ANALYZING MUSCULAR PAIN AND THE EFFECTS OF EXERCISE ON CHRONIC PAIN

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Abstract

Pain associated with chronic musculoskeletal disorders poses a major clinical problem and costs billions of dollars each year. The physical therapy profession has explored the physiology of exercise to control pain for many decades. Although we are far from understanding the underlying mechanism of exercise-induced analgesia, attempts have been made through body of this work to understand muscle pain and the role of exercise in mediating muscle pain utilizing animal models. The overall purpose of this dissertation was to examine muscle pain and the effect of aerobic exercise in reducing chronic pain induced by acidic saline in mice; this animal model mimics a human condition, fibromyalgia (FMS) and similar diseases that present with widespread hypersensitivity to mechanical stimuli**.** In addition, spinal activation of the immediate early gene, Fos, in response to gastrocnemius compression and hind paw palpation was investigated in the acid-induced pain model. Furthermore, the molecular basis by which exercise training can potentially control muscular pain in the chronic stage was investigated by exploring exercise-induced neurotrophin-3 (NT-3) synthesis.

All experiments were carried out in 2 different strains of female mice, C57Bl/6 and CF-1. Hypersensitivity to mechanical stimulation was tested in the cutaneous and muscle tissues utilizing standard methods of von Frey monofilaments, instrumented forceps and Fos expression of the dorsal horn spinal cells. The effect of aerobic exercise on chronic pain state was tested with behavioral (von Frey and instrumented forceps) and molecular (Reverse Transcription-Polymerase Chain

Reaction and Enzyme-Linked Immunosorbent Assay) measures. Two repeated intramuscular acid injections led to cutaneous mechanical hyperalgesia of bilateral hind paws in both strains of mice. In C57BL/6 mice, the effect of acid injections was limited to the contralateral muscle, indicating secondary muscle hyperalgesia. The CF-1 mice developed bilateral hind limb muscle hyperalgesia that lasted up to 2 weeks, suggesting chronic phase of muscle pain. This finding was confirmed with increased Fos activity in the corresponding spinal cord level. The increase in cutaneous hypersensitivity did not correlate with spinal Fos expression in either strain of mice. These results indicate that acid injections induced cutaneous and muscle hyperalgesia in female mice, yet the development of muscle pain is subjected to the variability of genetic background in both strains of mice.

The central projection of nociceptors from the paw and the gastrocnemious muscle evoked different Fos activation pattern in the spinal cord. Gastrocnemius compression resulted in rostrocaudal and centrolateral somatotopic organization of the ipsilateral dorsal horn in all laminae. In comparison, paw palpation resulted in Fos expression of mediocentral superficial laminae (I/II) of the ipsilateral side that was restricted to lumbar segments 4/5 (L4/5) and bilateral deep laminae (V/VI) of the entire lumbar spinal cord. Bilateral Fos activation was greater in C57BL/6 mice compared to CF-1 mice, demonstrating important baseline differences within the 2 strains.

NT-3 is known to act in an analgesic manner in the acid-induced model yet the molecular events of exercise training that cause upregulation of NT-3 have not

been investigated. Aerobic exercise training is likely to stimulate muscle spindle activity and blood flow to muscle cells, thus causing increase in NT-3 expression. Our results indicated that moderately intense level of aerobic exercise training was required to stimulate molecular changes in NT-3 expression, as observed in CF-1 mice. NT-3 expression did not change in response to mild exercise training with C57BL/6 mice, in the acute or the chronic stage. In addition, exercise training significantly decreased muscle pain and had positive effects on cutaneous sensitivity in CF-1 mice, whereas the effect of mild exercise training was less robust in C57BL/6 mice.

Some of the beneficial effects of exercise on chronic widespread musculoskeletal pain can be associated to increased NT-3 synthesis. The molecular events responsible for anti-analgesic actions of NT-3 are yet to be determined. However, we propose a central action of NT-3 by which exercise decreased widespread hyeralgesia since the exercise effect was noted bilaterally and on secondary hyperalgesia of multiple tissues. Thus, the explanation for pain modulation by exercise may be through mediating central sensitization by stimulating muscle afferents, as supported by our results. The lack of a strong analgesic effect of exercise training and NT-3 expression in C57BL/6 mice further strengthens the role of NT-3 as an anti-nociceptive molecule. In addition, similar to the exercise training, acid injections increased NT-3 expression within the muscle, suggesting a possible compensatory mechanism to reduce pain. This finding indicates that muscle has an endogenous system to protect against painful conditions.

In summary, the results of the present study suggest a novel finding of chronic muscle pain and the beneficial effect of aerobic exercise training on muscle pain in the acid-pain model. The study explores the role of NT-3 as a possible correlate by which aerobic exercise training may control the sensory aspect of pain processing. Our results also indicate different Fos activation patterns in response to mechanical perturbation to deep and cutaneous tissues which adds new information to our understanding of deep tissue nociception as well as its spinal distribution.

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Chapter 1

Introduction

1.1 Overview

Most people suffering from various chronic pain syndromes report deep tissue pain. However, our knowledge about chronic musculoskeletal pain is primarily based on rodent models of cutaneous pain. Most animal studies related to exercise training and its effect on hypersensitivity have exclusively focused on cutaneous pain models. The growing numbers and limited efficacy of current pharmacological interventions necessitate better understanding and management of deep tissue pain associated with chronic pain syndromes. Many human studies provide strong support for aerobic exercise training specifically for individuals with fibromyalgia syndrome (Goldenberg, Burckhardt, & Crofford, 2004; Meiworm, Jakob, Walker, Peter, & Keul, 2000; Redondo et al., 2004), and yet, very little is known about the central and peripheral neuromuscular mechanisms of exercise-induced analgesia.

Recent studies exploring the neuromuscular effects of physical training on healthy animals have indicated that selective neurotrophins, brain derived neurotriphin (BDNF) and NT-3 can be modulated by physical training (Gomez-Pinilla, Ying, Opazo, Roy, & Edgerton, 2001; Ying, Roy, Edgerton, & Gomez-Pinilla, 2003). The role of NT-3 in response to exercise in chronic pain has not been examined. In fact, very limited work has been conducted to show whether exercise can modulate sensory input in chronic muscular pain syndromes. The overall purpose of this dissertation was to analyze muscle hyperalgesia following acid injections, to explore activation of spinal cells in response to noxious mechanical stimulation, to tests the effect of exercise training on chronic widespread secondary

hyperalgesia, and to investigate molecular changes in NT-3 expression in response to exercise training using 2 strains of mice. We proposed that acid induces muscle pain and aerobic exercise training reduces the acid-induced muscle pain and increases NT-3 synthesis, providing a possible molecular link in the modulation of chronic muscle pain.

1.2 Pain

1.2.1 Historical Perspective

Nearly a century ago, Sherrington first described characteristics of pain sensory neurons and neurons with distinct threshold levels (Julius & Basbaum, 2001; Perl, 2007). During that time in the early 19th century, anatomists also reported the evidence of ascending pathway that originated from the spinal cord, with axons crossing over to the opposite side and ascended in the lateral white matter to the rostral brainstem. Since then, a substantial body of knowledge from basic science to clinical studies has accumulated in the field of pain. In recent years, much progress has been made in our understanding of processing and transmission of nociceptive information, particularly of the primary sensory neurons at the peripheral levels and the secondary neurons at the spinal level (Basbaum & Woolf, 1999).

1.2.2 Pain Generation

Tissue damage results in a release of chemicals triggering a nociceptive response through C- and A-delta afferent fibers. These afferents generate electrical

impulses that travel along nerve axons to the dorsal horn of the spinal cord. In the dorsal horn, an extremely complex chemical interaction, neurotransmission, takes place between the 1st and the 2nd order neurons. Many excitatory neurotransmitters are released and an influx of sodium and calcium ions take place, primarily in lamina I, II and V before information crosses over to the opposite side and travels to higher centers (Bolay & Moskowitz, 2002; Lawrence, Stroman, Bascaramurty, Jordan, & Malisza, 2004; Zigmond, 1999). Typically 3 distinct order of neurons are described to the generation of pain: 1st order neurons from the periphery to the spinal cord, 2nd order neurons from the spinal cord to the thalamus and 3rd order neurons from the thalamus to the cerebral cortex. The focus of this dissertation has been on the the 1st and 2nd order neurons.

1.2.3 Primary Afferents and Their Central Projections

Anatomically, there are 2 broad primary sensory neurons that convey painful information, A-fibers and C-fibers. A-fiber nociceptors are mainly Aδ that responds to noxious mechanical and chemical stimuli and thus are considered polymodal neurons (Julius & Basbaum, 2001; Treede, Meyer, & Campbell, 1998). Some Aδ fibers exclusively respond to heat stimulus. Aβ fibers mainly respond to mechanical stimuli but under certain conditions can elicit noxious chemicals. C-fiber nociceptors are most numerous in skin but also innervate other tissues. Most are polymodel as they respond to noxious chemical and heat stimuli. A small portion of C-fibers have a high threshold and respond to noxious mechanical stimuli (Light & Perl, 2003).

Beyond these two broad categories, there are suspected silent nociceptors that respond only to high-threshold or repeated stimuli and can be activated in acute inflammation (Mayer, Mao, Holt, & Price, 1999).

C-fibers are further divided into two distinct categories, peptidergic and purinergic, based on their responses to neurotrophic factors (Basbaum & Woolf, 1999) and their site of termination within the spinal cord (Bailey & Ribeiro-da-Silva; Hunt & Rossi, 1985; Nagy & Hunt, 1982). Peptidergic neurons synthesize and release neuropeptides, substance P and calcitonin gene-related peptide (CGRP), and express tyrosine kinase A (trkA) receptors for binding of nerve growth factor (NGF). These fibers terminate mainly in lamina I and the superficial layer of lamina II (Braz, Nassar, Wood, & Basbaum, 2005; Hunt & Mantyh, 2001; Levine, Fields, & Basbaum, 1993). This set of neurons is activated with inflammatory conditions and in clinical syndromes such as FMS (Basbaum & Woolf, 1999). Purinergic neurons express surface lectin receptors like IB4 and P2X3, and c-ret receptor for binding of glia cell derived neurotrophic factor (GDNF). These fibers terminate exclusively in the inner parts of lamina II, also called substantia gelatinosa (SG) (Bailey & Ribeiro-da-Silva; Bennett, 1999; Hunt & Rossi, 1985). Both groups of C-fibers respond to similar types of noxious stimulation and express the capsaicin-gated transient receptor potential venilloid 1 (TRPV1) receptor, which responds to noxious chemical and thermal stimulation (Caterina et al., 1997; Hunt & Mantyh, 2001). Besides these receptors, acid-sensing ion channels (ASIC) have also been identified and react to

the high level of acidity in inflamed tissue (Basbaum & Woolf, 1999; Molliver et al., 2005).

*1.2.4 S*econd Order Pain Neurons

The central projections of primary afferents connect with the dorsal horns neurons, called 2nd order neurons. Lamina I receives direct input from myelinated and unmyelinated nociceptor fibers, including heat and cold transduction, and contributes to the classic spinothalamic pathway that conveys information about the discrimination of pain. The 2nd order neurons that originate from lamina I and II are considered selective neurons. Another area of the spinal cord, located deep in lamina V-VII contain neurons that responds to a wide range of input from the periphery, called wide dynamic range (WDR) neurons. This area is considered diverse as multiple inputs from periphery converse here, and it acts as a relay center for numerous noxious and innoxious stimuli before the information reaches to the higher centers. The property of lamina I neurons is to signal the nature and the location of stimuli, whereas the response from the WDR neurons is not well defined (Perl, 2007).

It is worth describing the degree of response from these neurons. These afferent neurons respond to different intensities of stimuli called threshold. Low threshold neurons that respond to non-painful low-intensity stimuli are called mechanoreceptors Aα and Aβ when innervating the skin and type-I and type- II when innervating the muscle. In contrast, neurons that respond to high intensity threshold are considered Aδ and C nociceptors when innervating skin and III and IV

fibers when innervating muscle (Graven-Nielsen & Mense, 2001). Under pathological conditions, mechanoreceptors can behave as nociceptors or these nociceptors can lower their threshold, as discussed in the section of central sensitization.

1.2.5 Ascending Pain Pathways

Peripheral sites convey nociceptive information to the spinal cord. Information travels from the spinal cord to the thalamus or limbic system and then to the higher centers (Fig. 1a) (Hunt & Mantyh, 2001). In addition to the traditional spinothalamic pathway, multiple ascending pathways project from the spinal cord to the midbrain and perhaps to other cortical and subcortical areas in the chronic stage of pain. Five major ascending pathways have been identified and discussed in the literature (Almeida, Roizenblatt, & Tufik, 2004). The spinothalamic tract is the most prominent ascending tract. This track carries information from lamina I of nocicepive-specific and laminae V-VII from WDR neurons of the dorsal horn. These neurons project to the contralateral side and ascend in the anterolateral white matter terminating in the thalamus. These neurons provide the discriminatory information of pain stimuli. The spinomesencephalic tract or spino-parabrachial pathway also originates in lamina I and terminates in the limbic system. This tract innervates the areas of the brain that are concerned with affective and emotion components of pain, including the reticular formation, periaqueductal gray and parabrachial nucleus Fibers from the spinoreticular track connect information from the spinal cord to the

reticular formation of medulla. This tract comprises the axons of neurons in laminae VII-VIII. It ascends in the anterolateral quadrant of the spinal cord and terminates in both the reticular formation and the thalamus. Axons of this track do not cross the midline. The cervicothalamic tract arises from neurons in the lateral cervical nucleus, located in the lateral white matter of the upper two cervical segments of the spinal cord. The lateral cervical nucleus receives input from laminae III and IV. They cross the midline and ascend in the medial lemniscous of the brainstem with some projections through the dorsal columns of the spinal cord. The spinohypothalamic tract is made up of axons of neurons from laminae I, V, and VIII that projects directly to surpraspinal autonomic control centers and is thought to activate complex neuroendocrine and cardiovascular responses.

Of all these distinct pathways, the spinothalamic and spinomesencephalic tracts are considered the main pain processing pathways (Fig. 1a) (Hunt & Mantyh, 2001). The thalamus is a relay center where nociceptive input regarding type, temporal pattern, intensity and topographic location of pain is encoded prior to reaching limbic and other subcortical sites (Renn & Dorsey, 2005). Ultimately the nociceptive information reaches the cortex where it is integrated and undergoes cognitive and emotional interpretation. Regions of cortical and subcortical areas involved are somatosensory cortex (S1 and S2), anterior cingulated cortex, and medial prefrontal cortex (Craig, 2003b; Renn & Dorsey, 2005).

1.2.6 Descending Pain Pathways

Cells in the midbrain and forebrain invariably project directly or indirectly to the areas of the spinal cord that originate the ascending pathways. These neurons make up the descending pathways that originate from the amygdala and hypothalamus and project to periaqueductal grey (Hunt & Mantyh, 2001; Vanegas & Schaible, 2004). Neurons from this region project to the lower midbrain and then to the dorsal horns (Fig. 1b). In the dorsal horns, descending pathways interact with both pre-synaptic and post-synaptic cells to influence incoming information. It is now clear that descending influence from the rostral ventromedial medulla (RVM) acts in both an inhibitory and facilitatory fashions (Vanegas & Schaible, 2004). The inhibitory descending influence on the spinal dorsal horn nociception is well studied and supports the anti-analgesic effect of exercise training (Bement & Sluka, 2005; Hoffman et al., 2004; Sparling, Giuffrida, Piomelli, Rosskopf, & Dietrich, 2003). The existence of descending facilitatory pathway is also recognized but less understood.

Anatomical information of the nociceptive system is provided to reveal the complexity and difficulties associated with chronic pain syndromes. The multifaceted reciprocal nature of pain creates a system where connections at any level could fail and a painful condition could perpetuate into the chronic stage. The complex nature of pain puts many challenges on health professionals to implement intervention strategies. Therefore, it is vital to improve our understanding of pain processing and intervention techniques targeted toward the chronic phase.

1.3 Chronic pain

Chronic pain differs from acute pain in its onset, duration and underlying mechanisms (Carr & Goudas, 1999). As stated in the review by Wang et al. (Wang & Wang 2003), chronic pain may not have an apparent source of nociception or an identifiable ongoing injury or inflammation, and often responds poorly to pharmacological interventions. Normally, pain sensation results from specific activation, not hyperactivation, of the nociceptors in response to mechanical, thermal or chemical stimulus (Almeida et al., 2004). The physiological process of nociception in the spinal cord or supraspinal regions is altered in chronic pain that is characterized as maladaptive and persistent in nature (Almeida et al., 2004; Basbaum, 1999; Blomqvist & Craig, 2000; Bolay & Moskowitz, 2002; Holden & Pizzi, 2003; Kramis, Roberts, & Gillette, 1996; Mogil, Yu, & Basbaum, 2000; Sluka, 1996). In chronic conditions (McMahon & Jones, 2004) or with damage to the nervous system (Desmeules et al., 2003), a shift in the pain-signaling system occurs such that noxious stimuli applied to the injury site produce more pain, or the pain threshold necessary to generate pain decreases. This increased excitability of sensory neurons is called sensitization (McMahon & Jones, 2004; Sluka, 1996). The increased sensitivity of pain to previous noxious stimuli in the injured area is termed primary hyperalgesia (Adams & Sim, 2005; Blomqvist & Craig, 2000; Bolay & Moskowitz, 2002; McMahon & Jones, 2004; Sluka, 1996; Willis, Sluka, Rees, & Westlund, 1996) which results from either the peripheral or the central nervous system dysfunction (Bolay & Moskowitz, 2002). When pain sensation is perceived

beyond the injured area, it is considered secondary hyperalgesia, resulting from changes in the central nervous system (McMahon & Jones, 2004; Sluka, 1996; Sluka, Kalra, & Moore; Willis et al., 1996) and is similar to referred pain symptom in humans. The hypersensitivity becomes most pronounced when even a non-noxious stimulus, like light touch or pressure, causes a painful response and an avoidance behavior, indicating a state of allodynia (Blomqvist & Craig, 2000; McMahon & Jones, 2004). The occurrence of allodynia indicates that innocuous stimuli in some way have activated the nociceptive system (Blomqvist & Craig, 2000). Spinal sensitization is presumed to be a result of either increased release of excitatory substances from primary afferents, from spinal neurons or decreased activity of inhibitory descending pathways, resulting in enhanced activity of dorsal horn neurons. The sensitization associated with chronic pain state can occur as a result of one or more of the following mechanisms.

Hypersensitivity of WDR neurons: One possibility suggests that WDR neurons play a fundamental role in the spinal cord of central sensitization. WDR neuronal cells are located in lamina V (and I, II, IV and VI) of the dorsal horn and serve as relays for interaction of noxious and non-noxious stimuli. In general, these neurons assist in segmental suppression of pain because of the convergence of sensory afferents of A-δ, A-β and C-fibers (Almeida et al., 2004; Bolay & Moskowitz, 2002). However, once these neurons become activated and sensitized, they signal non-noxious stimuli as painful or the information is misinterpreted by supraspinal structures, and a perception of pain may be generated when for example,

only touch has occurred (Bennett, 1999; Kramis et al., 1996; Mogil et al., 2000). Convergence of sensory input from various tissues in the dorsal horn is the best anatomical explanation for referred pain and central sensitization seen in many clinical syndromes (Bennett, 1999).

Sprouting and reorganization of spinal cord neurons: Another explanation for central sensitization comes from studies of neuropathic pain (Blomqvist & Craig, 2000; Hoseini, Hoseini, & Gharibzadeh, 2006; Lekan, Carlton, & Coggeshall, 1996; Wilson & Snow, 1993; Woolf, Shortland, & Coggeshall, 1992). Neuropathic pain can lead to reorganization of spinal cord dorsal horn neurons, including induction of new genes, sprouting of primary afferent neurons of Aβ from lamina III and IV to laimna II or up-regulation of a various neurotransmitters, their receptors and second messengers. This rewiring leads to misinterpretation of non-noxious as noxious input. Hence, low-threshold stimuli activation of Aβ can cause central hyperexcitability.

Phenotypic switching of Aβ: In neuropathic pain, some large diameter low-threshold afferents that are normally not involved in nociception start to synthesize substance P and CGRP. Thus, they start communicating with spinal neurons that express substance P and contribute to central sensitization (Basbaum & Woolf, 1999). These changes lead to increased excitability of dorsal horn neurons and contribute to the spontaneous firing of an action potential (Basbaum & Woolf, 1999; Hoseini et al., 2006) seen in conditions of nerve injuries.

Altered input from descending pathway: Decreased activity of inhibitory pathways or increase activity of facilitatory pathways have also contributed to central sensitization. Many syndromes, such as FMS or low back pain where defined tissue pathology is absent may be associated with a potential dysfunction in the descending pathways (Bennett, 1999; Leffler, 2002). A recent article suggests the involvement of the facilitatory descending pathway in the origination and the maintenance of widespread hypersensitivity in the acid-induced animal model (Tillu, Gebhart, & Sluka, 2007). It is worth noting that the acid-induced chronic hyperalgesia model used in the present dissertation causes secondary or referred hyperalgesia, primarily measured for its cutaneous hypersensitivity. *In this dissertation, we attempt to assess deep tissue hyperalgesia in both the acute and chronic stages following acid injection and determine whether animals experience muscle hyperalgesia (Chapter 2).*

1.3.1 Differences between Muscle and Cutaneous Nociception

Muscle pain is different than cutaneous pain in many ways. The classification of sensory neurons is based on the conduction velocity and the cross-section of fiber diameter. The diameter and conduction velocities of afferent fibers vary slightly from tissue to tissue. In skin, sensory neurons that are myelinated are labeled as Aα, Aβ, Aδ, and those that are unmyelinated are called C-fibers or free nerve endings, all named from fastest to slowest conducting velocities. In deep tissue, sensory neurons are classified as I, II, III and IV, from largest to smallest diameter size (Sluka, 1996;

Almeida, 2004). The skeletal muscles are innervated by a large range of sensory axons, 60% myelinated and 40% unmyelinated (Graven-Nielsen and Mense, 2001). Primary muscle nociceptive afferents are group-III and group-IV, as classified based on the diameter size, where corresponding cutaneous Aδ and C-fiber are classified according to their conduction velocity.

Rather than a propagated action potential that occurs with cutaneous free nerve endings, the stimulation of muscle nociceptive nerve ending causes graded potential of receptors that primarily depend on the strength of the stimuli. In muscle, the local receptors build currents and then elicit an action potential if the stimulus is strong enough to depolarize the membrane to its threshold level. In humans, the conduction velocity is between 3.1 – 13.5 m/s for group III and in the range of .6 to 1.2 m/s for group IV (Graven-Nielsen & Mense, 2001).

Muscle fibers are considered high threshold and polymodal (Graven-Nielsen & Mense, 2001) and are able to respond to a variety of stimuli. Both peptidergic and purinergic expressing receptor type nociceptors are likely to be present in muscle tissue for many algesic substances like bradykinin, serotonin, prostaglandin, adenosine triphosphate, present in the muscle (Graven-Nielsen & Mense, 2001). However, the muscle fibers proper are spared from nociceptors, explaining why muscle degeneration does not cause muscle pain. Nerve endings are located in the lining of blood vessels and fascia connective tissue covering the muscle bundles (Mense, 1993; Mense; Mense & Meyer, 1985), providing the explanation for muscle pain when muscle is overly stretched or inflammed. Experimentally, muscle pain can

be elicited by squeezing a muscle in rats and humans (Graven-Nielsen & Mense, 2001). *This technique was applied in the present study to assess muscle hyperalgesia in mice subjected to acid injections.*

The perception of pain originating from deep tissues of muscle and joints and their central projections to the spinal cord is different compared to cutaneous pain. Cutaneous pain is sharp and specifically localized whereas muscle pain is characterized as aching, cramping, diffuse in nature, longer lasting and has referred localization due to its convergence of multiple input to WDR neurons in the spinal cord and brainstem (Graven-Nielsen & Arendt-Nielsen, 2002; Mense, 1991).

Even the mechanisms of pain and hyperalgesia induced by injecting inflammatory agents into different tissues (muscle, joint and skin) are thought to be different. Stimulation of C-fibers from a muscle nerve causes a long-lasting enhancement of the ventral root reflex compared to C-fibers from a cutaneous nerve. When capsaicin is injected into a muscle, it produces longer-lasting hyperalgesia. The central projection of the primary afferents from deep tissue mainly terminates in lamina I and deep regions of the dorsal horns. The central projection from cutaneous afferents is in lamina I and II (Graven-Nielsen & Arendt-Nielsen, 2002). This clearly indicates that the central projections for both tissues are different and should be recognized when conducting behavioral testing from cutaneous and deep tissues.

1.4 Chronic Pain Syndromes

A range of conditions fall under chronic musculoskeletal pain syndromes such as FMS, chronic low back pain, arthritis and headaches (Aggarwal, McBeth, Zakrzewska, Lunt, & Macfarlane, 2006; Goldenberg et al., 2004). Each year, approximately 97 million Americans suffer from chronic pain syndromes (*Brain Facts*, 1997) and about 12% of all adults in other developed countries. According to the International Association for the Study of Pain, pain is defined as, "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". This definition describes pain more as a *perception* rather than a true *sensation*; for many people who suffer from chronic pain conditions, this perception is real with or without obvious tissue damage. Cognition and emotion are integrated in the perception of pain that guides one's behavioral response to pain.

FMS is an example of such a persistent pain syndromes (Goldenberg et al., 2004) and is characterized by widespread musculoskeletal aching, altered biochemical substances, serotonin and substance P (Desmeules et al., 2003), and hypersensitivity to mechanical, thermal and electrical stimuli, suggesting abnormality in central pain mechanisms (Goldenberg et al., 2004; Jones, Clark, & Bennett, 2002; Staud, Price, Robinson, Mauderli, & Vierck, 2004; Whiteside, Hansen, & Chaudhuri, 2004). These characteristics could present with or without an obvious injury (Desmeules et al., 2003; Kramis et al., 1996) as people with FMS and other chronic musculoskeletal pain syndromes report similar symptoms that cannot

be traced to a specific structure. According to the American College of Rheumatology, nearly 6 million Americans (Jones et al., 2002), which is 2% of population or 1 every 73 people, present with FMS (Goldenberg et al., 2004). In addition, a substantial number of patients (~22%) with whiplash injuries develop widespread pain. Three main areas appear to play major roles in the development of FMS: central nervous system (CNS), musculoskeletal system and neuroendocrine system.

Central Nervous System

Adaptation in the CNS is the main culprit in the development of FMS. Several studies have reported that patients with FMS demonstrate altered levels of neurotransmitters, brain hormones and neuropeptides (Bennett, 1999; Russell, 1998a, 1998b; Staud et al., 2004; Weigent, 1998). Increased cerebral spinal fluid (CSF) level of substance P, a pro-nociceptive substance, and decreased serum levels of serotonin, an inhibitory substance, in patients with FMS suggest hyperexcitability of the dorsal horn spinal neurons and thus, suggests a mechanism of central sensitization (Russell, 1998a, 1998b; Weigent, 1998). Activation of neurons with release of excitatory amino acids (SP, CGRP, and dynorphin) can over time lead to functional alterations in the dorsal horn neurons and increase nociceptive transmission to the brain (Russell, 1998a; Weigent, 1998). In addition, substance P causes an increase in the size and number of receptive fields of nociceptive neurons and decrease in the threshold level of post-synaptic potentials (Weigent, 1998). This

alteration may lead to the classic sign of widespread muscle pain and lowered muscle threshold which is the primary symptom of FMS.

Musculoskeletal System

The presence of tender points has also been linked to the local metabolic changes found within the muscle and abnormalities in muscle energy metabolism. People with FMS have increased resting level of phosphocreatine, a molecule rich in energy that generates adenotriphosphate (ATP), and decreased level of cellular oxidative enzymes and tissue oxygenation, leading to perception of weakness and fatigue. Muscle biopsy reveals non-specific type II fiber atrophy with a moth-eaten and banded appearance of muscle fibers. At present, it is believed that the skeletal muscle findings are altered but are not the origin of FMS.

Neuroendocrine system

Evidence also suggests alteration of many endocrine-derived hormones, including growth hormone, cortisol, thyroid-stimulating hormone, and abnormalities in hypothalamic-pituitary adrenal axis (HPA) in patients with FMS (Staud, Vierck, Cannon, Mauderli, & Price, 2001; Tanriverdi, Karaca, Unluhizarci, & Kelestimur, 2007; Vierck, 2006; Wingenfeld et al., 2008). These hormones are important for tissue healing and stress responses. Thus caution must be taken to avoid tissue injury and overexertion when prescribing exercise training to people with FMS.

1.4.1 Referred Pain Symptom

Substantial clinical evidence exists regarding referred muscle pain from trigger point pressure in various musculoskeletal conditions. People with FMS have increased referred pain area and heightened temporal summation, suggestive of central sensitization. Sörensen et. al. first reported that people with FMS display musculoskeletal pain due to central sensitization. These people experienced pain in areas of lower limb muscles after intramuscular injection of hypertonic saline, where normally people with FMS do not experience ongoing pain, suggesting central sensitization (Sorensen, Graven-Nielsen, Henriksson, Bengtsson, & Arendt-Nielsen, 1998). Since then others have reported central sensitization in people with FMS and other musculoskeletal syndromes (Banic et al., 2004; Desmeules et al., 2003; Lidbeck, 2002; Staud, 2004). The symptom of referred pain is related to the secondary hyperalgesia observed in many animal models of pain. The underlying causes and characteristics of secondary hyperalgesia differ among various pain models.

1.5 Use of Animal Models in Studying Pain

Animal models of pain have provided many advances in our understanding of pain mechanisms. Many laboratory animal models of pain have been produced to mimic human painful conditions. These models of pain can be broadly divided into three types: 1) neuropathic pain that results from injury to the nervous system at central, spinal or peripheral levels; 2) nociceptive pain that rises from direct

stimulation of nociceptors; and 3) muscle pain by direct stimulation of muscle nociceptors. Here, the muscle is described as a separate entity since muscle nociception can be elicited by different means.

1.5. Models of neuropathic pain

Most neuropathic pain models are made by inducing diseases or causing injuries to spinal or peripheral nerves. The mechanism of neuropathic pain and its symptoms are quite different then other pain types. The description of neuropathic pain consists of burning, shooting, electrical shock or piercing quality. The direct evaluation of spontaneous pain is not possible in animal models. Therefore evoked pain, such as allodynia and hyperalgesia to thermal and mechanical stimuli, are observed (Wang & Wang, 2003; Wiesenfeld-Hallin et al., 1997).

1.5.1.1 Contusion / Weight Drop Model

This is the oldest model in which damage is induced by dropping a weight on a surgically exposed dorsal spinal surface at the thoracolumbar junction. This causes severe paraplegia and complete segmental necrosis. A complete transaction model has also been used. These models are quite traumatic to the nervous system and result in abnormal discharge of dorsal horn neurons with mechanical stimulation (Wang & Wang, 2003).

1.5.1.2 Neuroma Model

Complete nerve transaction at multiple sites along the sciatic nerve in rats and mice leads to development of a neuroma at the proximal nerve ending, mimicking the clinical condition of phantom limb pain secondary to amputation.

This causes symptoms of allodynia, hyperalgesia and autotomy behavior (self-mutilation of the denervated limb by animals) (Wang & Wang, 2003; Wiesenfeld-Hallin et al., 1997).

1.5.1.3 Chronic Constriction Injury Model (CCI)

This model was developed in 1988 by Bennett and Xie. By loosely tying the sciatic nerve bilaterally at mid thigh level, some afferents are spared. This type of nerve injury causes some damage to myelinated fibers and severe loss of C-fibers, leading to allodynia, hyperalgesia, cold allodynia and avoidance of weight bearing on limbs. These symptoms can lasts up to 2 months (Wang & Wang, 2003).

1.5.1.4 Partial Sciatic Nerve Ligation Model

This model was developed later (Seltzer, Dubner, & Shir, 1990) and simulates partial nerve injury in humans. The sciatic nerve is partially ligated at mid thigh level so that 1/3 to ½ thickness of the sciatic nerve is trapped in the ligature. Animals exhibit allodynia to a von Frey monofilament, cold and hyperalgesia to thermal and mechanical stimuli that lasts up to 7 months.

1.5.2 Models of Nociceptive Pain

There are many nociceptive pain models that mostly consist of injecting some type of inflammatory substance into cutaneous or deep tissues.

1.5.2.1 Monoarthritis or Polyarthritis

Several inflammatory agents like carageenan, kaolin or mustard oil, can be injected into the knee or the ankle joint to induce monoarthritis symptoms

demonstrated as decreased mechanical and thermal sensitivity in animals. An intradermal injection of complete Freund's adjuvant (CFA) into a hind paw results in polyarthritis that develops over time and lasts up to several months. CFA is an antigen emulsified in mineral oil to stimulate T-cell immunity. Animals display behavioral symptoms of hypersensitivity to mechanical pressure and heat, vocalization and edema.

1.5.2.1 Formalin

Diluted formalin is injected subcutaneously into a hind paw that gives rise to 2 stages of inflammatory pain in humans and animals, early and late phase. During the early phase, nociception is caused by direct chemical activation of nociceptors that results in excitation of the spinal cord neurons and lasts for 5 – 10 minutes. The late phase, lasting up to 15-60 minutes, is due to the hyperexcitability of the spinal cord neurons that release neuroactive mediators as a model of central sensitization. Animal behaviors of paw licking and paw flicking are noted during both phases of inflammation.

1.5.3 Models of Muscle Pain

Pain originating from cutaneous and muscle tissue has distinct features. Thus attempts have been made to develop animal models to mimic deep tissue pain and specific human painful conditions (Wang, 2003).

1.5.3.1 Chemical Stimulation

In humans and animals, intramuscular injection of capsaicin, potassium chloride or hypertonic saline as well as acid of pH 5.2 induces muscular pain. The model of hypertonic saline was first introduced by Kellgren and Lewis in 1930s and has been exclusively used to characterize acute clinical muscle pain (Graven-Nielsen & Mense, 2001; Graven-Nielsen & Mense, 2002).

1.5.3.2 Ischemic Contraction

This is a classic experimental muscle pain model. A tourniquet is usually applied around a limb for a time sufficient to cause ischemic muscle pain. Interestingly, ischemic contraction in animals affects a very small portion of muscle nociceptors, showing the functional diversity of muscle nociception (Graven-Nielsen, Arendt-Nielsen, & Mense, 2002; Graven-Nielsen & Mense, 2001).

1.5.3.3 Electrical Stimulation of Muscle Nociceptors

Physiologic properties and sensitivity of muscle nociceptors can be examined by use of intramuscular electrical stimulation. However, the major drawback of this method is that electrical stimulation bypasses receptor transduction and directly depolarizes the afferent fibers. Secondly the non-nociceptors with lower threshold are stimulated before nociceptors (Graven-Nielsen et al., 2002; Graven-Nielsen & Mense, 2001), opposite of the size recruitment principle that was first described by Sherrington. This poses a clinical problem in determining nociceptive properties in humans or animals.

1.5.3.4 Delayed Onset Muscle Pain (DOMS)

The mechanism underlying DOMS is different than ischemic contraction and characterized by inflammation and muscle weakness. DOMS is a result of ultrastructural damage to muscle fiber that causes release of algesic substances and inflammation. Eccentric treadmill training with high running speed for 10-15 minutes at downhill incline has been used in animal studies to mimic human DOMS. Muscles develop soreness 24 to 48 hours after the eccentric work load. The soreness is mainly caused by a peripheral mechanism. A classic feature of DOMS is absence of pain at rest but presence with muscle function or palpation (Graven-Nielsen & Mense, 2002; Kehl, 2003).

1.5.3.4 Model of Acid-Induced Hyperalgesia

An animal model of FMS-type pain mediated by acid injections has recently been developed (Sluka et al., 2001). Two repeated injections of low pH (4.0 saline) into the gastrocnemius muscle induce bilateral mechanical, but not thermal, hyperalgesia that is persistent for at least 4-6 weeks. The mechanical hypersensitivity develops within 4 hours following the 2nd injection (Sluka et al., 2001), demonstrating a shift in sensation perception from touch to pain. This widespread mechanical hyperalgesia is dose and time dependent; interinjection interval of 2 or 5 days, not 10 days, produces equal and significant decrease in mechanical withdrawal threshold bilaterally and the lowest pH (pH 4.0) produces the greatest hyperalgesia compared to pH level of 5.0, 6.0 and 7.2 (Sluka et al., 2001). This widespread somatic pain also extends to the visceral sensation that lasts up to 2 weeks. Increased electromyography (EMG) response from the external oblique muscle to colorectal

distention was reported in rats following acidic injections into the gastrocnemius muscle (Miranda, Peles, Rudolph, Shaker, & Sengupta, 2004).

It is hypothesized that when low pH acidic saline is injected into muscle fibers, the tissue pH drops to a maximum of 6.0 levels for approximately 6-8 minutes. The local change in the intramuscular pH is thought to activate acid-sensing ion channel-3 (ASIC-3) receptors (acid sensors), generating currents in small and large diameter primary muscle afferents (Hoheisel, Reinohl, Unger, & Mense, 2004; Lee, Lee, & Oh, 2005; Molliver et al., 2005; Sluka et al., 2003). The signaling of these afferents in turn increases the resting activity of spinal WDR neurons, increasing their responsiveness to mechanical stimuli and causing bilateral widespread hyperalgesia (Sluka et al., 2001). This is evident by an increase in the firing frequency of WDR neurons, an increase in the receptive field of WDR neurons including hypersensitivity of the contralateral paw in response to noxious and non-noxious stimuli (Sluka et al., 2003) and a decrease mechanical threshold. The WDR cells of the convergence region (lamina V) also receive input from visceral afferents (Fib 2). As a result of viscerosomatic convergence, a noxious somatic stimulus is also capable of altering visceral sensation as evident in acid-induced pain model (Miranda et al., 2004) and in patients with FMS (Kwan et al., 2005; Lubrano et al., 2001). Therefore, the mechanism seems to be related to the changes in the spinal cord nociception (Skyba, King, & Sluka, 2002; Skyba, Lisi, & Sluka, 2005; Sluka et al., 2001; Sluka et al., 2003; Sluka, Rohlwing, Bussey, Eikenberry, & Wilken, 2002).

