**Exploring the Metabolic, Activity, and
Musculoskeletal Changes in Neuropathic Pain**

by

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DEDICATION

To my amazing wife Julie.

"I can do all this through him who gives me strength." Philippians 4:13

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Introduction

Neuropathic pain is a significant adverse health condition. The mechanisms contributing to the altered processing of incoming stimuli are well studied. In addition, first line treatments, such as duloxetine, improve signs and symptoms of neuropathic pain. However, the metabolic alterations, physical activity and musculoskeletal changes associated with neuropathic pain remain unexplored. Also, the ability of antidepressants to prevent reductions in physical activity and resultant muscle atrophy by improving the processing of incoming nociceptive stimuli in preclinical models of neuropathic pain have yet to be studied. The purpose of this dissertation was to describe basic metabolic, activity, and musculoskeletal changes following neuropathic injury and how duloxetine, an antidepressant used in the treatment of diabetic peripheral neuropathic pain, may prevent these changes in a preclinical model of neuropathic pain.

Neuropathic pain, a form of chronic pain, affects an estimated 76.2 million people (U.S. Department of Health and Human Services, National Center for Health Statistics, 2006). Taylor (2006) reported the occurrence of specific types of neuropathic pain such as diabetic neuropathy and complex regional pain syndrome, but rates vary greatly among diseases. Using insurance data, Berger, Dukes, and Oster (2004) reported that 55,686 adults were diagnosed with a painful neuropathic disorder (back and neck pain, diabetic neuropathy). The authors did not report the total number of insured persons

sampled for the study, making overall prevalence difficult to determine. Similar rates of neuropathic pain have been reported in other countries.

In Europe, the prevalence of neuropathic pain ranges from 6.9-8.2% of adults surveyed (Bouhassira, Lantri-Minet, Attal, Laurent, & Touboul, 2008; Torrance, Smith, Bennett, & Lee, 2006). Dieleman, Kerklaan, Huygen, Bouma, and Sturkenboom (2008) reported an overall incidence of neuropathic pain of 8.2 per 1000 patient years. The authors extrapolated this figure to estimate the presence of 131,000 cases per year in a general population of 16 million (Dieleman et al., 2008). Regardless of incidence, neuropathic pain is more prevalent in women and older adults (age range: 50-64 years) (Berger et al., 2004; Bouhassira et al., 2008; Dieleman et al., 2008; Hans, Masquelier, & De Cock, 2007; Torrance et al., 2006). While the prevalence of neuropathic pain in the general population is less well known, mechanisms that alter the processing of incoming stimuli are well understood.

Pain is the interpretation of noxious (nociceptive) stimuli by information-processing neuronal tissue. Specialized nerve fibers in the peripheral nervous system, called nociceptors, detect noxious stimuli and generate action potentials along the length of the neuron, potentiate the signal, and transmit it to second-order neurons in the central nervous system (Baron, 2009; Castro-Lopez, Raja, & Schmelz, 2008; Costigan, Scholz, & Woolf, 2009). Ultimately, the interpretation of the stimulus is determined as painful (or not painful) by the conscious brain (Castro-Lopez et al., 2008).

Nociceptive pain can end with the cessation of incoming stimuli and/or tissue healing. However, with repeated nociceptor activation, a chronic pain state may develop. Neuropathic pain develops when somatosensory system tissue is damaged or diseased

(Treede et al., 2008) and alters the normal detection and transmission of incoming stimuli from the periphery. Within the peripheral and central nervous systems, neuropathic pain can develop from various initiating events and lead to key manifestations.

Peripheral neuropathic pain can arise from mechanical trauma, metabolic disease, or infection in the periphery. Conversely, spinal cord injury or multiple sclerosis can lead to central neuropathic pain (Costigan et al., 2009). Following these events, the processing of incoming stimuli is altered, resulting in three key manifestations: allodynia, hyperalgesia, and spontaneous pain.

Allodynia occurs when a stimulus that is not normally painful evokes a painful response (Basić-Kes et al., 2009; Castro-Lopez et al., 2008; Mellion, 2008). Even light touch or the slight bending of hairs on the surface of the skin may evoke severe pain (Baron, 2009; Basić-Kes et al., 2009; Taylor, 2001). In contrast, hyperalgesia is an amplified or exaggerated (pain) response to normally mild painful stimuli (Basić-Kes et al., 2009; Castro-Lopez et al., 2008; Mellion, 2008). Humans and animals with hyperalgesia can have increased response to heat, cold, or mechanical (pressure or pin-prick) stimuli. Finally, spontaneous pain occurs in the absence of a peripheral stimulus and is the result of ectopic action potential generation within nociceptive pathways (Costigan et al., 2009). Underlying these manifestations, neuropathic pain encompasses long-term anatomical and physiological changes that alter the processing of sensory input.

Many physiologic changes contribute to increased responsiveness of primary and secondary nociceptors. Sodium channel up-regulation contributes to increased neuronal excitability and spontaneous discharge (Baron, 2009; Campbell & Meyer, 2006; Costigan

et al., 2009; Mellion, 2008; Taylor, 2001). In addition, nerve growth factors released

during nerve degeneration stimulate channel and receptor expression on uninjured fibers,

leading to altered nociceptive function in healthy neurons (Baron, 2009). Abnormal

neurotransmitter release involving glutamate and substance P, as well as axonal

overgrowth, contribute to increased responsiveness of neurons in the central nervous

system to normal afferent input (Baron, 2009).

Neuropathic pain has a significant impact on quality of life. Patients with

neuropathic pain visit physicians more frequently, have poorer health-related quality of

life, and consume higher healthcare resources (Taylor et al., 2006). Neuropathic pain

also negatively impacts sleep and activities of daily living (Hans et al., 2007). De Sousa

and Frank (2007) found that pain limits several activities such as running, walking,

independently performing activities of daily living, and leisure activity. Importantly,

neuropathic pain that is inadequately treated may lead to an overall hypoactive lifestyle

and contribute to a reduction in physical fitness, leading to further hypoactivity (van den

Berg-Emons, Schasfoort, de Vos, Bussmann, & Stam, 2007). With reduced physical

fitness and decreased activity, muscle atrophy may develop in inadequately treated

neuropathic pain.

During periods of inactivity, protein synthesis often decreases from disuse,

contributing to muscle atrophy (Evans, 2010). "Decreased weight bearing and inactivity

produce a wide range of manifestations whose severity depend upon the degree and

duration of decreased activity" (Kasper, Maxwell, & White, 1996, pg. 133). Changes in

insulin signaling (O'Neill, Wilding, Kahn, Van Remmen, McArdle, & Jackson et al.,

2010) and impaired strength and exercise capacity (Kasper et al., 1996) necessitate

appropriate assessment and intervention. Specific rehabilitation strategies must be tailored to prevent additional damage of atrophied muscle (Kasper, Talbot, & Gaines, 2002). With potential effects on activity and muscle atrophy, the treatment of neuropathic pain is warranted, although challenging.

Treatment for neuropathic pain is often difficult and complex (Freynhagen & Bennett, 2009; Harden & Cohen, 2003). Clinical presentation of neuropathic pain is often under-recognized and not adequately treated with conventional analgesics (Hans et al., 2007; Harden & Cohen, 2003). Reasons for inadequate pain relief include inadequate dosage and/or use of first line treatment recommendations (O'Connor, 2009), variability in symptom presentation and response to treatment (Harden & Cohen, 2003), and the presence of multiple comorbidities (Berger et al., 2004). Inadequate treatment can lead to cardiovascular, gastrointestinal, and immunological changes (Dunwoody, Krenzischek, Pasero, Rathmell, & Polomano, 2008), and while the treatment of neuropathic pain is challenging, the benefits of effective management necessitate appropriate intervention.

Effective first-line treatment for neuropathic pain includes antidepressants with both norepinephrine and serotonin reuptake inhibitors, calcium channel blockers, and topical lidocaine (O'Connor, 2009). Within this treatment category, duloxetine hydrochloride, a selective serotonin and norepinephrine reuptake inhibitor is approved by the FDA for the management of diabetic peripheral neuropathic pain. In humans, duloxetine improved 24-hour average pain score vs. placebo, with efficacy beginning within 1 week of treatment initiation (Goldstein, Lu, Detke, Lee, & Iyengar, 2005; Raskin et al., 2005). Duloxetine also alleviates thermal (Bomholt, Mikkelsen, & Blackburn Munro, 2005) and mechanical hyperalgesia (Bomholt et al., 2005; Piesla et al., 2009) in

pre-clinical models of nociceptive and neuropathic pain. Though improvements in nociceptive processing have been demonstrated, the ability of duloxetine to prevent changes in physical activity and muscle atrophy by improving the processing of nociceptive stimuli remains unexplored.

The study of neuropathic pain has produced increasing information regarding the anatomical and physiological changes within the nervous system. However, little is known regarding metabolic alterations, physical activity, and musculoskeletal changes associated with neuropathic pain. Metabolic changes, specifically insulin resistance, are associated with acute, nociceptive pain (Greisen, Grfte, Hansen, Jensen, & Vilstrup, 1999; Greisen, Juhl, Grfte, Vilstrup, Jensen, & Schmitz, 2001). Increased inflammation may provide a link between neuropathic pain and metabolic changes. Inflammation is present in both insulin resistance and neuropathic pain (Bastard et al., 2006; Campbell & Meyer, 2006). Chronic inflammation that alters the processing of nociceptive input may also lead to deleterious effects on skeletal muscle structure and function, a primary tissue affected by insulin resistance (Wang et al., 2006). However, relationship between neuropathic pain and metabolic changes remains unexplored. On the contrary, changes in physical activity and muscle atrophy have been reported following neuropathic injury (Choe, Kim, An, Lee, & Heitkemper, 2011; Daemen et al., 1998). The underlying factors contributing to these changes, as well as the ability of duloxetine to prevent changes in activity and muscle atrophy by improving the altered processing of incoming stimuli have not been described.

