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The Effect of Chronic Constriction Injury on Cellular Systems Within Nociceptive Pathways in the Mouse

Michelle Hoot
Virginia Commonwealth University

THE EFFECT OF CHRONIC CONSTRICTION INJURY ON CELLULAR SYSTEMS

WITHIN NOCICEPTIVE PATHWAYS OF THE MOUSE

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

Michelle Renee Hoot
B.S., Wayne State University, 2004

Director: William L. Dewey, Ph.D.
Professor, Department of Pharmacology and Toxicology

Virginia Commonwealth University
Richmond, Virginia
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List of Abbreviations

2-AG	2-arachydonoyl glycerol
ACC	anterior cingulate cortex
AEA	anandamide
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CCI	chronic constriction injury
CFA	complete Freund's adjuvant
D9-THC	Delta-9-tetrahydrocannabinol
DAMGO	[d-Ala2,(*N*-Me)Phe4,Gly5-OH] enkephalin
DRG	dorsal root ganglion
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular regulated kinases 1 and 2
GPCR	G-protein coupled receptor
JNK	c-Jun NH_2-terminal kinase
LC/MS	Liquid Chromatography-Mass Spectrometry
LTP	long term potentiation
MT	medial thalamus
MAPK	mitogen activated protein kinase
MEK	MAPK kinase
MEKK	MEK kinase
NSAID	non-steroidal anti-inflammatory drug
p-ERK	phosphorylated ERK
PAG	periaqueductal grey
PBS	phosphate buffered saline
PSNL	partial sciatic nerve ligation
RVMM	rostroventral medial medulla
SNL	spinal nerve ligation
TBS	Tris-buffered saline
VTA	ventral tegmental area

Abstract

THE EFFECT OF CHRONIC CONSTRICTION INJURY ON CELLULAR SYSTEMS

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By Michelle R. Hoot, B.S.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Major Director: Dr. William L. Dewey
Professor, Interim Chair, Pharmacology and Toxicology

Chronic neuropathic pain is often difficult to treat due to its resistance to therapeutic intervention. This is due in part to the poor understanding of the physiological mechanisms involved in the establishment and maintenance of neuropathic pain states. The neuropathic pain model, chronic constriction injury of the sciatic nerve, produced robust pain hypersensitivity in our mice. It also induced significant changes in the mitogen activated protein kinase family, and the cannabinoid and μ-opioid systems in three different brain areas involved in the modulation or regulation of pain states.

CCI induced a 2.5 fold increase in mRNA of the kinase Raf-1 in the PAG of mice. Raf-1 is part of the ERK cascade in the MAP kinase family of proteins. The MAPK family of proteins has previously been shown to be involved in the establishment and maintenance of chronic neuropathic pain via central sensitization and the PAG is a critical regulator of nociceptive input and is part of the descending pain pathway, which has also been shown to have a role in central sensitization.

CCI also resulted in significant decreases in the μ-opioid receptor agonist DAMGO stimulated [^{35}S] GTPγS binding in the medial thalamus, and the cannabinoid receptor agonist, WIN 55, 212-2 stimulated [^{35}S] GTPγS binding in the anterior cingulate cortex. These effects were not due to an overall decrease in μ-opioid receptor or cannabinoid receptor 1 binding, suggesting that the chronic pain-like condition resulted in a desensitization of these receptors. Both the medial thalamus and the anterior cingulate cortex are brain areas involved in the medial pain pathway which, along with the limbic system, have been shown to be involved in the affective component of pain processing.

These data are the first to demonstrate changes in these three cellular systems in the respective brain areas of the mouse in response to chronic neuropathic pain. The novel findings presented in this dissertation provide new areas of investigation for the treatment of this debilitating disease.

Chapter I

Introduction

Introduction

Severe and chronic pain are debilitating conditions and an issue of such importance that U.S. Congress passed legislation (H.R. 3244) that was signed into law declaring 2000-2010 as the "Decade of Pain Control and Research". This is understandable when viewed from the perspective that 75 million Americans suffer from severe pain each year and 50 million of those people report their pain condition as being chronic (Berry, 2006). From a financial perspective, severe and chronic pain accounts for 150 billion US dollars in healthcare costs and 50 million lost work days per year (Berry, 2001;Turk, 2002). This type of loss is shouldered not only by patients and their families but by society as a whole. When taken into consideration that those over 65 years old suffer from severe and chronic pain ten times more often than those under the age of 30 (Stanford Medical Center, 2005), the cost of chronic pain is bound to grow significantly in the coming decade as the baby-boomer generation ages into their retirement years.

One may wonder why something like pain could cause such an enormous social and financial toll on society. Surveys of chronic pain sufferers shed some light on this issue. The issue does not rest in patients avoiding health care professionals; in fact 92% of patients have seen a healthcare professional about their pain (Association, 2004). Most pain sufferers seek treatment for their pain and the most common treatments are pharmacological, with non-sterioidal anti- inflammatory drugs (NSAIDs) and opioids being the most commonly used. NSAIDs are the most commonly utilized class of drugs due to the low cost and over the counter availability but only 12% of chronic pain sufferers report them working "very well" (Stanford Medical Center, 2005). Opioids are widely considered the most effective way to combat severe pain due to their direct central nervous effects and thus many chronic pain patients use them on a daily basis. However, only 23% of chronic pain sufferers rated opioids as "very effective" in treating their pain and almost 10% rated them as "ineffective". In addition, 51% of opioid users report having "only a little" or "no control" over their pain (Michaelson & Company, 2006). These statistics are disconcerting as this type of uncontrollable suffering can lead to

significantly lower quality of life that may cause comorbid psychological illness. In fact, a survey conducted by the American Chronic Pain Association (2004) found that 46% of chronic pain patients reported experiencing depression and 35% percent expressed feelings of the "inability to cope" as a result of their pain. Clearly the conventional treatments for chronic pain are lacking at best and chronic, uncontrollable pain can lead to potentially serious psychological illness which can lead to even greater healthcare costs and lost productivity. Therefore, alternative treatments are not only necessary from a humanitarian standpoint but also from a financial and societal perspective.

What Is Pain?

According to the International Association for the Study of Pain (IASP), pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." This definition speaks to the subjective nature of pain, and essentially, the experience of pain is unique to each individual. Pain is however, different from nociception in that nociception arises from the direct activation of nociceptor in either internal or external tissue. Nociceptor stimulation does not necessarily lead to the experience of pain, and pain is not necessarily caused by direct stimulation of nociceptors. Herein lies the difference between what is thought of as acute pain and chronic pain. Acute pain is a necessary component to the survival of an organism because it lets it know when noxious stimulation has occurred and helps an organism avoid further injury. It usually has a direct correlate to some type of tissue injury and nociceptor activation. Chronic pain, on the other hand, is defined not only by time course, but as "pain that extends beyond normal period of healing, disrupts sleep and normal living, ceases to serve a protective function, and instead degrades health and functional capability" (Berry, 2006).

Chronic pain can present itself in many ways and the most often studied are neuropathic and inflammatory pain. Neuropathic pain is caused by damage to or dysfunction of any part of the somatosensory system of the central nervous system (CNS). It can be caused by a variety of different events including peripheral nerve trauma, diabetes, multiple sclerosis, and CNS damage from stroke or viral infections. Clinically, neuropathic pain is characterized by multiple symptomologies. Symptoms commonly seen in humans are

motor weakness, numbness, and deficits or absence in deep tendon reflexes in the injured area (Gilron et al., 2006) as well as hyperalgesia, allodynia and stimulus-independent pain (Jensen et al., 2001). Hyperalgesia is classified as an "increased response to a stimulus which is normally painful" and allodynia is classified as "pain due to a stimulus which does not normally evoke pain" which, unlike hyperalgesia, "indicates loss of specificity of a sensory modality" (IASP, 1994). Stimulus independent-pain is spontaneous pain without an externally evoked stimulus. Hyperalgesia, allodynia, and stimulus-independent pain are considered the three "classic" signs of chronic pain and in both clinical and animal research are often used to obtain quantitative measures of the presence and intensity of chronic pain.

Chronic inflammatory pain includes the common conditions of rheumatoid arthritis and in many cases back pain and some types of visceral pain that arise from irritable bowel disease. It is characterized by two zones, the first being the zone of `primary' hyperalgesia, which encompasses the area of tissue damage itself and is characterized by spontaneous pain hypersensitivity to thermal, mechanical and chemical stimuli. The zone of `secondary' hyperalgesia does not include any tissue damage but displays hypersensitivity to mechanical but not thermal stimuli. (Millan, 1999)

Physiologically speaking, pain arises from the activation of mechanical, thermal or polymodal nociceptors. Unlike receptors that mediate non-noxious touch and pressure, nociceptors do not have specialized receptors but instead are located on free nerve endings within the skin or viscera. Mechanical and thermal nociceptors have thinly myelinated Aδ fibers that mediate fast, sharp pain while polymodal nociceptors have unmyelinated C fibers which are responsible for the conduction of pain that is described as slow, dull or burning (Kandel, 2000). The cell bodies of the nociceptive neurons are located within the dorsal root ganglion and are pseudo-unipolar neurons. Their afferents terminate in neurons in the dorsal horn of the spinal cord, and depending upon which lamina afferents terminate in, ascend through the spinal cord via one of several ascending pathways.

The spinothalamic tract is the main ascending tract that delivers information about the sensation of pain and is divided into two main branches, the medial and lateral

spinothalamic tracts. The lateral spinothalamic tract originates in both lamina I and V of the spinal cord and ascends contralaterally into the brainstem and synapses in the lateral nuclei of the thalamus and then into the primary and secondary somatosensory corticies (Figure 1). This part of the spinothalamic tract encodes for the sensory-discriminative aspect of pain which provides information such as localization and quality of a painful stimulus. The medial aspect of the spinothalamic tract originates primarily in lamina I and deep lamina of the spinal cord. It ascends contralaterally into the medial nuclei of the thalamus and then into the anterior and insular cortices which then project to the prefrontal cortex (Figure 2). The medial aspect of the spinal thalamic tract is responsible for encoding the affective-motivational aspect of pain. The affective-motivational component of pain is what is considered to be the emotional experience of "suffering" from pain as well as the desire to avoid and eliminate the painful experience. The differences in the two systems are highlighted within patients suffering from the condition pain asymbolia. Pain asymbolia patients show lesions to the medial pain pathway structure of the insular cortex and thus have deficits in the affective component of pain processing. These patients are able to accurately detect and locate painful stimuli and will even describe it as painful. However, they do not demonstrate withdrawal movements or express negative emotional responses to the stimuli. In fact, some of these patients were reported to smile and laugh at the painful stimulation (Berthier et al., 1988).

On the other hand, the descending pain pathways are responsible for modulation of pain processing. The most studied descending pain pathway originates in the periaqueductal grey (PAG) and descends through the rostroventral medial medulla (RVMM) to regulate the release of proinflammatory mediators in the dorsal horn of the spinal cord via inhibitory mechanisms. Interestingly, this system is also responsible for what is termed "descending facilitation" which is the potentiation of nociceptive neuronal responses in the spinal cord. It is hypothesized that the seemingly dichotomous function of the descending pain pathway is due to the release of many different types of

Figure 1: The Lateral Spinothalamic Tract

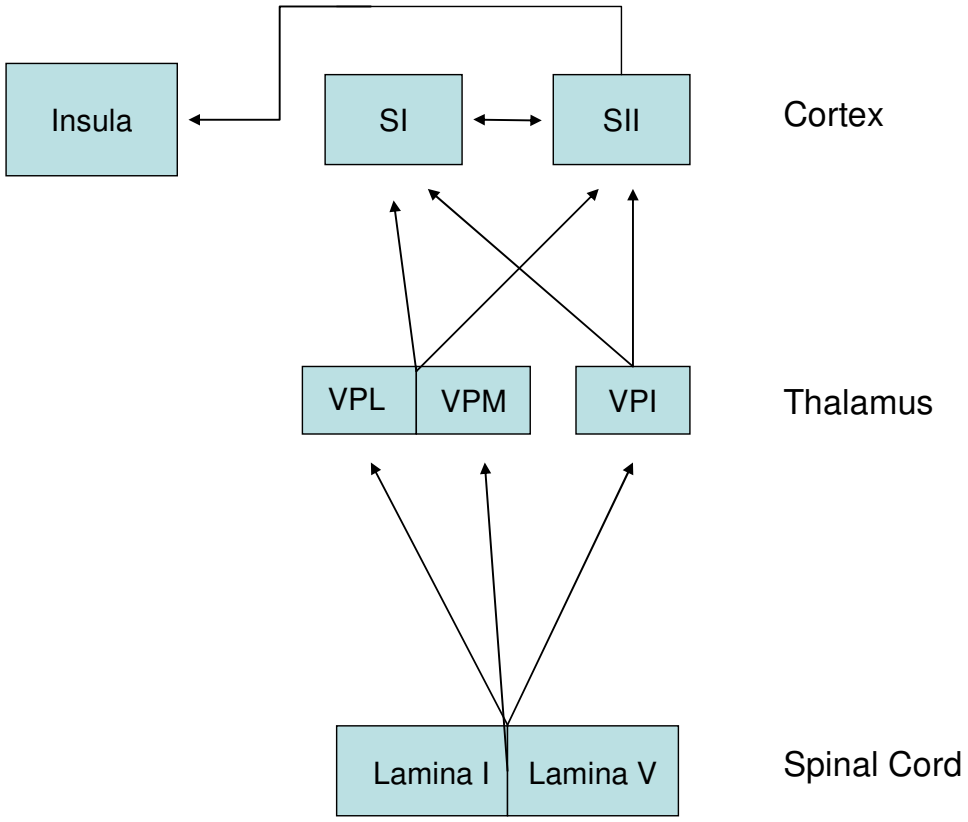

Figure 1: The lateral spinothalamic pain pathway, which transmits and processes information about the sensory-discriminative aspects of pain. SI, primary somatosensory cortex ; SII, secondary somatosensory cortex; VPI, ventral posterior inferior nucleus ; VPL ventral posterior lateral nucleus ; VPM ventral posterior medial nucleus. Adapted from Treede et al.,(1999).

Figure 2: The Medial Spinothalamic Tract

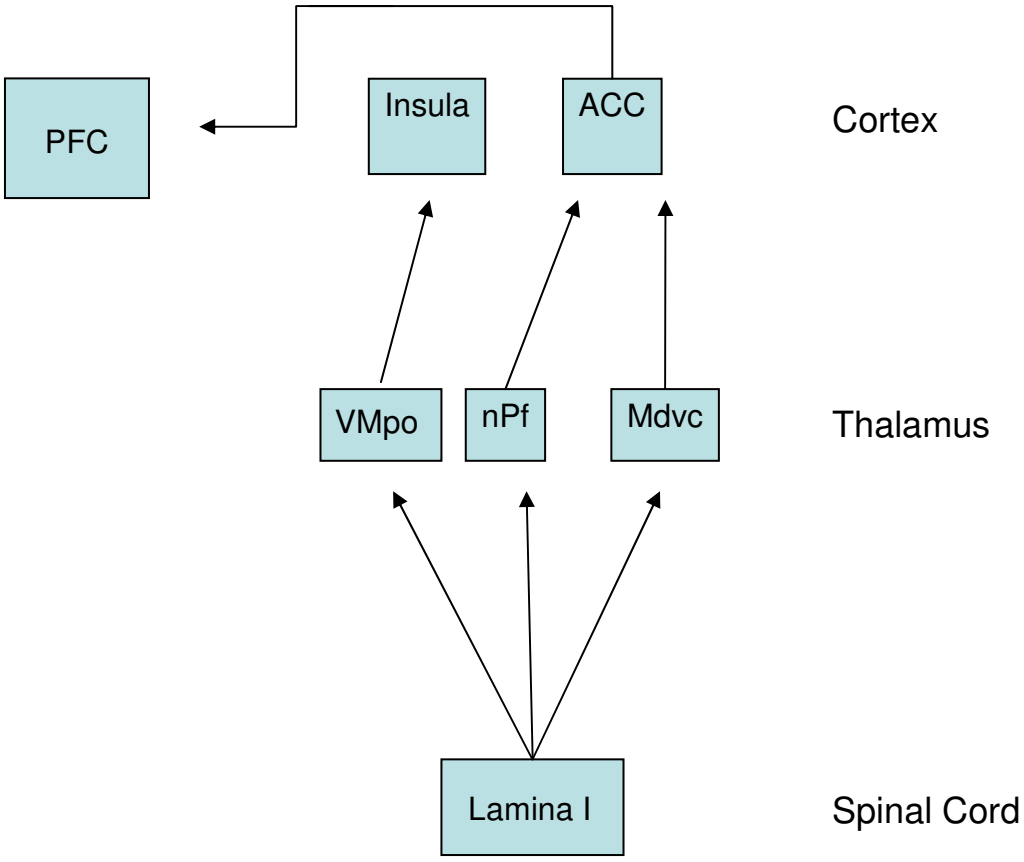

Figure 2: The medial spinothalamic tract which is involved in the affective-motivational component of pain processing. ACC, anterior cingulate cortex; MDvc, ventrocaudal part of the medial dorsal nucleus; nPf, parafascicular nucleus; PFC, prefrontal cortex; VMpo, posterior part of ventromedial nucleus. Adapted from Treede et. al, (1999).

neurotransmitters, which like in the case of serotonin, can be either inhibitory or excitatory depending upon which particular receptor subtype is activated (Millan, 2002).

Chronic Pain Models

To study the effects of chronic pain numerous animal models have been developed to mimic the various disease and traumatic states which can lead to chronic pain conditions in humans. These models induce chronic pain by causing either local or systemic inflammatory reactions in the animal or by causing trauma to various parts of the nervous system. There are a several chronic inflammatory models but the most well established and most often used model is the complete Freund's adjuvant (CFA) model of arthritis. This model involves the injection of heat deactivated *Mycobacterium butyricum* subcutaneously into the base of the tail or hind-paw. While this model produces robust and long-lasting thermal and mechanical hypersensitivity, it also produces a variety of abnormal behavioral and physical effects on animals. This includes nodular lesions on the ears, feet, tail and genitals, urethritis, diarrhea, as well as eye inflammation. Additionally, it was reported CFA animals stopped gaining weight at 1 week post CFA and were barely 50% of their control counterparts weight by the 4^{th} week post-CFA. The mobility of CFA animals was also affected, with CFA animals rated at mobility scores that ranged from a score of 0 "animal just lies down" or 1 "animal crawls" for several weeks post CFA (Calvino et al., 1987). Given the apparently severe systemic side-effects of this chronic inflammatory pain model, and that most other inflammatory models also utilize some type of adjuvant with similar side effects, we decided to use a neuropathic pain model.

There are several well-established and often used models of chronic neuropathic pain including chronic constriction injury (CCI) of the sciatic nerve, spinal nerve ligation (SNL) of the L5 or L6 spinal nerve and partial sciatic nerve ligation (PSNL). CCI involves the loose ligation of the sciatic nerve. This model produces robust mechanical and thermal hypersensitivity, which can be measured behaviorally, and induces spontaneous pain behaviors (Bennett et al., 1988). SNL is another commonly used model that involves tight ligation of the L5 and sometimes L6 afferent spinal nerves that, along with the efferent L4, merges peripherally to form the sciatic nerve. Behaviorally, this

model produces hypersensitivity to noxious heat and mechanical hypersensitivity in the affected foot as well as signs of spontaneous pain (Kim et al., 1992). The PSNL model involves tightly tying one-third to one half of the sciatic nerve which induces sympathetically maintained signs of spontaneous pain, touch-evoked hypersensitivity, and mechanical and thermal hypersensitivity in the surgerized and contralateral foot (Seltzer et al., 1990).

The chronic constriction injury (CCI) model of neuropathic pain was chosen here because this model produces robust thermal and mechanical hypersensitivity that has been shown to last over two months. Additionally, animals exhibit spontaneous pain behaviors such as guarding the paw, abnormal posture regarding the surgerized limb as well as shaking and licking of the paw (Bennett et al., 1988), making this model a close correlate to many of the classic symptoms seen in human chronic neuropathic pain patients. Of the neuropathic pain models, CCI is the least invasive as it involves only a small incision on the hindleg as opposed to SNL which require exposure of the spinal column. Additionally, this technique was previously established in this lab and reproducible behavioral data had already been generated from it.

Species

We chose to utilize male, Swiss-Webster mice for the studies presented in this dissertation. The decision to use mice as opposed to rats was due to several reasons. This laboratory has historically used mice and had already developed a protocol for CCI surgeries in mice. Additionally, we had planned to look into the genetic component of CCI and MAP kinases and technology such as shRNA and siRNA are developed for use in mice as opposed to rats. Lastly, since most research in some of the systems we examined in response to CCI had previously been conducted in rats, we were interested in examining potential species differences.

Conclusion

In conclusion, chronic pain is a debilitating condition which accounts for billions of dollars in healthcare costs and lost productivity. Due to limited understanding of the physiological mechanisms involved in the establishment and maintenance of chronic pain this condition is often difficult to treat. This is especially true for neuropathic pain conditions which are often resistant to traditional pharmacological treatments. The focus of this dissertation is to examine the effects of chronic neuropathic pain on three separate molecular systems known to be involved in regulation and maintenance of chronic pain states, within the lateral, medial, and descending pain pathways in the mouse.

