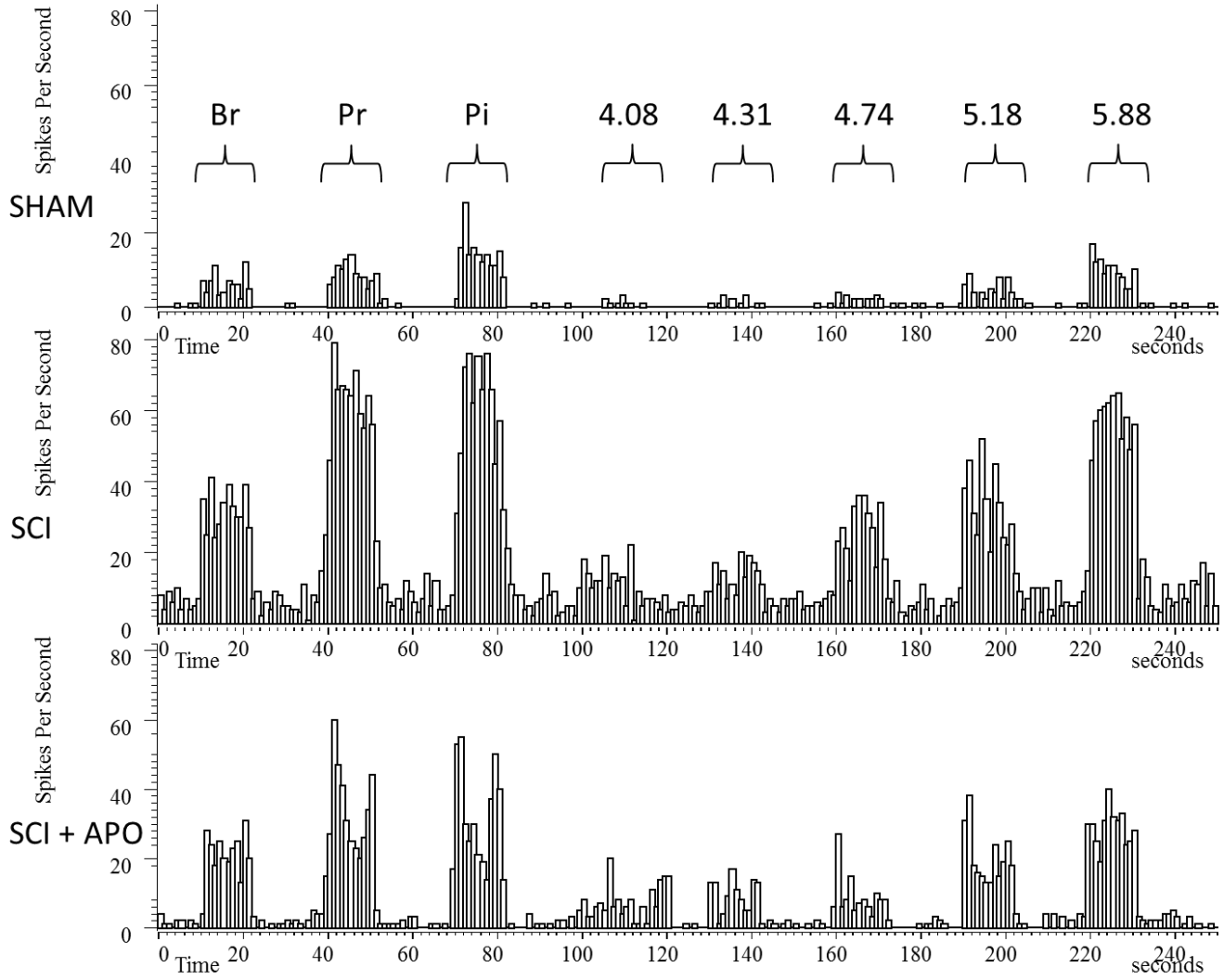

Figure 5.5 Representative stimulus histograms taken from chronic Sham, SCI, and Apocynin treated SCI animals.

The ipsalateral hindlimb paws of animals were stimulated with brush (Br), pressure (Pr), pinch (Pi), and Von Frey Filaments (4.08, 4.31, 4.74, 5.18, 5.88) during 10 second intervals. The neuron activity was recorded and displayed as the average number of spikes per second.