Figure 2 provides a schematic diagram to explain acid-induced hypersensitivity and central sensitization.

The central sensitization theory is further strengthened by other evidence: 1) anesthetic blockage to the ipsilateral grastrocnemius with lidocaine or elimination of the primary afferent input by ipsilateral dorsal rhizotomy does not alter mechanical threshold of contralateral hyperalgesia (Sluka et al., 2001), 2) spinal administration of μ- or δ- opioid receptors against N-methyl-D-aspartate (NMDA) or non-NMDA ionotropic glutamate receptor antagonists reverses cutaneous hyperalgesia (Skyba et al., 2002; Sluka et al., 2002), suggesting functional changes in the spinal neurons, 3) systemic administration of 10 mg/kg pregabalin increases the withdrawal threshold of paw and deep muscle (Yokoyama, Lisi, Moore, & Sluka, 2007), 4) it requires two injections to develop bilateral hyperalgesia; the 1st acid injection primes the nervous system in such a way that 2nd injection leads to widespread hypersensitivity, 5) intra-muscular acid injections increase phosphorylation of cAMP-response element binding protein (CREB) transcription factor in bilateral dorsal horns of the spinal cord (Hoeger-Bement & Sluka, 2003), and 6) acid injections increase the release of glutamate in the spinal cord (Skyba, et al., 2005).

Recent evidence suggests involvement of a supraspinal pain center. An injection of local anesthetic (ropivacaine) into the rostral ventromedial medulla (RVM) reversed secondary hyperalgesia. Rats failed to develop hypersensitivity when ropivacaine was administrated into nucleus raphe magnus (NRM) and the nucleus giganocellularis (Gi) prior to the 2nd acid injection, suggesting that

widespread hypersensitivity originates in RVM via facilitatory pathways (Tillu et al., 2007). All current evidence suggests that the mechanism that drives the development and maintenance of chronic hyperalgesia seems to be centrally oriented, either at the spinal or midbrain level.

However, ASIC-3 channels located on muscle primary afferent fibers are key features to inducing bilateral hyperalgesia as mice deficient in ASIC-3 fail to develop secondary hyperalgesia (Sluka et al., 2003). It should be noted that the structural and molecular mechanism to activate a nociceptive pathway by innocuous information beyond the involvement of ASIC-3 is not known in the development of acid-induced pain.

The concept of sensitization of muscle nociceptors is not new and may explain the reasons why some muscle injuries lead to Complex Regional Pain (CRP) syndrome. The mechanical sensitization associated with acid-injection is dose and time dependent. It is proposed that the 1st dose of acid is sufficient enough to cause sensitization of ASIC-3 receptors but not strong enough to lower their threshold. In addition, there might be some lingering effects of the primary or secondary afferents following the 1st acid injection. The 2nd dose of acid injection is needed to lower the threshold level to excite the receptors. The same concept is seen in human muscle inflammation in which first sensitization takes place and then excitation, unless the inflammatory substances are presented in high concentration. This explanation is provided to share some insight to the dose-dependency of the acid-model.

The time dependency of the acid-injection is difficult to comprehend, and it is not clear why the interinjection period needed to induce widespread pain in animals falls within 2-5 days apart. Many clinical studies suggest that central sensitization is time dependent. In humans, increased sensitization to pressure in a non-painful area has been associated with time. People with rheumatoid arthritis with pain symptoms greater than 5 years had increased secondary sensitization compared to those with pain less than 6 months (Leffler, Hansson, & Kosek, 2002). This fits well with the acid-model of central sensitization suggesting that a localized injury in the deep tissue causes somatosensory changes and leads to the development of central sensitization over time. This model explains the phenomenon of receptor sensitization that is seen in humans as a 2^{nd} dose of algesic substance such as bradykinin (BK) or prostaglandin (PGE) causes more pain than a single dose. If the theory of receptor sensitization is attributed to muscle pain, strategies to desensitize should also be examined with the same vigor.

Although several animal models (as described above) have been developed to mimic varying degree and symptoms of the chronic pain state, the work of this dissertation focuses on an acid model of chronic pain. This model of pain is classified as a non-inflammatory muscle pain model, making it a unique model to analyze exercise-induced behavioral and molecular changes at peripheral and central nervous system levels. Since the inception of the acid-induced pain model, it has been repeatedly shown that rats (Sluka et al., 2001) and mice (Gandhi, Ryals, & Wright, 2004) develop secondary cutaneous (Hoeger-Bement & Sluka, 2003; Skyba

et al., 2002; Skyba, Lisi et al., 2005; Sluka et al., 2001; Sluka et al., 2002) and visceral hyperalgesia (Miranda et al., 2004). The model is described as a muscle pain model based on the fact that injections are given into a muscle and the presence of ASIC-3 within muscle afferents are required for widespread hyperalgesia. However, it is unclear whether animals develop muscle hyperalgesia following intra-muscular injections. Therefore we assessed muscle hyperalgesia following acid injections in acute and chronic stage. This work is described in Chapter 2.

1.5.3.5 The Role of ASIC in Pain Generation

A decrease in intramuscular pH as a result of tissue injury or inflammation (Hoheisel et al., 2004), ischemia (Molliver et al., 2005) or manual injection of acidic solution into muscle activates nociceptors and produces pain sensation. A molecule that is responsible for proton sensitivity of nociceptors is ASIC, named for its ability to detect changes in the extracellular pH levels (Julius & Basbaum, 2001; Krishtal & Pidoplichko, 1981; Price et al., 2001). Lazdunski el al. first described a family of transmembrane protein ASICs belonging to the sodium ion channel family. The molecular mass of these channels is about 50 – 70 kDa. At present a total of 5 ASIC genes (1-5) are identified in animals (Lee et al., 2005). Of all ASICs, ASIC-3 is widely distributed in sensory neurons. Approximately 40% of DRG neurons express ASIC-3 (Molliver et al., 2005), diversely through both large and small diameter afferents (non-nociceptors and nociceptors) that innervate muscle (Molliver et al., 2005). They are considered important in muscle pain for both non-inflammatory

acid-induced muscle pain (Sluka et al., 2003) and inflammatory carrageenan-induced muscle pain (Ikeuchi, Kolker, Burnes, Walder, & Sluka, 2008; Sluka et al., 2007). ASIC-3 channels are not expressed in muscle spindles; rather they are expressed in small muscle afferents and lining of the muscle arterioles and blood vessels. These channels can open with even a small change in pH level (0.5 units) that occurs when a muscle is under metabolic stress or in an ischemic state. ASIC-3 channels respond to build-up of lactic acid following isometric exercise or when a muscle uses anaerobic metabolism providing a mechanism to explain exercise-induce pain. Based on nuclear magnetic resonance (NMR) of muscle cells, the intramuscular pH can drop up to 6.0 during ischemic or exhaustive exercise (Hoheisel et al., 2004; Pan, Hamm, Rothman, & Shulman, 1988). Similarly human studies show that intramuscular injections of acid at 5.2 pH level elicit intense pain (Steen, Reeh, Geisslinger, & Steen, 2000).

1.6 Methods of Pain Measures

Pain is an individual explaination that is often assessed by subjective reports. As animals are unable to report painful sensation, many behavioral measures are used to determine their nociceptive response; Bars and colleagues have provided a comprehensive review of all nociceptive measures used in animal pain models (Le Bars, Gozariu, & Cadden, 2001). Most models of pain rely on detecting a change in threshold to an applied stimulus. The following measures are considered reliable and therefore were used in the present studies.

1.6.1 Von Frey Monofilments

In 1884, Blix and Goldscheider first described discrete spots on skin that yielded different sensory experiences: touch, cold, warmth and pain (Craig, 2003). This concept was later supported by von Frey in 1896 (Craig, 2003). The stimulus of direct touch and tissue deformation is detected via ASIC. Beyond this ion channel family, other mechanically gated channels are not identified (Julius & Basbaum, 2001). Mice lacking these channels show reduced sensitivity to hair movement but normal response to noxious mechanical stimulus (Julius & Basbaum, 2001). Thus, the sensory processing of light touch is thought to be mediated through Aβ fibers and measured with von Frey monofilaments. Von Frey monofilaments are a series of approximately 2 cm length nylon monofilaments, with increasing diameter and known force (1-358 mN) and are often used in animal studies to test mechanical sensitivity. A filament is generally applied at the plantar surface of the hind paw to the point of bending. The animal's response of licking, withdrawing or shaking is recorded. Rats normally display high tolerance to even large bending force of monofilament but normal mice respond to 10 mN (a typical range for mice is 1-15) and their response is lowered to 1 mN with injury (Kehl & Fairbanks, 2003). This behavior from mice poses a challenge, and results should be interpreted with care. We used 1.0 g (10 mN) bending force with CF-1 mice and 0.6 g (6 mN) with black 57 mice to test their withdrawal responses to acid injection.

1.6.2 Muscle Compression Test

A common technique to assess deep tissue pain is to measure pain sensitivity to pressure. The direct method to measure deep tissue hyperalgesia of the knee joint was first established by Yu (Yu et al., 2002) and since, has been used to measure deep muscle hyperalgesia following acidic injections in rats (Yokoyama et al., 2007). We modified this method to fit a mouse model of acid-induced pain. Previous studies have shown that the mechanism of muscle withdrawal in acid-induced model is primarily based on the activation of muscle afferents as desensitization of skin overlying the muscle had no effect on the withdrawal response (Skyba, Radhakrishnan, & Sluka, 2005). It is difficult to compare thresholds from various pressure pain studies because different species, instrumentation and rates of pressure application have been used. All of these factors influence the animal's ability to sense the amount of pressure being applied. The small size and sharpness of the pressure area located in the center of the sensor used in the present study can excite more cutaneous receptors due to high shear forces. A standard technique should be used to measure pressure threshold of large muscle in animal studies.

In humans, a decrease in pressure pain threshold is seen with palpation to tenderness. Therefore, this forceps compression device is similar to the hand-held algometers used in clinical studies to measure threshold level of muscle tissue. The sensitized nociceptors display lowered mechanical threshold and longer response to noxious stimuli. The threshold level is measured with peak force response to applied compression. With innocuous deformation or light palpation, about 30% of group IV muscle afferents can be excited; the rest of group IV afferents respond to noxious or

tissue damaging pressure (Hoheisel et al., 2004) that our mice may have perceived with muscle compression test following acid-injections.

1.6.3 C-Fos

The biochemical analysis of muscle nociception is challenging because their peripheral sensory nerve endings are not localized to a particular anatomical location (Julius & Basbaum, 2001); in muscle tissues, nociceptors are located within nerve endings innervating the blood vessels and muscle fascia (Mense, 1993). However, genes like c-fos and c-jun are rapidly expressed in the dorsal horn neurons following different forms of stimulation and are therefore often used to assess nociception (Bon, Wilson, Mogil, & Roberts, 2002; Coggeshall, 2005; Harris, 1998; Munglani & Hunt, 1995; Naranjo, Mellstrom, Achaval, & Sassone-Corsi, 1991; You & Arendt-Nielsen, 2005). Once afferent fibers are simulated from their peripheral ends, an electrical current is initiated that reaches the cell bodies in the dorsal horn ganglia (DRG). The signals then activate and release certain nociceptive neurotransmitters, glutamate, substance P, CGRC, from their central terminals in the spinal cord. The post synaptic processing and increased concentration of Ca2+ in the spinal cord neurons activate the process of c-fos and c-jun transcription (Munglani & Hunt, 1995) in the 2nd order neurons. This occurs within minutes of stimulation and the accumulation of c-Fos mRNA reaches its peak within 30 – 40 minutes. The level of protein peaks about 2 hours following the gene transcription in the nucleus and can

be detected with immunohistochemistry. This technique has been extensively used in the field of pain since it was first reported by Hunt (Hunt, Pini, & Evan, 1987).

The laminar distribution of Fos expression depends on the nature of the sensory stimulus. The distribution of spinal Fos expression is different for noxious and nonnoxious (tactile) stimulation. Nonnoxious stimulation of brushing of hairs and gentle ankle joint manipulation (Hunt et al., 1987) as well as walking (Jasmin, Gogas, Ahlgren, Levine, & Basbaum, 1994) induces Fos reactivity in the inner part of lamina II (IIi) and the nucleus proprious (lamina III and IV) (Harris, 1998). In contrast, noxious stimulus normally results in Fos activity in the superficial layers of dorsal horns, laminae I and II. *This technique has been used to assess acid-induced muscular hyperalgesia and to map the spinal cord neurons in response to mechanical activation of the gastrocnemius muscle and hind paw afferents in the present study (Chapter 2).*

1.7 Role of Exercise in Controlling Pain

The complexity and chronicity of musculoskeletal pain syndromes place many challenges on clinicians who manage these patients. A recent review of the management of FMS reveals limited efficacy of current pharmacological interventions (Goldenberg et al., 2004; Wang & Wang, 2003). But there is growing evidence suggesting that aerobic exercise is an effective treatment for patients with FMS (Droste, Greenlee, Schreck, & Roskamm, 1991; Fulcher & White, 1997; Goldenberg et al., 2004; Gowans & deHueck, 2004; Gowans, Dehueck, Voss, Silaj,

& Abbey, 2004; Hoffman et al., 2004; Mannerkorpi, 2005; Meiworm et al., 2000; Redondo et al., 2004; Whiteside et al., 2004). The therapeutic benefit of aerobic exercise for individuals with FMS was 1st recognized approximately 20 years ago when patients randomized to 20 weeks of high-intensity exercise program showed improvement in tender point pain threshold, fitness level, and global functional ratings compared to a control group undergoing flexibility training (McCain, 1986). Since then an escalating number of randomized clinical trials have confirmed the benefits of exercise for individuals with FMS (Droste et al., 1991; Fulcher & White, 1997; Goldenberg et al., 2004; Gowans & deHueck, 2004; Gowans et al., 2004; Hoffman et al., 2004; Mannerkorpi, 2005; Meiworm et al., 2000; Redondo et al., 2004; Whiteside et al., 2004). These subsequent exercise programs, by and large have examined the benefit of moderately intense aerobic exercise as it is difficult to motivate patients to perform exercise training at maximum level.

Current literature suggests that long-term aerobic training at a moderate level, 50-60% maximum heart rate with gradual progression from pool walking to land jogging for 30 minutes, 3-5 times per week decreases symptom severity, improves physical function, and aspects of self-care in individuals with FMS (Gowans et al., 2004; Meiworm et al., 2000; Whiteside et al., 2004). These studies confirm that exercise has no serious side-effects, results in decreased pain, and improves aerobic fitness in people with FMS (Meiworm et al., 2000).

Despite growing human studies, the analgesic effect of exercise on chronic pain has not been extensively examined in animal models. There are few animal

studies of chronic pain (Bement & Sluka, 2005; Hutchinson, Gomez-Pinilla, Crowe, Ying, & Basso, 2004) conducted to assess sensory modulation in response to exercise. The proposed study is based on a recent experiment in which low-intensity exercise training reduced hyperalgesia in the acid-induced chronic pain (Bement & Sluka, 2005). The exercise-induced analgesia was attributed to the activation of the endogenous opioid system (Bement & Sluka, 2005). Rats were trained on a treadmill at low-intensity speed (10 - 20 ft/min) for 15-30 min for five days. Using varying force of monofilaments, behavioral testing was conducted before each acid injection, before and after each exercise training, and one day after the final exercise session. Five days of exercise at low intensity (10 – 20 ft/min for 15-30 minutes) reversed mechanical allodynia associated with chronic pain (Bement & Sluka, 2005). In another study, seven weeks of daily low intensity exercise (11-13 m/min) reversed allodynia associated with the spinal cord injury within 5 weeks (Hutchinson et al., 2004). These studies demonstrate that exercise training is capable of reducing or reversing hypersensitivity associated with chronic pain in various animal models. *The present study examined the efficacy of exercise training and mechanical sensation in response to aerobic exercise training after muscle pain induction (Chapter 3).*

1.8 The Sensory Nervous System and Neurotrophins

The sensory nervous system is primarily supported by neurotrophins. A family of four NTs has been identified in rodents: NGF, BDNF, NT-3, and

neurotrophin-4/5 (NT-4/5) (Lewin & Barde, 1996; Lu, Pang, & Woo, 2005; Malcangio, Garrett, Cruwys, & Tomlinson, 1997; Mendell, Munson, & Arvanian, 2001; Snider, 1994; Zweifel, Kuruvilla, & Ginty, 2005). NTs are a family of low-molecular weight proteins with similar biochemical characteristics. All neurotrophins are synthesized as precursor proteins in their pro-neurotrophin form of 30 kDa and are cleaved to the mature form of 13 kDa size. Neurotrophins and their precursors are extracellular signaling molecules important for survival and differentiation of neurons during embryonic development. They are important for repair and maintanence of adult nervous system. These neurotrophins are synthesized in target tissues and retrogradely transported to the cell body where they alter transcription of many neuropeptides, ion channels, and nociceptors influencing neuronal signaling and their functional capacity (Basbaum & Woolf, 1999; DiStefano et al., 1992).

Each neurotrophin has specificity for different type of sensory neurons and is associated with different sensory modalities. NGF primarily responds to small nociceptive sensory neurons; NT-3 responds to proprioceptive and mechanoreceptive sensory neurons; BDNF has overlapping function supporting of large and small diameter sensory neurons (tactile discrimination). In addition to their primary function, NGF and NT-3 also support sympathetic neurons, where BDNF supports parasympathetic and spinal motor neurons and plays a role in synaptic plasticity. During development, all neurotrophins are expressed in high levels in their target cells; NGF is expressed in skin and NT-3, BDNF and NT/4 are expressed in skeletal muscles (Zhao, Veltri, Li, Bain, and Fahnestock 2004). Their synthesis is drastically

decreased within the 1st few weeks after birth. Of all NTs, BDNF and NT-3 influence synaptic plasticity and thus are often subjected to changes through physical activity.

1.8.1 Neurotrophin Receptors

The diverse functions of neurotrophins are supported via two classes of receptors: p^{75} neurotrophin receptor (p^{75NTR}) with low affinity and tyrosine kinase receptor (trk) with high affinity (Friedman & Greene, 1999; Huang & Reichardt, 2003; Lu et al., 2005). These receptors are expressed selectively in different sensory neurons and allow preferential binding; NGF binds to trkA receptor, NT-3 to trkC (Lamballe, Klein, & Barbacid, 1991) and BDNF & NT4/5 to trkB. However, p^{75NTR}, expressed by 80% of DRG neurons (Karchewski, Kim, Johnston, McKnight, & Verge, 1999), is a common receptor for all neurotrophins (Roux & Barker, 2002), with low affinity to mature neurotrophins and high affinity to proneurotrophin. Under some circumstances, NT-3 also binds to trkB and trkA receptors (Karchewski, Gratto, Wetmore, & Verge, 2002; Rodriguez-Tebar, Dechant, Gotz, & Barde, 1992; Soppet et al., 1991). Binding of the ligand to trk receptors or p^{75NTR} leads to different functions.

These trk receptors have up to 5 distinct domains and almost 800 amino acids (Huang & Reichardt, 2003). Upon ligand binding, trk receptors auto-dimerize and the entire ligand-receptor-complex is internalized by endocytosis and retrogradely transported to the cell soma (Fig 3a) (Zweifel, Kuruvilla, & Ginty, 2005; Stock,

1996). Once the ligand is bound and dimerized, it becomes active, which in turn causes autophosphorylation of tyrosine residues on the cytoplasmic domain resulting in activation of a number of signaling cascades. These intracellular signaling cascades are mitogen-activated protein kinase (Ras/Raf/MAPK), phospholipase C-y (PLCy) and phosphoinositide 3-kinase (PI3K) (Fig 3 b) that eventually lead to gene transcription of cell differentiation, neuronal plasticity, and neuronal survival, respectively (Hennigan, O'Callaghan, & Kelly 2007). Since the sensory nervous system is primarily supported by neurotrophins, adult DRG neurons express their receptors at varying levels as discussed below; however, almost 80% DRG neurons express p75 receptors (Gratto & Verge, 2003).

1.8.2 Neurotrophins

Detailed information of each neurotrophin is provided to describe their individual role.

1.8.2.1 NGF

In mammals, about 70-80% of sensory neurons in the DRG that terminate in lamina I and II depend on NGF during development. However, almost 40% of mature DRG neurons express trkA receptors (Karchewski, et. al. 1999). The majorities of these nociceptors are polymodal in nature and respond to more than one type of noxious stimulus. During the embryonic state, the development of these polymodal neurons depends on availability of NGF. In adult rats, blockage of NGF with antibodies prevents both heat and mechanical hyperalgesia following tissue

inflammation, indicating that the adult nocicpetors do not require NGF for survival but rather are important for synaptic efficacy of nociceptor connections in the spinal cord.

1.8.2.2 BDNF and NT-4

In adults, approximately 35% of adult DRG neurons express trkB receptors (Karchewski et al., 1999). BDNF predominately supports large and medium size neurons, is anterogradely transported from the cell bodies to the spinal cord, terminates predominately in lamina I and II, and is upregulated in models of inflammatory and neuropathic pain. These neurons are primarily thought to be cutaneous neurons as no proprioceptive muscle afferent loss occurs in BDNF-/- animals (Carroll, Lewin, Koltzenburg, Toyka, & Thoenen, 1998; Perez-Pinera et al., 2008). The trkB receptors are also expressed in post-synaptic dorsal horn neurons, throughout the gray matter and in motor neurons (Scarisbrick, 1999; Tabuchi 2007). In addition to peripheral tissues, BDNF is predominately expressed in the CNS and plays a critical role in long-term potentiation (Tabuchi, 2007). NT-4 is dependent on muscle activity and its immunoreactivity can be detected in slow, type I muscle fibers.

1.8.2.3 NT-3

About 40% of adult DRG neurons express trkC receptors (Karchewski et al., 1999). During development, NT-3 is highly expressed in skeletal muscle and in skin (Ernfors, Lee, Kucera, & Jaenisch, 1994). Following birth, the intramuscular

expression of NT-3 is primarily restricted to muscle spindles where it supports proprioceptive neurons (Merlio, Ernfors, Jaber, & Persson, 1992; Simon, Terenghi, Green, & Coulton, 2000); thus spindle activity either via electrical stimulation or exercise is likely to stimulate NT-3 synthesis. The cutaneous expression of NT-3 is limited to Merkel cells whose primary afferents convey information about fine discriminatory touch.

Given its anatomical location, NT-3 in general is believed to play an important role in proprioception modulation (Wright, et. al. 2001; Hutchinson, et. al. 2004; Ernfors, et. al. 1995; Liebl & Tessarollo, 1997). Overexpression of NT-3 during embryonic development and the postnatal period increases the number of surviving proprioceptive neurons and muscle spindles (Wright, 2001). Similarly, intramuscular injection of NT-3 protects neuronal survival of muscle afferents following sciatic nerve crush (Wright, 2001). Additionally, mice lacking NT-3 gene show a severe loss of sensory neurons including muscle spindle and Golgi tendon organ afferents and concomitant gait abnormalities, providing further evidence for role of NT-3 in the survival and function of sensory neurons (Lewin & Barde, 1996; Tessarollo, Vogel, Palko, Reid, & Parada, 1994).

In the normal adult DRG, neither NGF nor NT-3 mRNA is present in detectable amounts, while their receptors are found in significant amounts. This suggests the retrograde transport mechanism by which these neurotrophins exert their effects. Contrary to this finding, a substantial amount of BDNF mRNA is

detected in the normal adult DRG. NT-3 is expressed in both muscle and skin in adult animals and their trkC receptor is expressed on spindle afferent fibers and motoneurons in the spinal cord (Munson, Johnson, & Mendell, 1999), suggesting the role of NT-3 in maintaining the normal physiological properties of muscle stretch receptor afferent fibers in adults.

BDNF gene transcription is dependent upon Ca^{2+} influx and is regulated by CRE-bind protein (CRAB) transcription factor. The molecular mechanisms that control NT-3 gene transcription are not fully understood and may be different than those involved in BDNF transcription. The NT-3 gene consists of 2 untranslated exons IA (EIA) and IB (EIB) and a common exon II. Two transcription factors, specificity proteins (Sp) 3 and 4 bind to Sp1 sequences within the promoter region and regulate NT-3 gene expression. Recent evidence suggest that NT-3 is independent of extracellular Ca^{2+} influx and functions through IP3 or Ca^{2+}/Calmodulin-dependent Kinase II (CaMKII) signaling pathways (He, Yang, Xie, & Lu, 2000). Figure 4 represents a schematic diagram for BDNF and NT-3 gene transcription (Tabuchi, 2007).

It is apparent from animal models that neurotrophins are essential for growth and survival of certain motor, sympathetic and sensory neurons in both the central and peripheral nervous systems during embryonic development (Snider, 1994; Watanabe, Endo, Kimoto, Katoh-Semba, & Arakawa, 2000; Wright, 2001). However, in adult life, neurotrophins have two primary functions: 1) to provide

trophic support for neurons and 2) to support activity dependent plasticity that is essential for maintenance and repair (Frostick, Yin, & Kemp, 1998; Gomez-Pinilla, Ying, Roy, Hodgson, & Edgerton, 2004; Hennigan, O'Callaghan, & Kelly, 2007; McAllister, Katz, & Lo, 1999). The concept of activity dependent neuroplasticity is evident in many clinical conditions of neurogenerative diseases and provides support for exercise training.

Since neurotrophins support sensory neurons, their role in pain modulation is currently being explored and has clinical importance. Both BDNF and NT-3 are important for the regulation of sensorimotor function (Molteni, 2004). *Of the four neurotrophins, this dissertation focuses primarily on NT-3 as it plays an important role in suppressing chronic pain triggered from intramuscular acid injections (Gandhi et al., 2004). It is hypothesized that sensory neurons responsive to NT-3, and those projecting to skeletal muscles play a key role in mediating exercise-induced analgesia.*

1.9 The Role of NT-3 and Sensory Modulation

Recently, efforts have been made to determine the clinical significance of NT-3 for pain modulation.

1.9.1 NT-3 and Pain

The role of NGF in promoting inflammatory pain is well understood (Wilson-Gerwing, Dmyterko, Zochodne, Johnston, & Verge, 2005). Recently, evidence is emerging about the role of NT-3 as a pain modulator. NT-3 has been shown to

modulate mechanical (Gandhi et al., 2004) and thermal (Wilson-Gerwing et al., 2005) hyperalgesia in various animal models of injury. The affect of NT-3 on mechanical hyperalgesia developed in response to acid injection was assessed by Gandhi, et al. (Gandhi et al., 2004). Mice with either high overexpression of NT-3 (high-OE myo/NT-3) or with intramuscular injection of NT-3 demonstrated *transient* mechanical hyperalgesia in contrast to mice with low-OE myo/NT-3, wild-type or saline treated who developed *persistent* hyperlagesia. This study demonstrated that NT-3 is a potent pain modulator in acid-induced hyperalgeia and *provides the reason for exploring its role endogenously via exercise training to mitigate muscle hyperalgesia (Chapter 3).*

Additional support comes from an experiment by Malcangio and colleagues. A single systemic dose of NT-3 into hyperalgesic rats from injection of NGF resulted in mechanical but not thermal hypoalgesia 24 hours after administration. In addition, NT-3 either systemically injected for two weeks or acutely applied to the spinal cord significantly reduced electrically evoked release of substance P from central terminals of the primary afferents, suggesting possible anti- nociceptive action of NT-3 involving substance P (Malcangio et al., 1997). Intrathecal administration of NT3 prevented thermal hyperalgesia and suppressed the injury-induced overexpression of TRPV1 receptors in the DRG and the spinal cord in a sciatic nerve injury model (Wilson-Gerwing et al., 2005). A local injection of NT3 into the skin of the hind paw transiently reversed inflammatory hyperalgesia associated with complete Freund's adjuvant (CFA) (Watanabe et al., 2000). *These findings suggest a*

link between NT-3 and hypoalgesia and guide the hypothesis for Chapter 3. It should be noted that the mechanism by which NT-3 modulates pain is not known.

1.9.2 Exercise and NT-3

Abundant evidence demonstrates that physical activity improves motor function following neurological impairments in clinical and experimental settings (Hutchinson et al., 2004; Johnson & Mitchell, 2003; Johnson, Rhodes, Jeffrey, Garland, & Mitchell, 2003; Ying, Roy, Edgerton, & Gomez-Pinilla, 2005). Recently, evidence suggests that exercise can also modulate sensory function in healthy (Molteni, Zheng, Ying, Gomez-Pinilla, & Twiss, 2004) and injured animals (Bement & Sluka, 2005; Hutchinson et al., 2004). The cellular and molecular mechanisms of exercise impact on neuronal function are mainly unexplored. Some studies have demonstrated that exercise may modulate various neurotrophins at central (Cotman & Berchtold, 2002), spinal (Ying et al., 2003, 2005), and peripheral levels (Gomez-Pinilla et al., 2001; Hutchinson et al., 2004; Molteni et al., 2004; Ying et al., 2003, 2005). The effect of exercise on neurotrophin-dependent nerve regeneration (Molteni et al., 2004), neuronal excitability and synaptic efficacy (Hutchinson, 2004; Ji, R-R., Kohno, T., Moore, K. A. & Woolf, C. J., 2003) of sensory and motoneurons and signal transduction receptor (Ying et al., 2003) have been reported. Thus, activation of NTs by exercise leads to improved neuronal networking.

The concept of exercise and NT-3 modulation is fairly novel. Few recent studies by Gómez-Pinilla and colleagues have proven that voluntary exercise is

capable of altering the level of NT-3 mRNA and NT-3 protein in the spinal cord as well as in the skeletal muscle (Gomez-Pinilla et al., 2001; Ying et al., 2003) in healthy animals. In the soleus muscle of healthy rats, a significant increased in NT-3 mRNA following 1 and 5 days of treadmill training at 27m/min and 3% incline (Gomez-Pinilla et al., 2001) and 3 days of voluntary wheel running (Ying et al., 2003) have been reported. However, NT-3 mRNA reduced to the control levels after 7 days of wheel running and no change in NT-3 protein was reported in response to 3 or 7 days of wheel running (Ying et al., 2003). In contrast to wheel running, treadmill training showed a progressive increase in mRNA NT-3 by day 1 and 5 as well as mRNA and protein levels of BDNF by day 5 (Gomez-Pinilla et al., 2001).

In the spinal cord of healthy rats, the levels of NT-3 mRNA and protein were significantly elevated after 7 days of wheel running (Ying et al., 2003) and 1 and 5 days of treadmill training (Gomez-Pinilla et al., 2001). The increase in NT-3 imunostaining was localized to substantia gelatinosa and motoneurons (Ying et al., 2003). Ying and colleague reported that motorneuron axons projecting to muscle show NT-3 immunostaining, suggesting that NT-3 is involved in spinal cord-skeletal muscle interactions. In the same study, 3 and 7 days of wheel running increased the expression of the trkC receptor in the spinal cord (Ying et al., 2003). These studies demonstrate the upregulation of NT-3 mRNA in soleus and spinal cord in response to acute stage of exercise (1-7 days) in healthy rats.

Similar results have been seen in the spinal cord and sciatic nerve crush injury models. Twenty-eight days of wheel running counteracted the reduced

expression of BDNF and increased NT-3 mRNA in the lumbar spinal cord in rats subjected to mid-thoracic hemisection. The increased level of mRNA NT-3 was only seen after 28 days and not after 3 or 7 days of wheel running, suggesting that the injured spinal cord requires long-term exercise training (Ying et al., 2005). Additionally, there was no change in the protein level of NT-3 in response to 28 days of exercise period (Ying et al., 2005). These authors did not analyze the effect of exercise on NT-3 in skeletal muscle. Neurons of DRG and sciatic nerve from three-day exercised animals showed significant longer neuritis and more axons respectively, compared to the sedentary animals; seven-days exercised animals showed even longer neuritis in DRG in vitro, suggesting increased neuronal outgrowth with longer periods of exercise (Molteni et al., 2004). In the same study, 3 and 7 days of running wheel activity resulted in increased expression of mRNA of NT-3, BDNF, and Synapsin I, a member of a family of nerve terminal-specific phosphoproteins and involved in neurotransmitter release (Molteni et al., 2004) supporting successful sensory stimulation from exercise training (Molteni et al., 2004). Although these experiments provided insight into exercise-induced modulation of neurotrophins and their connection to motor and sensory neurons, they are limited to biochemical and histological assessment and lack functional analysis of animal behavior associated with injury.

1.9.3 NT-3, Pain and Exercise

Many animal and human studies have provided clear evidence that voluntary exercise stimulates neurogenesis and improves neuronal survival, learning and

mental performances (Cotman & Berchtold, 2002). Locomotion activity in particular is proven to be beneficial in maintaining and improving neuronal function following insult (Wernig, Nanassy, & Muller, 1999). The effect of exercise on NT-3 production in pain-induced animals is limited to only one study in which 7 weeks of treadmill training reversed allodynia and restored normal sensation after experimental spinal cord injury (Hutchinson et al., 2004). Thus, exercise has been shown to affect the expression of NT3 (Gomez-Pinilla et al., 2001; Ying et al., 2003), and NT3 plays a central role in modulating hyperalgesia associated with acid-induced pain (Gandhi et al., 2004). These results provide a physiological basis to investigate exercise as the means to induce endogenous NT-3 and modulate sensitivity in acid-pain model (Chapter 3). All these experiments are based on the male rat model and various nerve injuries. No experiment has been conducted to examine NT-3 production in female mice in response to exercise and its effect on chronic muscle pain. The proposed study has addressed this issue by using acid-induced chronic muscle pain using a mouse model.

1.10 Significance of the Present Work

The complexity of chronic pain syndromes in reference to its physiology, diagnosis and intervention is well recognized in literature. Many attempts are being made to understand the underlying pathology, yet the present studies are limited to cutaneous sensation in regards to pain measures and exercise-induced effects in rodent models. Additionaly basic science research and its clinical applications are

needed to define muscle pain and to manage people with chronic pain syndromes. *This was the basis of the present dissertation.*

Despite growing literature on FMS and its management, limited empirical evidence exists for the molecular basis of exercise that is routinely used in the management of the condition. In fact most intervention techniques are based on clinical observation and lack empirical support. Recent advances in the role of exercise training on neurotrophins in healthy and various nerve injury animal models have provided insights into how activity-dependent plasticity can affect neuronal biology. However, the same effects have not been examined in chronic muscle pain which affects 97 million people in the United States (*Brain Facts*, 1997). Recently, the association between NT-3 and pain had begun to unfold. Logically, the association between exercise and NT-3 to modulate chronic muscle pain was the next step in understanding exercise-induced effects. The present study is the first to examine the chronic nature of muscle pain and exercise-induced changes in neurobiology of the peripheral and spinal cord tissues in a chronic muscle animal model of widespread pain.

The results of this study highlight important features of muscle pain and provide a molecular basic for exercise training to mitigate nociception using an animal pain model. In the absence of animal models, defining muscle pain and understanding mechanisms of exercise-induced analgesia would not be possible. Future studies should utilize animal studies to improve our knowledge base. Other important findings of this dissertation are that we were able to demonstrate

variability in the development of muscle hyperalgesia in different strains of mice, reproduce acid-induced hypersensitivity in female mice, and analyze the effect of exercise training using female-mouse model. These are important issues to consider, which we hope will guide the future experiments using animal models.