Chapter II

General Methodology

General Methodology

CCI Surgery

The CCI model, adapted from Bennet and Xie (1988), to accommodate the mouse was used in these studies. Mice were anesthetized under 2.5% isofluorane before having the lower back and right thigh shaved. The shaved area was then cleansed with 2% povodine iodine and rinsed with 70% ethyl alcohol. A linear skin incision was made along the lateral surface of the biceps femoris and tweezers were inserted into the muscle belly to split the muscle fibers and expose the sciatic nerve. The tips of the tweezers were passed gently under the sciatic nerve and lifted to pass two 5-0 chromic gut sutures under the nerve, 1mm apart. The suture was then tied loosely around the nerve and knotted twice to prevent slippage. The incision was cleansed and the skin was closed with 2–3 ligatures of 5-0 dermalon. The mice were then allowed to recover on a warmed surface covered by paper towels. Following recovery, they were returned to their home cage and checked routinely for 72 hours. A separate control group of sham-operated mice underwent the exact same surgical procedure with the exception of the ligation of the sciatic nerve.

Hyperalgesia Testing

Thermal hypersensitivity was assessed using a radiant heat source under a plexi-glass surface applied to the plantar surface of each hindpaw (Hargreaves et al., 1988). For two days prior to CCI surgery, mice were placed on the plexi-glass floor of the apparatus (Plantar Test, Ugo Basile, Comerio, Italy) and covered with an inverted clear plastic tube in order to familiarize the mice with the test. On the day prior to surgery, baseline measures of paw withdrawal latency were gathered. Mice and rats were allowed to acclimate on the Plantar Test apparatus for approximately 20 minutes before the start of the radiant heat test. Five measures of paw-withdrawal latency were conducted with at least five minutes between each test with the average latency used for statistical purposes. Mice were again tested for paw-withdrawal latency at either 1, 3, or 10 days post CCI or sham surgery. Rats were tested at 10 days post-surgery. Paw-withdrawal latencies were expressed as relative values (%) to baseline latencies for each animal, as well as group means ± S.E.M. Behavioral data was analyzed via Student's t-test with differences

considered statistically significant at $p < 0.05$. Any CCI mouse or rat that failed to demonstrate a statistically significant post-CCI withdrawal latency from baseline was excluded from further analysis.

Chapter III.
The Effect of Chronic Constriction Injury on the Extracellular Regulated Kinases

Introduction

The mitogen-activated protein kinase (MAPK) family has been implicated in recent years in pain processing and the establishment of chronic pain and this system may be a novel target for pharmacological treatments. The mammalian MAPK family is a highly conserved pathway whose functions have been shown to include regulation of gene expression, inflammation, cell growth and differentiation, apoptosis, and development. The MAPKs are the endpoint of a three-kinase cascade and are activated via dual phosphorylation by a MAPK kinase (MEK). MEKs in turn must be activated via dual-phosphorylation by a MAPK kinase kinase (MEKK) which is activated by a variety of extracellular signals (Pearson et al., 2001). While there is some cross-reactivity within the MAPK cascade subfamilies, most members within an individual cascade are specific to that cascade and do not react with other MAPK subfamilies. There are many MAPKs but the most well characterized are the extracellular regulated kinases 1 and 2 (ERK), the p38 enzymes, and the c-Jun NH_2-terminal kinase (JNK) subfamilies (Figure 5).

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Up to seven types of ERKs have been identified but the most well characterized are ERK 1 and 2. ERK 1 and 2 are 42 and 44 kD proteins with an 83% sequence homology and due to this are generally studied and referred to together as ERK. ERK proteins are phosphorylated by MEK 1 and 2 (MEK 1/2) which are activated primarily by the Raf family of proteins (Roux et al., 2004). A variety of extracellular stimuli have been shown to activate the ERK cascade including growth factors, viral infection, cytokines, ligands for G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (Trks) (Johnson et al., 2002). ERKs have been shown to have many substrates in the membrane, nucleus as well as the cytoplasm and include phospholipase A2 (PLA2) and the transcription factors Elk-1, cAMP-response-element-binding-protein (CREB) and c-Fos (Yoon et al., 2006). These transcription factors then go on to bind to CRE or SRE in gene promoter regions which activate transcription of both immediate early and late genes.

Figure 3: Pathway of the MAP Kinase Family of Proteins

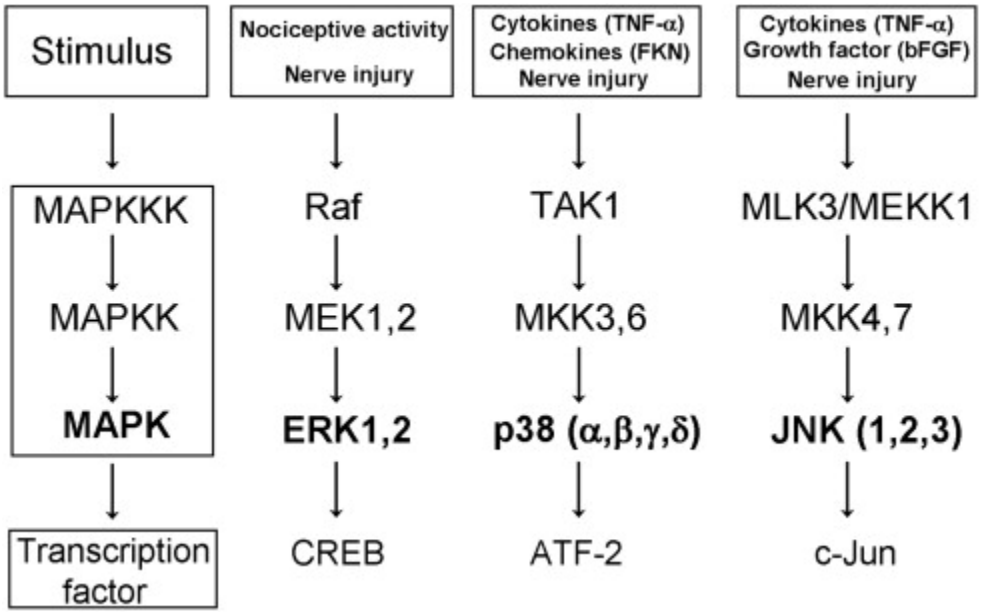

Figure 5: The MAP kinase family of proteins by specific pathway. Taken from Ji et al. (2009).

The Effect of Chronic Pain on ERK

The chronic constriction injury model (CCI) is a neuropathic pain model that involves the loose ligation of the sciatic nerve (Bennet and Xie, 1988). This model produces robust mechanical and thermal hypersensitivity, which can be measured behaviorally, as well as spontaneous pain behaviors. Using this model in the rat an increase in phosphorylated ERK (p-ERK) in ipsilateral L4/L5 dorsal root ganglion (DRG) was observed. The time course showed significant elevations in p-ERK at the third day post-CCI and this elevation continued to day 14 post-CCI. The relevance of the effects on ERK 1/2 in relation to the pain hypersensitivity in CCI animals was assessed using a MEK 1/2 inhibitor (MI) . Significant decreases in mechanical hypersensitivity induced by CCI were observed in MI treated CCI rats (Obata et al., 2004).

Using the spinal nerve ligation (SNL) model, it was found that p-ERK was significantly increased in L5 dorsal horn ipsilateral to SNL in the rat. This significant increase was observed from day one until day 21 post-SNL however, the cellular localization of the elevated levels of p-ERK changed throughout the time course. Ten minutes post SNL showed significant p-ERK co-localization in L5 dorsal horn neurons while the second day post-SNL showed p-ERK primarily expressed in microglia which was maintained at day 10 along with significant co-localization in astrocytes. At day 21, significant p-ERK activation was seen in only in astrocytes. Significant attenuation of mechanical hyperalgesia was observed with administration of a MEK 1/2 inhibitor at 2, 10 and 21 days post-SNL (Zhuang et al., 2005).

Significant up regulation of p-ERK was also seen in the injured L5 DRG in the SNL model, albeit in different populations of DRG neurons (Obata et al., 2004). Changes in ERK were also observed in another neuropathic pain model, partial sciatic nerve ligation (PSNL). This model involves tightly tying one-third to one half of the sciatic nerve which leads to sympathetically maintained signs of spontaneous pain, touch-evoked hypersensitivity, and mechanical and thermal hypersensitivity in the surgerized and contralateral foot (Seltzer et al., 1990). Significant up-regulation of p-ERK in the ipsilateral L4/L5 dorsal horn and nucleus gracilis was observed in PSNL rats when

compared to contralateral anatomical sites. This up-regulation was seen almost exclusively in glial astrocytes in both the dorsal horn and nucleus gracilis (Ma et al., 2002).

In summary p-ERKs have been shown to upregulated in response to chronic neuropathic pain in the rat. This effect was observed in multiple neuropathic models in the dorsal horn and DRG. Additionally, the majority of these studies showed the upregulation of these proteins to be in glial cells as opposed to neurons and blockade of the ERK pathway caused at least partial attenuation of pain hypersensitivity.

MAPK Regulation of Chronic Pain Induced Cellular and Molecular Changes

The persistent and preferential up-regulation of MAPKs in chronic injury in microglia and astrocytes, and not in neurons, may seem puzzling because traditional theory has focused on neuronal sensitization as the major contributor to the establishment and maintenance of chronic pain. However, new research is showing that glial cells play an important role in pain states. Microglia and astrocytes have been shown to regulate the expression of inflammatory cytokines such as IL-1, IL-6 and TNFα as well as COX-2 and reactive oxygen species (ROS) (Banati et al., 1993; Bauer et al., 1997; Lieberman et al., 1989; Wieseler-Frank et al., 2005). In fact, a study that found significant inhibition of mechanical hypersensitivity in the spinal cord model of inflammation (SCI) by the microglial inhibitor minocycline, also found that the drug caused significant decreases in SCI induced elevated levels of TNFα, IL-1, and IL-10 (Ledeboer et al., 2005). Interestingly, cytokines like IL-1 and IL-6 as well as TNFα, and COX-2 which are produced by glia have also been shown to be positively regulated by ERK (Clark et al., 2003; Koj, 1996). Considering that the same cytokines that are involved in the production of pain are regulated by MAPKs and inhibition of MAPKs has been shown to attenuate pain hypersensitivity, MAPKs may play a critical role in the induction and maintenance of pain.

In addition to affecting the release of cytokines and chemokines, the MAPK pathway has also been shown to be involved in the regulation of receptors involved in chronic pain. Cannabinoids have been shown to be effective in relieving chronic pain at the both the

spinal and supraspinal level (Walker et al., 2005). However, the effect of chronic pain on the cannabinoid system has yet to be fully elucidated. The upregulation of cannabinoid receptor type 1 (CB1) has been shown to occur in the thalamus, a major integration center in pain processing, in response to chronic neuropathic pain (Siegling et al., 2001). Additionally, CCI caused a significant, time dependent, increase in CB1 protein in the dorsal horn of the spinal cord compared to sham operated animals. This CCI mediated increase in CB1 protein was blocked with twice daily administrations of the MEK 1/2 inhibitor, PD98059. This ERK 1/2 mediated blockade of CB1 did not affect the development of pain hypersensitivity, but rather, blocked the antinociceptive effect of the potent CB1 agonist WIN 55, 212-2 at a dose that was observed to attenuate both thermal and mechanical hypersensitivity in CCI rats (Lim et al., 2003). The up-regulation of CB1 in the brain and the spinal cord may play a role in the efficacy of cannabinoid agonists in attenuating pain behaviors, a theory that has been proposed due to research that found that cannabinoid agonists not only reversed CCI induced hypersensitivity, but caused hyposensitivity at doses that did not alter pain perception in non-CCI animals (Herzberg et al., 1997).

Long Term Potentiation and Pain

Long term potentiation (LTP) is a long lasting enhancement and efficacy of synaptic transmission following stimulation. It was first characterized over 30 years ago in the hippocampus (Bliss et al., 1973) and is thought to be a major contributor to synaptic plasticity in the brain. While the majority of LTP research has focused on its involvement in the hippocampus with regards to learning and memory, recent research has shown that LTP is also involved in other CNS structures involved in pain processing like the spinal cord, amygdala and cerebral cortex. In regards to pain research, the phenomena of central sensitization, which is increased responsiveness of dorsal horn nociceptive neurons which outlasts peripheral stimulation (Woolf, 1983) has been theorized to be a form of LTP. This phenomenon can lead to reductions in threshold of the nociceptive neurons, increases in receptive field size as the ability of low-threshold sensory fibers associating with and activating high-threshold nociceptive neurons of the dorsal horn (Ji et al., 2003) all of which are thought to be involved in the transition of

acute pain to chronic pain. In support of this theory, a recent study found that high frequency stimulation (HFS), which is an established method of inducing LTP *in vitro* and in animal models, of the cutaneous skin produced both lasting hyperalgesia and allodynia in humans (Klein et al., 2004).

At the cellular and molecular level, hippocampal LTP and central sensitization share a great deal of common pathways and mediators. Many of the proteins involved in the establishment of LTP are regulated by the transcription factors CREB and Elk-1, which also regulate many of the proteins involved in central sensitization such as prodynorphin, NK1 and COX-2. Additionally, CREB and Elk-1 are direct targets of MAPK activated ERK1/2, of which production is upregulated in both hippocampal LTP and central sensitization. Additionally, a major contributor to establishment of LTP and central sensitization is the glutamatergic system, which includes NMDA receptors.
ERK 1/2 may indirectly regulate the function of the NMDA receptor via potassium channels. The voltage-dependent Kv4.2 potassium channel has recently been implicated in mediating LTP through modulation of NMDA receptor voltage detection (Sweatt, 2004). Additionally, inhibition of A-type K+ current, which is mediated by the Kv4.2 K+ channels, has been shown to increase neuronal excitability, a hallmark of central sensitization. This has been found in the dorsal horn of the spinal cord and the hippocampus and this increased excitability has been shown to be mediated in part by ERK 1/2 (Hu et al., 2003; Yuan et al., 2002). The mechanism by which ERK 1/2 regulates Kv4.2 has been shown to be via direct phosphorylation Kv4.2 channels. Site directed mutations to mimic phosphorylation of these ERK 1/2 sites in Kv4.2 causes an increase in the voltage required to activate the channels as well as slower recovery from inactivation inducing the overall effect of decreased A-type K+ current (Schrader et al., 2006). Further evidence of Kv4.2 involvement in central sensitization is the localization of Kv4.2 in the dendrites of excitatory interneurons in lamina II of the dorsal horn, which is involved in ascending pain pathways (Huang et al., 2005). Behaviorally speaking, genetic knock-outs (KO) of Kv4.2 were shown to have this same increased neuronal excitability in the dorsal horn neurons and these KO mice were also observed to have significantly increased mechanical and thermal hypersensitivity which was mediated in

part by ERK 1/2(Hu et al., 2006). This evidence points to the involvement of the Kv4.2 receptor, which is meditated by ERK 1/2 in central sensitization of dorsal horn neurons which has behavioral implications in regards to pain hypersensitivity. In conclusion, MAPKs play a role in the establishment of the LTP like phenomenon of central sensitization.

Summary

ERKs have been shown to be upregulated in response to chronic pain. The majority of these studies showed the upregulation of these proteins to be in glial cells as opposed to neurons and blockade of one of these pathways caused at least partial attenuation of pain hypersensitivity induced by nerve injury. Additionally, the MAPK system has multiple effects on several different receptors such as CB1 and Kv4.2. Therefore, the MAPK pathway may be a novel target for pharmaceutical or gene therapy in the treatment of chronic pain. Further research is needed, however, to completely elucidate the role of this protein kinase family in the initiation and maintenance of chronic pain states. As the majority of studies have focused on the role of ERK in neuropathic pain states at the level of the DRG and spinal cord, the focus of this study is to examine the effects of neuropathic pain on ERK cascades expression and activation in brain areas involved in the processing and modulation of pain, particularly those sites identified within the medial and lateral pain pathways.

Materials and Methods

Animals

For all studies male, Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) were utilized. Mice weighing 25-30g were housed 6 to a cage in animal care quarters on a 12h light-dark cycle. For the p-ERK ELISA study, male Sprague-Dawley rats weighing 200-250g were housed 2 to a cage were also used. Food and water were available *ad libitum.* Protocols and procedures were approved by the Institutional Animal Care and Use

Committee (IACUC) at Virginia Commonwealth University Medical Center and comply with recommendations of the International Association for the Study of Pain (IASP).

CCI of the Sciatic Nerve

The CCI model, which we adapted from Bennet and Xie (1988), to accommodate the mouse was used in these studies (described in detail on page 11) . A separate control group of sham-operated mice and rats underwent an identical surgical procedure with the exception of the ligation of the sciatic nerve.

Behavioral Testing

Thermal hypersensitivity was assessed using a radiant heat source under a plexi-glass surface applied to the plantar surface of each hindpaw (Hargreaves et al., 1988). Mice were again tested for paw-withdrawal latency at either 1, 3, or 10 days post CCI or sham surgery. Rats were tested at 10 days post-surgery. Paw-withdrawal latencies were expressed as relative values (%) to baseline latencies for each animal, as well as group means ± S.E.M. Behavioral data was analyzed via Student's t-test with differences considered statistically significant at $p < 0.05$. CCI mice or rats that failed to demonstrate statistically significant post-CCI withdrawal latency from baseline were excluded from further analysis.

RT-PCR Array

At day 10 post-surgery, CCI and sham mice were tested for pain hypersensitivity and then sacrificed and their PAGs were dissected out. RT^2-Profiler MAP Kinase Signaling Pathway PCR Arrays (SuperArray) which contain 86 MAP kinase pathway genes as well as housekeeping and control genes were used to assess for changes in the PAG following CCI surgery. Total RNA was isolated from pooled mouse PAGs (3-4) using a two-step protocol with TRIzol® Reagent (Invitrogen) followed by additional clean-up with the Qiagen RNeasy® Mini Kit. RNA concentration and purity was verified using UV Spectophotometry. cDNA was then reverse transcribed from 1.5µg RNA using RT^2 PCR Array First Strand Kit (SuperArray). cDNA was then added to SYBR Green Master Mix

(SuperArray) to generate the experimental cocktail and 25µl of this cocktail was added to each of the 96 wells in the array plate. RT-PCR was then performed.

ERK 1/2 Total Protein Analysis

At day 10 post-surgery, mice were sacrificed and the spinal cord, medulla and periaqueductal grey (PAG) and thalamus were dissected out and frozen at -80°C until analysis. Brain regions were sonicated in homogenization buffer (25mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, 10mM ß-mercaptoethanol, 20mM ß-glycerol, 50 mM sodium pyrophosphate) and prepared for Western Immunoblotting analysis via SDS-PAGE electrophoresis (Bio-Rad Laboratories, Inc., CA). Nitrocellulose blots were blocked overnight at 4ºC in Tris buffered saline (TBS) with 0.05% Tween-20 plus 1% BSA solution. Blots were then probed with ERK 1/2 polyclonal antibody (Promega, Madison, WI) at 1: 5,000 for 2 hours at room temperature followed by horseradish peroxidase (HRP) secondary staining (Promega) at 1: 5,000 dilution for 1 hour at room temperature. Protein levels were detected via chemiluminescence (ECL Plus™, Amersham) and visualized on the Storm 860 Molecular Imager (GMI).

Phosphorylated-ERK 1/2 ELISA

To determine levels of phosphorylated-ERK (p-ERK) the PathScan® Phospho-p44/p42 MAPK Sandwich ELISA Kit (Cell Signaling, Inc, MA) was used. At day 10 post-surgery mice were euthanized and the spinal cord, medulla, PAG, medial thalamus and anterior cingulate cortex (ACC) were dissected and flash frozen in liquid nitrogen before storage at -80°C until further use. On the day of the assay brain regions were homogenized in Cell Lysis Buffer (Cell Signaling, Inc) that contained a 1% solution of Phosphatase Inhibitor Cocktail III (Calbiochem, NJ). Protein levels were determined via the Bradford (1976) assay and 2-7µg/µl of protein were added to each well of the ELISA kit and incubated overnight at 4ºC. Wells were washed with Wash Buffer and p-ERK antibody was added to the wells and incubated at 37ºC for 1hr followed by HRP-linked secondary antibody for 30 min. TMB substrate was then added and wells and absorbance was measured with a plate reader at 450nm.

P-ERK Confocal Microscopy

Mice were deeply anesthetized with sodium pentobarbital and perfused with 4% paraformaldehyde. Brains were removed and allowed to further fix in 4% paraformaldehyde for 4 hrs and then cryoprotected in a 20% sucrose solution for at least 24 hrs before being stored at -80ºC until further use. On day of assay brains were sliced on a cryostat at a thickness of 20µM. Brain slices were incubated as free-floating sections in 5% normal goat serum in PBS + 0.03% Triton X for 1 hr at room temperature. Slices were then incubated overnight at 4ºC with p-ERK antibody (1:400) (Cell Signaling, MA) followed by secondary staining with Alexa Fluor 488 (1:400) (Invitrogen, CA) at room temperature for 2 hrs. Brain slices were then mounted using Vectashield Soft Set (Vector Laboratories, CA). Slices were imaged via confocal microscopy using Leica TCS-SP2 AOBS. Images were analyzed for fluorescence using Image J software and data is expressed as percent of fluorescence per region of interest (ROI).

Results

Thermal Hyperalgesia Induced by CCI

CCI mice exhibited a 55% decrease in paw withdrawal latency from radiant heat stimulus in the ipsilateral hindpaw at 10 days post surgery when compared to baseline measures ($t=23.50$, $p < 0.0001$) and a 54% decrease when compared to sham operated controls ($t=19.91$, $p < 0.0001$). CCI rats also showed a significant decrease in paw withdrawal latency in the ipsilateral hindpaw compared to the contralateral hindpaw ($t=20.23$, $p < 0.0001$) (Figure 4).