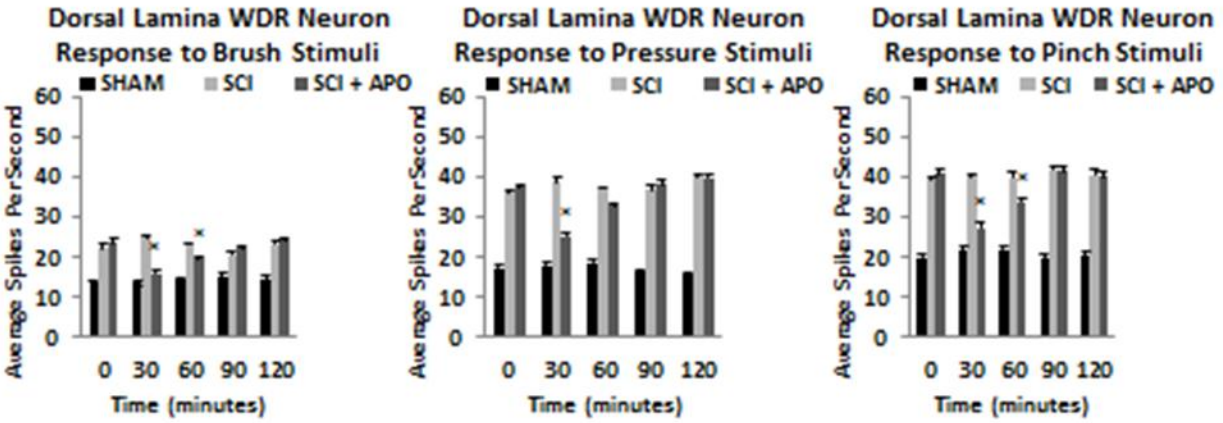

Figure 5.6 Average Spiking Activity in Sham, SCI, and Apocynin treated SCI dorsal lamina WDR neurons during stimulation with brush, pressure, and pinch stimuli.

Application of Apocynin (0.1mg/kg) to the exposed spinal cord during electrophysiological recording attenuates the hyperexcitability found in dorsal lamina neurons after SCI. All Sham group mean values are significantly different compared to the SCI group mean values. Data are plotted as mean ± SEM. *$p<0.05$ compared to SCI group mean values.

Von Frey filaments were used to stimulate the receptor fields of dorsal lamina WDR neurons in chronic SCI rats in conjunction with application of Apocynin. Figure 5.7 represents the average spike activity during stimulation of the hind ipsalateral paw with Von Frey filament stimuli. All average spike activity values from the SCI groups were significantly increased when compared to the Sham group measures ($p < .05$). The average spike activity measures for the SCI + APO group during the 30 (15.35 ± 1.18) minute time points for 6.0g Von Frey stimuli were significantly decreased when compared to the SCI group measures. The average spike activity measures for the SCI + APO group during the 30 (23.21 ± 1.26), and 60 (27.12 ± 1.32) minute time points for 15.0g Von Frey stimuli were significantly decreased when compared to the SCI group measures. The average spike activity measures for the SCI + APO group during the 30 (28.18 ± 2.61), and 60 (37.41 ± 3.27) minute time points for 60.0g Von Frey stimuli were significantly decreased when compared to the SCI group measures ($*p < .05$).

The average background activity of dorsal lamina WDR neurons was found to be higher in chronic SCI animals when compared to Sham animals. Figure 5.8 represents the average spike activity of the Sham, SCI, and SCI + APO groups without stimulation of the ipsalateral hindpaw. The data is analyzed using recordings taken just prior to the 30 minute time point of each animal. The SCI (4.85 ± 0.95) group measures are significantly increased when compared to Sham (1.23 ± 0.41) group measures and the SCI + APO (2.15 ± 0.28) group measures are decreased different when compared to the SCI group measures ($*p < .05$).

