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THE ROLE OF IONOTROPIC GLUTAMATE RECEPTORS IN CHRONIC CENTRAL PAIN AFTER SPINAL CORD INJURY

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THE ROLE OF IONOTROPIC GLUTAMATE RECEPTORS IN CHRONIC CENTRAL PAIN AFTER SPINAL CORD INJURY

by
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Dissertation
Presented to the Faculty of The University of Texas Graduate School of
Biomedical Sciences at Galveston
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

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May, 2007
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Key words: spinal cord injury, neuropathic pain, glutamate

ACKNOWLEDGEMENTS

I wish to express my gratitude to those who made this dissertation possible. I cannot express a greater thanks to those who were responsible for my education.

I wish to thank:

My supervising professor, Dr. Claire Hulsebosch, for her enthusiasm and nurturing mentorship.

Dr.William Willis who despite numerous obligations always had time to answer my questions.

Dr. Volker Neugebauer for being approachable and giving his insight and expertise on glutamate receptors.

Dr. Mark Evers who despite his many clinical obligations always has time to offer guidance.

Dr. Martin Grabois, my off-campus committee member from Baylor College of Medicine, for giving me future perspective on my professional career.

Dr. Woltering and Dr. Anthony, for giving me ideas and insight into my education.

Dr. Bryan Hains and Dr. Charles Mills for their guidance and friendship in and out of the Hulsebosch lab.

Debbie Pavlu, Dr. Crowne, Dr. Gwak, Mike Carter, Bryan Rooney, and Kathia, members of the Hulsebosch lab, for providing intellectual conversation and comedic relief.

Dr. Blankenship, Lonnell Simmons, and Cynthia Cheatham for their hard work in the NGP and their student advocacy.

Mom, Dad, and Huaiann for believing in me and taking a keen interest in my education. My parents have always helped me and been extremely supportive every step of the way.

Cherie Atkinson for taking care of me, having confidence in me, and making high-protein meals.

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Publication No._____

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Spinal cord injury (SCI) results in both loss of function and chronic central pain syndromes. In the clinical population, pain is characterized based on anatomical location: 1) Below-level pain – located at dermatomes corresponding to spinal segments caudal to the injury site, 2) At-level pain – located at dermatomes corresponding to spinal segments immediately adjacent to the injury site, 3) Above-level pain – located at segments rostral to the injury site (in area of sensory preservation).

A contusion model of SCI was first characterized behaviorally and electrophysiologically. A contusion at spinal segment T10 at 150 kdynes of force and a 1 second dwell time resulted in the pain like behavior in the hindlimbs, thoracic region, and forelimbs 35 days post-injury. These contused animals exhibited spinal hyperexcitability during extracellular single-unit electrophysiological spinal recordings from the dorsal horn of the lumbar enlargement (below-level), thoracic cord (at-level; immediately rostral to injury site), and brachial enlargement (above level).

In models of peripheral injury, increased ionotropic glutamate receptor mediated activity results in spinal central sensitization. Extracellular single-unit recordings from all three regions of the spinal cord (lumbar enlargement, thoracic cord, and brachial enlargement) were made on both contused and non-contused animals during ionotropic glutamate antagonist treatment (D-AP5 or NBQX). The thoracic cord, which is nearest to the site of injury, showed the greatest increase in ionotropic glutamate receptor mediated activity.

Calcium-calmodulin protein kinase II (CaMKII) has been shown to be responsible for enhancing ionotropic glutamate receptor mediated activity. CaMKII also has been

shown to be a molecular intermediate in both long-term potentiation (LTP) and peripherally induced central sensitization. After contusive SCI, the segments immediately rostral to the injury site show an increase in activated CaMKII. Application of CaMKII inhibitor, KN-93, during recording 35 days post injury, reduces spinal hyperexcitability induced by SCI.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 CHRONIC CENTRAL PAIN AFTER SPINAL CORD INJURY

Spinal cord injury (SCI) is a severe injury that often results in a loss of motor and somatosensory function below the level of the lesion. Because the standard of medical care has greatly improved, more victims survive the initial injury (Lundqvist et al. 1991) and find that they must face many other complications due to the chronic pathological consequences of the initial injury (Cairns et al. 1996; Ragnarsson 1997; Rintala et al. 1998; Summers et al. 1991). One example of the under recognized consequences of SCI are the chronic central pain (CCP) syndromes that often develop months to years after SCI (Balazy 1992; Beric et al. 1988; Davidoff et al. 1987; Jack and Lloyd 1983; Rintala et al. 1998). In fact, the percentage of SCI patients that develop CCP has been estimated to be as high as 75% (Christensen and Hulsebosch 1997). Roughly one third of patients describe the pain as severe (Siddall et al. 1999). CCP negatively influences the quality of life, frequently resulting in depression, and negatively impacts rehabilitative capabilities (Jensen et al. 2005; Siddall and Loeser 2001; Werhagen et al. 2004). Thus, understanding the mechanisms that contribute to CCP and the interventions that ameliorate CCP is important for improving the quality of life in individuals with SCI.

SCI patients that develop pain report rapid onset of pain at the time of injury or within the first year (New et al. 1997; Stormer et al. 1997) and usually described as worsening during the first year. Pain syndromes after SCI have been characterized by patients as burning, aching, stabbing, tingling, dull, sharp, hyperesthetic, and visceral cramping (Siddall and Loeser 2001). Pain has also been reported to have a greater impact on quality of life scores compared to the extent of SCI (Lundqvist et al. 1991) and is ranked as an area of highest priority for improving the quality of life by the clinical population with SCI (Anderson 2004). Patients with CCP have also been found to complain of sleep disturbances that include difficulty initiating and maintaining sleep that

consequently contribute to difficulties performing activities of daily living (Biering-Sorensen and Biering-Sorensen 2001; Rintala et al. 1998) .

CCP can be categorized based on the occurrence of pain during peripheral stimuli: (1) persistent pain – occurs independently of peripheral stimuli, is spontaneous, increases intermittently, and is described as numbness, burning, cutting, or piercing (Davidoff et al. 1987); and (2) peripherally evoked pain – occurs in response to either normally innocuous or noxious stimuli. CCP syndromes are characterized by the presence of persistent pain with concomitant changes in peripheral somatosensory responses (Hulsebosch et al. 2000; Lenz et al. 1994; Mills et al. 2001a; Mills et al. 2001b; Mills et al. 2000; Vierck et al. 2000).

Neuropathic CCP is anatomically categorized based on dermatomal region of involvement (Siddall 2002; Sjolund 2002): (1) below level pain – occurs in the dermatomes below the level of the spinal cord lesion.; (2) at level pain – occurs at the dermatomes immediately adjacent to the spinal cord lesion; and (3) above level pain – occurs several segments rostral of the spinal cord lesion (area of sensory preservation). 'Below-level' pain is described as occurring the most frequent, followed by 'at-level' pain (Stormer et al. 1997). Below-level pain is usually bilateral and described as diffusely located (Siddall 2002). Descriptors of below-level pain include: burning, tingling, numbness, aching, and throbbing. The pain is usually spontaneous in nature and responds poorly to opioids. At-level pain is characterized by the development of allodynia and hyperalgesia and is refractory to opioid therapy. At-level pain has been described as burning or stabbing. Above-level pain includes pains that may not be specific to SCI. Above-level pain includes peripheral neuropathies that are a result of overuse. Above-level pain occurs the least frequently and occurs in a very small minority of patients (Stormer et al. 1997). Given the different location of these pains relative to the lesion site, the etiology of these three pain (below-level, at-level, and above-level pain) types may be the result of different pathologies.

1.2 RODENT MODELS OF CHRONIC PAIN AFTER SPINAL CORD INJURY

Pain is a subjective, emotional sensory experience, that is impossible to measure objectively. When measuring pain in animal models, assumptions are made that a stimulus which produces pain in human subjects will produce similar painful effects and responses in animals (Christensen and Hulsebosch 1997; Woolf 1984). Animal models of CCP rely on the assumption that changes in central nociceptive pathways result in changes to response threshold during peripheral stimuli (Vierck 1991). Several animal models of SCI induced CCP exist. Each model represents a different aspect of SCI that reflects differences seen in the clinical SCI patients. The models of SCI induced pain include: 1) the excitotoxic model, 2) the ischemic model, 3) the hemisection model, and 4) the contusion model.

Glutamate has been deemed responsible for much of the secondary damage seen after SCI (Liu et al. 1999). An excitotoxic model of SCI generates the SCI without the mechanical trauma by creating glutamate agonist induced neurotoxicity. Following injection of quisqualic acid (AMPA and metabotropic glutamate receptor agonist), overgrooming/autotomy develops in dermatomes that border the lesion, indicating the presence of abnormal tonic sensations at the level of injury (Yezierski et al. 1998; Yezierski and Park 1993; Yezierski et al. 1993). Cavitation within the cord occurs after the quiqualic acid injection, and the animals exhibit thermal hyperalgesia and mechanical allodynia that was most robust at 27 days after injury and was maintained through to 35 days (Yezierski et al. 1998). Electrophysiological spinal changes, such as increased background activity, increased activity in response to innocuous and noxious stimuli, and increased afterdischarge activity are present from 7 through 36 days post injury induction (Yezierski and Park 1993).

Another model of SCI utilizes spinal ischemia to cause the initial injury. Mechanically induced spinal cord ischemia is achieved by applying an altered arterial clip to clamp the spinal cord at the T13 spinal segment for one minute. This clamping causes hindlimb and trunk mechanical allodynia that is maintained up to four weeks (Bruce et al. 2002). Another model of spinal ischemia is created using a photochemically

induced spinal lesion (Hao et al. 1991). Mechanical allodynia at dermatomes bordering the injury (at level) are present from days to 1.5 months after the injury (Hao et al. 1991; Xu et al. 1992). In the photochemically induced spinal ischemia model, dorsal horn neurons of injured and allodynic animals exhibit higher spontaneous hyperexcitability, increased afterdischarges, as well as increased responsiveness to non-noxious and noxious stimuli as compared to control animals (Hao et al. 2004).

The hemisection model is a pentration wound that may be analogous to spinal cord injury from a discharged firearm or knife wound. The hemisection model involves only cutting one entire side of the laminectomized spinal cord with a sharp scalpel. The hemisected animal develops bilateral hindlimb and forelimb mechanical allodynia and thermal hyperalgesia (Christensen et al. 1996). At-level allodynia that develops 10 to 14 days after hemisection results in parallel increases in dorsal horn spontaneous activity and responsiveness of WDR neurons to innocuous and noxious stimuli and HT neurons to noxious stimuli (Wang et al. 2005).

The contusion model involves mechanical blunt trauma, such as from a weight drop, onto the exposed spinal cord. Most injuries in humans are produced by vertebral or disc displacements that impinge upon the spinal cord; therefore, the contusion model of SCI may more closely parallel the types of injuries that occur in the majority of the clinical population (Bunge 1994; Bunge et al. 1993; Ramer et al. 2000). The NYU contusion device creates a SCI by dropping a 10g blunt tipped rod onto the laminectomized exposed cord surface. The NYU contusion at the thoracic spinal cord generates mechanical allodynia seen from 2 through 10 weeks in the forelimbs (Mills and Hulsebosch 2002), trunk (Hulsebosch et al. 2000), and hindlimbs (Lindsey et al. 2000). Thoracic contusion results in increased neuronal hyperexcitability caudal to the injury site (Drew et al. 2004; Hains et al. 2003a).

These models clearly utilize different modalities of injury: chemical, ischemic, sharp disruption, and blunt mechanical. These models of injury all result in the development of abnormal pain-like behavior and exhibit electrophysiological changes

that reflect increased nociceptive processing. The contusion model best mimics the injuries seen in the clinical SCI population.

1.3 BIOCHEMISTRY OF SPINAL CORD INJURY

The destructive pathophysiology of spinal cord injury includes the initial mechanical trauma and a progression of increase in the damaged area over time due to secondary effects. A primary cause of this secondary damage is excitatory amino acid (EAA) toxicity. Following the initial injury (e.g. ischemia in the brain or mechanical injury of the spinal cord), a large amount of glutamate and aspartate is briefly released at the injury site (Liu et al. 1991; Liu et al. 1999; McAdoo et al. 1999; Panter et al. 1990). This release may be due to exocytosis, inhibited or reversed uptake (Rothstein et al. 1996; Tanaka et al. 1997), blood brain barrier breakdown, mechanical disruption, and cell lysis. Energy supplies become depleted, and membrane permeability changes allow an influx of Ca^{2+} and Na^{+} resulting in depolarization. Injury induced depolarization leads to a further increase in Na^{+} influx, which activates Na^{+}, K^{+}-ATPase that depletes ATP stores. As energy reserves are depleted, failure of the Na^{+} pump to maintain balance with the inflow causes the level of intracellular Na^{+} to keep escalating. As the membrane further becomes more depolarized, more Ca^{2+} enters, and more glutamate is released into the extracellular space (Juurlink and Paterson 1998). This release of glutamate activates glutamate receptors, which potentiate the release of more excitatory amino acids (EAAs) and contribute to the loss of ionic homeostasis. These glutamate receptors are divided into two major types: (i) ionotropic glutamate receptors (iGluRs): N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainic acid (KA) receptors and (ii) metabotropic glutamate receptors: metabotropic glutamate receptors (mGluRs), groups I, II and III.

1.4 IONOTROPIC GLUTAMATE RECEPTORS

The ionotropic glutamate receptors are ligand-gated ion channels that mediate excitatory neurotransmission in the central nervous system. Activated ionotropic glutamate receptors regulate positive ionic currents involving sodium (Na^+), potassium (K^+), and sometimes calcium (Ca^{2+}) ions. The three recognized classes of ionotropic glutamate receptors are named according to their selective agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate. Because, some members of both AMPA and kainate classes of receptors can be activated by both AMPA and kainate, the ionotropic glutamate receptors are also commonly grouped as NMDA receptors and non-NMDA receptors.

NMDA receptors are thought to be multimeric assemblies of individual subunits, but the tetrameric composition is thought to be the most likely (Kew and Kemp 2005; Parsons et al. 1998; Szekely et al. 2002). The exact number of subunits is still unknown. The following subunits may compose an NMDA receptor: NR1, NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B. The NR2 subunits are believed to be the glutamate binding site. Channels coupled to NMDA receptors exhibit highest permeability to Ca^{2+} compared to both $Na^+$and K^+. Functional NMDA receptors are believed to be formed by a combination of both NR1 and NR2 subunits (Kew and Kemp 2005; Parsons et al. 1998). NMDA receptors are gated in a voltage-dependent manner by magnesium (Mg^{2+}).

AMPA receptors are most likely tetrameric (Kew and Kemp 2005; Parsons et al. 1998; Szekely et al. 2002). The following subunits may compose an AMPA receptor: GluR1, GluR2, GluR3, and GluR4 which are also referred to as GlurRA, GluRB, GluRC, and GluRD. AMPA and Kainic acid (KA) recptors are coupled to channels that are permiable to $Na^+$and K^+. AMPA receptors without the GluR2 subunit are Ca^{2+}-permeable (Fundytus 2001; Kew and Kemp 2005). KA receptors are most likely tetrameric and are composed of the following subunits: GluR5, GluR6, GluR7, KA-1, and KA-2 (Parsons et al. 1998).

1.5 CENTRAL SENSITIZATION, PAIN, AND SPINAL CORD INJURY

Central sensitization occurs when central nociceptive neurons become more responsive to both noxious and innocuous stimuli. Central sensitization results in hyperexcitability of dorsal horn neurons, which can be measured electrophysiologically. Central sensitization occurs in many models of peripheral neuropathy and inflamation which manifests as an increase in pain behavior. These peripherally induced central changes have been described in animal models of peripheral neuropathy (Palecek et al. 1992), arthritis (Neugebauer et al. 1994) and intradermal capsaicin (Dougherty and Willis 1992; Neugebauer et al. 1999; Neugebauer et al. 2000). In these models, WDR interneurons and identified STT cells are characterized by their responses to the presentation of peripheral stimuli. The background activity is determined, and the receptive field is mapped. In general, when central sensitization occurs, the background activity of WDR cells increases, the neurons develop expanded receptive fields, afterdischarges, and increased responses to normally non-painful (innocuous) mechanical stimuli. The mechanisms that have been proposed to lead to central sensitization include: 1) a large and prolonged discharge in nociceptive C fibers (Willis 1993; Woolf 1983), 2) ectopic discharges from dorsal root ganglion cells (Burchiel 1984; Wall and Devor 1981), 3) denervation supersensitivity (Nakata et al. 1979; Wright and Roberts 1978), 4) disinhibition (Basbaum and Wall 1976; Devor and Wall 1981), 5) increased efficacy of previously ineffective synapses (Basbaum and Wall 1976; Devor and Wall 1981), 6) release of nociceptive processing from descending inhibition (Sweet 1991), 7) permanent increase in receptor activation (Coderre 1992; Coderre and Melzack 1992) triggered by the acute increase in the extracellular concentrations of excitatory amino acids (EAAs) induced by SCI trauma (Faden and Simon 1988; Liu et al. 1991), 8) deafferentation hyperexcitability of spinal neurons and/or thalamic neurons (Lenz et al. 1994; Rinaldi et al. 1991; Tasker and Dostrovsky 1989), and 9) intraspinal sprouting (McNeill et al. 1990; McNeill et al. 1991).

Ionotropic glutamate receptors have been shown to be involved in pain states as a result of central sensitization (Baranauskas and Nistri 1998; Coderre and Melzack 1992; Woolf and Thompson 1991). Intrathecal delivery of ionotropic glutamate receptor agonists, AMPA and NMDA, increases pain responses (Kontinen and Meert 2002). Antagonism of the central ionotropic glutamate receptors during abnormal pain states due to central sensitization reduces pain-like behavior after the hemisection induced SCI (Bennett et al. 2000). Ionotropic glutamate receptor antagonism has been shown to decrease hyperexciteability of the sensitized spinal cord (Leem et al. 1996; Neugebauer et al. 1994; Neugebauer et al. 1993a; Rygh et al. 2001; Spraggins et al. 2001; Stanfa and Dickenson 1999; Zahn et al. 2005). An important component of these changes may involve chronic upregulation of ionotropic glutamate receptors, both NMDA, non-NMDA, and/or increased activation state of these receptors.

The changes associated with these models of central sensitization involve an initial primary peripheral injury or insult that results in a secondary central change. These secondary changes are believed to be a result of EAAs release from the primary afferents into the dorsal horn. Peripheral inflammatory injury leads to elevated levels of glutamate in the spinal dorsal horn (Sluka and Westlund, 1992).

Spinal cord injury leads to an abnormal pain state. A peripheral noxious stimuli creates central changes mediated by an increase in extracellular glutamate. As mentioned previously (1.3 Biochemistry of SCI), the initial mechanical injury leads to an increase in extracellular EAA concentration. The secondary damage is mainly caused by EAA toxicity. Spinal cord injury leads to secondary rises in extracellular glutamate that may sensitize surrounding neurons. One mechanism of the SCI pain may involve the ionotropic glutamate receptor mediated development of permanent hyperexcitability of dorsal horn neurons or central sensitization.

In work done by our laboratory, segments immediately rostral to site of contusion injury show increased NR1 (NMDA receptor subunit) and increased phosphorylated NR1 subunit expression. Immunocytochemistry also revealed that the increased expression of the NR1 and pNR1 subunits occurred in both the white and gray matter of the entire

cross-section of the spinal cord. The increased expression of the NR1 and pNR1 subunits are indicative of glutamate receptor mediated central sensitization.

1.6 CAMKII AND SPINAL CORD INJURY

A potential mechanism for the abnormal pain sequela after SCI is due to the post injury increase in excitatory amino acid concentrations (Liu et al. 1991; McAdoo et al. 1999) that leads to increases in intracellular calcium levels at and around the site of injury (Faden and Simon 1988; Tator and Fehlings 1991). Calcium increases lead to changes in transcription factors and receptor activation states that, in turn, cause the development of hyperexcitability of spinal neurons in the pain pathways through central sensitization (Bennett et al. 2000; Christensen and Hulsebosch 1997; Hains et al. 2002; Mills and Hulsebosch 2002; Mills et al. 2002; Vera-Portocarrero et al. 2002; Willis 2002; Woolf 1983; Woolf and Thompson 1991). This process is hypothesized to be the synaptic equivalent of long-term potentiation (LTP), which recently has been accepted as occurring in neuronal populations in many areas of the brain and spinal cord, rather than exclusively in the hippocampus (Bliss and Lomo 1973; Ji et al. 2003; Sandkuhler 2000; Willis 2002).

An important initiating event during LTP is the activation of calcium-calmodulin protein kinase II (CaMKII). Activation of the postsynaptic NMDA receptor via glutamate allows a Ca^{2+}influx, which activates calmodulin and in turn leads to the phosphorylation (activation) of CaMKII. Activated CaMKII can also phosphorylate the GluR1 AMPA subunit enhancing the conductance of the AMPA receptor. CaMKII also induces rapid trafficking and incorporation of GluR1 containing AMPA receptors into the postsynaptic membrane.

Intradermal capsaicin injection, a peripheral noxious input, results in increased expression and phosphorylation of CaMKII rat spinal dorsal horn (Fang et al. 2002). Spinal administration of KN-93 (a CAMKII inhibitor) results in decreased activity in hyperexcitable neurons from rat spinal cord in capsaicin injected animals. Work from

our lab shows spinally contused rats at T10 show an increased expression of phosphorylated CaMKII immediately rostral to the injury site compared to their non-contused counterparts 35 days post contusion injury. This increase in pCaMKII expression is localized to the dorsal horn as well as the entire grey matter. The increase in pCaMKII is also found in spino-thalamic tract cells.

1.7 SUMMARY AND EXPERIMENTAL DESIGN

The characterization of a rodent model of central pain following SCI will allow an assessment of the mechanisms that lead to the development of chronic pain. Here, we characterize a contusive model of SCI behaviorally and electrophysiologically. We also explore the contributions of ionotropic glutamate receptors in the injury state. It is well known that ionotropic glutamate receptor hyperfunction is seen in many abnormal pain states. Thus we tested the contributions of the ionotropic glutamate receptors in a model of SCI induced pain. CaMKII inhibitor has been shown to decrease spinal cord activity from sensitized neurons; consequently we tested to see if the CaMKII inhibitor, KN-93, holds the same attenuative capabilities in a model of SCI induced pain.

Hypothesis 1: Spinal cord contusion injury results in pain-like behavior that is paralleled by electrophysiologic changes at three spinal levels (below-level, at-level, and above-level).

> ***Specific Aim 1.1:*** To show that <u>below-level</u> hypersensitivity develops by 35 days post injury.
>
> > ***<u>Experiment 1.1a:</u>*** Tactile withdrawal to von Frey stimulation and thermal withdrawal threshold on <u>hindlimb paws</u> were tested before and after spinal cord contusion/sham surgery and in non-contused animals.
> >
> > ***<u>Experiment 1.1b:</u>*** Spinal recordings from the <u>lumbar enlargement</u> during peripheral stimulation of hind paws with mechanical and temperature stimuli.

Specific Aim 1.2: To show that at-level hypersensitivity develops by 35 days post injury.

Experiment 1.2a: Tactile withdrawal to von Frey stimulation on the trunk (thoracic) region were tested before and after spinal cord contusion/sham surgery and non-contused animals.

Experiment 1.2b: Spinal recordings were made from the thoracic spinal cord, immediately rostral to the site of injury, during peripheral stimulation of hind paws with mechanical and temperature stimuli.

Specific Aim 1.3: To show that above-level hypersensitivity develops by 35 days post injury.

Experiment 1.3a: Tactile withdrawal to von Frey stimulation and thermal withdrawal threshold on forelimb paws were tested before and after spinal cord contusion/sham surgery and non-contused animals.

Experiment 1.3b: Spinal recordings were made from the brachial enlargement during peripheral stimulation of hind paws with mechanical and temperature stimuli.

Hypothesis 2: Spinal cord contusion injury results in changes in ionotropic glutamate receptor activity that is dependent on the location of recording (below-level, at-level, and above-level).

Specific Aim 2.1: To show that below-level activity of contused versus non-contused animals is differentially modulated by ionotropic glutamate receptors.

Experiment 2.1: Extracellular single-unit recordings are measured from the lumbar enlargement of contused and non-contused animals during ionotropic glutamate receptor antagonist application.

Specific Aim 2.2: To show that at-level activity of contused versus non-contused is differentially modulated by ionotropic glutamate receptors.

Experiment 2.2: Extracellular single-unit recordings are measured from the thoracic cord of contused and non-contused animals during ionotropic glutamate receptor antagonist application.

Specific Aim 2.3: To show that above-level activity of contused versus non-contused is differentially modulated by ionotropic glutamate receptors.

Experiment 2.3: Extracellular single-unit recordings are measured from the brachial enlargement of contused and non-contused animals during ionotropic glutamate receptor antagonist application.

Hypothesis 3: CaMKII is involved in the hyperexciteability of the thoracic cord.

Specific Aim 3.3: To show that at-level hyperexciteability can by modulated with CaMKII inhibition.

Experiment 3.3: Extracellular single-unit recordings are measured from the thoracic cord of contused animals during KN-93 administration.

CHAPTER 2

BEHAVIORAL AND ELECTROPHYSIOLOGICAL CHARACTERIZATION OF A RODENT MODEL OF PAIN AFTER SCI

2.1 INTRODUCTION

Spinal cord injury (SCI) patients suffer from a variety of abnormal pain syndromes including musculoskeletal, visceral, and neuropathic pain (Siddall 2002). The percentage of patients that develop chronic pain is as high as 75% (Christensen and Hulsebosch 1997). Chronic central pain (CCP) develops months to years after SCI and are maintained and are generally refractory to current treatment strategies (Balazy 1992; Beric et al. 1988; Davidoff et al. 1987; Finnerup et al. 2005; Jack and Lloyd 1983; Kvarnstrom et al. 2004; Rintala et al. 1998; Siddall and Loeser 2001).