The purpose of this dissertation was to describe the metabolic, physical activity, and musculoskeletal changes associated with neuropathic pain. Specifically, using an

established pre-clinical model of neuropathic pain, the chronic constriction injury (CCI; Bennett & Xie, 1988), basic metabolic outcomes, activity, and musculoskeletal changes were investigated in an initial exploratory study. Rats from this study did not exhibit changes in metabolic indices, but did develop decreased activity and muscle atrophy of the injured hindlimb. The purpose of a subsequent study was to determine if repeated duloxetine administration prevented decreases in activity and muscle atrophy by improving the processing of nociceptive stimuli. A thorough review of the literature related to neuropathic pain, metabolism, activity changes, muscle atrophy, and the treatment of neuropathic pain is presented in Chapter 2. Information on selected research methods used is also provided in Chapter 2.

Neuropathic pain is a significant health problem and should be treated aggressively, yet appropriately, to prevent exacerbation and additional unintended consequences such as decreased activity and muscle atrophy. Nurses must be encouraged to assess and manage neuropathic pain; implementing effective strategies - both pharmacologic and non-pharmacologic - to effectively improve pain control and quality of life (Mann, 2008). Interventions aimed at reducing neuropathic pain symptoms could also improve activity and prevent changes in muscle. By treating conditions using a variety of complementary approaches, nurses can potentially improve multiple aspects of their patients' health.

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CHAPTER 2

Review of Literature and Methods

Pain management is a vital component of nursing practice. Painful conditions affect millions of patients and have a significant impact on daily activities and overall quality of live. Many physiologic processes are altered during the progression from acute, nociceptive pain to chronic, neuropathic pain. In addition to pathological changes within the nervous system, other potential systemic changes in metabolism, activity, and muscle function add to the importance of aggressive intervention. Changes within the nervous system in conditions of neuropathic pain are well documented. However, the effects of neuropathic pain on muscle functioning have yet to be described. To explore the relationships between neuropathic pain and muscle functioning, metabolic, activity, and musculoskeletal changes in the presence of neuropathic pain will be investigated.

Pain

Nociceptive pain

Pain is defined as, "An unpleasant sensory and emotional experience arising from actual or potential tissue damage or described in terms of such damage" (Merskey & Bogduk, 1994, pg. 213). The definition of pain, however, does not fully encompass the physiologic phenomena occurring in the peripheral and central nervous systems (PNS and CNS, respectively), termed nociception. Pain is not a stimulus (Castro-Lopez, Raja, & Schmelz, 2008). Rather, pain is the end result of a complex network of information-

processing neuronal tissue. Whether or not a particular stimulus is *perceived* as painful depends not only on the nature of the stimulus, but also the context within which the stimulus is experienced (2008).

A nociceptive experience begins with a network of specialized sensory neurons, called primary afferent nociceptors, in the PNS. Specifically, a special class of unmyelinated, high-threshold primary sensory nerve fibers- C-fibers- or myelinated A-delta fibers detect noxious stimuli and generate action potentials along the length of the neuron, potentiate the signal, and transmit it to second-order neurons in the CNS (Castro-Lopez et al., 2008; Baron, 2009; Costigan, Scholz, & Woolf, 2009). Ultimately, the interpretation of the stimulus is determined as painful (or not painful) by the conscious brain (Castro-Lopez et al., 2008). Though often referred to as painful stimuli, pain represents the interpretation of noxious, nociceptive stimuli.

Nociception can be the result of natural or artificial events. Natural events can be mechanical, thermal, or chemical (Castro-Lopez et al., 2008; Mellion, 2008). Artificial events are those such as electric shock (Castro-Lopez et al., 2008). Nociceptive pain can also be acute or chronic in duration. In acute nociceptive pain, the stimuli typically resolve concurrent with the cessation of the initiating event or with tissue healing. Nociceptive pain can also persist for longer periods of time. However, chronic pain develops when changes in the PNS and CNS alter the normal detection of and response to noxious stimuli. A chronic pain state may develop following repeated nociceptor activation through several mechanisms, including peripheral and central sensitization. Many neuronal cytoarchitectual and physiological changes present in chronic pain states are similar to those seen in neuropathic pain (Voscopoulos & Lema, 2010). For the

remainder of the discussion, 'chronic' and 'neuropathic' pain will be used interchangeably, as there is significant pathophysiological overlap between the two.

Neuropathic Pain

While nociceptive pain refers to the transmission of acute, noxious stimuli, neuropathic pain represents a form of chronic pain, characterized by a wide range of causes and sensations. A key hallmark of neuropathic pain, which may differ from other chronic pain states, is nerve damage. Neuropathic pain is defined as, "Pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" (Treede et al., 2008, pg. 1631). Knowledge pertaining to the pathological changes that occur in neuropathic pain conditions continues to expand.

Neuropathic pain can originate in either the PNS or CNS, depending on the location of the lesion or disease (Castro-Lopez et al., 2008; Basić-Kes et al., 2009; Mellion, 2008). Peripheral neuropathic pain may arise following mechanical trauma, metabolic disease, neurotoxic chemicals, or infection in the periphery (Costigan et al., 2009). Other events such as spinal cord injury or multiple sclerosis can lead to central neuropathic pain (Costigan et al., 2009), which is often burning in quality, and sometimes paroxysmal, stabbing, buzzing, or electric shock-like (Castro-Lopez et al., 2008; Baron, 2009; Basić-Kes et al., 2009). Following initial events, long-term anatomical and physiological changes contribute to three key manifestations of neuropathic pain: allodynia, hyperalgesia, and spontaneous pain.

Allodynia occurs when a stimulus that is not normally painful evokes a painful response (Castro-Lopez et al., 2008; Basić-Kes et al., 2009; Mellion, 2008). An example of primary allodynia in humans would be experiencing pain from lightly touching the

skin of someone with sunburn. Even light touch or the slight bending of hairs on the

surface of the skin may evoke severe pain (Baron, 2009; Basić-Kes et al., 2009; Taylor,

2001). Secondary allodynia is a similar response that occurs away from the initial injury,

as in the case of pain evoked from touching the shoulder when the wrist has been

crushed.

Allodynia is the result of reduced peripheral nociceptor terminal threshold

(primary allodynia) or Aβ-fiber activation on altered secondary nociceptors (secondary

allodynia) (Woolf & Mannion, 1999). Aβ-fibers normally transmit innocuous stimuli,

such as light touch and proprioception to deeper laminae of the spinal cord (Taylor, 2001;

Voscopoulos & Lema, 2010). In neuropathic pain, Aβ-fibers can sprout into outer

laminae normally innervated by C-fibers, which normally transmit noxious stimuli

(Taylor, 2001). As a result, normally benign stimuli are transmitted via nociceptive

pathways in the CNS and are interpreted as painful in central processing centers of the

brain.

In contrast to allodynia, hyperalgesia is an amplified or exaggerated response to

normally mild painful stimuli (Castro-Lopez et al., 2008; Basić-Kes et al., 2009; Mellion,

2008). Clinically, hyperalgesia is exhibited during hyperactivity to pin-prick. Normally,

stimulation with a pin-prick would cause the patient to startle or withdraw slightly. A

hyperalgesic response, however, would elicit a much greater reaction. Increased

responses to heat, cold, or mechanical pressure can also be demonstrated with

hyperalgesia.

In hyperalgesia, nociceptive threshold for transmitting noxious stimuli is lowered

(Castro-Lopez et al., 2008), resulting in increased sensitivity to external stimuli. This

sensitization of neurons is present at the site of neuronal insult and in uninjured areas, termed primary and secondary hyperalgesia, respectively.

Primary hyperalgesia occurs within the area of injury, which is thought to be attributable to the sensitization of C-fibers by the release of chemical mediators during cell damage or the inflammatory response (Basić-Kes et al., 2009) and sodium channel upregulation (Costigan et al., 2009). Consequently, exaggerated and prolonged responses to noxious stimuli are present in the area of injury (Latremoliere & Woolf, 2009).

Secondary hyperalgesia occurs in undamaged nociceptors surrounding the injury site and often away from the injury site, and contributes to dorsal horn neuronal sensitization (Basić-Kes et al., 2009). Sodium channel upregulation (Costigan et al., 2009), lack of disinhibitory descending pathways (Woolf & Mannion, 1999), excessive glutamate and substance P release, and axonal overgrowth in the CNS can result in increased sensitivity to incoming stimuli in the spinal cord dorsal horn. These disruptions affect how the incoming stimulus is processed and, ultimately, interpreted by the brain. The underlying mechanisms involved in primary and secondary hyperalgesia can also occur in primary and secondary allodynia, except that the initiating stimulus is innocuous (Costigan et al., 2009). These phenomena demonstrate why individuals with neuropathic pain can experience painful sensations both at the site of injury and in areas away from the injury.

A third characteristic of neuropathic pain is spontaneous pain. Spontaneous pain occurs in the absence of a peripheral stimulus. Spontaneous pain can be shooting or electric-shock-like (Baron, 2009), originate at any point along the peripheral nerve up to and including the dorsal root ganglion (Taylor, 2001), and result from ectopic action

potential generation within nociceptive pathways (Costigan et al., 2009). In animal

models of neuropathic pain, spontaneous pain is evident by increased grooming of the

effected limb or sudden withdraw in the absence of locomotion (Bennett & Xie, 1988).

These manifestations of neuropathic pain reflect long-term anatomical and physiological

changes following nerve injury.

An increased inflammatory response is often observed in conditions of

neuropathic pain (Campbell & Meyer, 2006; Costigan et al., 2009; Latremoliere &

Woolf, 2009; Maletic & Raison, 2009; Miller, Jung, Bhangoo, & White, 2009).

Concurrent with nerve and tissue injury, the inflammatory response, involving bradykinin

and cytokines, induces ectopic impulses along nociceptive pathways (Costigan et al.,

2009). In nociceptive pain, the inflammatory response often subsides along with

nociceptive sensations. In neuropathic pain, however, the inflammatory response,

"changes the functional properties of the neurons…to reduce pain threshold, increase the

magnitude and duration of responses to noxious input, and permit normally innocuous

inputs to generate pain sensation" (Latremoliere & Woolf, 2009, pg. 913). These

changes may remain long after the inflammatory response diminishes. Increased

sensitivity to incoming stimuli, changes in neurotransmitter release, and axonal growth

within the CNS also contribute to altered nociceptive processing.