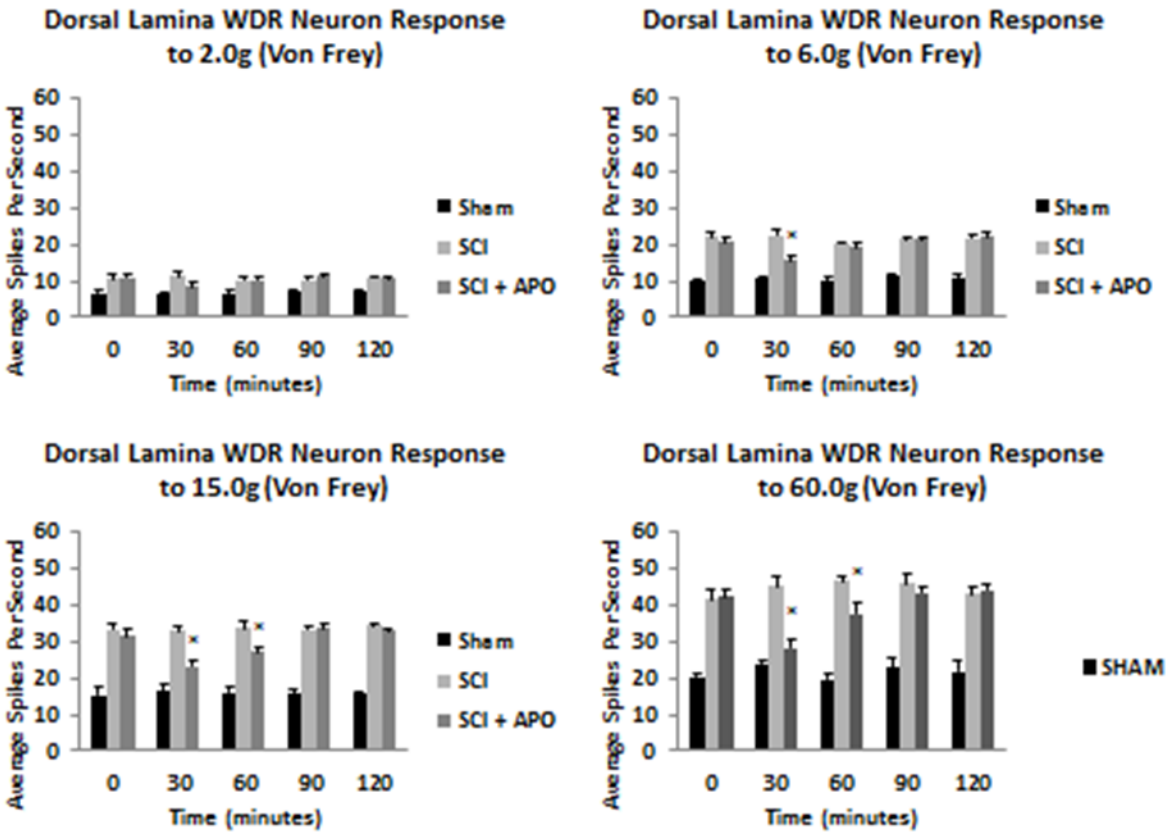

Figure 5.7 Average Spiking Activity in Sham, SCI, and Apocynin treated SCI dorsal lamina WDR neurons during stimulation with Von Frey filaments.

Application of Apocynin (0.1mg/kg) to the exposed spinal cord during electrophysiological recording attenuates the hyperexcitability found in dorsal lamina neurons after SCI. All Sham group mean values were significantly different compared to the SCI group mean values. Data are plotted as mean ± SEM. *$p<0.05$ compared to SCI group mean values.