Neuropathic CCP is anatomically categorized based on the dermatomal region of involvement (Siddall 2002; Sjolund 2002): (1) below level pain – occurs in the dermatomes below the level of the spinal cord lesion.; (2) at level pain – occurs at the dermatomes immediately adjacent to the spinal cord lesion; and (3) above level pain – occurs several segments rostral of the spinal cord lesion (area of sensory preservation). The pain etiology of these three pain regions may be the result of different pathologies.

The objective of this study is to behaviorally and electrophysiologically characterize a rodent model of contusive SCI which is known to demonstrate similar pathophysiology in the clinical SCI population (Bunge 1994; Bunge et al. 1993; Hulsebosch et al. 2000) at 3 levels (below-level, at-level, and above-level).

2.2 MATERIALS AND METHODS

2.2.1 Experimental animals

Subjects were male Sprague-Dawley rats (n=60), 225-250 g, obtained from Harlan Sprague-Dawley, Inc., Indianapolis, IN, housed with a 12-h light/12-h dark cycle and fed *ad libitum*. All procedures involving rats were reviewed by the University of

Texas Medical Branch Animal Care and Use Committee. Sixty subjects were divided into three groups: (naïve; n= 15), sham (laminectomy only; n= 15), and contused (n= 30).

2.2.2 Spinal contusion injury

Subjects were anesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg). Anesthesia was considered complete when there was no flexor withdrawal in response to noxious foot pinch. The subjects' backs were shaven and a laminectomy was performed. Spinal cord contusion injury at T10 was produced using the Infinite Horizon (IH) injury device (Infinite Horizon, LLC). A motor driven cylindrical tip, 2.0mm in diameter, was driven to a force of 150 kilodynes, allowed to compress the cord for 1 second (1 second dwell time), and retracted. Following the contusion injury, the musculature was sutured, the skin was autoclipped, and the animals were allowed to recover from anesthesia. Following contusion, bladders were manually expressed twice daily until bladder function recovered, usually within 10 days post injury. Prophylactic antibiotic (Baytril, 30 mg/kg, subcutaneously) was given twice daily until bladder function returned.

2.2.3 Locomotor recovery

The Basso, Beattie, and Bresnahan (BBB) open-field locomotor test (Basso et al., 1995) was used to evaluate locomotor recover after contusive SCI. The BBB scale ranges from 0 (no hindlimb movement) to 21 (normal movement-coordinated gait with parallel paw placement). Scores ranging from 1 to 7 indicate the return of isolated movements in three joints (hip, knee, and ankle). Scores from 8 to 13 reflect the return of paw placement and coordinated movements with the forelimbs. Scores from 14 to 21 describe the return of toe clearance during stepping, predominant paw position, trunk stability, and tail position. BBB scores were measured before surgery and on 1-14, 28, and 35 days after surgery.

2.2.4 Responses to mechanical stimuli

Paw withdrawal threshold in response to von Frey filament (Stoehlting, Inc.) stimulation of the glabrous surface of the forelimbs and hindlimbs was used to quantify mechanical sensitivity. The von Frey filaments were applied in a stereotyped fashion for 4 seconds. The von Frey filaments were applied in a step wise manner starting with the lightest filament and proceeding to the next higher value. The filament that triggered withdrawal, accompanied by complex supraspinally mediated behavior (such as head turns with avoidance posturing, biting the filament, etc.) was recorded. Forelimb and hindlimb von Frey filament mechanical sensitivities were tested at two time points, before injury (baseline) and 35 days post injury. Prior to injury, the animals were acclimated for 30 minutes in a plexiglass cubical testing area and tested in the above mentioned manner for 3 testing periods on different days. For each animal, the hindlimb data (6 values; 2 hindlimbs and 3 testing days) were averaged to yield the baseline withdrawal threshold. The same was done for the forelimbs. On the second time point of evaluation, 35 days post injury, the animals are allowed to acclimate for 30 minutes and were tested. The resulting values are averaged for a post injury withdrawal threshold. Trunk sensitivity was also assessed in the same manner. A response to trunk stimulation included trunk muscle contraction, vocalization, or movement that demonstrated avoidance behavior consistent with a supraspinal response to a noxious stimulus.

Data were expressed as percent of baseline withdrawal threshold. Each animal group's final 35 day threshold value was divided by its baseline threshold value and multiplied by 100 to yield the final threshold value as a percent of baseline threshold.

2.2.5 Responses to thermal stimuli

Forelimb and hindlimb response latency to thermal stimuli was measured using a radiant heat stimulating system (PAW Thermal Stimulator, UC San Diego). The rats were placed on a glass plate over a light box and a radiant heat stimulus was applied by aiming a beam of light through a hole in the light box onto the glabrous surface of the paw through the glass plate. The glass plate was heated from 30°C to 50°C, linearly,

over 15 seconds. The light beam was discontinued automatically as soon as the rat withdraws its paw. Thermal latency is measured by the device and is defined as the length of the time between the onset of the beam to the withdrawal of the rat limb. The paw withdrawal must be accompanied by supraspinally mediated behaviors (e.g. head turning and licking of the paw) to be recorded as a response. Five minutes were allowed between each pair of trials. Prior to injury, the animals were acclimated for 30 minutes in plexiglass cubical and tested in the above mentioned manner for 3 testing periods, each on different day. For each animal the hindlimb data (6 values; 2 hindlimbs and 3 testing days) was averaged to yield the baseline thermal latency. The same was done for the forelimbs. At the second time point of evaluation (35 days post injury), the animals were allowed to acclimate for 30 minutes and were tested. The resulting values were averaged for a post injury withdrawal latency.

Data were expressed as percent of baseline withdrawal latency. Each animal group's final 35 day withdrawal latency value was divided by its baseline withdrawal latency value and multiplied by 100 to yield the final threshold value as a percent of baseline threshold.

2.2.6. Electrophysiologic recordings

After final behavioral testing, extracellular single-unit recordings were made from contused (35 days post injury), sham (age-matched), and non-contused (age-matched) animals. Rats were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneal) and supplemented with sodium pentobarbital (5 mg/kg/h) infused intravenously through a jugular vein catheter. Adequacy of anesthesia was monitored by the lack of withdrawal reflexes to noxious stimuli and the absence of corneal blink reflexes. Core temperature was maintained at 37°C by a thermostatically controlled heating blanket with a rectal probe.

Neurons were isolated from the dorsal horn of the brachial enlargement (C7-T1), thoracic segments (T8-T9), or the lumbar enlargement (L3-L5). Cells were sought in areas that corresponded to the dermatomes subjected to peripheral mechanical and

thermal stimulation. Cells were sought from the lumbar and brachial enlargements and thoracic spinal cord up to a depth of 1000μm. All cells recorded from the lumbar enlargement, thoracic cord, and brachial enlargement responded to stimulation of the glabrous surface of the hind paw, shaved hairy skin of the thorax, and the glabrous surface of the forelimb, respectively. Three to four neurons per animal were sought. The dura was opened and the exposed cord was covered with warm mineral oil. Extracellular single-unit recordings were made with a low impedance (0.4-0.8 MΩ at 1KHz; Kation Scientific) glass carbon fiber microelectrode.

Once a neuron was identified, background activity was measured followed by cutaneous receptive field mapping with von Frey filaments and brief pinches. Peripheral stimuli during electrophysiology included the following: brush (with a makeup brush), pressure (with large arterial clip at 144 g/mm^2), pinch (with small arterial clip at 583 g/mm^2), von Frey filament application (with 5 filaments: 0.6g, 2g, 6g, 15g, and 60g), and three temperature stimuli (5°C, 25°C, and 45°C). Temperature stimuli were administered with a Physitemp NTE-2A temperature stimulus probe. Each stimulus was applied for 10 seconds.

Electrical signals were amplified and input to a window discriminator, displayed on analog and digital storage oscilloscopes, processed by a data collection system (CED 1401+; Cambridge Instruments, Cambridge, UK; Pentium computer, Dell, Austin, TX, USA), and stored on a computer to construct peristimulus time histograms. The stored digital record of unit activity was analyzed offline with Spike 2 software (v4.07,Cambridge Electronic Design, Cambridge, UK). Responsiveness to peripheral stimuli was calculated by subtracting the baseline activity from evoked activity to calculate a net increase due to a stimulus evoked response. Responsiveness to thermal stimuli was calculated by subtracting the response to the 25°C (ambient temperature) to both 5°C and 45°C to discount the response from the pressure attributed to the temperature probe on the skin of the animal. Cumulative time histograms were constructed to compare group responses between non-contused and contused animals.

Cells were classified as low threshold (LT), wide dynamic range (WDR), or high threshold (HT) (Chung et al. 1986). Cells responding best to brush stimuli were classified as LT cells. Cells responding maximally to pinch, but with brush response greater than 10% of the maximal response, were classified as WDR cells. Neurons with the largest response to pinch stimulus, but with either no brush response or a brush response <10% of the pinch response, were classified as HT cells. Afterdischarge activity after pinch stimulus (most noxious) was measured at 5 second intervals up to 20 seconds post pinch stimulus for the WDR and HT cells. Cell depth and distance from midline was recorded and mapped.

2.2.7 Statistical analysis

Results are expressed as the means ± SEM. Statistical analysis were evaluated at the α-level significance of 0.05. Statistical analysis comparing spinal activity between groups were done by means of a one way ANOVA followed by Tukey's HSD test. Afterdischarge activity between groups were evaluated using a two-way repeated measures ANOVA for each 5 second time interval. Values of $P<0.05$ were considered as statistically significant. Data management and all statistical analysis were performed using Jandel SigmaStat (version 2.03). All values were graphed using Jandel SigmaPlot (version 9.0).

2.3 RESULTS

Results were obtained from 15 naïve, 15 sham, and 30 contused animals. Because sham and naïve animals demonstrated no significant differences in either behavioral outcome measures and electrophysiological response, both are grouped together in the results as the 'non-contused' group.

2.3.1 Locomotor Function

All groups had baseline BBB scores of 21 ± 0.0. Both naïve and sham (non-contused) operated animals showed no change in BBB scores (Figure 2.1). On post surgical day (PSD) 1, contused animals had a BBB score of 0.3 ± 0.1. By PSD 28 and 35 contused animals achieve BBB scores of 10.1 ± 0.3 and 10.2 ± 0.2, respectively. Thus, the injury delivered by this device yielded consistent and reproducible injuries as determined by locomotor outcome.

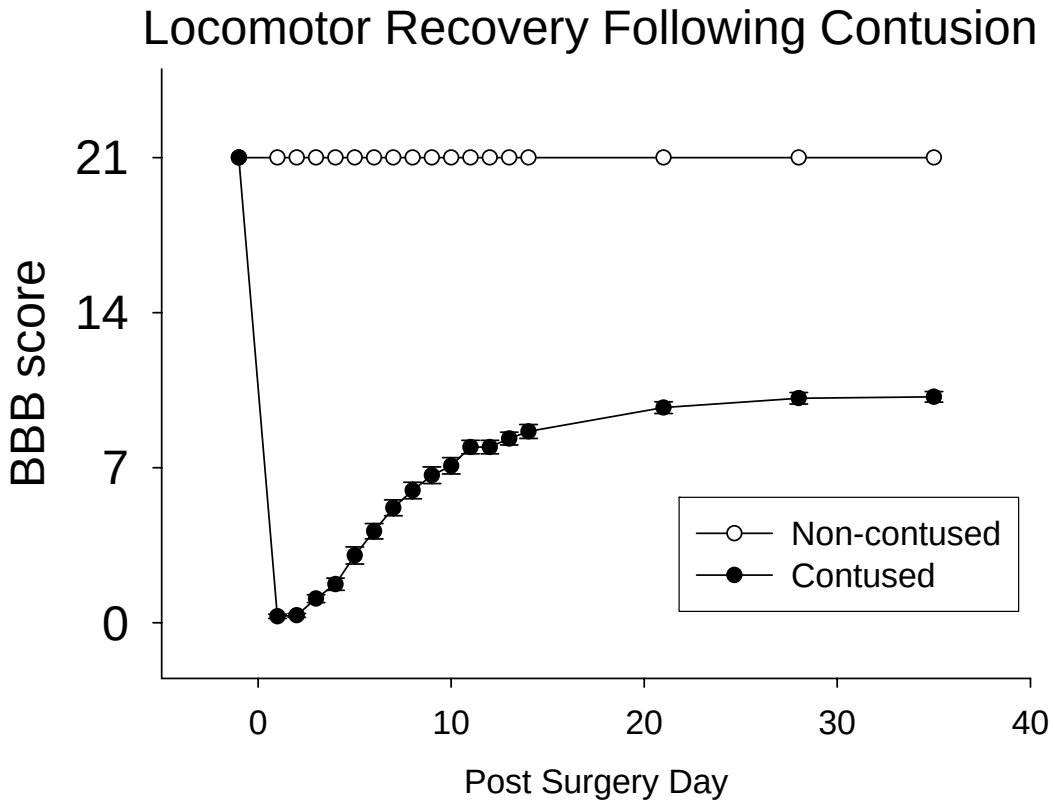

Figure 2.1. BBB scores for hindlimbs from baseline to 35 days after contusion. The BBB scores are plotted with post surgery days on the x-axis where -1 is the day before contusion (baseline). BBB scores of contused animals were maximally decreased immediately following SCI, and then rose to reach plateau by post surgery day 21. Data are expressed as means ± SEM.

2.3.2 Hindlimb behavioral response

Thirty-five days post contusion, von Frey withdrawal threshold in contused animals decreased to 44.54 ± 4.59% of the baseline presurgery value (Figure 2.2A). The contused animals showed a statistically significant difference in withdrawal threshold ($p<0.001$) compared to their non-contused counterparts. The decrease in the withdrawal threshold demonstrates the development of enhanced sensitivity to mechanical stimulation of the hindlimbs.

Hindlimb thermal withdrawal latencies of contused animals, 35 days post injury, were signicantly lower than hindlimb thermal withdrawal latencies of age matched non-contused ($p<0.001$; Figure 2.2B). This behavior is consistent with the development of enhanced responsiveness to thermal stimulus applied at the hindlimbs.

2.3.3 Trunk behavioral response

The von Frey filament threshold decreased to 40.12 ± 6.25% from baseline value, 35 days after injury (Figure 2.3). The contused animals exhibited a significant ($p<0.001$) decrease in threshold compared to non-contused animals. The significant difference in the percentage change of withdrawal threshold is consistent with the development of enhanced sensitivity to mechanical stimulation of the trunk.

2.3.4 Forelimb behavioral response

The contused animals showed a statistically significant decrease in forelimb von Frey filament withdrawal threshold ($p<0.001$) from baseline compared to the non-contused group (Fig. 2.4A). The significant difference in the percentage change of withdrawal threshold is consistent with the development of enhanced sensitivity to mechanical stimulation of the forelimbs.

The decrease of the forelimb thermal withdrawal latency 35 days post injury is statistically significant compared to the age matched non-contused group ($p<0.001$). This behavior is consistent with the development of enhanced responsiveness to thermal stimuli applied to the forelimbs.

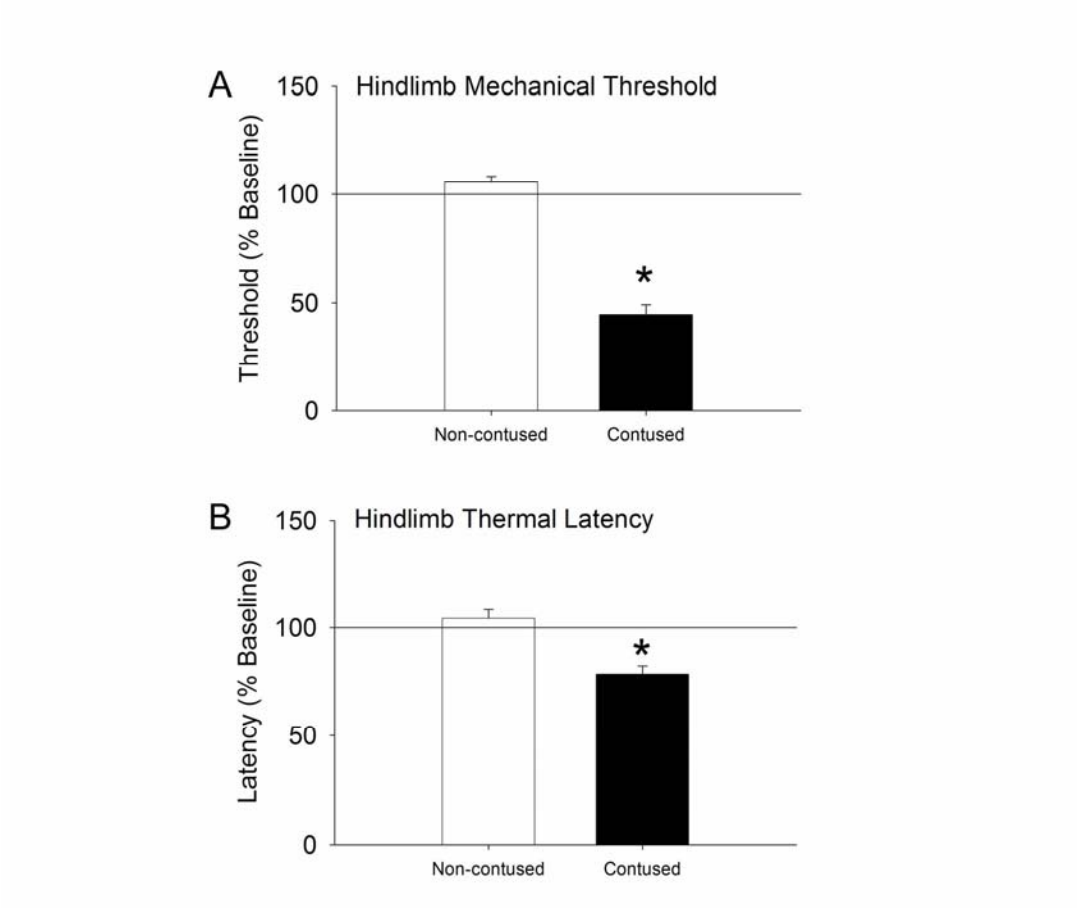

Figure 2.2. Hindlimb mechanical withdrawal threshold and thermal withdrawal latency reported as percent of baseline value obtained at the 35 post surgery day.
The contused group showed a significant decrease in both mechanical withdrawal threshold (A) and thermal withdrawal latency (B) compared to the non-contused group . An * indicates a statistically significant difference ($p<0.001$) compared to the non-contused group. Data are expressed as means ±SEM (n=30 for each group).

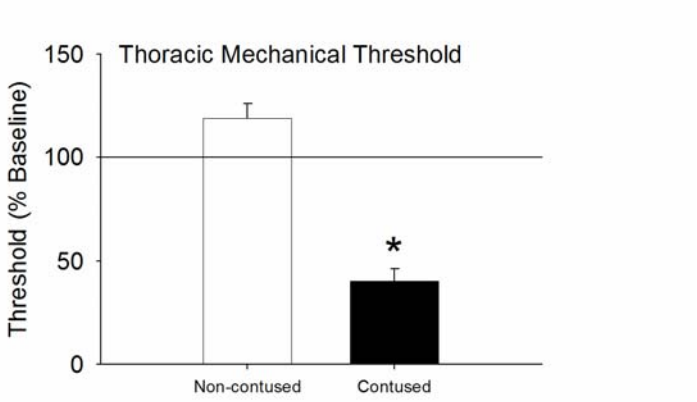

Figure 2.3. Thoracic mechanical threshold reported as percent of baseline value obtained 35 days post surgery. The contused group showed a significant decrease in both mechanical withdrawal threshold and thermal withdrawal latency compared to the non-contused group . An * indicates a statistically significant difference ($p<0.001$) compared to the non-contused group. Data are expressed as means ±SEM (n=30 for each group).

2.3.5 Electrophysiology of the lumbar enlargement

Ten animals from each group, non-contused and contused, were utilized to isolate 65 neurons in the dorsal horn of the lumbar enlargement. The number of neurons observed and the mean recording depths are shown in table 2.1. The recording sites are depicted on a crossectional map of the lumbar enlargement in figure 2.5.

Figure 2.6 illustrates single unit activity from single units of both non-contused and contused animals. The single cell activity from a WDR neuron of a contused animal exhibits heightened background and increased responses to brush, press, pinch, von Frey filament, and temperature stimuli.

Lumbar WDR cells in contused animals show significantly increased background activity (spontaneous activity), response to brush, press, and pinch when compared to lumbar WDR cells of non contused animals ($p<0.05$; Figure 2.7A). WDR cells in contused animals also exhibit significantly increased responses to 0.6, 2.0, 6.0, 15, and 60g von Frey filaments as compared to the cells from non-contused animals ($p<0.05$; Figure 2.7B). WDR responses to 5°C and 45°C temperature probe are also significantly elevated as compared to responses from cells of non-contused animals (Figure 2.7C).

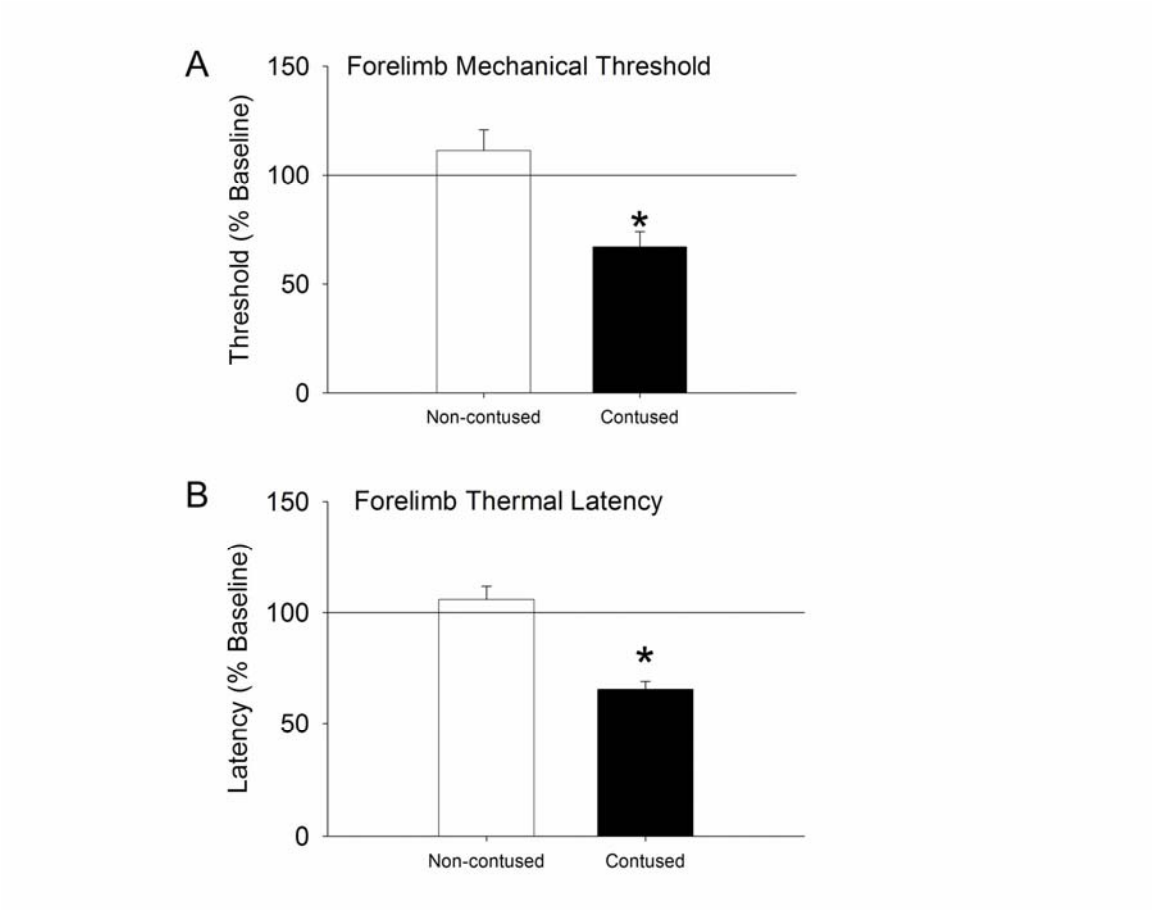

Figure 2.4. Forelimb mechanical withdrawal threshold and thermal withdrawal latency reported as percent of baseline value obtained at the 35 post surgery day. The contused group showed a significant decrease in both mechanical withdrawal threshold (A) and thermal withdrawal latency (B) compared to the non-contused group . An * indicates a statistically significant difference ($p<0.001$) compared to the non-contused group. Data are expressed as means ±SEM (n=30 for each group).

Lumbar Enlargement		
Non-contused	No. of Neurons	Depth (μm)
WDR	10	573 ± 68
HT	8	317 ± 43
LT	15	651 ± 50
Contused		
WDR	15	545 ± 52
HT	8	305 ± 43
LT	10	553 ± 90

Table 2.1. Number of cells sampled in the dorsal horn of the lumbar enlargement.
Depth is represented as mean ± SEM.

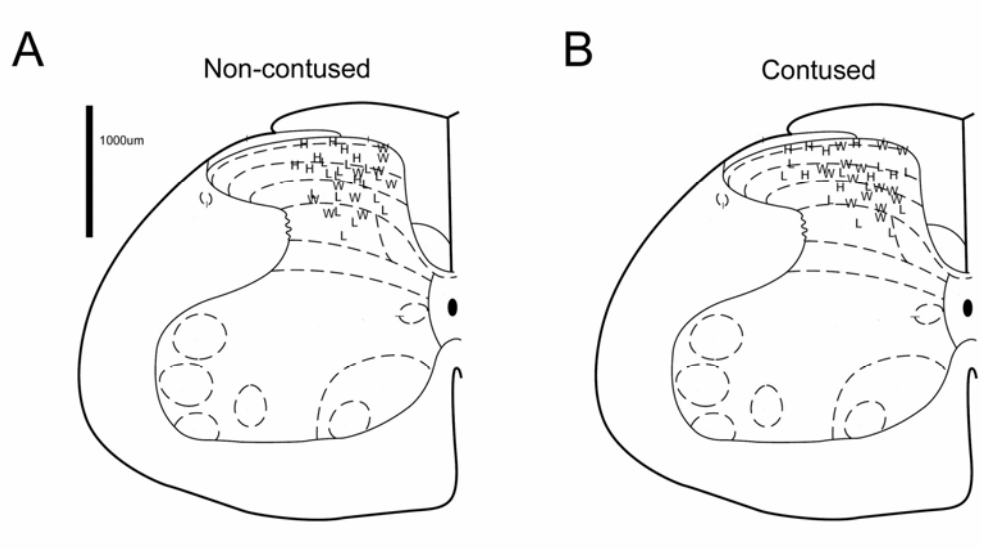

Figure 2.5. Representative recording sites of neurons from the dorsal horn of the lumbar enlargement. In the lumbar enlargement of both non-contused animals (A) and contused animals (B) recordings were made up to a depth of 1000μm. WDR, HT and LT neurons are labeled as W, H, and L, respectively.