Sensitization is an altered response to normal and noxious sensory input, resulting

from intense, repeated, and sustained noxious stimuli. Altered processing often follows

tissue injury with damage to actual nerves (lesion, neuroma). (Latremoliere & Woolf,

2009). Sensitization can occur through similar mechanisms in both the PNS and CNS.

Peripheral sensitization is an increased responsiveness of primary nociceptors to stimulation of their receptive field (Baron, 2009). Upregulation of voltage-gated sodium channels associated with increased electrical excitability and spontaneous activity is a key mechanism involved in peripheral sensitization (Baron, 2009; Campbell & Meyer, 2006; Costigan et al., 2009; Mellion, 2008; Taylor, 2001). Interestingly, nerve growth factors released during nerve (Wallerian) degeneration stimulate channel and receptor expression on uninjured fibers (Baron, 2009) in partial nerve lesions. These growth factors can lead to altered nociceptive function in un-injured neurons (secondary allodynia/hyperalgesia). Peripheral sensitization can also involve a disinhibition of primary afferent neurons, evoking a greater response to noxious stimuli (Woolf & Mannion, 1999) and allowing more action potentials to reach the CNS.

In contrast to peripheral sensitization, central sensitization is the end result of maladaptive processes in both the PNS and CNS. Central sensitization occurs predominantly in the dorsal horn of the spinal cord (Baron, 2009). Like peripheral sensitization, sodium channel upregulation in second-order neurons may lead to a lowered threshold potential and spontaneous ectopic discharge (Taylor, 2001). Glutamate, substance P, and axonal overgrowth are also involved in central sensitization.

Normally, glutamate acts as a primary excitatory neurotransmitter, relaying the signal from the periphery to the CNS (Taylor, 2001; Woolf & Mannion, 1999). Increased glutamate release from sensitized C-fibers acting on N-methyl-D-aspertate receptors can lead to central sensitization. Substance P can accentuate this phenomenon by depolarizing membranes and removing the voltage-dependent block (Mg^{2+}). Subsequent calcium entry activates intracellular pathways that maintain central sensitization (Baron,

2009; Latremoliere & Woolf, 2009). These changes in the CNS can result in increased responsiveness to normal afferent input (Baron, 2009).

Axonal overgrowth within the dorsal horn can also contribute to central sensitization. Axon terminals of C-fibers can atrophy, while Aβ-fibers sprout into the outer laminae of the dorsal horn normally innervated by C-fibers (allodynia; Latremoliere & Woolf, 2009; Woolf & Mannion, 1999). Other mechanisms can also contribute to central sensitization, such as a decreased inhibitory signaling (Costigan et al., 2009; Latremoliere & Woolf, 2009) and the involvement of inflammatory cytokines (Campbell & Meyer, 2006; Costigan et al., 2009; Maletic & Raison, 2009; Miller et al., 2009).

The study of neuropathic pain has predominantly focused on changes in the nervous system. The pathophysiological mechanisms involved in neuropathic pain have been well documented. In addition, conditions of neuropathic pain, such as diabetic neuropathy and fibromyalgia, have received similar attention. However, very little is known about muscle function under conditions of neuropathic pain, which may impact overall activity and metabolism. Given the close relationship between nerve and muscle function, the lack of attention as to how neuropathic pain may affect multiple systems is startling.

In human studies, there is limited evidence describing the systemic metabolic, activity, and musculoskeletal changes accompanying neuropathic pain. Fasting glucose and total cholesterol are higher in individuals with severe pain compared with individuals experiencing no pain (Nilsson, Kandell-Colln, & Andersson, 1997). However, individuals with severe pain also had a higher body mass index, a known contributor to

insulin resistance (National diabetes fact sheet, 2005), compared to individuals with moderate and no pain.

Changes in metabolism, specifically insulin resistance, have been reported in various painful conditions such as trauma, burns, and surgery (Black, Brooks, Bessey, Wolfe, & Wilmore, 1982; Thorell, Efendic, Gutniak, Hggmark, & Ljungqvist, 1993; Wolfe, Durkot, Allsop, & Burke, 1979). In these studies, insulin resistance was measured following pain initiating events. However, the authors were unable to isolate pain as the primary antecedent for metabolic changes from among potentially confounding variables such as inflammation or adrenergic response.

A pivotal study conducted by Greisen, Juhl, Grfte, Vilstrup, Jensen, and Schmitz, (2001) provides the strongest evidence for a relationship between nociception and metabolic changes. Greisen et al. found that 30 minutes of intense (8/10 pain scale) electrical stimulation was sufficient to decrease insulin sensitivity measured with the hyperinsulinemic euglycemic clamp technique (2001). Oxidative and non-oxidative glucose disposal were both decreased following painful stimulation. Pain also increased levels of plasma free fatty acids, epinephrine, growth hormone, and s-cortisol (2001). The authors concluded that nociceptive pain *per se* contributes to increased insulin resistance. While this study supports a relationship between nociceptive pain and metabolic changes, there have been no studies characterizing the metabolic and activity changes in neuropathic pain.

Metabolism

Limited evidence supports a relationship between nociceptive pain and changes in metabolism (Greisen et al., 2001; Nilsson et al., 1997). In addition, no studies have

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characterized the metabolic or activity changes in conditions of neuropathic pain. Metabolic changes, specifically insulin resistance, may result from neuropathic pain, either directly from pain, or indirectly through changes in activity.

Insulin Resistance

Insulin resistance refers to the body's inability to respond to and use the insulin it produces (American Diabetes Association, 2010). Insulin resistance can also be defined as the impairment of insulin action in insulin-target tissues, including muscle (Abdul-Ghani & DeFronzo, 2010), resulting in elevated glucose levels, diminished hepatic suppression of glucose production and disinhibition of adipocyte lipolysis. Although insulin resistance develops as the result of multiple, overlapping antecedents, physical inactivity is an important risk factor (Esposito, Ciotola, Maiorino, & Giugliano, 2008).

Insulin resistance can be the result of decreased activity. Hindlimb immobilization and unloading in animals result in insulin resistance and decreased glucose uptake (Henriksen & Tischler, 1988; Ploug, Ohkuwa, Handberg, Vissing, & Galbo, 1995). Humans subjected to bed rest have decreased insulin sensitivity compared to baseline, measured by the oral glucose tolerance test (Hamburg et al., 2007; Stuart, Shangraw, Prince, Peters, & Wolfe, 1988) and hyperinsulinemic euglycemic clamp (Alibegovic et al., 2009). While whole body insulin resistance is evident following bed rest, skeletal muscle appears to be the primary target of insulin resistance (Stuart et al., 1988). Because glucose transport increases with muscle contraction, the absence of contraction may contribute to insulin resistance. Should neuropathic pain contribute to decreases in activity, insulin resistance and musculoskeletal changes may also develop.

Free Fatty Acids

Neuropathic pain may contribute to other metabolic changes, such as elevated free fatty acids (FFA). Much of the literature pertaining to fat and neuropathic pain has focused on dietary intake of polyunsaturated fatty acids alleviating pain (Prez et al., 2004; Prez, Ware, Chevalier, Gougeon, & Shir, 2005; Shapiro, 2003). Knitza, Clasen, and Fischer (1979) reported a 40% increase in plasma FFA following a strong painful stimulus. Acute electrical stimulation also elevates FFA in humans (Greisen, Grfte, Hansen, Jensen, & Vilstrup, 1999; Greisen et al., 2001), though this increase is transient. While not strongly supported in the literature, changes in FFA would broaden the characterization of metabolic effects of neuropathic pain. Limited available evidence supports only a transient increase in FFA following nociceptive pain. No studies have examined the effects of neuropathic pain on FFA over time.

Contrary to the relationship between neuropathic pain and FFA, there is ample evidence to support a relationship between insulin resistance and elevated plasma FFA levels (Kraegen, Cooney, Ye, & Furler, 2002), whether produced by artificial lipid/heparin infusion (Boden, Chen, Ruiz, White, & Rossetti, 1994; Boden, Chen, Rosner, & Barton, 1995; Dresner et al., 1999; Griffin et al., 1999; Homko, Cheung, & Boden, 2003; Kelley, Mokan, Simoneau, & Mandarino, 1993; Roden et al., 1996), fasting (Salgin et al., 2009), or as found in Type 2 Diabetic (T2D) adults (Kelley et al., 1996). Despite strong evidence to support a relationship between elevated FFA and insulin resistance, disagreement exists on the mechanisms contributing to this phenomenon.

Some postulate that elevated FFA contributes to insulin resistance via impaired glucose transport and/or phosphorylation in skeletal muscle (Belfort et al., 2005; Dresner

et al., 1999; Griffin et al., 1999; Kelley et al., 1996; McGarry, 2002). Others argue that altered glucose metabolism within skeletal muscle contributes to FFA induced insulin resistance (Boden et al., 1995; Kelley et al., 1993). Despite differences in the mechanisms contributing to FFA induced insulin resistance, many authors conclude FFA must be elevated for a significant period of time for impaired glucose uptake to occur (Boden et al., 1994; Boden et al., 1995; Dresner et al., 1999; Griffin et al., 1999; Homko et al., 2003; Kelley et al., 1993; Roden et al., 1996; Salgin et al., 2009). High levels of FFA may lead to the development of insulin resistance by overcoming compensatory increases in insulin- which is often the case in T2D. However, metabolic changes such as elevated FFA concentration and insulin resistance have not been investigated in neuropathic pain.

Activity

Individuals experiencing neuropathic pain may also experience decreased activity. Decreases in activity may support an indirect link between neuropathic pain and metabolic and musculoskeletal changes. Neuropathic pain can have a wide-ranging impact, including decreased ambulation, lack of exercise and mobility, and ultimately, deconditioning (Barkin, Barkin, & Barkin, 2005). Other studies have examined the effects of several painful conditions on activity.

Chronic back pain negatively affects activities of daily living (Rudy, Weiner, Lieber, Slaboda, & Boston, 2007). Pain, as a secondary complication to spinal cord injury, is also a barrier to physical activity participation (Tawashy, Eng, Lin, Tang, & Hung, 2009). Patients with chronic pain determined to be of neuropathic origin have more mobility and daily activity restrictions than those with non-neuropathic pain symptoms (Smith, Torrance, Bennett, & Lee, 2007; Toth, Lander, & Wiebe, 2009). De

Sousa and Frank (2007) found that pain limited activities such as running, walking, independently performing activities of daily living, and leisure activity. With multiple detriments to activity, inadequately treated neuropathic pain could negatively impact muscle function as well.