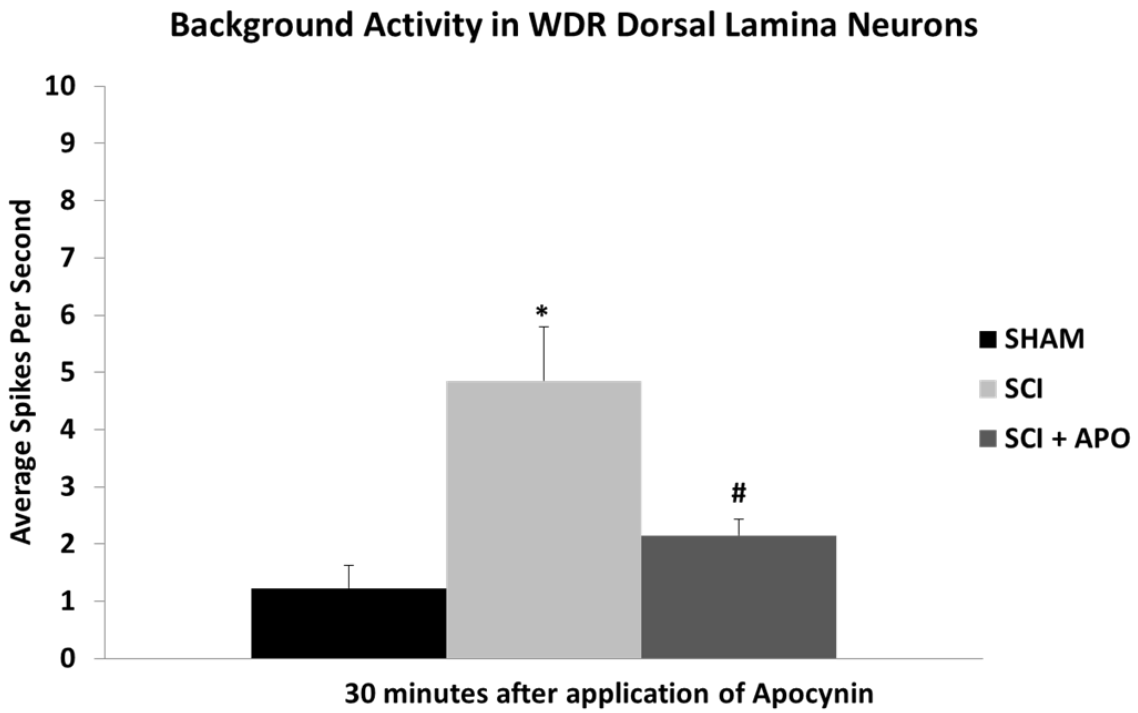

Figure 5.8 Non-evoked background activity in spinal cord dorsal lamina WDR neurons in Sham, SCI, and Apocynin treated SCI animals.

The background spiking activity of SCI dorsal lamina neurons is significantly higher when compared to Sham average spiking measures. This increased background spiking activity in SCI is significantly reduced when Apocynin (0.1mg/kg) is applied to the spinal cord.

5.3 DISCUSSION

The purpose of the study was to determine if measures of chronic neuropathic pain could be reduced when concentrations of ROS are inhibited. To inhibit ROS we applied Apocynin, a NADPH-oxidase inhibitor, which would inhibit the production of superoxide, the precursor molecule for ROS. In this study it was important to determine if application of Apocynin was actually reducing concentrations of ROS in a chronic SCI neuropathic pain model. Immunohistochemistry was used to investigate spinal tissue for expression of 4-HNE, a lipid peroxidation product, which is a common indicator of downstream ROS activity. We compared expression of 4-HNE in sham and chronic SCI tissue and found that chronic SCI tissue has increased intensity of 4-HNE staining figure 5.1, and that treatment of Apocynin can reduce the intensity of 4-HNE staining. The 4-HNE staining observed was diffuse and not limited to the dorsal lamina of the spinal cord; however, there were concentrations of 4-HNE staining observed in the central sulcus of the spinal cord. It is likely that this is due to the layer of glial cells that line this sulcus taking in the 4-HNE, as the small molecule diffuses along the spinal cord through this sulcus. The spinal cord tissue was co-stained for NeuN, a common neuron marker. The central sulcus concentrations of 4-HNE were not stained for NeuN, making it likely that the cells were glial. More study is required to determine if the observed concentration of 4-HNE at the central sulcus is a result of housekeeping activities of glial cells, or also may play a role in chronic neuropathic pain. Apocynin administration reduces expression of 4-HNE in chronic SCI animals, however, this finding does not provide any information on whether this has any functional consequences for neuropathic pain. To address this we performed behavioral assays that measure nocioception.