1.11 Goal of the Study

The purpose of this study was to assess muscle pain and determine the role of aerobic exercise in reducing chronic pain states in mice. Furthermore, the central projections of the gastrocnemius and the hind paw in response to mechanical perturbation in a chronic state were determined. Also, a possible mechanism by which exercise influences pain was investigated. This was attempted by utilizing an animal model of acid-induced mechanical hyperalgesia in 2 different strains of mice. The use of acid-model is ideal to analyze muscle pain and study exercise related effects on behavioral and molecular levels for 2 reasons: one, acid produces long-term chronic pain condition without inflammation or tissue damage (Sluka et al., 2001); and two, it resembles the characteristics of clinical condition of FMS and other related syndromes that display referred hypersensitivity to mechanical stimuli. *The overall goal of this dissertation was to define muscle pain and to investigate the efficacy of exercise training of 2 different intensities in reducing chronic muscle pain at behavioral and molecular levels.*

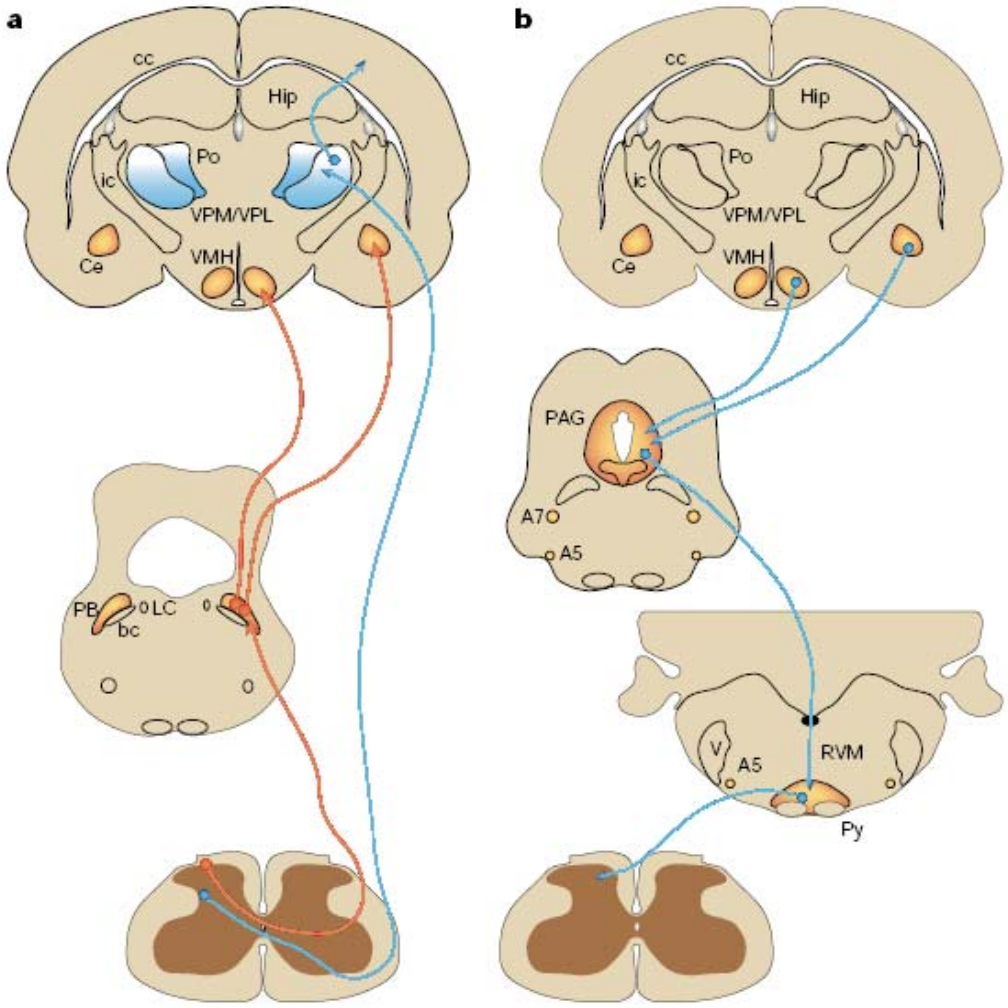
The effects of exercise-induced analgesia in patients with chronic pain are complex and poorly understood (Vierck et al., 2001). Exercise-induced analgesia

may involve activation of proprioceptive and muscle afferents to inhibit central pain circuitry and modulate descending inhibitory pathways (Hoffman et al., 2004). Animal models allow the investigation of such potential mechanism. The proposed research seeks to explore this by measuring changes in mechanical sensitivity in response to exercise and exercise-induced NT-3 levels. Additionally, we determined whether expression of NT-3, a proprioceptive sensory modulator (Ernfors et al., 1994; Farinas, Jones, Backus, Wang, & Reichardt, 1994; Klein et al., 1994; Tessarollo et al., 1994; Wright, 2001) and a potent pain modulator (Gandhi et al., 2004) occurred peripherally in muscle or centrally in the spinal cord. This was attempted to localize anatomical sites involved in pain modulation from exercise. This information is important to neuroscientists and clinicians as it adds to the role of NT-3 in pain and provides insights into the potential molecular changes that occur in response to exercise training.

Concerning the use of acid-induced pain model, Sluka and others from our laboratory have reported secondary hyperalgesia in animals, but not all later studies have been able to reproduce this finding (Ambalavanar, Yallampalli, Yallampalli, & Dessem, 2007). We examined the acid-induced hypersensitivity in 2 different strains of mice and analyzed the spinal Fos pattern in response to mechanical perturbation. Injections of acidic saline led to increased mechanical hyperalgesia to skin and muscle at different degrees in each strain of mice.

Our findings may identify a specific subpopulation of DRG neurons that are activated with exercise training and mediate analgesic effect through upregulation of

NT-3. These neurons express trkC and ASIC-3 and innervate vasculature in skeletal muscles. The sensory neurons that respond to NT-3 in the skeletal muscle are mainly Aα, Aβ and Aδ. A previous study from our lab demonstrated that the Aδ fibers are the ones that attenuate the function of exogenous NT-3 in reversing acid-induced cutaneous pain. *The present study focused on the endogenous mean of NT-3 synthesis through exercise training and its role in reducing deep muscle pain induced by acid injections*. Our results also indicate a possible compensatory mechanism by which NT-3 mitigates muscle pain under the condition of physiological stress of tissue acidosis. Together these data strengthens the role of NT-3 as an anti-nociceptive neurotrophin in treating muscle pain and provide further support for exercise training as a therapeutic intervention for the growing field of pain medicine.



Ascending **Descending**

Figure 1: The main ascending (a) and descending (b) pain pathways.

Figure a demonstrates 2 primary ascending pathways: spinoparabrachial (left) and spinothalamic (right). Figure b demonstrates the descending pathways from the origin sites of amygdale and hypothalamus to periaqueductal gray (PAG); from PAG to lower midbrain (rostroventral medulla – RVM); from lower midbrain to the dorsal horn (DH) (Hunt & Mantyh, 2001).

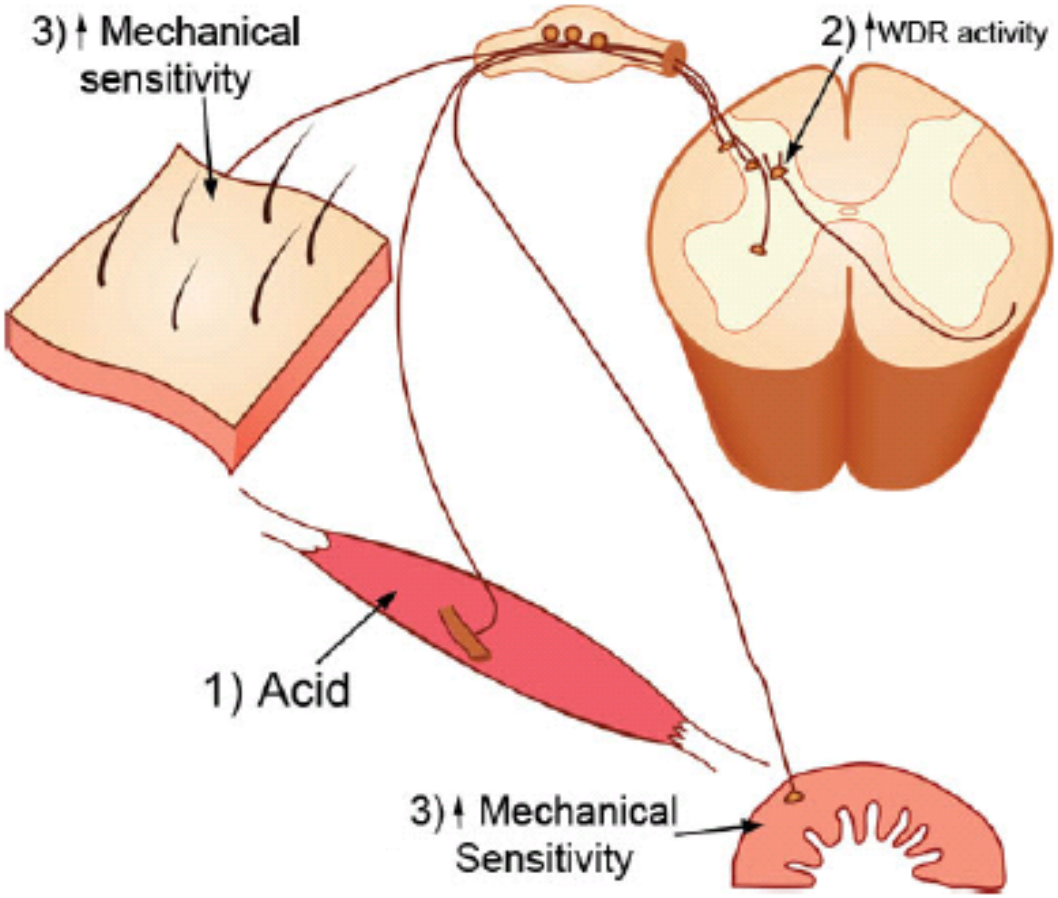


Figure 2 demonstrates the central projections of the primary afferents from the cutaneous and deep tissues. The convergence of these afferents in deeper laminae of the dorsal horn of the spinal cord provides anatomical explanation for secondary hyperalgesia induced by acid injections.

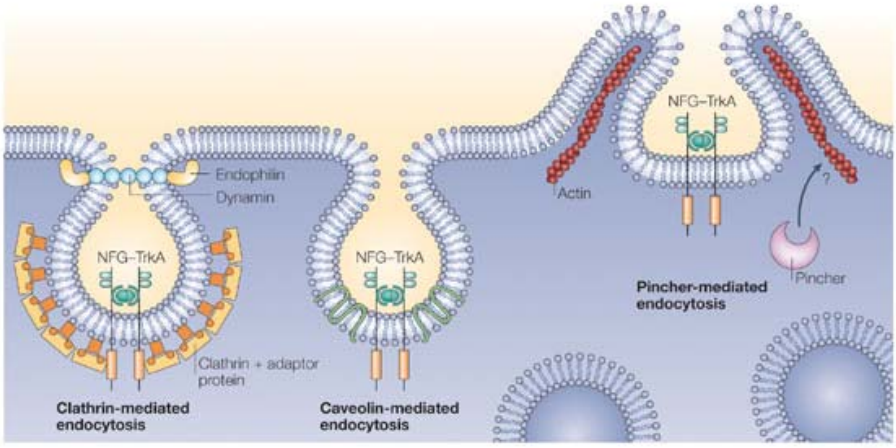


Figure 3a: A schematic figure demonstrating internalization of neurotrophin and neurotrophin receptor (Zweifel et al., 2005)

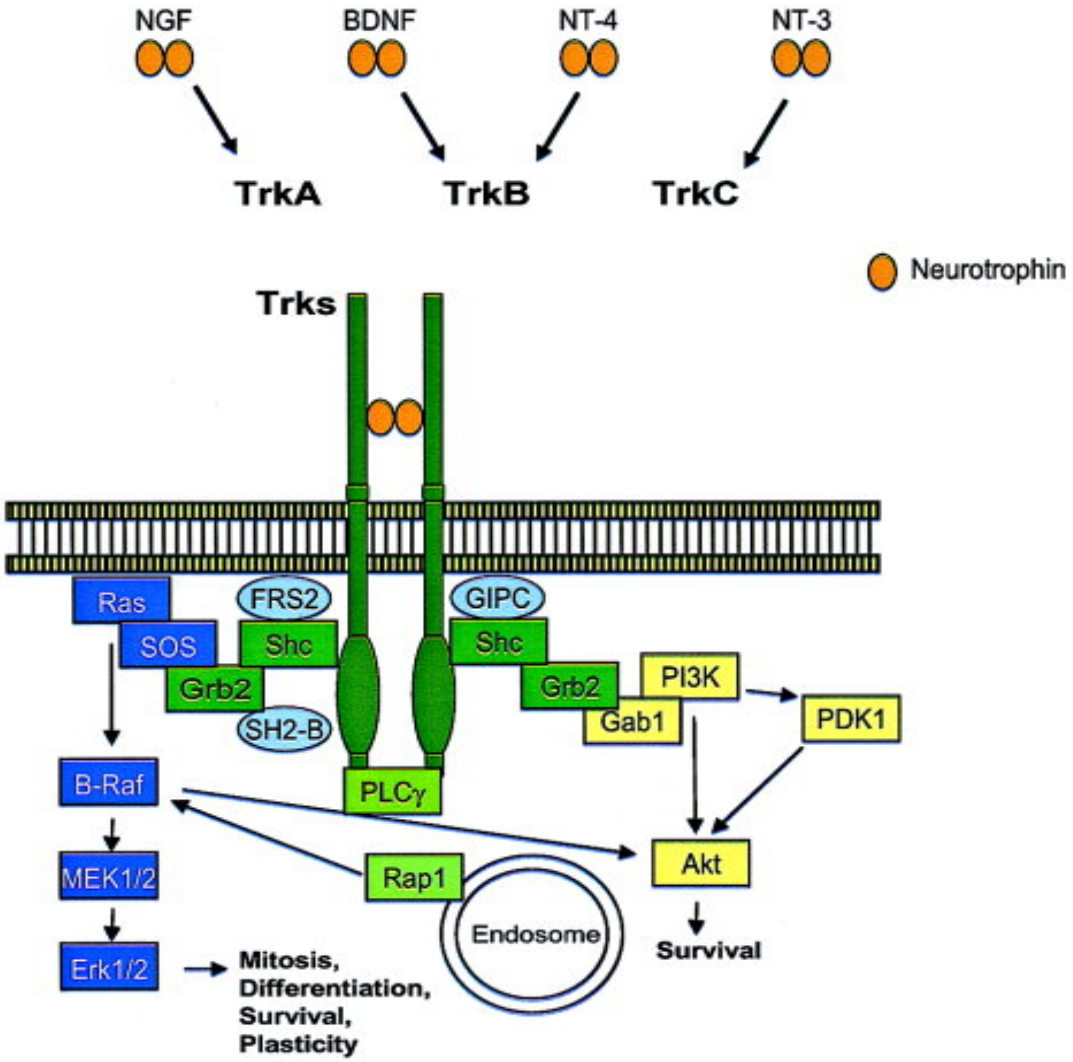
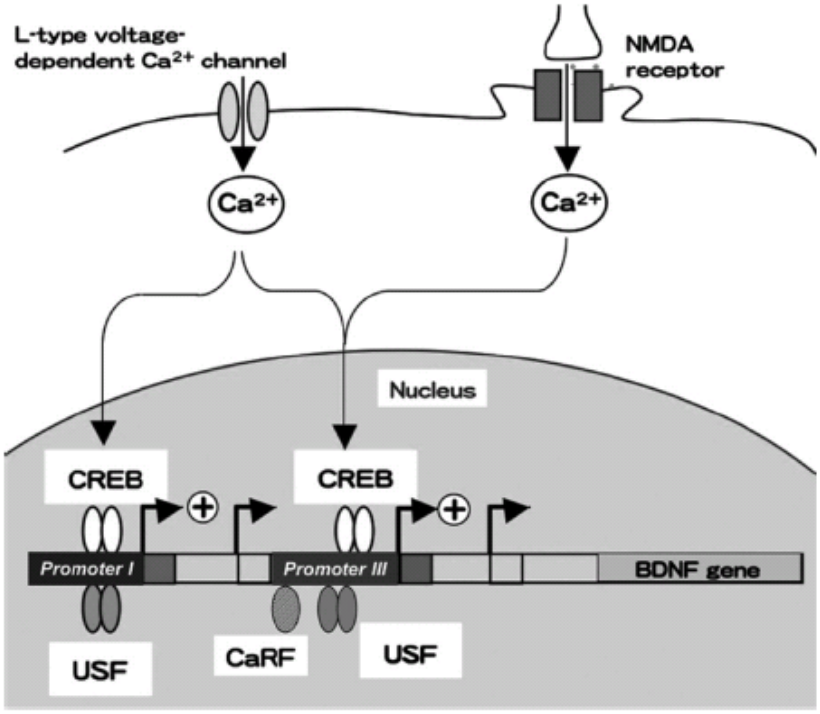
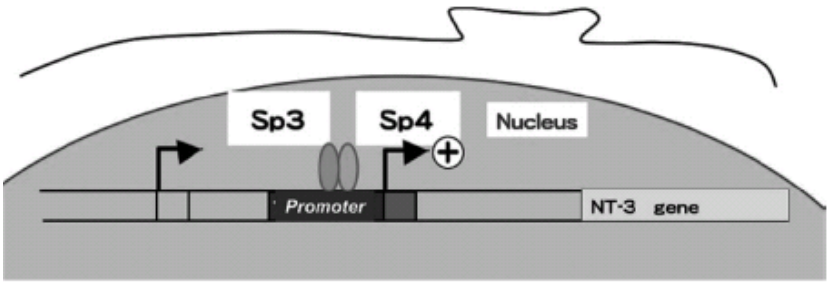


Figure 3b: A photograph showing trk receptors, ligand binding and intracellular pathways downstream from trk receptors (Chevrel, Hohlfeld, and Sendtner, 2006).



A: BDNF gene transcription



B: NT-3 Gene transcription

Figure 4: A diagram depicting BDNF and NT-3 gene transcriptions (Tabuchi, 2007).

Chapter 2

Strain-Specific Variability in the Development of Acid-Induced Muscle and Cutaneous Mechanical Hyperalgesia in Mice

Neena K. Sharma, Janelle M. Ryals, Hongzeng Liu, Wen Liu, and Douglas E. Wright. Strain-Specific Variability in the Development of Acid-Induced Muscle and Cutaneous Mechanical Hyperalgesia in Mice

(Submitted, Journal of Pain, June, 2008)

2.1 Abstract

To test the hypothesis that animals develop muscular hyperalgesia following intramuscular acidic injections, acid-induced muscle hyperalgesia was analyzed in 2 strains of mice and was compared to secondary cutaneous hyperalgesia in the hind paw that develops following intramuscular acid injection. Two acid (pH 4.0) injections were administrated into the gastrocnemius of female C57BL/6 and CF-1 mice. Mechanical sensitivity to paw and muscle stimulation was examined using behavioral measures and spinal Fos expression. Compared to C57BL/6, CF-1 mice developed a robust hypersensitivity bilaterally in hind paws and gastrocnemius muscles that lasted up to 2 weeks. In CF-1 mice, muscle hyperalgesia correlated well with Fos activity. Cutaneous hind paw palpation stimulated ipsilateral Fos expression in the superficial spinal laminae at L4/L5 levels, and bilaterally in deep laminae at L2-L5 spinal levels. In contrast, muscle compression stimulated widespread Fos expression in all regions of the ipsilateral dorsal horn within L2-L6 spinal segments. These findings identify that there are important mouse strain differences in response to acid, that acid injection induces muscle hyperalgesia, and finally that the patterns of Fos expression in response to muscle versus cutaneous stimulation are strikingly different.

PERSPECTIVE

This study assesses muscular pain, which is the primary complaint of people with musculoskeletal conditions. The results suggest that critical strain differences

exist related to nociception in this model and that the spinal patterns activated by noxious mechanical stimuli to the gastrocnemius is different than paw. This information should aid our understanding of spinal processing of muscular pain and approaches to test nociception arising from muscle.

2.2 Introduction

Pain associated with chronic musculoskeletal disorders poses a major clinical problem, affecting nearly one-third of the world's population and costing approximately $100 billion each year (Bergman, 2007; Holden & Pizzi, 2003). Clinically, people report pain arising from joint, muscle, fascia or visceral tissues (L. J. Kehl & Fairbanks, 2003). However, most of our knowledge concerning the mechanism and measurement of pain is inferred from studies assessing cutaneous tissue. Little is known about muscle pain, in part because of the complexity of muscle nociception (L. J. Kehl & Fairbanks, 2003) and the lack of reliable laboratory measures. Clinically, muscle pain is measured by the presence of edema and bruising, loss of strength, and pain with palpation. In animals, muscle strength tests have been used for testing muscle pain, (L. J. Kehl, Trempe, & Hargreaves, 2000; Schafers, Sorkin, & Sommer, 2003) but are currently limited to assessing forelimb muscles. An effective way to test hind limb deep tissue pain may be direct muscle compression, (Schafers et al., 2003; Skyba, Radhakrishnan et al., 2005; Yu et al., 2002) which parallels clinical assessment by muscle palpation. Yu et al. (Yu et al., 2002) first described a study in which a compression was applied to the deep hind

limb tissues using an instrumented forceps to control the compressive force applied. This device has become a widely accepted and beneficial tool for measuring deep tissue pain in various pain models (Schafers et al., 2003; Skyba, Radhakrishnan et al., 2005; Yu et al., 2002).

Animal models of muscle pain are induced by ischemia (Graven-Nielsen et al., 2002; Graven-Nielsen & Mense, 2001); various irritant chemicals (Hoheisel, Mense, Simons, & Yu, 1993; Hunt et al., 1987; L. J. Kehl et al., 2000; Sluka, 2002); muscle contraction via either electrical stimulation (Taguchi, Matsuda, Tamura, Sato, & Mizumura, 2005; S. F. Wang, Chen, Liao, & Shyu, 2005) or exercise (L.J. Kehl, 1996); and acidic saline (Sluka et al., 2001). The model of acid hyperalgesia is unique in that it causes no muscle damage and the widespread hypersensitivity has been proposed to mimic fibromyalgia (Sluka et al., 2001). Muscle hyperalgesia induced by acid injection is dependent on the presence of acid sensing ion channel-3 (ASIC3) (Sluka et al., 2003) and mediated by central sensitization of spinal (Hoeger-Bement & Sluka, 2003; Miranda et al., 2004; Skyba et al., 2002; Skyba, Lisi et al., 2005; Sluka et al., 2001) and supraspinal mechanisms (Tillu et al., 2007). Following 2 acidic injections, animals develop widespread secondary hyperalgesia that spreads to bilateral cutaneous (Gandhi et al., 2004; Hoeger-Bement & Sluka, 2003; Miranda et al., 2004; Skyba et al., 2002; Skyba, Lisi et al., 2005; Sluka et al., 2001) and visceral tissues (Miranda et al., 2004). The development of cutaneous hyperalgesia has been reported in many studies, but it is not clear whether animals develop muscle hyperalgesia following intramuscular acid injection (Tillu et al., 2007).

Fos has been extensively used to examine neuronal activation following cutaneous application of noxious chemical, (Bon et al., 2002; Hunt et al., 1987; Jinks et al., 2002; Li, Lighthall, Liang, & Clark, 2004; Svendsen, Edwards, Lauritzen, & Rasmussen, 2005) thermal, (Hunt et al., 1987; Todd, Spike, Young, & Puskar, 2005; Williams, Evan, & Hunt, 1990), and electrical stimuli (Lawrence et al., 2004; Taguchi et al., 2005). The activation and expression of Fos in the dorsal horn in response to gastrocnemius muscle stimulation is limited to two studies utilizing chemical or electrical stimuli (Hunt et al., 1987; S. F. Wang et al., 2005). Here, we used immunohistochemistry to map the distribution Fos in the dorsal horn in response to mechanical stimulation of the gastrocnemius muscle and hind paw in the acid-induced pain model. The purpose of the present study was to 1) compare the responses of 2 strains of mice to intramuscular acid injection, 2) assess muscle hyperalgesia using an instrumented forceps device and Fos activation in the acid pain model and 3) compare patterns of Fos expression in response to either hind paw palpation and gastrocnemius muscle compression. Characterizing the features of pain in different strains of mice using the acid-induced model of muscle pain may provide a broader understanding of muscle nociception and add to our understanding of clinical testing of muscle palpation.

2.3 Materials and Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center and adhered to the University's animal care guidelines. Eighteen female C57BL/6 mice and twenty female CF-1 mice were randomly assigned to either acid or saline groups. Two 20 μl injections of either acidic (pH 4.0) or normal (pH 7.4) saline were administered 2 days apart into the right gastrocnemius muscle to induce wide spread hypersensitivity. Injections were made with a 1 ml latex-free insulin syringe with 10 μl increments (Becton Dickinson, Franklin Lakes, NJ). The examiner was blinded to group assignment for all experiments.

Behavioral Assessments

Cutaneous Sensitivity: Cutaneous sensation of both hind paws was measured using 0.6 g (C57BL/6) and 1.0 g (CF-1) von Frey monofilaments according to a previous study (Gandhi et al., 2004). Two different filaments were used due to the differences in mechanical sensitivity between the 2 strains of mice. The 0.6 g filament for female C57BL/6 mice was chosen based on unpublished data from our laboratory that suggested a 1.0 g filament produced a ceiling effect and did not adequately allow assessments of hyperalgesia in these mice. For all animals, the monofilament was applied 5 times to each hind paw with 15-30 seconds between each application for a total of 3 trials. Three days prior to the initial testing, animals were acclimated under small plastic chambers on wire mash table for 30 minutes each day until the day of testing. The numbers of positive responses were recorded,

and the percent withdrawal response for each paw was calculated. A positive response was defined as retraction of the paw. In C57BL/6 mice, the response was measured at pre-injection and 1 and 3 days following the 2nd injection. In CF-1 mice, testing was performed pre-injection and 1, 10 and 16 days following the 2nd acid injection to examine the chronic stage of muscle hyperalgesia.

Muscle Sensitivity: To assess whether animals experience deep tissue hyperalgesia following acid injections, we used a forceps compression device similar to the one described by Yu et. al. (Yu et al., 2002). Our device is a modified version, built internally in our Neuromuscular Research Laboratory. The device consists of a forceps, a pressure sensor, a signal amplifier, and a laptop computer. Yu et. al. (Yu et al., 2002) used a strain gage sensor affixed in the middle portion of a forceps' arm. The deformation of the forceps arm was measured during the experiment and later converted to the compressive force applied at the tip of the forceps. Here, we used a commercial pressure sensor, (LCKD subminiature compression load cells, Omega Engineering, Inc. Stanford, CT, USA), which was mounted on the inner tip flat surface of the forceps. This modification makes the device more reliable and allows a direct measure of the compressive force applied to the tissue through the tip of the forceps. The signal from the load cells was amplified through a DMD-465 Bridgesensor AC Powered Signal Conditioner (Omega Engineering, Inc. Stanford, CT, USA), digitized using an A/D board, and stored in a laptop computer. The examiner applied manual force from a marked area using the forceps (Fig. 1). The

contact area of the pressure sensor measured the direct compressive force applied to the tissue. The recorded force signal was processed using a custom-written Mat-Lab computer program (Matlab 6.5, The MathWorks Inc., Boston, MA, USA) to identify the force peaks and the respective loading time. The peak force was defined as the amount of force when the animal either withdrew its hind limb or vocalized.

Three days prior to each testing session, mice were acclimated in a 50 ml restraining tube for 10 minutes 3 times a day. Each gastrocnemius muscle was compressed 3 to 5 times in 1 trial for a total of 3 trials. In C57BL/6 mice, the response was measured prior to acid injection and 5 days following the 2nd injection. In CF-1 mice, testing was conducted prior to acid injection and 3 and 16 days post-second acid injection. The mean of 9-11 peak forces over 3 trials was calculated for each hind limb. Only peak forces with a loading time (defined as the beginning of loading to the point of peak) of less than 1.5 (C57BL/6) or less than 1.0 (CF-1) seconds were used for the final calculations.

Paw Palpation and Muscle Compression to Induce Fos Expression: In order to produce a strong enough mechanical stimulus to induce Fos expression in the spinal cord, groups of mice were either stimulated with paw palpation of the right hind paw or manual compression of the right gastrocnemius muscle. For paw palpation, mice were restrained in 50 ml conical tubes with their hind paws exposed, and the right hind paw was manually compressed with a circular motion for 2 minutes (Gandhi, et al., 2004). For muscle compression, mice were similarly

restrained and the right gastrocnemius was manually compressed with a circular motion for 2 minutes. The manual compression to hind paw and gastrocnemius muscle was applied by the same individual who was blinded to the group assignments.

Immunohistochemistry: Two hours following muscle or hind paw compression (Harris, 1998; Hunt et al., 1987; S. F. Wang et al., 2005), mice were deeply anesthetized with 500 µl 1.25 % Avertin (20 ul / g; 100% Avertin in 10 g 2,2,2-tribromoethyl alcohol and 10 ml *tert*-amyl alcohol) and perfused intracardiacally with 50 ml 1x PBS followed by 500 ml 4% paraformaldehyde (pH 7.4). The lumbar spinal cord was removed and the ventral surface of the spinal cord was nicked on one side to identify the right and left sides. The cord was postfixed overnight then cryoprotected overnight with 30% sucrose. Serial 20 µm sections of frozen lumbar cord were cut and mounted on microscope slides. Approximately every 3rd slide was chosen for Fos immunocytochemistry. The sides were incubated in 0.5% Triton X-100 for 20 minutes, followed by 2 washes and 10% normal horse serum for 10 minutes at room temperature. Sections were incubated overnight with a rabbit polyclonal Fos primary antibody (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C in a humidified tray. The sections were washed 3 times and incubated with the secondary antibody (donkey anti-rabbit CY3; 1:200 in 0.1M PBS; Jackson ImmunoResearch) for one hour at 4°C. Sections were washed in PBS and coverslipped before viewing.

To assess the rostrocaudal extent of spinal Fos activation in response to muscle or paw stimulation, the lumbar spinal cord was subdivided into 3 segments: L2/3 (rostral), L4/5 (central) and L6 (caudal). Furthermore, each dorsal horn was divided into 3 regions on each side: lamina I and II (superficial region); lamina III and IV (intermediate region); lamina V and VI (deep region). Every 3rd section on each slide was used to count Fos-positive cells. The dorsal horns were photographed using a Nikon E800 microscope attached to a Magnafire digital camera (Optronics, Goleta, CA). A person blinded to the animals' grouping manually counted positive cells in each area of the ipsilateral and contralateral dorsal horns.

Statistical Analysis

For all measures, ipsilateral and contralateral sides were analyzed separately. A repeated measures analysis of variance (RM ANOVA) was used to analyze von Frey withdrawal responses and muscle compression withdrawal peak forces before and after acid injections. In addition, a t-test was conducted to examine the group differences related to cutaneous and muscle hyperalgesia at different time points when group differences or interactions (time*acid) were significant using RM ANOVA. Group differences in the number of Fos-positive cells in L4/L5 segment were also analyzed between acid and saline groups using RM ANOVA to account for possible laminar interactions. Further analysis was conducted using t-tests to examine differences in specific laminae when either group or interactions were significant with RM ANOVA. Additionally, correlations were performed between

behavioral measures and the number of Fos-positive cells in the ipsilateral superficial L4/L5 dorsal horn using Pearson's Correlation Coefficients. Values were considered significant at α level $P < 0.05$.

2.4 Results

Behavioral Assessments

Cutaneous Sensitivity: Acidic injections into the right gastrocnemius muscle produced secondary cutaneous hyperalgesia in both hind paws in both strains of mice as assessed by paw withdraw in response to von Frey monofilaments. In saline-injected mice, withdrawal responses remained relatively constant on the ipsilateral and contralateral sides in both strains of mice (Fig. 2). In C57BL/6 mice, the withdrawal responses in the ipsilateral paw of acid-injected mice increased from 29.63 ± 6.60 SEM to 60.74 ± 4.36 SEM ($P < 0.05$) 3 days following the 2nd acid injection (Fig. 2A). The % withdrawal responses in the contralateral paw of acid-injected C57BL/6 animals also increased from 49.63 ± 7.21 SEM to 69.63 ± 7.12 SEM ($P < 0.05$) 3 days following the 2nd acid injection (Fig. 2B).

In comparison, CF-1 mice displayed a robust and long-lasting mechanical hypersensitivity in response to intramuscular acid injection. In CF-1 mice, the % withdrawal response in the ipsilateral paw of acid-injected mice increased from 22.67 ± 5.28 SEM to 66.00 ± 7.20 ($P < 0.05$) 16 days following acid injection in CF-1 mice (Fig. 2C). The % withdrawal response in the contralateral paw of acid-injected CF-1 animals also increased from 21.33 ± 4.07 SEM to 65.33 ± 5.33 SEM

($P < 0.05$) 16 days following acid injection (Fig. 2D). These results indicate changes in the withdrawal threshold level of ipsilateral and contralateral sides in animals following acid injections and confirm the development of cutaneous hyperalgesia in the acid-induced pain model. Importantly, these findings reveal that CF-1 mice develop a comparatively stronger response to intramuscular acid injection than C57BL/6 mice.

Muscle Sensitivity The assessment of muscle hyperalgesia was tested with a forceps compression device as shown in Fig 1. In the ipsilateral limb of saline-injected C57BL/6 mice, the compression withdrawal thresholds increased from 73.2g ± 18.7 SEM pre-injection to 124.1g ± 12.0 SEM post injection ($P < 0.05$, Fig. 3A), suggesting the saline-injected mice became acclimated to the test and were able to endure greater compression at later tests. The ipsilateral limb of acid-injected C57BL/6 mice displayed a similar increase in withdrawal thresholds (72.2g ± 14.9 SEM to 133.6g ± 17.1 SEM) following the 2nd acidic injection. The compression withdrawal thresholds in the contralateral limb of saline-injected mice also increased (66.7g ± 10.1 SEM pre-injection to 140.6g ± 19.4 SEM post-second injection, $P < 0.05$). However, the withdrawal thresholds in the contralateral limb of acid-injected C57BL/6 mice did not increase comparably to the saline-injected mice (80.2g ± 8.1 SEM pre-injection to 89.8g ± 13.1 SEM post-injection, Fig. 3B).

In comparison, CF-1 mice developed robust muscle hypersensitivity in response to acid-injection compared to C57BL/6 mice (Figs 3C, D). In the ipsilateral

limb of acid-injected CF-1 mice, compression withdrawal thresholds significantly decreased from 129.39g ± 10.59 SEM pre-injection to 103.47g ± 6.13 SEM ($P < 0.01$) 16 days following acid-injections. In the contralateral limb of acid-injected CF-1 mice, the compression withdrawal thresholds were also significantly decreased after acid injection (63.43g ± 6.79 SEM to 61.09 ± 3.82 SEM ($P < 0.05$) 16 days post-acid injections. These decreases in compression withdrawal thresholds were also evident 3 days post acid injection in both limbs and were significantly different from saline-injected mice ($P < 0.05$, Fig. 3C, D), These results indicate that CF-1 mice developed bilateral muscle hyperalgesia that lasted up to 16 days.

Acid-Induced Spinal Fos Expression From Hind Paw and Gastrocnemius

C57BL/6 Mice: The early immediate gene c-Fos is upregulated in spinal cells following the administration of peripheral noxious stimuli. Here, the number of Fos-positive neurons in L4/5 segment was quantified in saline and acid-injected animals in response to either rigorous hind paw palpation or compression of the gastrocnemius. Both stimuli were performed in the leg ipsilateral to saline- or acid injection. In C57BL/6 mice, hind paw or muscle compression was performed 5 days following the second acid injection. As shown in Table 1 and Fig. 4, hind paw palpation induced the expression of Fos-positive cells (30.63 +2.27 SEM total Fos-positive cells). In comparison, acid-injection did not stimulate additional Fos expression in the ipsilateral dorsal horn in C57BL/6 mice (35.00 ± 2.09 SEM total Fos-positive cells).

In comparison to paw palpation, ipsilateral muscle compression of the gastrocnemius resulted in a comparable number of Fos-positive cells (Table 1, Fig. 4). In saline-injected C57BL/6 mice, muscle compression induced a total of 35.84 $\pm$ 3.50 SEM total Fos-positive cells in the ipsilateral dorsal horn, whereas the number of Fos-positive cells in acid-injected C57BL/6 mice totaled 32.84 $\pm$ 1.55 SEM Fos-positive cells. Thus, in C57BL/6 mice, acid-injection had no impact on Fos expression either following paw palpation or muscle compression, a finding that is consistent with the mild to moderate changes in mechanical sensitivity observed following acid-injection.

CF-1 Mice: In CF-1 mice, paw or muscle compression was performed 16 days following the second acid injection. As shown in Table 1 and Fig. 4, paw palpation induced the expression of a total of 39.89 $\pm$ 2.84 SEM Fos-positive cells. In comparison, acid-injection of CF-1 mice induced 46.49 $\pm$ 2.88 SEM Fos-positive cells. This number was increased compared to saline-injected C57BL/6 mice, but this increase was not statistically significant ($P > 0.05$).

In contrast to paw palpation, muscle compression of the gastrocnemius in CF-1 mice resulted in 25.80 $\pm$ 1.35 SEM Fos-positive cells (Table 1, Fig. 4). However, Fos expression in acid-injected CF-1 mice was higher than saline-injected CF-1 mice (36.24 $\pm$ 1.50 SEM Fos-positive cells), although this increase was not statistically significant ($P = 0.054$). Further analysis revealed that the effects of acid on increased Fos expression in CF-1 mice were mainly restricted to the superficial laminae ($P < 0.05$). Moreover, even though no significant differences were observed

following paw palpation, a similar trend was observed in that acid increased Fos expression in the superficial laminae only, suggesting laminar-specific changes in response to acid-induced hyperalgesia. Finally, this increased expression of Fos in acid-injected CF-1 mice is consistent with the robust mechanical hypersensitivity observed in these mice and suggests that this mouse strain is perhaps more sensitive to acid-injection and develops a chronic mechanical muscular hyperalgesia.

Fos Expression Patterns in Response to Mechanical Compression of Paw or Muscle

As part of the analysis of Fos expression, we mapped the rostrocaudal and ipsilateral-contralateral expression of Fos-positive cells in response to either paw palpation or muscle compression. The number of Fos-positive cells from 3 different lumbar segments within each lamina from both ipsilateral and contralateral sides were quantified and represented schematically in Fig. 5. First, paw palpation induced Fos expression predominantly in deeper laminae in lumbar segments L2-L5; the greatest number of Fos-positive cells were located in laminae V/VI. The pattern of Fos expression in deeper laminae in response to paw palpation was similar in both C57BL/6 and CF-1 mice. Second, paw palpation induced a noticeable bilateral expression of Fos that was greater in C57BL/6 mice compared to CF-1 mice. Finally, paw palpation induced Fos expression in superficial laminae that was restricted to lumbar segments L4/L5. These rostrocaudal patterns were similar in both strains of mice.

In comparison to paw palpation, muscle compression led to a very different pattern of Fos expression (Fig. 5). First, muscle compression induced a relatively uniform pattern of Fos expression with superficial to deep laminae. This is in contrast to paw palpation in which the predominant pattern was in deep layers of the dorsal horn. Second, muscle compression in C57BL/6 mice led to a slight bilateral pattern of Fos expression similar to paw palpation, but to lesser degree. Finally, muscle compression led to a much broader rostrocaudal pattern of expression that stretched from L2-L6. This broad expansion of Fos-positive cells was evident in both C57BL/6 and CF-1 mice. These results reveal that there are differences in the patterns of Fos expression with the dorsal horn (superficial to deep) in response to paw or muscle compression; there are important differences within the 2 strains related to the bilateral nature of Fos expression; and finally, there are important differences in the rostrocaudal limits to Fos expression induced by paw or muscle compression.