Treatment guidelines for neuropathic pain should seek to maintain normal function and activity, not only to relieve symptoms. Neuropathic pain that is inadequately treated may lead to an overall hypoactive lifestyle and contribute to a reduction in physical fitness, leading to further hypoactivity (van den Berg-Emons, Schasfoort, de Vos, Bussmann, & Stam, 2007). Changes in activity that result from neuropathic pain may not only indirectly contribute to metabolic changes, but changes in muscle composition and function as well.

Muscle Atrophy

Muscle mass is maintained when the balance of protein synthesis and degradation is achieved. During periods of inactivity, protein synthesis often decreases from disuse, contributing to muscle atrophy (Evans, 2010). Bed rest immobility is commonly used to simulate conditions of inactivity. Leg muscle mass decreases following periods of bed rest, largely due to decreased protein synthesis (Ferrando, Lane, Stuart, Davis-Street, & Wolfe, 1996; Shangraw, Stuart, Prince, Peters, & Wolfe, 1988; Stuart, Shangraw, Peters, & Wolfe, 1990; Symons, Sheffield-Moore, Chinkes, Ferrando, & Paddon-Jones, 2009). In animal models of limb immobilization, significant muscle atrophy develops within two weeks (Frimel, 2005; Han, Zhu, Ma, & Du, 2007). Following space flight and whole body suspension, rats develop muscle atrophy as well (Musacchia, Steffen, Fell, & Dombrowski, 1990). Muscle atrophy can also occur as the result of nerve damage.

Following sciatic nerve crush injury, Beehler, Sleph, Benmassaoud, and Grover (2006) reported atrophy of both the soleus (SOL) and extensor digitorum longus (EDL) muscles. Muscle atrophy is also present in animals with induced spinal cord injury (Hutchinson, Linderman, & Basso, 2001). Rats with chronic constriction injury (a preclinical model of neuropathic pain) develop atrophy of the SOL, Gastrocnemius (Choe, Kim, An, Lee, & Heitkemper, 2011; Daemen et al., 1998) and rectus femoris (Daemen et al., 1998). Daemen et al. attributed muscle atrophy to motor denervation of the sciatic (ligated) and femoral (non-ligated) nerves, rather than to hypokinesia. While this hypothesis is supported in the literature, muscle atrophy in neuropathic pain cannot be entirely attributed to denervation. In conditions of neuropathic pain, muscle atrophy may develop as a combination of decreased activity and/or nerve damage. More investigation into the mechanisms associated with muscle atrophy in neuropathic pain is needed.

Neuropathic Pain Treatment

Standard treatment of neuropathic pain with conventional analgesic therapy does not often result in effective relief. Newer therapies and algorithms have been developed that utilize a combination therapy approaches (Freynhagen & Bennett, 2009). Adequate treatment not only achieves pain relief, but may also improve activity and prevent metabolic and muscle function.

Because of the heterogeneous nature of neuropathic pain syndromes, no single therapy or treatment regimen can be extrapolated to every individual (Harden & Cohen, 2003; O'Connor & Dworkin, 2009). However, combination therapy may provide the best

opportunity for pain relief. Of the available treatments, three classes of medications are recommended:

> Antidepressants with both norepinephrine and serotonin reuptake inhibition (TCAs and selective serotonin and norepinephrine reuptake inhibitors [SSNRIs]), calcium channel and α_2-δ ligands (gabapentin and pregabalin), and topical lidocaine (lidocaine patch 5%). Opioids and tramadol were recommended as generally second-line treatments, except in certain specific clinical situations in which it was recommended that first-line use could be considered. A number of medications were considered third-line choices (O'Connor & Dworkin, 2009, pg. 24).

Opioid agonists have antinociceptive effects. In preclinical models of neuropathic pain, morphine attenuates mechanical allodynia (Erichsen, Hao, Xu, & Blackburn-Munro, 2005; Nakazato-Imasato & Kurebayashi, 2009) in a dose dependent fashion (De Vry, Kuhl, Franken-Kunkel, & Eckel, 2004; Hama & Borsook, 2005) after a single drug administration. In addition, single (De Vry et al., 2004) and repeated (Backonja, Miletic, & Miletic, 1995; Chu, Chen, Kang, & Tsai, 2000) morphine administration attenuates thermal hyperalgesia. However, major drawbacks of opioid use involve a dose dependent effect on motor function and somnolence (Erichsen et al., 2005), which may confound measures of activity. Response to opioids may also vary and no single drug/dose/route will be effective for each situation (Dellemijn, 1999).

Antiepileptics have also been studied in preclinical models of neuropathic pain. Sodium channel blockers, such as carbamazepine, have strong effects on thermal hyperalgesia (Hunter et al., 1997), but to a lesser degree on mechanical allodynia (De Vry et al., 2004). This class of drugs may also have sedative effects and changes in activity (De Vry et al., 2004; Hunter et al., 1997).

While the use of opioids and antiepileptics are effective drugs in the treatment of neuropathic pain, tricyclic antidepressants (TCAs) are considered first line treatment.

Amitriptyline, a commonly prescribed drug, increases tolerance to thermal stimulus (Bomholt, Mikkelsen, & Blackburn Munro, 2005; De Vry et al., 2004), but may or may not affect mechanical allodynia (Bomholt, Mikkelsen, & Blackburn Munro, 2005; De Vry et al., 2004; Garcia, del Valle, Escribano, Domenech, & Queralt, 2010). Importantly, repeated amitriptyline administration attenuates mechanical allodynia and thermal hyperalgesia for days after the last injection (Berrocoso et al., 2011; Yasuda et al., 1999).

Duloxetine hydrochloride, an SSNRI is approved for the treatment of diabetic peripheral neuropathic pain. In multiple double-blinded placebo controlled phase III clinical trials, duloxetine improved 24-hour average pain scores vs. placebo following 12 weeks of treatment, with efficacy beginning as early as one week post-treatment. Participants in these studies also used less over the counter analgesics while taking duloxetine (Goldstein, Lu, Detke, Lee, & Iyengar, 2005; Raskin et al., 2005). Significant antinociceptive effects of duloxetine have also been demonstrated in pre-clinical models of nociceptive and neuropathic pain.

In animals with neuropathic pain, duloxetine alleviates both thermal (Bomholt, Mikkelsen, & Blackburn Munro, 2005) and mechanical hyperalgesia (Bomholt, Mikkelsen, & Blackburn Munro, 2005; Piesla et al., 2009). While some have not found effects of duloxetine on mechanical hyperalgesia/allodynia (Pedersen & Blackburn Munro, 2006), methodological differences may account for this discrepancy. Others have reported that using a place escape/avoidance paradigm, duloxetine attenuates aversive behavior (Pedersen & Blackburn Munro, 2006), but does not affect general gait or motor performance hyperalgesia (Bomholt, Mikkelsen, & Blackburn Munro, 2005; Piesla et al., 2009).

The study of duloxetine in preclinical models of neuropathic pain has predominantly focused on the short-term antinociceptive effects. Larger doses (30 mg/kg) have an antinociceptive effect between 3-6 hours after administration (Bomholt, Mikkelsen, & Blackburn Munro, 2005; Iyengar, Webster, Hemrick Luecke, Xu, & Simmons, 2004). However, the effects of repeated duloxetine administration on neuropathic pain have not been investigated.

The study of neuropathic pain has produced increasing information regarding the anatomical and physiological changes within the nervous system. However, the characterization of metabolic, activity, and musculoskeletal changes following neuropathic pain remain unexplored. Moreover, the ability of duloxetine to prevent changes in activity and muscle atrophy by improving the processing of incoming nociceptive stimuli has not been investigated. The following methods were used to explore basic metabolic, activity, and musculoskeletal changes in a reliable model of neuropathic pain. In addition, the ability of duloxetine to improve the processing of nociceptive stimuli and prevent changes in activity and muscle atrophy was explored.

Methods

Neuropathic Pain

Chronic Constriction Injury (CCI) model of neuropathic pain

Various animal models have been developed and are useful to understand the pathology of neuropathic pain (Bomholt, Mikkelsen, & Blackburn-Munro, 2005; Dowdall, Robinson, & Meert, 2005; Dworkin et al., 2003). The CCI model of neuropathic pain was originally developed by Bennet and Xie (1998). This method employs tying four chromic gut ligatures around the sciatic nerve with sufficient tension to produce mild constriction (leg twitch). Bennet and Xie propose that the ligatures

retard but do not arrest epineural circulation which produces edema; but due to the ligatures, the nerve self-strangulates (Bennett & Xie, 1988; Bennett, 1993). Although other models have been utilized in the study of neuropathic pain (Dowdall et al., 2005; Jaggi, Jain, & Singh, 2009; Jarvis & Boyce-Rustay, 2009; Kluskov & Dubov, 2009), current experience with the CCI model, as well as the model's demonstrated efficacy to induce signs of neuropathic pain, support its use to investigate the metabolic, activity, and muscle-related changes associated with neuropathic pain.

The CCI model characterizes many of the signs of neuropathic pain often seen in humans. Behaviorally, animals develop altered mobility, limping, favoring the uninjured side while sitting or standing, and exaggerated hindlimb guarding and licking (Bennett & Xie, 1988; Jaggi et al., 2009). While humans do not necessarily 'groom' painful extremities or regions, the common occurrence of massaging or rubbing the affected area in response to epicritic pain (dull ache) is often seen clinically. Specifically related to neuropathic pain, the CCI model effectively induces mechanical and cold allodynia, thermal hyperalgesia, (Bennett & Xie, 1988; Dowdall et al., 2005; Jarvis & Boyce-Rustay, 2009; Tal & Bennett, 1994), and spontaneous pain (Bennett, 1993). The onset of the various signs ranges from the first day postoperatively through two months; with peak incidence occurring within the first seven (±3) days (Bennett & Xie, 1988; Dowdall et al., 2005; Jaggi et al., 2009; Jarvis & Boyce-Rustay, 2009).