CCI rats exhibited a 61% decrease in paw withdrawal latency from radiant heat stimulus in the ipsilateral hindpaw at 10 days post surgery when compared to baseline measures ($t = 4.301$, $p < 0.01$) and a 50% decrease when compared to sham operated controls ($t = 4.710$, $p < 0.01$). CCI rats also showed a significant decrease in paw withdrawal latency in the ipsilateral hindpaw compared to the contralateral hindpaw ($t=3.359$, $p < 0.01$) (Figure 5).

Figure 4: Comparison of Paw Withdrawal Latency to Radiant Heat in the Mouse at 10 Days Post Surgery

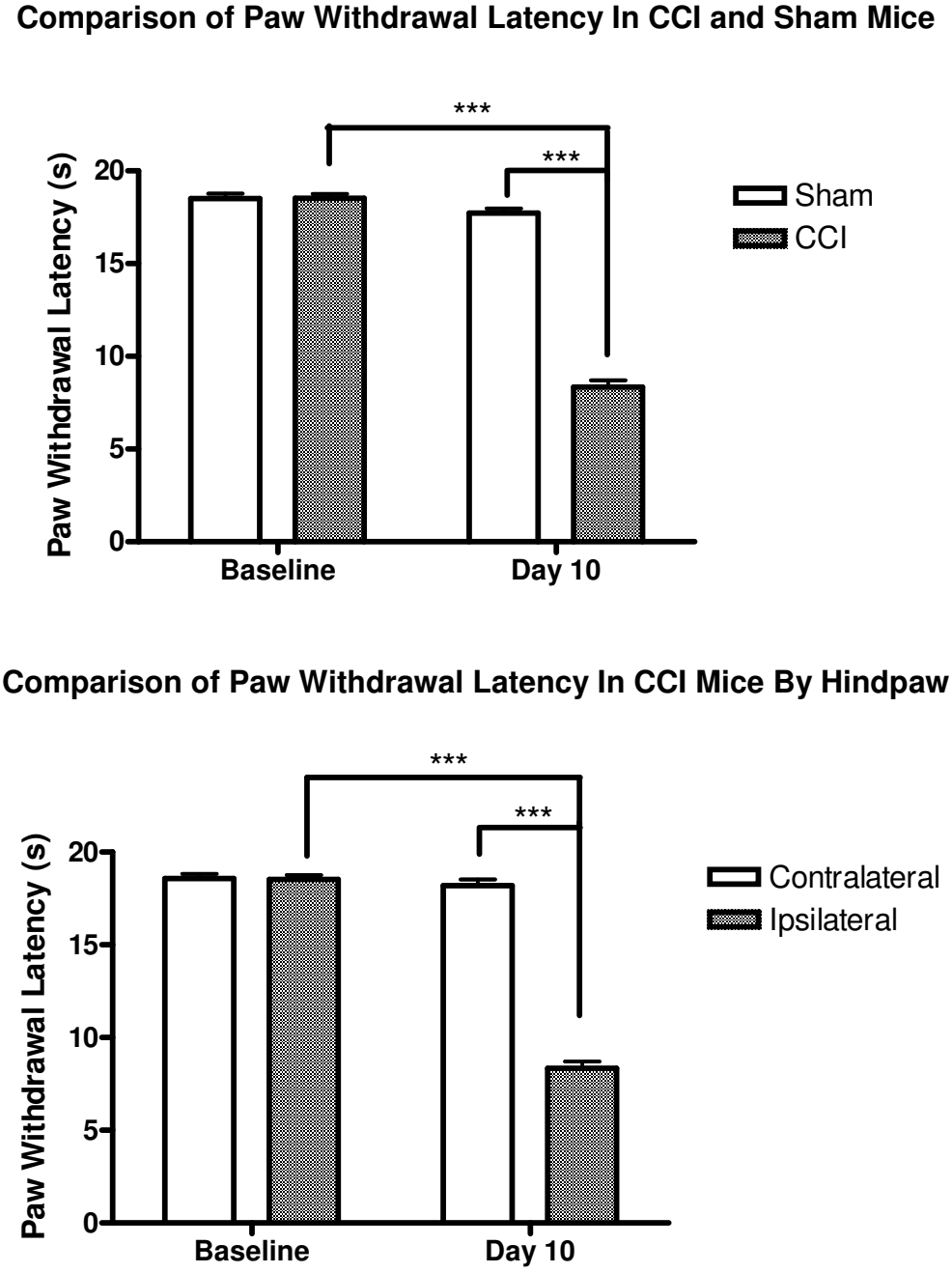

Figure 4: Effect of CCI on surgery on paw withdrawal latency (s) from a radiant heat source in Swiss-Webster mice. CCI induced a significant decrease in paw withdrawal latency when compared to baseline, sham, and contralateral hindpaw measures (***$p < 0.0001$).

Figure 5: Comparison of Paw Withdrawal Latency to Radiant Heat in the Rat at 10 Days Post Surgery

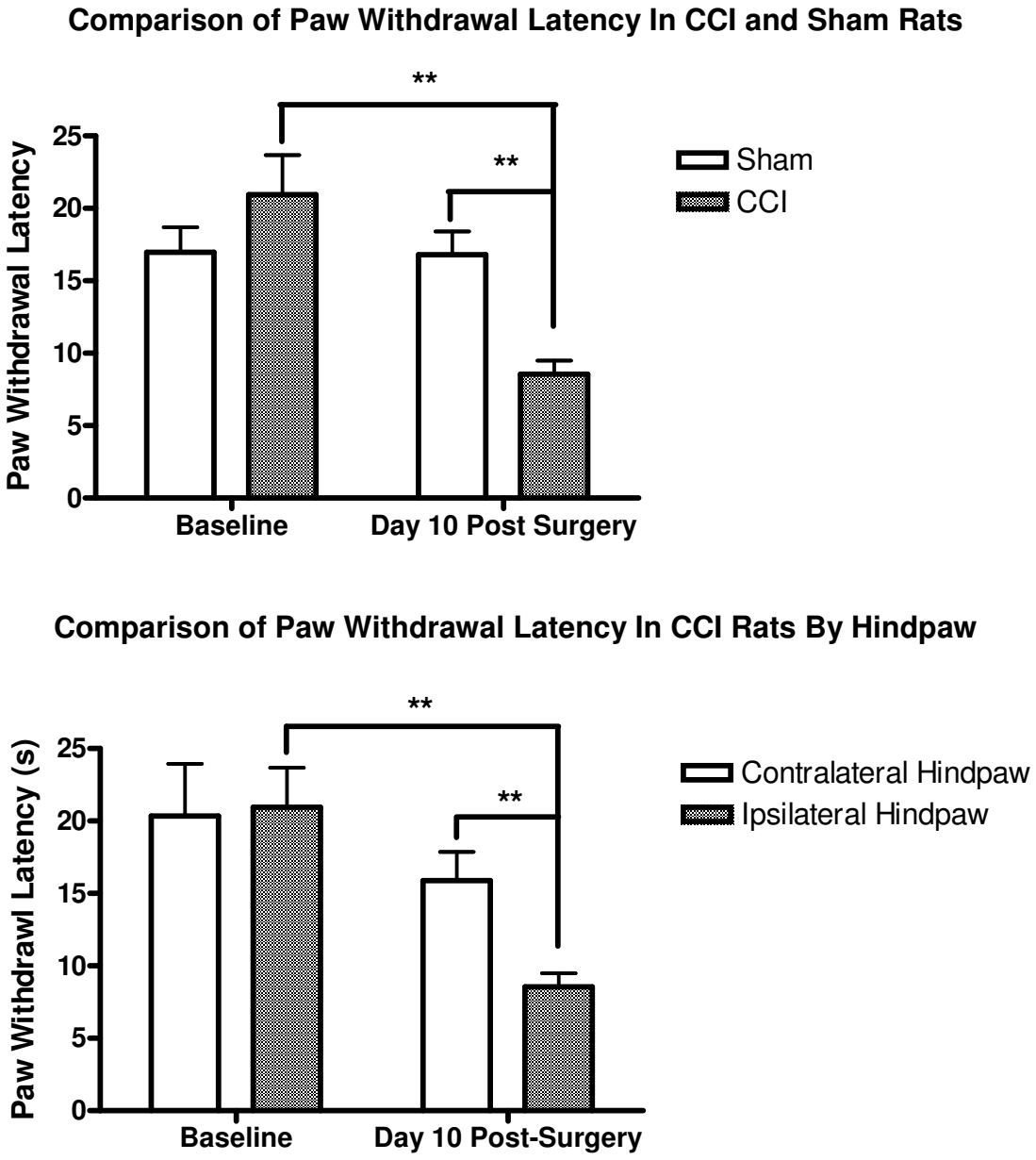

Figure 5: Effect of CCI on surgery on paw withdrawal latency (s) from a radiant heat source in Sprague-Dawley rats. CCI induced a significant decrease in paw withdrawal latency when compared to baseline, sham, and contralateral hindpaw measures (n = 4) (**$p < 0.01$).

RT-PCR Array

Since neuropathic pain induced p-ERK upregulation occuring in the DH and DRG has been shown to play a role in central sensitization we decided to look at the effect of CCI on the mRNA expression of MAPK genes in the PAG. The PAG is part of the descending pain pathway and has been shown to have a role in central sensitization following neuropathy (Suzuki et al., 2005). Of the genes in the PCR Array three genes were significantly upregulated in the PAGs of CCI mice when compared to sham-operated controls (Table 1). The three genes that were upregulated were Raf-1, Sfn, and Rb1. Sfn and Rb1 encode for the stratifin and retinoblastoma 1 proteins, respectively and are both regulators of the cell cycle. Raf-1 encodes for the MAPK3 protein. MAPK3 proteins are kinases involved in the phosphorylation and activation of the ERK1/2 cascade. Since p-ERK has been shown to be implicated in the establishment and maintenance of chronic pain in the spinal cord (Obata et al., 2004; Zhuang et al., 2005), an investigation of ERK proteins in the PAG as well as other brain areas involved in the pain pathway was pursued.

Western Immunoblotting for total ERK

We began our analysis by immunostaining for total protein levels of ERK in the PAG as well as the spinal cord, medulla and thalamus. There were no significant differences between CCI and naïve or sham-controls in the Western blotting analysis of total ERK protein in the spinal cord ($p = 0.79$), medulla ($p = 0.34$), (Figure 6) PAG ($p = 0.92$), and thalamus ($p = 0.52$) (Figure 7) when compared by Student's t-test.

Phospho-ERK ELISA

Previous studies showed that neuropathic pain increased the activity of ERK via phosphorylation so we decided to examine the effect of CCI on p-ERK levels. There were no significant differences in levels of phosphorylated ERK 1/2 between CCI and sham mice at 10 days post surgery in the L4-6 spinal cord ($p = 0.06$), medulla ($p = 0.77$),

Table 1: Increase of mRNA Expression in the PAG of Mice Following CCI

Gene	Fold Increase	p value
Raf-1	2.53	0.04
Sfn	2.94	0.04
Rb1	2.78	0.004

Table 1: CCI (n = 6) caused a significant increase in mRNA levels of three genes in the PAG when compared to sham mice (n = 6). Most notably Raf-1,which is an upstream kinase in the ERK 1/2 cascade, showed a 2.5 fold increase.

Figure 6: Western Blotting Analysis of Total ERK Protein Levels

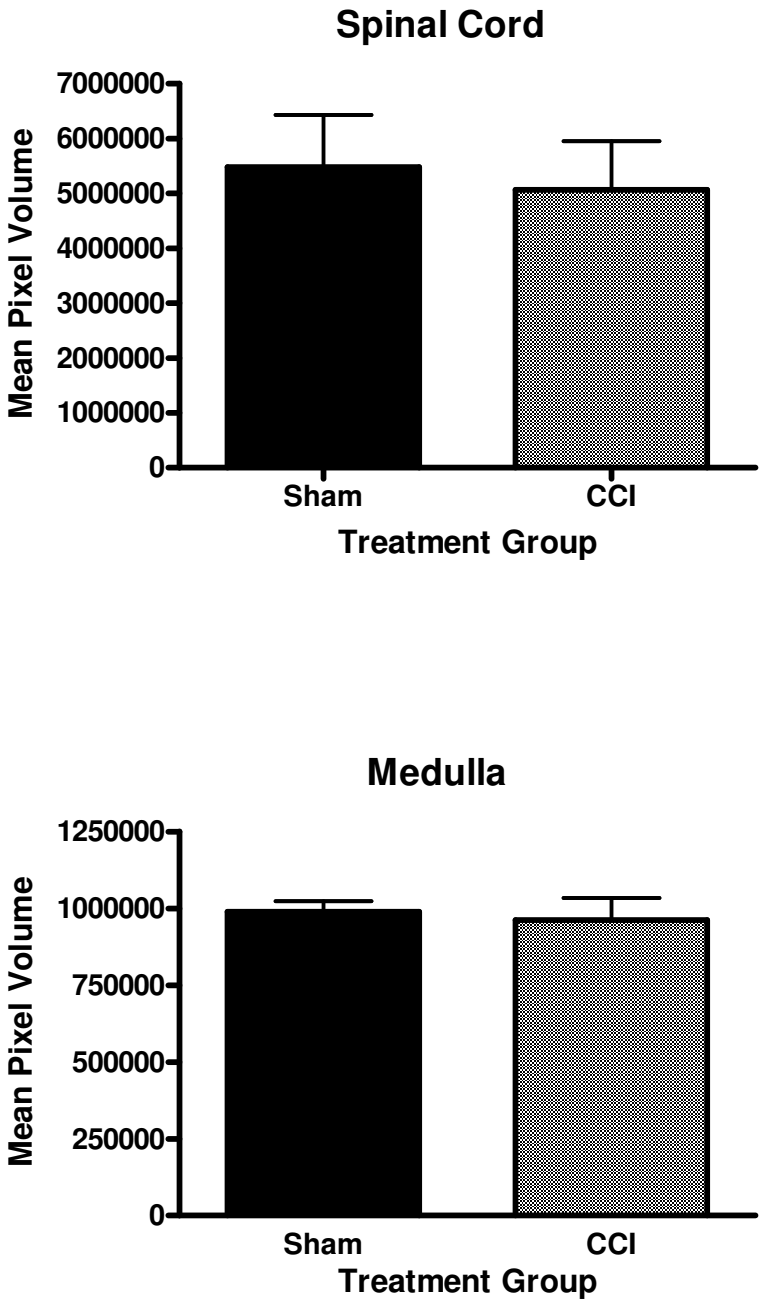

Figure 6: There were no significant differences in total ERK protein levels in the spinal cord (n = 4) or medulla (n = 5) of the mouse as detected by Western Immunoblotting.

Figure 7: Western Blotting Analysis of Total ERK Protein Levels

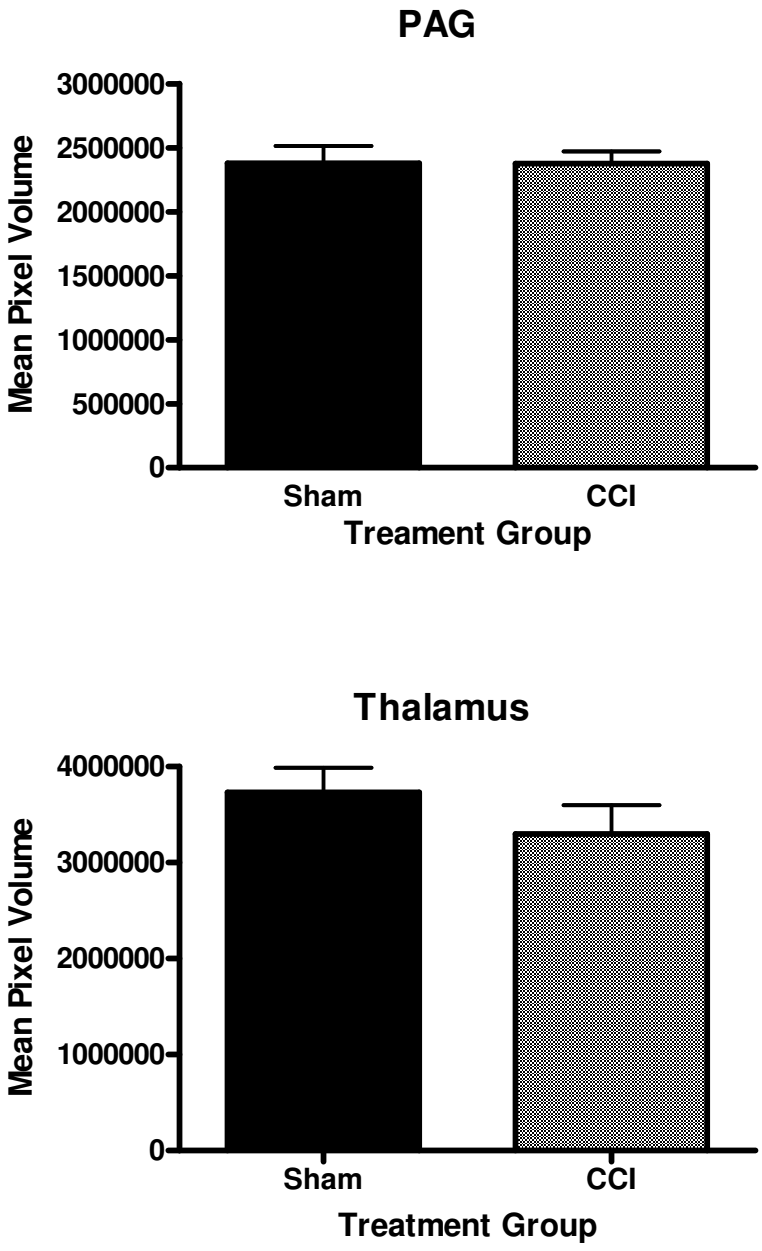

Figure 7: There were no significant differences in total ERK protein levels in the PAG (n = 5) or thalamus (n = 4) of the mouse as detected by Western Immunoblotting.

PAG ($p = 0.11$), medial thalamus ($p = 0.41$) or ACC ($p = 0.59$) (Figure 8). Since significant upregulation in levels of phosphorylated-ERK following neuropathic pain have been widely reported in the rat, further analysis of the spinal cord was conducted, this time comparing only tissue from the side of the L4-6 spinal cord ipsilateral to the surgerized leg and there were no significant differences detected ($p = 0.59$) (Figure 9). Since the majority of studies conducted on neuropathic pain and the MAP kinases were in the rat, we then conducted an analysis of p-ERK in L4-6 spinal cord sections ipsilateral to the surgerized leg of CCI and sham operated rats and again, there were no significant differences ($p = 0.46$) (Figure 9).

P-ERK Confocal Microscopy

We also utilized confocal microscopy to assess for changes in p-ERK levels in the decending pain pathway structures the PAG and medulla. As determined with the ELISA assay, there were no significant differences in p-ERK immunofluorescence between CCI and sham mice in the medulla ($p = 0.69$) or the PAG ($p = 0.94$). (Figure 10).

Discussion

CCI induced a 2.5 fold increase in mRNA of the ERK cascade kinase Raf-1 in the PAG of CCI mice (Table 1). This finding lead us to investigate the effects of CCI on the protein levels and activity of ERK in PAG. Additionally, we analyzed total ERK expression and activity in the spinal cord, medulla, and which are part of the lateral pain pathway, as well as the medial thalamus and ACC, which are part of the medial pain pathway. Our results did not show any significant differences in ERK levels or activity in any of those CNS regions in the mouse.

Figure 8: The Effect of CCI on p-ERK Expression in the Mouse Brain

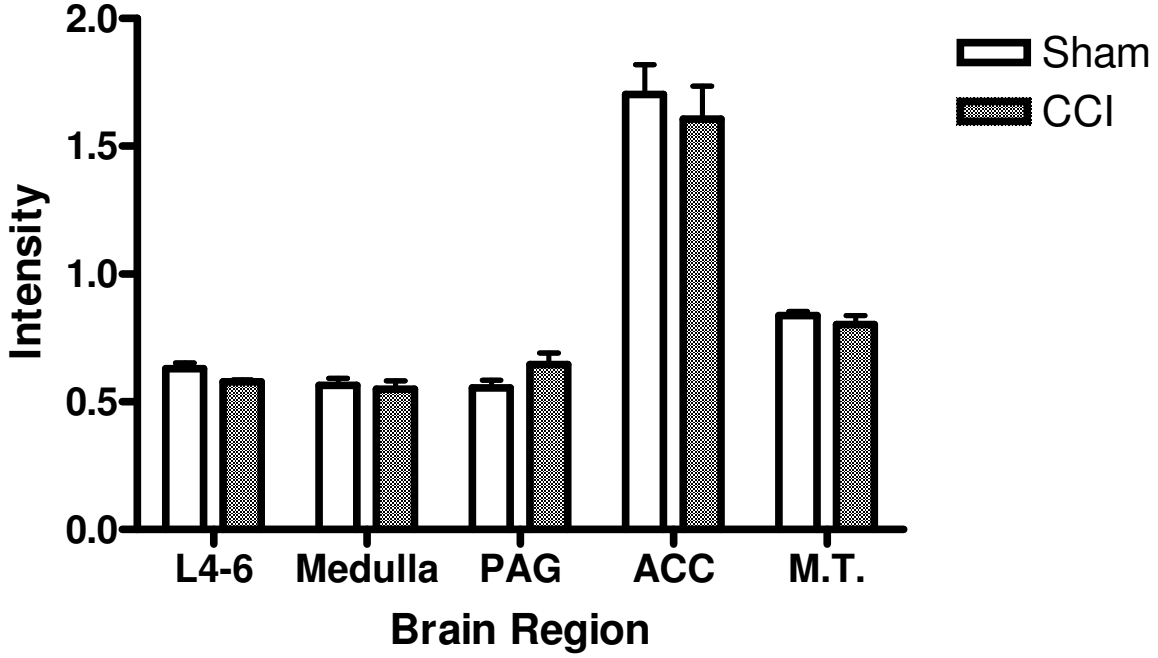

Figure 8: Measures of p-ERK expression were expressed as mean fluorescent intensity ± S.E.M. CCI did not induce any significant differences in p-ERK expression in the L4-L6 spinal cord (n = 6), medulla (n = 6), PAG (n = 6), ACC (n = 6) or medial thalamus (M.T.) (n = 5).