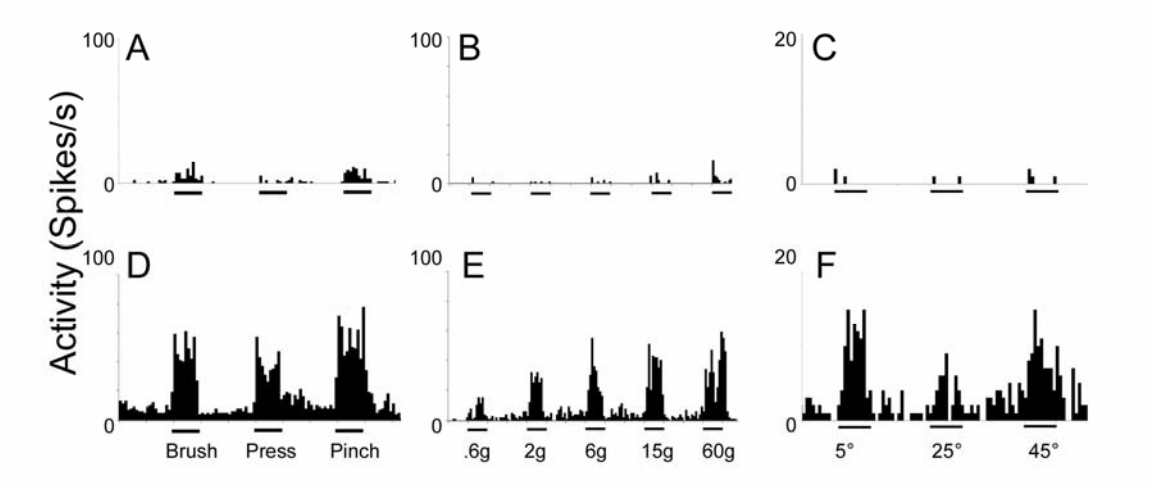

Figure 2.6. Representative single unit peristimulus time histograms (spikes/1 second bin) of WDR neurons from the lumbar enlargement of non-contused and contused animals. Recordings were made from non-contused (A-C) and contused (D-F) subjects. The recordings were taken during peripheral: brush, press, and pinch (A and D), von Frey filament application (B and E), and temperature stimuli (C and F).

Lumbar HT cells of contused animals exhibit significantly increased responses to press and pinch compared to the HT cells of noncontused animals ($p<.05$; Figure 2.7D). Responses to 15g and 60g von Frey filament stimulation are also significantly increase ($p<0.05$; Figure 2.7E). The response to both 5°C and 45°C temperature probe is increased in HT cells from contused animals ($p<0.05$; Figure 2.7F).

Lumbar low threshold neurons from contused animals only exhibit heightened responses to brush compared to the LT cells from non-contused animals ($p<0.05$; Figure 2.7G). LT neurons from contused animals exhibited no difference during von Frey filament application and temperature stimuli compared to LT neurons from non-contused animals (Figure 2.7H and 2.7I).

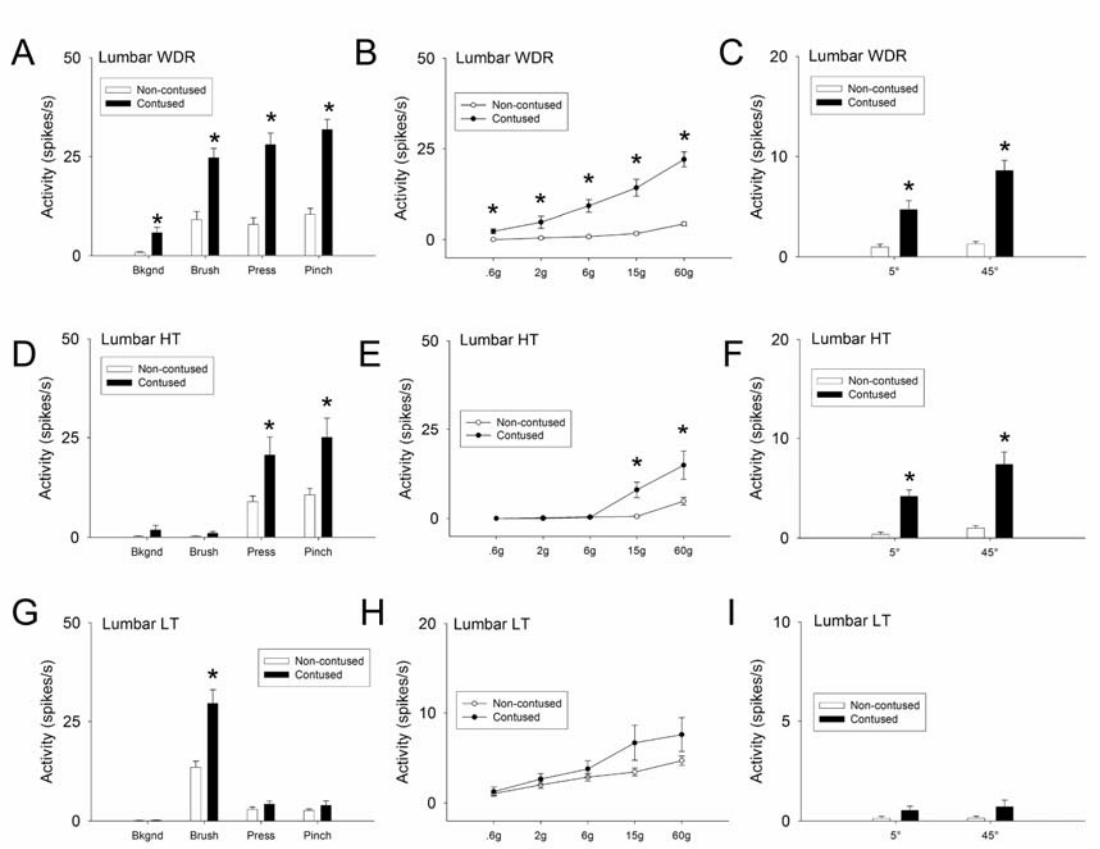

Figure 2.7. Responses of dorsal horn neurons of the lumbar enlargement (spinal segments L3-5) of contused and non-contused animals to hindpaw stimulation.
Recordings are made from WDR (A-C), HT (D-F), and LT (G-H) cells during background (no stimulus), brush, press, and pinch (A, D, G), von Frey stimulation (B, E, H), and temperature stimuli (C, F, I). Responses to peripheral stimuli are shown as discharge rate above background activity. Temperature stimuli are depicted as discharge rate above discharge rate during 25°C stimulation. Lumbar WDR neurons from contused animals exhibit enhanced background activity, exaggerated responses to mechanical stimuli, 5°C, and 45°C compared to the WDR neurons from the non-contused animals. Lumbar HT neurons of contused animals exhibit enhanced activity to noxious mechanical stimuli, 5°C, and 45°C compared to the HT cells from non-contused animals. Lumbar LT neurons from contused animals exhibit enhanced response to brush only compared to LT cells from the non-contused group. An * indicates a statistically significant difference ($p<0.05$) compared to the non-contused group. Data are expressed as means ±SEM.

Lumbar WDR cells (n=15) of contused animals showed increased afterdischarge activity compared to the WDR cells (n=10) of their non-contused counterparts ($p<0.001$; Figure 2.8). Lumbar HT cells (n=7) of contused animals showed no significant difference of afterdischarge activity compared to HT cells (n=8) from the non-contused group (Figure 2.8B).

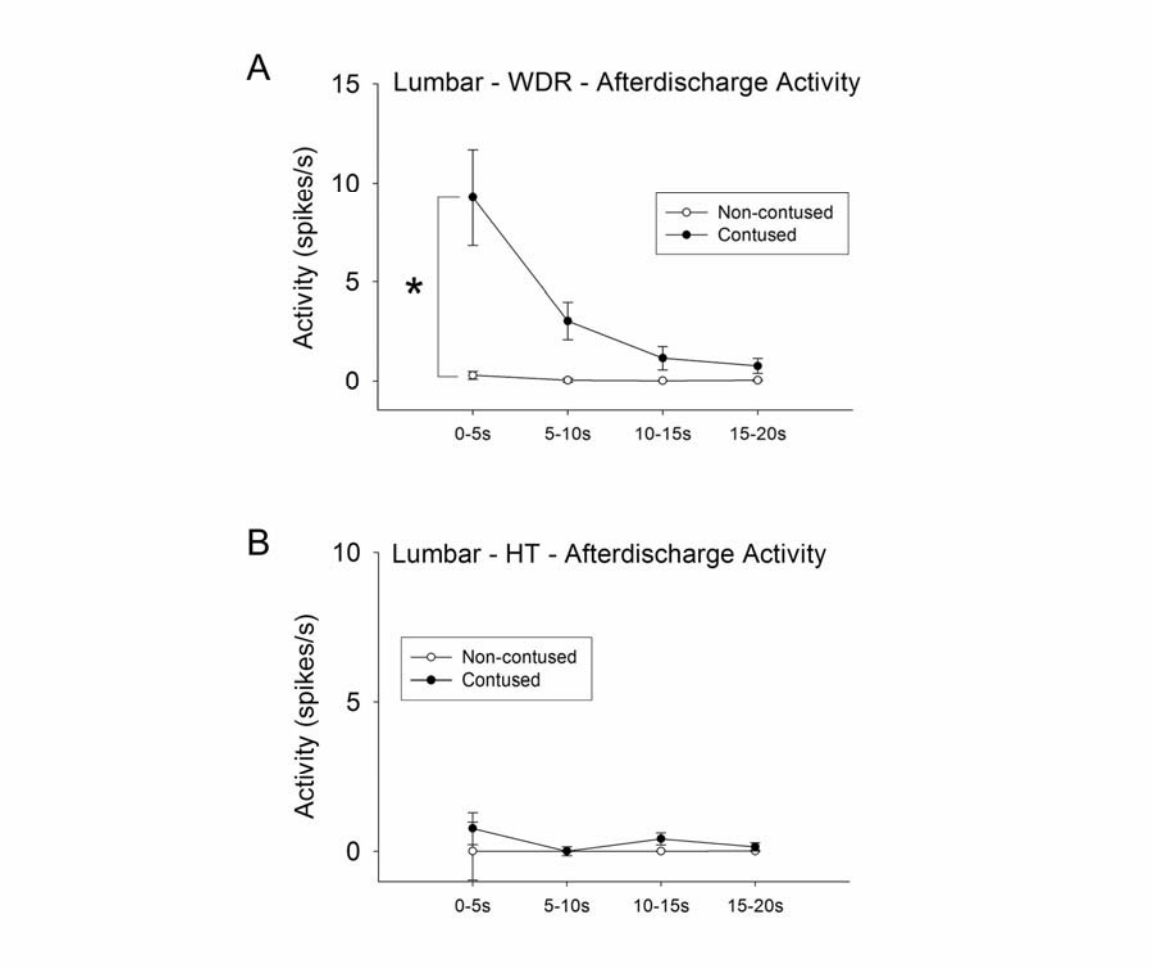

Figure 2.8. Afterdischarge activity of lumbar neurons immediately after pinch stimulus. The x-axis shows four time interval groups (0-5s, 5-10s, 10-15s, 15-20s). The y-axis shows the activity (average discharge rate) of the neuron during the 5 second interval. WDR cells (A) from contused animals have a significant difference compared to cells from the non-contused controls. HT cells (B) showed no difference between groups. An * indicates a statistically significant difference ($p<0.001$) compared to the non-contused group. Data are expressed as means ±SEM.

2.3.6 Electrophysiology of the thoracic cord

Ten animals from each group, non-contused and contused, were utilized to isolate 75 neurons from the dorsal horn of the lumbar enlargement. The number of neurons observed and the mean recording depths are shown in table 2.2. The recording sites are depicted on a cross-sectional map of the thoracic spinal cord in figure 2.9.

Thoracic Cord		
Non-contused	No. of Neurons	Depth (μm)
WDR	13	474 ± 45
HT	10	275 ± 40
LT	14	582 ± 50
Contused		
WDR	17	425 ± 55
HT	8	254 ± 55
LT	13	593 ± 47

Table 2.2. Number of cells sampled in the dorsal horn of the thoracic cord. Depth is represented as mean ± SEM.

Thoracic WDR cells of contused animals exhibit significantly increased background and increased responses to brush, press, pinch, 2g, 6g, 15g, and 60g filament, 5ºC cold, and 45º heat stimuli compared to WDR cells from the non-contused animals ($p<0.05$; Figure 2.10A-C).

Thoracic HT cells of contused animals also exhibit increased responses to press, pinch, 6g, 15g, and 60g von Frey filament stimuli, and both temperature stimuli (Figure 2.10D-F) compared to HT cells the non-contused animals.

Thoracic LT cells of contused animals showed no significant differences in background or during peripheral stimuli compared to LT cells from non-contused animals (Figure 2.10G-I).

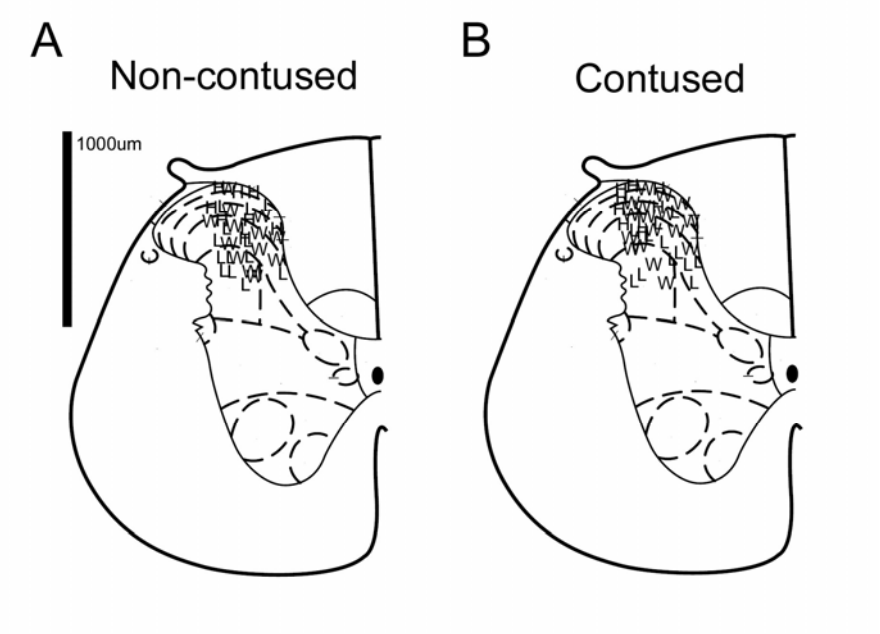

Figure 2.9. Representative recording sites of neurons from the dorsal horn of the thoracic cord. In the thoracic cords of both non-contused animals (A) and contused animals (B) recordings were made up to a depth of 1000μm. WDR, HT and LT neurons are labeled as W, H, and

Thoracic WDR cells of contused animals showed increased afterdischarge activity compared to the WDR cells of their non-contused counterparts ($p<0.005$; Figure 2.11A). Thoracic HT cells of contused animals showed no significant difference of afterdischarge activity compared to the afterdischarge activity of HT cells from non-contused animals (Figure 2.11B).

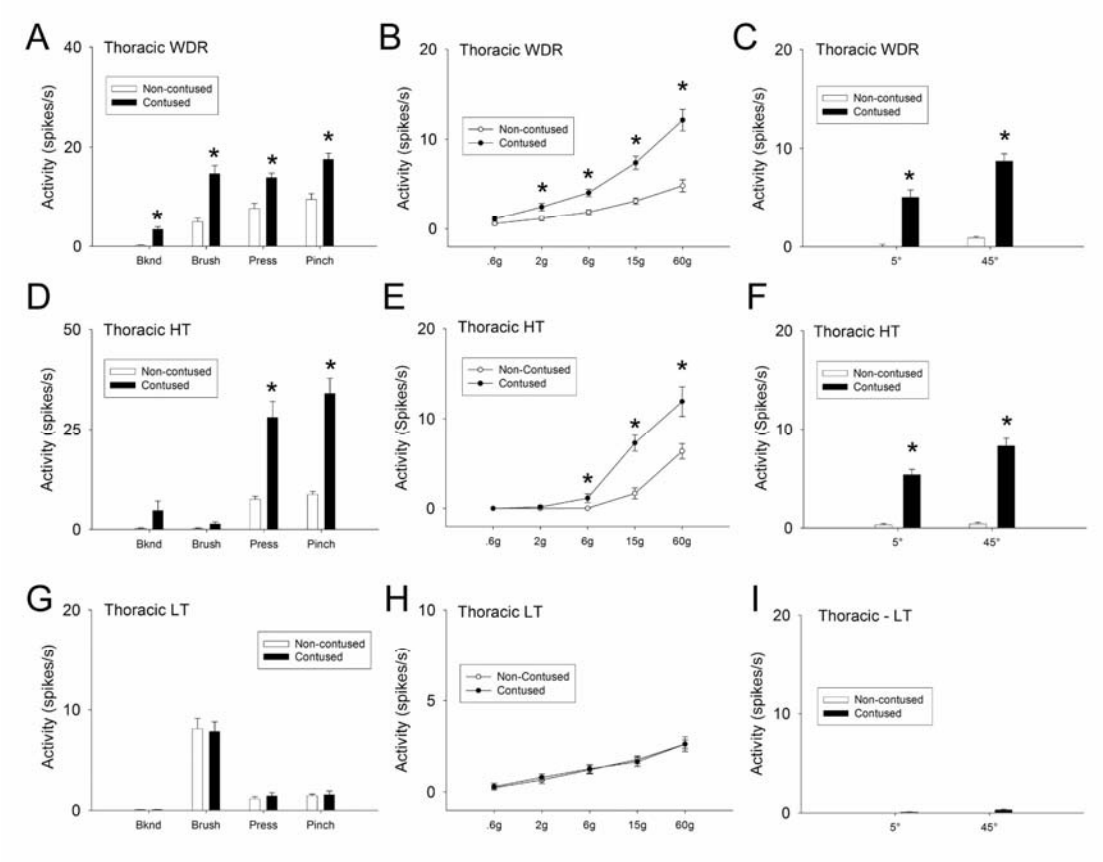

Figure 2.10. Responses of dorsal horn neurons of the thoracic cord (spinal segments T8-9; immediately rostral to injury) of contused and non-contused animals to hindpaw stimulation. Recordings are made from WDR (A-C), HT (D-F), and LT (G-I) during background (no stimulus), brush, press, and pinch (A, D, G), von Frey stimulation (B, E, H), and temperature stimuli (C, F, I). Responses to peripheral stimuli are shown as discharge rate above background activity. Temperature stimuli are depicted as discharge rate above discharge rate during 25°C stimulation. Thoracic WDR neurons from contused animals exhibit enhanced background activity, exaggerated responses to mechanical stimuli, 5°C, and 45°C compared to the WDR neurons from the non-contused animals. Thoracic HT neurons of contused animals exhibit enhanced activity to noxious mechanical stimuli, 5°C, and 45°C compared to the HT cells from non-contused animals. Thoracic LT neurons from contused animals showed no differences compared to LT cells from the non-contused group. An * indicates a statistically significant difference ($p<.05$) compared to the non-contused group. Data are expressed as means ±SEM.

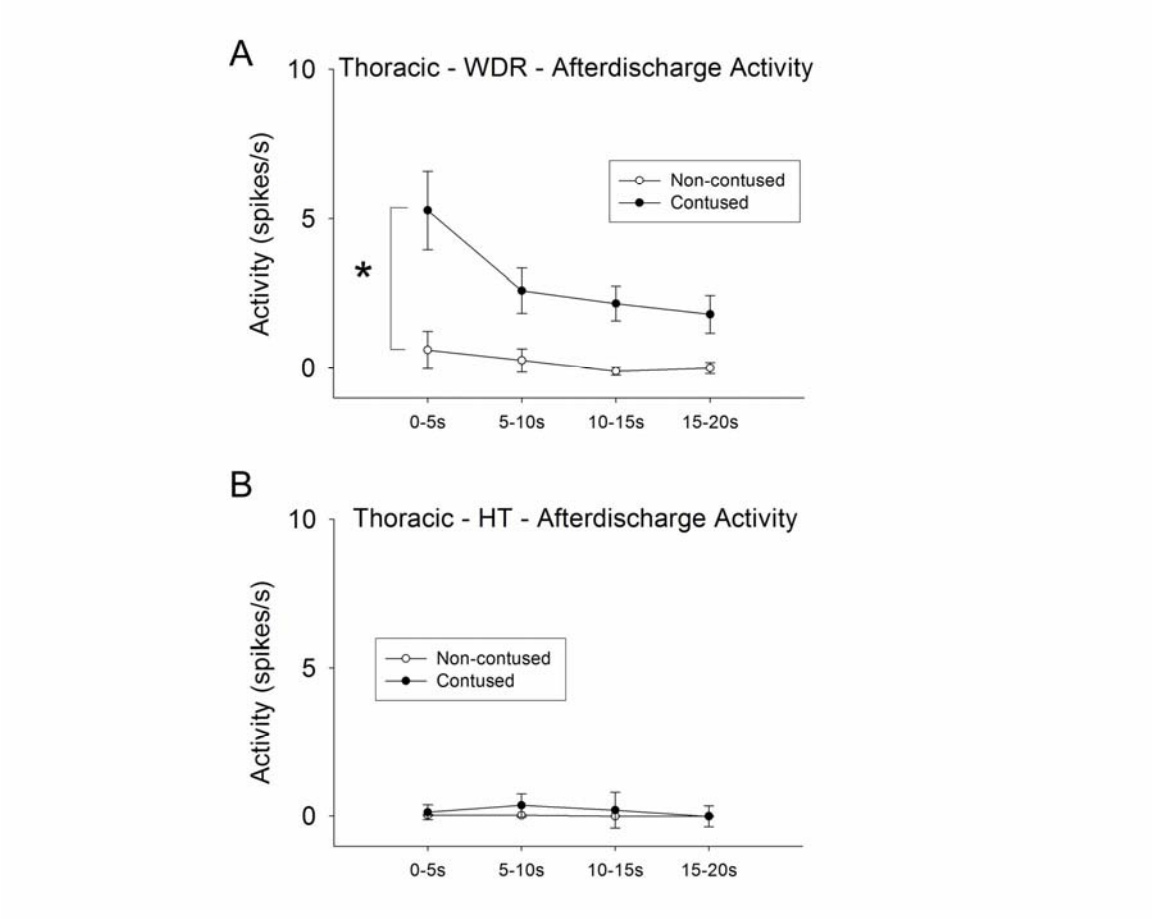

Figure 2.11. Afterdischarge activity of thoracic neurons after immediately after pinch stimulus. The x-axis shows four time interval groups (0-5s, 5-10s, 10-15s, 15-20s). The y-axis shows the activity (average discharge rate) of the neuron during the 5 second interval. Only the WDR cells (A) from contused animals have a significant difference compared to cells from their non-contused control. HT cells (B) show no significant differences between groups. An * indicates a statistically significant difference ($p<0.005$) compared to the non-contused group. Data are expressed as means ±SEM.

2.3.7 Electrophysiology of the brachial enlargement

Ten animals from each group, non-contused and contused, were utilized to isolate 70 neurons from the dorsal horn of the brachial enlargement. The number of neurons observed and the mean recording depths are shown in table 2.3. The recording sites are depicted on a cross-sectional map of the thoracic spinal cord in figure 2.12.

Brachial Enlargement		
Non-contused	No. of Neurons	Depth (μm)
WDR	15	622 ± 43
HT	12	341 ± 27
LT	9	631 ± 87
Contused		
WDR	13	545 ± 57
HT	8	364 ± 45
LT	13	559 ± 59

Table 2.3. Number of cells sampled in the dorsal horn of the brachial enlargement. Depth is represented as mean ± SEM.

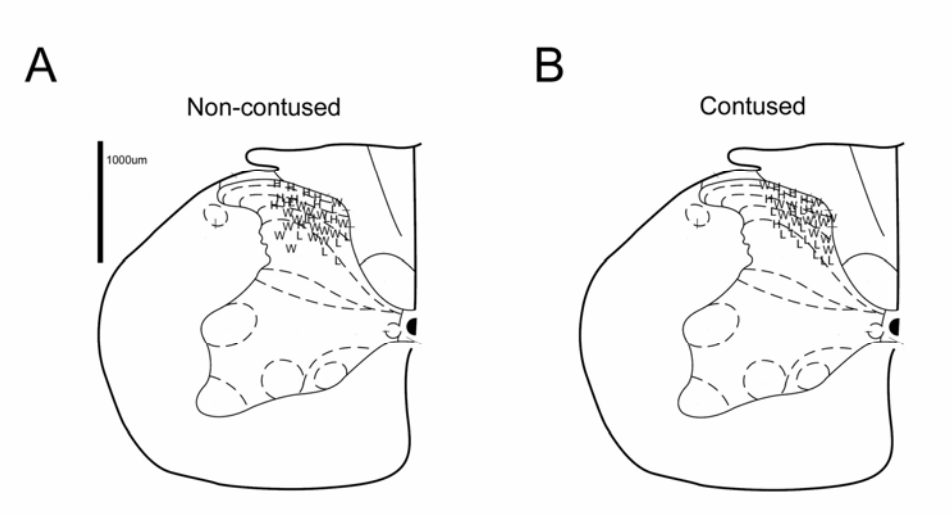

Figure 2.12. Representative recording sites of neurons from the dorsal horn of the brachial enlargement. In the brachial enlargement of both non-contused animals (A) and contused animals (B) recordings were made up to a depth of 1000μm. WDR, HT and LT neurons are labeled as W, H, and L, respectively.