Various cautions should be mentioned when utilizing this model of neuropathic pain. The first concern is the consistency with which the ligatures are placed on the sciatic nerve. Utilizing a single individual to reliably place ligatures of the same tension minimizes variability in outcome (Dowdall et al., 2005; Jaggi et al., 2009; Kluskov &

Dubov, 2009). Others have reported that the type of material used for the ligatures effects the animal's response to neuropathic pain testing (Clatworthy, Illich, Castro, & Walters, 1995; Maves, Pechman, Gebhart, & Meller, 1993). The type of suture material used may suggest a chemical/inflammatory component to the model, but overall, does not drastically affect the outcome, namely, the presence of neuropathic pain.

Variability in testing can also lead to aberrant results. The hot plate test, for example, can be difficult to standardize as the heat stimulus is not delivered in a controlled fashion (Jarvis & Boyce-Rustay, 2009). Other methods such as the cold water bath or pin-prick (for mechanical hyperalgesia) also introduce sources of variability. One method of testing for thermal hyperalgesia involves a heat lamp with a precise beam aimed at the dorsal surface of the foot. While contracture induced by the CCI surgery may inhibit the animal's ability to maintain proper positioning, heat lamp testing represents a reliable and valid method of testing for thermal hyperalgesia in rats (Yeomans & Proudfit, 1994). Unfortunately, no single model manifests the full range of mechanisms associated with neuropathic pain (Jeong & Holden, 2008).

Another model of neuropathic pain produces behavioral signs of evoked and ongoing pain with a similar time course yet has a distinct use from the CCI (Kim, Yoon, & Chung, 1997). Developed by Wall et al. (1979), the sciatic nerve is completely transected. This model produces signs of neuropathic pain similar to phantom limb pain following amputation. In contrast, the CCI mimics neuropathic pain with intact peripheral nerves. Allodynia and hyperalgesia are difficult to measure in deafferented limbs of the transection model due to complete lack of nociceptive transmission in the periphery (Kluskov & Dubov, 2009). Autotomy (self-mutilation) is also difficult to

analyze in the transection model, although Bennet (1993) is unsure if autotomy is in fact a painful response.

In addition to the CCI and nerve transection models, two other models are widely employed to investigate the pathological changes of neuropathic pain. These models involve "partial or complete ligation of the sciatic nerve or of the spinal L5-L6 nerves" and are similar to the CCI (Jeong & Holden, 2008, pg. 358). Seltzer, Dubner, and Shir (1990) developed the partial sciatic nerve ligation (PSL), which involves a single ligature transecting one-third of the sciatic nerve. The PSL produces nerve contusion (Kluskov & Dubov, 2009) and acts as a surrogate for human causalgia (Jaggi et al., 2009). Difficulty in standardizing ligation diameter is a major drawback of the PSL. Response to behavioral stimuli may vary between animals and diminish over time, resulting from decreases in tension, making the PSL model less suitable for long-term study (Dowdall et al., 2005).

A more invasive model involves transection of the L5-L6 (or L5 alone) spinal nerve (Kim & Chung, 1992). The spinal nerve ligation is accurate and repeatable, but requires extensive and invasive surgery, which may damage surrounding nervous tissue (Kluskov & Dubov, 2009). Comparatively, the CCI model is minimally invasive and reproducible with adequate personnel training (Bennett, 1993), making it and excellent model for the study of neuropathic pain.

Thermal Hyperalgesia

Thermal hyperalgesia is an increased sensitivity to heat and is one sign of neuropathic pain. Measurement of thermal hyperalgesia is often accomplished by use of a tail flick thermal analgesimeter (Ugo Basile, Italy) or similar method. Alternative

methods include the hot plate and tail immersion tests (Ibironke & Aji, 2011), but the current discussion will focus on the tail flick method.

Thermal hyperalgesia, as measured by the tail flick method, involves the projection of a radiant heat source on the hairy surface of the hindlimb (Jeong & Holden, 2009; Yeomans & Proudfit, 1994) or surface of the tail in a rodent (Isabel, Wright, & Henry, 1981). Radiant heat increases both surface and subsurface temperatures that contribute to increased nociceptor activity (Yeomans & Proudfit, 1994). A computer records the time (latency, in seconds) the animal takes to withdraw from the stimulus. Withdrawal latency decreases with increasing stimulus intensity (Berge, Garcia-Cabrera, & Hole, 1988; Isabel et al., 1981; Tjolsen, Lund, Berge, & Hole, 1989; Yeomans & Proudfit, 1994). In contrast, lower intensity stimuli produce longer latencies and increased variability (Ness, Jones, & Gebhart, 1987). Overall, the tail flick method accurately measures nociceptive threshold in normal animals, changes in nociceptive threshold produced by antinociceptive drugs (Yeomans & Proudfit, 1994), and is useful for long-term monitoring of pain threshold (Martinez-Gomez, Cruz, Salas, Hudson, & Pacheco, 1994). Several covariates, however, deserve mention.

Because radiant stimuli heat the skin of either the tail or foot, variations in skin temperature may produce variable results. Yeomans and Proudfit (1994) found that skin temperature (surface and subsurface) increases in a non-linear and linear (respectively) fashion after prolonged heating and concluded that nociceptive thresholds depend on the rate of noxious skin heating. Others have reported negative correlations between tail skin temperature and withdrawal latency (Berge et al., 1988; Tjolsen et al., 1989), stressing the importance of continual skin temperature monitoring (Han & Ren, 1991) and

consistent location and intensity measurement (Ness et al., 1987). Some have found no

relationship between skin temperature and withdrawal latency (Lichtman, Smith, &

Martin, 1993), but determined this after administering antinociceptive drugs. Other

important considerations when using the tail flick method include time of day, estrous

cycle, and stimulus location (Martinez-Gomez et al., 1994).

Mechanical Allodynia

Changes in mechanical sensitivity can be measured in multiple ways. The von

Frey test, considered the gold standard (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994),

is often used to assess mechanical allodynia in both humans (Tena et al., 2011) and

animals (Chaplan et al., 1994; Moller, Johansson, & Berge, 1998; Pitcher, Ritchie, &

Henry, 1999). While variability in testing is of chief concern, von Frey testing has

proven to be a reliable and valid method.

Specifically in rats, filaments of increasing diameter (stiffness) are applied to the

mid-plantar surface of the hindlimb, avoiding the less sensitive footpad (Chaplan et al.,

1994). Different techniques and protocols can be used to assess mechanical allodynia

(Dixon, 1980; Ren, 1999), but determining the withdrawal threshold of the hindlimb is

consistent across methods. Newer methods have also been validated against the von Frey

technique with varying reliability (Gabriel, Marcus, Walenkamp, & Joosten, 2009; Tena

et al., 2011; Vrinten & Hamers, 2003).

Variability between and within methods may be due to several factors. To enable

adequate access to the hindlimb, animals are often tested in cages with wire-mesh floors.

However, Pitcher et al. (1999) found that the surface on which rats with nerve injury are

tested can impact response to von Frey filaments. Humidity and temperature can also

affect the bending forces of the filaments, and may reduce their reliability over time (Vrinten & Hamers, 2003). Successive testing can also bias mechanical thresholds (Chaplan et al., 1994). Mechanical threshold determination can also vary greatly among pain models (Gabriel et al., 2009).

Despite the caveats to von Frey testing, this method has been validated in both humans (Tena et al., 2011) and rats (Chaplan et al., 1994). The internal validity of this method can be increased by performing all testing by a trained observer in a consistent manner, in a temperature and humidity controlled environment with adequate (24-48 hours) rest between testing sessions (S. Dorsey, 2011, personal communication).

Activity

Several methods can be used to measure activity in rats. Some methods require expensive, specialized equipment, such as photoelectric beam arrays (Sable Systems International). This method, however, is limited by photo beam sensitivity to specific movement. Alternatively, total spontaneous activity can be determined by measuring "downward force exerted by the rat's activity, recorded as a change in weight on a top-loading electronic balance" (Biesiadecki, Brand, Koch, & Britton, 1999, pg. 66). In this procedure, rats are housed in their home cage and placed on an electronic precision balance interfaced with a computer. Movements are recorded by the balance as changes in weight and transmitted to the computer at 10 Hz. Data are analyzed on-line using laboratory data acquisition software to derive the absolute value of the difference in weight between consecutive samples, and the one-second average of the absolute values are calculated. The measurement can be carried out for an extended period of time with minimum interruption of the home cage activity of the rat. This simple method has been

shown to be precise and reliable (Biesiadecki et al., 1999). A main limitation of this method is the inability to distinguish between specific movements. An alternative method for measuring activity involves videotaping and/or direct observation.

Animal activity can be classified by a human observer using a video recording or direct observation. While this method does introduce human variability, it has been shown to be a reliable method for activity measurement (Choe et al., 2011). While direct observation is less feasible for long-term assessment, it does provide a simple and cost-effective activity method. No literature has documented the reliability and validity of direct observation with other quantitative methods.

Metabolism

Free Fatty Acids

Multiple methods are currently utilized to determine the concentration of FFA (non-esterified fatty acids, [NEFA]), including titratic (Dole, 1956), colorimetric (Duncombe, 1964), chromatographic or liquid chromatography (MacGee & Allen, 1974) and enzymatic (Kiziltun & Akay, 1998; Mizuno, Toyosato, Yabumoto, Tanimizu, & Hirakawa, 1980; Shimizu, Inoue, Tani, & Yamada, 1979). Of these, the enzymatic method is an accurate method for determining the presence of FFA with minimal interference (Okabe, Uji, Nagashima, & Noma, 1980). It relies on the breakdown of long chain, non-esterified fatty acids into Acyl-CoA and key derivatives: hydrogen peroxide, inorganic phosphate, and AMP.