Figure 9: Effect of CCI on p-ERK Expression in the L5/L6 Spinal Cord of Rats and Mice

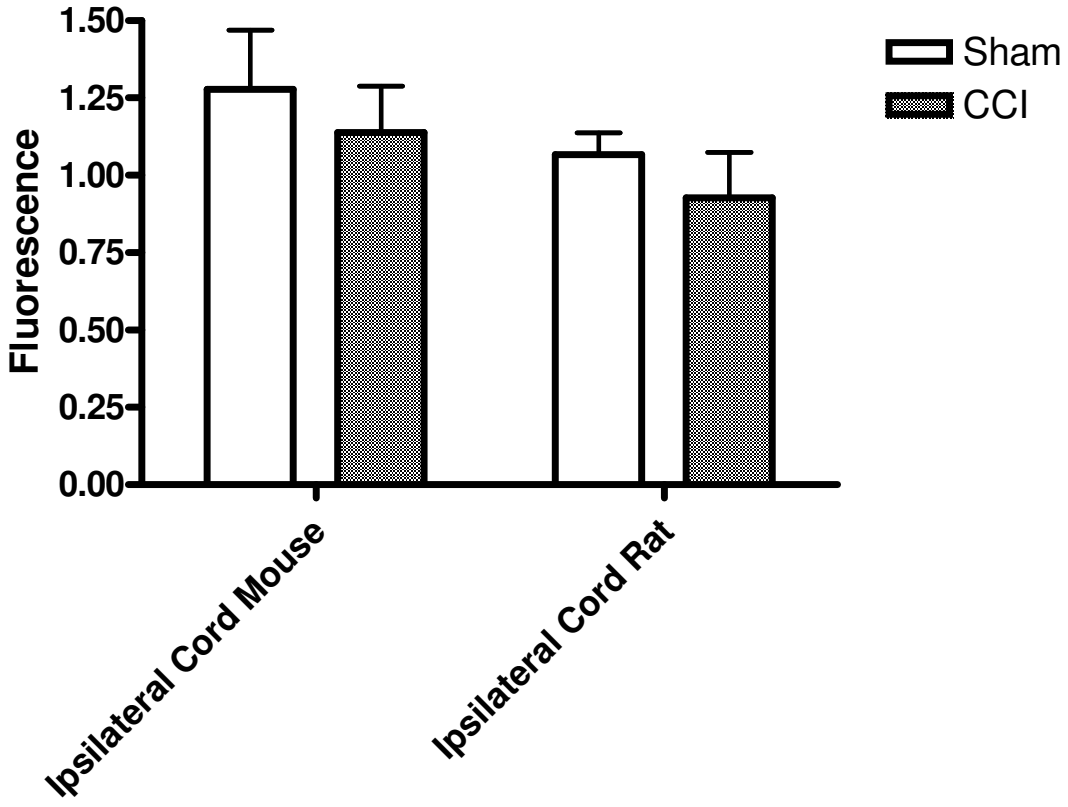

Figure 9: Measures of p-ERK expression were expressed as mean fluorescent intensity ± S.E.M. There were no significant differences in p-ERK expression in ipsilateral L4-6 spinal cord (n = 7) of mice or in the ipsilateral L4-6 spinal cord of rats (n = 4) when comparing CCI and sham at day 10 post-surgery.

Figure 10: The Effect of CCI on p-ERK Immunoreactivity in the Medulla and PAG

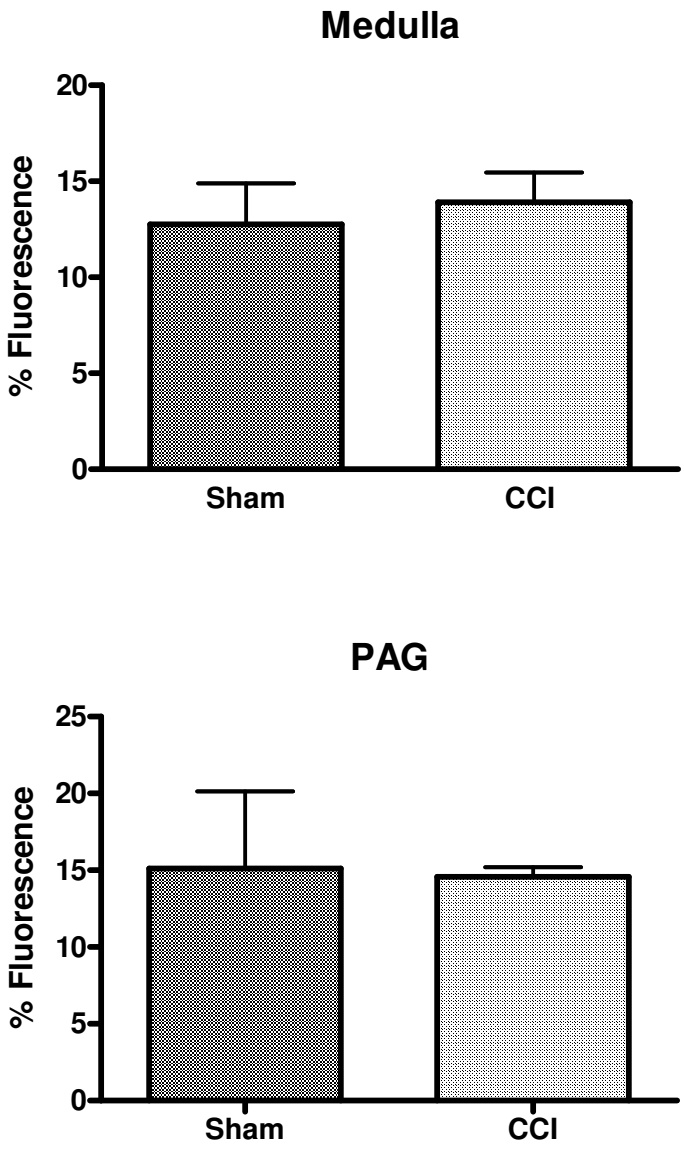

Figure 10: Confocal measures of p-ERK are expressed as percent fluorescence per region of interest. There are no significant differences in p-ERK staining in the PAG (n = 3) or medulla (n = 3) when comparing CCI and sham mice at day 10 post-surgery.

The data which were generated from these studies differs from that of previously published works in the rat that assessed for changes in MAP kinases in the spinal cord in response to neuropathic pain. Our CCI mice did not show significant upregulation of p-ERK expression in the L4/L5 section of their spinal cord. However, to date, there are no published works utilizing neuropathic pain models in the mouse assessing changes in ERK in the spinal cord.

Since the majority of published studies on MAPK and chronic pain utilized rats, we decided to investigate the effects of CCI on p-ERK expression in the ipsilateral L4-6 spinal cord of Sprague-Dawley rats. As determined in the mouse, there were no significant differences between CCI and sham rats. There are only two published studies utilizing the CCI model of neuropathic pain in rats which found that there was a statistically significant increase in lumbar spinal cord levels of p-ERK and those studies used immunohistochemistry (Song et al., 2005; Tseng et al., 2007) . One of those two studies also used Western blotting techniques using whole sections of the lumbosacral spinal cord and while the CCI group showed increases in p-ERK expression across many different time points, those differences were not reported as statistically significant (Song et al., 2005). Tseng et al. (2007) conducted an immunohistochemical study using the CCI model in Sprague Dawley rats which also found significant increases in p-ERK activation in the dorsal horn ipsilateral to the surgerized leg when compared to the contralateral dorsal horn. Thus, there are no published papers utilizing CCI that found significant differences in p-ERK using techniques which require whole tissue dissection as the ELISA assay we utilized to detect p-ERK expression does. The ELISA assay has not been previously utilized in any published works on neuropathic pain and MAPK expression. However, optimization of the ELISA assay in this lab showed it is sensitive enough to detect linear increases in levels of p-ERK (Figure 11) and the ELISA data generated in the medulla and PAG of our mice matches the data generated from the confocal slice staining for p-ERK in the PAG and medulla of mice. CCI has been reported to have a much greater variability in behavioral outcomes when compared to other neuropathic pain models (Kim et al., 1997) One reason why we failed to see

Figure 11: Optimization of p-ERK ELISA Assay

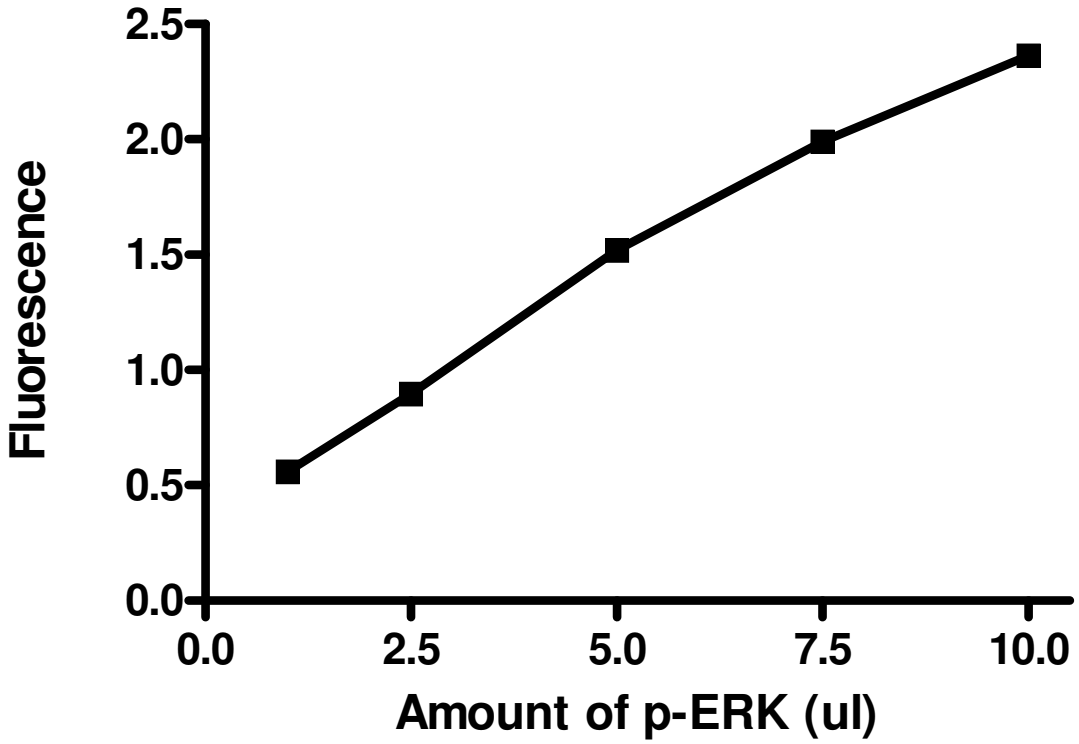

Figure 11: This ELISA assay shows a linear increase in fluorescence in response to increased levels of p-ERK protein.

differences in the levels of p-ERK in the spinal cord of rats is that there seemed to be much greater variability in the levels of p-ERK expression in the ipsilateral spinal cord data than that of other brain regions examined with ELISA.

There are published reports of differences in levels p-ERK in the spinal cord of rats analyzing gross tissue sections, but those studies did not use the CCI model of neuropathic pain. All neuropathic pain models have different behavioral outcomes and thus different physiological manifestations. A study conducted comparing SNL and PSNL models of neuropathic pain and CCI in the rat found similar time courses of evoked pain responses amongst all three models, but differences in magnitude of those responses. Additionally, CCI animals showed significantly greater responses to measures of spontaneous pain than SNL or PSNL rats (Kim et al., 1997). Similar sensitivity differences were shown again in a comparison of the SNL, PSNL, CCI and two other models of peripheral nerve injury in the rat (Dowdall et al., 2005). Behavioral differences were also described in three different neuropathic pain models in the mouse, with CCI mice showing greater sensitivity to cold allodynia (Walczak et al., 2006). Another study assessing for physiological changes in response to these three models showed that only in the SNL model did sympatheic fiber sprouting post injury differ significantly from controls (Lee et al., 1998). Thus, in these peripheral nerve injury models, which all involve ligation or transection of the spinal nerves leading to or the sciatic nerve itself, different behavioral and physiological outcomes are present in rats as well as mice which could explain the lack of significant differences in p-ERK expression in the spinal cord of our CCI rats.

In a neuropathic model of digit amputation in the rat immunohistochemical analysis of the ACC showed a significant increase in p-ERK labeled neurons in the bilateral layers I and II of the ACC. This increase in p-ERK labeling in the amputation group was also increased in amputation rats that had, less than 15 minutes prior to sacrifice, been delivered a noxious stimulus to their amputated hindpaw (Wei et al., 2008). Our study in CCI mice did not show any significant differences in p-ERK expression in the ACC. The Wei et. al., (2008) study differed from our own in species as well as pain model, again

highlighting the differences in physiological outcomes amongst species and models in pain research.

In conclusion, we have shown that CCI in the mouse produces an upregulation of mRNA levels of Raf-1 in the PAG, but does not produce changes in ERK protein levels or activity in the spinal cord, medulla, PAG, thalamus or ACC. Other studies have shown upregulation of p-ERK in the spinal cord following chronic neuropathic pain but those studies differed from our own in both species and neuropathic pain model. Thus, our data sheds doubt on previous data reported with CCI in the rat and warrants a more thorough investigation of the activity of ERK in this species. We conclude that CCI in the mouse does not have an effect on levels of ERK or its activity in the brain areas we examined that have been identified to be involved in the pain pathway.

Chapter III.

The Effect of CCI on the Cannabinoid System in the Mouse Brain

Introduction

The endogenous cannabinoid system is comprised of two distinct receptors, the cannabinoid receptors 1 (CB1) and 2 (CB2), as well several endocannabinoid lipids including anandamide and 2-arachydonoyl glycerol. CB1 is expressed primarily in the central nervous system including many areas involved in nociceptive transmission and processing including the PAG, anterior cingulate cortex (ACC), amygdala, medial thalamus MT) as well as the dorsal horn of the spinal cord and dorsal root ganglion of the rodent (Farquhar-Smith et al., 2000; Glass et al., 1997; Herkenham, 1991; Herkenham et al., 1991; Hohmann et al., 1999). CB1 receptors are G-protein coupled receptors (GPCRs) of the $G_{i/o}$ variety and are primarily localized on the presynaptic terminals of neurons but not the soma or dendrites (Katona et al., 2001; Katona et al., 2000). The overall effect of activated $G_{i/o}$ coupled receptors is inhibition of adenylyl cyclase and subsequently cyclic AMP, as well as activation of the MAP kinases and inhibition of both potassium and calcium ion channels. The overall effect appears to be due to cellular inhibition of the presynaptic terminal which leads to inhibition of neurotransmitter release.

In contrast to the CB1 receptor, CB2 receptors are localized primarily on immune cells such as B- and T-lymphocytes, macrophages and organs such as the spleen and thymus (Pertwee et al., 2002). Like the CB1 receptor the CB2 receptor is coupled to $G_{i/o}$ proteins and thus has an overall inhibitory effect. Since CB2 receptors are localized on inflammatory cytokine producing immune cells it is thought that they may modulate inflammatory responses involved in pain. In addition to being expressed in the immune system, CB2 receptors have recently been found to be expressed in several brain regions involved in the transmission of nociceptive information including the PAG, thalamus and amygdala (Gong et al., 2006; Onaivi, 2006; Onaivi et al., 2006).

There are five endogenous cannabinoid ligands produced in the central nervous system; anandamide (AEA), 2-arachydonoyl glycerol (2-AG) and 2-arachydonoyl glyceryl ether (noladin ether), virodhamine and N-arachydonoyldopamine (NADA). Anandamide

(AEA) and 2-AG are the most widely studied of the endocannabinoids. AEA and 2-AG have affinity for both the CB1 and CB2 receptors but AEA has a four-fold greater affinity for the CB1 receptor (Felder et al., 1995). 2-AG has a lower affinity for the CB1 receptor than AEA does, but is found in much higher concentrations in the CNS than AEA (Childers et al., 1998).

The CCI model has been shown to affect the endogenous cannabinoid system within the CNS. Following CCI, levels of the endocannabinoid lipids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG),were shown to have significantly increased in the spinal cord as well as the dorsal raphe, periaqueductal grey, and rostral ventral medulla (RVM) (Palazzo et al., 2006); Petrosino et. al. 2007). In respect to the effects of neuropathic pain on the cannabinoid receptors themselves, at the level of the spinal cord, CCI was shown to produce a significant upregulation of CB1 protein levels in the ipsilateral dorsal horn when compared to sham rats (Jones et al., 2004; Wang et al., 2007). Furthermore, CB1 receptors were upregulated in the spinal cord of mice with chronic constriction of the saphenous nerve (Walczak et al., 2006) and CB1 receptor mRNA has been shown to be upregulated in the contralateral thalamus in response to transection of the tibial nerve in the rat (Siegling et al., 2001). This indicates that neuropathic pain in and of itself can induce changes within the cannabinoid system in a variety of different models and at differing levels of the CNS. However, few reports have appeared in neuropathic pain on cannabinoid receptors in mice.

There are a variety of compounds that activate both the CB1 and CB2 receptors. CB1 agonists Delta-9-tetrahydrocannabinol (D9-THC), WIN 55,212-2 and CP 55,940 are commonly used in research, as are the CB2 selective agonists JWH-133 and GW405833. Various cannabinoid agonists have been shown to reduce or block behavioral as well as physiological responses to neuropathic pain models. CCI in the rat invokes increases in spontaneous firing and noxious evoked heat responses of nociceptive neurons in the dorsal horn of the spinal cord. These CCI induced changes are blocked via administration of the cannabinoid agonist WIN 55, 212-2 (Liu et al., 2006). Repeated low dose administration of WIN 55, 212-2 after CCI has also been shown to reduce hyperalgesia

while concurrently reducing levels of the inflammatory mediators nitric oxide and prostaglandin E2 in the rat (Costa et al., 2004). Both the cannabinoid agonists D9-THC and CP 55,940 are effective in reducing thermal and mechanical hyperalgesia induced by CCI in the rat (De Vry et al., 2004). Fatty acid amide hydrolase (FAAH) is an enzyme that cleaves the endocannabinoids anandamide and 2-AG. Reduction of FAAH via administration of FAAH inhibitors results in an increase in spinal anandamide levels and decreases hyperalgesia induced by CCI in the rat (Russo et al., 2007).

Intrathecal administration of the CB2 selective agonist JWH-133 in mice with partial sciatic nerve ligation caused a significant reduction in mechanical allodynia (Yamamoto et al., 2008). These anti-allodynic effects have also been demonstrated by other CB2 selective agonists such as A-836339 and MDA7 in several different rodent models of neuropathic pain (Naguib et al., 2008; Yao et al., 2009). The CB2 receptor has also been implicated in the regulation of neuropathic pain. CCI treated mice lacking the CB2 receptor showed an increase in mechanical as well as thermal hyperalgesia as did CCI wild-type mice when compared to sham operated controls. However, ligated CB2 knockout mice showed significant levels of hyperalgesia in the contralateral paw when compared to wild type CCI mice and controls. This hyperalgesia was concurrent with a bilateral increase in activity of immune cells in the ipsi- and contralateral spinal cords of CCI CB2 knockout mice and these behavioral and physiological changes were absent in transgenic CCI mice that over-express the CB2 receptor (Racz et al., 2008). Neuropathic pain was also shown to cause changes in neurons in the thalamus of spinal nerve ligated rats. Recordings in the thalamus demonstrated that administration of JWH-133 caused a significant reduction in spontaneous as well as mechanically induced firing of neurons which was not observed in sham-operated control rats (Jhaveri et al., 2008).

In human clinical trials cannabinoids have been shown to have limited analgesic potential in acute pain states yet, in neuropathic pain conditions, cannabinoids have been shown to be highly potent and in some instances rival the analgesic efficacy of morphine (Ashton et al., 2008). While most clinical trials involving cannabinoids and neuropathic pain have focused on subjective pain ratings as reported by patients, one study did assess for the

effects of cannabinoids on evoked pain responses. Wilsey et al., (2008) reported that while cannabinoids were effective in reducing pain ratings of neuropathic pain patients, they were not effective in reducing levels of evoked pain, suggesting a central rather than a peripheral mechanism. Studies in the animal previously mentioned have found increases in cannabinoid receptor protein in response to neuropathic pain and we hypothesize that neuropathic pain also induces increases in CB1 receptor density in the medial pain pathway which could account for the differences in cannabinoid mediated analgesia in acute versus chronic pain states.

Materials and Methods

Animals

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25-30g were housed 6 to a cage in animal care quarters on a 12h light-dark cycle. Food and water were available *ad libitum.* Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University Medical Center and comply with recommendations of the International Association for the Study of Pain (IASP).

CCI of the Sciatic Nerve

Surgical technique is described previously (page 11). Briefly, mice were anesthetized under 2.5% isofluorane before having the lower back and right thigh shaved. Two 5-0 chromic gut sutures were used to loosely ligate the sciatic nerve of the right hindleg. A separate control group of sham-operated mice underwent the exact same surgical procedure with the exception of the ligation of the sciatic nerve.