Brachial enlargement WDR neurons of contused animals exhibited significantly increased responses to brush, press, pinch, von Frey filament stimulation, 5ºC cold stimulus, and 45ºC heat stimulus compared to the WDR cells from the non-contused animals ($p<0.05$; Figure 2.13A-C).

Brachial enlargement HT cells of contused animals also exhibit increased responses to press, pinch, 15g and 60g von Frey filament stimulation, and both temperature stimuli compared to responses of HT cells of non-contused animals ($p<0.05$; Figure 2.13D-F).

Brachial LT cells of contused animals showed no significant differences in background or during peripheral stimuli compared to LT cells from non-contused animals (Figure 2.13G-I).

Brachial WDR cells of contused animals showed increased afterdischarge activity compared to the WDR cells of their non-contused counterparts ($p<0.05$; Figure 2.14A). Brachial HT cells of contused animals showed no significant difference of afterdischarge activity compared to the afterdischarge activity from cells of non-contused animals (Figure 2.14B).

2.4 DISCUSSION

2.4.1 Summary

The data from this study demonstrated behavioral and electrophysiological differences between contused and non-contused animals. The hindlimbs, trunk (thorax), and forelimbs of contused animals develop decreased threshold to mechanical von Frey filament stimulation consistent with the development of mechanical allodynia in the same animals. The hindlimbs and forelimbs of contused animals develop decreased thermal withdrawal latency consistent with the development of thermal hyperalgesia.

WDR neurons from all three sites (lumbar enlargement, rostral to injury, and brachial enlargement) of spinally contused animals showed greater neuronal activity in response to brush, press, pinch, von Frey stimulation and both temperature stimuli (5°C and 45°C) compared to the responses from WDR neurons from the non-contused group. Increased response of WDR neurons to both non-noxious and noxious stimuli is consistent with the development of the facilitation of nociceptive signal transduction.

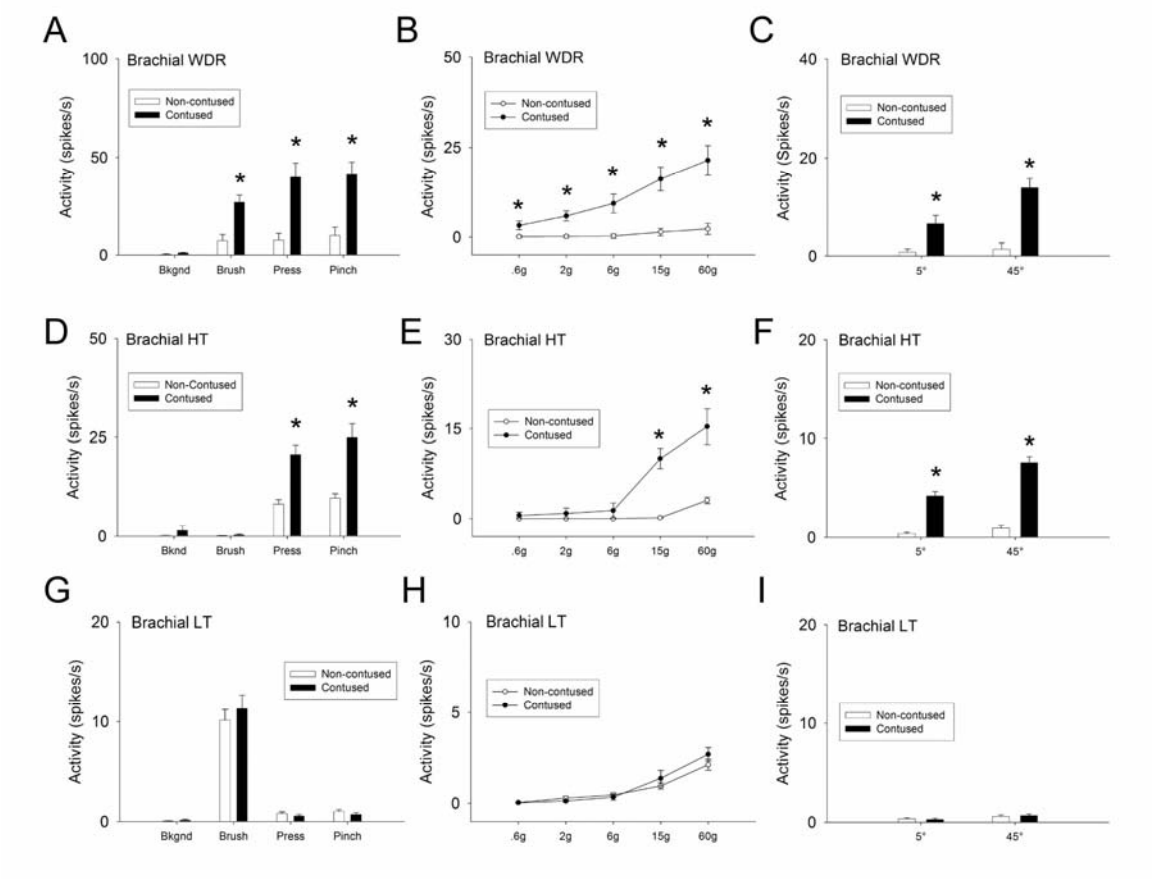

Figure 2.13. Responses of dorsal horn neurons of the brachial enlargement (spinal segments C7-T1) of contused and non-contused animals to hindpaw stimulation.
Recordings are made from WDR (A-C), HT (D-F), and LT (G-I) during background (no stimulus), brush, press, and pinch (A, D, G), von Frey stimulation (B, E, H), and temperature stimuli (C, F, I). Responses to peripheral stimuli are shown as discharge rate above background activity. Temperature stimuli are depicted as discharge rate above discharge rate during 25°C stimulation. Brachial enlargement WDR neurons from contused animals exhibit exaggerated responses to mechanical stimuli, 5°C, and 45°C compared to the WDR neurons from the non-contused animals. Brachial enlargement HT neurons of contused animals exhibit enhanced activity to noxious mechanical stimuli, 5°C, and 45°C compared to the HT cells from non-contused animals. Brachial LT neurons from contused animals showed no differences compared to LT cells from the non-contused group. An * indicates a statistically significant difference ($p<.05$) compared to the non-contused group. The data are presented as mean ± SEM.

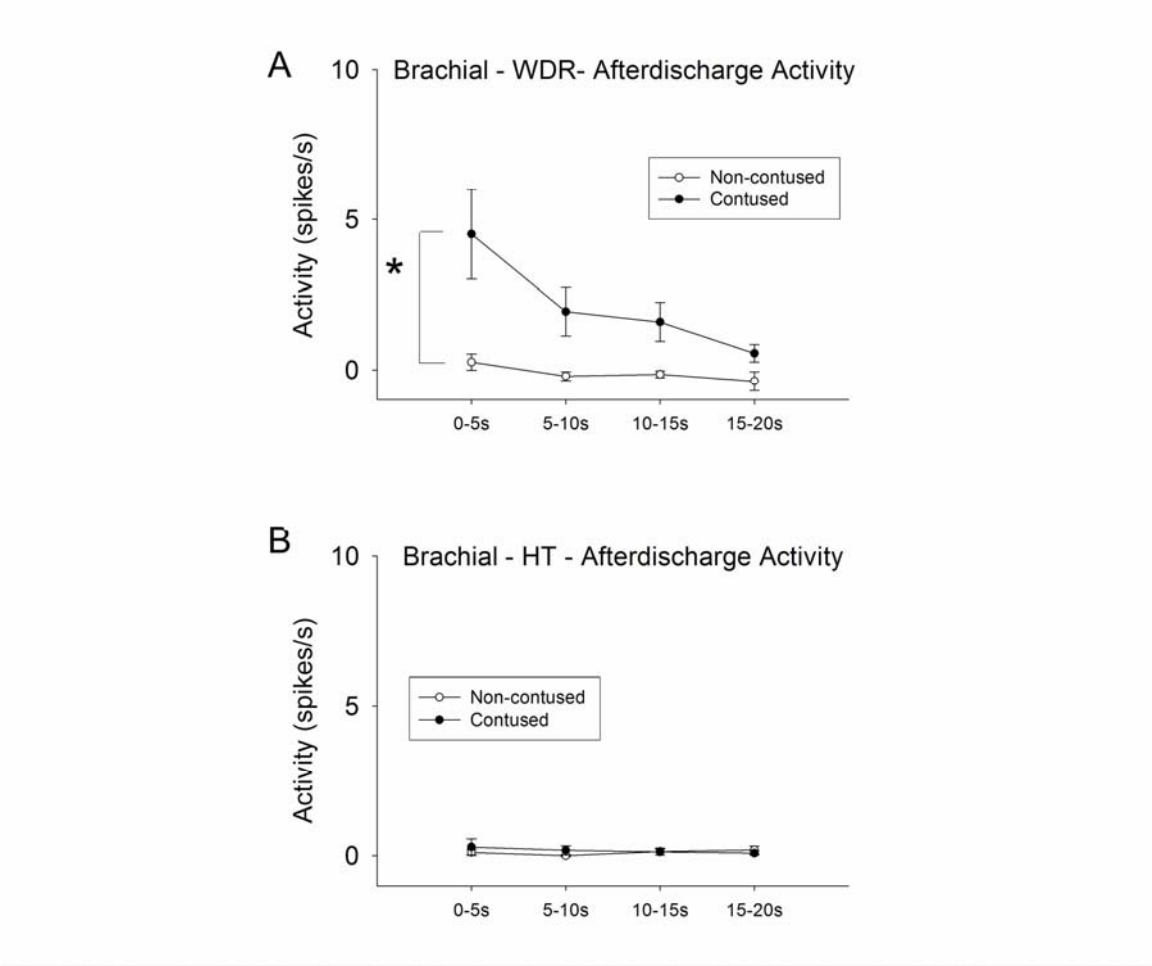

Figure 2.14. Afterdischarge activity of brachial enlargement neurons after immediately after pinch stimulus. The x-axis shows four time interval groups (0-5s, 5-10s, 10-15s, 15-20s). The y-axis shows the activity (average discharge rate) of the neuron during the 5 second interval. Only the WDR cells (A) from contused animals have a significant difference compared to cells from their non-contused control. HT cells (B) show no difference between groups. An * indicates a statistically significant difference ($p<.05$) compared to the non-contused group. Data are expressed as means ±SEM.

HT neurons of contused animals from all three sites also showed increased responses to press, pinch, and temperature stimuli as compared to responses from HT cells from their non-contused counterparts. In the contused animals, only WDR neurons from the lumbar enlargement and thoracic cord showed increased spontaneous activity compared to the responses of WDR cells of their respective non-contused group counterparts. The increased background activity potentially explains the phenomena of persistent (non-evoked) pain in the clinical SCI population.

Only the low threshold neurons of the lumbar enlargement of contused animals exhibited an increase in response to brush as compared to the non-contused group. Table 2.2 summarizes the recording data. Afterdischarge activity after pinch was greater in only the WDR neurons, but was increased in all three regions (below-level, at-level, and above-level). The behavioral and electrophysiologic findings of this SCI model are consistent with presence of neuropathic pain in three distinct areas in the clinical SCI population: below-level, at-level, and above-level (Siddall and Loeser 2001; Siddall et al. 1997; Siddall 2002).

		Bknd	Br	Pr	Pi	von Frey	Cold	Heat
Lumbar Enlargement	LT		+					
	WDR	+	+	+	+	+	+	+
	HT			+	+	+	+	+
Thoracic Cord	LT							
	WDR	+	+	+	+	+	+	+
	HT			+	+	+	+	+
Brachial Enlargement	LT							
	WDR		+	+	+	+	+	+
	HT			+	+	+	+	+

Table 2.2. Summary of Electrophysiological Activities During Various Stimuli. '+' is indicative of significant differences between cells from the non-contused and contused groups. Abbreviations are as follows: Bknd = background, Br = brush, Pr = press, and Pi = pinch.

2.4.2 Clinical descriptions of neuropathic pain after SCI

Below-level neuropathic pain pain is a result of the spinal cord trauma/ischemia and is termed central dysesthesia syndrome, central pain, or phantom pain. The pain is described as tingling, lancinating, numbness, aching, throbbing, squeezing and sickening (Siddall and Loeser 2001). Below-level pain has been reported to occur in both areas with totally anesthetic regions and areas with preserved function.

At-level neuropathic pain may be the result of SCI sequelae such as nerve root entrapment, syringomyelia, or segmental deafferentation as a direct result of trauma/ischemia (Siddall and Loeser 2001; Sjolund 2002). Nerve root entrapment (compression) leads to radicular pain in the distribution (dermatome) of a single nerve root. Pain that results from nerve root entrapment has been described as lancinating, burning, or stabbing (Siddall and Loeser 2001). Syringomyelia is characterized by the development of a cavitation (syrinx) in the spinal cord. Patients describe a constant, burning pain that may be associated with allodynia (Siddall and Loeser 2001). Segmental deafferentation pain is also known as transitional zone pain or girdle zone pain. This type of pain occurs at an area between normal sensation and anesthetic skin. Transitional zone pain has been described as burning and aching and is associated with allodynia and hyperpathia (Siddall and Loeser 2001).

Above-level neuropathic pain is often due to either compressive mononeuropathies or complex regional pain syndromes (CRPS) (Siddall and Loeser 2001). Peripheral nerve compression (compressive neuropathy) can be a result of overuse; such overuse is commonly seen in quadriplegic patients relying more on their upper body for activities of daily living after losing function in their lower limbs. Both compressive neuropathy and CRPS pain have been described as numbness, burning, or tingling.

2.4.3 Animal models of neuropathic pain after SCI

Animal models of below level pain behavior are demonstrated in SCI delivered contusion (Hains et al. 2003a; Hulsebosch et al. 2000; Lindsey et al. 2000), hemisection

(Christensen et al. 1996; Hains et al. 2003b), and spinal ischemic clip-compression (Bruce et al. 2002) . Tactile allodynia and thermal hyperalgesia develop by 28 days post injury. Hemisection of thoracic spinal cord leads to dorsal horn neuronal hyperexcitability below the level of the lesion in the lumbar enlargement (Hains et al. 2003c). Thoracic contusion results in increased neuronal hyperexcitability in the lumbar enlargement during peripheral stimulation (Drew et al. 2004; Hains et al. 2003a) and hyperexcitability during noxious stimuli located 1 to 2 segments caudal to the injury site (Drew et al. 2004).

At-level pain-like behaviors are demonstrated in SCI with spinal excitotoxic lesions (Yezierski and Park 1993), contusive injury (Drew et al. 2001; Hoheisel et al. 2003; Hulsebosch et al. 2000; Siddall et al. 1995), hemisection injury (Wang et al. 2005) and ischemic injury (Bruce et al. 2002; Hao et al. 2004). Quisqualic acid injected animals developed increased background activity, increased activity in response to peripheral stimuli, and increased afterdischarge activity that were maintained up to 36 days post injury (Yezierski and Park 1993). Contusive SCI results in pain-like behavior and enhanced responses to brush and pinch and increased afterdischarge activity in WDR neurons (Drew et al. 2001) and increased background activity (Hoheisel et al. 2003). At-level allodynia 10 to 14 days after hemisection results in parallel increases in dorsal horn spontaneous activity and responsiveness of WDR neurons to innocuous and noxious stimuli and HT neurons to noxious stimuli (Wang et al. 2005). In the spinal ischemia model where spinal ischemia is chemically induced via a photochemical reaction, dorsal horn neurons of injured and allodynic animals exhibited higher spontaneous hyperexcitability, increased afterdischarges, as well as increased responsiveness to non-noxious and noxious stimuli as compared to control animals (Hao et al. 2004).

Above level pain-like behavior has been demonstrated in SCI in both spinal contusion (Hulsebosch et al. 2000) and hemisection (Christensen et al. 1996) models. Animals contused at spinal segment T8 developed forelimb mechanical allodynia and thermal hyperalgesia at day 14 which was maintained through day 33 (Hulsebosch et al.

2000). In a spinal hemisection model at T13, animals developed forelimb mechanical allodynia and thermal hyperalgesia, which lasted through 160 days post injury.

2.4.4 Mechanisms of neuropathic pain

The development of post SCI chronic central neuropathic pain is thought to have different etiologies based on anatomical location. Each location represents potentially different pathological changes in neural circuitry. Hyperexcitable dorsal horn neurons below the level of the lesion may be a result of disruption of descending supra-spinal inhibitory pathways (Hains et al. 2003b). Abnormal sodium channel expression post spinal cord injury may lead to neuronal hyperexciteability associated with central neuropathic pain (Hains et al. 2003a). Changes at the level of the injury are thought to be governed by different circumstances given the proximity to the initial injury and localized secondary injury cascades. At the level of spinal injury, deafferentation can be a cause of neuropathic pain. Extracellular recordings from paraplegic patient suffering from chronic pain exhibited hyperactive firing patterns similar to this in the deafferented cat spinal cord and a primate cortical epileptic focus (Loeser et al. 1968). Ectopic spinal cord firing could be the initiator of persistent pain. In the hemisection model changes in calcitonin gene-related peptide (CGRP) expression in GAP-43 expressing neurons of C8, T13, and L5 suggest that SCI neuropathic pain above level, at level, and below level may be due to neural reorganization in response to hemisection at T13 (Ondarza et al. 2003). The contusion model of SCI also shows an increased mGluR1 expression at both the site immediately rostral to the site of injury and at the brachial enlargement (Mills and Hulsebosch 2002). While each model has its strength, the most physiologically relevant model of SCI is the contusion model because the pathophysiology is similar to SCI in the clinical population (Bunge 1994; Bunge et al. 1993).

2.4.5 Conclusions

Other studies have focused on one region of pain and either behavior and/or electrophysiology; this study is the first to examine simultaneously characterize both

behavioral and electrophysiological characteristics of contusive SCI in three distinct regions: below level, at level, and above the level of injury. In summary, contusion injury at T10 produced by a 150 kdyne force with a 1 second dwell time results in a moderate injury and the development of enhanced withdrawal behavior to mechanical and thermal stimuli below the level of injury, at the level of injury, and above the level of injury. Spinally contused animals also have increased dorsal horn responses to peripheral mechanical and thermal stimuli in all three sites. The increase in background activity in segments rostral to the site of injury and below the level of injury could explain the phenomenon of persistent pain. The development of dorsal horn neuronal hyperexcitability potentially explains the development of chronic neuropathic pain behavior after SCI. The presence of elevated afterdischarges, increased hyperexcitability of the spinal spinal cord, and the increased mechanical and thermal responsiveness, are consistent with a model of centrally induced chronic neuropathic pain after SCI.

CHAPTER 3

THE ROLE OF IONOTROPIC GLUTAMATE RECEPTORS IN PAIN AFTER SPINAL CORD INJURY

3.1 INTRODUCTION

Spinal cord injury (SCI) is a severe injury that often results in a loss of motor and somatosensory function below the level of the lesion. As mentioned previously, neuropathic pain after SCI can be anatomically categorized based on dermatomal region of involvement (Lundqvist et al. 1991; Siddall 2002; Sjolund 2002): (1) above level pain – occurs above the level of the spinal cord lesion; (2) at level pain – occurs at the dermatomes immediately rostral to the spinal cord lesion; and (3) below level pain – occurs in the dermatomes below the level of the spinal cord lesion. Given the different locations of the pain with respect to the dermatomes of involvement, the pathophysiology of the pain may be due to differing etiologies and mechanisms. Thus, we wanted to begin to test potential mechanisms of neuropathic pain at the cellular level at these three regions.

Ionotropic glutamate receptors are involved in the transmission of nociceptive information in both normal and neuropathic conditions (Dougherty et al. 1992; Dougherty and Willis 1991; Neugebauer et al. 2000; Neugebauer et al. 1993b; Rygh et al. 2001; Stanfa and Dickenson 1999). Spinal administration of glutamate antagonists have been shown to reduce pain-like behaviors. Intrathecal delivery of NMDA receptor antagonists (Fairbanks et al. 2000; Hama et al. 2003) and non-NMDA receptor antagonists have been shown to alleviate pain-like behaviors in models of peripheral injury. Intrathecal delivery of NMDA receptor antagonists (Fairbanks et al. 2000) and non-NMDA receptor antagonists have also been shown to alleviate pain-like behaviors in spinal cord injury (Bennett et al. 2000). Ionotropic glutamate receptors include: (1) N-methyl-D-aspartate (NMDA) receptors (selectively activated by NMDA, and demonstrated to be involved in responses to noxious mechanical, thermal, and chemical stimuli) and (2) non-NMDA ionic receptors (selectively sensitive to α-amino-3hydroxy-

5-methyl-4-isoxazolepropionic acid (AMPA) or to kainate, demonstrated to play a role in signaling both innocuous and noxious stimuli).

Our lab has characterized a contusion model of spinal cord injury which results in pain-like behavior by 35 days post injury. The injury results in the development of a decrease von Frey withdrawal threshold on the forelimbs, trunk, and hindlimbs and a decrease of thermal withdrawal latency in the forlimbs and hindlimbs. Our lab has demonstrated that the background activity of wide dynamic range (WDR) neurons in the lumbar enlargement and thoracic spinal cord (segments immediately rostral to the site of injury) have been shown to be increased in contused animals (30 days post-contusion; T10 contusion) compared to their age-matched non-contused. Activity of WDR neurons of all three regions (lumbar enlargement, thoracic cord, and lumbar enlargement) during peripheral brush, pinch, von Frey filament stimulation, 5°C and 45°C were found to be increased compared to WDR neurons from their non-contused counterparts. Activity of high threshold (HT) neurons of all three regions during peripheral pinch, von Frey filament stimulation, 5°C and 45°C was found to be increased compared to the activity of neurons from their non-contused counterparts. The low threshold (LT) neurons of the lumbar enlargement display hyperexcitability and only during brush stimulus.

An important component of these changes may involve chronic upregulation of ionotropic glutamate receptors, both NMDA, non-NMDA, and/or increased activation state of these receptors. Congruent with other pain models, abnormal pain syndromes may be due to aberrant glutamate receptor mediated signal transduction. The objective of this study was to characterize further electrophysiologically and pharmacologically the role of ionotropic glutamate receptor contributions 35 days post SCI in three different regions (below-level, at-level, and above-level) in a rodent model of spinal cord injury.

3.2 MATERIALS AND METHODS

3.2.1 Experimental animals

Subjects were male Sprague-Dawley rats (n=156), 225-250 g, obtained from Harlan Sprague-Dawley, Inc., Indianapolis, IN, housed with a 12-h light/12-h dark cycle

and fed *ad libitum*. All procedures involving rats were reviewed by the University of Texas Medical Branch Animal Care and Use Committee. One hundred and fifty-six subjects were divided into three groups: naïve (n= 38), sham (laminectomy only; n= 37), and injured (n= 81).

3.2.2. Spinal contusion injury

Subjects were anesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg). Anesthesia was considered complete when there was no flexor withdrawal in response to noxious foot pinch. The subjects' backs were shaven and a laminectomy was performed and spinal cord contusion injury was produced at spinal segment T10 using the Infinite Horizon (IH) injury device (Infinite Horizon, LLC). A motor driven cylindrical tip, 2.0mm in diameter, was driven to a force of 150 kilodynes, allowed to compress the cord for 1 second (1 second dwell time), and retracted. Following the contusion injury, the musculature was sutured, the skin was autoclipped, and the animals were allowed to recover from anesthesia. Following contusion, bladders were manually expressed twice daily until bladder function recovered, usually within 10 days post injury. Prophylactic antibiotic (Baytril, 30 mg/kg, subcutaneously) was given twice daily until bladder function returned.

3.2.3 Electrophysiologic recordings

Extracellular single-unit recordings were made from contused (35 days post injury), sham (age-matched), and non-contused (age-matched) animals. Rats were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneal) and supplemented with sodium pentobarbital (5 mg/kg/h) infused intravenously through a jugular vein catheter. Adequacy of anesthesia was monitored by the lack of withdrawal reflexes to noxious stimuli and the absence of corneal blink reflexes. Core temperature was maintained at 37°C by a thermostatically controlled heating blanket with a rectal probe.

Neurons were isolated from the dorsal horn of the brachial enlargement (C7-T1), thoracic segments (T8-T9), or the lumbar enlargement (L3-L5). Cells were sought in

areas that corresponded to the dermatomes subjected to peripheral mechanical and thermal stimulation. Cells were sought from the lumbar and brachial enlargements, and thoracic cord up to a depth of 1000μm. All cells recorded from the lumbar enlargement, thoracic cord, and brachial enlargement responded to stimulation of the glaborous surface of the hind paw, shaved hairy skin of the thorax, or the glaborous surface of the forelimb, respectively. The dura was opened and the exposed cord was covered with warm mineral oil. Extracellular single-unit recordings were made with a low impedance (0.4-0.8 MΩ at 1KHz; Kation Scientific) glass carbon fiber microelectrode.

Once a neuron was identified, background activity was measured followed by cutaneous receptive field mapping with von Frey filaments and brief pinches. Cells were classified as low threshold (LT), wide dynamic range (WDR), or high threshold (HT) (Chung et al. 1986). Cells responding best to brush stimuli were classified as LT cells. Cells responding maximally to pinch, but with brush response greater than 10% of the maximal response, were classified as WDR cells. Neurons with the largest response to pinch stimulus, but with either no brush response or a brush response <10% of the pinch response, were classified as HT cells. Peripheral stimuli during electrophysiology included the following: brush (innocuous stimuli), pinch (noxious stimuli), von Frey filament application (with 5 filaments: 0.6g, 2g, 6g, 15g, and 60g), and three temperature stimuli (5°C and 45°C). Each stimulus was applied for 10 seconds. Cell depth and distance from midline was recorded and mapped.

Temperature stimuli were administered with the Physitemp NTE-2A temperature stimulus probe. Activity during temperature stimuli was presented as the difference from 25°C to account for the pressure created by the temperature probe.