The NEFA-HR (2) is an enzymatic, colorimetric procedure developed by Wako Chemicals USA, Inc. Non-esterified fatty acids in serum, when treated with acyl-CoA synthetase in the presence of ATP and CoA, form the thiol esters of CoA known as acyl

CoA along with the byproducts AMP and pyro-phosphate. In the second portion of the procedure, the acyl CoA is oxidized by added acyl-CoA oxidase to produce hydrogen peroxide which in the presence of added peroxidase allows for the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-alanine with 4-aminoantipyrine to form a purple colored product which can be determined from the optical density measured at 550 nm. Sensitivity of the assay is 0.10 mEq/L. Inter-assay variabilities are 5.2% at 1.8mEq/L and 4.4% at 0.97mEq/L. This is beneficial when determining total FFA content. However, it does not permit the analysis of specific free fatty acids (palmitic, oleic, etc.).

The enzymatic method has established reliability and validity. It is highly correlated with gas-liquid chromatography ($r = 0.992$), and the colorimetric method ($r = 0.976$-0.98) (Mulder et al., 1983; Okabe et al., 1980). The coefficient of variation for this method is also very low, both intra- and interassay (≤ 2-5%; Mizuno et al., 1980; Mulder, Schouten, & Popp-Snijders, 1983; Okabe et al., 1980)). The sensitivity of this method has also been reported, with samples as small as 4 μl achieving accurate results (Jeevanandam, Hsu, Ramias, & Schiller, 1989). Others have found a linear detection relationship ranging from 0.01-4.0 mEq/L with a minimum detectable level of 0.0014 mEq/L (WAKO Industries). While the enzymatic method has many advantages, several cautions should also be mentioned.

The enzymatic method has several advantages over other methods. First, there is no need for an extraction step compared to chromatographic and colorimetric methods, making the enzymatic method simpler, less time consuming, and eliminating the need for highly skilled personnel. There is also minimal equipment to purchase and small sample

volumes can be used with consistent readings, even when run up to four weeks after collection (Mulder, 1983; WAKO Industries). Cost savings may be offset with the use of expensive reagents however (1983). Incongruence does exist concerning sample collection and interference that may skew FFA analysis.

When collecting serum, various circumstances may alter the precision and consistency of FFA determination. Controversy exists pertaining to the use of heparinized collection tubes. The rationale surrounding the use of heparin is the potential activation of lipoprotein lipase (LPL) in the sample, thus resulting in an overestimation of FFA (Visser, Zuurbier, van Wezel, van der Vusse, & Hoek, 2004). Some product specifications recommend non-hepranized tubes (WAKO Industries), while others claim that heparin does not interfere with the enzymatic method (Jeevanandam et al., 1989; Okabe et al., 1980). Jeevanadam et al. (1989) reported that FFA concentration was lower when heparinized tubes were used; but the tubes were also chilled, which may have arrested or at least attenuated LPL activity.

Another potential concern with the enzymatic method is possible interference by other substances. While some have reported a lack of interference (Kiziltun & Akay, 1998; Shimizu et al., 1979), ascorbic acid, albumin, bilirubin, and catecholamines have been found to interfere with FFA results (Carr, Humphreys, & Frayn, 1995; Okabe et al., 1980; Shimizu, Tani, Yamada, Tabata, & Murachi, 1980) but only at higher concentrations. Despite these potential confounding variables, the enzymatic method is an excellent method for determining FFA concentration.

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Glucose

Monitoring blood glucose is an important strategy in the treatment of diabetes. Various portable glucometers allow patients to monitor their blood glucose between health care provider visits. Despite small deviation from laboratory reference values (<10%), variation does exist between manufacturers (Solnica, Naskalski, & Sieradzki, 2003). Laboratory glucose assays, however, provide very reliable results.

Numerous manufacturers produce chemistry analyzers and reagent kits to conduct glucose assays. Plasma or serum can be used for glucose analysis. In the hexokinase method, glucose is phosphorylated by hexokinase (HK) in the presence of adenosine triphosphate (ATP) and magnesium to form glucose-6-phosphate (G-6-P) and adenosine diphosphate (ADP). G-6-P is then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) in the presence of nicotinamide adenine dinucleotide (NAD) producing 6-phosphogluconate and NADH. The formation of NADH causes an increase in absorbance at 340 nm which is directly proportional to the concentration of glucose in the sample.

The hexokinase method is highly correlated with the glucose oxidase method ($r^2 = 0.99$) and is the method recommended by the CDC (Jia & Zhang, 2010). Intra-assay variability is 2% at 84 and 283 mg/dl. Inter-assay variability is 4.1% at 92 mg/dL and 3.2% at 310 mg/dL. While the presence of ascorbic and uric acids do not affect results (Purcell, Behenna, & Walsh, 1979), others have reported interference with the sulfonylurea drug gliclazide (Nakashima et al., 1995). Overall, the hexokinase method for determining glucose concentration is valid and reliable.

Insulin

Various methods are available to measure insulin in serum and plasma. The most commonly used are chromatographic and immunoassay techniques, including radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA). These methods have wide variability in precision and cross-reactivity (Marcovina et al., 2007), but are well correlated ($r^2 > 0.98$) with each other (Ackermans et al., 2008).

Chromatographic methods are tedious, requiring large sample volumes, and preparation. Increasingly popular are the ELISA reagent kits, which involve primary and secondary antibody binding, with a conjugated enzyme bound to the secondary antibody. This enzyme can be assayed with a colored fluorescent or chemilimunescent substance for quantification (Golla & Seethala, 2004). While ELISAs are reliable, several intermediate wash steps introduce potential sources of error (Golla & Seethala, 2004). Alternatively, RIA utilizes two monoclonal antibodies which have led to improvements in specificity and sensitivity (Golla & Seethala, 2004).

Radioimmunoassay involves a radio-labeled isotope bound to the target antigen. This radio-labeled antigen is also bound to a specific antibody. When added to a sample, the target antigen displaces the radio-labeled antigen and binds to the antibody. A gamma counter is then used to measure the unbound antigen (Millipore). Several reagent kits are commercially available that involve an insulin tracer, rat insulin standard, and various primary and secondary antibodies. This method has a limit of sensitivity of 1 µU/ml and acceptable inter-assay and intra-assay variability at 30.5 µU/ml (11.2% and 3.2%, respectively). This method involves higher cost as well as hazards involved with

radioactive material and waste (Daijo & Sportsman, 1999; Golla & Seethala, 2004), but provides accurate and reliable results overall.

Insulin Resistance

Insulin resistance refers to the body's inability to respond to and use the insulin it produces (American Diabetes Association, 2010), resulting in elevated glucose levels, impaired hepatic suppression of glucose production (hepatic insulin resistance), disinhibition of adipocyte lipolysis, and impaired response to insulin in skeletal muscle and glucose uptake (peripheral insulin resistance) (Pham, Utzschneider, & de Boer, 2011). As insulin resistance develops, pancreatic β-cells increase insulin release to maintain glucose levels within normal range. Hyperglycemia and eventually T2D develop when pancreatic β-cell function becomes insufficient (Pham et al., 2011). Insulin resistance can be measured by both dynamic and static methods, of which a detailed description is presented in Pham et al. (2011).

Static insulin resistance measurement occurs after a fasting period in which insulin sensitivity is determined by the regulation of hepatic glucose production, reflecting hepatic insulin resistance (Pham et al., 2011). The homeostasis model assessment of insulin resistance (HOMA-IR) and the quantitative insulin sensitivity check index rely on fasting blood samples and reflect hepatic insulin sensitivity. These values are used to calculate insulin resistance using a standard formula: [fasting plasma insulin (μUnits/ml) x fasting plasma glucose (mmol/l)]/22.5 (Matthews et al., 1985; Wallace, Levy, & Matthews, 2004). While the HOMA-IR has been validated in humans (Bonora et al., 2000; Mather et al., 2001; Sarafidis et al., 2007; Yokoyama et al., 2004) and rodents (Cacho, Sevillano, de Castro, Herrera, & Ramos, 2008; Lee et al., 2008;

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Muniyappa et al., 2009; Tran et al., 2003), it does not reflect peripheral insulin resistance as a dynamic method would.

Dynamic testing reflects peripheral insulin resistance because the body disposes of glucose in skeletal muscle in response to a glucose or insulin challenge. What is considered to be the 'gold' standard for measuring insulin sensitivity is the hyperinsulinemic euglycemic clamp technique (DeFronzo, Tobin, & Andres, 1979). During the procedure, high dose insulin and glucose infusions maintain glucose concentration at a steady level and provide a measure of whole body insulin sensitivity (Pham et al., 2011). However, this method is costly, complex, invasive, and labor intensive (Groop, 1999; Pham et al., 2011). Other dynamic methods use a glucose challenge (intravenous or oral ingestion) with frequent sampling of blood glucose for up to four hours and are simple yet valid methods to calculate insulin sensitivity (Frequently Sampled IntraVenous Glucose Tolerance Test [FSIVGTT], Bergman, Ider, Bowden, & Cobelli, 1979; and Oral Glucose Tolerance Test [OGTT]. Alternatively, an insulin challenge may be used.

The insulin tolerance test (ITT) is a valid measure for insulin resistance. The procedure is similar to glucose challenge methods except that insulin is administered in place of glucose. Blood glucose is frequently sampled regularly over a predetermined period of time. In states of insulin sensitivity, blood glucose declines; conversely, blood glucose remains elevated in conditions of insulin resistance. Although less commonly used, this method has been validated with the hyperinsulinemic euglycemic clamp in rats (Ai et al., 2005). The main limitations of this method include improper absorption of insulin and errors in blood glucose measurement.

Muscle Atrophy

Numerous methods are used to measure muscle atrophy. Ubiquitin ligase activity – involved in protein breakdown – measures muscle atrophy on a cellular level (Clavel, Coldefy, Kurkdjiam, Salles, Margaritis, & Derijard, 2006). Conversely, dynamometers quantify whole-limb skeletal muscle strength in humans, which is proportional to cross-sectional area (Kasper, Talbot, & Gaines, 2002). Because muscle mass determines function (Seymour et al., 2009), smaller muscles have decreased functional ability. In humans, this can be assessed non-invasively (ultrasound, computerized tomography). In animal studies, muscle dissection and weight are acceptable surrogates for muscle atrophy.