Behavioral Testing

Thermal hypersensitivity was assessed using a radiant heat source under a plexi-glass surface applied to the plantar surface of each hindpaw (Hargreaves et al., 1988) (described in detail on page 11). Mice were again tested for paw-withdrawal latency at

either 1, 3, or 10 days post CCI or sham surgery. Paw-withdrawal latencies were expressed as relative values (%) to baseline latencies for each animal, as well as group means ± S.E.M. Behavioral data were analyzed via Student's t-test with differences considered statistically significant at $p < 0.05$. Any CCI mouse that failed to demonstrate statistically significant post-CCI withdrawal latency from baseline was excluded from further analysis.

Cannabinoid-Stimulated [^{35}S] GTPγS Binding

On day 1, 3, or 10 post surgery and following thermal hyperalgesia testing, mice were euthanized, their brains removed and the PAG, medial thalamus and cingulate cortex were dissected out. Tissue was immediately flash-frozen in liquid nitrogen and stored at -80°C until further processing. On the day of the assay, tissue was thawed and sonicated in 5ml of membrane buffer (50 mM Tris, 3 mM MgCl2, 1 mM EGTA, 100 mM NaCl, pH 7.7). The homogenate was centrifuged at 50,000 x g at 4°C for 10 min and the resulting pellet was resuspended in 3-5ml of membrane buffer and resonicated. Protein levels were determined by the Bradford assay (1976) using BSA as a standard. Membranes were then incubated with 4 mU/ml adenosine deaminase for 35 min at 30°C. Membranes (8-10µg) were incubated in membrane buffer containing 30 µM GDP, 0.1 nM $^{[35S]}$GTPγS, and varying concentrations of the CB1/CB2 agonist WIN 55, 212-2 or the CB2 agonist JWH-133. Non-specific binding was assessed via the addition of 20µM unlabeled GTP and basal levels of binding were assessed via the omission of agonist. Membranes were incubated for 2h at 30°C with gentle agitation. The incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with ice-cold 50mM Tris, pH 7.2. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for ^{35}S after extraction of the filters in 4ml Budget Solve scintillation fluid and a 45 min shake cycle.

[^{3}H] SR141716A Binding

Brain membranes were diluted with buffer and prepared under the same conditions as for the [^{35}S]GTPγS binding assays. Saturation binding analyses were performed by incubating 10 µg of membrane protein with 0.1–2.5 nM [^{3}H] SR 141716A in the

presence and absence of 1 mM unlabeled SR141716A to determine nonspecific and specific binding, respectively. [^{3}H] SR141716A was diluted in assay buffer (50mM Tris, 3mM MgCl2, 1mM EGTA, 0.125% (wt/vol) BSA, pH 7.7) before addition to the assay. Assays were conducted in duplicate for 1.5 h at 30°C. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass-fiber filters that had been soaked in Tris buffer, pH 7.4, containing 0.5% (wt/vol) BSA, followed by three washes with the Tris buffer. Bound radioactivity was determined by liquid scintillation spectrophotometry after extraction of the filters in 4ml Budget Solve scintillation fluid and a 45 min shake cycle.

Liquid Chromatography-Mass Spectrometry Analysis (LC/MS)

At day 1 or 10 post surgery, the PAG and ACC were extracted from the brain from each mouse, weighed and flash frozen in dry ice and stored at -80°C until the time of processing. On the day of processing, tissues were weighed and homogenized with 1.4 ml chloroform: methanol (2:1 v/v containing 0.0348g PMFS/ml) after the addition of internal standards to each sample (2 pmole AEA –d8 and 1 nmole 2-AG-d8). Homogenates were then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed and then centrifuged for 10 min at 4,000 rpm at 4°C. The aqueous phase plus debris were collected and extracted again twice with 0.8 ml chloroform. The organic phases from the three extractions were pooled and the organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml chloroform and mixed with 1 ml cold acetone. The mixtures were then centrifuged for 5 min at 3000 rpm and 4 °C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml methanol and placed in autosample vials for analysis.

LC/MS/MS was used to quantify AEA and 2-AG. The mobile phase consisted of (10:90) water: methanol with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery ® HS C18, 4.6* 15 cm, 3 micron (Supelco, USA). The mass spectrometer was run in Electrospray Ionization in positive mode. Ions were analyzed in multiple reaction monitoring mode and the following transitions were monitored:

(348>62) and (348>91) for AEA ; (356>62) for AEA-d8; (379>287) and(279>269) for 2-AG ; (387>96) for 2AG-d8. A calibration curve was constructed for each assay based on linear regression using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.03 pm to 40 pm for AEA and from 0.05nm to
64 nm for 2-AG.

Data Analysis

For [^{35}S]GTPγS binding studies, the percentage of stimulation is expressed as (net-stimulated [^{35}S]GTPγS binding/basal) × 100%. Basal binding is defined as specific [^{35}S]GTPγS binding in the absence of drug and net-stimulated [^{35}S]GTPγS binding is defined as [^{35}S]GTPγS binding in the presence of drug minus basal. E_{max} and EC_{50} values were calculated from nonlinear regression analysis by fitting of the concentration-effect curves to the equation $E = E_{max} \times$ agonist concentration /(EC_{50} + agonist concentration) using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). For [^{3}H] SR141716A binding, B_{max} and K_D values were calculated by fitting of the saturation curves to the equation $B = B_{max} \times$ ligand concentration /(K_D + ligand concentration) using Prism 4.0 software (GraphPad Software Inc.). E_{max} and EC_{50} and B_{max} and K_D values were then analyzed via Student's t-test to assess for statistical significance between CCI and sham groups.

Results

Thermal Hyperalgesia Induced by CCI

Sciatic nerve ligation produced a significant reduction in paw withdrawal latency to thermal stimulus in the ipsilateral paw of CCI mice when compared to baseline measures and sham-operated controls. Differences were not observed at day 1 but were observed in the day 3 post-CCI surgery group when compared to baseline ($p < 0.001$, $t = 11.03$) and sham ($t = 6.938$, $p < 0.0001$) and also in the day 10 post-CCI group when compared to baseline ($p < 0.0001$, $t = 25.46$) and sham mice ($p < 0.0001$, $t = 19.35$) (Figure 12). Paw withdrawal latencies were also significantly different in the ipsilateral and contralateral paws of CCI mice at day 3 ($p < 0.01$, $t = 5.193$) and day 10 ($p < 0.0001$, $t = 41.99$)-post CCI

Figure 12: Paw Withdrawal Latency From Radiant Heat

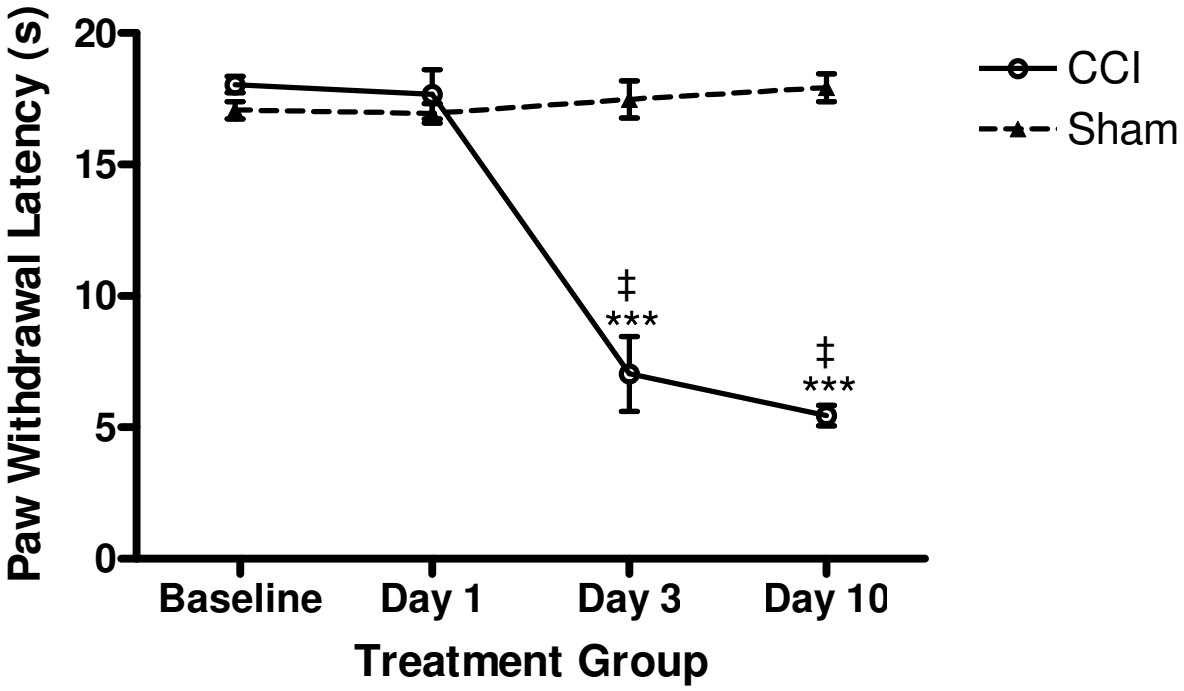

Figure 12: Sciatic nerve ligation produced a significant reduction in paw withdrawal latency to thermal stimulus in the ipsilateral paw of CCI mice when compared to sham control (***p< 0.0001) and baseline (‡ p< 0.0001) measures. This effect was first observed at Day 3 post-CCI surgery and continued until Day 10.

surgery (Figure 13). Since differences in withdrawal latencies were greatest at day 10 post-surgery, it was the time point that was selected for all binding experiments, unless otherwise noted.

Cannabinoid Stimulated [^{35}S] GTPγS Binding

We hypothesized that CCI would induce an increase in both WIN 55, 212-2-stimulated [^{35}S] GTPγS binding and subsequent receptor density. Binding experiments were conducted on the membranes of the PAG, medial thalamus and ACC of CCI and sham operated mice at day 10 post surgery. WIN 55, 212-2 (10^{-5}-10^{-8} M) produced a concentration dependent increase in binding in both CCI and sham groups in all brain areas (Figures 14-16). This efficacy (E_{max}) of WIN 55, 212-2 was significantly decreased by 20% in the ACC regions of CCI mice when compared to sham mice ($p < 0.01$, $t = 3.211$) (Figure 14). There were no significant differences in E_{max} values between sham and CCI mice in the PAG ($p= 0.38$) (Figure 15) or MT ($p= 0.29$) (Figure 16) and no statistically significant differences in EC_{50} values in any brain areas examined (Table 2).

To further investigate the decrease in E_{max} values in the ACC a time-course analysis was conducted assessing WIN 55, 212-2 stimulated [^{35}S] GTPγS binding at days 1 and 3 post CCI. Further time-course analysis of the ACC region showed that there were no statistically significant differences in E_{max} or EC_{50} observed at day 1 or 3 post surgery between CCI and sham mice (Figure 17). There were no statistically significant differences in basal levels of [^{35}S] GTPγS binding in any of the brain areas examined at any time point which indicates that CCI induced desensitization in WIN 55,212-2-stimulated [^{35}S] GTPγS binding are specific to the CB1 receptor and not due to an overall decrease in the ability of GTP to bind to GPCRs in the ACC.

Additionaly, JWH-133-stimulated [^{35}S] GTPγS binding experiments were conducted in the PAG and medial thalamus as these two areas have recently been shown to express functional CB2 receptors (Onaivi et al., 2006). There were not enough CB2 receptors in these areas of the mouse to generate a binding curve (data not shown), indicating that

Figure 13: Paw Withdrawal Latency From Radiant Heat By Hindpaw in CCI Mice

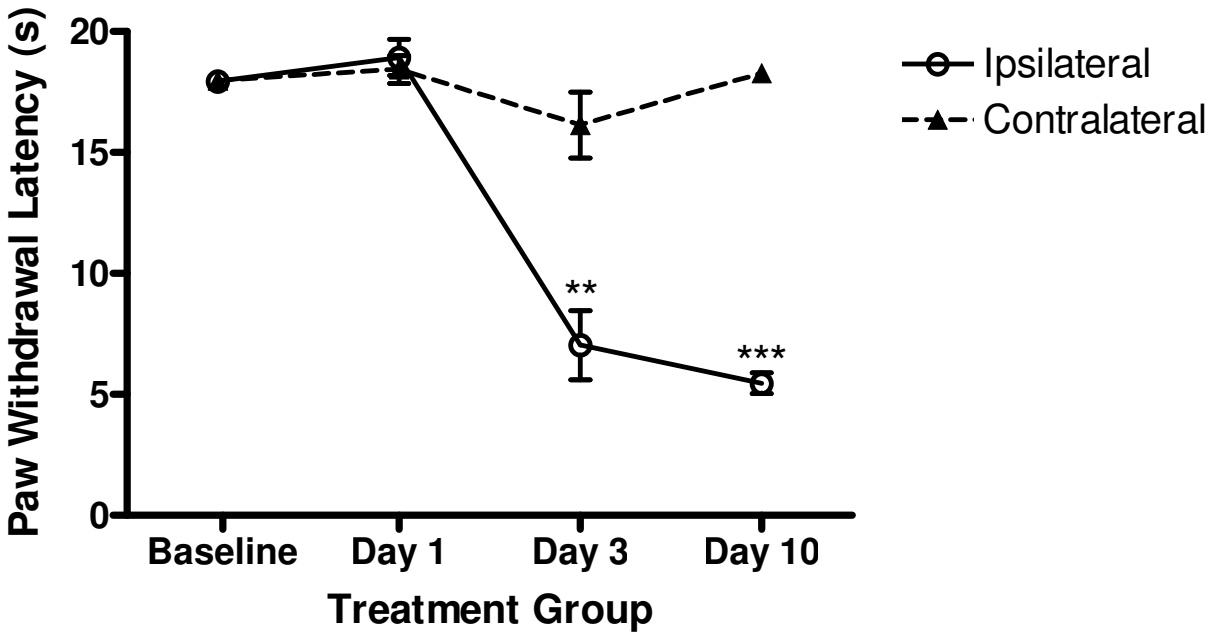

Figure 13: Sciatic nerve ligation produced a significant reduction in paw withdrawal latency to thermal stimulus in the ipsilateral paw of CCI mice when compared to the contralateral hindpaw. This effect was first observed at Day 3 post-CCI surgery and continued until Day 10. (**$p < 0.01$, ***$p < 0.0001$)

Figure 14: WIN 55, 212-2 stimulated [^{35}S] GTPγS Binding in the ACC

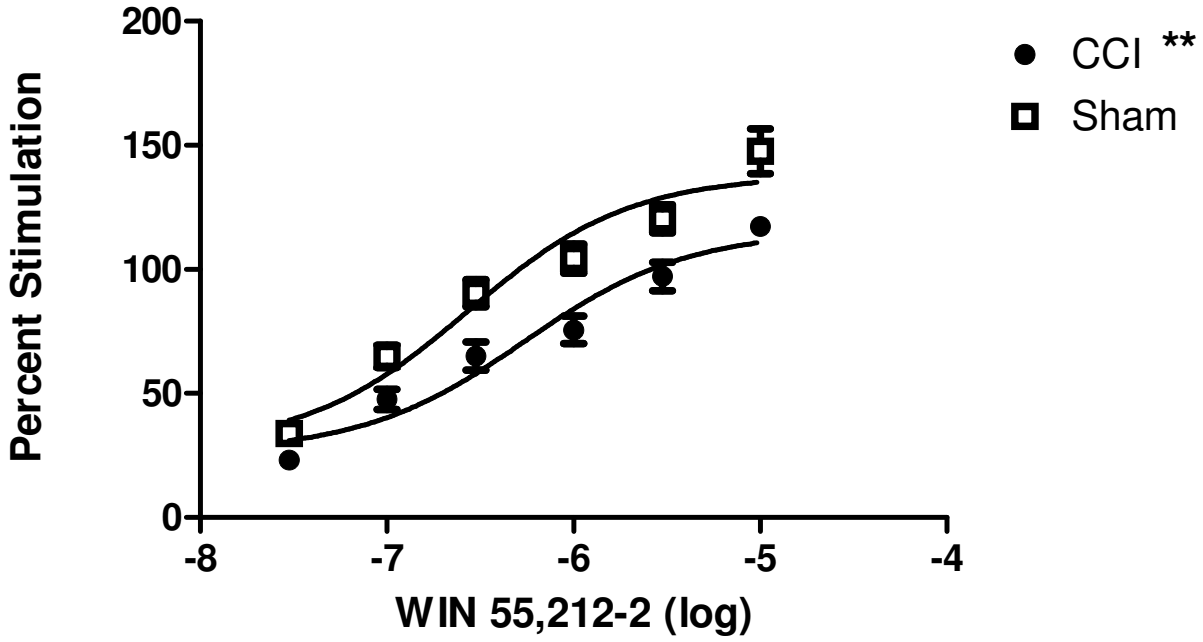

Figure 14: Data is expressed as percent net stimulated binding above basal binding. WIN 55, 212-2-stimulated [^{35}S] GTPγS binding in the ACC shows a significant decrease in E_{max} values in CCI mice at day 10 post surgery when compared to sham operated controls. (n = 8) (** $p<0.01$)

Figure 15: WIN 55, 212-2 Stimulated [^{35}S] GTPγS Binding in the MT

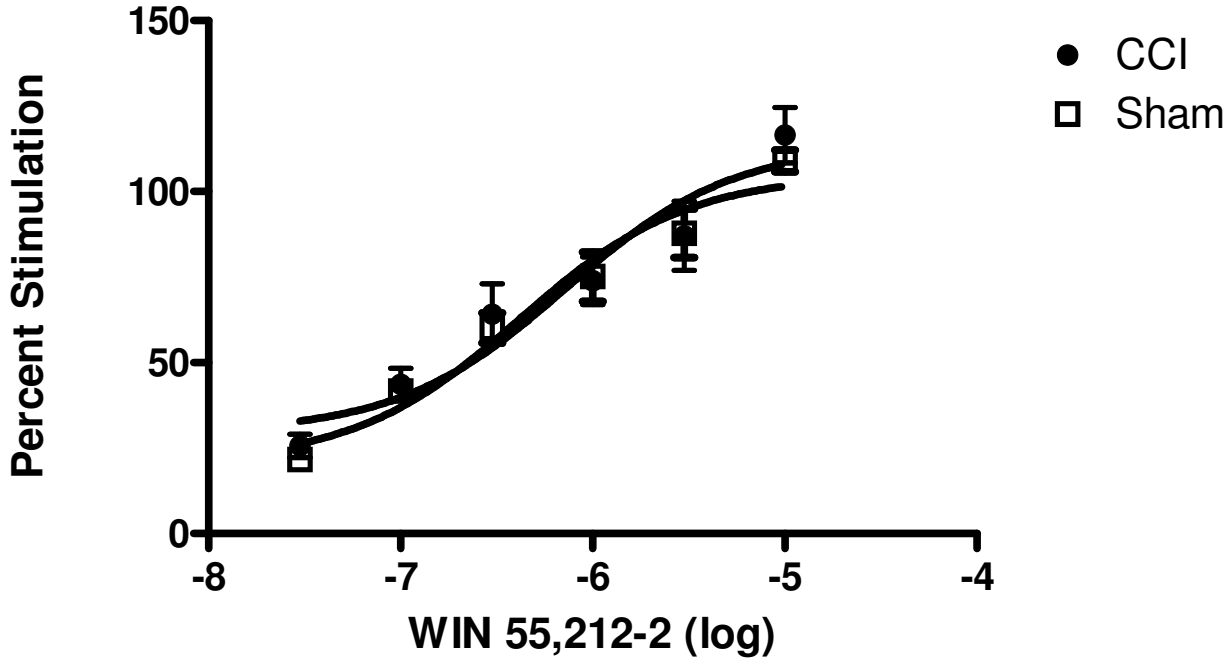

Figure 15: Data is expressed as percent net stimulated binding above basal binding. There were no significant differences in WIN 55, 212-2-stimulated [^{35}S] GTPγS binding in the MT between CCI and sham mice at day 10 post surgery ($p > 0.05$) ($n = 6$).

Figure 16: WIN 55, 212-2 Stimulated [^{35}S] GTPγS Binding in the PAG

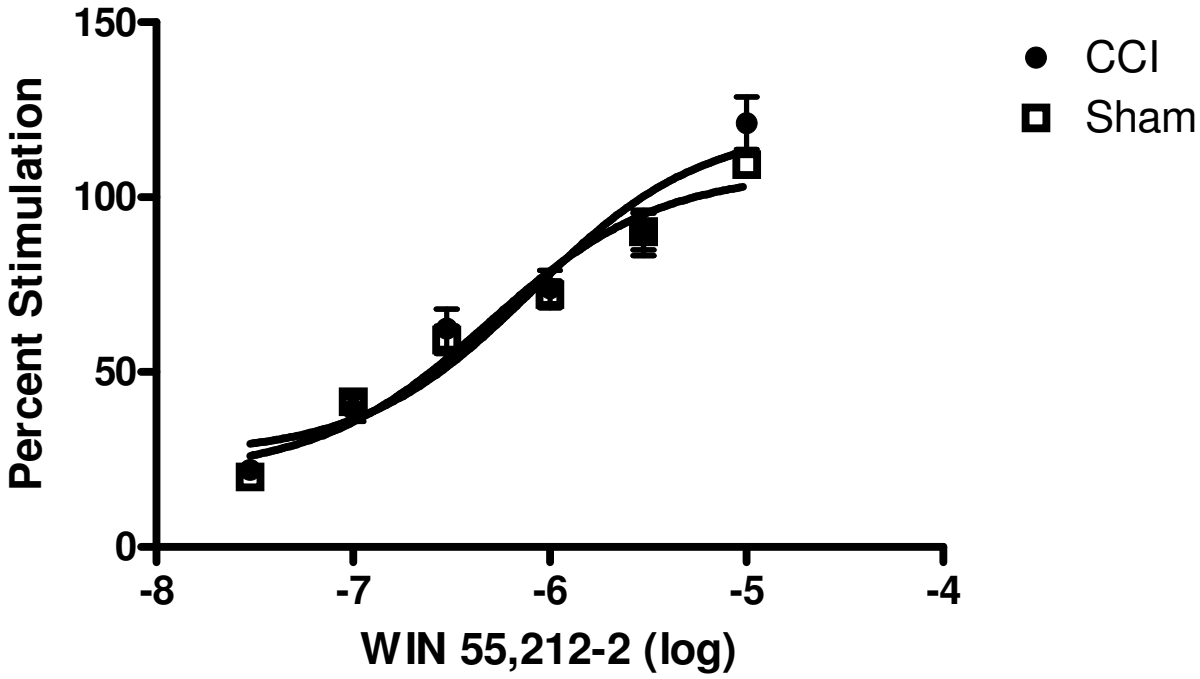

Figure 16: Data is expressed as percent net stimulated binding above basal binding. There were no significant differences in WIN 55, 212-2-stimulated [^{35}S] GTPγS binding in the PAG between CCI and sham mice at day 10 post surgery ($p > 0.05$) ($n = 6$).