Electrical signals were amplified and input to a window discriminator, displayed on analog and digital storage oscilloscopes, processed by a data collection system (CED 1401+; Cambridge Instruments, Cambridge, UK; Pentium computer, Dell, Austin, TX, USA), and stored on a computer to construct peristimulus time histograms. The stored digital record of unit activity was analyzed offline with Spike 2 software (v4.07,Cambridge Electronic Design, Cambridge, UK). Responsiveness to peripheral

stimuli was calculated by subtracting the baseline activity from evoked activity to calculate a net increase due to stimulus evoked response. Responsiveness to thermal stimuli was calculated by subtracting the response to the 25°C (ambient temperature) from both 5°C and 45°C to discount the response from the pressure attributed to the temperature probe on the skin of the animal. Cumulative time histograms were constructed to compare group responses between non-contused and contused animals.

3.2.4 Drug application

For spinal application, drugs were dissolved in phosphate buffered saline (PBS) and given in a volume of 20ul directly onto the exposed cord. D-2-amino-5-phosphonopentanoate (D-AP5) or 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (F) quinoxaline (NBQX) were used in doses of 1nmol, 15nmol, 30nmol, and 60nmol. Recordings were made 15 minutes after dosing. Fluid on top of the cord was vacuumed suctioned after application of each dose and reapplication of PBS and suctioned every 15minutes for an hour. One cell per animal was studied.

3.2.5 Statistical measures

Background activity and responses to brush, press, and pinch were graphed and analyzed as percentage of baseline value. This allowed direct comparison between the activities of non-contused and contused animals during drug administration. Activity during peripheral von Frey application was analyzed as raw values, because the discharges of cells from non-contused animals were variable to the response to lesser strength filaments; hence from a percentage standpoint, a decrease from an unresponsive cell would never occur. Activity during temperature stimuli was also analyzed in the raw format. The slight fluctuations of the activity in non-contused animal were grossly magnified when expressed as a percentage due to the already diminutive baseline response.

All data are presented as mean ± standard error of the mean (SEM). Comparison of responses between non-contused and contused animals at different drug doses were

compared using a two-way repeated measures (RM) ANOVA and post-hoc Tukey test. If significant effect of condition (non-contused versus contused) was not found then pairwise comparison between non-contused and contused groups at each drug dose was considered. Two-way RM ANOVA was also used to compare activity during von Frey stimulation for each given dose of drug. If a significant effect of dose was not found, then pairwise comparison at each von Frey strength between the activity during baseline (no-drug) and drug was considered. Comparison of drug response to baseline response during temperature stimulation was made with one-way RM ANOVA. Post-hoc analysis was carried out using the Tukey test. $P<0.05$ was considered as statistically significant. Statistical analyses were done with Jandel SigmaStat 3.1.

3.3 RESULTS

The number of each type of neuron observed and the mean recording depths are shown for both drug treatment groups (D-AP5 or NBQX) in table 3.1.

3.3.1 Background activity

The background activity of WDR cells from the lumbar enlargement, thoracic cord, and brachial enlargement of non-contused animals did not show a change in activity with increasing dose of D-AP5 (Figure 3.1A, B, and C). Because the background activity of lumbar WDR cells did not exhibit a significant effect of condition (two way RM ANOVA; $p>0.05$; Figure 3.1A), pairwise comparison at each drug dose was done. The pairwise comparison (two way RM ANOVA, Tukey test) showed that at the there was a significant difference between contused and non-contused groups at the 60nmol D-AP5 dose ($p<0.05$). The background activity of WDR cells from the thoracic cord of contused animals shows an overall significant difference of effect of condition (contused vs. non-contused; two way RM ANOVA; $p<0.001$) compared to the activity neurons in the non-contused thoracic cord (Figure 3.1B). The background activity of the WDR neurons from the brachial enlargement of the contused group had not been shown to be

hyperactive compared to the non-contused group; the activities between the contused and non-contused group during D-AP5 treatment are not different (two way RM ANOVA; Tukey test; $p>0.05$; Figure 3.1C).

A

D-AP5

Lumbar Enlargement

Non-contused	No. of Neurons	Depth (μm)
WDR	4	657 ± 100
HT	4	317 ± 63
LT	4	692 ± 100
Contused		
WDR	6	630 ± 106
HT	4	348 ± 48
LT	4	703 ± 105

Thoracic Cord

Non-contused	No. of Neurons	Depth (μm)
WDR	5	591 ± 83
HT	4	442 ± 71
LT	4	705 ± 74
Contused		
WDR	5	544 ± 78
HT	4	310 ± 63
LT	4	761 ± 82

Brachial Enlargement

Non-contused	No. of Neurons	Depth (μm)
WDR	5	630 ± 66
HT	4	347 ± 41
LT	4	653 ± 152
Contused		
WDR	5	575 ± 106
HT	4	425 ± 69
LT	4	602 ± 88

B

NBQX

Lumbar Enlargement

Non-contused	No. of Neurons	Depth (μm)
WDR	4	593 ± 102
HT	4	395 ± 60
LT	4	763 ± 116
Contused		
WDR	6	604 ± 77
HT	4	310 ± 63
LT	4	691 ± 85

Thoracic Cord

Non-contused	No. of Neurons	Depth (μm)
WDR	4	556 ± 84
HT	4	362 ± 45
LT	4	676 ± 68
Contused		
WDR	5	572 ± 95
HT	4	272 ± 33
LT	4	639 ± 40

Brachial Enlargement

Non-contused	No. of Neurons	Depth (μm)
WDR	5	656 ± 93
HT	4	454 ± 38
LT	4	613 ± 99
Contused		
WDR	5	618 ± 70
HT	4	393 ± 85
LT	4	682 ± 91

Table 3.1. Number of cells sampled in the dorsal horn of each spinal region for each treatment group. Neurons for each region (lumbar enlargement, thoracic cord, and brachial enlargement) for each treatment, D-AP5 (A) and NBQX (B) group show comparable depth values between non-contused and contused groups. Depth is represented as mean ± SEM.

Background activity of WDR cells from the lumbar enlargement (Figure 3.1D), thoracic segment (Figure 3.1E), and brachial enlargement (Figure 3.1F) from contused animals showed no statistical significant difference compared to background spinal activity of their non-contused counterparts during NBQX application (two way RM ANOVA; $p>0.05$) and did not show a significant difference at each dose of NBQX (Tukey test; $p>.05$).

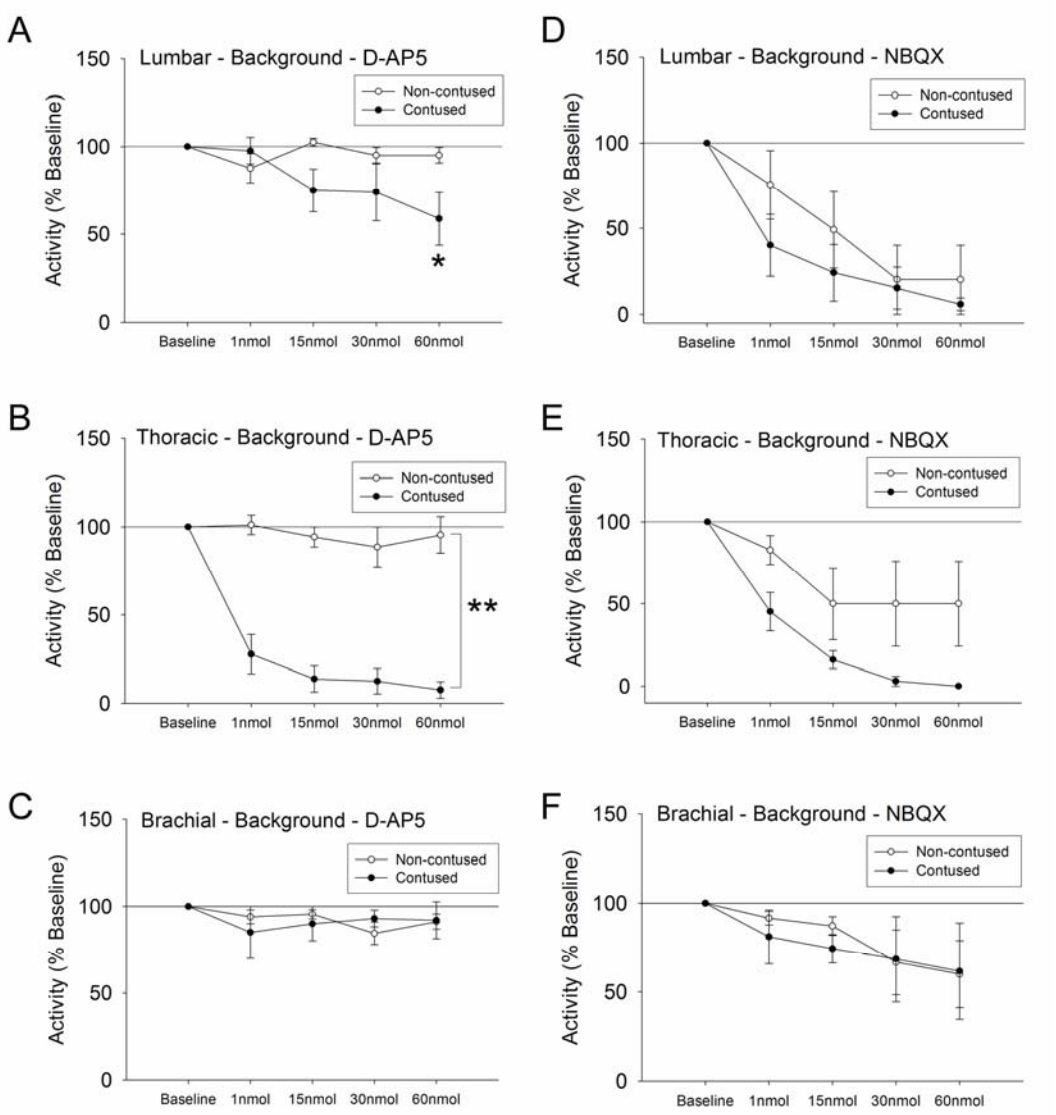

Figure 3.1. Background activity from WDR neurons of the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C,F) during D-AP5 or NBQX administration. The x-axis indicates the dose of drug and the y-axis represents the activity as a percent of baseline activity. The dark circles represent the mean activity from the contused group while the white circles represent the mean activity from the non-contused group. Lumbar background activity did not show a significant effect of condition (non-contused vs. contused), but was significantly different at the 60nmol D-AP5 dose. Neurons from the thoracic cord showed a significant decrease of background activity during D-AP5 administration compared to neurons from the non-contused group. Neurons from lumbar enlargement, thoracic cord, and brachial enlargement do not show a significant difference between contused and non-contused groups during NBQX treatment. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<0.05$) between the contused and non-contused group. An ** indicates a statistically significant difference ($p<0.001$) between the contused and non-contused group.

3.3.2 Activity during brush stimulus

The brush responses of WDR neurons from the lumbar enlargement (Figure 3.2A) and the brachial enlargement (Figure 3.2C) of contused animals, did not show an overall significant difference compared to WDR neurons from their non-contused counterparts during D-AP5 treatment (two way RM ANOVA; $p>0.05$). However, the WDR neurons from the lumbar enlargement of the contused animals did show a significant decrease at with both 30 and 60nmol treatments of D-AP5 (Tukey test; $p<0.05$). The WDR neurons from the brachial enlargement showed a significant difference at the 60nmol dose of D-AP5 between non-contused and contused animals (Tukey test; $p<0.05$). In contrast to both of these areas, the thoracic segment (Figure 3.3B) shows a significant difference of condition (contused vs. non-contused) during the D-AP5 application (two way RM ANOVA; $p<0.001$).

Neuronal activity from the neither lumbar enlargement (Figure 3.2D) nor brachial enlargement (Figure 3.2F) showed any significant difference during NBQX application (two way RM ANOVA; $p>0.05$) or any significant differences at each individual dose of NBQX (Tukey test; $p>0.05$). WDR neurons from the thoracic spinal cord (Figure 3.2E) of contused animals showed a significant inhibition of activity compared to the non-contused group (two way RM ANOVA; $p<0.05$).

In the prior chapter, it was established that only the LT neurons of the lumbar enlargement were hyperexcitable and they were only hyperexcitable to the brush stimuli. The brush activity of LT neurons from the lumbar enlargement (Figure 3.3A), thoracic spinal cord (Figure 3.3B), and the brachial enlargement (Figure 3.3C) of contused animals, did not show an overall significant difference compared to WDR neurons from their non-contused counterparts during D-AP5 treatment (two way RM ANOVA; $p>0.05$). However, the LT neurons from the lumbar enlargement of the contused animals did show a significant decrease with the 60nmol treatment of D-AP5 (Tukey test; $p<0.05$).

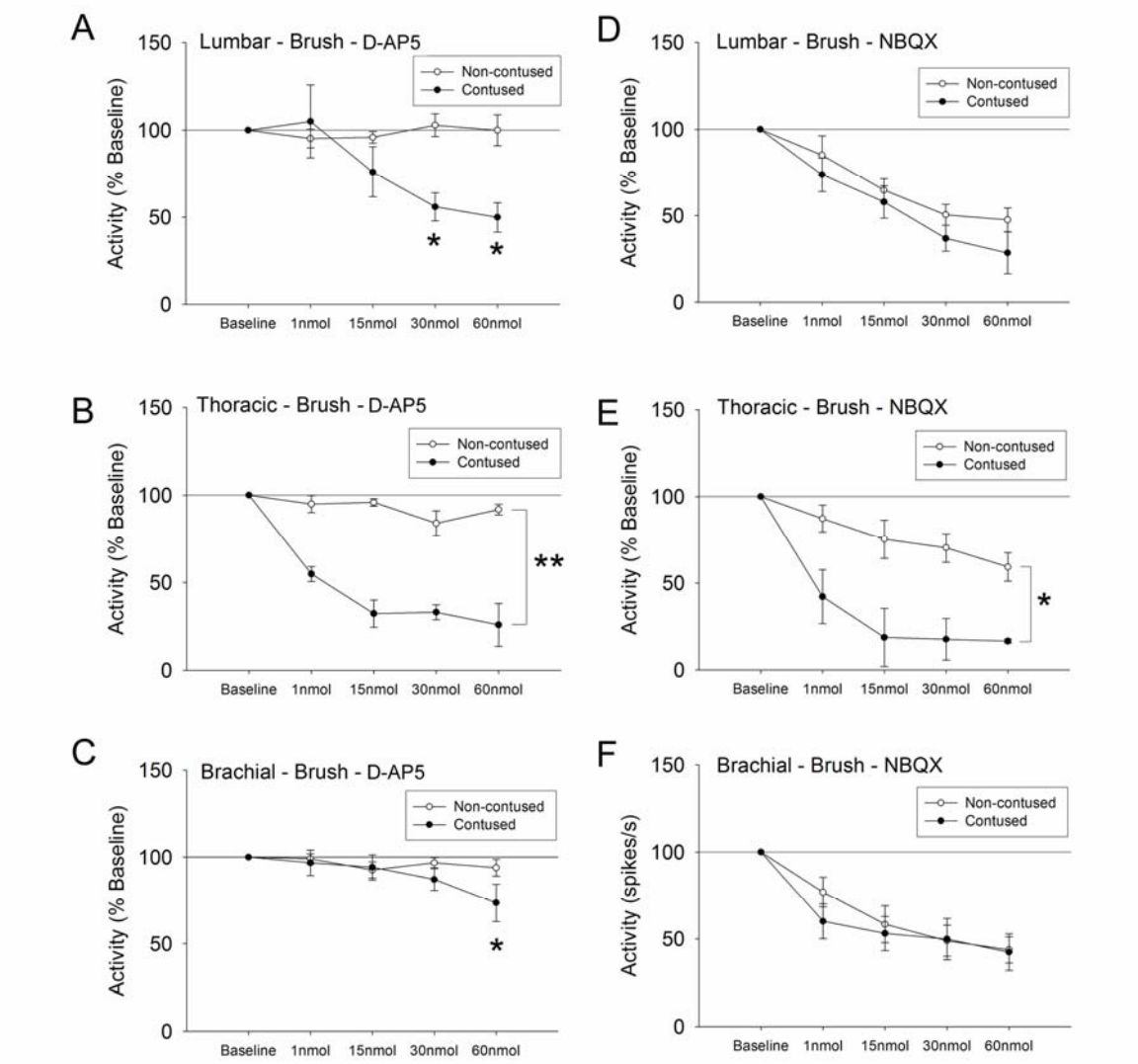

Figure 3.2. Activity during peripheral brush from WDR neurons of the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during D-AP5 (A-C) or NBQX (D-F) administration. Lumbar brush activity did not show a significant effect of condition (contused vs. non-contused), but was significantly different at the 30 and 60nmol D-AP5 dose. Neurons from the thoracic cord showed a significant decrease of activity during D-AP5 administration compared to neurons from the non-contused group. WDR neurons from the brachial enlargement did not show a significant effect of condition between groups, but showed significant difference between groups at the 60nmol D-AP5 dose. WDR neurons from both lumbar enlargement and brachial enlargement did not show a significant difference between contused and non-contused groups. WDR neurons from the thoracic cord of contused animals showed significantly decreased activity during NBQX. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<.05$) between the contused and non-contused group. An ** indicates a statistically significant difference ($p<.001$) between the contused and non-contused group.

Neuronal activity from the neither the thoracic spinal cord (Figure 3.3E) nor the brachial enlargement (Figure 3.3F) showed any significant difference compared to the LT cells from their non-contused counterparts during NBQX application (two way RM ANOVA; $p>0.05$) or any significant differences at each individual dose of NBQX (Tukey test; $p>0.05$). LT neurons from the lumbar enlargement (Figure 3.3D) of contused animals showed a significant inhibition of activity compared to the non-contused group at the 60nmol dose (two way RM ANOVA; Tukey test, $p<0.05$).

3.3.3 Activity during pinch stimulus

The pinch activity of WDR neurons from the lumbar enlargement (Figure 3.4A) and the brachial enlargement (Figure 3.4C) of contused animals, did not show an overall significant difference compared to WDR neurons from their non-contused counterparts during D-AP5 treatment (two way RM ANOVA; $p>0.05$) and did not show significant differences at any dose of D-AP5 (Tukey test; $p>0.05$). In contrast to both of these areas, the thoracic segment (Figure 3.4B) shows a significant difference of condition (contused vs. non-contused) during the D-AP5 application (two way RM ANOVA; $p<0.001$).

Neuronal activity from the lumbar enlargement (Figure 3.4D), thoracic cord (Figure 3.4E), and brachial enlargement (Figure 3.4F) did not show any significant difference during the application of NBQX (two way RM ANOVA; $p>0.05$) or any significant differences at each individual dose of NBQX (Tukey test; $p>0.05$).

The pinch activity of HT neurons from the lumbar enlargement (Figure 3.5A) and the brachial enlargement (Figure 3.5C) of contused animals, did not show an overall significant difference compared to WDR neurons from their non-contused counterparts during D-AP5 treatment (two way RM ANOVA; $p>.05$) and did not show significant differences at any dose of D-AP5 (Tukey test; $p>.05$). In contrast to both of these areas, HT cells from the thoracic segment (Figure 3.5B) shows a significant effect of condition (contused vs. non-contused) during the D-AP5 application (two way RM ANOVA; $p<.05$).

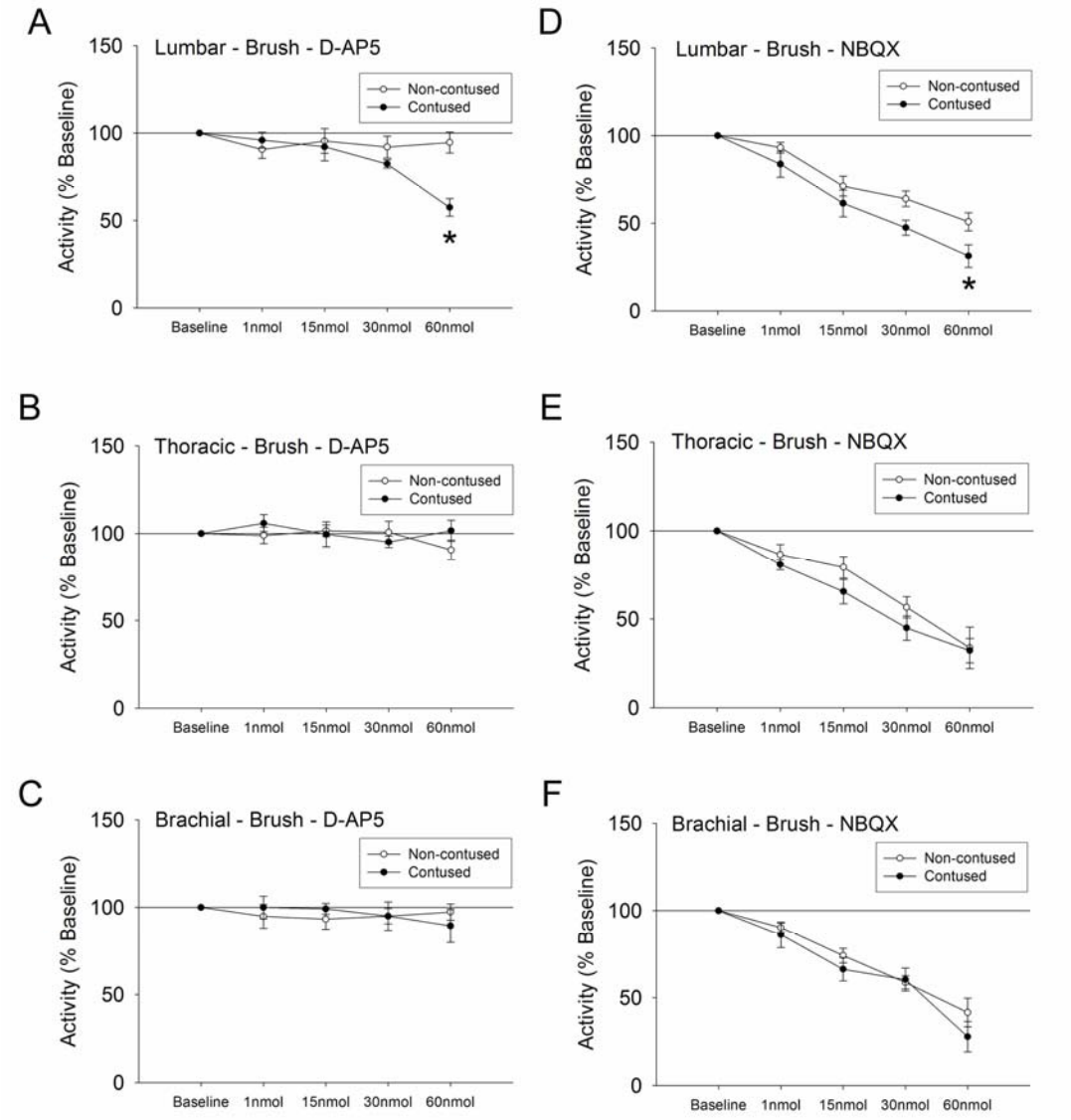

Figure 3.3. Activity during peripheral brush from LT neurons of the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during D-AP5 (A-C) or NBQX (D-F) administration. Lumbar brush activity did not show a significant effect of condition (contused vs. non-contused), but was significantly different at the 60nmol D-AP5 dose. LT neurons from the brachial enlargement and thoracic cord did not show a significant effect of condition between groups, and did not show significant difference between groups at any D-AP5 dose. LT neurons from all 3 regions did not show a significant difference between contused and non-contused groups; however, neurons from the lumbar enlargement of contused animals showed significantly decreased activity during the 60nmol NBQX dose. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<.05$) between the contused and non-contused group.

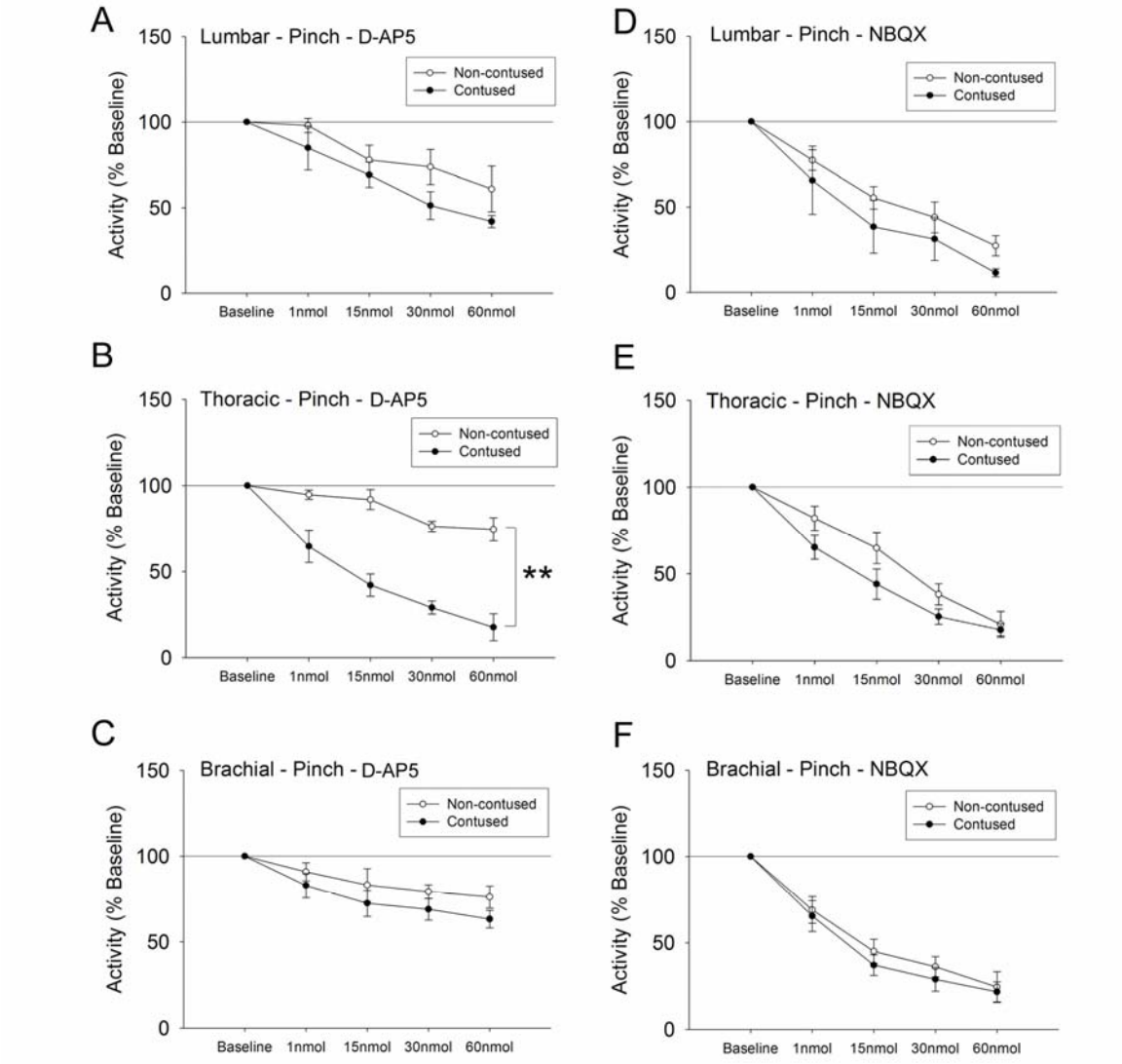

Figure 3.4. Activity during peripheral pinch from WDR neurons of the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during D-AP5 (A-C) or NBQX (D-F) administration. Lumbar activity (A) during pinch did not show a significant effect of condition (contused vs. non-contused) and was not significantly at any D-AP5 dose. Neurons from the thoracic cord (B) showed a significant decrease of activity during D-AP5 administration compared to neurons from the non-contused group. WDR neurons from the brachial enlargement (C) did not show a significant effect of condition between groups and did not show significant difference between groups at any D-AP5 dose. WDR neurons from the lumbar enlargement (D), thoracic cord (E), and brachial enlargement (F) of contused animals showed no significantly difference in activity during NBQX application. Data are represented as mean ± SEM. An ** indicates a statistically significant difference ($p<0.001$) between the contused and non-contused group.