Conclusion

Neuropathic pain is a major health problem. Increasing knowledge regarding the underlying mechanisms associated with neuropathic pain has led to advances in treatment. However, the effects of neuropathic pain on muscle function, metabolism, and activity have received less attention. Despite the challenges of pain management, several therapies have proven effective in the treatment of neuropathic pain. What is unknown, however, is the ability for drugs to prevent the development of activity changes and muscle atrophy by improving the processing of nociceptive input.

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CHAPTER 3

Neuropathic Pain Contributes to Decreased Activity and Skeletal Muscle Atrophy, But Not to Insulin Resistance

Abstract

While the study of neuropathic pain has focused on changes within the nervous system, little research has described systemic changes that may accompany neuropathic pain. In the present study, metabolic, activity and musculoskeletal changes were described using an established animal model of neuropathic pain, the chronic constriction injury (CCI). Male Sprague-Dawley rats were used in a pre-posttest quasiexperimental study. Thirteen rats received CCI, while age and weight matched rats served as sham surgery controls (SHAM; n = 5). Thermal testing verified the presence of neuropathic pain. Fasting glucose, insulin, and free fatty acids (FFA) were measured using standard techniques, and insulin tolerance testing (ITT) was conducted prior to (BASELINE) and 2 weeks after surgery. Data were also collected 1 week (n = 8) and 6 weeks (n = 5) in 2 separate CCI sub-cohorts. Spontaneous cage activity was measured gravimetrically prior to and following CCI (n = 4). Animals were euthanized and skeletal muscle was dissected and weighed to determine muscle atrophy.

Shorter foot withdrawal latency (sec) of the injured hindlimb (FWL-INJ) confirmed the presence of thermal hyperalgesia, a sign of neuropathic pain. Weight and FFA increased in both CCI and SHAM rats. Muscles of the injured hindlimb weighed significantly less than uninjured hindlimb muscles in CCI rats 2 and 6 weeks after

surgery. In addition, CCI rats had smaller injured hindlimb muscles that SHAM.

Spontaneous activity decreased following CCI ligation, but only trended towards

significance (p = 0.06). Fasting glucose, insulin, and insulin resistance did not change

following CCI ligation (p > 0.05). Overall, insulin resistance in this strain of rat with

neuropathic pain did not develop. Increases in FFA were likely due to natural growth and

not a consequence of neuropathic pain. Muscle atrophy observed in CCI rats may have

resulted from decreases in activity.

Key words: Neuropathic pain, thermal hyperalgesia, insulin resistance, spontaneous

activity, muscle atrophy.

Introduction

Metabolic changes are often seen in painful conditions such as trauma, surgery, and burns. These conditions are characterized by increased sympathetic activity and free fatty acid mobilization, both of which associate with insulin resistance, the body's inability to respond to and use the insulin it produces. These painful conditions may also lead to the development of chronic, neuropathic pain. The study of neuropathic pain has predominantly focused on changes within the nervous system. The effect of neuropathic pain, a common presenting symptom in Type 2 Diabetes (T2D), on metabolic, activity, and musculoskeletal changes remains unexplored.

Chronic pain, which is often of neuropathic origin, affects an estimated 76.2 million people (U.S. Department of Health and Human Services, National Center for Health Statistics, 2006). Patients with neuropathic pain visit clinicians more frequently, have poorer health related quality of life, and report impaired sleep, mobility, and activities of daily living (Hans, Masquelier, & De Cock, 2007; Smith, Torrance, Bennett, & Lee, 2007; Taylor, 2006), even with available treatments (O'Connor & Dworkin, 2009). In addition, neuropathic pain is often under-recognized clinically due in part to variations in patients' symptom presentation and response to treatment (Berger, Dukes, & Oster, 2004; Harden & Cohen, 2003). Despite the significant challenges related to the diagnosis and treatment of neuropathic pain, much is known regarding the pathophysiological changes associated with neuropathic pain.

Defined as, "Pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" (Treede et al., 2008, pg. 1631), neuropathic pain encompasses an array of pathological changes arising from the peripheral and central nervous systems

(PNS and CNS, respectively). Mechanical trauma, metabolic disease, neurotoxic chemicals, or infection can lead to neuropathic pain in the PNS (Costigan, Scholz, & Woolf, 2009). Other events such as spinal cord injury or multiple sclerosis can cause central neuropathic pain (Costigan et al., 2009). These traumatic events can lead to severe, long-lasting pain that can lead to the development of neuropathic pain through alterations in the processing of incoming noxious stimuli by the CNS.

In neuropathic pain, the transmission of noxious stimuli via specialized sensory neurons (C-fibers; Aδ-fibers) called primary afferent nociceptors is altered, increasing the responsiveness of nociceptors to normal afferent input (Baron, 2009). As a result of increased nociceptor sensitivity, neuropathic pain is characterized by several manifestations, including hyperalgesia, allodynia, and spontaneous pain (Baron, 2009; Campbell & Meyer, 2006; Taylor, 2001; Woolf & Mannion, 1999). Thermal hyperalgesia is an exaggerated response to a painful heat stimulus that can develop in as little as two weeks in an animal model (Bennet and Xie, 1988) and can last for years following the initial injury in humans (Niederberger, Khlein, & Geisslinger, 2008).

The study of neuropathic pain has predominantly focused on changes within the PNS and CNS. Little attention has been devoted to characterizing the metabolic, activity, and musculoskeletal changes that occur in neuropathic pain, although some studies have shown a relationship between acute, nociceptive pain and changes in metabolism.

Nociceptive pain contributes to changes in metabolic associates: catecholamines, FFA, and insulin resistance. Individuals with severe pain have higher fasting blood glucose (Nilsson, Kandell-Colln, & Anderson, 1979). In addition, Greisen et al., (2001) found that nociceptive pain in the form of electrical stimulation increases insulin

resistance, catecholamines, and FFA in humans. These authors concluded that nociceptive pain *per se* contributes to increases in insulin resistance and other metabolic indicators (2001), suggestive of a possible correlation between nociceptive pain and changes in metabolism. In contrast to nociceptive pain, neuropathic pain can be more severe, debilitating, and long-lasting, and the effects of neuropathic pain on metabolic changes, specifically insulin resistance, have yet to be explored.

Insulin resistance, which precedes T2D, represents a growing health problem in the United States. An estimated 24 million people in the U.S. have diabetes (U.S. Department of Health and Human Services [HHS], Centers for Disease Control and Prevention [CDC], 2008). Diabetes is the most common cause of peripheral neuropathy and neuropathic pain (Smith & Singleton, 2008), with approximately 60-70% of diabetics having mild to severe forms of nervous system damage, contributing to impaired sensation or pain in the feet or hands (HHS, CDC, 2008). Diabetic neuropathy is often studied as a consequence of insulin resistance and T2D. However, little is known about the effects of neuropathic pain on metabolic changes, specifically insulin resistance.

Insulin resistance is present in many painful, traumatic conditions that may ultimately result in neuropathic pain. Insulin resistance occurs in patients with trauma (Black, Brooks, Bessey, Wolfe, & Wilmore, 1982), surgery (Thorell, Efendic, Gutniak, Hggmark, & Ljungqvist, 1993; Thorell et al., 1999), and burns (Wolfe, Durkot, Allsop, & Burke, 1979). However, these studies could not isolate pain as the primary antecedent for insulin resistance from among potentially confounding variables such as inflammation, FFA mobilization, or increased adrenergic response, all of which are

associated with insulin resistance. Neuropathic pain may also contribute to metabolic changes through changes in activity.

Individuals with neuropathic pain report limitations in activities of daily living and mobility (Eggermont, Bean, Guralnik, & Leveille, 2009; Rudy, Weiner, Lieber, Slaboda, & Boston, 2007; Smith & Singleton, 2008; Toth, Lander, & Wiebe, 2009). In turn, decreased activity contributes to insulin resistance and altered glucose transport (Alibegovic et al., 2009; Hamburg et al., 2007; Henriksen & Tischler, 1988; Ploug, Ohkuwa, Handberg, Vissing, & Galbo, 1995). Decreases in activity, a potential consequence of neuropathic pain, may also have significant effects on skeletal muscle structure and function (Henriksen & Tischler, 1988; Ploug et al., 1995).

Neuropathic pain is often studied in the context of chronically elevated blood glucose. The pathophysiological mechanisms involved in neuropathic pain have been well documented in the literature. In addition, conditions of neuropathic pain, such as diabetic neuropathy and fibromyalgia, have received similar attention. However, metabolic responses, physical activity, and musculoskeletal alterations following neuropathic pain injury have yet to be explored. The purpose of this study was to describe basic metabolic, physical activity, and musculoskeletal changes in rats with neuropathic pain induced by ligation of the sciatic nerve.

Methods

Materials

Human recombinant insulin was purchased from the University of Michigan Health System pharmacy (Ann Arbor, MI).

Animals

Male Sprague Dawley rats (225-250g; Charles River) were used in a pre-posttest quasiexperimental design. Rats were housed in pairs in a humidity and temperature controlled room with 12:12 light/dark cycle (lights on at 0700). All procedures were approved by the University Committee for the Use and Care of Animals and meet Association for Assessment and Accreditation of Laboratory Animal Care standards.

Neuropathic Pain

The CCI was used to induce neuropathic injury, as described in Bennet and Xie (1988). Briefly, under isoflurane anesthesia, an incision was made through the left thigh exposing the common sciatic nerve. Four chromic gut ligatures were tied around the nerve proximal to the trifurcation with sufficient tension to produce a leg twitch. The wound was closed with sutures and sterile skin clips. A single injection of buprenorphine (0.05 mg/kg) was given immediately after CCI to minimize post-operative procedural pain. The CCI was performed 1 day after BASELINE metabolic measurements to minimize stress from multiple procedures, hypoglycemia, hypovolemia, and potential anesthesia-analgesia interactions (n = 13). Age and weight matched SHAM rats received identical surgery without sciatic nerve ligation (n = 5).