Correlations Between Behavioral Measures and Fos-Positive Cells

In addition, we performed an analysis of the correlation between behavioral responses and Fos expression within the superficial laminae of L4/L5 (Fig. 6). This lamina was chosen since it displayed the most significant changes in response to acid injections. Foremost, Fos expression in the dorsal horn correlates with measures of behavioral sensitivity, regardless of whether the animal is induced to a hypersensitive state. Related to paw palpation, C57BL/6 mice had poor separation

between saline-and acid-injected mice. Alternatively, the CF-1 displayed only a moderate correlation, but did display a clear distinction between saline- versus acid-injected mice. This is consistent with the view that these mice develop robust mechanical hypersensitivity in response to acid injections. Relative to muscle compression, C57BL/6 had neither a correlation to Fos expression and behavioral responses, nor a separation of saline- or acid-injected mice. In contrast, CF-1 had a strong correlation that was significant ($P<0.05$) and distinct separation between saline-and acid-injected mice. Again, this is consistent with the view that C57BL/6 mice respond comparatively poorly to the acid, whereas CF-1 mice are more sensitive to acid-induced mechanical hyperalgesia.

2.5 Discussion

Muscular pain is relatively understudied and approaches to quantify muscle pain are limited. Here, we compared muscle and cutaneous hyperalgesia that develops after acid injection into the gastrocnemius muscle. Our results suggest that different strains of mice develop primary and secondary hyperalgesia to different degrees, and that the patterns of Fos expression vary in response to muscle or paw stimulation. These results provide novel information about our understanding of the acid-induced model of pain and also provide new insight about the patterns of spinal activation that arise from mechanical stimulation of different anatomical sites.

Acid-Induced Secondary Hyperalgesia

The intramuscular acid model is proposed to mimic aspects of musculoskeletal pain associated with secondary hyperalgesia that spreads to the paws and viscera (Lawrence et al., 2004; Miranda et al., 2004). This model does not rely on peripheral inflammation to elicit changes, and central mechanisms likely contribute to the maintenance of the hyperalgesia (Hoeger-Bement & Sluka, 2003; Skyba et al., 2002; Sluka et al., 2001; Sluka et al., 2002). Convergent input in the spinal cord from muscle/paw fibers and receptive field plasticity likely underlie the acid-induced secondary hyperalgesia. For example, after acid injection, wide dynamic range (WDR) neurons that respond to multiple peripheral stimuli and have broad receptive fields. After acid injection, WDR neurons increase their responsiveness to mechanical stimuli and expand their receptive fields. These changes are thought to be critical for the appearance of the secondary hyperalgesia (Sluka et al., 2003; Treede, Meyer, Raja, & Campbell, 1992). Our assessment of secondary hyperalgesia in the paw is consistent with previous findings using this model, both from our laboratory and others (Gandhi et al., 2004; Sluka et al., 2001). However, information about primary hyperalgesia is comparatively lacking, and our studies provide new information that this acid model also induces hyperalgesia in muscle, both ipsilateral and contralateral to the site of acid injection.

Quantitative Approaches to Measuring Muscle Sensitivity

Muscle compression was used to activate muscle afferent nociceptors and to test whether acid injection alters the behavioral sensitivity of mice to muscle

compression. The use of quantitative approaches to measure deep tissue pain thresholds is relatively new, and no previous studies have examined deep tissue threshold levels in mice. Using similar measures in rats, investigators have reported average threshold around 15,000 – 23,000 mN (Skyba, et. al., 2005; Yu et al., 2002) under inflammatory conditions and 1500 mN following acid-induced hyperalgesia (Skyba, et al., 2005). All of these studies used a flat contact surface with a relatively large area of pressure applied to the animals' muscle belly. In comparison, Schafers et. al. (Schafers et al., 2003) reported an average threshold of 1000 mN when pressure was applied to the muscle belly using a small contact area.

Our study is the first to report changes in deep tissue pain thresholds using this approach in mice. Here, we used a small contact area to measure direct compressive force applied parallel to the muscle belly. We found the compression threshold values for mice were approximately 75 to 100 g (1000 mN), indicating that mice may have similar threshold levels as rats when using the same size contact area. It is important to note that the mechanical threshold levels were greater for ipsilateral compared to the contralateral sides in CF-1 mice. This difference may be due to the testing paradigm, as the ipsilateral side was always tested before the contralateral side. It is possible that animals anticipated testing of the contralateral side and responded to a smaller amount of compression force. Moreover, both strains of mice were able to tolerate greater force over time, and these threshold changes could be attributed to acclimation to repeated testing and handling. Both of these effects may need to be accounted for in future studies.

Acid-Induced Primary Site Muscle Hyperalgesia

In C57BL/6 mice, response threshold levels to deep tissue compression were unchanged on the ipsilateral side, but decreased on the contralateral side in acid-injected mice compared to their saline-injected counterparts, suggesting that primary muscle hyperalgesia failed to develop in acid-induced in C57BL/6 mice. In contrast to C57BL/6 mice, response thresholds to muscle compression significantly decreased bilaterally in CF-1 mice and remained decreased up to 2 weeks. Additional analysis of muscle hyperalgesia with Fos expression revealed no differences between saline- and acid-injected C57BL/6 mice, but significant differences in muscle sensitivity between saline- and acid-injected CF-1 mice. Differences in Fos expression mirrored the behavioral assessments and suggest that C57BL/6 mice did not develop ipsilateral deep tissue pain (primary hyperalgesia). However, the appearance of muscle hyperalgesia following acid injection in CF-1 mice suggests that acid injection does lead to a primary hyperalgesia in mice and is consistent with previous reports in rats that propose acid induces primary hyperalgesia in rats (Yokoyama, et al., 2007). Our results extend their findings as the muscle hyperalgesia lasted for over 2 weeks, suggesting that this hyperalgesia is not transient.

Patterns of Fos Expression In Response to Paw or Muscle Stimulation

Hind paw palpation stimulates nociceptive fibers from cutaneous tissue whereas muscle compression stimulates nociceptive and non-nociceptive fibers located within the deep tissue. Previous studies have reported that afferent fibers

from rat hind paw project predominantly to L4/5 spinal segments (Abbadie, Lombard, Morain, & Besson, 1992; Takahashi, Chiba, Kurokawa, & Aoki, 2003) with additional projections to adjacent segments (L3-L6) (Lawrence et al., 2004). Accordingly, we observed the highest Fos expression in superficial laminae within spinal L4/L5 segments. In addition, we observed substantial Fos expression in deeper layers (laminae IV/V) following paw palpation, suggesting that mechanical palpation activates Fos expression significantly in deeper layers of the dorsal horn.

In comparison, patterns of Fos expression following muscle stimulation are not well understood. Wang and colleagues (S. F. Wang et al., 2005) examined Fos expression following electrical stimulation to the gastrocnemius muscle and reported Fos expression predominantly in laminae I, II and V. Similarly, Hunt et. al. (Hunt et al., 1987) reported Fos expression in laminae I/II following the introduction of chemical irritants into the gastrocnemius. Our findings support the idea that muscle nociception activates Fos in laminae I, II and V, but also suggest that muscle compression induces Fos in intermediate laminae as well. However, it is plausible that cutaneous afferents were also activated overlying the gastrocnemius, as our muscle compression included the overlying skin.

One clear finding from this study is that compared to paw stimulation, muscle stimulation induced Fos expression in a much broader pattern within the rostrocaudal axis. In both strains of mice, we observed relatively equal Fos expression in L2 and extending back to L6, suggesting that muscle compression leads to a broad activation of spinal neurons. This rostrocaudal expansion of Fos intensity into all dorsal horn

layers within lumbar segments L2-L6 is in agreement with previous studies (Clement, Keay, Podzebenko, Gordon, & Bandler, 2000; Taguchi et al., 2005), supporting the idea that deep tissues project to multiple spinal segments compared to cutaneous projections.

Fos as a Correlate of Mechanical Sensitivity

Fos has been extensively used as a marker of activation of spinal cells in response to inflammatory (Abbadie & Besson, 1992; Abbadie, Honore, Fournie-Zaluski, Roques, & Besson, 1994; Bon et al., 2002; Hoheisel et al., 1993; Hunt et al., 1987; Jinks et al., 2002; Li et al., 2004) and neuropathic pain (Molander, Hongpaisan, & Grant, 1992; Munglani & Hunt, 1995). One important aspect of the current study is that we compared the von Frey -induced responses of mice with the expression of Fos following paw palpation. Interestingly, the correlation between these 2 measures of pain sensitivity was strong in C57BL/6 mice and moderate in CF-1 mice. These findings suggest that animals that react more frequently to monofilament stimulation also have greater nociceptive responses in spinal expression of Fos, regardless of whether they received saline- or acid-injection. In contrast, correlations between muscle compression and Fos expression following muscle compression were poor in C57BL/6 mice and stronger in CF-1 mice. Our finding of acid-induced muscular pain via behavioral measures and Fos activity indicates that the compression device used in the present study is a reliable tool to test deep tissue hyperalgesia in mice, but also reveals important differences between

the two strains of mice with regard to the correlation between withdrawal responses to noxious stimuli from different anatomical sites and the expression of Fos.

Genetic Differences in Nociception

One clear finding from this study is that C57Bl/6 and CF-1 mice respond differently to acid injection. As measured by most of our parameters, the C57Bl/6 mice developed only minor hypersensitivity to acid injections. In comparison, the CF-1 mice robustly responded to the acid injections by developing a clear primary and secondary hyperalgesia in response to intramuscular acid injection that lasted well over 2 weeks. As the use of intramuscular acid injections increases, reports are emerging that acid injection does not always generate demonstrable hypersensitivity. For example, a recent study by Ambalavanar et al. (Ambalavanar et al., 2007) reported that acid injection into the masseter muscle failed to induce nociceptive behavioral responses. This has led some to question the reliability of acid to induce hyperalgesia, but this result could also be explained simply by the inherent differences in various muscles to acid injection (masseter versus gastrocnemius). Our findings shed some additional light on this issue and suggest that there are important species differences related to the response to acid.

In addition, our studies revealed interesting differences between C57BL/6 and CF-1 mice related to the bilateral nature of Fos expression following peripheral stimulation. C57BL/6 displayed a greater bilateral expression Fos to both paw palpation and muscle compression compared to CF-1 mice. It is not clear why these

species differences exist, but our results do suggest that these spinal responses be considered in future studies assessing the role of bilateral activation and widespread pain.

2.6 Conclusion

The present study assessed acid-induced deep tissue hyperalgesia and spinal Fos expression following cutaneous and muscle nociception in 2 strains of mice. Our results suggest that CF-1 mice may be a better model to study acid-induced widespread pain due to their robust response to acid and the long-lasting hypersensitivity. Also, our results demonstrate that acid does induce primary muscle hyperalgesia, in addition to the cutaneous and visceral pain that has been reported (Gandhi et al., 2004; Miranda et al., 2004; Sluka et al., 2001). This study adds to the growing knowledge of muscle thresholds and muscle hyperalgesia in this model of widespread pain and provides new information about Fos-related patterns of spinal activation in response to mechanically stimulated gastrocnemius muscle afferent fibers.

Acknowledgements

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for helpful comments on the manuscript and Melissa Sindt for helping with behavioral testing.

Table 1

			Superficial	**Intermediate**	**Deep**	**Total**
C57BL/ 6 Mice	Paw Palpation	Saline	6.12 ± 2.07	9.05 ±1.91	15.46 ± 2.84	30.63 ± 2.27
		Acid	6.23 ± 1.9	9.48 ± 1.6	19.25 ± 2.45	34.97 ± 2.09
	Muscle Compression	Saline	11.60 ± 3.0	12.92 ± 4.64	11.32 ± 2.91	35.84 ± 3.50
		Acid	11.03 ± 1.21	9.97 ± 1.48	11.84 ± 2.0	32.84 ±1.55
CF-1 Mice	Paw Palpation	Saline	12.46 ± 3.0	8.32 ± 1.88	19.11 ± 3.65	39.89 ± 2.84
		Acid	16.19 ± 3.57	9.40 ± 1.88	20.90 ± 3.19	46.49 ± 2.88
	Muscle Compression	Saline	8.75 ± 2.47	5.31 ± 1.60	11.75 ± 1.21	25.80 ± 1.35
		Acid	*15.85 ± 2.10	7.12 ± 1.55	13.27 ± 1.58	*36.24± 1.50

The table illustrates the mean ± S.E.M number of Fos-positive cells in each L4/L5 spinal laminae ipsilateral to the acid-injected limb in response to either hind paw palpation or muscle compression. * denotes significant difference between saline and acid-injected mice.

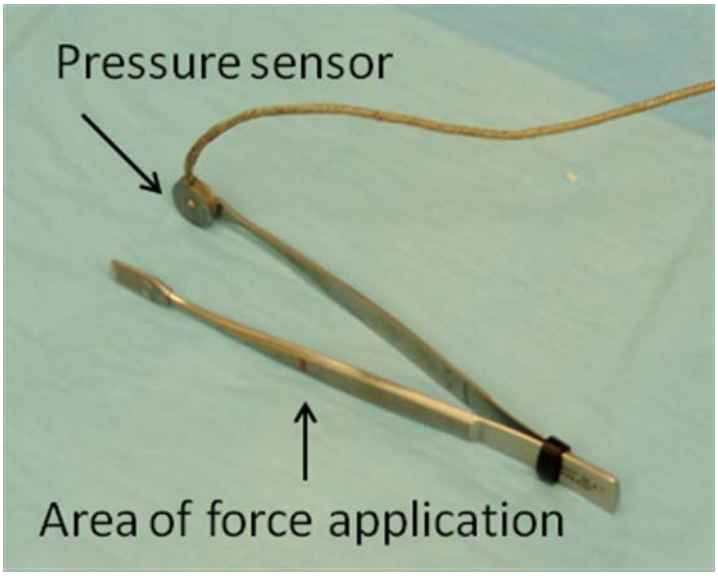

Figure 1: Photograph of the forceps device used to measure muscle compression threshold. Arrows indicate the pressure sensor (which is also the contact area with the tissue) and the site where force was applied by the examiner.

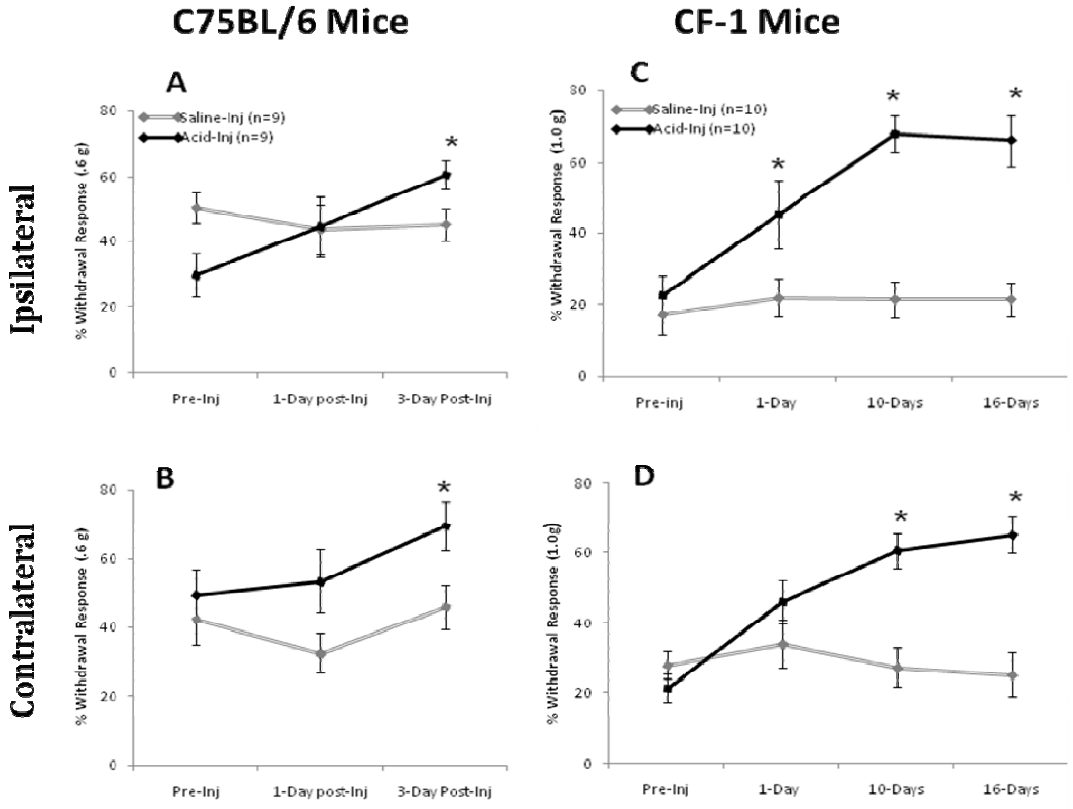

Figure 2: Withdrawal Responses to Von Frey Monofilament Application to the Hind Paw

Comparison of % withdrawal mean scores from pre-injection to post-injection periods in C57BL/6 mice. Arrows illustrate the time point at which acid was injected into the right gastrocnemius. Withdrawal responses on the ipsilateral (A) and contralateral (B) sides in C57Bl/6 mice. Withdrawal responses on the ipsilateral (C) and contralateral (D) sides of CF-1 mice. Data represented as mean $\pm$ S.E.M. Asterisks denote significant differences at specific time points between saline- and acid-injected mice ($P < 0.05$).

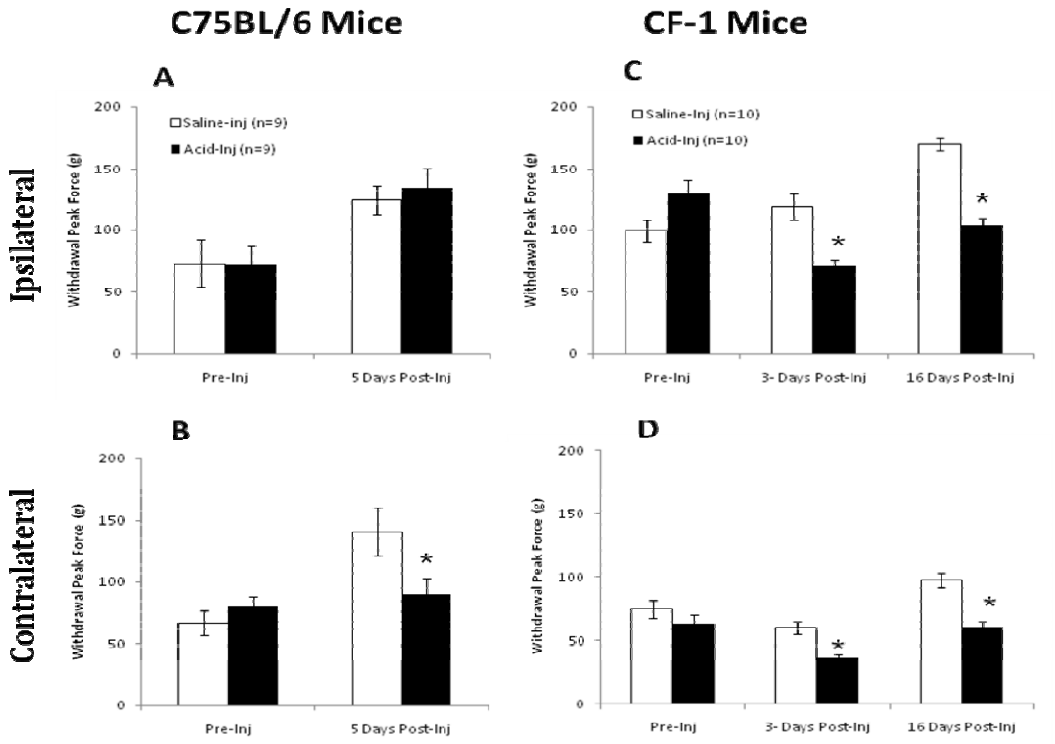

Figure 3: Withdrawal Responses to Muscle Compression of the Gastrocnemius Muscle

Comparison of mean withdrawal peak forces from pre-injection to post-injection in C57BL/6 and CF-1 mice. In C57BL/6 mice, the ipsilateral side (A) was not significantly different between saline- and acid-injected mice. However, the contralateral side (B) of acid-injected mice was significantly different following acid injection compared to saline-injected mice. In contrast, CF-1 mice displayed significant reductions in withdrawal thresholds on both ipsilateral (C) and contralateral (D) sides at both days tested. Data represented as mean $\pm$ S.E.M. Asterisks denote significant differences between saline- and acid-injected mice ($P < 0.05$).

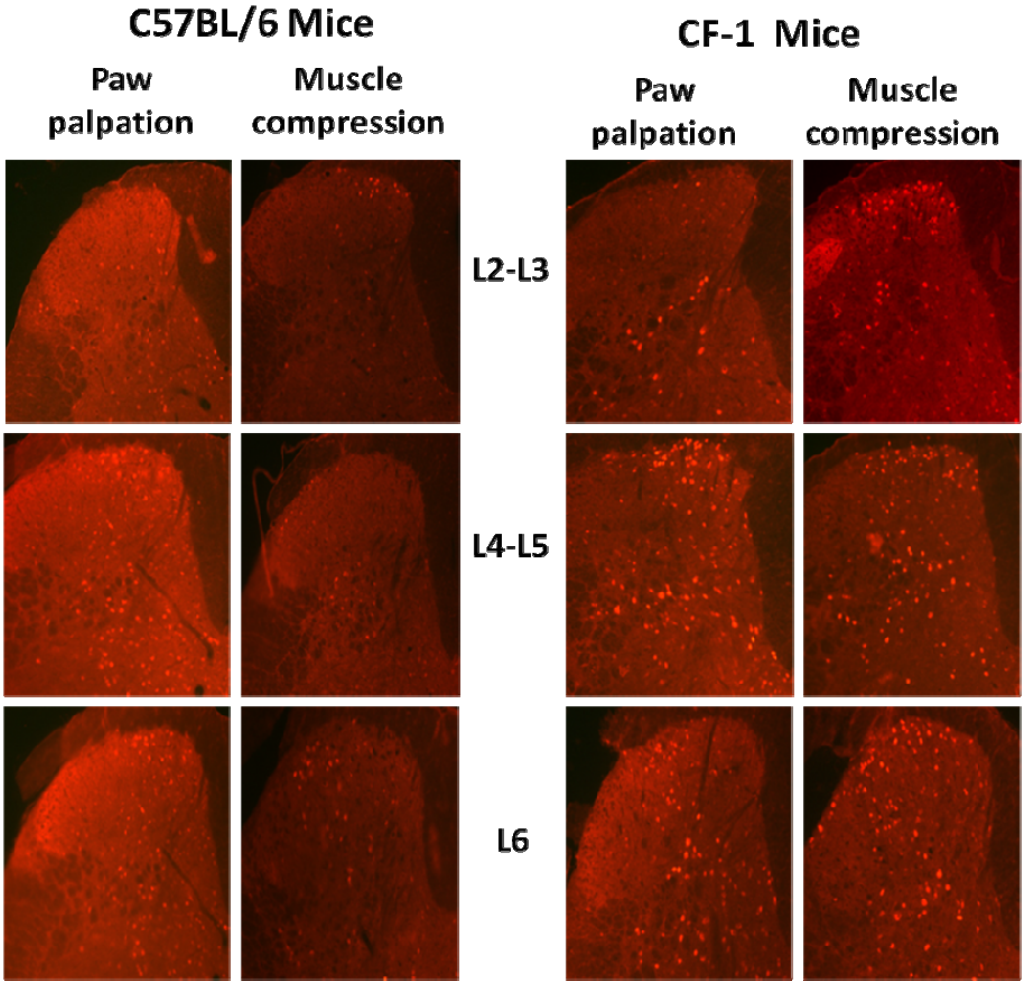

Figure 4: Fos Expression in Ipsilateral Lumbar Dorsal Horn Following Paw Palpation or Muscle Compression

Expression of Fos positive cells in L2/3, L4/5, and L6 dorsal horn in C57BL/6 mice (left side) and CF-1 mice (right side). Images are displayed for both hind paw palpation and muscle compression for each strain of mice. Scale bar equals 100 μms.

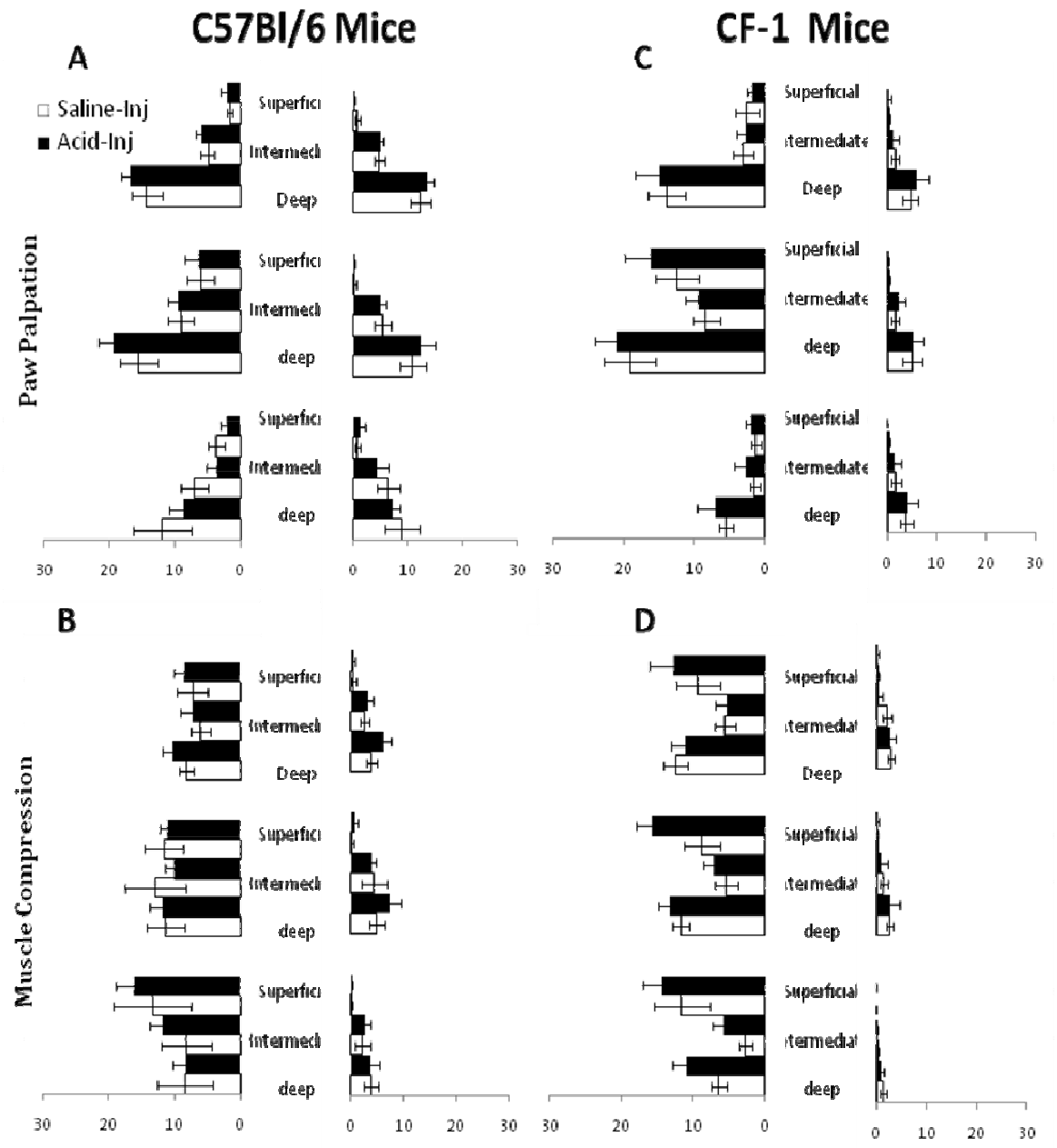

Figure 5: Schematic Representation of Fos Expression in the Lumbar spinal cord

A representation of the segmental distribution of the entire lumbar spinal cord following hind paw palpation (A, C) and gastrocnemius muscle compression (B, D).

Following paw palpation, the largest number of Fos-positive cells was concentrated in superficial dorsal horn of segment L4/5 on ipsilateral side and deeper layers from L2-L5. Following muscle compression, widespread Fos activity was observed throughout the rostrocaudal extent of the lumbar spinal cord, L2-L6. Note that C57BL/6 mice display greater Fos expression bilaterally following both paw palpation and muscle compression compared to CF-1 mice, particularly with regard to paw palpation.

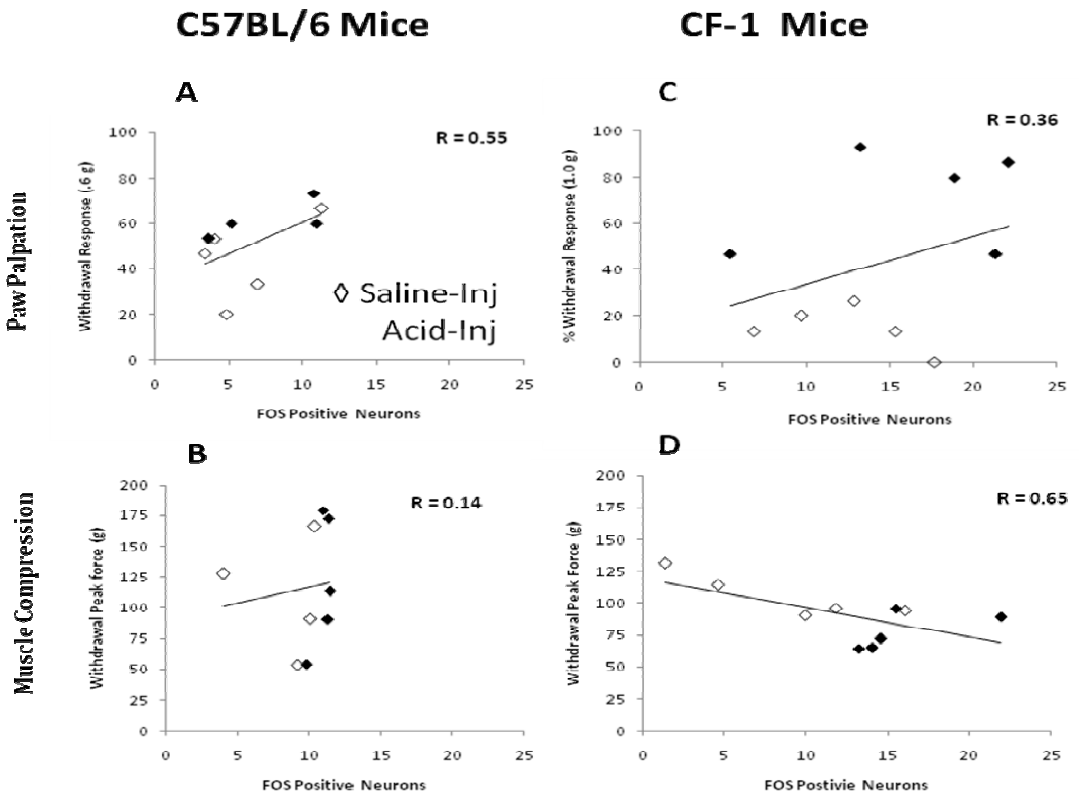

Figure 6: Correlation of Behavioral Responses and Fos Expression in Superficial Lamina Correlations between responses to von Frey application (A, C) and muscle compression (B, D) and Fos expression in the ipsilateral superficial dorsal horn in C57BL/6 (A, B) and CF-1 mice (C, D). A and C display correlations between von Frey-induced withdrawal responses and paw palpation-induced Fos expression. B and D display correlations between von Frey-induced responses and muscle compression-induced Fos expression. Related to paw palpation, strong (C57BL/6) and moderate (CF-1) correlations were noted between von Frey testing and Fos expression. Related to muscle compression, a weak correlation was noted in C57BL/6 mice, whereas a strong correlation was observed in CF-1 mice.

Chapter 3

Exercise-Induced Analgesia and NT-3 Synthesis in an Animal Model of Chronic Widespread Pain

Neena Sharma, Janelle M. Ryals, Byron Gajewski, and Douglas E. Wright.
Exercise-Induced Analgesia and NT-3 Synthesis in an Animal Model of Chronic Widespread Pain, Neuroscience (Submitted, 2008)

3.1 Abstract

Exercise is considered beneficial for neuronal function and pain reduction. Present knowledge is restricted to exercise-induced analgesia at the cutaenous level in healthy and nerve-injured rodent models. Exercise is believed to negatively affect pain transmission via stimulation of medium and large diameter muscle afferents. However, the molecular basis for this explanation remains poorly understood. A few recent studies suggest that neurotrophin-3 (NT-3) can be stimulated with exercise training in rats. Others have shown that NT-3 plays an important role in controlling neuropathic pain when delivered exogenously. Thus this study examined the effect of physical activity on muscle-derived NT-3 and deep tissue hyperalgesia. Two separate studies were conducted using female mice (C57BL/6, n=80 and CF-1, n=40). C57BL/6 mice performed mildly-intense exercise training whereas CF-1 mice performed moderately-intense exercise training for 3 weeks. Mice were injected with either saline (pH 7.2) or acidic (pH 4.0) solution to induce widespread hyperalgesia. Cutaneous (von Frey) and muscle (forceps compression) mechanical sensitivity was tested for measurements of hyperalgesia and the effect of exercise. The results demonstrate that aerobic exercise training reduced cutaneous and deep tissue mechanical hyperalgesia, induced by intramuscular acid saline and upregulated NT-3 synthesis in peripheral tissue. However, moderately-intense exercise training was required for NT-3 synthesis and the reduction of secondary hyperalgesia. The effect of exercise-induced NT-3 was more significant at the protein levels compared to mRNA expression. In addition, the protein levels were significant only in the

gastrocnemius and not in the soleus muscle, suggesting that exercise can preferentially target NT-3 synthesis in select muscles**.** In conclusion, this is the first study demonstrating the effect of exercise on deep tissue mechanical hyperalgesia in a rodent model of pain and provides a link between exercise, NT-3 and muscle pain.

3.2 Introduction

Chronic widespread pain is complex, poorly understood, and affects about 12% of the adult population in developed countries (Gran, 2003; Neumann & Buskila, 2003; Rohrbeck, Jordan, & Croft). Management of chronic pain syndromes places challenges on health care practitioners and pharmacological interventions offer limited efficacy (Goldenberg et al., 2004; L. X. Wang & Wang, 2003). Exercise training has been long suggested to reduce pain and improve functional activities (Goldenberg et al., 2004; Gowans & deHueck, 2004; Gowans et al., 2004; Hoffman et al., 2004; Mannerkorpi, 2005; Meiworm et al., 2000; Redondo et al., 2004; Whiteside et al., 2004). Surprisingly, the literature is mainly limited to human studies. Few animal studies have been conducted to address the effect and mechanism of exercise on sensory modulation in the chronic pain stage of pain. Bement and Sluka reported that low-intensity exercise training for 5 days (treadmill walking) increased the cutaneous mechanical withdrawal threshold in the same acid-pain model used in current study (Bement & Sluka, 2005). In another study, swimming exercise also decreased mechanical and thermal hypersensitivity associated with inflammation and peripheral nerve injury (Kuphal, Fibuch, & Taylor,

2007). These studies demonstrate that exercise training is capable of reducing or reversing hypersensitivity associated with chronic pain in various animal models. However, they are limited to examining cutaneous sensation, lacking the effect of exercise on deep tissue pain, which is a major clinical complaint of many people with chronic pain syndromes. There are no known animal studies that have evaluated the effect of exercise on muscle pain.

The molecular mechanisms by which exercise induces analgesia are unknown beyond the release of endogenous opioids. The concept of exercise-induced analgesia via modulation of neurotrophins is fairly novel. Few recent studies by Gómez-Pinilla and colleagues have reported that various exercise regimens (voluntary or forced) alter neurotrophins at the central and peripheral levels in healthy and injured animals (Gomez-Pinilla et al., 2001; Hutchinson et al., 2004; Ying et al., 2005). A significant increase in soleus and spinal cord levels of BDNF and NT-3 mRNA following 5 days of treadmill training (at speed of 27m/min and 3% incline) was reported (Gomez-Pinilla et al., 2001). Ying et al. (Ying et al., 2003) also examined NT-3 mRNA and protein levels in the spinal cord and soleus. These authors showed that voluntary wheel running increased NT-3 message and protein levels in the spinal cord in healthy rats. The up-regulated NT-3 mRNA levels in the soleus were transient and decreased to control levels within 7 days, and no change in NT-3 protein level in soleus was seen. In a separate study, Ying et al. (Ying et al., 2005) showed a similar effect of exercise training in a nerve-crush injury model. These studies provide the first line of evidence in support of neurotrophin

modulation after exercise training, yet are limited to biochemical assays without analysis of behavioral responses to exercise training. Only one study examined the effect of exercise on cutaneous hypersensitivity and correlated improved behavioral responses to exercise-induced BDNF levels in a spinal cord contusion model (Hutchinson et al., 2004). These scatted and conflicting results clearly necessitate further analysis of exercise training's effect on neurotrophins.

In recent years, evidence has emerged about the role of NT-3 as a pain modulator for thermal (Wilson-Gerwing et al., 2005) and mechanical (Gandhi et al., 2004) hyperalgesia. A local injection of NT-3 into the skin of the hind paw of rats transiently reversed inflammatory hyperalgesia in response to complete Freund's adjuvant (CFA) (Watanabe et al., 2000). NT-3 is effective in controlling neuropathic pain via alteration of trkA, substance P (SP), calcitonin gene related peptide (CGRP), pituitary cAMP-activated peptide (PACPL) and galanin levels (Wilson-Gerwing & Verge, 2006) and transient receptor potential vanilloid receptor-1 (TRPV1) via phosphor-p38 MAPK (Wilson-Gerwing et al., 2005). Furthermore, an earlier experiment from our lab (Gandhi et al., 2004) revealed that higher than normal levels of NT-3 (either genetically overexpressed or given intramuscularly) abolished mechanical hypersensitivity developed in response to acid injections. If exercise increases NT-3 synthesis and NT-3 reduces cutaneous and thermal pain, it is logical to test the hypothesis of whether exercise-induced analgesia can be achieved in a muscle pain model, specifically in the chronic stage. Thus, the goals of the present study were to 1) examine the effect of exercise training on muscle pain in a mouse

model of chronic pain and 2) test muscle-derived NT-3 synthesis in response to exercise, which is becoming a prime neurotrophic candidate of antinociception.