The behavioral experiments looked at measures of mechanical and thermal sensitivity using the Von Frey assay and a Hargreaves-like assay in which Apocynin is able to reduce mechanical and thermal sensitivity in the chronic SCI rats at about 30 minutes after intrathecal injection, as seen in figure 5.3. The Von Frey assay and Hargreaves assay are canonically used to measure evoked mechanical and thermal nociception (Willis and Coggeshall, 1991); however, neuropathic pain has a well-documented ability to produce spontaneous pain in chronic pain patients (Davidoff and Roth, 1991). To that end we modified a previously used activity assay (Mills et al, 2001) and looked for changes in the rats' diurnal activity, which is the time of day that the animals typically are resting. From many studies of chronic pain in humans, we know that a common aspect of chronic pain is loss of sleep and shorter periods of rest (Moldofsky, 2001). Other labs have also documented this behavior in rats by monitoring disturbances in EEG patterns in pain model rats (Landis et al, 1989; Sutton and Oop, 2014). In our assay of spontaneous pain, we measured activity of freely roaming rats during their diurnal, or "sleep" cycle. Chronic SCI animals are more active and travel further than their Sham counterparts and that when administered Apocynin, that this increased activity is reduced, as seen in figure 5.2. Chronic SCI rats were less active during their nocturnal, or "awake" cycle, when compared to the sham animals, although application of Apocynin did not significantly increase their activity. The Apocynin treatment did not decrease activity in the nocturnal trials, which could be considered evidence that Apocynin is reducing activity during the "sleep" cycle in the diurnal trials by an effect other than sedation.

A common consequence of SCI is hyperexcitability of spinal cord dorsal horn neurons, the neurons responsible for relaying pain information to the brain (Woolf and Salter, 2000). The hyperexcitability of dorsal horn neurons is considered the physiological correlate of neuropathic pain. For the current study, our chronic SCI animals displayed increased excitability in wide dynamic range (WDR) neurons, which is consistent with the previous studies of this lab and others (Drew et al., 2004; Hains et al., 2003; Yezierski and Park, 1993). We measured the activity of dorsal horn neurons in anesthetized chronic SCI rats in response to brush, pressure, pinch, and graded Von Frey filament stimuli. The hindlimb paws are stimulated and the resulting activities of the dorsal horn neurons are recorded. Treatment of Apocynin reduces the hyperexcitability of the dorsal horn neurons in the chronic SCI rats as seen in figures 5.4, 5.5, and 5.6. It is important to note that there was a significant increase in the background activity in the dorsal horn neurons of chronic SCI rats when compared to the sham rats, and that this background activity was reduced when the chronic SCI rats were treated with Apocynin figure 5.6. The increased background activity of the spinal cord dorsal neurons may be a correlate to the loss of descending inhibitory GABAergic input or weakening of GABAergic signal strength due to dysfunctional glial cells, which are often observed in CNP models (Gwak and Hulsebosch, 2011; Jiang et al, 2012).

In conclusion, ROS plays a major role in generating neuropathic pain in chronic SCI animals. The mechanisms that underlie this are not well understood and are deserving of further investigation. Apocynin has been extensively studied for its oxygen radical inhibiting properties and has shown great potential as a treatment for chronic SCI-induced neuropathic pain. Further study of compounds that reduce ROS or lipid

peroxidation products as treatments for chronic pain would increase the knowledge and options that physicians have in fighting neuropathic pain.

Chapter 6 Summary and Conclusions

6.1 SUMMARY

This project characterizes ROS and lipid peroxidation expression in a chronic model of contusion spinal cord injury. Immunihistochemical, mass spectrometry, and electrophysiological techniques were used to investigate ROS and lipid peroxidation products in a chronic model of SCI. ROS and lipid peroxidation products were found to be overexpressed in our chronic SCI animals. The ROS scavenger, PBN, was able to decrease the production of ROS and that this decrease in ROS resulted in a decrease in the neuronal hyperexcitability in the wide-dynamic range lumbar dorsal horn neurons in SCI animals. Hyperexcitability in dorsal horn neurons is an indicator that central sensitization has occurred in a neuropathic pain model, by reducing the hyperexcitability observed in these SCI animals dorsal horn neurons using a ROS scavenger, this result suggested that overexpression of ROS in chronic SCI animals may contribute to neuropathic pain following thoracic spinal contusion injury in rats. Lipid peroxidation products were also found to be overexpressed in our chronic model of SCI. Lipid peroxidation products are a downstream consequence of ROS and are a common measure of oxidative damage (Gutteridge, 1995). 4-HNE, and other lipid peroxidation products were overexpressed in spinal cord tissue in chronic SCI animals. The increased production of lipid peroxidation products provided evidence that the overproduction of ROS seen in our chronic SCI model is causing oxidative damage to lipids in spinal cord neurons. This characterization of ROS in a chronic model of SCI provides strong evidence for a functional role for ROS in chronic neuropathic pain. However, it was unknown if ROS contributes to pain behaviors in SCI animals.