Table 2: E_{max} and EC_{50} Values from WIN 55, 212-2 Stimulated [^{35}S] GTPγS Binding

Region	EC50 (μM)		Emax (%)	
	Sham	CCI	Sham	CCI
PAG	0.192 ± 0.024	0.227 ± 0.031	99.66 ± 4.12	106.6 ± 6.53
M.T	0.170 ± 0.014	0.215 ± 0.088	98.40 ± 5.19	102.8 ± 9.02
ACC	0.130 ± 0.022	0.193 ± 0.038	132.4 ± 6.94	106.3 ± 4.25 **

Table 2: Emax and EC50 values from WIN 55, 212-2-stimulated [^{35}S] GTPγS binding in brain areas of the mouse brain at day 10-post surgery (**$p < 0.01$).

Figure 17: Differences in Means of E_{max} and EC_{50} values Comparing WIN Stimulated [^{35}S] GTPγS Binding in the ACC by Post-Surgical Day

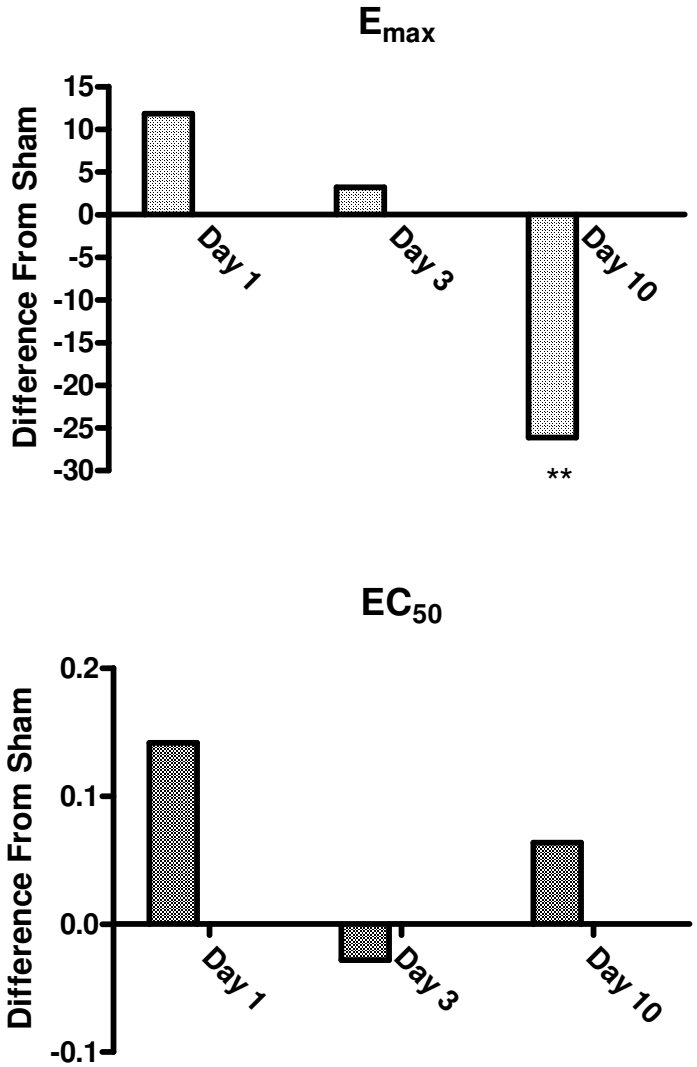

Figure 17: E_{max} and EC_{50} values are expressed as difference between the group means of CCI and sham mice. The time course shows that the E_{max} value of WIN 55, 212-2 was significantly decreased in CCI relative to sham mice only on day 10 and was not significantly different day 1 or 3 post surgery. There were no significant differences in EC_{50} values at any time point examined (**$p < 0.01$).

desensitization shown in the CB1/CB2 agonist WIN 55,212-2-stimulated binding experiments was due specifically to alterations in CB1 receptor function(s).

[^{3}H] SR141716A Receptor Binding

In order to determine whether the decrease in WIN 55, 212-2 stimulated [^{35}S] GTPγS binding in the ACC was due to desensitization of the CB1 receptor or a change in the levels of the CB1 receptor, a tritiated receptor binding experiment was conducted. [^{3}H] SR 141716A receptor binding was conducted on ACC membranes of mice at day 10 post CCI surgery. There were no significant differences in B_{max} ($p= 0.12$) or K_D ($p= 0.22$) values between CCI and sham mice (Figure 18) indicating that the decrease in GTP binding in the ACC of CCI mice was due to a desensitization of the CB1 receptor and not a decrease in the number of receptors.

LC/MS Analysis

We used LC/MS to test the hypothesis that the desensitization of CB1 receptors was due to an increase in the endogenous cannabinoids AEA and 2-AG. There were no significant differences between CCI and sham mice in endocannabinoids levels measured in the ACC at either day 1 ($p = 0.85$ for AEA, $p = 0.60$ for 2-AG) or day 10 ($p = 0.48$ for AEA, $p = 0.77$ for 2-AG) post surgery. (Figure 19). The PAG was utilized as a control area since significant differences in endocannabinoid levels were observed in that tissue following CCI in previous studies (Petrosino et al. 2007). Similarly, there were no significant differences in endocannabinoid levels in the PAG at either day 1 ($p = 0.72$ for AEA, $p = 0.13$ for 2-AG) or day 10 ($p = 0.84$ for AEA, $p = 0.70$ for 2-AG) post surgery (Figure 20). This indicates that the CCI induced desensitization of the CB1 receptor in the ACC was not caused by an increase in the endocannabinoids AEA or 2-AG.

Figure 18: [3H] SR 141716A Receptor Binding in the ACC at Day 10 Post-Surgery

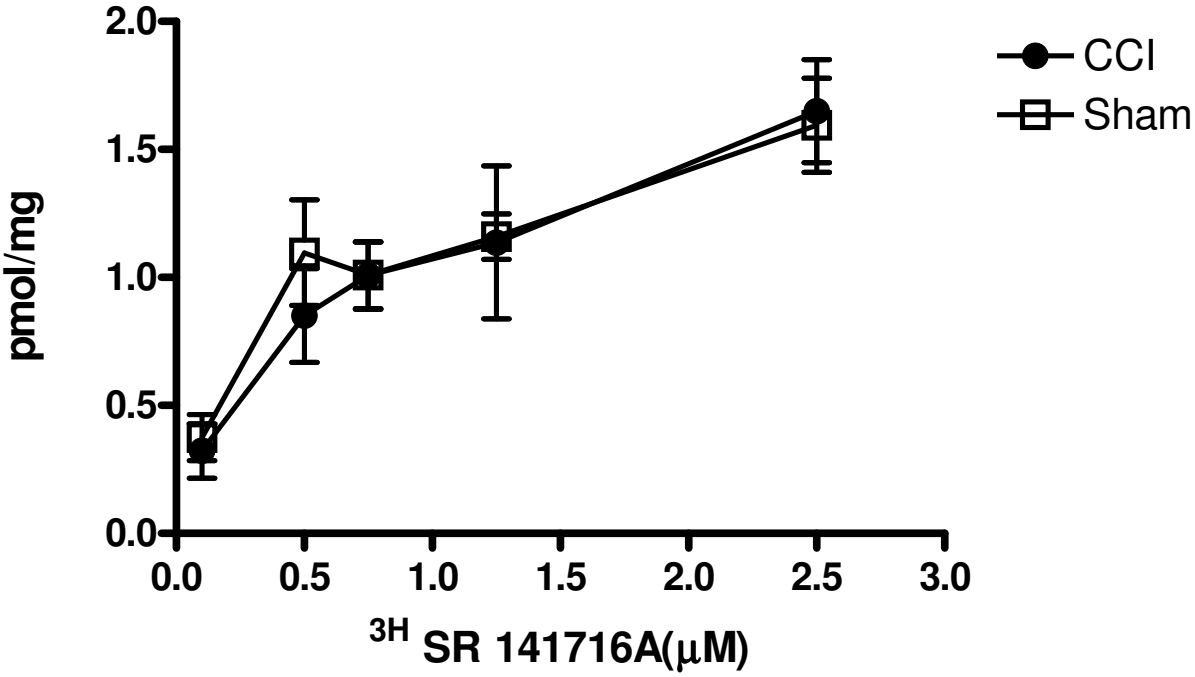

Figure 18: [^{3}H] SR 141716A receptor binding was conducted on the ACC at day 10 post CCI surgery. There were no significant differences in CB1 receptor binding between CCI and sham mice in the ACC (n = 5).

Figure 19: Levels of AEA and 2-AG in the ACC Following CCI in Mice

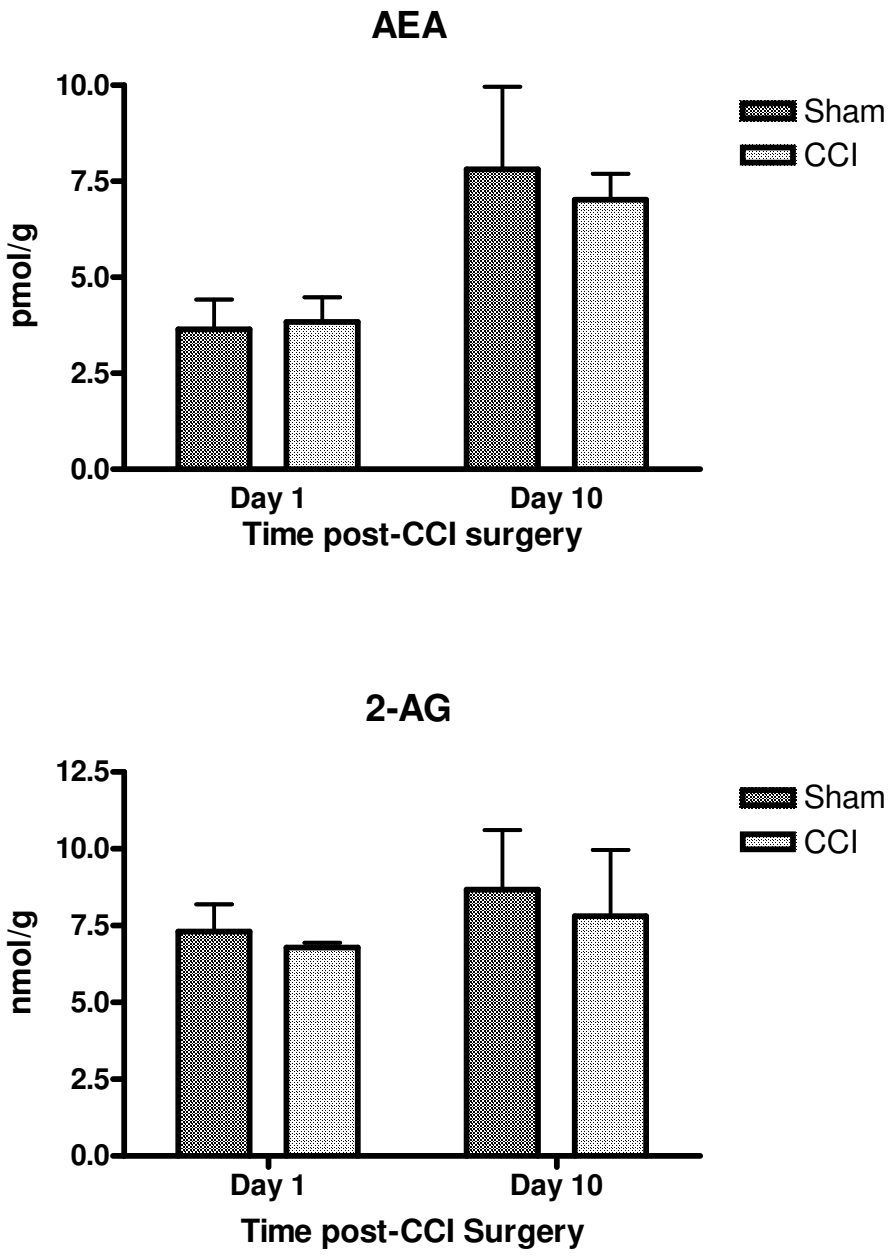

Figure 19: CCI did not induce significant differences between CCI and sham mice in levels of the endocannabinoids AEA or 2-AG in the ACC (n = 6).

Figure 20: Levels of AEA and 2-AG in the PAG Following CCI in Mice

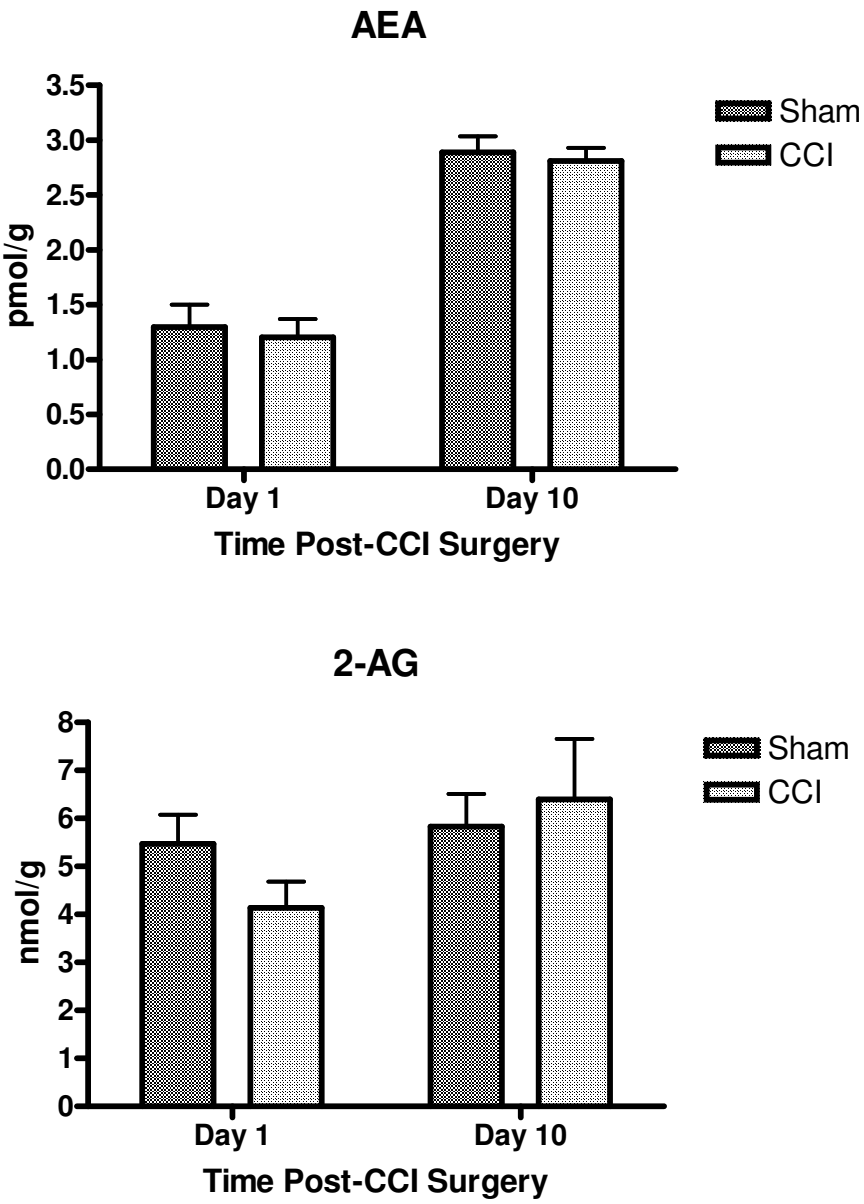

Figure 20: CCI did not induce significant differences between CCI and sham mice in levels of the endocannabinoids AEA or 2-AG in the PAG (n = 6).

Discussion

The decrease in WIN 55, 212-2 stimulated [^{35}S] GTPγS binding in the ACC coupled with a lack of differences in the MT was contrary to our hypothesis. Given that previously mentioned studies have shown an upregulation of CB1 receptor protein in the spinal cord in response to neuropathic pain, we were anticipating a similar effect in the MT or the ACC. The decrease in WIN 55,212-2 stimulated [^{35}S] GTPγS binding in the ACC was without a subsequent decrease in [^{3}H] SR141716A receptor binding, indicating that there was no change in the overall receptor density, but that the CB1 receptors were desensitized in response to CCI. Considering that prolonged exposure to the endocannabinoid, anandamide, has been shown to cause a decrease in [^{35}S] GTPγS binding in the murine brain (Rubino et al., 2000) and endocannabinoids were shown to be upregulated in supraspinal areas following CCI (Petrosino et al., 2007) we hypothesized that there was an increased level of AEA or 2-AG in the ACC following CCI in our mice to explain the WIN 55, 212-2 stimulated GTPγS binding data. However, our LC/MS studies did not show any significant differences in levels of 2-AG or AEA in the ACC between CCI and sham operated mice.

The reason that we did not find any differences in levels of AEA or 2-AG following CCI surgery may be attributed to the way endocannabinoids are synthesized and degraded. AEA and 2-AG are not stored in intracellular vesicles like many classic neurotransmitters but rather are synthesized "on demand" in response to specific stimuli as demonstrated in several different tissues (Panikashvili et al., 2001), (Basavarajappa et al., 2000), (Stella et al., 2001). Additionally, both AEA and 2-AG are rapidly internalized and degraded by the cell following release (Sugiura et al., 2002; Sugiura et al., 2002). Levels of both anandamide and 2-AG have even been shown have diurnal variation in the rodent (Valenti et al., 2004), making accurate measurement of concentrations of these lipids even more difficult.

Also, there are other endocannabinoids such as N-AEs—Nhomo-c-linolenoylethanolamine (HEA) and N-docosatetraenoylethanolamine (DEA) which are found in brain tissue and have been shown to have nanomolar affinity for the CB1 receptor (Hanus et al., 1993). Therefore, it could be that the desensitization of the CB1 receptor in the ACC of our CCI mice was mediated by an increase in other endocannabinoids which were not examined.

The PAG is an area in which differences in AEA and 2-AG were previously shown following CCI (Petrosino et al., 2007) and our data differed from those findings. This could be due to species difference since the Petrosino (2007) study was conducted in rats and not mice. There are many behavioral and physiological differences which have been noted in chronic pain research between these species and in different strains of the same species. In assessing differences in mechanical hypersensitivity and cold allodynia, it was found that C57BL/6J mice showed significant differences in response thresholds when compared to other mouse strains following spared nerve injury of the sciatic nerve (Leo et al., 2008). These differences in mice have also been reported in a variety of mouse strains when comparing all types of noxious stimuli, including acute, inflammatory and neuropathic pain with measured differences in pain tests as great as 54-fold between strains in some instances (Mogil et al., 1999; Smith et al., 2004) These effects have also been demonstrated amongst different strains of rats. Among four different rat strains, significant differences in basal sensitivity to mechanical or heat stimulation were observed. Following sciatic nerve injury, all strains of rats developed mechanical allodynia and thermal hyperalgesia however, the time-course and magnitude of the responses were significantly different even despite the fact that comparable nerve damage was observed in all the strains. Even more striking is that there were significant differences between sub-strains of Sprague-Dawley rats with one sub-strain developing cold allodynia while the other did not and the other sub-strain developing more severe mechanical allodynia than the other (Xu et al., 2001). These behavioral differences in nociceptive responses amongst strains also have physiological correlates. An increase in levels of BDNF following CCI in rats was shown to be greatest in rat strains that exhibited the most robust hypersensitivity (Herradon et al., 2007). Significant increases in

prodynorphin in the DRGs of CCI rat strains most resistant to were observed along with significant decreases in delta-opioid receptors in strains most sensitive to neuropathic pain ((Herradon et al., 2008)). These studies highlight the differing behavioral and physiological manifestations that occur amongst species and within strains of the same species in response to chronic pain and highlight the complexity of the disease itself.

Given the results of our studies, it is likely that the increased analgesic action of cannabinoids that has been reported to occur in neuropathic pain conditions is due to a change in a site outside of the ACC, MT or PAG. However, this study shows that CCI of the sciatic nerve alters the functional activity of the CB1 receptor in the ACC. This study is the first to show alterations to the cannabinoid system in response to neuropathic pain within the cortex of the mouse.

Chapter IV.