3.3 Experimental Procedures

Animals

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kansas Medical Center and adhered to the University's animal care guidelines. Two separate experiments were conducted; one with C57BL/6 mice and one with CF-1 mice to examine the effects of mildly- or moderately-intense exercise on secondary hyperalgesia and NT-3 synthesis (Table 1). Eighty female C57BL/6 mice (25 g weight) and forty female CF-1 mice (25 g weight) were purchased from Charles Rivers Laboratories. Mice were exposed to a 12-hour light/dark cycle and had excess to food and water ad libitum. Mice were daily transported to our laboratory for acclimation, exercise training and testing purpose. The examiner was blinded to group assignment of acid or saline but not for exercise or sedentary. Based on the random assignment, mice received two 20 µl injections either acid (pH=4.0 ±.1) or normal (pH=7.4 ±.1) saline 2 days apart into the right gastrocnemius muscle to induce chronic muscle hyperalgesia. Injections were made with a 1 ml latex-free insulin syringe with 10 µl increments (Becton Dickinson, Franklin Lakes, NJ). The hind paw plantar tactile sensitivity to von Frey test develops within 4 hours following the 2nd injection (Gandhi et al., 2004; Sluka et al., 2001).

Exercise training

All mice were acclimated and trained for 3-4 days on treadmill at the desired speed level before group assignment. The purpose of training all mice was to assign only those mice who run without prompting into the exercise groups. However since all mice ran naturally, they were randomly assigned to either exercise or sedentary groups. Two six lane motorized treadmills (Columbus Instruments) were utilized for exercise training. During the training sessions, occasional mild electrical shock stimulation and/or brushing were applied as necessary to maintain the running motivation. The speed of the treadmill and the duration of the training were different for each experiment as discussed below and in table 1. For each experiment, the desired speed and duration was gradually increased over time. Each mouse in the exercise group was assigned a daily running score (0, 1, 2, 3, 4) based on her performance level. The scoring was based on the amount of prompting mice required: 4 = no assistant was needed, 3 = some manual encouragement was given, 2 = mouse required some prodding to maintain running, 1 = mouse didn't complete full running period in which case the length of the run was recorded, and 0 = mouse did not run that session. Most mice did not require physical encouragement and ran spontaneously (97% of mice scored higher than 3 points on average). Although treadmill running in rodents is considered forced activity (Burghardt, Fulk, Hand, & Wilson, 2004), we observed that most animals ran without any promoting and were allowed to rest for short period. Animals did not seem overly fatigued, which is an

important consideration as continuous exercise of moderate level may result in fatigue and increased levels of lactic acid that may increase pain sensitivity.

Experiment design

C57BL/6 mice: This experiment was a time-course design over a 3-week period (Table 2). Mice were initially randomly assigned to either the exercise or the sedentary group to examine the acute effect of exercise training on NT-3 synthesis and the ability of exercise to alter the development of secondary hyperalgsia from acid injection. After one week of exercise training, the remaining mice were further randomly assigned to either acid-injected or control groups, resulting in Sedentary-control (SC), Sedentary-acid (SA), Exercise-control (EC) and Exercise-acid (EA) groups. As limited animal studies exist, this exercise protocol was chosen based on the human studies (Glass et al., 2004; Gowans et al., 2004; Meiworm et al., 2000; Whiteside et al., 2004) in which physiological and analgesic effects were noted with low-moderate intensity exercise protocol.

CF-1 mice: Based on the results from C57BL/6 mice, in which no change in NT-3 levels or tissue hypersensitivity at any time-point was noted, we increased the exercise protocol to longer duration and higher intensity (Table 1). CF-1 mice were initially randomly assigned to either acid or control group. After inducing muscle hyperalgesia with acid injections, animals were further assigned to either exercise or sedentary groups, resulting in Sedentary-control (SC), Sedentary-acid (SA),

Exercise-control (EC) and Exercise-acid (EA) groups. The assignment into exercise or sedentary groups was stratified within each group (acid or saline group) based on their post-injection von Frey scores as animals displayed some variability in their cutaneous sensitivity (with pre- and post-injections). All mice were sacrificed after completing 3-weeks of exercise training to examine the effect of exercise on chronic cutaneous and muscle sensitivity and NT-3 levels.

Behavioral Testing

For all behavioral testing, animals were acclimated for 3 days; 2 times a day for von Frey testing and 3 times a day for muscle squeeze testing. Each exercise testing was conducted in the afternoon, between 4-6 PM when animals are in their wake cycle. Behavioral testing was always conducted in the morning to avoid potential confounding factors such as stress-induced antinociception or acute effect of exercise training.

Mechanical Testing of Cutaneous Sensitivity: Mechanical hyperalgesia to innocuous stimulus was assessed using von Frey monofilaments. Clear plastic chambers (3 X 8 X 12) were placed in inverted position on a wire mesh-top table. Mice were placed under the plastic chambers and allowed to acclimate for 20 minutes prior to beginning of each test. A single von Frey monofilament (Stoelting, Wood Dale, IL) of 1.0 g bending force was applied to the plantar surface of each foot. A positive response to the von Frey stimuli was defined as retraction of paws or

retraction of paws with licking upon application of the von Frey monofilament. Responses to the single monofilament were recorded for the ipsilateral and the contralateral hind paw of each animal at baseline (pre-injections), 1 day following the 2nd acid injection and every week thereafter. Three trials, each consisting of 5 stimuli of repeated application (each 30 seconds apart) of von Frey monofilament to the plantar surface of the ipsilateral and the contralateral hind paws were conducted. The percent response for each hind limb was obtained by determining the number of withdrawals in response to 5 repeated stimuli of von Frey monofilament. For statistical analysis, data were averaged per group.

Mechanical Testing of Muscle Sensitivity: Mice were placed in 50 ml tube, and mechanical sensitivity to deep tissue was tested with a forceps compression device similar to the one described by Yu et al (Yu et al., 2002). A modified version of the device was built internally in our Neuromuscular Research laboratory to accommodate small size of the mouse species. The details of our modified version have been described previously (Sharma, submitted 6/2008). In brief, the device consisted of a forceps, a pressure sensor, a signal amplifier, and a laptop computer. A manual force was applied to each gastrocnemius muscle by the examiner blinded to group assignment and peak force to withdrawal response as well as time to peak force was recorded for each leg. The peak threshold level as demonstrated by withdrawal response or vocalization upon compression was measured bilaterally. Three consecutive squeezes on the right and left sides were conducted on each

mouse. A total of 3 trials were conducted only with CF-1 mice in experiment 2. The mean of 9 force peaks over three trials was calculated for each hind limb. Only force peaks with a loading time period, defined as the beginning of loading to the force peak, less than 1.0 second were used for the final calculation.

Biochemical Assays

In experiment 1 with C57BL/6 mice, 3 and 7 days following mild-exercise training, 11 mice from each group (the exercise and sedentary groups) were euthanized to analyze acute effect of exercise training on NT-3 synthesis. The remaining animals in the exercise group continued with treadmill training for 1 or 2 more weeks before termination. This time course design was chosen to determine changes in NT-3 expression over time following exercise training. A few animals were randomly chosen from each group at different time periods to analyze the biochemical effects of exercise. In experiment 2, CF-1 mice from all groups were terminated at the end of experiments, 24 hours after last exercise session. Two to three independent RT-PCR and protein assays per animals were performed. In experiment 1 (C57BL/6 mice), gastrocnemious and soleus muscle samples were homogenized together for message and protein assays; whereas both tissues were analyzed separately in experiment 2 (with CF-1 mice). Soleus is often chosen in many rat studies because of its high level of recruitment during treadmill training (Gomez-Pinilla et al., 2004; Hutchinson et al., 2004; Ying et al., 2003).

NT-3 mRNA: Total RNA was isolated using Trizol (SIGMA). Briefly, soleus and gastroctrocneimus tissue samples were removed from each animal, homogenized in 1 mL of Trizol reagent and precipitated with isopropanol. RNA pellets were washed with 75% ethanol and resuspended in DEPC water. RNA concentration was determined using a BioRed spectrophotometer. The amount of 0.653 ug reaction was reverse-transcribed using iScript TM cDNA synthesis kit (BIO-RAD). Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of NT-3 mRNA was done using .2 ug of total RNA and SYBR green master mix (BioRad iCycler). The thermal cycling conditions for PCR were set at 95º C and 60º C for 40 cycles (MyiQ real-time PCR detection system). Either Beta-actin (C57BL/6 mice) or glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (CF-1 mice) (Gomez-Pinilla et al., 2001; Ying et al., 2003, 2005) was used as a housekeeping gene. The primer sequences used for RT-PCR were as follows:

NT-3 forward: 5' AACGGACACAGAGCTACTACG-3'

NT-3 reverse: 5' CCATTAGGTATAAGGGAGGGGG-3'

Beta-actin forward: 5' AGTGTGACGTTGACATCCGTA-3'

Beta-actin reverse: 5' GCCAGAGCAGTAATCTCCTTCT-3'

GAPDH forward: 5' AGGTCGGTGTGAACGGATTTG-3'

GAPDH reverse: 5' TGTAGACCATGTAGTTGAGGTCA-3'

For standards, tissues from transgenic mice over-expressing NT-3 in skeletal muscle were used. Samples were run in duplicate or triplicate and control reactions were run with each amplification series. Each sample was analyzed 2 or 3 times

independently to reduce procedural variability. Only data with efficiency (90 – 105) and correlation > .98 were used to analyze group differences. Values were averaged and a relative change in RNA level was determined by subtracting threshold cycle values from the housekeeping gene (ΔCT). The group difference of ΔCT was calculated and the relative fold change was determined 2-ΔCT.

Agarose gel electrophoresis: To verify the size of PCR product and the primer specificity, 6 samples were randomly selected from experiment 1 and analyzed with electrophoresis. Ten µl of PCR product was directly sequenced (using bi-directional method) through 2% agarose gels, stained with DNA sample loading buffer, photographed, and analyzed for separation of DNA strain. Electrophoresis showed clear bands (Fig 5), and PCR product of NT-3 was approximately 100 bp and GAPDH was approximately 120 bp in length. Only samples with clear band of appropriate size were used for the final calculation.

Agilent Analysis: In addition, samples were tested for the quality of RNA using an electrophoretic separation technique (Agilent 2100 bioanalyzer tracer with the Eukaryote Total RNA Nano assay) prior to RT-PCR analysis. Only samples with RNA Integrity Number (RIN) above 7.0 were used for group comparison (Schroeder et al., 2006)]. Sample RNA integrity values for CF-1 mice ranged between from 8 to 9 on a 0-10 scale. The RIN range was around 7.0 for most C57BL/6 mice.

NT-3 protein analysis: The left gastrocnemius and soleus muscle either combined (C57BL/6 mice) or separately (CF-1 mice) were used to assess NT-3

protein levels using enzyme-linked immunosorbent assay (ELISA) methods. Frozen sections of samples were homogenized in homogenization buffer consisting 20 mM Tris-HCI, pH 8.0, 137 mM NaCl, 1% NP40, 1 mM PMSF, 10% Glycerol, 10 µg/ml aprotinin, 1 µg/ml Leupeptin, 0.5 mM Sodium Vanadate and 4% Triton X-100 using electrical homogenizers. The homogenates were centrifuged and supernatants were collected. The total protein concentration was measured using the Bradford method (protein reagent, Bio-Rad). NT-3 protein was quantified using ELISA kit (NT-3 Emax Immuno Assay System Kit, Promega, Madison, WI) (Ying, 2003; Ying 2005). Equal amounts of protein extracts (10 ug for soleus and 5 ug for gastrocnemius, when both tissues were analyzed separately in the 2nd experiment with CF-1 mice) were analyzed in duplicate using manufacturer's instructions. Only test samples with the linear part of the standard curve were used to determine the NT-3 concentration in pg/ml and compared for group differences.

Data Analysis

Hypersensitivity following the acid injections and the effect of exercise on behavioral measures was initially tested with a repeated measures analysis of variance (RM ANOVA) on ipsilateral and contralateral side separately**.** In addition, one-way ANOVA was conducted to examine the group differences related to cutaneous and muscle hyperalgesia at different time points when interactions (time*acid or time*exercise) were significant. Post hoc analysis of behavioral measures was used to conduct pair wise comparisons when group was significant

using RM ANOVA. In experiment 1 with C57BL/6 mice, a linear mixed model was used to compare cutaneous measures among groups over time. Mixed model is equivalent to RM ANOVA yet accounts for imbalance design (Verbeke & Molenberghs, 2000) as experiment 1 was a time-course study, scarifying certain numbers of animals for NT-3 synthesis and remaining fewer numbers over time. NT-3 mRNA measures were analyzed with Pfaffl method to determine pair wise comparisons between animals from different groups (Pfaffl, 2001; Pfaffl, Horgan, & Dempfle, 2002). NT-3 protein levels were analyzed with one-way ANOVA for group difference. Values were considered significant at α level $p < 0.05$.

3.4 Results

Behavioral assessments

The effect of mild intensity exercise training on secondary hyperalgesia was tested in experiment 1 with C57BL/6 mice. These mice were tested for the cutaneous hypersensitivity only. We did not test these mice for deep tissue pain. For the effect of moderate intensity level of exercise, we switched to CF-1 mice in experiment 2. This was based on a previous experiment conduced in our lab, which demonstrated a more robust effect of acid on the cutaenous and muscle hyperalgesia in CF-1 strain [Sharma, submitted 6/2008].

Cutaneous mechanical hypersensitivity

C57BL/6 mice: Behavioral data from C57BL/6 mice were considered inconclusive as a trend toward increased sensitivity was noted in all animals (regardless of group assignment) as the testing progressed with 1.0 g bending force of von Frey monofilament. Nevertheless, data are presented in Fig. 1. On the ipsilateral side, the pre-injection withdrawal threshold was 25% and increased to approximately 55% 3-weeks post-injection in all mice; mice in the exercise-con group displayed less ceiling effect of testing. On the contralateral side, the pre-injection withdrawal threshold of mice was at varying levels (from 15% to 35%) and increased to 60% 3-weeks post-injection in all mice; again, mice in the exercise-con group had less ceiling. These results indicate that exercise training prior to acid-injection had no effect on cutaneous hypersensitivity that normally develops following acid injection; that C57BL/6 mice in Sed-acid (SA) group did not develop secondary cutaneous hyperalgesia following acid injections; and that mildly-intense exercise training did not have significant effect on the cutaneous sensitivity (Fig. 1 A, B $p > 0.05$).

CF-1 mice: In comparison to C57BL/6 mice, CF-1 mice displayed a robust increase in secondary hyperalgesia in response to acid injection. Additionally, moderate intensity exercise decreased cutaneous hypersensitivity induced by acid injection (Fig 2, A, B). The cutaneous withdrawal response remained fairly unchanged on ipsilateral and contralateral sides in both control groups of mice (Fig. 2 A). In contrast to the control groups, the % withdrawal response increased on

ipsilateral and contralateral sides in both acid-injected groups. The % withdrawal response on the ipsilateral side in SA mice increased from 22.67 ± 5.2-8 to 45.33 ± 9.73 after 1-day and to 60.33 ± 4.05 after 3 weeks post-acid injection ($p < 0.05$). The % withdrawal response on the ipsilateral side in Ex-acid (EA) mice also increased from 30 ± 7.18 SEM to 50 ± 8.62 1-day after acid injection but then decreased to 41.33 ± 5.43 after 3 weeks of exercise training (Fig. 2 A, $p < 0.05$).

Likewise, the % withdrawal response on the contralateral side in SA mice increased from 21.33 ± 4.07 pre-injection level to 46 ± 6.24 1-day and 52.67 ± 5.92 3-weeks post-acid injection ($p < 0.05$). However, the % withdrawal response on the contralateral side in EA mice increased from 26.67 ± 7.50 of pre-injection level to 44.33 ± 9.43 SEM 1-day after the acid injection but then remained at 43.33 ± 6.46 after 3 weeks of exercise training (Fig. 2, B, $p > 0.05$). These results indicate that acid induced a robust cutaneous hyperalgesia in bilateral hind limbs of CF-1 mice. Furthermore, exercise training significantly reduced the heightened cutaneous hypersensitivity on the ipsilateral side. The effect of exercise training on the contralateral side was also positive but didn't reach the significance level. These results are consistent with previous studies (Bement & Sluka, 2005; Hutchinson et al., 2004; Kuphal et al., 2007) and indicate that long term exercise training is effective in altering cutaneous hypersensitivity.

Muscle mechanical hypersensitivity:

Muscle hypersensitivity was assessed only in CF-1 mice. As with the cutaneous sensitivity, CF-1 mice displayed a robust muscle hyperalgesia that lasted up to 2 weeks post-acid injections, and moderately-intense exercise minimally decreased muscle hyperalgesia (Fig. 2 C, D). Muscle withdrawal threshold increased on ipsilateral and contralateral sides in both control groups. Muscle withdrawal threshold on the ipsilateral side in the Sed-con (SC) mice increased from 99.58g ±8.74 pre-injection levels to 170.27g ± 5.04 and in the Ex-con group from 102.13g ±6.14 to 171.77g ±8.12 2-weeks post-saline injection. Likewise, muscle withdrawal threshold on the contralateral side in the SC mice increased from 74.64g ± 7.12 to 97.71g ± 5.59 and in the Ex-con (EC) group from 67.62g ± 4.93 to 99.26g ±5.55 2-weeks post-saline injection. These results suggest the control mice became acclimated to the muscle compression test and were able to tolerate greater compression over time.

In contrast to the control groups, muscle withdrawal threshold decreased after acid injections in both acid-injected groups (Fig. 2 C, D). Muscle withdrawal threshold on the ipsilateral side in SA mice decreased from 129.39g ±10.59 pre-injection level to 71.59g ±3.87 post-acid injection and remained decreased to 103.47g ± 6.13 levels 2 weeks later. Muscle withdrawal threshold on the ipsilateral side in EA mice also decreased from 98.62g ±6.36 pre-injection level to 69.18g ±5.93 after acid injection but then increased to 123.12g ±7.26 after 2 weeks of exercise training (Fig. 2 C, $p < 0.05$). Likewise, muscle withdrawal response on the contralateral side in SA mice decreased from 63.43g ±6.79 pre-injection level to

36.43g ±2.85 post-injection and returned to 61.09 ± 3.82 2-weeks later ($p > 0.05$). However, muscle withdrawal threshold on the contralateral side in EA mice also decreased from 53.94g ±4.78 of pre-injection level to 38.49g ±4.50 after the acid injection but then increased to 75.63g ±4.62 after 2 weeks of exercise training (Fig. 2, D, $p < 0.05$). These results indicate that acid induced a robust muscle hyperalgesia in bilateral hind limbs of CF-1 mice. Furthermore, exercise training minimally but significantly increased the muscle hypersensitivity on ipsilateral and contralateral sides. These results for the first time indicate that exercise training increased muscle withdrawal threshold in the chronic stage in animal model of acid pain.

Biochemical Assessments

C57BL/6 mice: To investigate whether mild intensity level of exercise training can stimulate NT-3 synthesis, a time course analysis was performed with C57Bl/6 mice. Both mRNA and protein levels of combined soleus and gastrocnemius muscle were analyzed after 3 and 7 days of exercise training to examine the acute effect of exercise. We also analyzed mRNA and protein levels following 2 and 3 weeks of exercise training (one week after the acid-injections were administrated). No significant difference was noted at any time point for message or protein levels (Fig. 3, $p > 0.05$) indicating mild intensity exercise training was not sufficient to synthesize NT-3 in mice**.**

CF-1 mice Since no change in message or protein levels were noted from experiment 1 with C57Bl/6 mice, the exercise training was increased to moderate

intensity level in the 2nd experiment with CF-1 mice, and lower limb muscle (soleus and gastrocnemius) were analyzed separately, as previous studies have analyzed exercise-induced neurotrophic synthesis in soleus (Hutchinson et al., 2004; Ying et al., 2003 2001).

NT-3 mRNA: the exercise training had no effect on mRNA in soleus muscle (Fig. 4 A, $p > 0.05$). In comparison, the exercise training increased mRNA level of the gastrocnemius muscle to 1.7 fold (SC versus EC), but the increased level of message was not significant (Fig. 4 B, p=.06). In addition, when the two groups of acid-injected (SA versus EA) were compared, no significant change in mRNA was noted; in fact, the EA had less message level. Interestingly we noticed up-regulation of NT-3 message in the sedentary mice that received acid injections (SC versus SA) (Fig. 4 B, p=.044), suggesting a possible interaction between acid and NT-3.

NT-3 protein: In contrast to mRNA, significant increase in protein levels was noted following 3 weeks of moderate-level of exercise training in CF-1 mice. In the soleus muscle, an upward trend was noted in both exercise groups but the difference was not significant (Fig. 4 C, $P > 0.05$). However, a more robust effect was noted in the protein levels of the gastrocnemius muscle. Both exercise groups (EC and EA) had significantly greater amount of NT-3 protein compared to the sedentary control group (Fig 4 D, $P < 0.05$). The results were consistent with each separate assay and Fig. 4 is a representation of one assay. These results indicate that exercise performance at a high level is required to stimulate NT-3 synthesis in the skeletal muscle. Additionally, exercise can preferentially increase NT-3 synthesis in select

muscles, preferentially muscle with higher number of spindles. We didn't test for NT-3 synthesis in the spinal cord, and this should be examined in the future.

3.5 Discussion

There is a significant gap in our knowledge about how exercise alters deep tissue hypersensitivity, as many experiments are limited to cutaneous sensation. Here we analyze the effect of mild and moderate levels of exercise training on secondary hyperalgesia induced by acid injection and NT-3 synthesis in skeletal muscle. There are 2 main findings of the present study. One, we have demonstrated that exercise training doesn't reverse acid-induced secondary hyperalgesia but significantly reduces muscle hypersensitivity, especially in the chronic stage in the acid-induced pain model. Two, moderately intense treadmill running is required to cause an increase in muscle-derived NT-3 levels in mice species that is preferentially greater in the gastrocnemius muscle. These results show for the first time that exercise decreases chronic muscle hyperalgesia in rodents, support the growing literature indicating the role of NT-3 in pain modulation, and provide a possible explanation for exercise-induced analgesia in chronic muscle pain.

Chronic Acid-Induced Muscle Pain Model

The acid model is a non-inflammatory muscle pain model and is considered analogues to human fibromyalgia. Acid saline produces mechanical hypersensitivity of cutaneous (Hoeger-Bement & Sluka, 2003; Skyba et al., 2002; Sluka et al., 2001;

Sluka et al., 2003; Sluka et al., 2002), visceral (Miranda, 2004) and muscle tissue (Yokoyama, Maeda, Audette, & Sluka, 2007a; Sharma, submitted 6/2008) that lasts up to 3-4 weeks. This model does not rely on on-going input from the periphery to elicit hypersensitivity, and central mechanisms likely contribute to the development and maintenance of the hyperalgesia. (Hoeger-Bement & Sluka, 2003; Skyba et al., 2002; Sluka et al., 2001; Sluka et al., 2002; Tillu et al., 2007). Convergent input in the spinal cord from muscle and paw fibers and receptive field plasticity of wide dynamic range (WDR) neurons is believed to cause the widespread secondary hyperalgesia (Sluka et al., 2001). No muscle tissue damage or gross motor / sensory loss is associated with this model of pain (Sluka et al., 2001), thus making it ideal for the present exercise and pain study.

Aerobic exercise attenuates mechanical hyperalgesia associated with acid-injections

Clinical studies using various types of exercise interventions for people with chronic pain syndromes are increasing (Gowans, 2004; Glass, 2004; Hoffman, 2004; Meiworm, 2000; Mannerkorpi, 2005; Whiteside, 2004; Droste, 1991; Fulcher, 1997; Redondo, 2004; Goldenberg, 2004). Although human experiments provide important treatment avenues, their basis are theoretical rather than empirical. More animals studies are needed to extend our knowledge about exercise-induced analgesia and its mechanisms. A limited number of animal studies have attempted to address this issue. Bement and Sluka recently reported that low intensity treadmill training (3.05 m/min speed for 30 minutes) for 5 days reversed acid-induced cutaneous mechanical

allodynia in rats, suggesting opioid-mediated effects of exercise training on cutaneous pain (Bement & Sluka, 2005) . However, this study examined an immediate effect of exercise, as seen in most animal studies (Koltyn, 2000).

Another study reported a decrease in responses of licking/flinching following 9 days of swimming. They associated the beneficial effects of exercise by decreasing C-fiber activity that causes phase 2 symptoms following formalin injection (Kuphal et al., 2007). In the same study, the authors demonstrated a decrease in cold and heat hyperalgesia associated with nerve injury in response to long term exercise. The goal of the present study was to examine the long-term effect of exercise training on muscle hypersensitivity, which is the predominant complaint in people with various chronic pain syndromes. The results suggest that moderately intensity exercise is more effective in reducing chronic nature of secondary hyperalgesia. These results are consistent with previous studies (Bement & Sluka, 2005; Hutchinson et al., 2004; Kuphal et al., 2007) and provide additional information about the role of exercise in negating muscle pain.

Moderately intense exercise training is needed to induce NT-3 synthesis

No consensus currently exists on specifics of the level or the type of activity required for NT-3 synthesis in adult rodents. In addition, studies examining the effect of exercise on NT-3 have been performed exclusively using rat models. Thus, we examined 2 different intensities of exercise training on 2 mouse strains. Contrary to

Hutchinson's finding where NT-3 mRNA was up-regulated with general physical activity (low intensity treadmill training at 11-13 m/min, swimming or standing), we report that a moderately intense exercise is required to stimulate NT-3 synthesis mice. The differences in the exercise training protocol and in the choice of species may explain such disparity in results. Here, the exercise training in experiment 1 was not vigorous enough to alter gene express of NT-3 in C57Bl/6 mice. Therefore, exercise training was increased to a moderate level in the 2nd experiment with CF-1 mice, resulting in significantly increased NT-3 levels. This finding is consistent with Ying's study in which 28 days of wheel running was required to stimulate NT-3 synthesis in the spinal cord under a condition of injury (Ying et al., 2005). In adult life, NT-3 is primarily synthesized in peripheral tissues, especially in skeletal muscle and in muscle spindles. A higher level of exercise is likely to activate the nervous system and stimulate muscle-derived NT-3 synthesis via spindle activity. Besides muscle spindles, NT-3 is also synthesized in smooth muscle cells along the lining of vasculature that innervate muscle tissues (unpublished data from our lab). Moderately intense running is required to stress the cardiovascular system and to promote blood circulation, thus leading to increased NT-3 synthesis in the peripheral tissues. Our findings are consistent with those who used a higher level of exercise regimen for NT-3 levels (Ying et al., 2005). An in vitro experiment provides further support for activity-dependent expression of NT-3. Xenopus developing muscle cultured cells treated with an electrical stimulation (for 1-hour, 6 Hz, just above the threshold of muscle contraction level) or with acetylcholine (Ach) significantly

increased NT-3 RNA (Xie, Wang, Olafsson, Mizuno, & Lu, 1997). These authors suggested activity-dependent NT-3 expression via Ca2+ influx that was restricted to immature muscle cells.

Additionally, the increase in NT-3 was mainly observed at the protein levels and not at message levels. This could be attributed to the experimental design where animals were sacrificed 24 hours after the last exercise bout. We might have missed the period when the message was upregulated. The detectable protein levels in the muscle are either a result of in-situ synthesis or its cellular transport from the spinal cord, as exercise has been shown to increase spinal NT-3 (Ying et al., 2003). However, we believe that the earlier explanation is more plausible as NT-3 is primarily synthesized in skeletal muscle postnatally.

Consistent with previous rat studies (Gomez-Pinilla et al., 2004; Hutchinson et al., 2004; Ying et al., 2003, 2005), we observed an upward trend in NT-3 protein synthesis in the soleus muscle. However, the group difference between exercise and sedentary animals was not significant. This could be due to the small size of the soleus muscle in mice, compared to rats. Additionally although soleus is a postural muscle and likely to be more active during treadmill running, the running pattern of mice is different than rats. Mice often jump, run backwards, and climb on the walls of the treadmill; all which stimulate spindle activity in the gastrocnemius as well. In addition, it is unclear whether NT-3 is preferentially more expressed in slow-twitch

or fast-twitch fibers in adult rodents under normal conditions. NT-3 has been shown to favorably improve reinnervation of fast twitch fibers (gastrocnemius and extensor digitorum longus) over the slow twitch fiber muscle, soleus (Simon, Mann, Coulton, & Terenghi, 2002; Simon et al., 2000) and myo/transgenic mice showed overproduction of NT-3 and spindle counts in gastrocnemius and plantaris muscle compared to the soleus muscle (Wright, Zhou, Kucera, & Snider, 1997). Collectively, these data suggest that NT-3 is synthesized in higher levels in fast-twitch fibers and support the finding of the present study in which aerobic exercise training had greater effect of NT-3 synthesis in the gastrocnemius over soleus.

Interestingly, acid saline also increased NT-3 expression in the gastrocnemius muscle in SA mice. This suggests a possible interface between acid and NT-3, which was not expected. No link between ASIC3 channels that are responsible for sensing the change in intracellular pH levels and NT-3 has been reported in the literature to our knowledge. Our finding suggests that acid may not selectively activate ASIC3 and might cause activation of other factors that lead to an increase in NT-3 synthesis. This finding may indicate an interaction between acid and NT-3 levels in the condition when the muscle is metabolically challenged (either with acidosis or exercise). There could be also be a more simple explanation such as animals in the acid group could have been more active in their cages under painful conditions, which might have resulted in an increase in NT-3 synthesis.

There may be different adaptive mechanisms in response to exercise or activity in different strains of rodents. The differences observed in the current study could also be due to different genetic backgrounds of the 2 strains of mice used in the experiment. A previous study from our lab [unpublished data] show genetic variability in the development of muscle pain with acid injections. Exercise training has also been shown to be subject to genetic variability.

Potential mechanisms underlying anti-nociceptive effect of exercise-induced NT-3 on chronic muscular pain

The effects of exercise-induced analgesia in patients with chronic pain are poorly understood. Limited information is available to determine the role and mechanism of exercise in relieving chronic pain. Two possible mechanisms have been reported in support for exercise-induced analgesia. The most widely accepted explanation of exercise-induced analgesia involves modulation of central opioid receptors (Bement & Sluka, 2005; Hoffman et al., 2004; Skyba et al., 2002; Sluka et al., 2002). However, this effect is short-term and often a result of intense exercise training. Another theory of central mechanisms proposes that activation of proprioceptive and muscle afferents inhibit central pain circuitry and may affect descending inhibitory pathways (Hoffman et al., 2004). We propose this explanation in support for our results over other mechanisms of exercise induced analgesia as the beneficial effect of exercise on muscle pain was bilateral and extended to cutaneous tissue. In adult life, NT-3 is primarily synthesized in peripheral tissues, preferentially

binds to trkC receptors located on about 75% of muscle afferents (Watanabe et al., 2000) and retrogradely transported to cell bodies of the DRG (Malcangio et al., 1997; Munson et al., 1999; Watanabe et al., 2000; Wright, 2001). Given its anatomical location and ability to be altered under physiological stress (i.e. exercise), NT-3 could provide an indirect measure of neuroplasticity associated with inhibition of central pain circuitry. Additionally, NT-3 may also be expressed by C-fibers and/or A-δ fibers (Gandhi et al., 2004; Wilson-Gerwing et al., 2005) which could provide additional support for its ability to modulate pain.

Besides trkC receptors, NT-3 is also capable of activating trkA and trkB receptors after certain injuries (Saragovi & Gehring, 2000; Wilson-Gerwing et al., 2005), providing another possible mechanism by which exercise alters muscle pain. NT-3 has been shown to reduce the expression of trkA receptors and substance P that are involved in the inflammatory response in intact neurons (Gratto & Verge, 2003). NT-3 either systemically injected for 2 weeks or acutely applied to the spinal cord, significantly reduces electrically evoked release of substance P from the central terminals of the primary afferents (Malcangio et al., 1997) under normal conditions. In addition, the antagonistic role of NT-3 to secondary hyperalgesia has been tested in many pain models. Intrathecal administration of NT-3 prevents thermal hyperalgesia and suppresses the injury-induced overexpression of transient receptor potential vanilloid (TRPV1) receptors in the DRG and the spinal cord in rats receiving a chronic constructive injury (CCI) of the sciatic nerve (Wilson-Gerwing et

al., 2005). A single systemic dose of NT-3 into hyperalgesic rats (by injection of NGF) results in mechanical but not thermal hypoalgesia 24 hours after the administration. Either overexpression of NT-3 or intramuscular injection of NT-3 negatively affects mechanical hyperalgesia from acid injection (Gandhi et al., 2004). In many of these studies, continuous infusion of exogenous NT-3 was required for its positive effects. If exercise provides a continuous source of endogenous NT-3, it could explain its positive effect on muscle pain, at least in part.

The activity-induced muscle-derived NT-3 could have antinociceptive effects on muscular pain at 3 possible sites. One, NT-3 may have local effects on muscle afferents. Two, it may have general influences on gene expression of various neuropeptides and neurotransmitters that are involved in the process of nociception after it reaches the soma via retrograde transport. Three, NT-3 is likely to influence the spinal circuitry of nociception through central projections of muscle afferents that terminate in lamina III and IV (Woolf, 1987). The interneurons of lamina III and IV have direct connections with wide dynamic range (WDR) neurons whose hyperactivity leads to the acid-induced hyperalgesia (Sluka et al., 2001). These questions should be addressed in future studies.

3.6 Conclusion

Our study is the first to report the effect of exercise training on muscular pain. We have demonstrated that long-term exercise training did not reverse, but significantly reduced cutaneous and deep tissue mechanical hypersensitivity induced

from acid injection. The data also demonstrate activity-dependent NT-3 synthesis in selective peripheral tissues as a result of higher intensity exercise training. We associate the decrease in mechanical hypersensitivity to elevated levels of NT-3 protein as mild intensity level of exercise training failed to increase NT-3 expression and thus decrease secondary hyperalgesia. This further supports the notion that NT-3 is an anti-nociceptive neurotrophin. We also show a positive correlation between exercise, activity-induced NT-3 and modulation of deep tissue pain. These findings indicate a beneficial effect of exercise and a potential role of NT-3 in the treatment of muscular pain. However, the mechanism by which NT-3 modulates mechanoreceptors is unknown and remains to be investigated. The findings of exercise's effects on NTs are restricted to animal models; however, these studies give insight into the potential explanation for exercise-induced relief of muscle pain reported in clinical studies.

Acknowledgements

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Table1. Experimental design

	C57BL/6 Mice	CF-1 Mice
Exercise protocol:	12 m/min, 25', 3 weeks	1st week: 13 m/min, 30'
		2nd week: 14m/min for 40'
		3rd week: 15-16 m/min for 45'
Acid Injections:	1 week after start of Ex	2 days before s of Ex
Behavioral measures:	Cutaneous hypersensitivity	Cutaneous hypersensitivity
		Muscle hypersensitivity
Biochemical measures:	NT-3 mRNA and protein	NT-3 mRNA and protein
	gastrocnemius & soleus combined	Soleus
		Gastrocnemius separately

Table 2: Animal assignment

Experiment 1: C57BL/6 mice

	3 days Ex Remaining mice (n=58)	**7 days Ex Remaining mice (n=36)**		**14 days Ex Remaining mice (n=20)**	**21 days Ex**
			Acid-Injection		
Sedentary (n=40)	-11 for NT-3 analysis	-11 for NT-3 analysis		-8 for NT-3 analysis	
			Sed-Con (n=9)	N=9	N=5
			Sed-Acid (n=9)	N=9	N=5
Exercise (n=40)	-11 for NT-3 analysis	-11 for NT-3 analysis		-8 for NT-3 analysis	
			Ex-con (n=9)	N=9	N=5
			Ex-Acid (n=9)	N=9	N=5

Experiment 2: CF-1 mice

Random Assignment	**Stratified Assignment**
Acid (n=20)	Sedentary-Acid (n=10)
	Exercise-Acid (n=10)
Control (n=20)	Sedentary-Control (n=10)
	Exercise-Control (n=10)

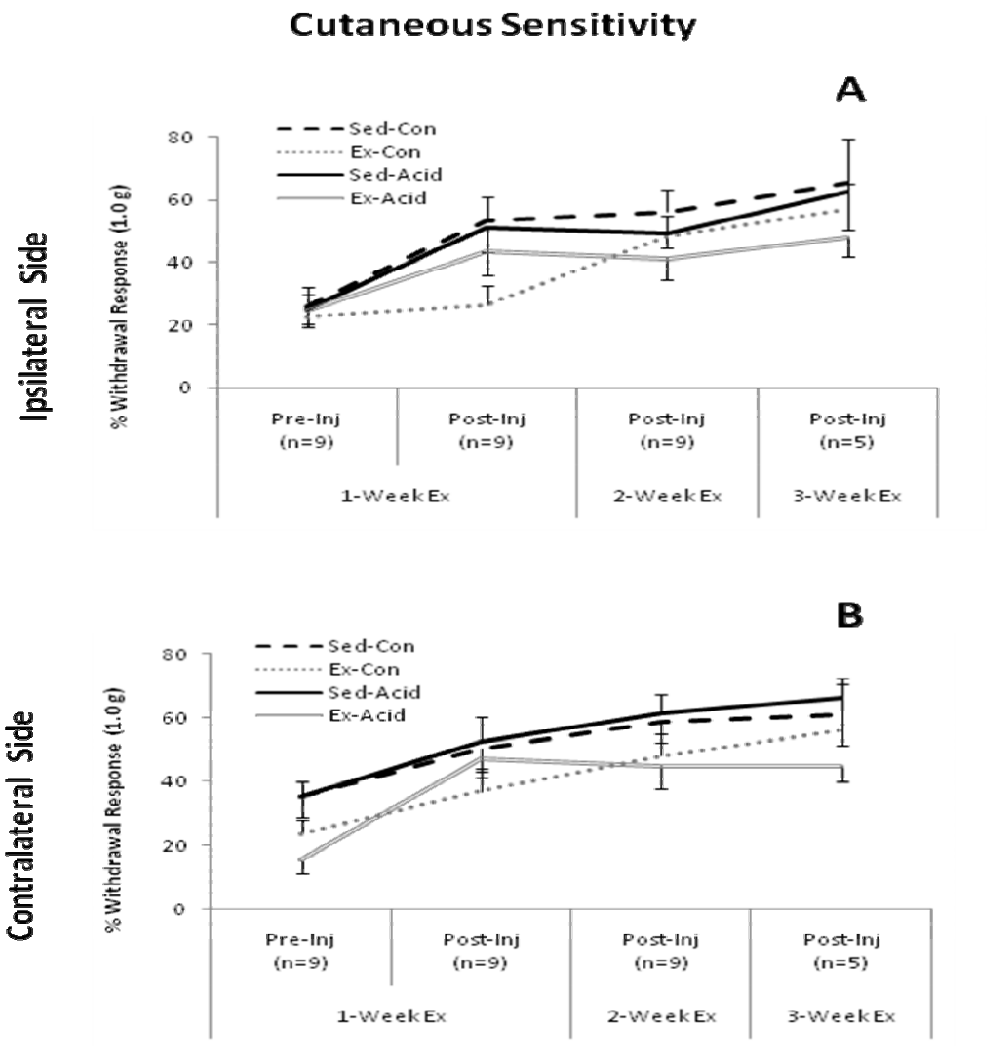

Figure 1: The effect of mildly-intense exercise training on cutaneous hyperalgesia

Mice did not development cutaneous hyperalgesia and mildly-intense exercise had no effect on cutaneous sensitivity on the ipsilateral or contralateral sides (group; interaction $p > 0.05$). Mice in all groups developed hypersensitivity with 1.0 g force; thus these data are considered inconclusive. Data are shown in mean ± SEM.