Thus, we used ROS and lipid peroxidation inhibitors resulted in a decrease in neuropathic pain like behaviors observed in our chronic model of SCI. To do this we measured the mechanical sensitivity (Von Frey Filaments) of chronic SCI animals in conjunction with intrathecal administration of the ROS and lipid peroxidation inhibitors. Broad inhibition of superoxide production (progenitor of ROS) using Apocynin, resulted in reducing SCI-induced mechanical hypersensitivity. Other inhibitors of ROS and lipid peroxidation products were tested, such as Tirilazad, 4-OXO-TEMPO, and U-83836E. These compounds have varying degrees of efficacy indicating that multiple mechanisms may contribute to the production of neuropathic pain behaviors via overexpression of ROS and lipid peroxidation products. This data confirms that overexpression of ROS and lipid peroxidation products are contributing to the neuropathic pain behaviors observed in chronic SCI animals and that inhibition of these reactive molecules reduces neuropathic pain behaviors.

We tested whether Apocynin could inhibit thermal and non-evoked measures of neuropathic pain in SCI rats and hyperexcitability in SCI dorsal lamina WDR neurons. To accomplish this we used a Hargreaves-like test of thermal allodynia, a novel activity assay, and whole cell electrophysiology. We used immunohistochemistry to test whether Apocynin was able to reduce lipid peroxidation product, 4-HNE, in chronic SCI spinal cord tissue. Apocynin reduced paw withdraw latency in response to noxious thermal stimuli, reduced hyperactivity during the animals sleep cycle, and reduced hyperexcitability in dorsal horn neurons in SCI animals. It was also confirmed that Apocynin is able to reduce overexpression of 4-HNE in spinal cord tissue from chronic SCI animals. These experiments further confirmed that ROS and lipid peroxidation products contribute to neuropathic pain syndromes in chronic SCI animals.

6.2 CONCLUSIONS

There is a robust role for ROS and lipid peroxidation production in chronic central pain mechanisms, however, it is likely that ROS is involved in peripheral mechanisms of chronic pain as well (Barriere et al., 2012; Kallenborn-Gerhardt et al., 2012). ROS activates TRP1A and TRP1V receptors which are highly expressed in peripheral sensory neurons (Nishio et al., 2013), which indicates that ROS plays a role in chronic models of peripheral pain. The development of a ROS or lipid peroxidation product inhibitor that can be administered topically via a cream or lotion could provide a valuable therapy for patients that suffer from peripheral allodynia and hyperalgesia as well as would make an excellent future direction for the investigation of ROS mechanisms in a peripheral model of chronic pain.

These studies have shown a novel role for ROS and lipid peroxidation products as contributors of neuropathic pain in chronic models of SCI and indicate that ROS are likely involved in the molecular mechanisms that regulate maintenance of hyperexcitability in dorsal horn neurons. The pathological effects of ROS and lipid peroxidation products that occur acutely after injury have been well established (Lewen et al., 2000; Sullivan et al., 2004), and are a major target for pharmaceutical strategies. However, ROS and lipid peroxidation product production at chronic time points in models of neurotrauma have received very little attention. This project has found novel evidence of ROS overexpression in a chronic SCI model and that this overexpressed ROS contributes to neuropathic pain behaviors. These novel findings indicate that ROS and lipid peroxidation products are a viable target for therapies that seek to treat neuropathic pain in patients that develop chronic neuropathic pain months to years after a serious injury.

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Vita

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This dissertation was typed by Shayne Hassler.