Neuronal activity from the lumbar enlargement (Figure 3.5D), thoracic cord (Figure 3.5E), and brachial enlargement (Figure 3.5F) did not show any significant difference during the application of NBQX (two way RM ANOVA; $p>.05$) or any significant differences at each individual dose of NBQX (Tukey test; $p>.05$).

3.3.4 Activity during von Frey filament stimulation

Activity during von Frey filament stimulation (Figure 3.6) was not graphed as a percentage of baseline activity. Rather, it was graphed for the raw activity without normalization. Because not all of the baseline activity showed a response at the lowest filament, comparing these values during drug administration would give a false indicator of not having a decrease, as it is not possible to decrease from no activity.

WDR lumbar enlargement cells of non-contused animals of the lumbar enlargement (Figure 3.6A), thoracic cord (Figure 3.6B), and brachial enlargement (Figure 3.6C) showed no significant attenuation during D-AP5 treatment (two way RM ANOVA-Tukey test; $p>0.05$). WDR cells from the lumbar enlargement (Figure 3.6D) and brachial enlargement (Figure 3.6F) of contused animals exhibited attenuation at 30 and 60nmol D-AP5 (two way RM ANOVA – Tukey test; $p<0.05$). In contrast, cells from the thoracic cord showed (Figure 3.6E) attenuation at the 1nmol, 15nmol, 30nmol, and 60nmol doses (two way RM ANOVA – Tukey test; $p<0.05$).

WDR lumbar enlargement cells from the lumbar enlargement (Figure 3.7A) of non-contused animals show attenuation during the 15 and 60nmol dose of NBQX (two way RM ANOVA – Tukey test; $p<0.05$). Neurons from the thoracic cord (Figure 3.7B) and brachial enlargement (Figure 3.7C) showed attenuation during NBQX treatment only at the 60nmol dose (two way RM ANOVA – Tukey test; $p<0.05$). WDR cells from the lumbar enlargement (Figure 3.7D) and brachial enlargement (Figure 3.7F) of contused animals exhibited attenuation at 30 and 60nmol D-AP5 (two way RM ANOVA – Tukey test; $p<0.05$). In contrast, cells from the thoracic cord (Figure 3.7E) showed attenuation at the 1nmol, 15nmol, 30nmol, and 6nmol doses (two way RM ANOVA – Tukey test; $p<0.05$).

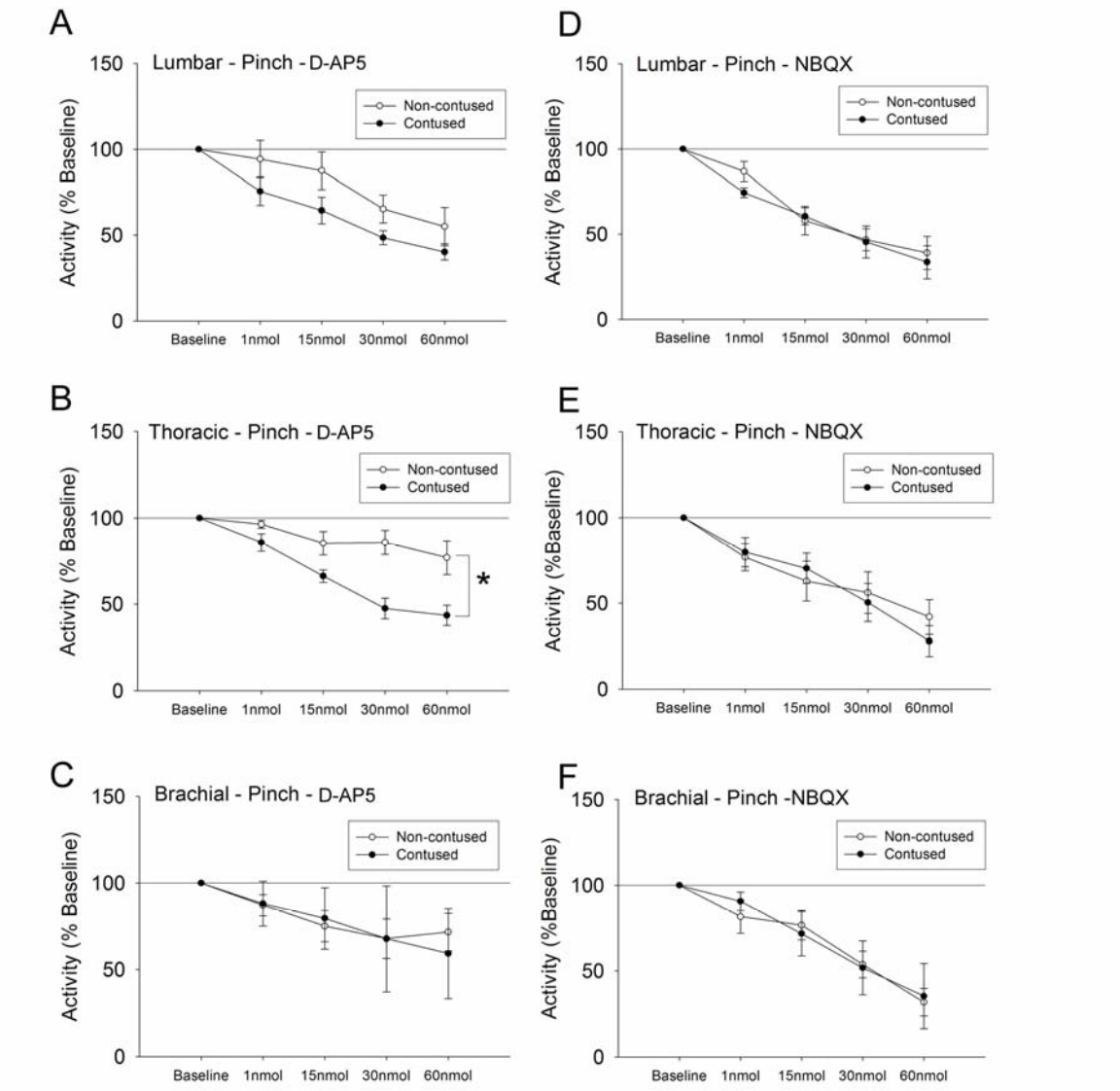

Figure 3.5. Activity during peripheral pinch from HT of the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during D-AP5 (A-C) or NBQX (D-F) administration. Lumbar activity (A) during pinch did not show a significant effect of condition (contused vs. non-contused) and was not significant at any D-AP5 dose. Neurons from the thoracic cord (B) showed a significant decrease of activity during D-AP5 administration compared to neurons from the non-contused group. HT neurons from the brachial enlargement (C) did not show a significant effect of condition between groups and did not show significant difference between groups at any D-AP5 dose. HT neurons from the lumbar enlargement (D), thoracic cord (E), and brachial enlargement (F) of contused animals showed no significantly difference in activity during NBQX application. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<0.05$) between the contused and non-contused group.

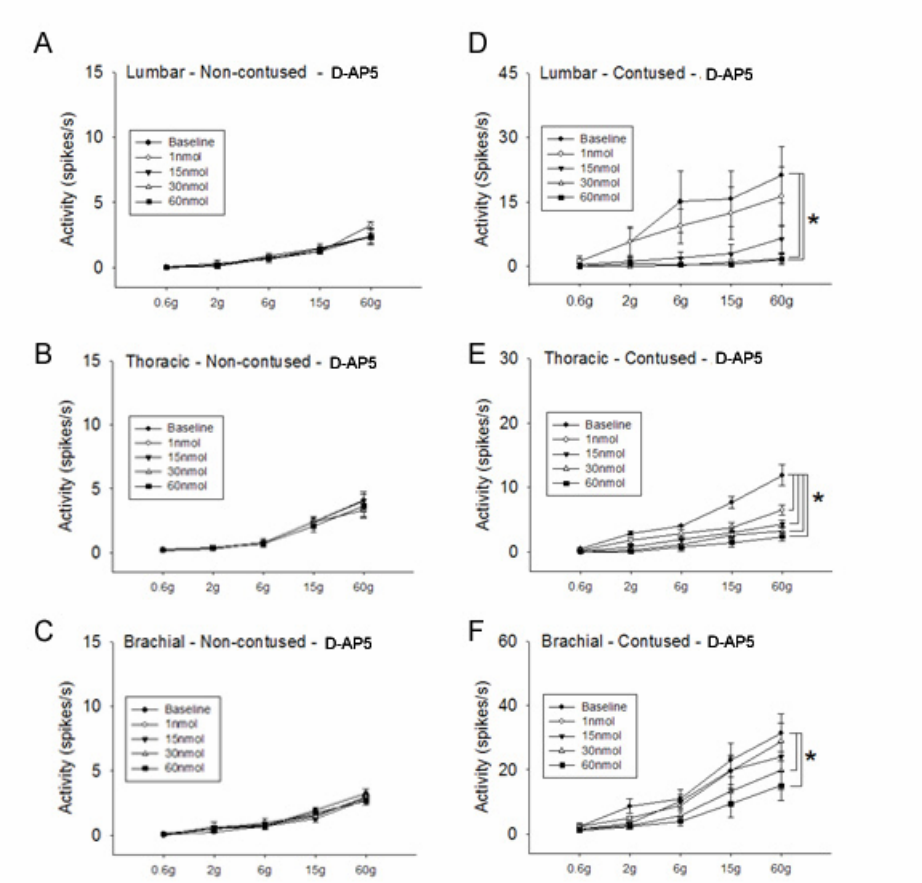

Figure 3.6. Activity during peripheral von Frey filament stimulation from WDR neurons of the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during D-AP5 administration. The activities shown are from cells from non-contused (A-C) and contused animals (D-F) at different von Frey stimulation strengths. Activity from WDR neurons of non-contused animals (A) show no signs of attenuation at all doses of D-AP5 administration. Cells from both the lumbar enlargement (D) and brachial enlargements (F) of contused animals during both D-AP5 treatment showed a significant decrease from baseline at 30 and 60nmol doses. Cells from the thoracic cord of contused animals (E) showed a significant decrease from the lowest dose 1nmol to 60nmol. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<0.05$) compared to baseline.

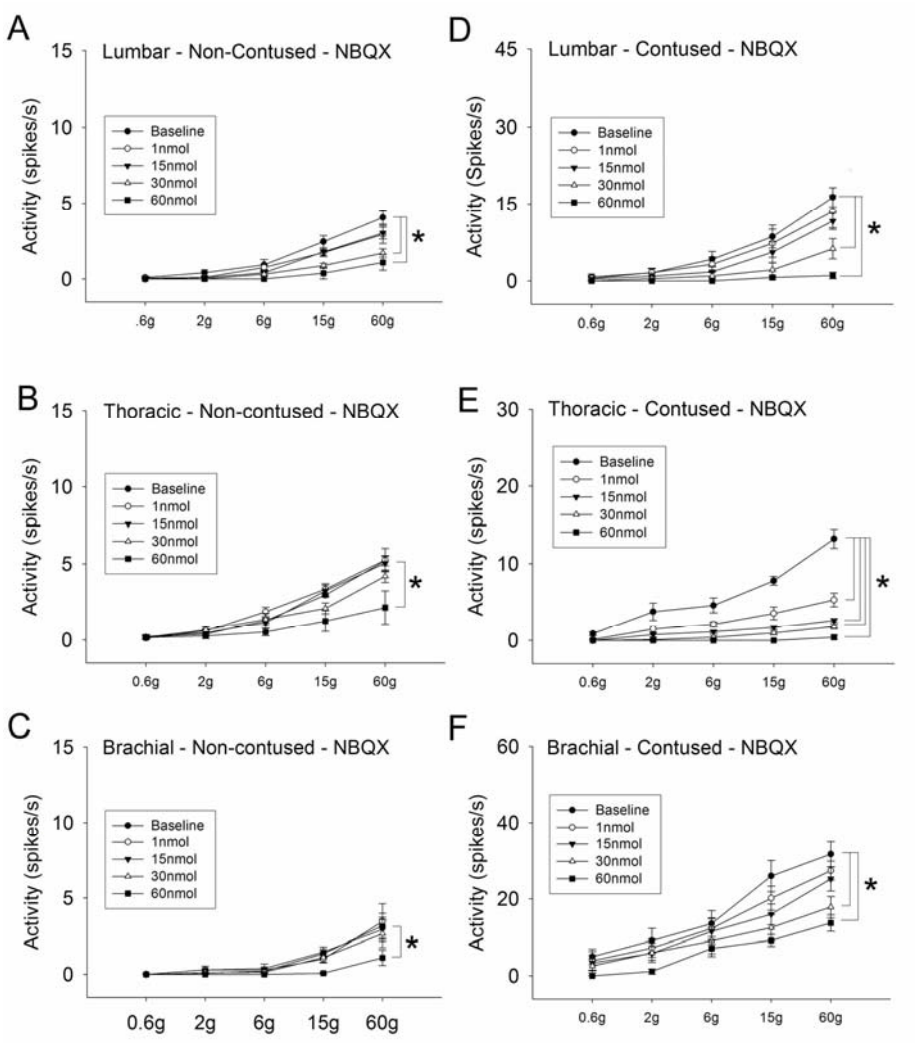

Figure 3.7. Activity during peripheral von Frey filament stimulation from WDR neurons of the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during administration. The activities shown are from cells from non-contused (A-C) and contused animals (D-F) at different von Frey stimulation strengths. Activity from WDR neurons lumbar enlargement of non-contused animals (A,) show attenuation 30 and 60nmol doses of NBQX administration. Activity from the thoracic cord (B) and brachial enlargement (C) show attenuation at the 60nmol dose of NBQX. Cells from both the lumbar enlargement (D) and brachial enlargements (F) of contused animals during both D-AP5 treatment showed a significant decrease from baseline at 30 and 60nmol doses. Cells from the thoracic cord of contused animals (E) showed a significant decrease from the lowest dose 1nmol to 60nmol. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<0.05$) compared to baseline.

HT lumbar enlargement cells of non-contused animals of the lumbar enlargement (Figure 3.8A), thoracic cord (Figure 3.8B), and brachial enlargement (Figure 3.8C) showed no significant attenuation during D-AP5 treatment (two way RM ANOVA – Tukey test; $p>0.05$). HT cells from the lumbar enlargement (Figure 3.8) and brachial enlargement (Figure 3.8F) of contused animals exhibited attenuation at 30 and 60nmol D-AP5 (two way RM ANOVA – Tukey test; $p<0.05$). In contrast, cells from the thoracic cord showed (Fig. 3.8E) attenuation at the 1nmol, 15nmol, 30nmol, and 60nmol doses (two way RM ANOVA – Tukey test; $p<0.05$).

Lumbar enlargement HT cells of contused animal show a significant effect of dose at the 30nmol and 60nmol dose of NBQX during von Frey filament stimulation (Figure 3.9D; $p<0.05$). Brachial enlargement HT cells of contused animals show a significant effects of dose only at the 60nmol dose (Figure 3.9F; $p<0.05$). Thoracic enlargement HT cells show the most susceptibility of inhibition by showing significant effects of dose of NBQX from the 15, 30, and 60nmol dose (Figure 3.9E, $p<0.05$).

3.3.5 Activity evoked by temperature stimuli

Activity during 5°C stimulation (cold) (Figures 3.10 and 3.11) and 45°C (heat) (Figures 3.12 and 3.13) were not graphed as a percentage of baseline activity. Rather, the activities were represented as raw activities (spikes/s) without normalization. Because the baseline activities of the non-contused group were markedly of less magnitude compared the contused group, minor fluctuations in activity were magnified when expressed as a percentage. Cells from non-contused animals showed no significant change in activity when administered D-AP5 orNBQX (data not shown; $p>0.05$; one way RM ANOVA) during cold or heat stimulation.

WDR cells from both lumbar (Figure 3.10A) and brachial enlargements (Figure 3.10C) of contused animals did not show a significant decrease from baseline at any dose of D-AP5 ($p>0.05$; one way RM ANOVA). Only the WDR cells of the thoracic segments from contused animals showed a significant decrease as compared to the

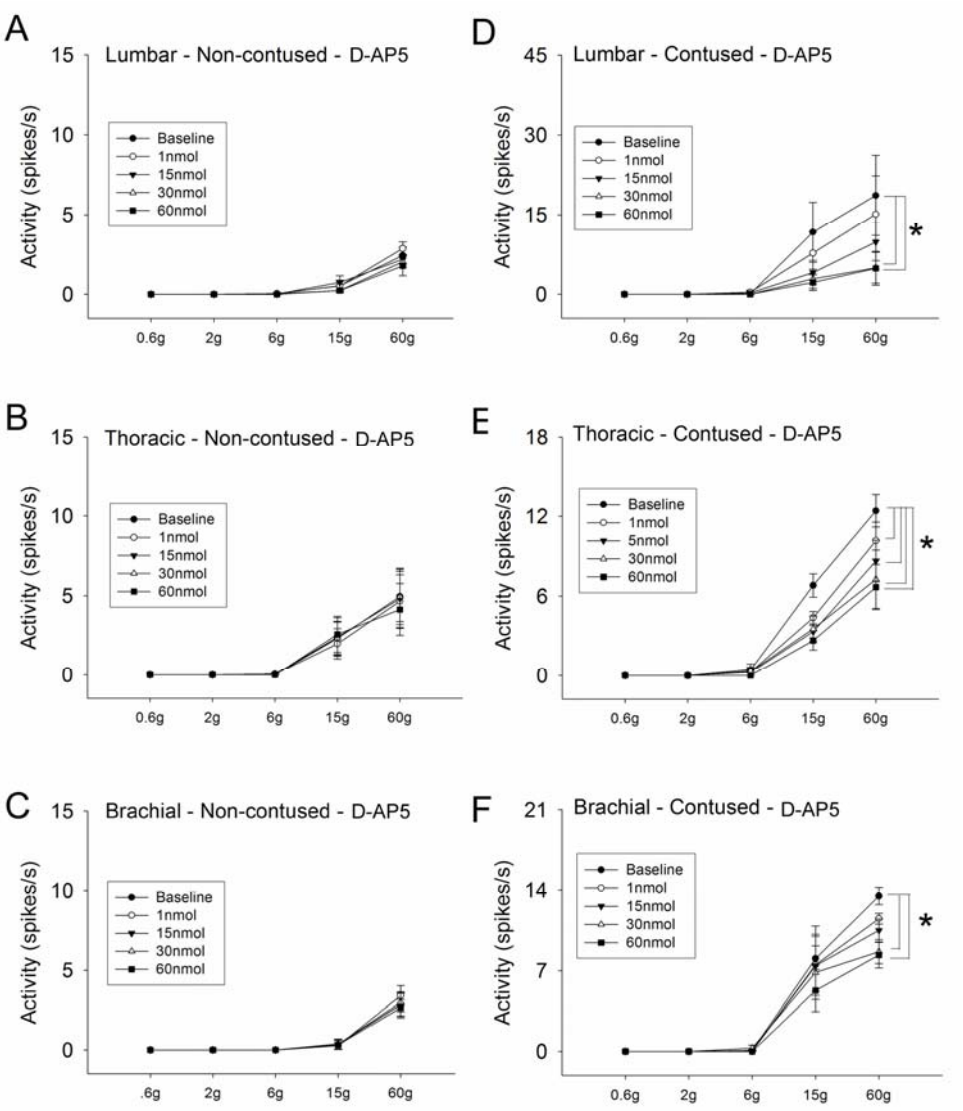

Figure 3.8. Activity during peripheral von Frey filament stimulation from HT neurons of the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during D-AP5 administration. The activities shown are from cells from non-contused (A-C) and contused animals (D-F) at different von Frey stimulation strengths. Activity from HT neurons of non-contused animals (A) show no signs of attenuation at all doses of D-AP5 administration. Cells from both the lumbar enlargement (D) and brachial enlargements (F) of contused animals during both D-AP5 treatment showed a significant decrease from baseline at 30 and 60nmol doses. Cells from the thoracic cord of contused animals (E) showed a significant decrease from the lowest dose 1nmol to 60nmol. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<.05$) compared to baseline.

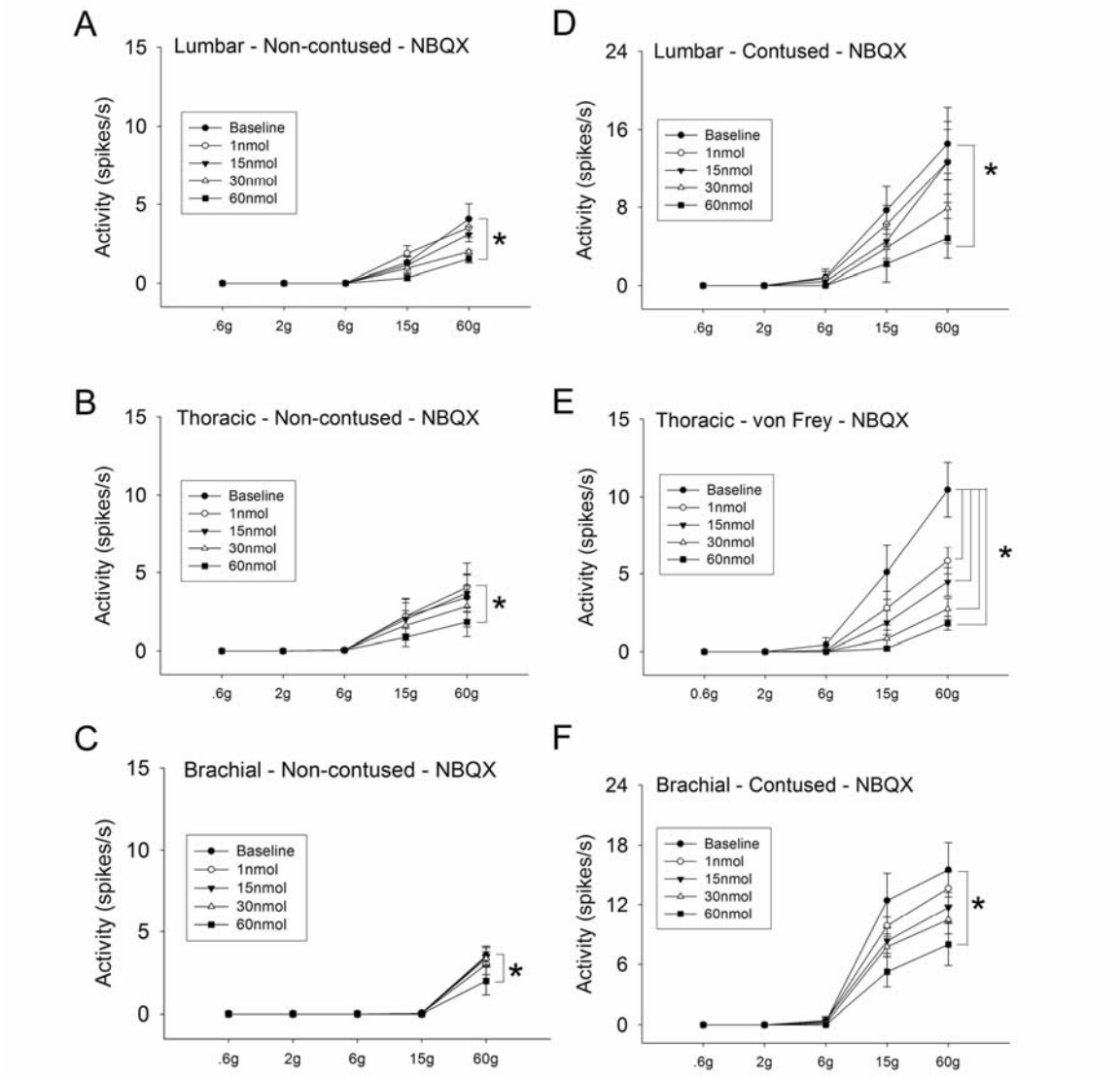

Figure 3.9. Activity during peripheral von Frey filament stimulation from HT neurons of the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during administration. The activities shown are from cells from non-contused (A-C) and contused animals (D-F) at different von Frey stimulation strengths. Activity from HT neurons of lumbar enlargement (A), thoracic cord (B) , and brachial enlargement (C) from the non-contused animals show attenuation during 60nmol doses of NBQX administration. Cells from both the lumbar enlargement (D) and brachial enlargements (F) of contused animals during both NBQX treatment showed a significant decrease from baseline at the 60nmol dose. Cells from the thoracic cord of contused animals (E) showed a significant decrease from the lowest dose 1nmol to 60nmol. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<0.05$) compared to baseline.

baseline value of activity in response to the 15, 30 and 60nmol D-AP5 application (Figure 3.10B; $p<0.05$; one way RM ANOVA) during the 5°C cold stimulus.