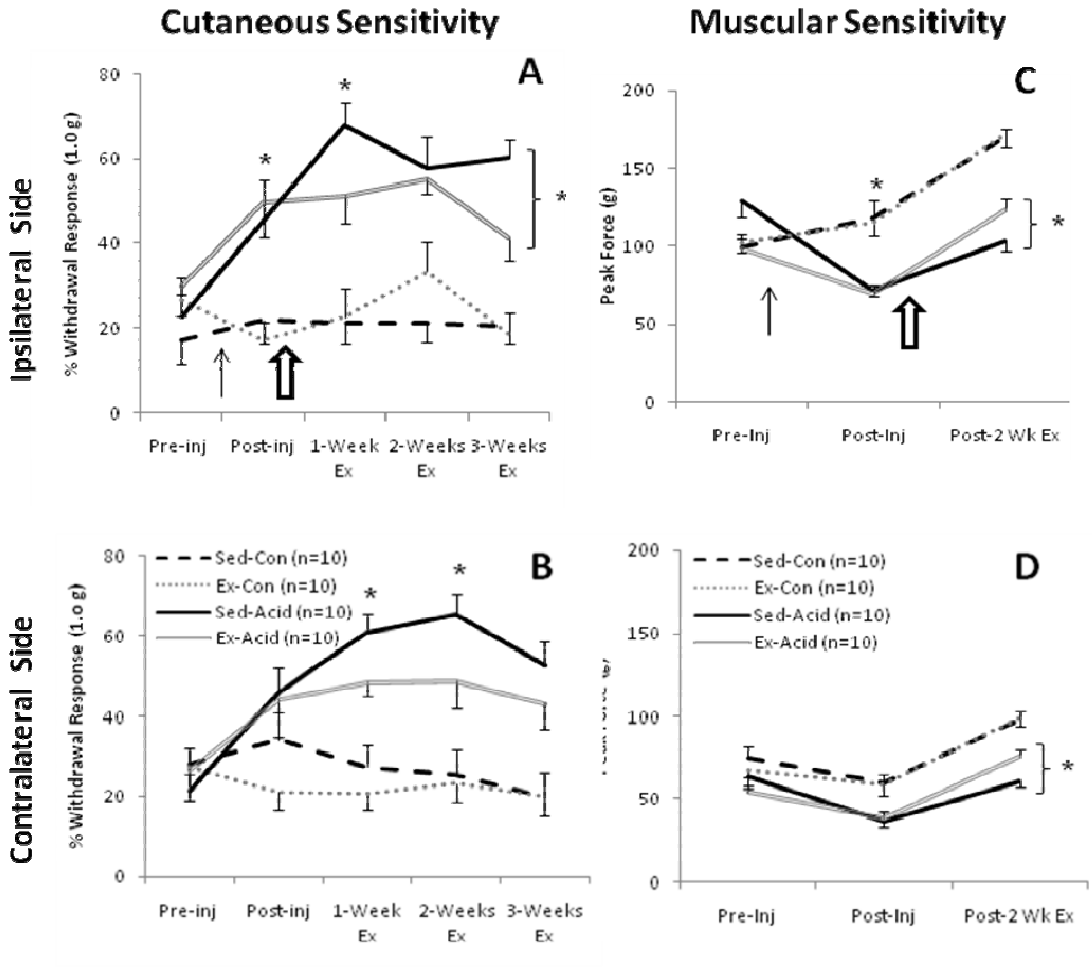

Figure 2: The effect of moderately-intense exercise on cutaneous and muscle hyperalgesia

Acid injections significantly increased cutaneous withdrawal response of the ipsilateral and contralateral sides. Moderately-intense exercise significantly decreased withdrawal response (A, $p < 0.05$) on the ipsilateral side. Similar effect was noted the contralateral but was not significant (B, $p > 0.05$). Likewise, acid injections significantly decreased muscle withdrawal threshold. Exercise training had minimal but significant effect on muscle withdrawal threshold.

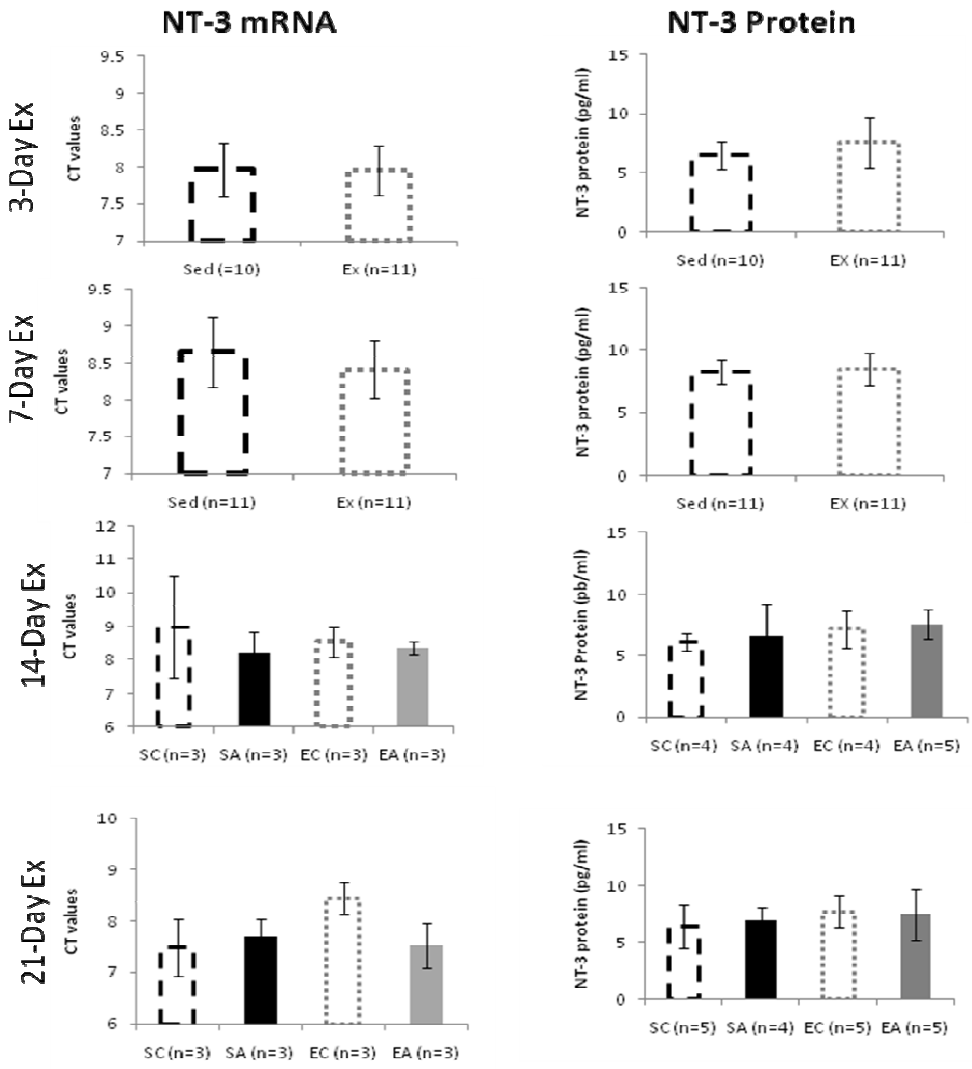

Figure 3: NT-3 mRNA and Protein Levels in C57BL/6 Mice

No significant differences were noted at mRNA or protein levels in combined tissue of the gastrocnemius and soleus muscle following 3, 7, 14, or 21 days of mildly-intense exercise training. Data are represented in mean ± S.E.M. mRNA was analyzed with Pffafl pair wise comparisons ($p > 0.05$). Protein level was analyzed with t-test ($p > 0.05$) following 3 and 7 days of exercise and ANOVA ($p > 0.05$) following 14 and 21 days of exercise training.

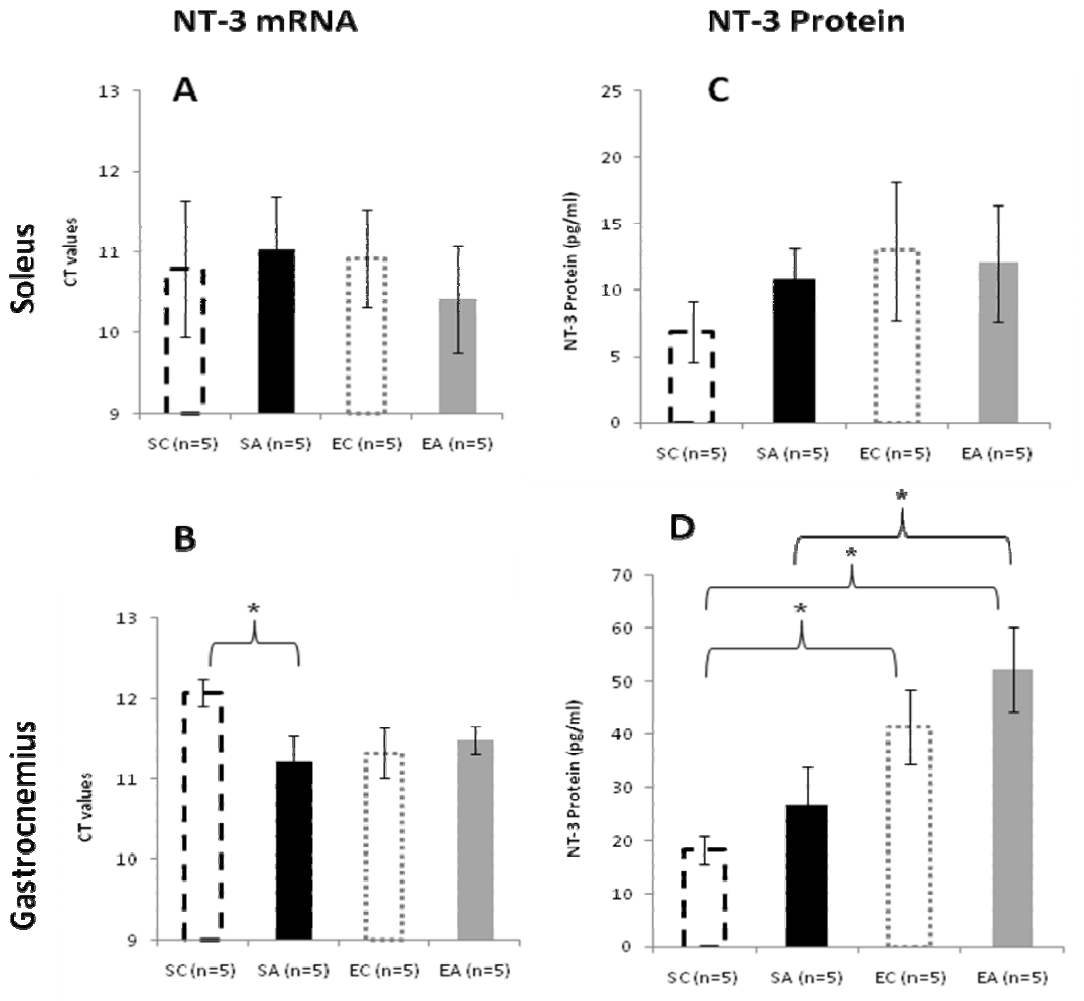

Figure 4: NT-3 mRNA and Protein Levels in CF-1 Mice

Moderately intense exercise training did not change NT-3 expression in soleus or the gastrocnemius muscle (A, B, $p > 0.05$). Acid injection significant increased NT-3 mRNA in the gastrocnemius muscle (B, $p < 0.05$). In contrast, the effect of exercise training was greater at the protein levels (C and d). The increased NT-3 protein levels were not significant in soleus (C, $p > 0.05$) but were significant in the gastrocnemius muscle (D, $p < 0.05$).

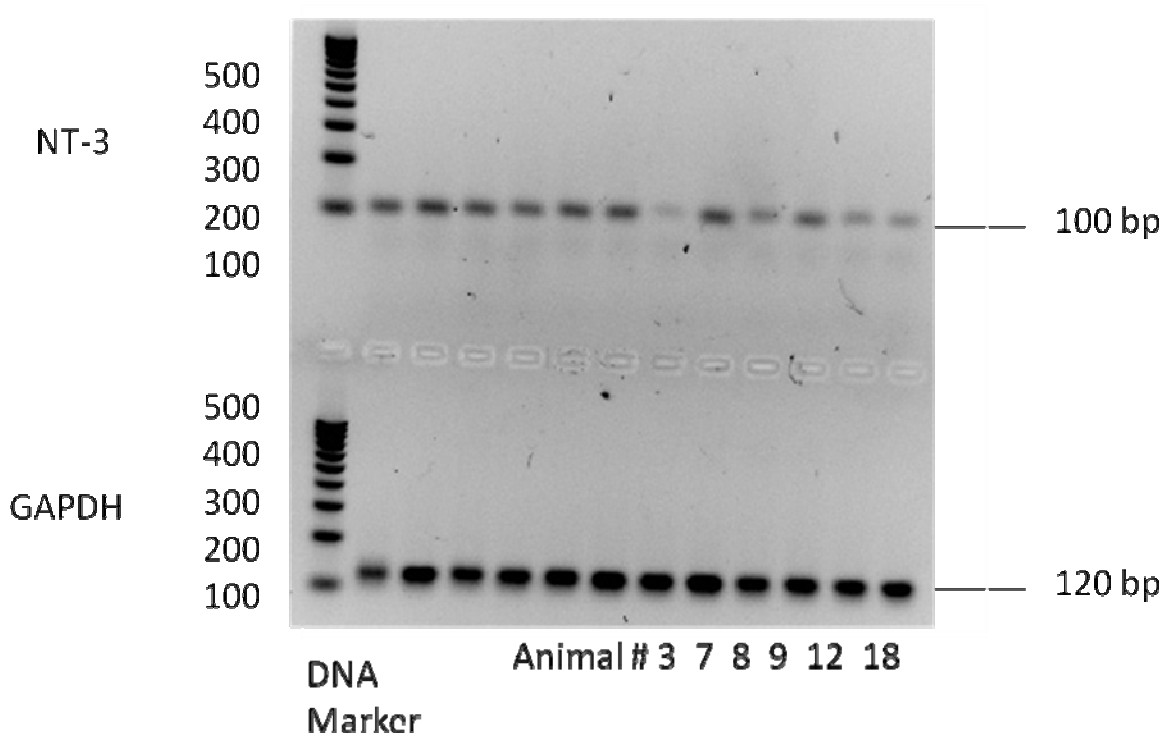

Figure 5: Agarose Gel Electrophoresis of RT-PCR

Six animals from C57BL/6 mice, as labeled, were randomly selected to verify the primer size. NT-3 primer resulted in 100 bp and GAPDH resulted in120 bp size of RT-PCR produce, as expected from each primer size.

Chapter 4

Summary

5.1 Summary of Findings

The goal of the present study was to use a referred pain animal model of musculoskeletal structures to study muscle pain and to investigate the efficacy of exercise training in negating centrally driven widespread musculoskeletal pain. The identification of muscle pain and its assessment is a relatively recent development in the field of pain research. Previously most pain related experiments have exclusively used various cutaneous and neuropathic pain models. Even to this date, very little is known about physiological events and the underlying mechanisms that lead to muscular pain. We have measured muscle pain in 2 strains of female mice and assessed the hypothesis that animals develop chronic muscle hyperalgesia following acid injection. The results suggested variability in the development of muscle hyperalgesia in C57BL/6 versus CF-1 mice, as observed by different measures, presumably due to the genetic background. The 2 strains of mice also differed in Fos expression of the spinal cord cells, which was analyzed by applying mechanical stimulation to the cutaneous and muscle tissues. Cutaneous palpation and muscle compression resulted in different pattern of Fos expression in the dorsal horn cells of the spinal cord.

Secondly, despite many human studies, the impact of exercise on the pain threshold and its underlying mechanism has received limited attention in basic science experiments. The anti-nociceptive effect of exercise at the cellular and molecular level is largely unexplored. Here, the effect of 2 levels of aerobic exercise-training, mild and moderate, in negating widespread hyperalgesia and over-

expression of NT-3 was analyzed using an animal model of chronic pain. The results indicated that moderate intensity exercise was most beneficial in decreasing the cutaneous and muscle hyperalgesia induced by acid injections. Furthermore, the moderate intensity exercise was required to cause the necessary physiological events that led to over-expression of NT-3 in mice. Thus, the study also provides a molecular link between exercise training and muscular pain hypersensitivity.

5.2 Limitation of study

Characterization of muscular pain is difficult in rodent models of pain. Previous electrophysiological studies are limited to a specific set of dorsal horn neurons; the Fos measure provides a broader image of all possible neurons that are activated to a given stimulation. However one criticism is that this measure does not distinguish between facilitatory and inhibitory cell populations. Another limitation of the Fos measure is that it is difficult to separate brainstem input from the dorsal horn activity. Additionally, since the compressive force was manually delivered to induce Fos expression in the present study, it is difficult to compare results of the present experiment across different studies (Chapter 2). A quantifiable method needs to be considered in future studies. The forceps compression device used for behavioral testing in the present study is ideal to apply long-duration noxious stimulus needed for Fos expression.

The duration of the exercise protocol with CF-1 mice (Chapter 3) may be a limitation of this study. The experiment should have been extended from 3 weeks to

4 weeks, as acid has been shown to induce chronic hyperalgesia that lasts up to 4 weeks (Sluka et al., 2001). The significant effect of exercise on cutaneous hyperalgesia started about 3 weeks post-exercise. Likewise, the effect of exercise on muscle hyperalgesia should have been analyzed every week for 4 weeks. The present study assessed the effect of exercise on muscle hyperalgesia 2 weeks post-exercise training, which displayed minimal effect. Thus it is difficult to comprehend the full effect of exercise training on the chronic stage of pain. One additional week of exercise training would have provided a better understanding of its effect on secondary hyperalgesia in the chronic state.

The efficacy of exercise training on the chronic nature of pain was analyzed with behavioral measures. A complimentary biomarker of a pain measure, such as Fos, could confirm the behavioral finding and should be utilized in future studies. One must be cautious as Fos can be evoked with exercise alone (Jasmin et al., 1994). However, Fos dissipates after 5-6 hours following stimulation (Harris, 1998), thus should be utilized as a biomarker to examine the effect of exercise on hypersensitivity at least 24 hours following the exercise training.

The physiological significance of increased NT-3 level in the periphery is contingent upon the availability of trkC receptors or other receptors responsive to NT-3. In the present study, we measured the expression of NT-3 at peripheral and central levels (only mRNA in the spinal cord, unpublished data) and report that activity can modulate its synthesis. We did not examine the effect of exercise on trkC receptors and assumed that over-expressed NT-3 is being taken up by these

receptors. However, a previous study reported upregulation of trkC receptors peripherally and centrally from 7 days of exercise training (Ying et al., 2003). Considering the lack of studies in this field, future studies should determine if activity could modulate the expression of its signal transduction receptor, trkC in skeletal muscle and in the spinal cord to confirm that NT-3 is being taken up via its receptors to exert its effect on functional levels.

The mode of exercise training, treadmill training chosen in the present study could have been a potential confounding factor in decreasing mechanical hypersensitivity. Previous studies have suggested that stress is often associated with forced treadmill running that may not be due to physical exhaustion (Burghardt et al., 2004). To counter this, a 12-hour lag period between exercise training and behavioral testing was allowed. Also, animals were permitted to rest on shocker during the exercise training, as shockers were kepted turned off. Additionally, it may be difficult to relate the results of the present study to clinical syndromes of chronic pain in humans. Many people with FMS suffer from multiple symptoms and thus have less motivation to perform their exercise training regularly. These patients have poor level of physical fitness, exemplified by lesser capability to perform exercise and a tendency to fatigue with usual activities (Adams & Sim, 2005; Jones et al., 2002). These people present with reduced physical performance capacity but not reduced aerobic capacity (Mannerkorpi, 2005). Unlike the animals, humans cannot be forced to exercise at researcher-determined exercise training. However basic science experiments, as proposed in the present study are necessary to examine

possible molecular changes occurring in response to exercise training. The correlation of pain modulation to molecular changes through exercise training can provide the necessary research information to clinicians to support exercise prescriptions and to guide these patients.

5.3 Characteristics of Acid-Induced Model

The acid model is an important pain model that allows for assessment and intervention of hyperalgesia in deep tissues. Tissue acidosis occurs in many physiological (exercise, ischemia) (Hoheisel et al., 2004; Molliver et al., 2005) and pathological conditions (such as arthritis, inflammation and cancer) (Hoheisel et al., 2004; Issberner, Reeh, & Steen, 1996) and is thought to contribute to the experience of pain. The acid-pain model was first developed in the rat by Sluka (Sluka et al., 2001) and has been extensively used to assess widespread musculoskeletal hyperalgesia of cutaneous structures. Since then, the model has been tested in a mouse species by an earlier experiment in our lab. Gandhi et al. demonstrated that the acid-model can be reproduced in CF-1 mice and pH 4.0 is most effective in causing secondary hyperalgesia, compared to pH 5.0 or 6.0 (Gandhi et al., 2004; Sluka et al., 2003), similar to initial rat studies (Sluka et al., 2001). In the present studies, the acid-model was repeated in 2 distinct strains of female mice; C57BL/6 inbred, a commonly used strain in the field of pain, and CF-1 outbred. We have demonstrated that the acid-model does not work well in C57BL/6 female mice; CF-1 is the best strain for studying acid-induced hyperalgesia. In CF-1 mice, Gandhi et al.

reported a 30% increase in cutaneous sensitivity with a 1.4 g monofilament following acid injections; in the present studies, a 30% increase was also found with a 1.0 g bending force, resulting in 30% pre-injection to 60% post-acid injection withdrawal response. Collectively, these results indicate consistent and reproducible findings in the cutaneous tissue and favor the use of CF-1 strain in acid-pain model.

It has been repeatedly shown that acid induces widespread cutaneous hyperalgesia (Gandhi et al., 2004; Sluka et al., 2001) and visceral hyperalgesia (Miranda et al., 2004). However, it is unclear whether animals continue to experience muscular pain, which is a clinically important issue. Concurrent to our study of muscular pain assessment, Yokoyama et. al. demonstrated that rats develop muscle hyperalgesia 24 hours following the acid-injections (Yokoyama et al., 2007), indicating the acute state of muscle pain in rats. We demonstrated that intramuscular acid not only produced bilateral muscle hyperalgesia but also muscle hyperalgesia was maintained for 2 weeks, indicating the chronic state of muscle pain. More importantly, our study demonstrated strain differences in the development of muscle hyperalgesia; C57Bl/6 female mice did not develop robust cutaneous hypersensitivity, nor did they develop primary muscle hyperalgesia to mechanical stimulation. In contrast, the mechanical hyperalgesia related to the cutaneous and muscle tissue was robust and long-lasting in CF-1 female mice. The increase in the primary muscle hyperalgesia was strongly correlated with an up-regulation of Fos expression in the corresponding superficial dorsal horn laminae in the spinal cord. Thus the chronic phase of muscle hyperalgesia was evident by behavioral and

neurochemical marker analyses. These results for the first time demonstrate that animals develop chronic muscle hyperalgesia following acid injections. It is worth noting that the ipsilateral muscle hypersensitivity is being called as the primary hyperalgesia; it may also be referred to as secondary hyeralgesia since acid does not induce any tissue injury (Sluka et al., 2001). The present experiment extends the results of previous studies conducted in rats (Sluka et al., 2001) and mice (Gandhi et al., 2004) and additionally shows the development of chronic muscle hyperalgesia.

Genetic variability

This is the first evidence demonstrating possible genetic differences in the development of muscle hyperalgesia. Many clinical studies have pointed out gender and genetic differences that contribute to human pain sensitivity (Berkley, 2006; Limer, Nicholl, Thomson, & McBeth, 2008). The same is true for animals. Jeff Mogil has published a number of articles indicating the naturally occurring variations in pain measures attributed to genetic differences. One might think that inbred mice will have less variability then outbred. However, Mogil et al. tested 11 inbred strains of mice with 12 commonly used nociceptive measures and reported significant strain variability within the inbred mice (Mogil et al., 1999). In this study, C57BL/6 mice displayed resistance to acid saline with von Frey testing. Besides this reported finding, the specific information related to the strains of mice used in the present experiments is not available, i.e. the variability in the number of ASIC3 receptors, their variants, the variability in nociceptive behavior of these 2 mouse strains or their

susceptibility to pain. In general, the genetic variability associated with nociception involves the promoter regions of genes or other regulatory regions such as their loci, their variants and the product they code for, which ultimately leads to functional changes in protein and enzymes involved in nociception rather than a point mutation in specific genes (Mogil et al., 2000). Mogil identified quantitative trait loci (QTL) on chromosome 4, which accounts for the inconsistency in hot-plate nociception measure among male and female C57BL/6 mice. The variability was mainly in the gene that encodes the delta-opioid receptor (Mogil & Belknap, 1997). The fact that we showed differences in deep tissue hyperalgesia by 2 distinct measures, behavior and Fos, which indicated that C57Bl/6 female mice were less susceptible to acid-induced effects. Thus, we suggest that CF-1 female mice should be used to perform any further testing in an acid-induced pain model. This knowledge also provides an explanation for the baseline variability that we have encountered with each nociceptive measure in C57Bl/6 mice and previously reported in QTL experiments among female mice (Mogil et al., 1997). We relate the variability in pain measures to the genetic background since the environment is often controlled in the laboratory setting. However, there may be some environmental factors that could have skewed the results. Future studies should examine how two strains are different in their genetic make-up resulting in different responses to acid.

Spinal Neuronal Activation Pattern

The spinal activation for centrtal projection of deep tissue structures has not been studied in depth. Previous studies of the gastrocnemius muscle hyperalgesia caused by inflammatory agents (Hunt et al., 1987) or an electrical stimulation to sensory nerves (S. F. Wang et al., 2005) suggested the activation pattern of Fos in the lateral aspect of the dorsal horn, which is similar to the present finding. These studies have provided important information regarding the dorsal horn activation following primary muscle afferents but are limited to inflammation and individual nerve stimulation. The intention of on of the present studies was to examine the spinal cord Fos expression of the gastrocnemius muscle under a chronic non-inflammatory condition with a clinical test of "palpation". Therefore, to obtain a complete spatial distribution of all primary afferents originating from the gastrocnemius muscle, we examined the activation of the entire lumbar spine, including both dorsal horns, in response to a functional / clinical stimulus of compression. This knowledge provides an increased understanding of the dorsal horn circuitry, involved in mechanical nociception. Gastrocnemius compression resulted in rostrocaudal, centrolateral somatotopic organization of the ipsilateral dorsal horn including all laminae. In comparison, paw palpation resulted in Fos expression of mediocentral superficial laminae (I/II) of the ipsilateral side restricted to the L4/5 segment and bilateral deep laminae (V/VI) involving multiple segments (L2-L5). These results indicate that paw tissues mainly contain nociceptors (C-fibers and Aδ) whose projections are bilateral in the deeper regions, in addition to the known ipsilateral superficial layers. Whereas muscle tissues contain both nociceptors and mechanoreceptors**,** and their orientation

is in rostrocaudal direction that is limited to the ipsilateral side only; unexpectedly minimum to no Fos straining was noted on the contralateral side following muscle compression. These findings indicate the different neuronal activation pattern in response to mechanical perturbation to deep and cutaneous tissues and add new information to our understanding of deep tissue nociception and its spinal distribution.

5.4 Female Pain Syndromes and Exercise Training

Numerous studies and clinical observations have demonstrated that women develop widespread pain syndromes at a greater rate than age-matched men (Berkley, 1997; Berkley, Zalcman, & Simon, 2006; Gran, 2003; Greenspan et al., 2007). However, most animal models examining the mechanism of pain have exclusively utilized male rat models. The present study demonstrated that acid-induced chronic muscle hyperalgesia can be induced in female mice. Future experiments should address the issue of female prevalence of chronic pain syndromes.

Exercise training has been long suggested to people who experience chronic pain. We examined the effect of 2 different intensities, low and moderate aerobic trainings. Unlike previous rat studies (Ying et al., 2003, 2005 2001), our findings in mice showed that low-intensity treadmill training was not sufficient to up-regular NT-3 or to decrease secondary hyperalgesia induced by acid injection. Few previous studies examining the effect of exercise on various neurotrophins have exclusively

used a rat model under healthy or neurologic conditions (Hutchinson et al., 2004; Ying et al., 2003, 2005 2001). At present there is no established exercise protocol to determine the level of physical activity required to stimulate NT-3 synthesis in skeletal muscle. A great deal of inconsistencies in the intensity of the exercise, from 6 m/min speed (Bement & Sluka, 2005) to 27 m/min (Hutchinson et al., 2004) as well as in the duration of the exercise from 5 days (Bement & Sluka, 2005) to 7 weeks (Hutchinson et al., 2004) exist in rodent experiments utilizing treadmill training. As limited animal studies exist, our exercise protocol was chosen based on the human studies (Droste et al., 1991; Fulcher & White, 1997; Glass et al., 2004; Goldenberg et al., 2004; Gowans & deHueck, 2004; Gowans et al., 2004; Hoffman et al., 2004; Mannerkorpi, 2005; Meiworm et al., 2000; Redondo et al., 2004; Whiteside et al., 2004) in which physiological and analgesic effects were noted with low-moderate intensity exercise protocol. Most animal studies utilize a running wheel (Kuphal et al., 2007; Ying et al., 2003, 2005); however, humans are normally engaged in walking or running. Secondly, treadmill training, as opposed to wheel running (Ying et al., 2003, 2005) or swimming (Hutchinson et al., 2004; Kuphal et al., 2007), was chosen as a mode of aerobic exercise based on some recent animals studies (Bement & Sluka, 2005; Gomez-Pinilla et al., 2001; Hutchinson et al., 2004) in which treadmill training increased NT-3 production and reduced allodynia. These scattered findings suggest that expression of NTs can be altered in response to mechanical and metabolic changes. Nevertheless, we report that in the mouse

species, at least moderately intense exercise training was required to up-regulate NT-3 expression.

Inconsistent exercise designs may be responsible for the unpredictable results found in the literature. The mode and the intensity of exercise were considered prior to designing the present experiments. The exercise training should optimize physical performance and benefits as their effects are dose-dependent; however, intense-level of exercise without rest period may cause exhaustion, fatigue and ultimately increase painful condition (Aguiar, Tuon, Pinho, Sliva, Andreazza, Kapczinski, Quevedo, Streck, Pindo, 2008; Yokayama, 2007b). Thus the effect of low-level treadmill training was first investigated. The subsequent treadmill training with CF-1 mice was considered moderate as animals were allowed to take frequent breaks to eliminate possible over-exhaustion. Only exhaustive levels of exercise induce oxidative damage (Sastre et al., 1992). Thus an incremental running program was used to progressively increase running intensity to 16 m/min over 3 weeks. Surprisingly, the exercise training did not result in an increase in citrate synthase activity [unpublished data]. Citrate synthase is one of the key regulator mitochondrial enzymes in the energy- generating metabolism and is frequently used as a metabolic biomarker in assessing oxidative capacity of the cell (Bahi et al., 2004; Siu, Donley, Bryner, & Alway, 2003). However, inconsistant results have been reported in CS activity from various exercise protocols (unpublished data from rat experiments conducted in the diabetes laboratory at KUMC).

One way to distinguish aerobic versus anaerobic exercise intensity is to quantify lactate levels in blood sample (Aguiar, Tuon, Pinho, Sliva, Andreazza, Kapczinski, Quevedo, Streck & Pindo, 2007; Aguiar, et. al. 2008) and make a comparison between trained and untrained animals. Aguiar et al. reported that trained animals displayed lower lactate values indicating improvement of the oxidative metabolism (Aguiar, et. al., 2007). Aguiar el al. conducted a study utilizing male CF-1 mice that ran 16.5 m/min for 45 minutes for 8 weeks. This training regimen resulted in low blood level lactate, indicating aerobic fitness of mice and thus was considered at an intense level. The exercise training used in the present study was less intense than one used by Aguiar and colleagues (Aguiar, et. al., 2007) and is therefore considered at a moderate high level in study two.

5.5 Mechanisms of Exercise-Induced Analgesia

The effects of exercise-induced analgesia in patients with pain are poorly understood. Limited information is available to determine the role and mechanism of exercise in relieving pain. Several possible mechanisms have been postulated in support for exercise-induced analgesia. The theory of exercise-induced release of central opioids is the most studied and widely accepted explanation for exercise training (Hoffman, 2004; Bement & Sluka, 2005). Exercise training can release B-endorphin and catecholamines in central and peripheral tissues as evident by increased plasma concentration of these substances with short-term or long-term exercise training (L. J. Kehl & Fairbanks, 2003). A similar result was noted with a

low-intensity exercise training (10-20 ft/min for 15-30 min, 5 days for 1 week) that reversed mechanical allodynia associated with acid-induced hyperalgesia (Bement & Sluka, 2005). These studies are limited to immediate effects or intense exercise training. It is a less likely mechanism in support for the present finding as the effect of exercise training was tested in the chronic stage.

Another theory of central mechanism proposes that activation of proprioceptive and muscle afferents inhibit central pain circuitry that may affect descending inhibitory pathways (Hoffman et al., 2004). This theory has gained interest in physical therapy rehabilitation and has been supported by studies in which resistive exercise (Hoffman et al., 2004) and electric stimulation of afferent nerves (Pertovaara, Kemppainen, & Leppanen, 1992) produce an analgesic effect. However, based on randomized experiments, there is limited evidence supporting this. The present study showed that aerobic exercise indeed synthesized a trophic factor, which exerts its effect by activating large and medium diameter muscle afferents. Does this finding support the well known "classic" hypothesis that exercise stimulates activity of medium and large diameter afferents that might be involved in closing the gate for painful input at the spinal cord? Future experiment should address this question.

In addition, a peripheral mechanism that involves the local release of circulating factors, potassium, hydrogen or prostaglandins that act on receptor sites in response to exercise has also been postulated. However, it is a less supported theory as these substances activate nociceptors. Another possibility suggests that

exercise serves as a distracter, and attention modulates pain perception. However this is not a complete explanation as human studies show that alternation in pain perception does not occur in all humans and at all intensities of exercise (Hoffman et al., 2004). Another system besides endogenous opioids, involved in exercise-induced analgesia may be endocannabinoids (Elmes, Jhaveri, Smart, Kendall, & Chapman, 2004; Sparling et al., 2003). Endurance training (Long term running or cycling at 70-80% of maximum heart rate for 6 months for the duration of 30 minutes, 4 days /week) resulted in elevated anandamide levels, a ligand for CB1 receptor, in the circulation of healthy male adults (Sparling et al., 2003). This possibility was examined in healthy subjects who were able to perform high intensity exercise training for a long period of time and is limited to one study.

Exercise is considered as an endogenous way of controlling pain. But, there have been reports of exercise interventions in which exercise training in fact increased the pain perception (Mathes & Kanarek, 2001; Smith & Lyle, 2006). These exercise trainings utilized wheel running in which animals voluntarily exercised for 6-8 weeks while they received exogenous opioids. These studies reported that chronic exercise training, which tended to upregulate opioids, caused tolerance to opioid receptors (Mathes & Kanarek, 2001; Smith & Lyle, 2006). This phenomenon explains the concept seen in patients who develop a greater degree of sensitivity to opioids with higher dosages of morphine. *Among all these theories, the present study provides support for the hypothesis of activation of large diameter mechanoreceptors, via NT-3 synthesis, to overcome sensory input from nociceptors.*

5.6 Proposed Mechanisms of Exercise-Induced, NT -3 Linked Analgesic Effect

The regulation of NT expression and NT receptors on sensory nerves in adult life is not fully understood. However, NT expression on sensory neurons is influenced by injury (Hennigan et al., 2007) and activity (Gomez-Pinilla et al., 2001; Hutchinson et al., 2004 2003; Molteni et al., 2004; Ying et al., 2005)**.** Muscle activity provides signals to innervating neurons and can alter synthesis of neurotrophic factors (Gomez-Pinilla et al., 2001; Hutchinson et al., 2004 2003; Molteni et al., 2004; Xie et al., 1997; Ying et al., 2005).