The Effect of CCI on the μ-Opioid Receptor in the Mouse Brain

Introduction

Opiates are a class of drugs which mimic the effects of the endogenous opioid peptide ligands, enkephalin, endorphin, and dynorphin within the nervous system. Opiates activate the opioid receptor subtypes κ, δ, and μ which are highly, yet differentially, expressed in the central nervous system (Chen et al., 1993; Yasuda et al., 1993). Morphine and other opiates most commonly prescribed for moderate to severe pain have the highest efficacy for the μ-opioid receptor. The majority of μ-opioid receptors in the CNS are localized in the plasma membrane of the cell bodies of neurons as well as on pre- and postsynaptic terminals (Garzon et al., 2001; Wang et al., 2001). These receptors are found in significant quantities in peripheral nerve terminals, dorsal horn and dorsal root ganglion as well as the structures in both the medial and lateral spinothalamic tracts and limbic system, including the anterior cingulate, insular cortex, medial thalamus and amygdala (Arvidsson et al., 1995; Ding et al., 1996) and thus are highly involved in the regulation of nociceptive processing.

The opioid receptors belong to the G-protein coupled family of receptors (GPCR). The μ-opioid receptor is coupled to the $G_{\alpha i}$ subtype protein. Activated $G_{\alpha i}$ receptors inhibit the function of adenylyl cyclase, which is a regulator of the formation of the second messenger cyclic AMP. Additionally, N and P/Q type voltage gated Ca^{++} channels, which modulate the presynaptic release of glutamate, are inhibited by μ-opioid receptor activation as are inwardly rectifying K^+ channels are activated, which causes a hyperpolarization of the cell (Pan et al., 2008). Thus, the overall analgesic effect of opioids could be considered to by via cellular inhibition.

Morphine and other opiates have long been the therapeutic drug of choice in treating various acute pain conditions. However, in human and animal chronic neuropathic pain states morphine and other opioids have been shown to have limited analgesic potency and efficacy (Arner et al., 1988; Ossipov et al., 1995; Przewlocka et al., 1999). Studies have focused on the potential physiological changes that may contribute to the limited efficacy of opioids and evidence points to changes that occur at the spinal and supraspinal level.

At the level of the spinal cord, physiological changes in the μ-opioid system have been shown to occur in animal models of neuropathic pain. In a model of sciatic nerve ligation, a downregulation of κ, δ, and μ receptor mRNA was shown in the dorsal root ganglion, which was not observed in a model of inflammatory pain (Obara et al., 2009). Other studies have shown an increase in phosphorylation of the μ-opioid receptor in the dorsal horn of the spinal cord following sciatic nerve ligation (Narita et al., 2004). Phosphorylation of GPCRs including the μ-opioid receptor is a major mechanism contributing to the desensitization of the receptor (Kelly et al., 2008). This is evidenced in a model of alcohol induced neuropathy which resulted in a decrease in the μ-opioid receptor specific agonist DAMGO stimulated GTPγS binding in the dorsal horn, without corresponding decreases in μ-opioid receptor protein levels (Narita et al., 2007). Supraspinally, sciatic nerve ligation in the mouse results in decreases in μ-opioid stimulated GTPγS binding in both the amygdala and the ventral tegmental area (Narita et al., 2006; Ozaki et al., 2003), which are two areas in the limbic system known to be involved in reward as well as the affective component of pain processing.

Both the medial thalamic nuclei (MT) as well as the cingulate cortex (ACC) are part of the medial pain pathway which is implicated in both chronic as well as the affective dimension of pain processing (Treede et al., 1999). The medial thalamus has been shown to be involved in processing of nociceptive information and has multiple connections to the ACC (Hsu et al., 2000; Kung et al., 2002). Human case studies report that damage to the medial thalamic nuclei produces chronic pain (Boivie et al., 1989; Montes et al., 2005) Direct chemical or electrolytic lesion of the rostro-medial, ventro-median, and parafascicular nuclei of the thalamus was reported to decrease neuropathic pain-like behaviors in the rat (Saade et al., 2007) and ablation of medial thalamic nuclei alleviates chronic intractable pain in humans (Jeanmonod et al., 1994; Uematsu et al., 1974; Young et al., 1995). The ACC has been implicated in pain processing in animal and human chronic pain states. Electrophysiological as well as imaging studies have shown activation of the ACC in response to nociceptive stimuli (Kuo et al., 2005; Lorenz et al., 2005; Sikes et al., 1992; Talbot et al., 1991). Also, lesions of the ACC have been

demonstrated to reduce chronic pain in humans (Cohen et al., 1999; Foltz et al., 1968; Hurt et al., 1974). Additionally, the ACC as well as the MT have been shown to be involved in mu-opioid mediated pain suppression. Morphine injected directly into MT nuclei has been reported to suppress vocalizations, which are considered to be measures of the affective component of pain, in response to noxious stimuli (Carr et al., 1988; Harte et al., 2000; Yeung et al., 1978). fMRI studies in the rat have shown that brain activation of the ACC induced by noxious stimuli is suppressed in rats following systemic morphine administration (Chang et al., 2001; Tuor et al., 2000).

The focus of this study is to determine the effect of a chronic neuropathic pain-like state on the u-opioid receptor in the medial pain pathway, which is known to be involved in both affective pain processing and u-opioid mediated pain suppression, in order to further elucidate potential mechanisms of lack of analgesic efficacy of morphine and other opioids in chronic neuropathic pain states.

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Materials and Methods

Animals

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25-30g were housed 6 to a cage in animal care quarters on a 12h light-dark cycle. Food and water were available *ad libitum.* Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University Medical Center and comply with recommendations of the International Association for the Study of Pain (IASP).

CCI of the Sciatic Nerve

Surgical technique is described previously (page 11). Briefly, mice were anesthetized under 2.5% isofluorane before having the lower back and right thigh shaved. Two 5-0 chromic gut sutures were used to loosely ligate the sciatic nerve of the right hindleg. A separate control group of sham-operated mice underwent the exact same surgical procedure with the exception of the ligation of the sciatic nerve.

Behavioral Testing

Thermal hypersensitivity was assessed using a radiant heat source under a plexi-glass surface applied to the plantar surface of each hindpaw (Hargreaves et al., 1988). Mice were again tested for paw-withdrawal latency at either 1, 3, or 10 days post CCI or sham surgery. Paw-withdrawal latencies were expressed as relative values (%) to baseline latencies for each animal, as well as group means ± S.E.M. Behavioral data were analyzed via Student's t-test with differences considered statistically significant at $p < 0.05$. Any CCI mouse that failed to demonstrate a statistically significant post-CCI withdrawal latency from baseline was excluded from further analysis.

Agonist Stimulated [^{35}S] GTPγS Membrane Binding

On the final day of behavioral testing, mice were euthanized, their brains removed and the PAG, medial thalamus and cingulate cortex were dissected out and weighed for consistency between samples. Tissue was immediately flash-frozen in liquid nitrogen and stored at -80°C until further processing. On the day of the assay, tissue was thawed and sonicated in 5 ml of membrane buffer (50 mM Tris, 3 mM MgCl2, 1 mM EGTA, 100 mM NaCl, pH 7.7). The homogenate was centrifuged at 50,000 x g at 4°C for 10 min and the resulting pellet was resuspended in 3-5ml of membrane buffer and resonicated. Protein levels were determined by the Bradford assay (1976) using 1mg/ml BSA as a standard. Protein was then diluted to reach a standard concentration between all samples. Membranes were then incubated with 4 mU/ml adenosine deaminase for 35 min at 30°C. Membranes (8-10µg) were incubated in membrane buffer containing 30 µM GDP, 0.1 nM [^{35}S] GTPγS, and varying concentrations of the µ-opioid agonist [d-Ala2,(*N*-Me)Phe4,Gly5-OH] enkephalin (DAMGO). Non-specific binding was assessed via the addition of 20µM unlabeled GTP and basal levels of binding were assessed via the omission of agonist. Membranes were incubated for 2h at 30°C in a water bath with gentle agitation. The incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters, followed by three washes with ice-cold 50mM Tris, pH 7.2. Bound radioactivity was determined by liquid scintillation spectrophotometry at

95% efficiency for ^{35}S after extraction of the filters in 4ml Budget Solve scintillation fluid and a 45 min shake cycle.

[^{3}H] Naloxone Binding

Membranes from the medial thalamus were diluted with membrane buffer and prepared under the same conditions as for the $^{[35S]}$GTPgS binding assays. Saturation binding analyses were performed by incubating 30 µg of membrane protein with 0.1–7.5 nM [^{3}H]naloxone in the presence and absence of 1 mM unlabeled naloxone to determine nonspecific and specific binding, respectively. Assays were conducted in duplicate and incubated for 1.5 h in 30°C water bath. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass-fiber filters that had been soaked in Tris buffer, pH 7.4, followed by three washes with ice-cold Tris buffer. Bound radioactivity was determined by liquid scintillation spectrophotometry after extraction of the filters in 4ml Budget Solve scintillation fluid and a 45 min shake cycle.

Data Analysis

For [^{35}S]GTPγS binding studies, the percentage of stimulation is expressed as (net-stimulated [^{35}S]GTPγS binding/basal) × 100%. Basal binding is defined as specific [^{35}S]GTPγS binding in the absence of drug and net-stimulated [^{35}S]GTPγS binding is defined as [^{35}S]GTPγS binding in the presence of drug minus basal. E_{max} and EC_{50} values were calculated from nonlinear regression analysis by fitting of the concentration-effect curves to the equation $E = E_{max} \times$ agonist concentration /(EC_{50} + agonist concentration) using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). For [^{3}H]naloxone binding, B_{max} and K_D values were calculated by fitting of the saturation curves to the equation $B = B_{max} \times$ ligand concentration /(K_D + ligand concentration) using Prism 4.0 software (GraphPad Software Inc.). E_{max} and EC_{50} and B_{max} and K_D values were then analyzed via Student's t-test to assess for statistical significance between CCI and sham groups.

Results

Thermal Hyperalgesia Induced by CCI

Sciatic nerve ligation produced a significant reduction in paw withdrawal latency to thermal stimulus in the ipsilateral paw of CCI mice when compared to baseline measures and sham-operated controls. Differences were not observed at day 1 but were observed in the day 3 post-CCI surgery group when compared to baseline ($p < 0.001$, $t=9.079$) and sham ($t=6.938$, $p< 0.0001$) and also in the day 10 post-CCI group when compared to baseline ($t=18.27$, $p < 0.0001$) and sham mice ($p < 0.001$, $t=20.32$) (Figure 21). Paw withdrawal latencies were also significantly different in the ipsilateral and contralateral paws of CCI mice at day 3 ($p < 0.01$, $t=5.193$) and day 10 ($p <0.0001$, $t=39.84$)-pose CCI surgery (Figure 22). Since differences in withdrawal latencies were greatest at day 10 post-surgery, it was the time point that was selected for all binding experiments, unless otherwise noted.

DAMGO Stimulated [^{35}S] GTPγS Membrane Binding

DAMGO stimulated [^{35}S] GTPγS binding was conducted on the membranes prepared from the PAG, medial thalamus and ACC of CCI and sham operated mice at day 10 post surgery. The PAG was utilized as a control brain region which is involved in pain processing, but is outside of the medial pain pathway. DAMGO (10^{-5}-10^{-8} M) produced a concentration dependent stimulation of [^{35}S] GTPγS binding in both CCI and sham groups in all brain areas examined (Figures 23-25). The efficacy (E_{max} value) of DAMGO was significantly decreased by 15% in the medial thalamus of CCI mice when compared to sham mice ($t=2.525$; $p < 0.05$) (Figure 23). There were no significant differences in E_{max} values between sham and CCI mice in the PAG ($p= 0.41$) (Figure 24) or ACC ($p= 0.42$) (Figure 25) and no statistically significant differences in EC_{50} values in any brain areas examined (Table 3).

Figure 21: Paw Withdrawal Latency From Radiant Heat Source

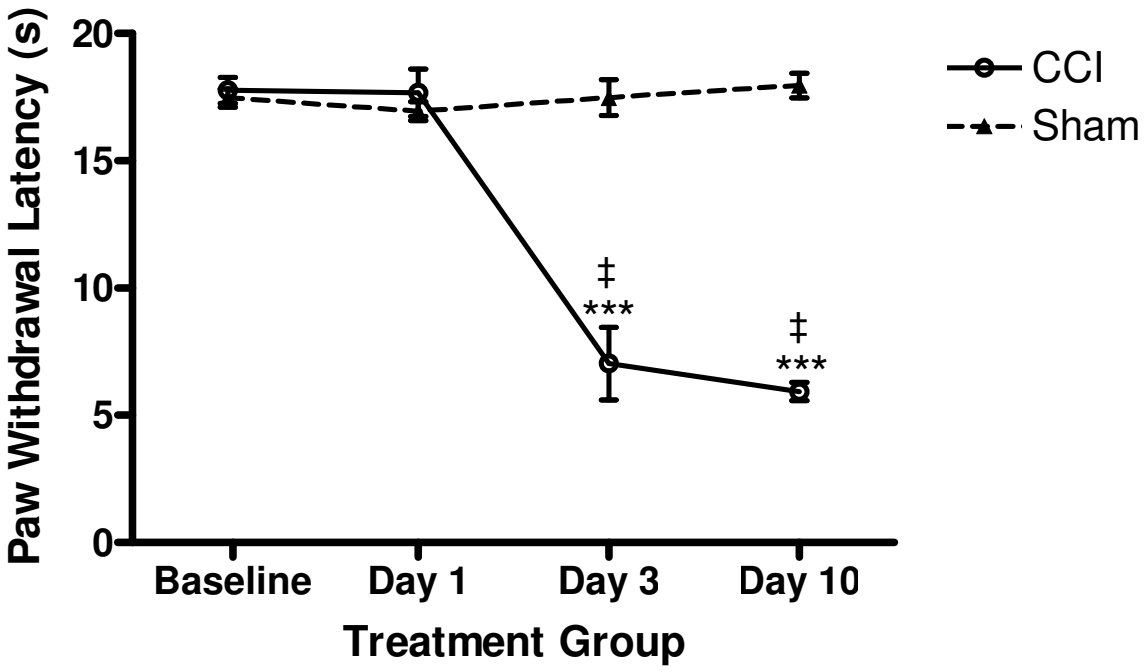

Figure 21: Sciatic nerve ligation produced a significant reduction in paw withdrawallatency to thermal stimulus in the ipsilateral paw of CCI mice when compared to sham operated control (*$p < 0.0001$) and within group baseline measures (‡$p < 0.0001$). This effect was first observed at Day 3 post-CCI surgery and continued until Day 10.

Figure 22: Withdrawal Latency From Radiant Heat in CCI Mice by Paw

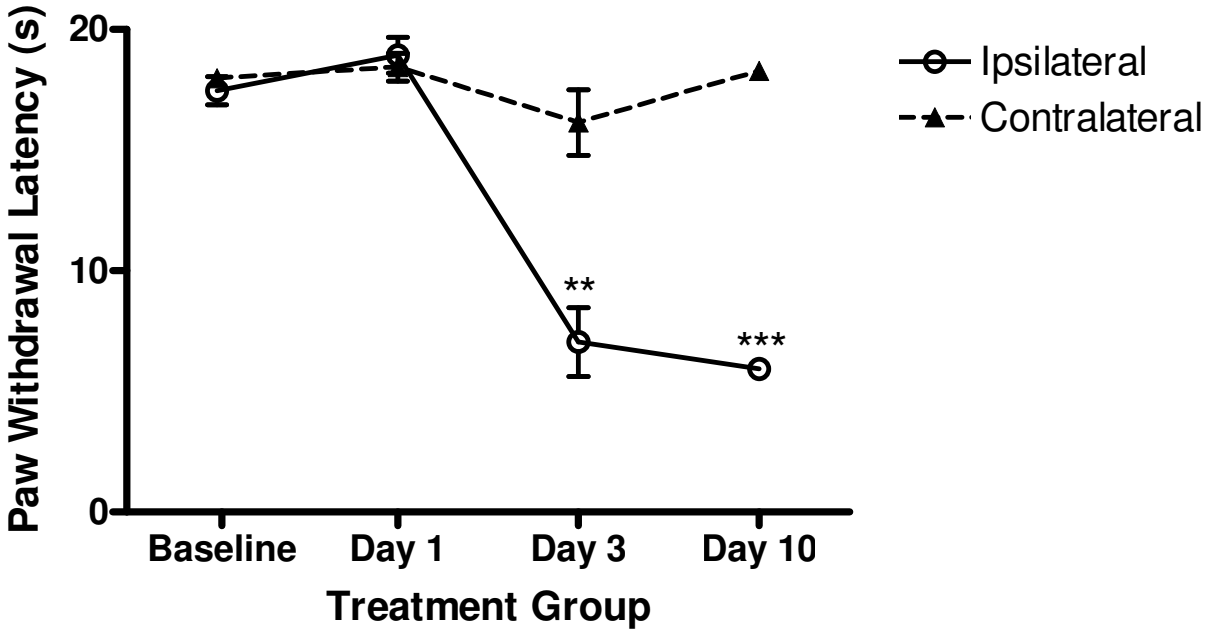

Figure 22: Sciatic nerve ligation produced a significant reduction in paw withdrawallatency to thermal stimulus in the ipsilateral paw of CCI mice when compared to the contralateral paw. This effect was first observed at Day 3 post-CCI surgery and continued until Day 10 (**$p < 0.01$, ***$p < 0.0001$).

Figure 23: DAMGO Stimulated [^{35}S] GTPγS Binding in the Medial Thalamus

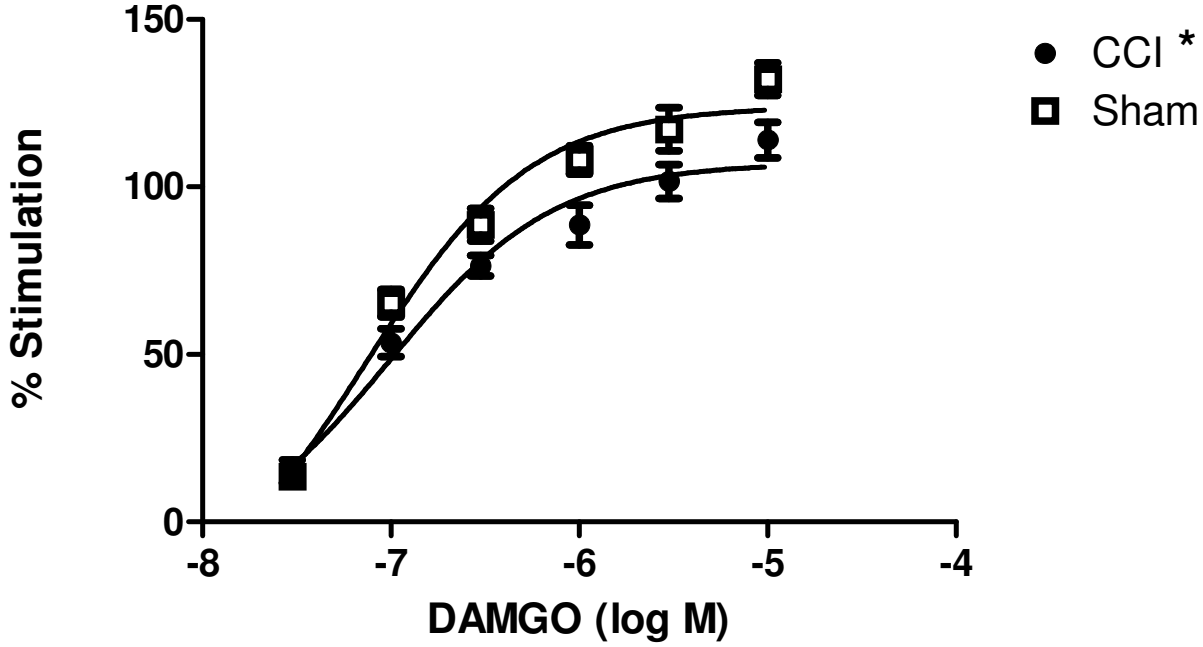

Figure 23: Data is expressed as percent net stimulated binding above basal binding. The efficacy (Emax value) of DAMGO was significantly decreased in the medial thalamus region in CCI mice on Day 10 in post surgery when compared to sham mice (*$p < 0.05$) ($n = 6$).