WDR cells from neither lumbar (Figure 3.10D) nor brachial enlargements (Figure 3.10F) of contused animals showed a significant change from baseline at any dose of NBQX. Only the WDR cells of the thoracic segments from contused animals showed a significant decrease as compared to the baseline value of activity in response to the 15, 30, 60 nmol NBQX application (Figure 3.10E; $p<0.05$; one way RM ANOVA) during the 5°C cold stimulus.

HT cells from both lumbar (Figure 3.11A) and brachial enlargements (Figure 3.11C) of contused animals did not show a significant decrease from baseline at any dose of D-AP5 ($p>0.05$; one way RM ANOVA) during cold stimulus application. Only the HT cells of the thoracic segments from contused animals showed a significant decrease as compared to the baseline value of activity in response to the 30 and 60nmol D-AP5 application (Figure 3.11B; $p<0.05$; one way RM ANOVA) during the 5°C cold stimulus.

HT cells from neither lumbar (Figure 3.11D) nor brachial enlargements (Figure 3.11F) of contused animals showed a significant change from baseline at any dose of NBQX ($p>0.05$; one way RM ANOVA). Only the HT cells of the thoracic segments from contused animals showed a significant decrease as compared to the baseline value of activity in response to the 30 and 60 nmol NBQX application (Figure 3.11E; $p<0.05$; one way RM ANOVA) during the 5°C cold stimulus.

WDR cells from both lumbar (Figure 3.12A) and brachial enlargements (Figure 3.12C) of contused did not show a significant decrease from baseline at any dose of D-AP5 ($p>0.05$; one way RM ANOVA). Only the WDR cells of the thoracic segments from contused animals showed a significant decrease as compared to the baseline value of activity in response to the 15, 30 and 60nmol D-AP5 application (Figure 3.12B; $p<0.05$; one way RM ANOVA) during the 45°C thermal stimulus.

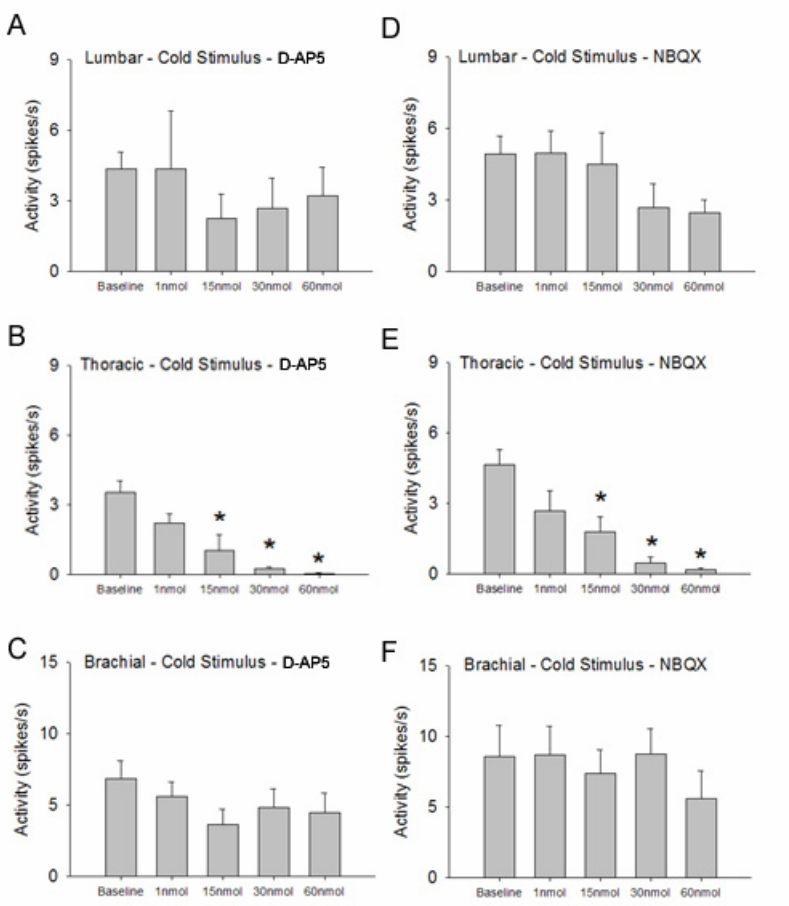

Figure 3.10. Activity during peripheral cold stimulation of 5C° from WDR neurons of contused animals from the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during D-AP5 (A-C) and NBQX (D-F) administration.
Neurons from the lumbar enlargement (A) and the brachial enlargement (C) were not reduced during D-AP5 application. Neurons from the lumbar enlargement (D) and brachial enlargement (F) were also not affected during NBQX application. Only neurons from the thoracic region were attenuated by both D-AP5 (B) and NBQX (E) at the 15nmol, 30nmol, and 60nmol doses. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<0.05$) compared to baseline.

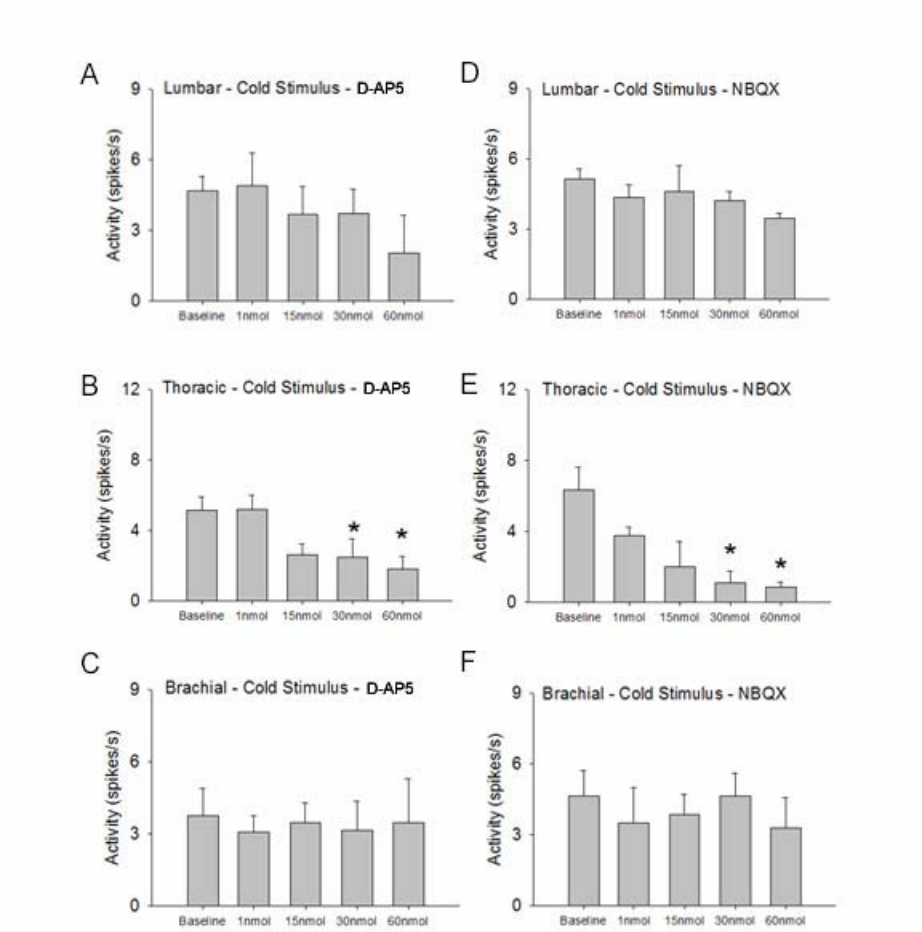

Figure 3.11. Activity during peripheral cold stimulation 5C° from HT neurons of contused animals from the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during D-AP5 (A-C) and NBQX (D-F) administration.
Neurons from the lumbar enlargement (A) and the brachial enlargement (C) were not reduced during D-AP5 application. Neurons from the lumbar enlargement (D) and brachial enlargement (F) were also not affected during NBQX application. Only neurons from the thoracic region were attenuated by both D-AP5 (B) and NBQX (E) at the 30nmol, and 60nmol doses. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<0.05$) compared to baseline.

WDR cells from neither lumbar (Figure 3.12D) nor brachial enlargements (Figure 3.12F) of contused animals showed a significant change from baseline at any dose of NBQX. Only the WDR cells of the thoracic segments from contused animals showed a significant decrease as compared to the baseline value of activity in response to the 1, 15, 30, 60 nmol NBQX application (Figure 3.12E; $p<0.05$; one way RM ANOVA) during the 45°C thermal stimulus.

HT cells from lumbar (Figure 3.13A) and brachial enlargements (Figure 3.13C) of contused did not show a significant decrease from baseline at any dose of D-AP5 ($p>0.05$; one way RM ANOVA) during 45°C thermal stimulus. Only the HT cells of the thoracic segments from contused animals showed a significant decrease as compared to the baseline value of activity in response to the 30 and 60nmol D-AP5 application (Figure 3.13B; $p<0.05$; one way RM ANOVA) during the 45°C thermal stimulus.

HT cells from neither lumbar (Figure 3.13D) nor brachial enlargements (Figure 3.13F) of contused animals showed a significant change from baseline at any dose of NBQX. Only the WDR cells of the thoracic segments from contused animals showed a significant decrease as compared to the baseline value of activity in response to the 30 and 60nmol NBQX application (Figure 3.13E; $p<0.05$; one way RM ANOVA) during the 45°C thermal stimulus.

3.4 DISCUSSION

3.4.1 Summary

Table 3.2 summarizes the results of this study. Our results show that, the cells of the thoracic cord (immediately rostral to injury site) undergo the most robust changes. The hyperexciteable background discharge of WDR neurons of the thoracic spinal cord from the contused animals is attenuated significantly compared to the WDR neurons from non-contused animals with D-AP5. Only the neurons of the thoracic cord showed a significant effect of condition (non-contused vs. contused) during D-AP5 treatment. D-AP5 also preferentially attenuated the brush response in the contused group while leaving

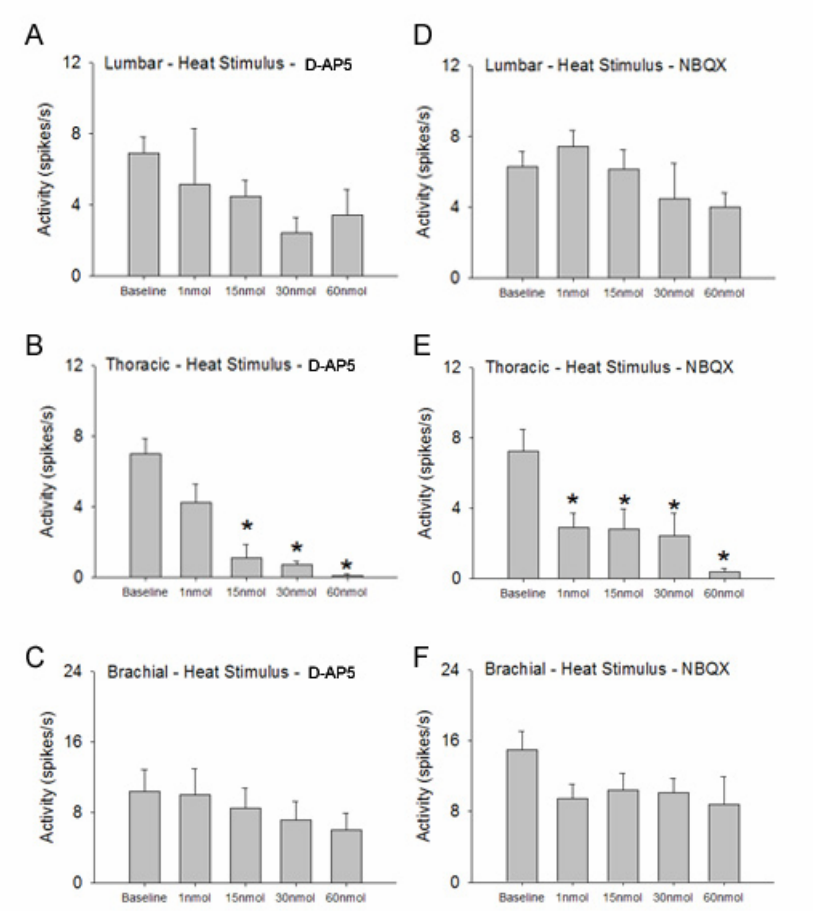

Figure 3.12. Activity during peripheral thermal stimulation 45C° from WDR neurons of contused animals from the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during D-AP5 (A-C) and NBQX (D-F) administration.
Neurons from the lumbar enlargement (A) and the brachial enlargement (C) were not reduced during D-AP5 application. Neurons from the lumbar enlargement (D) and brachial enlargement (F) were also not affected during NBQX application. Only neurons from the thoracic region were attenuated by D-AP5 (B) at the 15nmol, 30nmol, and 60nmol doses. Neurons from the thoracic region were also attenuated by NBQX (E) at the 1nmol, 15nmol, 30nmol, and 60nmol doses. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<0.05$) compared to baseline.

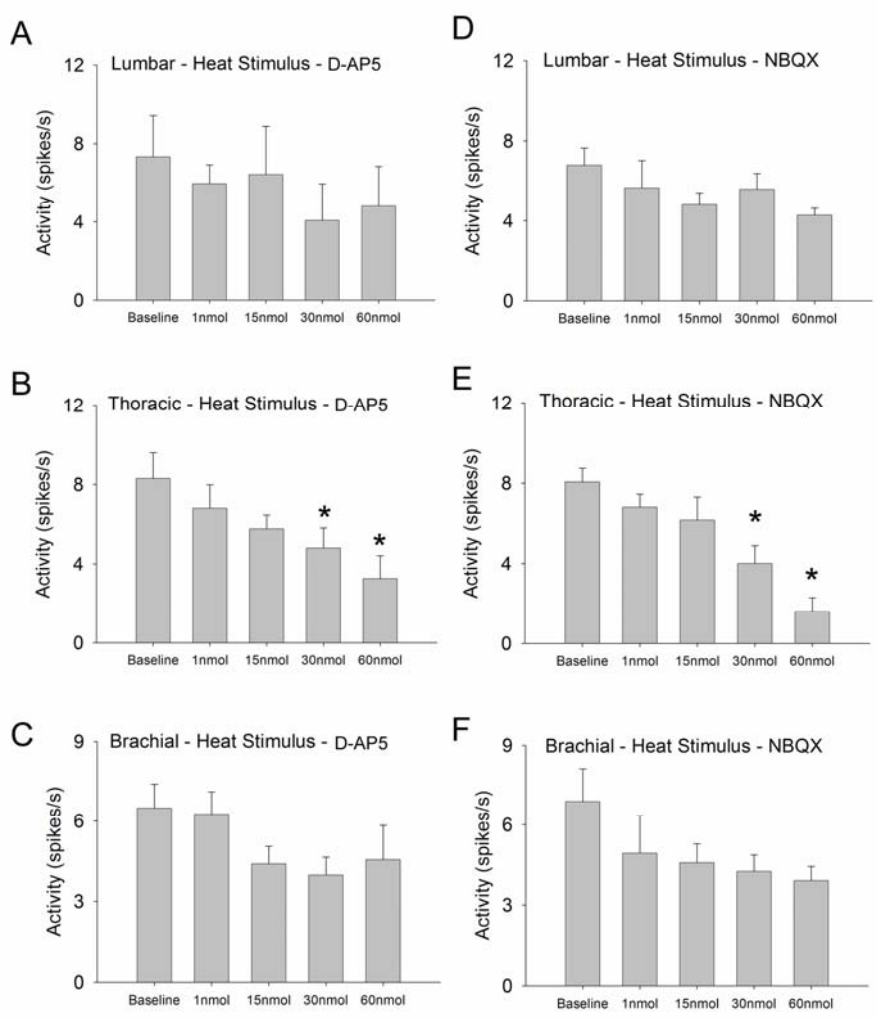

Figure 3.13. Activity during peripheral 45C° heat stimulation from HT neurons of contused animals from the lumbar enlargement (A, D), thoracic cord (B, E), and brachial enlargement (C, F) during D-AP5 (A-C) and NBQX (D-F) administration.
Neurons from the lumbar enlargement (A) and the brachial enlargement (C) were not reduced during D-AP5 application. Neurons from the lumbar enlargement (D) and brachial enlargement (F) were also not affected during NBQX application. Only neurons from the thoracic region were attenuated by both D-AP5 (B) and NBQX (E) at the 30nmol, and 60nmol doses. Data are represented as mean ± SEM. An * indicates a statistically significant difference ($p<0.05$) compared to baseline.

the non-contused unchanged. D-AP5 also attenuated the pinch response greater in the WDR and HT neurons of the contused group. All doses (1nmol, 15nmol, 30nmol, and 60nmol) of D-AP5 and NBQX showed significant attenuation during von Frey

stimulation in WDR neurons of contused animals. NBQX suppressed the brush response of both non-contused and contused groups; however, the overall response of the contused group was greater. D-AP5 and NBQX suppressed the response to both 5°C (cold) stimulus and 45°C (heat) at the thoracic cord. These changes involve a heightened response to both NMDA and AMPA receptor antagonism and suggest an increase in the ionotropic glutamate receptor mediated activity. Because the inhibitory activity was most apparent in the thoracic region, the thoracic region may show the greatest potential for ionotropic glutamate receptor therapy.

Neurons from the lumbar enlargement also show a greater attenuation by the ionotropic glutamate receptor antagonists in the contused group compared to the non-contused groups. But these effects were only confined to the higher doses (30nmol or greater) and did not result in a significant effect of condition (contused vs. non-contused). The location of the lumbar enlargement (L3-5) is 5-7 spinal segments away from the injury site (T10); however, because the spinal segments do not numerically correlate with the vertebra towards the caudal end, it lies only 2 vertebral gaps caudal (T10 to T13). The proximity of the lumbar enlargement to the injury site may render the lumbar enlargement susceptible to sensitization by increases in extracellular glutamate.

Neurons from the brachial enlargement did show differences at the 60nmol in activity between contused and non-contused during brush stimulation during WDR neuron recording. Both lumbar and brachial enlargement neurons showed a greater attenuation by the ionotropic glutamate receptor antagonists in the contused group compared to the non-contused groups during von Frey filament application, but these effects were only noticeable at higher doses.

While only neurons of the thoracic cord exhibited significant effects of condition (non-contused vs. contused) at the doses of drug given, both brachial enlargement and lumbar enlargement show some signs of glutamate mediated sensitization. The latter is

more readily explained by the proximity to the injury site. The thoracic region appears to be most susceptible to glutamate mediated sensitization.

Background Activity		D-AP5	NBQX
WDR	Lumbar	+	
	Thoracic	++	
	Brachial		
Brush Response			
WDR	Lumbar	+	
	Thoracic	++	++
	Brachial	+	
LT	Lumbar	+	
	Thoracic		
	Brachial		
Pinch Response			
WDR	Lumbar		
	Thoracic	++	
	Brachial		
HT	Lumbar		
	Thoracic	++	
	Brachial		

von Frey Response		D-AP5	NBQX
WDR	Lumbar	**	
	Thoracic	****	***
	Brachial	**	*
HT	Lumbar	**	
	Thoracic	****	***
	Brachial	**	
Cold Response			
WDR	Lumbar		
	Thoracic	#	#
	Brachial		
HT	Lumbar		
	Thoracic	#	#
	Brachial		
Heat Response			
WDR	Lumbar		
	Thoracic	#	#
	Brachial		
HT	Lumbar		
	Thoracic	#	#
	Brachial		

Table 3.2. Summary of Electrophysiological Results During D-AP5 or NBQX Treatment. A '++' denotes a significant effect of condition (contused vs. non-contused). A '+" denotes the presence of a significant effect only at one or two doses of drug. A '*' indicates the difference between the quantity of doses among non-contused and contused animals that were effective for attenuating von Frey filament response. A '#' denotes significant attenuation during drug dosing.

3.4.2 Glutamate and SCI

Different secondary events in the anatomic region of the spinal cord relative to the initial mechanical injury may determine the pathophysiology of the regional pain (below-level, at-level, and above-level). The lumbar region is exposed to a removal of

descending inhibition as certain descending influences are disconnected from caudal sites. The brachial region is not removed from descending inhibition and is several spinal segments away from the injury site. The thoracic region, by nature of its proximity to the injury site, is more susceptible to changes caused by local events secondary to mechanical injury.

Following immediately after CNS injury (brain ischemia or spinal cord injury), there is a brief but large amount of glutamate (and aspartate) released at the injury site (Liu et al. 1991; McAdoo et al. 1999; Panter et al. 1990). The surge in extracellular glutamate causes secondary cellular damage and cell death. In a quisqualate model of SCI injury, quisqualate, a potent AMPA and metabotropic glutamate receptor agonist, is used to chemically induce SCI (Yezierski et al. 1998). The quisqualate model of SCI shows injection of quiqualic acid directly into the spinal cord result in spinal cavitation and the development of pain like behavior. Intrathecal delivery of ionotropic glutamate receptor agonists, AMPA and NMDA, increases pain responses (Kontinen and Meert 2002). Quisqualate injection also sensitizes spinal sensory neurons to mechanical stimuli and increases afterdischarges response as observed electrophysiologically (Yezierski and Park 1993).

3.4.3 Central Sensitization and Ionotropic Glutamate Receptors

Peripheral injury models also produce an increase in extracellular glutamate concentrations in the spinal cord (Sluka and Westlund 1992; Sorkin and McAdoo 1993). It is believed that the increase in glutamate sensitizes the spinal cord (Coderre 1993). The phenomenon of increased activity in the spinal cord is termed ‘central sensitization’. Central sensitization causes hyperexcitability of dorsal horn neurons, which can be measured electrophysiologically. Central sensitization has been demonstrated in many models of peripheral neuropathy (Palecek et al. 1992), arthritis (Neugebauer et al. 1993b), intradermal capsaicin (Dougherty and Willis 1992; Neugebauer et al. 1999; Neugebauer et al. 2000), and incisional pain (Zahn et al. 2005). Both peripheral injury

and contusive SCI lead to an increase in extracellular glutamate in the spinal cord, painlike behaviors, and a hyperexcitable state of the spinal cord.

Ionotropic glutamate receptor antagonism has been shown to decrease activity of the sensitized spinal cord (Leem et al. 1996; Neugebauer et al. 1994; Neugebauer et al. 1993a; Rygh et al. 2001; Spraggins et al. 2001; Stanfa and Dickenson 1999; Zahn et al. 2005). Ionotropic glutamate antagonism has also been shown to affect cells of the sensitized cord more effectively than the non-sensitized cord (Neugebauer et al. 1993b; Rygh et al. 2001; Stanfa and Dickenson 1999). One potential mechanism of SCI pain involves the ionotropic glutamate receptor mediated development of permanent hyperexcitability of dorsal horn neurons or central sensitization. Ionotropic glutamate receptor antagonism is a potential pain therapy, best affecting the segments close to the injury site.

3.4.4 Conclusions

In summary, this study shows that NMDA and non-NMDA receptors play an important role in the processing of afferent input after contusive SCI. Both NMDA and non-NMDA receptors are involved in the processing of noxious input in the normal un-injured state. Both receptor types are involved in the maintenance of hyperexciteability 35 days post-injury. The NMDA and non-NMDA receptor antagonism show the greatest change in activity at the site immediately rostral to the site of injury, the thoracic cord. This finding is reasonable given the proximity to the rise in glutamate at the injury site after SCI. The data suggests that ionotropic glutamate receptor antagonism is potentially most effective at treating at-level evoked and spontaneous pain.

CHAPTER 4

CAMKII AND SCI

4.1 INTRODUCTION

A potential mechanism for the abnormal pain sequelae after SCI is the post injury increase in excitatory amino acid concentrations (Liu et al. 1991; McAdoo et al. 1999) that leads to increases in intracellular calcium levels at and around the site of injury (Faden and Simon 1988; Tator and Fehlings 1991). Calcium increases lead to changes in transcription factors and receptor activation states that, in turn, cause the development of hyperexcitability of spinal neurons in the pain pathways through a process known as central sensitization (Bennett et al. 2000; Christensen and Hulsebosch 1997; Hains et al. 2002; Mills and Hulsebosch 2002; Mills et al. 2002; Vera-Portocarrero et al. 2002; Willis 2002; Woolf 1983; Woolf and Thompson 1991). This process is hypothesized to be the synaptic equivalent of long-term potentiation (LTP), which recently has been accepted as occurring in neuronal populations in many areas of the brain and spinal cord, rather than exclusively in the hippocampus (Bliss and Lomo 1973; Ji et al. 2003; Sandkuhler 2000; Willis 2002).

An important initiating event during LTP is the activation of calcium-calmodulin dependent protein kinase II (CaMKII). Activation of the postsynaptic NMDA receptor via glutamate allows a Ca^{2+}influx, which activates calmodulin and in turn leads to the phosphorylation (activation) of CaMKII. The CaMKII in turn binds to the NR2B subunit of the NMDA receptor to maintain a state of persistent activity. Activated CaMKII can

also phosphorylate the GluR1 AMPA subunit enhancing the conductance of the AMPA receptor. CaMKII also induces rapid trafficking and incorporation of GluR1 containing AMPA receptors into the postsynaptic membrane. The activated CaMKII phosphorylates transcription factors which include cyclic AMP responsive element binding protein (CREB). CREB is known to be important in the formation of long-term memory through its role in the late phase of LTP. CaMKII has already been shown to regulate CREB's role in the central sensitization (Fang et al. 2005; Ji and Rupp 1997). CaMKII's importance in this cascade lies in its role in initiating a sequence of events which lead to long term changes.