These neurotrophins are synthesized by target tissues, skeletal muscles, and the receptors are found on neurons innervating muscle tissue; thus exercise is likely to mediate its effect on the sensory nervous system through mobilization of neurotrophins. Among all NTs known to this date, NT-3 plays a crucial role in pain perception. Emerging evidence suggests that NT-3 is capable of negating thermal hyperalgesia (Wilson-Gerwing et al., 2005) and mechanical hyperalgesia (Gandhi et al., 2004). There are two lines of evidence reported in the literature that signifies the role of NT-3 as a pain modulator: 1) its effect via trkC receptors (Wilson-Gerwing, & Verge, 1996; Karchewski, 2002) and 2) its ability to bind to trkA receptors (Gratto & Verge, 2003; Wilson-Gerwing et al., 2005; Wilson-Gerwing & Verge, 2006). In all of these studies, NT-3 was exogenously delivered into a muscle or spinal cord tissues. These authors didn't find a significant influence of NT-3 on the baseline thermal or mechanical sensitivity. We propose that NT-3 is likely to affect deep tissue pain via trkC receptors as there is no indication that acid-infusion alters trkA

susceptibility. Gandhi et al. examined the anti-nociceptive effect of all neurotrophins in the development and maintenance of acid-induced hyperalgesia. NT-3 was the only neurotrophin capable of reversing acid-induced mechanical hyperalgesia. Other NTs (NGF, BDNF and GDNF) had no physiological effect on mechanical hyperalgesia. This study clearly suggested that NT-3 played an important role in mitigating mechanical hyperalgesia related to musculoskeletal tissues and guided the hypothesis of the present study (Chapter 3). In the present study, moderate intensity exercise training resulted in overexpression of NT-3 in the skeletal muscle. *It is, therefore, suggested that increased muscle and cutaneous thresholds in response to exercise training are attributable to increased peripheral levels of NT-3, but its mechanism is different than suggested by Gandhi et al., as described below.*

A large number of studies highlight the importance of exercise-induced neuroplasticity via a BDNF-linked mechanism in the hippocampus to fight against declining brain function. Thus exercise-induced neurotrophins, specifically NT-3 to negate nociception at the peripheral or spinal level is an equally important phenomenon to investigate. Exercise has been shown to affect the expression of NT-3 in soleus and spinal cord (Gomez-Pinilla et al., 2001; Ying et al., 2003). However, the effect of exercise on NT-3 production in pain-induced animals is limited to only one study in which 7 weeks of treadmill training reversed allodynia and restored normal sensation after experimental spinal cord injury (Hutchinson et al., 2004). Post-developmentally, NT-3 is synthesized in peripheral tissues and retrogradely transported by cutaneous (A-α, A-β) and nociceptive (group III and IV) and

proprioceptive (group I and II) muscle afferents to the cell bodies within the DRG (Malcangio et al., 1997; Munson et al., 1999; Watanabe et al., 2000; Wright, 2001). The cell bodies of group III terminate in lamina III- VI of the spinal cord (Malcangio et al., 1997; Woolf, 1987). This suggests that the anti-nociceptive action of NT-3 via exercise can be at 3 possible sites: 1) peripheral terminals of the primary afferents innervating muscular tissue, or 2) at cell bodies of the sensory neurons in the DRG, or 3) centrally in the spinal cord. See figure one.

5.6.1 Peripheral Terminal Level

Overexpression of NT-3 could decrease muscle hypersensitivity by locally blocking the activity of the primary afferents (Gandhi et al., 2004). However, in the present study, this explanation for the exercise-induced analgesia is less likely. An earlier study from our lab identified a subset of sensory neurons (Aδ fibers projecting to muscle tissues) that respond to NT-3 and thus mediate the nociceptive effect of acid-induced hyperalgesia. The study also demonstrated that NT-3 is highly expressed in muscle spindles and smooth muscle cells along the lining of the blood vessels [unpublished data]. Exercise training, particularly treadmill training, is likely to stimulate muscle spindles and increase blood flow, thus resulting in over expression of NT-3, as observed in the present study. NT-3 expression in muscle spindles supports the longstanding understanding that proprioception neurons, group Ia and II spindle afferent fibers, respond to NT-3 (Wright et al., 1997). Additionally, the expression of NT-3 within the arterioles contributes to the anti-nociception role

of NT-3. Arterioles are innervated by sympathetic fibers, C-fibers and A-δ fibers. A-δ fibers express trkC, the receptors for NT-3 [Funfschilling, 2004]. These findings identified the anatomical sites for NT-3 expression within the skeletal muscle which are also stimulated with exercise training. The study by Gandhi demonstrated that the delivery of exogenous NT-3 was required concurrently with acid-injections for its protective effect. In addition, once NT-3 protection against the development of mechanical hyperalgesia occurred, mice did not require continued delivery of NT-3 to reduce sensitization (Gandhi et al., 2004) suggesting the role of NT-3 in suppressing the initial events that lead to hypealgesia. Mice in the present study started exercise training 3-4 days following the 2nd acidic injection. By this time, animals had developed widespread hyperalgesia of cutaneous and muscle tissues. Thus it is less likely that NT-3 had anti-nociceptive effect via the peripheral mechanism suggested by Gandhi et. al. (Gandhi et al., 2004). Yet, together, these findings suggest that over expression of NT-3 in muscle, either delivered exogenously or induced by exercise training, can lessen chronic muscular pain.

5.6.2 DRG Level

Chronic pain processing signals are complex and require upregulation of many pain substances and their corresponding receptors (Bolay & Moskowitz, 2002; Waxman, Cummins, Dib-Hajj, & Black, 2000). The anti-nociceptive effects of exogenous NT-3 are being explored in various pain models. NT-3 has been shown to regulate many proteins in the DRG, including voltage-gated potassium (Park et al.,

2003) and sodium (Wilson-Gerwing, Stucky, McComb, Verge, 2008) channels, substance p (Wilson-Gerwing et al., 2005) and trkA receptors (Karchewski, 2002; Gratto, 2003). Intrathecal administration of NT-3 blocked the development of thermal but not mechanical hyperalgesia in rats subjected to chronic constructive injury (CCI) (Gratto & Verge, 2003). Exogenous infusion of NT-3 for 7 days prevented thermal hyperalgesia and suppressed the injury-induced overexpression of TRPV1 receptors in the DRG and spinal cord in rats receiving a CCI of the sciatic nerve (Wilson-Gerwing et al., 2005). It is also known that NT-3 can mediate certain anti-nociceptive effects by altering trkA expression (receptor for NGF) in the DRG neurons in healthy animals and animals subjected to neuropathic pain (Karchewski, 2002; Gratto, 2003). Intrathecal delivery of NT-3 for one week reduced neuronal galanin expression and pituitary adenylate cyclase-activating polypeptide (PACAP) in CCI-injury model; both of these peptides are associated with neuropathic pain (Wilson-Gerwing & Verge, 2006). NT-3 also effects BDNF, substance P and CGRP and TRPV1 via p38 MAPK (Wilson-Gerwing et al., 2005). Many of these molecules are involved in nociception. By regulating their expression, protein synthesis and functional signaling in the DRG, NT-3 could influence thermal and mechanical sensitivity. *Thus it is possible that exercise-induced NT-3 upregulation observed in the present study exerted its anti-nociceptive effect indirectly by regulating the expression of other molecules involved in nociception through retrograde transport of NT-3 from the gastrocnemius muscle to the DRG.*

5.6.3 Central or Spinal Level

The central nervous system contains modulatory circuits whose main function is to regulate the perception of pain. The initial site of pain modulation in the CNS is within the dorsal horns where interconnections between nociceptive and nonnociceptive afferent pathways can control the transmission of incoming nociceptive information prior to reaching higher centers (Bennett, 1999; Lima, 1998). The time dependent changes and cronicity observed after tissue injury may be a result of an imbalance between the facilitatory and inhibitory descending pathways, as observed in many pain models (Desmeules et al., 2003; Tillu et al., 2007; Wiesenfeld-Hallin et al., 1997). Various types of tissue injuries, inflammatory versus neuropathic, result in a different pattern of facilitation or inhibition of descending control over time (Tillu et al., 2007). A recent study demonstrated the involvement of NRM and Gi nuclei of RVM in initiating and facilitating chronic acid-induced pain; both nuclei NRM and Gi project bilaterally to laminae VII, VIII and lamina I, II, V respectively (Tillu et al., 2007). Furthermore many clinical studies have also shown deficits in the descending pain inhibitory systems in the chronic stage of pain (Bennett, 1999; Desmeules et al., 2003; Wiesenfeld-Hallin et al., 1997). Many exercise-induced analgesic effects are also considered to be under descending control (Bement & Sluka, 2005; Hoffman et al., 2004; Sparling et al., 2003). Thus the involvement of descending pathways in the development and maintenance of acid-induced pain and the attenuation of hyperalgesia from exercise training cannot be ignored. *In fact, we suggest that the exercise-induced analgesic effect on muscular pain detected in the present study is centrally driven, via NT-3 retrograde transport.*

This is largely due to the fact that exercise training was initiated 3-4 days after the central sensitization had developed, and the reduction in muscular pain was bilateral and extended to the cutaneous tissue.

In adult life, the majority of muscle afferents (75%) (Watanabe et al., 2000) and half of the DRG neurons (approximately 40%) express trkC receptors, mainly medium and large diameters (Gratto & Verge, 2003). TrkC receptors are also expressed in many areas in the CNS (Merlio et al., 1992); importantly in the dorsal horns of the spinal cord (Bradbury, King, Simmons, Priestley, & McMahon, 1998; Scarisbrick, Isackson, & Windebank, 1999; Watanabe et al., 2000), in 2^{nd} order projection sensory neurons (Bradbury et al., 1998) and in primate descending serotoninergic system (Arvidsson et al., 1994; Bechade, Mallecourt, Sedel, Vyas, & Triller, 2002). Thus, it is plausible to suggest that the action of exercise-induced NT-3 was mediated through spinal mechanisms. In addition, the general spatial arrangement of central projections of the primary afferents in the spinal cord allows direct contact between nociceptors and mechanoreceptors. A-δ fibers terminate in I and V layers; C-fibers mainly terminate in lamina II and Aβ terminate in lamina III – V (Craig, 2003; Leigh A. Lamont, 2000); however, C-fibers and Aβ also terminate in lamina II – IV from a dorsal to ventral direction. Our results (Chapter 2) suggest that muscle sensory afferents (noxious and non-noxious) terminate in all lamina and multiple lumbar segments. Previous knowledge suggests that the input to laminae II-IV is arranged in 2 horizontal sheets of afferent terminals. The superficial sheet represents terminals of C-fibers and the deep sheet represents terminals of A-β fibers

(Woolf, 1987). Woolf and Fitzgerald also demonstrated that cells responding to low-threshold mechanical stimuli (A-β) can be found in all laminae (Woolf, 1987). There are interneurons located within lamina II, III, and IV, making direct connections with lamina I and V (Lima, 1998) and are likely to influence nociception. This arrangement allows complex neurochemical connection between nociceptors and mechanoreptors that result in either facilitation or inhibition of painful sensation. *Thus we believe this is likely the mechanism by which exercise-induced NT-3 exerted its anti-nociceptive effect.*

Acid and NT-3 - a Compensatory Mechanism

In addition to the evidence of co-expression of trkC and ASIC-3 that occupies a small portion of mechanoreceptors, there is no information available that links NT-3 and acidosis. The co-expression of trkC and ASIC-3 propose a possible signaling mechanism through which NT-3 could influence ASIC-3 channel activity and increase mechanical threshold sensitivity. *An interesting finding from this study seems to suggest a possible interaction between the physiological alteration in pH level that activates ASIC and an up-regulation of NT-3 (Chapter 3).* The finding illustrates that acid itself regulates NT-3 expression in muscle up to 3 weeks after the initial injections were delivered. This suggests a possible interaction between NT-3 and ASIC-3 receptors of the primarily sensory neurons whose activity, in the chronic stage, is primarily driven by central sensitization. This finding could be a cofounding factor or may suggest an intrinsic compensatory mechanism by which body attempts

to negate tissue acidosis and nociception. Does this finding imply another mechanism by which exercise modulates pain when muscle is physiologically challenged either by acid or exercise? Future investigations should address this question by examining ASIC-3 expression in skeletal muscle in exercised and acid-injected animals.

5.7 Future Directions

The present studies have established a correlation between exercise, NT-3 and pain modulation in a chronic musculoskeletal pain model. Future studies should focus on solidifying the physiological processes involved in exercise-induced pain modulation. The exact mechanism by which activity induces NT-3 synthesis and the subsequent events that lead to muscular analgesia is beyond the scope of this study and remains to be investigated. However some thoughts about investigating these questions are shared below. A series of cause and effect hypotheses/experiments are suggested to understand mechanisms underlying exercise-induced analgesia through NT-3.

NT-3 Effects Mediated by TtrkC Receptors

If exercise-induced NT-3 expression reduces secondary hyperalgesia, presumably through trkC receptors, then blocking NT-3 ligand binding to trkC receptor with antibody against trkC should reverse the analgesic effect of NT-3. This may be the next logical step to investigate. *Clear anti-nociceptive action of NT-3 on sensory nerves should be determined by examining the signaling cascade in sensory*

nerves. By blocking messages from the periphery or by retrograde transport to cell bodies that influence gene transcription of many neuropeptides and ion channels involved in pain transduction, one could analyze the anti-nociceptive action of NT-3.

Target Subpopulation of Sensory Neurons

There is a significant co-expression of trk receptors and ASIC-3 receptors on sensory neurons. Approximately, 50% of all small sensory neurons that innervate muscle tissue and 28% of small sensory neurons that innervate skin express ASIC-3 channels (McIlwrath, Lawson, Anderson, Albers, & Koerber, 2007; Molliver et al., 2005). Additionally, approximately 68% of ASIC co-express trkA receptors (receptor for NGF), and 25% co-express trkC receptor (receptor for NT-3). Interestingly, ASIC3+/trkC+ subpopulation of neurons are non-nociceptive mechanoreceptors rather than proprioceptors. *These mechanoceptors are probably the target neurons for NT-3 action induced by exercise training*. NT-3 may also be expressed by C-fibers and/or A-δ fibers (Gandhi et al., 2004; Wilson-Gerwing et al., 2005) and could mitigate nociception via these afferents. *In future, a double-labeling immunohistochemistry technique can be utilized to identify a subset of neurons responsive to ASIC-3 and trkC receptors under exercise conditions.*

Moreover, recent evidence indicates that intrathecally delivered NT-3 binds to trkA receptors in adult DRG neurons (Gratto & Verge, 2003). Since NT-3 preferentially binds to its trkC receptors, we hypothesize its exercise-induced analgesic effect through trkC receptors by increased activity of large diameter fibers.

However, as mentioned earlier, NT-3 is capable of binding multiple trk receptors (both trkA and trkB) in certain injuries (Wilson-Gerwing, 2005); this is not surprising since about 50% DRG neurons co-express trkA and trkC receptors and provides further support for its ability to modulate pain. Therefore it is important to examine its influence on both trk receptors to determine its effect on muscle pain with exercise training. *To examine the effect mediated via NT-3, again techniques of colocalization (radioautography or in situ hybridization, can be applied to visualize and quantify trkA, trkC, trkB, SP, p75, BDNF and ASIC3 in DRG neurons.*

5.7.1 Solidify Peripheral versus Central Mechanism of Exercise-Induced Analgesia

The next step may be to solidify peripheral or central effects of anti-nociception associated with NT-3. Because neurotrophins are expressed by target tissue and exert their impact via sensory neurons, their action at specific anatomical sites is critical to the understanding of possible associated mechanisms. In vivo expression patterns and specific transport sites of NT-3 in the spinal cord should reveal its anti-nociceptive action induced by exercise training. The present study demonstrated over expression of exercise-induced NT-3 in peripheral tissues. However, its action is likely to be within the CNS.

To assess whether NT-3 protein is being retrogradely transported to DRG and then anterogradely to the spinal cord (X. Wang, Butowt, Vasko, & von Bartheld, 2002) a technique of retrograde tracer may be employed. An earlier study from our lab utilized mice with lacZ gene inserted into a protomoter region of the trkC gene to

trace peripheral terminal sites for NT-3 synthesis. In that experiment, 5 ul of 2% solution of Fast Blue (Sigma, St. Louis, MO) was injected into the right gastrocnemius muscle to retrogradely label muscle afferent neurons and cell bodies of neurons positive for NT-3 in the DRG. The same technique can be utilized to trace the physiological source of NT-3 from exercise training. Approximately 5 days prior to scarifying animals, retrograde labeling should be injected into the gastrocnemius muscle, and animals should be allowed to continue with the exercise training. The DRG and spinal cord (L4/5) should be processed with the technique of immunohistochemistry utilizing anti-ASIC3 and anti-mouse trkC antibodies and viewed under a microscope to identify the type of neurons and their specific locations in the spinal cord. This procedure would ensure the retrograde transport and identify trkC+/ASIC3+ neurons.

The classic view states that neurotrophins are produced in peripheral target tissues and retrogradely transported to the CNS (DiStefano et al., 1992); however, increasing evidence now suggests that NTs are also expressed locally in the spinal cord, specifically in response to exercise (Gomez-Pinilla et al., 2001; Hutchinson et al., 2004; Ying et al., 2003, 2005). Exercise provides systemic stimuli which may either cause in-situ synthesis or translocation of NT-3 protein from periphery via retrograde transport. Both long-term (28 days) and short-term (7 days) exercise training have shown to increase trkB and BDNF expression in the spinal cord (Macias et al., 2007). In addition, exercise training has also been shown to upregulate NT-3 expression in the spinal cord (Ying et al., 2003). Our results indicated that 3

weeks of treadmill training at moderate intensity caused a 1.5 fold increase in the spinal cord NT-3 mRNA expression in the exercise control group, which was not statistically significant (unpublished data). However together these findings suggest that exercise could induce local increase in NT-3 expression in the spinal cord. The method of RT-PCR used in the present studies is sensitive and beneficial to detect changes in gene transcription but does not provide cell specific information. A technique such as in situ hybridization and immunohistochemistry should be utilized to localize laminae and cell type expressing NT-3 in the spinal cord.

To detect cell specific changes in the mRNA expression, in-situ hybridization is often used (Gratto & Verge, 2003; Wilson-Gerwing et al., 2005; Wilson-Gerwing & Verge, 2006). A DNA (cDNA) probe of NT-3 complementary to the coding sequence of mature NT-3 should be generated to read the NT-3 sequence. Two probes, the sense and the antisense with specific RNA polymerase will be needed. pRNT3-1 with 392 bp insert (nucleotides 481 – 873) has been used to encode mature rat NT-3 in the spinal cord (Scarisbrick et al., 1999). Following immunohistochemistry, slides with spinal enlargement should be processed with hybridization buffer and cRNA hybridization can be visualized by film or emulsion autoradiography for the relative amount of cRNA hybridization. The details of this procedure have been described by Sarisbrick and colleagues (Scarisbrick et al., 1999).

Besides the mRNA expression, changes in the protein levels within the spinal cord should be examined. In the present study, we did not examine the protein levels

of NT-3 in the spinal cord. A previous study demonstrated an upregulation of NT-3 protein and mRNA and trkC mRNA in the spinal cord from 3 and 7 days wheel running (Ying et al., 2003); this upregulation of NT-3 was mostly seen in the substantial gelatinosa (SG) region (Ying et al., 2003), suggesting either localized increase in protein or retrograde transport from the primary afferent as the central projections of mechanoreceptors innervate lamina III or IV. The increase in NT-3 from exercise training in the spinal cord could suggest a means of sensory modulation within the spinal cord. The study by Ying et. al. (Ying et al., 2003) showed that exercise training regulated the expression of NT-3 and its cognate receptor trkC in the spinal cord. However, this finding is limited to only one study and the cellular localization of trkC receptors in the spinal cord was not determined. Further studies are needed to confirm this finding.

If exercise provides a physiological stimulus that leads to a mechanism of pain modulation and if the spinal cord circuitry is involved in negating central sensitization, then a detailed mapping of the cellular expression of NT-3 and trkC receptors within the spinal cord would be a valuable approach. This knowledge is of crucial importance, particularly for understanding the anti-nociceptive role of NT-3 and exercise-induced analgesic mechanisms at the spinal level. Exercise may change the mRNA level of the trkC receptor or the number of cells expressing trkC receptors. The majority of previous studies examining NT-3 expression focused on the ventral horn motor nuclei as NT-3 primarily supports proprioceptive function. A mapping of NT-3 and trkC receptor of sendentary and exercised-mice in the dorsal

horns should be important for central sensitization. This will also suggest whether the dorsal horn neurons in lamina III and IV receive input from A-β relays on the trophic support from the peripheral sensory neurons or produce their own source of NT-3. Based on a few previous studies, exercise training is likely to have some effect on spinal neuronal networks. Thus, in future experiments, one might expect to find NT-3 in lamina III-IV or deep layers where Aβ fibers from smooth muscle cells of arterioles and group II from muscle spindle terminate. The interneurons in laminae V-VI are also involved in motor control and therefore may be activated during locomotion (Bannatyne, Edgley, Hammar, Jankowska, & Maxwell, 2006).

The trk receptors, especially trkB and trkC are found in many areas of the brain, including the neocortex, hippocampus, thalamic and hypothalamic nuclei, brainstem nuclei, cerebellum and spinal cord motoneurons (Merlio et al., 1992). Many of these regions influence pain processing, directly or indirectly. Given the diversity of trk receptors, it is possible that exercise could influence NT-3 expression in the CNS and pain processing at supraspinal levels.

5.7.2 Human Studies

The findings of exercise's effects on NTs are restricted to animal models; however, these studies give insight into the potential explanation for exercise-induced relief of muscular pain reported in clinical studies. It will be interesting to find out whether these observations hold true in human skeletal muscle. Blood

sample and muscle biopsy following exercise training might provide some insight into exercise effects on select NTs in human experiments.

Future investigations into these questions should provide a better understanding of how physical activity controls pain. It is likely that exercise may affect pain in multiple ways; through activation of large diameter primary afferents or through activation of descending inhibitory pathways. Many questions remain unanswered and opportunities for multiple experiments for further studies regarding the effect of exercise exist.

5.8 Clinical Significance

Pain is multidimensional. Although the sensory aspect represents only a fraction of the entire pain experience, we have combined different stimulation and assessment approaches to gain better understanding of the chronic nature of pain. Direct muscle sensitivity, referred pain (secondary hyperalgesia) pattern and spinal activation pattern have been tested through this dissertation. Although this is an animal study and generalization is limited, a major advantage of animal models is that aspect of pain and intervention mechanisms can be investigated in a standardized setting without confounding factors. Considering the high prevalence rate of chronic musculoskeletal pain conditions in women, female mice were used in all experiments to understand muscle pain and the analgesic effect of exercise on muscle pain.

The recent appreciation of deep tissue pain by the scientific community has started to shift the focus toward understanding muscle pain. Unfortunately, our understanding of the treatment of deep tissue pain with exercise training and other physical therapy interventions lags behind. An editorial report in *Rheumatology* identified chronic musculoskeletal pain as an international issue (Rheumatology. 2005). After osteoarthritis, Fibromyalgia (FMS) is the second most prevalent disorder observed by rheumatologists (Goldenberg et al., 2004). The persistent nature of pain associated with FMS leads to decreased functional activity and pain with even light activities (Gowans & deHueck, 2004) effecting overall function and fitness level (Fulcher & White, 2000). These individuals have limited success with current pharmacological interventions (Goldenberg et al., 2004; L. X. Wang & Wang, 2003) and depend on other avenues to manage their symptoms. A growing numbers of human studies provide measurable, physiological and functional proof of exercise benefits (Droste et al., 1991; Fulcher & White, 1997; Goldenberg et al., 2004; Gowans & deHueck, 2004; Gowans et al., 2004; Hoffman et al., 2004; Mannerkorpi, 2005; Meiworm et al., 2000; Redondo et al., 2004; Whiteside et al., 2004) with little to no molecular explanation. The clinical importance of exercise-induced analgesia warrants an investigation to explore possible molecular changes involved in controlling pain. It is important to determine whether exercise facilitates a continuous upregulation of NT-3 and can be used as an intervention to control pain. This work expands our understanding of exercise-induce analgesia, and allows clinicians to better guide these individuals while improving their function and quality

of life. The study also adds evidence to the current knowledge of exercise-induced sensory alteration in the chronic pain model which is limited to only a few experiments.

5.9 Conclusion

In summary, we have used behavioral, anatomical, and molecular approaches to identify muscle pain and to evaluate the efficacy of exercise training in modulating chronic musculoskeletal pain induced by intramuscular acid injection in 2 different strains of mice. Our data demonstrated that acid injection into the gastrocnemius muscle resulted in persistent, long-lasting, mechanical hyperalgesia of cutaneous and muscle tissues in CF-1 mice. Mice with a C57BL/6 background did not develop secondary hyperalgesia consistently and therefore should not be used when studying acid-induced pain. Future studies should consider genetic differences when studying pain mechanisms and therapeutic interventions. Our data also revealed that exercise training, when performed at moderately high intensity produced over expression of NT-3 in skeletal muscle and in the spinal cord to a lesser extent. Exercise training did not reverse the acid-induced hyperalgesia but significantly reduced it, which is consistent with many clinical studies in which exercise training does not cure a painful condition but rather lessens it. We contribute the positive effect of exercise training to over-expressed NT-3 levels. Because many chronic pain syndromes occur in females, we tested the acid-model in female mice and demonstrated that the acid-model was reproducible in female

gender. Since the acid model was sensitive to female mice, produced widespread hyperalgesia and did not cause inflammation or tissue damage, made this model unique to study the effects of exercise training. Future experiments should focus on clarifying peripheral versus central actions of exercise-induced NT-3 and its underlying mechanisms in mediating nociception.

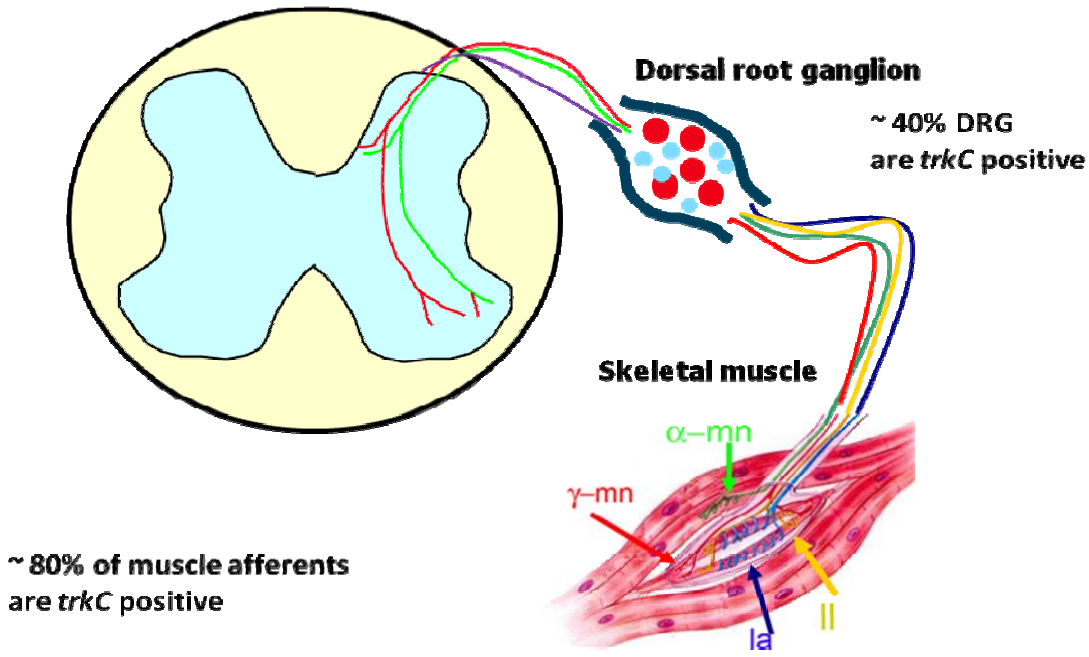

Figure 1: represents a schematic diagram showing possible anatomical sites for anti-nociceptive actions of NT-3, at peripheral levels, in DRG via retrograde transport or in the spinal cord dorsal horn neurons.

References

Abbadie, C., & Besson, J. M. (1992). c-fos expression in rat lumbar spinal cord during the development of adjuvant-induced arthritis. *Neuroscience, 48*(4), 985-993.

Abbadie, C., Honore, P., Fournie-Zaluski, M. C., Roques, B. P., & Besson, J. M. (1994). Effects of opioids and non-opioids on c-Fos-like immunoreactivity induced in rat lumbar spinal cord neurons by noxious heat stimulation. *Eur J Pharmacol, 258*(3), 215-227.

Abbadie, C., Lombard, M. C., Morain, F., & Besson, J. M. (1992). Fos-like immunoreactivity in the rat superficial dorsal horn induced by formalin injection in the forepaw: effects of dorsal rhizotomies. *Brain Res, 578*(1-2), 17-25.

Adams, N., & Sim, J. (2005). Rehabilitation approaches in fibromyalgia. *Disabil Rehabil, 27*(12), 711-723.

Aggarwal, V. R., McBeth, J., Zakrzewska, J. M., Lunt, M., & Macfarlane, G. J. (2006). The epidemiology of chronic syndromes that are frequently unexplained: do they have common associated factors? *Int J Epidemiol, 35*(2), 468-476.

Aguiar, A. S., Tuon, T., Pinho, C. A., Sliva, L. A., Andreazza, A. C., Kapczinski, F., Quevedo, J., Streck, E. L., Pindo, R. A., (2008). Intense exercise induces mitochondrial dysfunction in mice brain. *Neurochem Res, 33*(1), 51-8.

Aguiar, A. S., Tuon, T., Pinho, C. A., Sliva, L. A., Andreazza, A. C., Kapczinski, F., Quevedo, J., Streck, E. L., Pindo, R. A., (2007). Mitochondrial IV complex and brain neurotrophic derived factor responses of mice brain cortex after downhill running. *Neuro Lett, 426*(3), 171-4

Almeida, T. F., Roizenblatt, S., & Tufik, S. (2004). Afferent pain pathways: a neuroanatomical review. *Brain Res, 1000*(1-2), 40-56.

Ambalavanar, R., Yallampalli, C., Yallampalli, U., & Dessem, D. (2007). Injection of adjuvant but not acidic saline into craniofacial muscle evokes nociceptive behaviors and neuropeptide expression. *Neuroscience, 149*(3), 650-659.

Arvidsson, U., Risling, M., Frisen, J., Piehl, F., Fried, K., Hokfelt, T., et al. (1994). trkC-like immunoreactivity in the primate descending serotoninergic system. *Eur J Neurosci, 6*(2), 230-236.

Bahi, L., Koulmann, N., Sanchez, H., Momken, I., Veksler, V., Bigard, A. X., et al. (2004). Does ACE inhibition enhance endurance performance and muscle energy metabolism in rats? *J Appl Physiol, 96*(1), 59-64.

Bailey, A. L., & Ribeiro-da-Silva, A. (2006). Transient loss of terminals from non-peptidergic nociceptive fibers in the substantia gelatinosa of spinal cord following chronic constriction injury of the sciatic nerve. *Neuroscience, 138*(2), 675-690.

Banic, B., Petersen-Felix, S., Andersen, O. K., Radanov, B. P., Villiger, P. M., Arendt-Nielsen, L., et al. (2004). Evidence for spinal cord hypersensitivity in chronic pain after whiplash injury and in fibromyalgia. *Pain, 107*(1-2), 7-15.

Bannatyne, B. A., Edgley, S. A., Hammar, I., Jankowska, E., & Maxwell, D. J. (2006). Differential projections of excitatory and inhibitory dorsal horn interneurons relaying information from group II muscle afferents in the cat spinal cord. *J Neurosci, 26*(11), 2871-2880.

Basbaum, A. I. (1999). Distinct neurochemical features of acute and persistent pain. *Proc Natl Acad Sci U S A, 96*(14), 7739-7743.

Basbaum, A. I., & Woolf, C. J. (1999). Pain. *Curr Biol, 9*(12), R429-431.

Bechade, C., Mallecourt, C., Sedel, F., Vyas, S., & Triller, A. (2002). Motoneuron-derived neurotrophin-3 is a survival factor for PAX2-expressing spinal interneurons. *J Neurosci, 22*(20), 8779-8784.

Bement, M. K., & Sluka, K. A. (2005). Low-intensity exercise reverses chronic muscle pain in the rat in a naloxone-dependent manner. *Arch Phys Med Rehabil, 86*(9), 1736-1740.

Bennett, R. M. (1999). Emerging concepts in the neurobiology of chronic pain: evidence of abnormal sensory processing in fibromyalgia. *Mayo Clin Proc, 74*(4), 385-398.

Bergman, S. (2007). Management of musculoskeletal pain.

Berkley, K. J. (1997). Sex differences in pain. *Behav Brain Sci, 20*(3), 371-380; discussion 435-513.

Berkley, K. J., Zalcman, S. S., & Simon, V. R. (2006). Sex and gender differences in pain and inflammation: a rapidly maturing field. *Am J Physiol Regul Integr Comp Physiol, 291*(2), R241-244.

Blomqvist, A., & Craig, A. D. (2000). Is neuropathic pain caused by the activation of nociceptive-specific neurons due to anatomic sprouting in the dorsal horn? *J Comp Neurol, 428*(1), 1-4.

Bolay, H., & Moskowitz, M. A. (2002). Mechanisms of pain modulation in chronic syndromes. *Neurology, 59*(5 Suppl 2), S2-7.

Bon, K., Wilson, S. G., Mogil, J. S., & Roberts, W. J. (2002). Genetic evidence for the correlation of deep dorsal horn Fos protein immunoreactivity with tonic formalin pain behavior. *J Pain, 3*(3), 181-189.

Bradbury, E. J., King, V. R., Simmons, L. J., Priestley, J. V., & McMahon, S. B. (1998). NT-3, but not BDNF, prevents atrophy and death of axotomized spinal cord projection neurons. *Eur J Neurosci, 10*(10), 3058-3068.

Brain Facts. (1997). Paper presented at the Society of Neuroscience.

Braz, J. M., Nassar, M. A., Wood, J. N., & Basbaum, A. I. (2005). Parallel "pain" pathways arise from subpopulations of primary afferent nociceptor. *Neuron, 47*(6), 787-793.

Burghardt, P. R., Fulk, L. J., Hand, G. A., & Wilson, M. A. (2004). The effects of chronic treadmill and wheel running on behavior in rats. *Brain Res, 1019*(1-2), 84-96.

Carr, D. B., & Goudas, L. C. (1999). Acute pain. *Lancet, 353*(9169), 2051-2058.

Carroll, P., Lewin, G. R., Koltzenburg, M., Toyka, K. V., & Thoenen, H. (1998). A role for BDNF in mechanosensation. *Nat Neurosci, 1*(1), 42-46.

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., & Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature, 389*(6653), 816-824.

Chevrel, G., Hohlfeld, R., & Sendtner, M. (2006). the role of neurotrophins in muscle under physiological and pathological conditions. *Muscle & Nerve,* 33, 462-476.

Clement, C. I., Keay, K. A., Podzebenko, K., Gordon, B. D., & Bandler, R. (2000). Spinal sources of noxious visceral and noxious deep somatic afferent drive onto the ventrolateral periaqueductal gray of the rat. *J Comp Neurol, 425*(3), 323-344.

Coggeshall, R. E. (2005). Fos, nociception and the dorsal horn. *Prog Neurobiol, 77*(5), 299-352.

Cotman, C. W., & Berchtold, N. C. (2002). Exercise: a behavioral intervention to enhance brain health and plasticity. *Trends Neurosci, 25*(6), 295-301.

Craig, A. D. (2003a). Pain mechanisms: labeled lines versus convergence in central processing. *Annu Rev Neurosci, 26*, 1-30.

Craig, A. D. (2003b). A new view of pain as a homeostatic emotion. Trends in Neurosciences, 26(6), 303-307

Desmeules, J. A., Cedraschi, C., Rapiti, E., Baumgartner, E., Finckh, A., Cohen, P., et al. (2003). Neurophysiologic evidence for a central sensitization in patients with fibromyalgia. *Arthritis Rheum, 48*(5), 1420-1429.

DiStefano, P. S., Friedman, B., Radziejewski, C., Alexander, C., Boland, P., Schick, C. M., et al. (1992). The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. *Neuron, 8*(5), 983-993.

Droste, C., Greenlee, M. W., Schreck, M., & Roskamm, H. (1991). Experimental pain thresholds and plasma beta-endorphin levels during exercise. *Med Sci Sports Exerc, 23*(3), 334-342.

Elmes, S. J., Jhaveri, M. D., Smart, D., Kendall, D. A., & Chapman, V. (2004). Cannabinoid CB2 receptor activation inhibits mechanically evoked responses of wide dynamic range dorsal horn neurons in naive rats and in rat models of inflammatory and neuropathic pain. *Eur J Neurosci, 20*(9), 2311-2320.

Ernfors, P., Lee, K. F., Kucera, J., & Jaenisch, R. (1994). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell, 77*(4), 503-512.

Farinas, I., Jones, K. R., Backus, C., Wang, X. Y., & Reichardt, L. F. (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. *Nature, 369*(6482), 658-661.

Friedman, W. J., & Greene, L. A. (1999). Neurotrophin signaling via Trks and p75. *Exp Cell Res, 253*(1), 131-142.

Frostick, S. P., Yin, Q., & Kemp, G. J. (1998). Schwann cells, neurotrophic factors, and peripheral nerve regeneration. *Microsurgery, 18*(7), 397-405.