Figure 24: DAMGO Stimulated [^{35}S] GTPγS Binding in the PAG

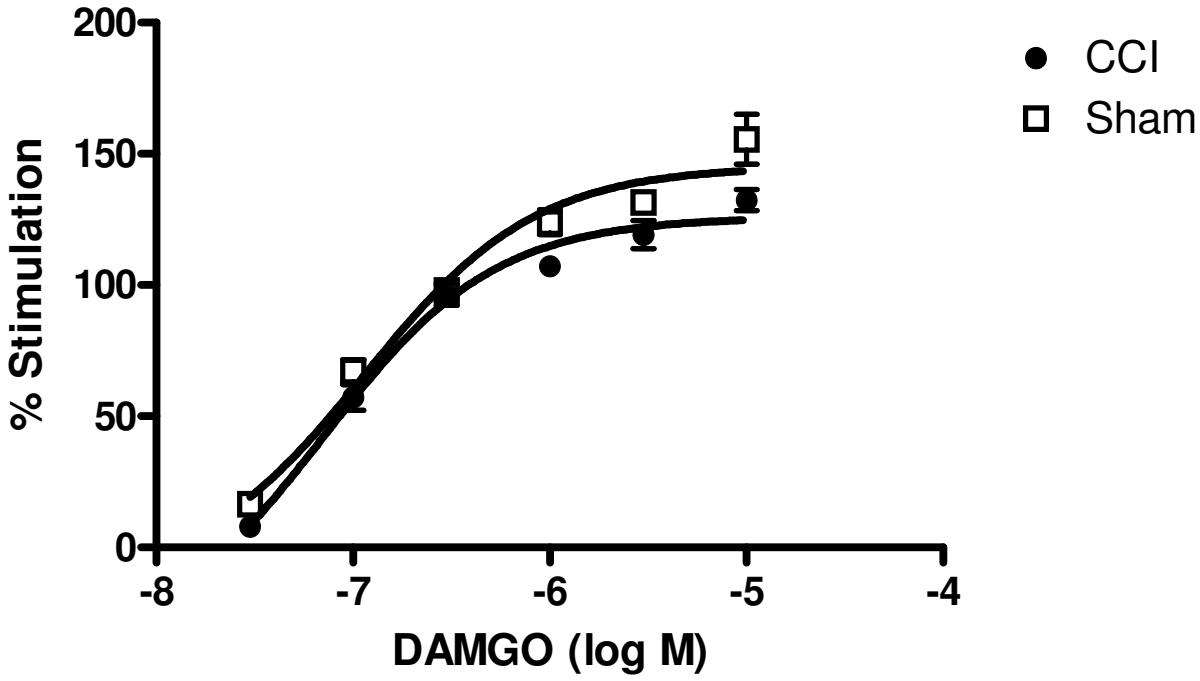

Figure 24: Data is expressed as percent net stimulated binding above basal binding. CCI did not induce any significant differences in DAMGO Stimulated GTPγS Binding in the PAG. ($p > 0.05$) ($n = 6$)

Figure 25: DAMGO Stimulated [^{35}S] GTPγS Binding in the ACC

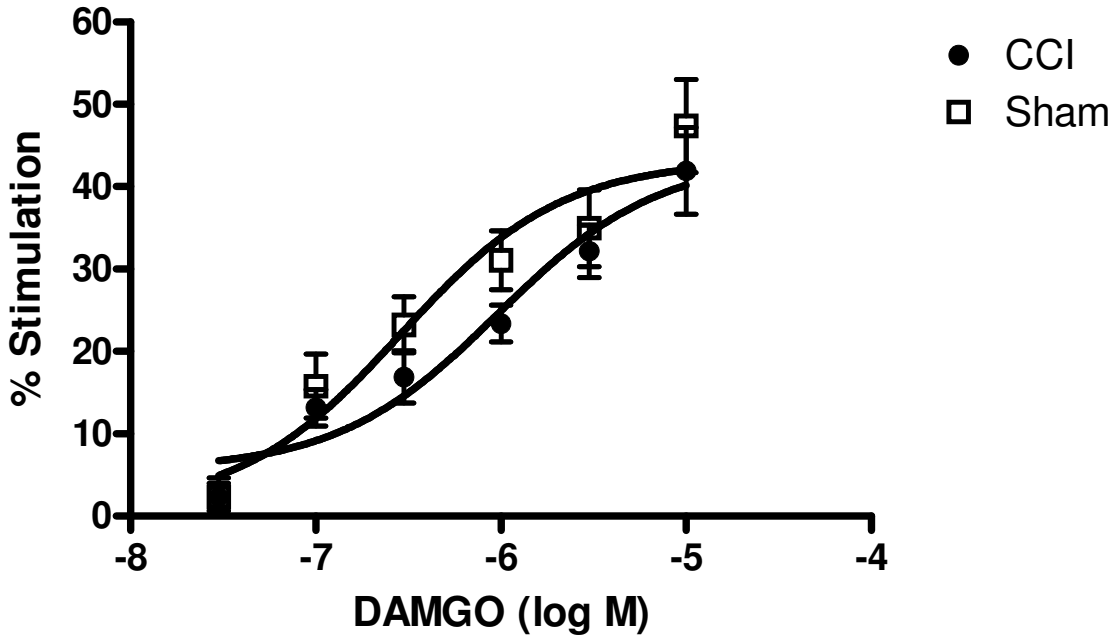

Figure 25: Data is expressed as percent net stimulated binding above basal binding. CCI did not induce any significant differences in DAMGO Stimulated GTPγS Binding in the ACC. ($p > 0.05$) ($n = 5$)

Table 3: E_{max} and EC_{50} Values from DAMGO Stimulated [^{35}S] GTPγS Binding by Brain Area

Region	EC50 (μM)		Emax (%)	
	Sham	CCI	Sham	CCI
PAG	0.139 ± 0.015	0.124 ± 0.012	139.3 ± 8.17	127.9 ± 3.81
M.T	0.130 ± 0.010	0.132 ± 0.016	127.2 ± 5.01	108.6 ± 5.41*
ACC	0.280 ± 0.069	0.441 ± 0.10	42.74 ± 4.39	39.33 ± 4.36

Table 3: The E_{max} and EC_{50} values of DAMGO stimulated [^{35}S] GTPγS binding by brain area in mice at 10 days post-surgery. (*$p<0.05$).

To further investigate the effect of CCI on DAMGO stimulated G-protein activity in the medial thalamus, a time-course analysis was conducted. The time course showed that the E_{max} value of DAMGO was significantly decreased in CCI relative to sham mice only on day 10 and was not significantly different day 1 or 3 post surgery (Figure 26). This indicates that the desensitization of the μ-opioid receptor is due an upstream effect. There were no differences in EC_{50} values at any time point examined. There were no statistically significant differences in basal levels of [^{35}S] GTPγS binding in any of the brain areas examined at any time point which indicates that CCI induced desensitization in DAMGO-stimulated [^{35}S] GTPγS binding are specific to the μ-opioid receptor and not due to an overall decrease in the ability of GTP to bind to GPCRs in the medial thalamus.

[^{3}H] Naloxone Receptor Binding

In order to determine whether the decreases in DAMGO stimulated [^{35}S] GTPγS binding in the medial thalamus were due to a desensitization of the μ-opioid receptor or a decrease in the overall receptor density, [^{3}H] Naloxone receptor binding was conducted. In medial thalamus membranes of mice at day 10 post CCI surgery there were no significant differences in B_{max} ($p= 0.12$) or KD ($p= 0.22$) values between CCI and sham mice (Figure 27) indicating that there were no differences in overall μ-opioid receptor density between CCI and sham mice.

Discussion

Chronic constriction injury of the sciatic nerve produced a significant decrease in DAMGO stimulated [^{35}S] GTPγS binding in the medial thalamus of mice when compared to sham operated controls. This effect was seen at day 10 post CCI surgery, but not at days 1 or 3. The reduction in GTPγS binding in the CCI mice does not appear to be due to an overall reduction in μ-opioid receptor density, as there were no significant differences in [3H] DAMGO binding between day 10 post-CCI and sham groups. This pattern of decrease in μ-opioid receptor function without a decrease in μ-opioid receptor levels is similar to that reported by Ozaki et al. (2003) and Narita et al. (2006) in the

Figure 26: Differences in Means of E_{max} and EC_{50} values Comparing DAMGO Stimulated [^{35}S] GTPγS Binding in the MT by Post-Surgical Day

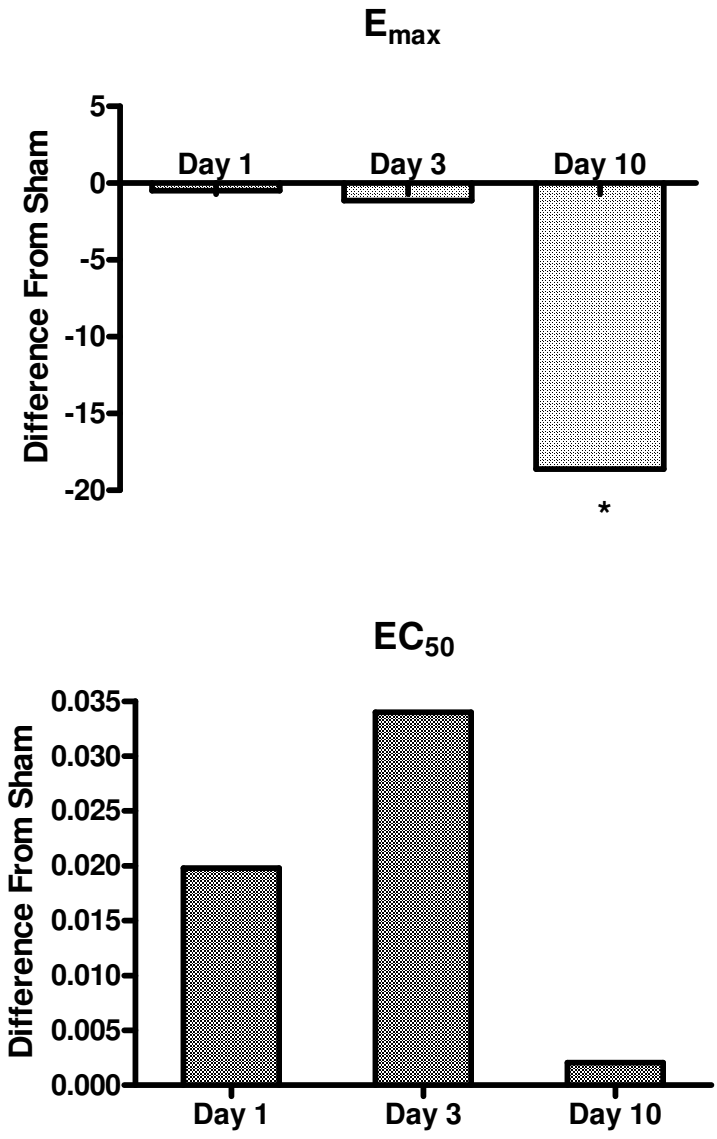

Figure 26: E_{max} and EC_{50} values are expressed as difference between the group means of CCI and sham mice. The time course shows that the E_{max} value of DAMGO was significantly decreased in CCI relative to sham mice only on day 10 and was not significantly different day 1 or 3 post surgery. There were no significant differences in EC_{50} values at any time point examined.

Figure 27: [3H] Naloxone Receptor Binding in the MT at Day 10 Post-Surgery

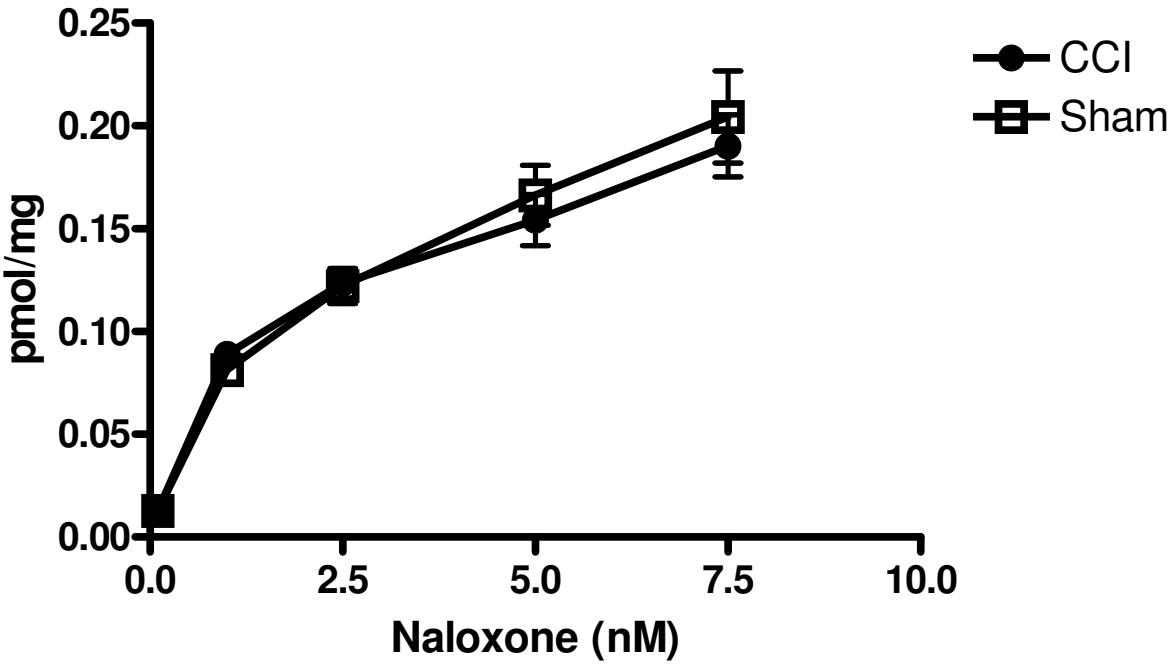

Figure 27: In medial thalamus membranes of mice at day 10 post CCI surgery there were no significant differences in B_{max} (p= 0.12) or KD (p= 0.22) values between CCI and sham mice (n = 6).

amygdala and ventral tegmental area following CCI in the mouse. Both of these brain areas have been reported to be involved in the affective component of pain processing (Sotres-Bayon et al., 2001) and morphine directly injected into the amygdala and VTA has been shown to produce analgesia (Altier et al., 1998; Nandigama et al., 2003). The lack of significant differences in density in our study as well as the study of Ozaki et al. (2003) and Narita et al. (2006) is contrary to human imaging studies in chronic pain patients. PET studies μ-opioid receptor binding demonstrate that μ-opioid receptor binding decreases in several brain areas including the cortex and medial thalamus in human peripheral as well as central neuropathic pain patients (Jones et al., 2004; Maarrawi et al., 2007; Willoch et al., 2004) for at a minimum of 3 months, a time period considerably longer than that experienced by mice in the CCI model. Thus, it is possible that neuropathy-induced changes in opioid receptor binding are time dependent. Moreover, all of these chronic pain patients had, at some point, been given a regimen of opioid analgesics which may account for the differences in our findings.

There were no significant differences in DAMGO stimulated GTPγS binding in the PAG which is consistent with other reports of opioid stimulated GTPγS binding in this region following CCI (Narita et al., 2008). This may indicate that the reduced efficacy of opioids following chronic neuropathic pain-like state is specific to changes in supraspinal brain areas involved in the affective component of pain processing rather than pain modulation.

A potential mechanism to explain the reduced efficacy of opioids in the medial thalamus of CCI mice could be that there is an increase in endogenous opioid release following nerve injury that causes desensitization of medial thalamic μ-opioid receptors. Considering that the reduction in G-protein activation in CCI mice was observed only at day 10 and not days 3 or 1 post surgery supports this hypothesis. Prolonged acute pain has been reported to increase levels of ß-endorphin in several brain areas including the medial thalamus (Porro et al., 1988; Porro et al., 1991) Elevated levels of endomorphin, another putative endogenous ligand for the mu-opioid receptor, were also observed in the

rat brain following CCI surgery (Sun et al., 2001). Chronic neuropathic pain was demonstrated to cause a reduction in tolerance to the pharmacological effect of morphine and increased phosphorylation of μ-opioid receptors in the striatum of the mouse. However, in ß-endorphin knockout mice these effects were abolished (Petraschka et al., 2007). Additionally, Nikkura et al. (2008), reported that sciatic nerve ligation induced a decrease in DAMGO-stimulated [^{35}S] GTPγS binding in the VTA of wild-type mice but not in ß-endorphin knockout mice.

In conclusion, we have demonstrated that CCI resulted in decreased μ-opioid receptor mediated G-protein activation in the medial thalamus of mice, an area which has been shown to be involved in the affective component of pain processing. This effect was not due to an overall decrease in μ-opioid receptor density suggesting that the chronic pain-like condition produced by CCI resulted in a desensitization of the μ-opioid receptor. These data coupled with a lack of significant differences in opioid-induced G-protein activity in the PAG suggests that the reduced analgesic efficacy of opioids in chronic neuropathic pain may be at least partially attributed to reduced μ-opioid receptor mediated signaling in the medial thalamus.

General Discussion

Chronic pain is a significant public health problem. It generates an extraordinary financial burden to society and inflicts millions with daily suffering that is often undertreated or resistant to therapeutic interventions. This is due, in part, to our poor understanding of the pathophysiological mechanisms that underlie the development and maintenance of chronic pain states. Therefore, strategies directed toward unraveling the complex biology of chronic pain are of preeminent clinical importance. To this end, the series of studies presented in this dissertation were designed to examine the effects chronic neuropathic pain on the expression and function of molecular targets implicated in the processing and modulation of pain. These studies indicate that chronic constriction injury of the sciatic nerve in the mouse increases mRNA expression of the ERK cascade kinase, raf-1 in the PAG which is part of the descending pain pathway and alters function of the μ-opioid and cannabinoid receptors in different brain areas of the medial pain pathway

CCI induced a 2.5 fold increase in mRNA of the kinase Raf-1 in the PAG of mice. Raf-1 is part of the ERK cascade in the MAP kinase family of proteins. The PAG is a critical regulator of nociceptive input and is part of the descending pain pathway. The descending pain pathway has been shown to have the ability to both inhibit and facilitate nociceptive transmission and has been implicated in central sensitization, contributing chronic pain states (Heinricher et al., 2009; Vanegas et al., 2004). Upregulation of MAP kinases including ERK in the spinal cord have also been implicated in the establishment and maintenance of central sensitization (Hu et al., 2003). Additionally, pharmacologic inhibition of MAP kinases has been shown to reduce allodynia and hyperalgesia in neuropathic pain states, yet MAP kinase inhibitors do not appear to have an effect on basal levels of pain perception, giving further evidence of their specific role in chronic pain states (Ji et al., 2007). These data are the first to show a significant increase in levels of mRNA of a MAP kinase pathway protein in the PAG in response to neuropathic pain. Given that MAP kinase activation has been shown to contribute to the development of central sensitization spinal cord, and the PAG can facilitate the development of central

sensitization in the spinal cord, this upregulation of Raf-1 mRNA may indicate a similar pro-nociceptive role for MAP kinases in the PAG.

The increase in Raf-1 mRNA in the PAG lead us to investigate the effects of CCI on the protein levels and activity of ERK in the PAG as well as the spinal cord and medulla, which are part of the lateral and descending pain pathways, and the medial thalamus and ACC, which are part of the medial pain pathway. We did not detect any significant differences in ERK levels or activity in any of those CNS regions in the mouse. Previous studies have shown an upregulation of p-ERK in the spinal cord of rats using differing methodology as well as differing pain models. These findings highlight the differences amongst species as well as individual neuropathic pain models in this field of research. Species differences in pain research are an issue which has been the subject of ongoing debate in the field. Differences amongst species and strains amongst the same species have been found to occur in behavior as well as physiological outcomes in response to painful states (Max et al., 2008; Mogil, 2009).

Chronic constriction injury of the sciatic nerve produced a significant decrease in DAMGO stimulated [^{35}S] GTPγS binding in the medial thalamus and WIN 55, 212-2 stimulated [^{35}S] GTPγS binding in the ACC of mice when compared to sham operated controls. The μ-opioid receptor and the cannabinoid receptor systems have well established roles in the modulation of nociceptive processing and pain states and these studies were the first to show a change in μ-opioid and cannabinoid receptor functioning in the medial pain pathway in response to neuropathic pain. Most animal research on the establishment and maintenance of chronic pain has been focused on spinal mechanisms and descending mechanisms that are classically involved in the processing and modulation of nociceptive input. However, chronic pain is a disease state and recent human imaging studies have shown that patterns of brain activity differ greatly in human chronic pain patients when compared to patterns of brain activity in response to acute pain. These differences are also specific to the disease state or precipitating cause of chronic pain as patterns of brain activation have been shown to be unique amongst

differing chronic pain conditions (Apkarian et al., 2005; Baliki et al., 2009; Geha et al., 2007).

The medial pain pathway is involved in the affective component of pain processing and is altered in chronic pain states. Several studies conducted in human chronic pain patients have shown that changes in brain chemistry, activity and receptor density are altered in the patients in areas in the medial pain pathway such as the prefrontal cortex, cingulate cortex and thalamus. In some instances these changes in brain chemistry were correlated to the perceptual quality of pain in patients (de Leeuw et al., 2005; Grachev et al., 2000) and studies have shown a decrease in opioid receptor binding in the medial pain pathway of chronic pain patients (Jones et al., 2004; Maarrawi et al., 2007; Willoch et al., 2004). A limitation of imaging studies in human pain patients is that patients being studied have been suffering from their ailments for months to years and have undergone in many cases multiple pharmacological treatments. Animal studies have shown that chronic opioid treatment can induce desensitization of μ-opioid receptors in multiple brain areas including the areas involved in nociception (Martini et al., 2007; Sim-Selley et al., 2007). Thus, it is not entirely possible to determine whether CNS changes in μ-opioid receptor found in these patients are due to the chronic pain condition itself or to desensitization and downregulation due to pharmalogical treatment or if chronic pain patients have pre-existing differences in μ-opioid receptor levels. The studies presented in this dissertation show that neuropathic pain itself induces significant desensitization of μ-opioid receptor in brain areas critical to the regulation and processing of chronic pain states. Opioids have been shown to have reduced analgesic efficacy in both chronic neuropathic pain patients and animal models and our data suggest that this could be partially attributed to the desensitization of the μ-opioid receptor in response to neuropathic pain.

In conclusion, chronic neuropathic pain is hallmarked by extensive physiological changes throughout CNS, which makes treatment of this disease such a significant challenge. The data presented in this dissertation demonstrate the ability of a chronic neuropathic pain like condition to induce significant changes in the mRNA of proteins and activity of proteins shown to be involved in the establishment or regulation of chronic pain states.

These changes were observed in three separate supraspinal brain areas involved in either the descending or medial pain pathways, highlighting the extensive physiological alterations which occur in response to chronic neuropathic pain. The novel findings presented in this dissertation provide new areas of investigation for the treatment of this debilitating disease.

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Vita

Michelle Renee Hoot was born on June, 11th, 1982 in Southfield, Michigan and is a United States citizen. She received her Bachelor's of Science in Psychology with Honors, Cum Laude from Wayne State University in 2004. She received her Ph.D. in Neuroscience from Virginia Commonwealth University in June, 2009 and will be starting a postdoctoral fellowship at Johns Hopkins Hospital in Baltimore, MD in August, 2009.