Our lab has already characterized a rodent model of SCI induced pain that results in maintained animal hypersensitivity to mechanical and thermal stimuli 35 days post-injury. The abnormal hypersensitivity is paralleled by electrophysiological changes which include increased background activity and increased response to both innocuous and mechanical stimuli. The cells from the segments immediately rostral to the injury site (those that are close to the injury site), have already been established as having increased background discharge, increased responses to brush (innocuous stimulous), press, pinch, von Frey filament stimulation, 5°C stimulus, and 45°C stimulus. Our lab has also shown the injured rats show a greater CaMKII activation or pCaMKII expression in the spinal segments immediately rostral to the segments of injury (Crown et al. 2005). Immunocytochemistry experiments have also shown that the increase in pCaMKII was present in both the dorsal horn gray matter and in spinothalamic tract cells. The purpose

of this study is to further examine the role of CaMKII in the central sensitization produced by traumatic SCI.

4.2 METHODS

4.2.1 Spinal cord contusion injury

Subjects were anesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg). Anesthesia was considered complete when there was no flexor withdrawal in response to noxious foot pinch. The subjects' backs were shaven and a laminectomy was performed at vertebral segment T10. Spinal cord contusion injury was produced using the Infinite Horizon (IH) injury device (Infinite Horizon, LLC). A motor driven cylindrical tip, 2.0mm in diameter, was driven to a force of 150 kilodynes, allowed to compress the cord for 1 second (1 second dwell time), and retracted. Following the contusion injury, the musculature was sutured, the skin was autoclipped, and the animals were allowed to recover from anesthesia. Following contusion, bladders were manually expressed twice daily until bladder function recovered, usually within 10 days post injury. Prophylactic antibiotic (Baytril, 30 mg/kg, subcutaneously) was given twice daily until bladder function returned.

4.2.2 Electrophysiology

Extracellular single-unit recordings were made from contused rats (n=10). Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and supplemented with sodium pentobarbital (5 mg/kg/h) infused intravenously through a jugular vein catheter. Adequacy of anesthesia was monitored by the lack of withdrawal reflexes to noxious stimuli and the absence of corneal blink reflexes. Core temperature was maintained at

37°C by a thermostatically controlled heating blanket. Laminectomy was performed to expose the thoracic spinal segments T8-T10. Cells were isolated from thoracic segments (T8-T9), which lay immediately rostral to the site of contusion injury (T10). Extracellular single-unit recordings were made with a low impedance (0.4-0.8 MΩ at 1KHz; Kation Scientific) glass carbon fiber microelectrode.

Once a cell was identified, background activity was measured followed by cutaneous receptive field mapping with von Frey filaments and brief pinches. Five wide dynamic range (WDR) neurons, responsive to both innocuous (brush) and noxious (press and pinch) were sought.

Peripheral stimuli during electrophysiology included the following: brush, pressure (with large arterial clip at 144 g/mm^2), pinch (with small arterial clip at 583 g/mm^2), von Frey filament application (with 5 filaments: 0.6g, 2g, 6g, 15g, and 60g), and three temperature stimuli (5°C, 25°C, and 45°C). Temperature stimuli were administered with the Physitemp NTE-2A temperature stimulus probe. Each stimulus was applied for 10 seconds.

Electrical signals were amplified and input to a window discriminator, displayed on analog and digital storage oscilloscopes, processed by a data collection system (CED 1401+; Cambridge Instruments, Cambridge, UK; Pentium computer, Dell, Austin, TX, USA), and stored on a computer to construct peristimulus time histograms. The stored digital record of unit activity was analyzed offline with Spike 2 software (v4.07, Cambridge Electronic Design, Cambridge, UK). Responsiveness to peripheral stimulus was calculated by subtracting the baseline activity from evoked activity to calculate a net

increase in discharge rate. Responsiveness to thermal stimuli was calculated by subtracting the response to the 25°C from responses to both 5°C and 45°C to discount the response from the pressure attributed to the temperature probe on the skin of the animal.

KN-93 (EMD Biosciences, San Diego), a CaM Kinase II inhibitor, was dissolved in vehicle (saline). The effects of topical spinally applied KN-93 (100uM in 50uL) or vehicle were tested on cells immediately rostral to the site of contusion. Cells from this area have been described as having increased responsiveness to peripheral stimuli. Recordings were made prior to KN-93 or vehicle application and 15, 45, and 75 minutes after application.

4.2.3 Statistical measures

Neuronal responses to von Frey stimuli and temperature probes applied just rostral to the site of injury were analyzed using 1 between-1within analyses of covariance (ANCOVAs; with baseline reactivity as the covariate). Post hoc tests were performed using Duncan's New Multiple Range Test. In all cases, the alpha level for statistical significance was set at $p < 0.05$.

4.3 RESULTS

The mean recording depth for the vehicle treated WDR cells from contused rats was 442 ± 53 (SEM). The mean recording depth for the KN-93 treated WDR cells from contused animals was 497 ± 83 (SEM).

4.3.1 Change in Neuronal Background Activity and Responses to Brush, Press, and Pinch Stimuli

Figure 1A-D shows peristimulus time histogram of single cell recordings from one cell at baseline (prior to KN-93 treatment) and 15, 45, and 75 minutes after KN-93 treatment, respectively. The maximum effect of treatment in the series was seen 15 minutes post KN-93 treatment. By 75 minutes, the activity had recovered to baseline value.

In order to make direct comparisons between vehicle treated and KN-93 treated animals (Fig. 1E-gG), the activities during their respective stimulus application was normalized to their baseline levels. This was done by dividing the raw value by its baseline (pre-drug) value and expressed as a percentage. The KN-93 treatment showed a significant depression of background, brush, press, and pinch activity (Fig. 1E-G; one way RM ANOVA; $p<.05$) compared to the group treated with vehicle. This shows that in addition to reducing background activity, KN-93 is able to reduce background activity as well as neuronal response to innocuous (brush) and noxious (press and pinch stimuli).

4.3.2 Change in Neuronal Response to von Frey stimulation

In addition to the impact of CaMKII inhibition on reactivity to brush, press, and pinch, we examined the effects of KN-93 on reactivity to graded von Frey stimuli applied to the dermatome immediately rostral to the site of SCI. In the case of von Frey stimulation, KN-93 produced a significant inhibition of neuronal firing that was graded according to the applied force (Fig. 2A-D). KN-93 failed to elicit change in reactivity to the 0.6 g von Frey stimulus ($p>.05$; data not shown). This is due to the situation that not all of the WDR neurons tested were responsive at the 0.6g von Frey filament. However, KN-93 reduced neuronal firing to the 2, 6, 15, and 60g von Frey stimuli (Figure 2E-H).

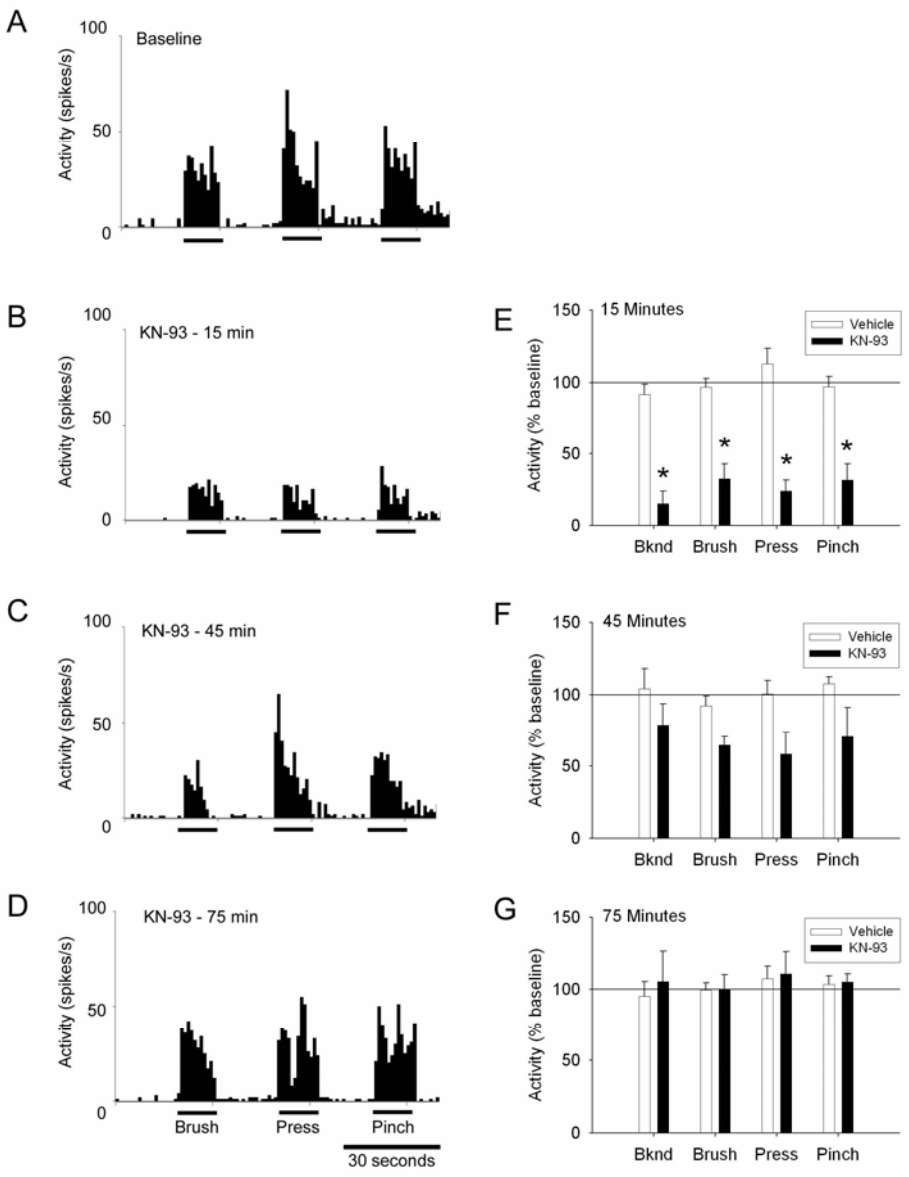

Figure 4.1. Peristumulus time histograms and mean discharge rates during peripheral brush press and pinch. Activity was recorded at baseline (pre-drug), 15, 45, and 75 minutes after KN-93 application. Single-unit recordings (A-D) shows maximal inhibition of background, brush, press, and pinch at 15minutes with return of activity by 75 minutes. Mean discharge rates are expressed as a percentage of baseline activity (E-G). Mean discharge rates (background, brush, press, and pinch) are attenuated during KN-93 compared to vehicle treated groups at 15 minutes post treatment (E). Data are expressed as mean ± SEM. An * indicates statistical significance ($p<0.05$) compared to the vehicle treated group.

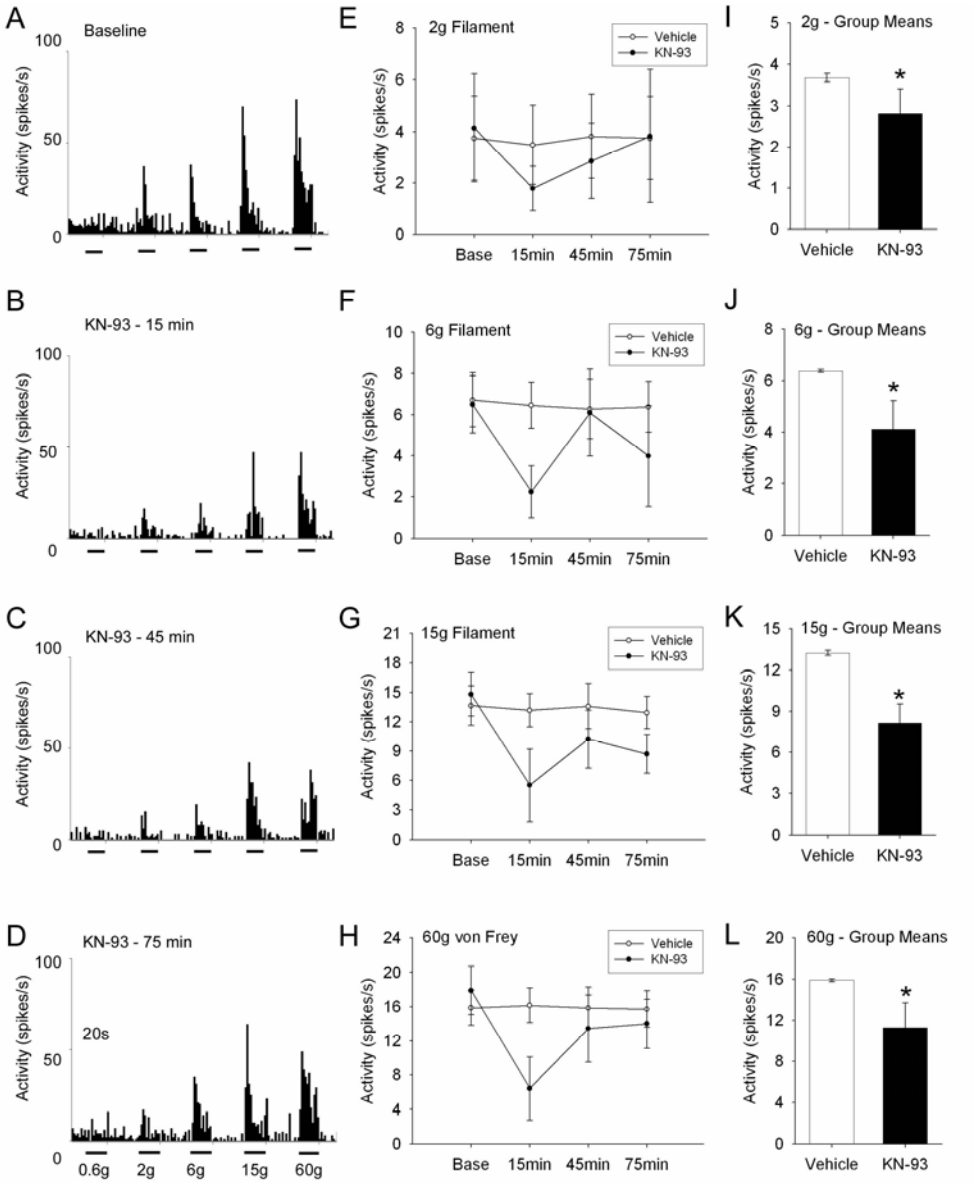

Figure 4.2. Peristumulus time histograms and mean discharge rates during von Frey filament application. Activity was recorded at baseline (pre-drug), 15, 45, and 75 minutes after KN-93 application. Single-unit recordings (A-D) shows maximal inhibition of background, brush, press, and pinch at 15minutes with return of activity by 75 minutes. Mean discharge rates are shown for each von Frey filament at baseline, 15, 45, and 75 minutes (E-G). Mean discharge rates for the 2, 6, 15, 60g von Frey filament are attenuated during KN-93 compared to vehicle treated groups at 15 minutes post treatment (E-H). Group means (I-L) show a difference between vehicle treated and KN-93 treated groups. Data are expressed as mean ± SEM. An * indicates statistical significance ($p<0.05$) compared to the vehicle treated group.

4.3.3 Changes in Neuronal Response to Heat and Cold

CaMKII inhibition with KN-93 also significantly diminished neuronal responses to heat (45°C) and cold (5°C) stimuli applied just rostral to the site of SCI (Figure 3). KN-93 produced a significant inhibition of neuronal firing in response to 5, 25, and 45°C 15 minutes after application (Fig. 2A-D). The mean discharge rates shown (Fig. 2E-G) are normalized to the discharge rate during 25°C stimulation. This is done to adjust for the activity that may be due to the pressure exerted from the temperature stimulus probe. Significant attenuation of activity from both 5°C and 45°C stimuli occurs at 15minutes post application of KN-93 ((Fig. 2E-G; $p<.05$).

4.4 DISCUSSION

4.4.1 Summary

A previous study (Chapter 3) has shown that the spinal segments, rostral to the site of injury, are hyperexcitable and neurons have increased mechanical sensitivity. The results from this study show that the hyperexciteable background and responses to mechanical stimulation (brush, press, pinch, and von Frey filament) and temperature stimuli (cold and heat) are attenuable by CaMKII inhibition with KN-93. Work from our lab also has shown that an upregulation of pCaMKII occurs 35 days post SCI in the dorsal horn of contused animals. Our lab has also shown that phosphorylated (and thus activated) forms of ERK 1/2 and p-38 MAPK were upregulated in similar regions of the spinal cord over the same time course in injured rats. Taken together, these finding further implicate cascades associated with LTP and central sensitization in the chronic central neuropathic pain seen after spinal cord injury.

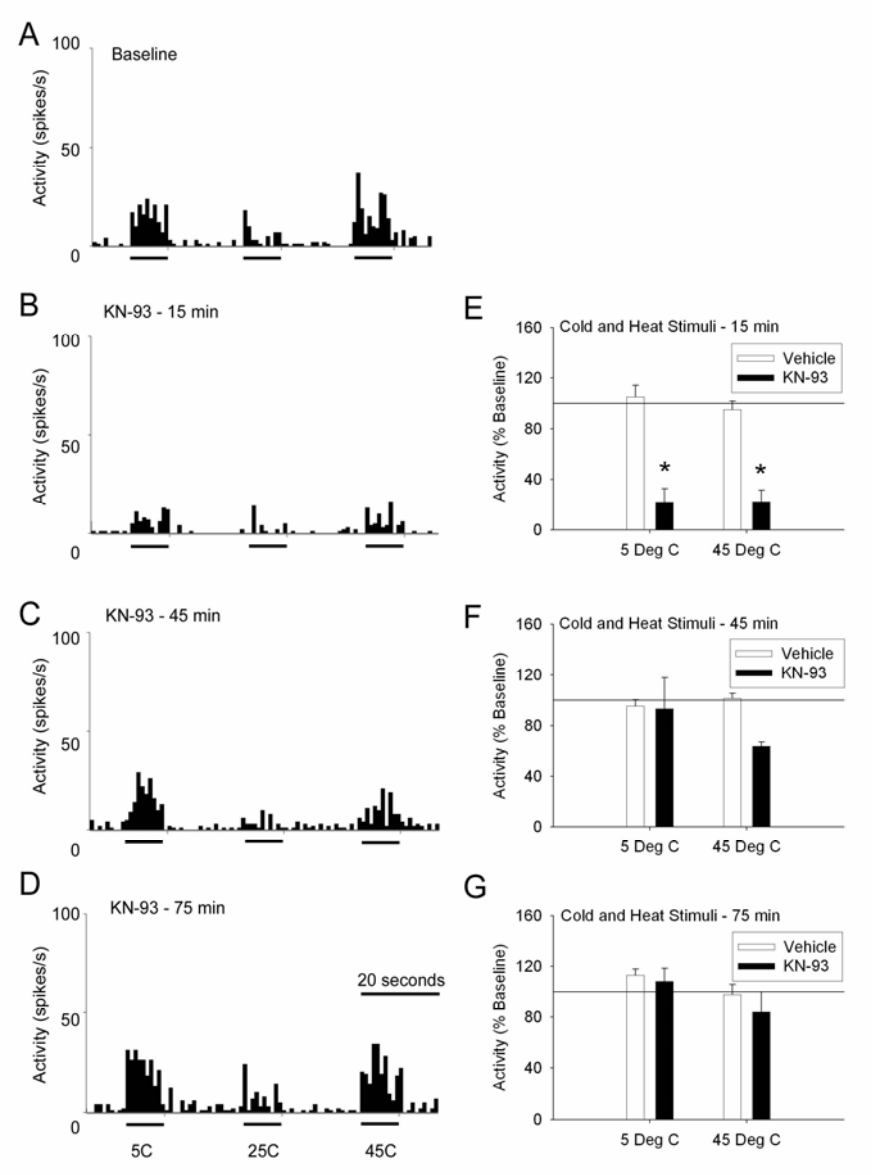

Figure 4.3. Peristumulus time histograms and mean discharge rates during temperature stimulation. Activity was recorded at baseline (pre-drug), 15, 45, and 75 minutes after KN-93 application. Single-unit recordings (A-D) shows maximal inhibition of 5, 25 and 45°C at 15minutes with return of activity by 75 minutes. Mean discharge rates are shown for 5 and 45°C stimuli (E-G). Each discharge rate is normalized by subtracting the discharge rate at 25°C. Mean discharge rates for 5 and 45°C stimuli are attenuated during KN-93 compared to vehicle treated groups at 15 minutes post treatment (E-H). Group means (I-L) show a difference between vehicle treated and KN-93 treated groups. Data are expressed as mean ± SEM. An * indicates statistical significance ($p<0.05$) compared to the vehicle treated group.

4.4.2 LTP and central sensitization

The current data support the idea that activation of intracellular signaling cascades associated with LTP and central sensitization is related to the behavioral manifestation of at-level tactile allodynia. Recent evidence suggests that LTP and central sensitization share a number of molecular similarities. Both phenomena have an early activity-dependent phase and a later transcription-dependent phase that relies on the formation of new proteins (Ji et al. 2003; Nguyen and Woo 2003). In addition, both the late phase LTP and central sensitization have been linked to activation of the NMDA receptor, followed by subsequent activation of downstream intracellular enzymatic cascades involving adenylyl cyclase, protein kinase A, protein kinase C, and/or Ca^{2+}/calmodulin kinase (CaMK). Stimulation of these cascades leads to activation of a number of MAPKs, including ERK 1/2, JNK, and p38 MAPK which then, in turn, can lead to phosphorylation of transcription factors and changes in gene transcription (Ji et al. 2003). Late phase LTP depends not only on activation of signaling cascades involving PKA, PKC, nitric oxide (NO) and/or CaMK II, but on subsequent cyclic AMP responsive element binding protein (CREB)-mediated synthesis of mRNA and protein (Nguyen and Woo 2003). The current data suggest even broader implications for the activity of these cascades in chronic central, as well as peripheral, neuropathic pain.

4.4.3 Conclusions

Central sensitization and LTP share common molecular signaling pathways in the molecular development of abnormal pain states. CaMKII is only one of these molecular intermediates. Spinal cord injury produces an abnormal pain state, which parallels molecular changes akin to those occurring during central sensitization and LTP. Spinal cord hyperexcitability is attenuable by the CaMKII inhibitor KN-93, which reaffirms the hypothesis of the involvement CaMKII in the spinal cord injured state.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 SUMMARY

Spinal cord injury results not only in a loss of function, but also results in chronic pain syndromes. These pain syndromes are quite debilitating and greatly decrease the quality of life. Spinal cord injury results in pain located in three distinct anatomical locations: 1) below-level pain – located in dermatomes caudal to the injury site, 2) at-level pain – located in dermatomes bordering the injury site, and 3) above-level pain – located in dermatomes rostral to the injury site (area of complete sensory preservation).

The initial spinal cord injury is followed by a surge of excitatory amino acids (EAAs), which include glutamate, from the injured cells. In peripherally induced pain models, peripheral nociceptive injury results in increased extracellular spinal levels of glutamate. Peripheral injury models, that result in allodynia and hyperalgesia, have been shown to develop electrophysiologic spinal hyperexcitability indicative of enhanced nociceptive transmission.

Spinally contused animals show abnormal development of pain-like behavior by 35 days post-injury. Increased responses to mechanical and thermal stimuli mimic the development of allodynia and hyperalgesia in the clinical population. These changes are not confined to one location. These changes occur in the hindlimbs, thoracic dermatomes, and the forelimbs. These changes parallel the development of hindlimb, at-level, and forelimb pain. The development of mechanical and thermal hypersensitivity is paralleled by electrophysiological changes in the spinal cord at all three sites. Background activity of contused animals was elevated in the WDR neurons from the lumbar enlargement (below-level) and thoracic cord (at-level). The WDR neurons from all three sites of contused animals exhibit increased response to brush, press, pinch, von Frey filament stimulation, a cold stimulus (5°C), and a heat stimulus (45°C). The HT neurons from all three sites of contused animals exhibited increased activity during press,

pinch, von Frey filament stimulation, cold stimulus (5°C), and heat stimulus. Only the LT neurons from the lumbar enlargement of contused animals show a hyperexcitable response to brush.

The contusion model of SCI with a 150kD impact and a 1 second dwell time, results in measurable changes in behavior and electrophysiology that reflects the development of an abnormal pain state. Because the injury is confined to a focal impact, it is conceivable that the spinal changes are due to different mechanisms. One such potential mechanism is central sensitization due to increased extracellular glutamate. As mentioned above, increased extracellular spinal glutamate occurs in both peripheral injury and spinal cord injury. Both result in the development of pain-like behavior. Peripheral injury models show an increased role of ionotropic glutamate receptor dependent activity.

Of the three areas, the thoracic cord shows the greatest increase in ionotropic glutamate receptor mediated activity. It stands to reason that based on the anatomical location of the thoracic cord (immediately rostral to injury), this area has the highest levels of extracellular glutamate. The curious outcome of the study is that the lumbar and brachial enlargements show a very small increase in ionotropic glutamate receptor mediated activity as compared to their respective non-contused groups. While the lumbar enlargement (recording site; L3-L5) is 6 spinal segments away from the injury site (T10), it is located only 3 vertebral segment away (T13 and T10). The location of the lumbar enlargement may be disposed to a milder elevation of glutamate compared to the injury site. The brachial enlargement is located many more segments away from the injury site. The changes observed in the contused animals could potentially be a result of peripheral sensitization. Certainly as the animals recover from injury, they regain much locomotor function but remain more dependent on forelimb function.

CaMKII is an enzyme which plays a role in pain states. CaMKII plays a role in the maintenance of ionotropic glutamate receptor activity. Segments rostral to injury show an increase in pCaMKII expression 35 days after injury. Electrophysiologic recordings show that the CaMKII inhibitor, KN-93, is able to attenuate at-level

hyperexcitability. As peripherally induced sensitization has been shown to have common molecular pathways as long-term potentiation (LTP), it appears that SCI induced pain/sensitization also related.

5.2 CONCLUSION

In these studies, we showed that SCI results in pain-like behavior. The pain-like behavior is accompanied by spinal hyperexcitability. Ionotropic glutamate receptor activity plays an increased role after SCI. CaMKII plays a role in at-level SCI induced pain-like behavior. These findings give insight into potential therapeutic interventions for spinal cord injury induced pain.

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Abstracts

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