Fulcher, K. Y., & White, P. D. (1997). Randomised controlled trial of graded exercise in patients with the chronic fatigue syndrome. *Bmj, 314*(7095), 1647-1652.

Fulcher, K. Y., & White, P. D. (2000). Strength and physiological response to exercise in patients with chronic fatigue syndrome. *J Neurol Neurosurg Psychiatry, 69*(3), 302-307.

Gandhi, R., Ryals, J. M., & Wright, D. E. (2004). Neurotrophin-3 reverses chronic mechanical hyperalgesia induced by intramuscular acid injection. *J Neurosci, 24*(42), 9405-9413.

Glass, J. M., Lyden, A. K., Petzke, F., Stein, P., Whalen, G., Ambrose, K., et al. (2004). The effect of brief exercise cessation on pain, fatigue, and mood symptom development in healthy, fit individuals. *J Psychosom Res, 57*(4), 391-398.

Goldenberg, D. L., Burckhardt, C., & Crofford, L. (2004). Management of fibromyalgia syndrome. *Jama, 292*(19), 2388-2395.

Gomez-Pinilla, F., Ying, Z., Opazo, P., Roy, R. R., & Edgerton, V. R. (2001). Differential regulation by exercise of BDNF and NT-3 in rat spinal cord and skeletal muscle. *Eur J Neurosci, 13*(6), 1078-1084.

Gomez-Pinilla, F., Ying, Z., Roy, R. R., Hodgson, J., & Edgerton, V. R. (2004). Afferent input modulates neurotrophins and synaptic plasticity in the spinal cord. *J Neurophysiol, 92*(6), 3423-3432.

Gowans, S. E., & deHueck, A. (2004). Effectiveness of exercise in management of fibromyalgia. *Curr Opin Rheumatol, 16*(2), 138-142.

Gowans, S. E., Dehueck, A., Voss, S., Silaj, A., & Abbey, S. E. (2004). Six-month and one-year followup of 23 weeks of aerobic exercise for individuals with fibromyalgia. *Arthritis Rheum, 51*(6), 890-898.

Gran, J. T. (2003). The epidemiology of chronic generalized musculoskeletal pain. *Best Pract Res Clin Rheumatol, 17*(4), 547-561.

Gratto, K. A., & Verge, V. M. (2003). Neurotrophin-3 down-regulates trkA mRNA, NGF high-affinity binding sites, and associated phenotype in adult DRG neurons. *Eur J Neurosci, 18*(6), 1535-1548.

Graven-Nielsen, T., & Arendt-Nielsen, L. (2002). Peripheral and central sensitization in musculoskeletal pain disorders: an experimental approach. *Curr Rheumatol Rep, 4*(4), 313-321.

Graven-Nielsen, T., Arendt-Nielsen, L., & Mense, S. (2002). Thermosensitivity of muscle: high-intensity thermal stimulation of muscle tissue induces muscle pain in humans. *J Physiol, 540*(Pt 2), 647-656.

Graven-Nielsen, T., & Mense, S. (2001). The peripheral apparatus of muscle pain: evidence from animal and human studies. *Clin J Pain, 17*(1), 2-10.

Greenspan, J. D., Craft, R. M., LeResche, L., Arendt-Nielsen, L., Berkley, K. J., Fillingim, R. B., et al. (2007). Studying sex and gender differences in pain and analgesia: a consensus report. *Pain, 132 Suppl 1*, S26-45.

Harris, J. A. (1998). Using c-fos as a neural marker of pain. *Brain Res Bull, 45*(1), 1-8.

He, X., Yang, F., Xie, Z., & Lu, B. (2000). Intracellular Ca(2+) and Ca(2+)/calmodulin-dependent kinase II mediate acute potentiation of neurotransmitter release by neurotrophin-3. *J Cell Biol, 149*(4), 783-792.

Hennigan, A., O'Callaghan, R. M., & Kelly, A. M. (2007). Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. *Biochem Soc Trans, 35*(Pt 2), 424-427.

Hoeger-Bement, M. K., & Sluka, K. A. (2003). Phosphorylation of CREB and mechanical hyperalgesia is reversed by blockade of the cAMP pathway in a time-dependent manner after repeated intramuscular acid injections. *J Neurosci, 23*(13), 5437-5445.

Hoffman, M. D., Shepanski, M. A., Ruble, S. B., Valic, Z., Buckwalter, J. B., & Clifford, P. S. (2004). Intensity and duration threshold for aerobic exercise-induced analgesia to pressure pain. *Arch Phys Med Rehabil, 85*(7), 1183-1187.

Hoheisel, U., Mense, S., Simons, D. G., & Yu, X. M. (1993). Appearance of new receptive fields in rat dorsal horn neurons following noxious stimulation of skeletal muscle: a model for referral of muscle pain? *Neurosci Lett, 153*(1), 9-12.

Hoheisel, U., Reinohl, J., Unger, T., & Mense, S. (2004). Acidic pH and capsaicin activate mechanosensitive group IV muscle receptors in the rat. *Pain, 110*(1-2), 149-157.

Holden, J. E., & Pizzi, J. A. (2003). The challenge of chronic pain. *Adv Drug Deliv Rev, 55*(8), 935-948.

Hoseini, S. S., Hoseini, M., & Gharibzadeh, S. (2006). Sprouting phenomenon, a new model for the role of A-beta fibers in wind up. *Med Hypotheses, 66*(4), 805-807.

Huang, E. J., & Reichardt, L. F. (2003). Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem, 72*, 609-642.

Hunt, S. P., & Mantyh, P. W. (2001). The molecular dynamics of pain control. *Nat Rev Neurosci, 2*(2), 83-91.

Hunt, S. P., Pini, A., & Evan, G. (1987). Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. *Nature, 328*(6131), 632-634.

Hunt, S. P., & Rossi, J. (1985). Peptide- and non-peptide-containing unmyelinated primary afferents: the parallel processing of nociceptive information. *Philos Trans R Soc Lond B Biol Sci, 308*(1136), 283-289.

Hutchinson, K. J., Gomez-Pinilla, F., Crowe, M. J., Ying, Z., & Basso, D. M. (2004). Three exercise paradigms differentially improve sensory recovery after spinal cord contusion in rats. *Brain, 127*(Pt 6), 1403-1414.

Ikeuchi, M., Kolker, S. J., Burnes, L. A., Walder, R. Y., & Sluka, K. A. (2008). Role of ASIC3 in the primary and secondary hyperalgesia produced by joint inflammation in mice. *Pain*.

Issberner, U., Reeh, P. W., & Steen, K. H. (1996). Pain due to tissue acidosis: a mechanism for inflammatory and ischemic myalgia? *Neurosci Lett, 208*(3), 191-194.

Jasmin, L., Gogas, K. R., Ahlgren, S. C., Levine, J. D., & Basbaum, A. I. (1994). Walking evokes a distinctive pattern of Fos-like immunoreactivity in the caudal brainstem and spinal cord of the rat. *Neuroscience, 58*(2), 275-286.

Ji, R. R., Kohno, T., Moore, K. A., & Woolf, C. J. (2003). Central sensitization and LTP: do pain and memory share similar mechanisms? TRENDS in Neurosciences, 26(12), 696-705.

Jinks, S. L., Simons, C. T., Dessirier, J. M., Carstens, M. I., Antognini, J. F., & Carstens, E. (2002). C-fos induction in rat superficial dorsal horn following cutaneous application of noxious chemical or mechanical stimuli. *Exp Brain Res, 145*(2), 261-269.

Johnson, R. A., & Mitchell, G. S. (2003). Exercise-induced changes in hippocampal brain-derived neurotrophic factor and neurotrophin-3: effects of rat strain. *Brain Res, 983*(1-2), 108-114.

Johnson, R. A., Rhodes, J. S., Jeffrey, S. L., Garland, T., Jr., & Mitchell, G. S. (2003). Hippocampal brain-derived neurotrophic factor but not neurotrophin-

3 increases more in mice selected for increased voluntary wheel running. *Neuroscience, 121*(1), 1-7.

Jones, K. D., Clark, S. R., & Bennett, R. M. (2002). Prescribing exercise for people with fibromyalgia. *AACN Clin Issues, 13*(2), 277-293.

Julius, D., & Basbaum, A. I. (2001). Molecular mechanisms of nociception. *Nature, 413*(6852), 203-210.

Karchewski, L. A., Gratto, K. A., Wetmore, C., & Verge, V. M. (2002). Dynamic patterns of BDNF expression in injured sensory neurons: differential modulation by NGF and NT-3. *Eur J Neurosci, 16*(8), 1449-1462.

Karchewski, L. A., Kim, F. A., Johnston, J., McKnight, R. M., & Verge, V. M. (1999). Anatomical evidence supporting the potential for modulation by multiple neurotrophins in the majority of adult lumbar sensory neurons. *J Comp Neurol, 413*(2), 327-341.

Kehl, L. J. (1996). Eccentric exercise induces muscle hyperalgesia in rats. *Am. Pain Soc.*

Kehl, L. J., & Fairbanks, C. A. (2003). Experimental animal models of muscle pain and analgesia. *Exerc Sport Sci Rev, 31*(4), 188-194.

Kehl, L. J., Trempe, T. M., & Hargreaves, K. M. (2000). A new animal model for assessing mechanisms and management of muscle hyperalgesia. *Pain, 85*(3), 333-343.

Klein, R., Silos-Santiago, I., Smeyne, R. J., Lira, S. A., Brambilla, R., Bryant, S., et al. (1994). Disruption of the neurotrophin-3 receptor gene trkC eliminates la

muscle afferents and results in abnormal movements. *Nature, 368*(6468), 249-251.

Koltyn, K. F. (2000). Analgesia following exercise: a review. *Sports Med, 29*(2), 85-98.

Kramis, R. C., Roberts, W. J., & Gillette, R. G. (1996). Non-nociceptive aspects of persistent musculoskeletal pain. *J Orthop Sports Phys Ther, 24*(4), 255-267.

Krishtal, O. A., & Pidoplichko, V. I. (1981). Receptor for protons in the membrane of sensory neurons. *Brain Res, 214*(1), 150-154.

Kuphal, K. E., Fibuch, E. E., & Taylor, B. K. (2007). Extended swimming exercise reduces inflammatory and peripheral neuropathic pain in rodents. *J Pain, 8*(12), 989-997.

Kwan, C. L., Diamant, N. E., Pope, G., Mikula, K., Mikulis, D. J., & Davis, K. D. (2005). Abnormal forebrain activity in functional bowel disorder patients with chronic pain. *Neurology, 65*(8), 1268-1277.

Lamballe, F., Klein, R., & Barbacid, M. (1991). trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell, 66*(5), 967-979.

Lawrence, J., Stroman, P. W., Bascaramurty, S., Jordan, L. M., & Malisza, K. L. (2004). Correlation of functional activation in the rat spinal cord with neuronal activation detected by immunohistochemistry. *Neuroimage, 22*(4), 1802-1807.

Le Bars, D., Gozariu, M., & Cadden, S. W. (2001). Animal models of nociception. *Pharmacol Rev, 53*(4), 597-652.

Lee, Y., Lee, C. H., & Oh, U. (2005). Painful channels in sensory neurons. *Mol Cells, 20*(3), 315-324.

Leffler, A. S., Hansson, P., & Kosek, E. (2002). Somatosensory perception in a remote pain-free area and function of diffuse noxious inhibitory controls (DNIC) in patients suffering from long-term trapezius myalgia. *Eur J Pain, 6*(2), 149-159.

Leigh A. Lamont, D., William J. Tranquilli, DVM, MS, and Kurt A. Grimm, DVM. (2000). Physiology of Pain. *Management of Pain, 30*(4), 703 -715.

Lekan, H. A., Carlton, S. M., & Coggeshall, R. E. (1996). Sprouting of A beta fibers into lamina II of the rat dorsal horn in peripheral neuropathy. *Neurosci Lett, 208*(3), 147-150.

Levine, J. D., Fields, H. L., & Basbaum, A. I. (1993). Peptides and the primary afferent nociceptor. *J Neurosci, 13*(6), 2273-2286.

Lewin, G. R., & Barde, Y. A. (1996). Physiology of the neurotrophins. *Annu Rev Neurosci, 19*, 289-317.

Li, X., Lighthall, G., Liang, D. Y., & Clark, J. D. (2004). Alterations in spinal cord gene expression after hindpaw formalin injection. *J Neurosci Res, 78*(4), 533-541.

Lidbeck, J. (2002). Central hyperexcitability in chronic musculoskeletal pain: a conceptual breakthrough with multiple clinical implications. *Pain Res Manag, 7*(2), 81-92.

Liebl D . J., Tessarollo, L., Palko, M. E., & Parada, L. E., (1997). Absence of sensory neurons before target innervations in brain-derived neurotrophic factor-, neurotrophin -, and trkC- deficient embryonic mice. J Neurosci, 17(23), 9113-21.

Light, A. R., & Perl, E. R. (2003). Unmyelinated afferent fibers are not only for pain anymore. *J Comp Neurol, 461*(2), 137-139.

Lima, D. (1998). Anatomical basis for the dynamic processing of nociceptive input. *Eur J Pain, 2*(3), 195-202.

Limer, K. L., Nicholl, B. I., Thomson, W., & McBeth, J., (2008). Exploring the genetic susceptibility of chronic widespread pain: the tender points in gender association studies. Rheumatology, 47(5), 572-5

Lu, B., Pang, P. T., & Woo, N. H. (2005). The yin and yang of neurotrophin action. *Nat Rev Neurosci, 6*(8), 603-614.

Lubrano, E., Iovino, P., Tremolaterra, F., Parsons, W. J., Ciacci, C., & Mazzacca, G. (2001). Fibromyalgia in patients with irritable bowel syndrome. An association with the severity of the intestinal disorder. *Int J Colorectal Dis, 16*(4), 211-215.

Macias, M., Dwornik, A., Ziemlinska, E., Fehr, S., Schachner, M., Czarkowska-Bauch, J., et al. (2007). Locomotor exercise alters expression of pro-brain-

derived neurotrophic factor, brain-derived neurotrophic factor and its receptor TrkB in the spinal cord of adult rats. *Eur J Neurosci, 25*(8), 2425-2444.

Malcangio, M., Garrett, N. E., Cruwys, S., & Tomlinson, D. R. (1997). Nerve growth factor- and neurotrophin-3-induced changes in nociceptive threshold and the release of substance P from the rat isolated spinal cord. *J Neurosci, 17*(21), 8459-8467.

Mannerkorpi, K. (2005). Exercise in fibromyalgia. *Curr Opin Rheumatol, 17*(2), 190-194.

Mathes, W. F., & Kanarek, R. B. (2001). Wheel running attenuates the antinociceptive properties of morphine and its metabolite, morphine-6-glucuronide, in rats. *Physiol Behav, 74*(1-2), 245-251.

Mayer, D. J., Mao, J., Holt, J., & Price, D. D. (1999). Cellular mechanisms of neuropathic pain, morphine tolerance, and their interactions. *Proc Natl Acad Sci U S A, 96*(14), 7731-7736.

McAllister, A. K., Katz, L. C., & Lo, D. C. (1999). Neurotrophins and synaptic plasticity. *Annu Rev Neurosci, 22*, 295-318.

McCain, G. A. (1986). Role of physical fitness training in the fibrositis/fibromyalgia syndrome. *Am J Med, 81*(3A), 73-77.

McIlwrath, S. L., Lawson, J. J., Anderson, C. E., Albers, K. M., & Koerber, H. R. (2007). Overexpression of neurotrophin-3 enhances the mechanical response properties of slowly adapting type 1 afferents and myelinated nociceptors. *Eur J Neurosci, 26*(7), 1801-1812.

McMahon, S. B., & Jones, N. G. (2004). Plasticity of pain signaling: role of neurotrophic factors exemplified by acid-induced pain. *J Neurobiol, 61*(1), 72-87.

Meiworm, L., Jakob, E., Walker, U. A., Peter, H. H., & Keul, J. (2000). Patients with fibromyalgia benefit from aerobic endurance exercise. *Clin Rheumatol, 19*(4), 253-257.

Mendell, L. M., Munson, J. B., & Arvanian, V. L. (2001). Neurotrophins and synaptic plasticity in the mammalian spinal cord. *J Physiol, 533*(Pt 1), 91-97.

Mense, S. (1991). Considerations concerning the neurobiological basis of muscle pain. *Can J Physiol Pharmacol, 69*(5), 610-616.

Mense, S. (1993). Nociception from skeletal muscle in relation to clinical muscle pain. *Pain, 54*(3), 241-289.

Mense, S. (1996). Group III and IV receptors in skeletal muscle: are they specific or polymodal? *Prog Brain Res, 113*, 83-100.

Mense, S., & Meyer, H. (1985). Different types of slowly conducting afferent units in cat skeletal muscle and tendon. *J Physiol, 363*, 403-417.

Merlio, J. P., Ernfors, P., Jaber, M., & Persson, H. (1992). Molecular cloning of rat trkC and distribution of cells expressing messenger RNAs for members of the trk family in the rat central nervous system. *Neuroscience, 51*(3), 513-532.

Miranda, A., Peles, S., Rudolph, C., Shaker, R., & Sengupta, J. N. (2004). Altered visceral sensation in response to somatic pain in the rat. *Gastroenterology, 126*(4), 1082-1089.

Mogil, J. S., & Belknap, J. K. (1997). Sex and genotype determine the selective activation of neurochemically-distinct mechanisms of swim stress-induced analgesia. *Pharmacol Biochem Behav, 56*(1), 61-66.

Mogil, J. S., Richards, S. P., O'Toole, L. A., Helms, M. L., Mitchell, S. R., Kest, B., et al. (1997). Identification of a sex-specific quantitative trait locus mediating nonopioid stress-induced analgesia in female mice. *J Neurosci, 17*(20), 7995-8002.

Mogil, J. S., Wilson, S. G., Bon, K., Lee, S. E., Chung, K., Raber, P., et al. (1999). Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. *Pain, 80*(1-2), 67-82.

Mogil, J. S., Yu, L., & Basbaum, A. I. (2000). Pain genes? natural variation and transgenic mutants. *Annu Rev Neurosci, 23*, 777-811.

Molander, C., Hongpaisan, J., & Grant, G. (1992). Changing pattern of c-FOS expression in spinal cord neurons after electrical stimulation of the chronically injured sciatic nerve in the rat. *Neuroscience, 50*(1), 223-236.

Molliver, D. C., Immke, D. C., Fierro, L., Pare, M., Rice, F. L., & McCleskey, E. W. (2005). ASIC3, an acid-sensing ion channel, is expressed in metaboreceptive sensory neurons. *Mol Pain, 1*, 35.

Molteni, R., Zheng, J. Q., Ying, Z., Gomez-Pinilla, F., & Twiss, J. L. (2004). Voluntary exercise increases axonal regeneration from sensory neurons. *Proc Natl Acad Sci U S A, 101*(22), 8473-8478.

Munglani, R., & Hunt, S. P. (1995). Molecular biology of pain. *Br J Anaesth, 75*(2), 186-192.

Munson, J. B., Johnson, R. D., & Mendell, L. M. (1999). Neurotrophin-3 and maintenance of muscle afferent function. *Prog Brain Res, 123*, 157-163.

Nagy, J. I., & Hunt, S. P. (1982). Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. *Neuroscience, 7*(1), 89-97.

Naranjo, J. R., Mellstrom, B., Achaval, M., & Sassone-Corsi, P. (1991). Molecular pathways of pain: Fos/Jun-mediated activation of a noncanonical AP-1 site in the prodynorphin gene. *Neuron, 6*(4), 607-617.

Neumann, L., & Buskila, D. (2003). Epidemiology of fibromyalgia. *Curr Pain Headache Rep, 7*(5), 362-368.

Pan, J. W., Hamm, J. R., Rothman, D. L., & Shulman, R. G. (1988). Intracellular pH in human skeletal muscle by 1H NMR. *Proc Natl Acad Sci U S A, 85*(21), 7836-7839.

Park, S. Y., Choi, J. Y., Kim, R. U., Lee, Y. S., Cho, H. J., & Kim, D. S. (2003). Downregulation of voltage-gated potassium channel alpha gene expression by axotomy and neurotrophins in rat dorsal root ganglia. *Mol Cells, 16*(2), 256-259.

Perez-Pinera, P., Garcia-Suarez, O., Germana, A., Diaz-Esnal, B., de Carlos, F., Silos-Santiago, I., et al. (2008). Characterization of sensory deficits in TrkB knockout mice. *Neurosci Lett, 433*(1), 43-47.

Perl, E. R. (2007). Ideas about pain, a historical view. *Nature Reviews*, 8, 71-80.

Pertovaara, A., Kemppainen, P., & Leppanen, H. (1992). Lowered cutaneous sensitivity to nonpainful electrical stimulation during isometric exercise in humans. *Exp Brain Res, 89*(2), 447-452.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res, 29*(9), e45.

Pfaffl, M. W., Horgan, G. W., & Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res, 30*(9), e36.

Price, M. P., McIlwrath, S. L., Xie, J., Cheng, C., Qiao, J., Tarr, D. E., et al. (2001). The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. *Neuron, 32*(6), 1071-1083.

Redondo, J. R., Justo, C. M., Moraleda, F. V., Velayos, Y. G., Puche, J. J., Zubero, J. R., et al. (2004). Long-term efficacy of therapy in patients with fibromyalgia: a physical exercise-based program and a cognitive-behavioral approach. *Arthritis Rheum, 51*(2), 184-192.

Renn, C. L., & Dorsey, S. G. (2005). The physiology and processing of pain: a review. *AACN Clin Issues, 16*(3), 277-290; quiz 413-275.

Rodriguez-Tebar, A., Dechant, G., Gotz, R., & Barde, Y. A. (1992). Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *Embo J, 11*(3), 917-922.

Rohrbeck, J., Jordan, K., & Croft, P. (2007). The frequency and characteristics of chronic widespread pain in general practice: a case-control study. *Br J Gen Pract, 57*(535), 109-115.

Roux, P. P., & Barker, P. A. (2002). Neurotrophin signaling through the p75 neurotrophin receptor. *Prog Neurobiol, 67*(3), 203-233.

Russell. (1998a). Advances in Fibromyalgia: Possible Role for Central Neurochemicals. *Am J Med Sci, 316*(6), 377-384.

Russell. (1998b). Neurochemical Pathogenesis of Fibromyalgia. *Z Rheumatol, 57*(2), 63-66.

Saragovi, H. U., & Gehring, K. (2000). Development of pharmacological agents for targeting neurotrophins and their receptors. *Trends Pharmacol Sci, 21*(3), 93-98.

Sastre, J., Asensi, M., Gasco, E., Pallardo, F. V., Ferrero, J. A., Furukawa, T., et al. (1992). Exhaustive physical exercise causes oxidation of glutathione status in blood: prevention by antioxidant administration. *Am J Physiol, 263*(5 Pt 2), R992-995.

Scarisbrick, I. A., Isackson, P. J., & Windebank, A. J. (1999). Differential expression of brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 in the adult rat spinal cord: regulation by the glutamate receptor agonist kainic acid. *J Neurosci, 19*(18), 7757-7769.

Schafers, M., Sorkin, L. S., & Sommer, C. (2003). Intramuscular injection of tumor necrosis factor-alpha induces muscle hyperalgesia in rats. *Pain, 104*(3), 579-588.

Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., et al. (2006). The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol, 7*, 3.

Seltzer, Z., Dubner, R., & Shir, Y. (1990). A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain, 43*(2), 205-218.

Simon, M., Mann, D., Coulton, G., & Terenghi, G. (2002). Differential tyrosine kinase C mRNA distribution in extensor digitorum longus and soleus motoneurons in adult rats: effect of axotomy and neurotrophin-3 treatment. *Neurosci Lett, 320*(1-2), 9-12.

Simon, M., Terenghi, G., Green, C. J., & Coulton, G. R. (2000). Differential effects of NT-3 on reinnervation of the fast extensor digitorum longus (EDL) and the slow soleus muscle of rat. *Eur J Neurosci, 12*(3), 863-871.

Siu, P. M., Donley, D. A., Bryner, R. W., & Alway, S. E. (2003). Citrate synthase expression and enzyme activity after endurance training in cardiac and skeletal muscles. *J Appl Physiol, 94*(2), 555-560.

Skyba, D. A., King, E. W., & Sluka, K. A. (2002). Effects of NMDA and non-NMDA ionotropic glutamate receptor antagonists on the development and

maintenance of hyperalgesia induced by repeated intramuscular injection of acidic saline. *Pain, 98*(1-2), 69-78.

Skyba, D. A., Lisi, T. L., & Sluka, K. A. (2005). Excitatory amino acid concentrations increase in the spinal cord dorsal horn after repeated intramuscular injection of acidic saline. *Pain, 119*(1-3), 142-149.

Skyba, D. A., Radhakrishnan, R., & Sluka, K. A. (2005). Characterization of a method for measuring primary hyperalgesia of deep somatic tissue. *J Pain, 6*(1), 41-47.

Sluka, K. A. (1996). Pain mechanisms involved in musculoskeletal disorders. *J Orthop Sports Phys Ther, 24*(4), 240-254.

Sluka, K. A. (2002). Stimulation of deep somatic tissue with capsaicin produces long-lasting mechanical allodynia and heat hypoalgesia that depends on early activation of the cAMP pathway. *J Neurosci, 22*(13), 5687-5693.

Sluka, K. A., Kalra, A., & Moore, S. A. (2001). Unilateral intramuscular injections of acidic saline produce a bilateral, long-lasting hyperalgesia. *Muscle Nerve, 24*(1), 37-46.

Sluka, K. A., Price, M. P., Breese, N. M., Stucky, C. L., Wemmie, J. A., & Welsh, M. J. (2003). Chronic hyperalgesia induced by repeated acid injections in muscle is abolished by the loss of ASIC3, but not ASIC1. *Pain, 106*(3), 229-239.

Sluka, K. A., Radhakrishnan, R., Benson, C. J., Eshcol, J. O., Price, M. P., Babinski, K., et al. (2007). ASIC3 in muscle mediates mechanical, but not heat, hyperalgesia associated with muscle inflammation. *Pain, 129*(1-2), 102-112.

Sluka, K. A., Rohlwing, J. J., Bussey, R. A., Eikenberry, S. A., & Wilken, J. M. (2002). Chronic muscle pain induced by repeated acid Injection is reversed by spinally administered mu- and delta-, but not kappa-, opioid receptor agonists. *J Pharmacol Exp Ther, 302*(3), 1146-1150.

Smith, M. A., & Lyle, M. A. (2006). Chronic exercise decreases sensitivity to mu opioids in female rats: correlation with exercise output. *Pharmacol Biochem Behav, 85*(1), 12-22.

Snider, W. D. (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell, 77*(5), 627-638.

Soppet, D., Escandon, E., Maragos, J., Middlemas, D. S., Reid, S. W., Blair, J., et al. (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. *Cell, 65*(5), 895-903.

Sorensen, J., Graven-Nielsen, T., Henriksson, K. G., Bengtsson, M., & Arendt-Nielsen, L. (1998). Hyperexcitability in fibromyalgia. *J Rheumatol, 25*(1), 152-155.

Sparling, P. B., Giuffrida, A., Piomelli, D., Rosskopf, L., & Dietrich, A. (2003). Exercise activates the endocannabinoid system. *Neuroreport, 14*(17), 2209-2211.

Staud, R. (2004). Fibromyalgia pain: do we know the source? *Curr Opin Rheumatol, 16*(2), 157-163.

Staud, R., Price, D. D., Robinson, M. E., Mauderli, A. P., & Vierck, C. J. (2004). Maintenance of windup of second pain requires less frequent stimulation in fibromyalgia patients compared to normal controls. *Pain, 110*(3), 689-696.

Staud, R., Vierck, C. J., Cannon, R. L., Mauderli, A. P., & Price, D. D. (2001). Abnormal sensitization and temporal summation of second pain (wind-up) in patients with fibromyalgia syndrome. *Pain, 91*(1-2), 165-175.

Steen, A. E., Reeh, P. W., Geisslinger, G., & Steen, K. H. (2000). Plasma levels after peroral and topical ibuprofen and effects upon low pH-induced cutaneous and muscle pain. *Eur J Pain, 4*(2), 195-209.

Stock, J., (1996). Receptor signaling: dimerization and beyond. *Curr Biology, 6*(7), 835-827.

Svendsen, O., Edwards, C. N., Lauritzen, B., & Rasmussen, A. D. (2005). Intramuscular injection of hypertonic saline: in vitro and in vivo muscle tissue toxicity and spinal neurone c-fos expression. *Basic Clin Pharmacol Toxicol, 97*(1), 52-57.

Tabuchi, A. (2007). Synaptic plasticity-regulated gene expression: a key event in the long-lasting changes of neuronal function. *Biol. Pharm. Bull., 31*(3), 327-335.

Taguchi, T., Matsuda, T., Tamura, R., Sato, J., & Mizumura, K. (2005). Muscular mechanical hyperalgesia revealed by behavioural pain test and c-Fos

expression in the spinal dorsal horn after eccentric contraction in rats. *J Physiol, 564*(Pt 1), 259-268.

Takahashi, Y., Chiba, T., Kurokawa, M., & Aoki, Y. (2003). Dermatomes and the central organization of dermatomes and body surface regions in the spinal cord dorsal horn in rats. *J Comp Neurol, 462*(1), 29-41.

Tanriverdi, F., Karaca, Z., Unluhizarci, K., & Kelestimur, F. (2007). The hypothalamo-pituitary-adrenal axis in chronic fatigue syndrome and fibromyalgia syndrome. *Stress, 10*(1), 13-25.

Tessarollo, L., Vogel, K. S., Palko, M. E., Reid, S. W., & Parada, L. F. (1994). Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons. *Proc Natl Acad Sci U S A, 91*(25), 11844-11848.

Tillu, D. V., Gebhart, G. F., & Sluka, K. A. (2007). Descending facilitatory pathways from the RVM initiate and maintain bilateral hyperalgesia after muscle insult. *Pain*.

Todd, A. J., Spike, R. C., Young, S., & Puskar, Z. (2005). Fos induction in lamina I projection neurons in response to noxious thermal stimuli. *Neuroscience, 131*(1), 209-217.

Treede, R. D., Meyer, R. A., & Campbell, J. N. (1998). Myelinated mechanically insensitive afferents from monkey hairy skin: heat-response properties. *J Neurophysiol, 80*(3), 1082-1093.

Treede, R. D., Meyer, R. A., Raja, S. N., & Campbell, J. N. (1992). Peripheral and central mechanisms of cutaneous hyperalgesia. *Prog Neurobiol, 38*(4), 397-421.

Vanegas, H., & Schaible, H. G. (2004). Descending control of persistent pain: inhibitory or facilitatory? *Brain Res Brain Res Rev, 46*(3), 295-309.

Verbeke G, & Molenberghs G (2000). *Linear mixed models for longitudinal data.* NY: Springer.

Vierck, C. J., Jr. (2006). Mechanisms underlying development of spatially distributed chronic pain (fibromyalgia). *Pain, 124*(3), 242-263.

Vierck, C. J., Jr., Staud, R., Price, D. D., Cannon, R. L., Mauderli, A. P., & Martin, A. D. (2001). The effect of maximal exercise on temporal summation of second pain (windup) in patients with fibromyalgia syndrome. *J Pain, 2*(6), 334-344.

Wang, L. X., & Wang, Z. J. (2003). Animal and cellular models of chronic pain. *Adv Drug Deliv Rev, 55*(8), 949-965.

Wang, S. F., Chen, C. C., Liao, W. S., & Shyu, B. C. (2005). Different types of variant muscle nociception after intermittent and continuous neuromuscular stimulation in rats. *J Biomed Sci, 12*(3), 467-479.

Wang, X., Butowt, R., Vasko, M. R., & von Bartheld, C. S. (2002). Mechanisms of the release of anterogradely transported neurotrophin-3 from axon terminals. *J Neurosci, 22*(3), 931-945.

Watanabe, M., Endo, Y., Kimoto, K., Katoh-Semba, R., & Arakawa, Y. (2000). Inhibition of adjuvant-induced inflammatory hyperalgesia in rats by local injection of neurotrophin-3. *Neurosci Lett, 282*(1-2), 61-64.

Waxman, S. G., Cummins, T. R., Dib-Hajj, S. D., & Black, J. A. (2000). Voltage-gated sodium channels and the molecular pathogenesis of pain: a review. *J Rehabil Res Dev, 37*(5), 517-528.

Weigent. (1998). Current Concepts in the Pathophysiology of Abnormal Pain: Perception in Fibromyalgia. *Am J Med Sci, 315*(6), 408-412.

Wernig, A., Nanassy, A., & Muller, S. (1999). Laufband (treadmill) therapy in incomplete paraplegia and tetraplegia. *J Neurotrauma, 16*(8), 719-726.

Whiteside, A., Hansen, S., & Chaudhuri, A. (2004). Exercise lowers pain threshold in chronic fatigue syndrome. *Pain, 109*(3), 497-499.

Wiesenfeld-Hallin, Z., Aldskogius, H., Grant, G., Hao, J. X., Hokfelt, T., & Xu, X. J. (1997). Central inhibitory dysfunctions: mechanisms and clinical implications. *Behav Brain Sci, 20*(3), 420-425; discussion 435-513.

Williams, S., Evan, G. I., & Hunt, S. P. (1990). Changing patterns of c-fos induction in spinal neurons following thermal cutaneous stimulation in the rat. *Neuroscience, 36*(1), 73-81.

Willis, W. D., Sluka, K. A., Rees, H., & Westlund, K. N. (1996). Cooperative mechanisms of neurotransmitter action in central nervous sensitization. *Prog Brain Res, 110*, 151-166.

Wilson-Gerwing, T. D., Dmyterko, M. V., Zochodne, D. W., Johnston, J. M., & Verge, V. M. (2005). Neurotrophin-3 suppresses thermal hyperalgesia associated with neuropathic pain and attenuates transient receptor potential vanilloid receptor-1 expression in adult sensory neurons. *J Neurosci, 25*(3), 758-767.

Wilson-Gerwing, T. D., & Verge, V. M. (2006). Neurotrophin-3 attenuates galanin expression in the chronic constriction injury model of neuropathic pain. *Neuroscience, 141*(4), 2075-2085.

Wilson-Gerwing, T. D., Stucky, c. L., McComb, G. W. & Verge, V. M. (2008). Neurotrophin-3 significantly reduces sodium channel expression linked to neuropathic pain states. *Exp Neurol, 14.*

Wilson, P., & Snow, P. J. (1993). Morphology of A beta hair follicle afferent collaterals in dorsal horn of cats with neonatal chronic denervation of digits. *J Neurophysiol, 70*(6), 2399-2410.

Wingenfeld, K., Heim, C., Schmidt, I., Wagner, D., Meinlschmidt, G., & Hellhammer, D. H. (2008). HPA axis reactivity and lymphocyte glucocorticoid sensitivity in fibromyalgia syndrome and chronic pelvic pain. *Psychosom Med, 70*(1), 65-72.

Woolf, C. J. (1987). Central terminations of cutaneous mechanoreceptive afferents in the rat lumbar spinal cord. *J Comp Neurol, 261*(1), 105-119.

Woolf, C. J., Shortland, P., & Coggeshall, R. E. (1992). Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature, 355*(6355), 75-78.

Wright, D. E. (2001). Muscle-Drived Neurotrophin-3 Reduces Injury-Induced Proprioceptive Degeneration in Neonatal Mice.

Wright, D. E., Zhou, L., Kucera, J., & Snider, W. D. (1997). Introduction of a neurotrophin-3 transgene into muscle selectively rescues proprioceptive neurons in mice lacking endogenous neurotrophin-3. *Neuron, 19*(3), 503-517.

Xie, K., Wang, T., Olafsson, P., Mizuno, K., & Lu, B. (1997). Activity-dependent expression of NT-3 in muscle cells in culture: implications in the development of neuromuscular junctions. *J Neurosci, 17*(9), 2947-2958.

Ying, Z., Roy, R. R., Edgerton, V. R., & Gomez-Pinilla, F. (2003). Voluntary exercise increases neurotrophin-3 and its receptor TrkC in the spinal cord. *Brain Res, 987*(1), 93-99.

Ying, Z., Roy, R. R., Edgerton, V. R., & Gomez-Pinilla, F. (2005). Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. *Exp Neurol, 193*(2), 411-419.

Yokoyama T., Maeda, Y., Audette, K. M., Sluka, K. A. (2007a). Pregabalin reduces muscle and cutaneous hyperalgesia in two models of chronic muscle pain in rats. J Pain, 8(5), 422-29.

Yokoyama, T., Lisi, T. L., Moore, S. A., & Sluka, K. A. (2007b). Muscle fatigue increases the probability of developing hyperalgesia in mice. *J Pain, 8*(9), 692-699.

You, H. J., & Arendt-Nielsen, L. (2005). Unilateral subcutaneous bee venom but not formalin injection causes contralateral hypersensitized wind-up and after-

discharge of the spinal withdrawal reflex in anesthetized spinal rats. *Exp Neurol, 195*(1), 148-160.

Yu, Y. C., Koo, S. T., Kim, C. H., Lyu, Y., Grady, J. J., & Chung, J. M. (2002). Two variables that can be used as pain indices in experimental animal models of arthritis. *J Neurosci Methods, 115*(1), 107-113.

Zhao, C., Veltri, K., Li, S., Bain, J. R. and Fahnestock, M. (2004). NGF, BDNF, NT-3, and GDNF mRNA expression in rat skeletal muscle following denervation and sensory protection. *J Neurotrauma, 21*(10), 1468-1478.

Zigmond, M. J. B., F.E; Landis, S. C.; Roberts, J. C.; Squire, L. R. (1999). Fundamental Neuroscience. 761-787.

Zweifel, L. S., Kuruvilla, R., & Ginty, D. D. (2005). Functions and mechanisms of retrograde neurotrophin signalling. *Nat Rev Neurosci, 6*(8), 615-625.