The characterization of neuropathic pain in the CCI model is well known. The onset of thermal hyperalgesia, a sign of neuropathic pain, ranges from the first day postoperatively through two months, with peak incidence occurring within the first seven (±3) days (Bennett & Xie, 1988; Dowdall, Robinson, & Meert, 2005; Jaggi, Jain, & Singh, 2009; Jarvis & Boyce-Rustay, 2009). In an attempt to align potential changes in metabolism and activity with the onset of neuropathic pain, metabolic outcomes were

measured on all rats at BASELINE and 2 weeks after surgery. After preliminary

analysis, CCI rats were not exhibiting changes in metabolic outcomes 2 weeks after

surgery (n = 8). Metabolic outcomes were again measured 6 weeks after surgery in a

separate CCI sub-cohort (n = 5) to determine if extending the duration of neuropathic

pain affected metabolic outcomes. A different sub-cohort of CCI rats also had metabolic

outcome measurement 1 week after surgery (n = 8). Age and weight matched SHAM rats

had metabolic outcome measurements at BASELINE and 1 and 6 weeks after surgery.

Thermal Hyperalgesic Testing

To confirm the presence of neuropathic pain, changes in nociceptive threshold

(pain sensitivity) were measured using the tail flick analgesiometer (Ugo Basile,

Collegevile, PA). The foot withdrawal latency (FWL, sec) measures the reflexive

withdrawal of the hind foot in response to noxious thermal infrared heat stimulus. Under

light sodium pentobarbital anesthesia (35 mg/kg, Intraperitoneal [IP]), thermal

hyperalgesic testing was conducted one day after final blood draw and ITT, 2 or 6 weeks

after surgery. Testing occurred every 5 min for 45 min. Shorter latencies demonstrate

increased sensitivity to heat, or thermal hyperalgesia, a sign of neuropathic pain.

Fasting Protocol

Metabolic outcomes were measured following a 6 hour fast. Food was removed

daily at 0800 and returned at 1400. To ensure adequate hydration, water was not

removed. During non-fasting hours, animals had ad- libitum access to standard rat chow

and water. The fasting protocol began 5 days prior to BASELINE metabolic outcome

measurement to acclimate animals and minimize potential effects of food restriction on

stress hormones. The fasting protocol continued for 2 weeks after surgery. Rats tested 6

weeks after surgery were fasted for the initial 2 weeks after surgery and 1 week prior to the final metabolic outcome measurement (6 weeks after surgery).

Metabolic Outcomes

Following a 6 hour fast, rats had BASELINE fasting blood samples taken under isoflurane anesthesia to determine levels of glucose, insulin, and FFA, which served as metabolic indicators. Fasting blood was also drawn 2 weeks after surgery. In addition, 2 separate CCI sub-cohorts had blood drawn 1 and 6 weeks after surgery. SHAM rats had fasting blood draws at BASELINE and 2 and 6 weeks after surgery. Approximately 1 ml of blood was drawn from the saphenous vein into EDTA coated tubes and centrifuged at 3500 rpm for 15 min at 4°C. Plasma was used for estimation of insulin and FFA using standard radioimmunoassay and colorimetric techniques, respectively, at the Michigan Diabetes Research and Training Center chemistry lab. Glucose was estimated using glucose oxidase method (Home Diagnostics, Liverpool, NY).

Insulin tolerance testing (ITT) was conducted immediately after each fasting blood draw. After obtaining an initial blood glucose, insulin (1.5 Units/kg, IP) was given and blood samples were taken via tail prick every 5 min for 30 min using a glucometer. Area above the curve was calculated to estimate insulin resistance. Decreases in area above the curve/sustained elevation of blood glucose during ITT indicate increased insulin resistance.

Activity Monitoring

Home cage activity was determined in a sub-group of individually housed rats (n=4) prior to and following CCI. Spontaneous home cage activity was measured gravimetrically as described previously (Biesiadecki, Brand, Koch, & Britton, 1999).

The rat housed in its home cage was placed on an electronic precision balance (Sartorius BP6100) interfaced with a computer. Movements were recorded by the balance as changes in weight and transmitted to the computer at 10 Hz. Data were analyzed on-line using a laboratory data acquisition software (DASYlab 9.0) to derive the absolute value of the difference in weight between consecutive samples, and the one-second average of the absolute values was calculated. The measurement was carried out continuously for up to 48 hours with a minimum of interruption of the home cage activity of the rat.

Activity monitoring required repeated transport of animals between housing facilities. Repeated measures ANOVA was conducted between animals that did and did not receive activity monitoring. There were no significant differences in glucose, insulin, FFA and CCI rat data were combined and used in subsequent analysis for these measures.

Muscle Atrophy

Forty eight hours after thermal hyperalgesic testing, rats were euthanized and the extensor digitorum longus (EDL) and soleus (SOL) muscles were removed, weighed, frozen in liquid nitrogen, and stored at -70°C. These muscles were selected based on their anatomical and physiological properties: SOL is involved in posture, while the EDL is a non-load bearing muscle. Differences between these muscles may reflect differential effects of neuropathic pain on muscle atrophy and activity.

Statistical Analysis

Data are presented as Mean ± SEM. Two-way (Group x Time) repeated measures ANOVA with Bonferroni post hoc tests was used to compare means. Paired t-test was used to compare spontaneous activity and the effect of 4 additional weeks of neuropathic pain on metabolic outcomes.

Results

Rats with CCI-induced neuropathic pain exhibited thermal hyperalgesia. Six weeks after surgery, FWL was significantly shorter in CCI rats compared with SHAM rats (5.04 ± 0.99 vs. 8.17 ± 0.4 sec; $F_{(1,8)} = 29.5$, $p < 0.05$; see Figure 3.1) indicating increased sensitivity to thermal stimulus. In addition, CCI rats tested 6 weeks ($n = 5$) after surgery had longer FWL than CCI rats tested 2 weeks after surgery, but this trend was not significant (5.04 ± 0.99 vs. 4.02 ± 0.19 sec, $p > 0.05$).
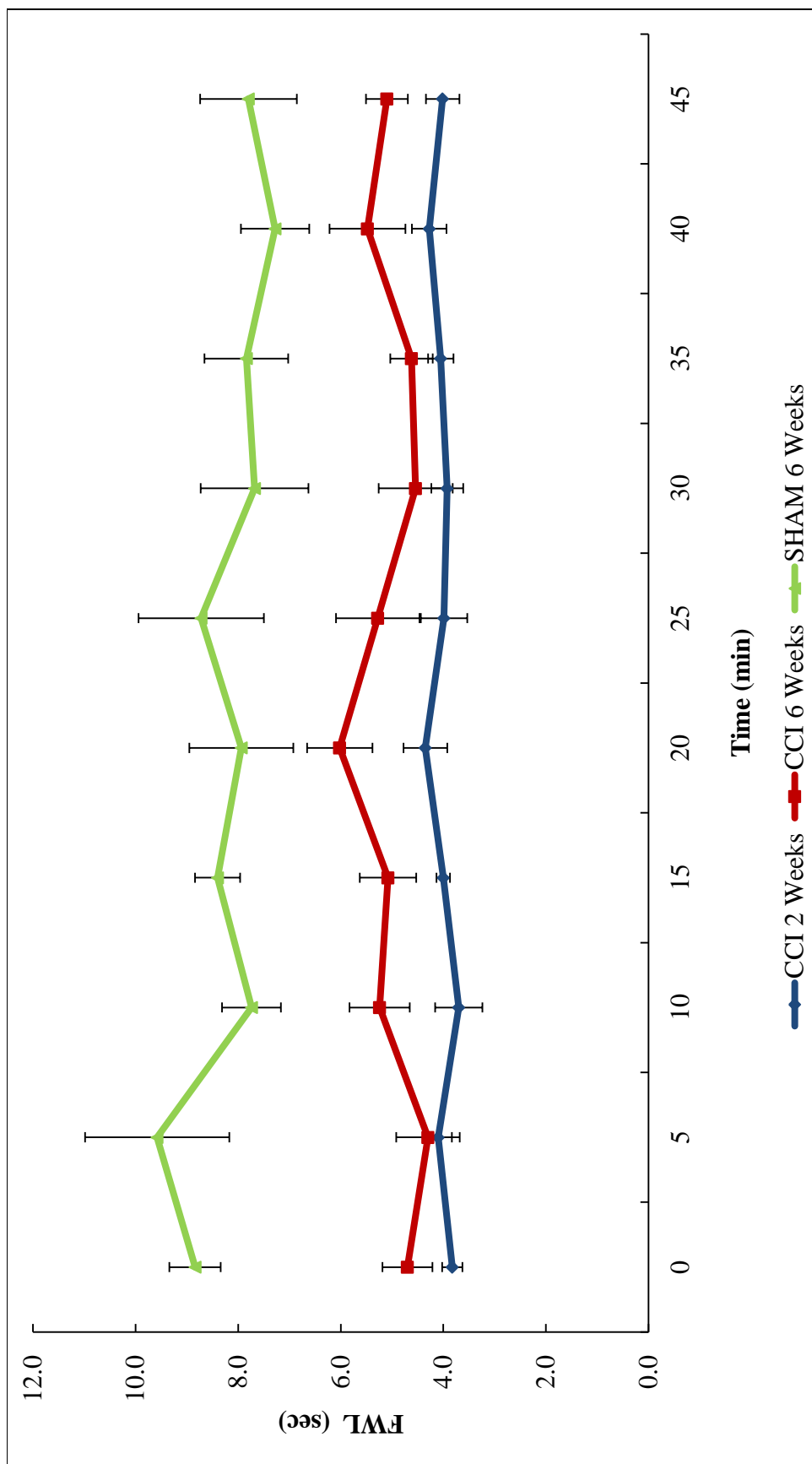
Figure 3.1. Rats with CCI-induced neuropathic pain exhibited thermal hyperalgesia. Data are Mean ± SEM for each group (n = 5-8). Note: 2 Weeks or 6 Weeks: thermal hyperalgesic testing conducted 2 or 6 weeks after surgery, respectively.

Weight increased significantly in CCI and SHAM rats after surgery ($F_{(3,30)}$ = 496.29, $p < 0.05$; see Figure 3.2). CCI rats that were monitored for activity (Activity CCI; $n = 4$) gained weight while waiting to be transported to separate housing and were significantly heavier at BASELINE ($F_{(2,10)} = 96.4$, $p < 0.05$). All other CCI rats (Non-Activity CCI) gained weight at a similar rate ($n = 9$). Weight was not significantly different between CCI and SHAM rats 6 weeks after surgery ($p > 0.05$).
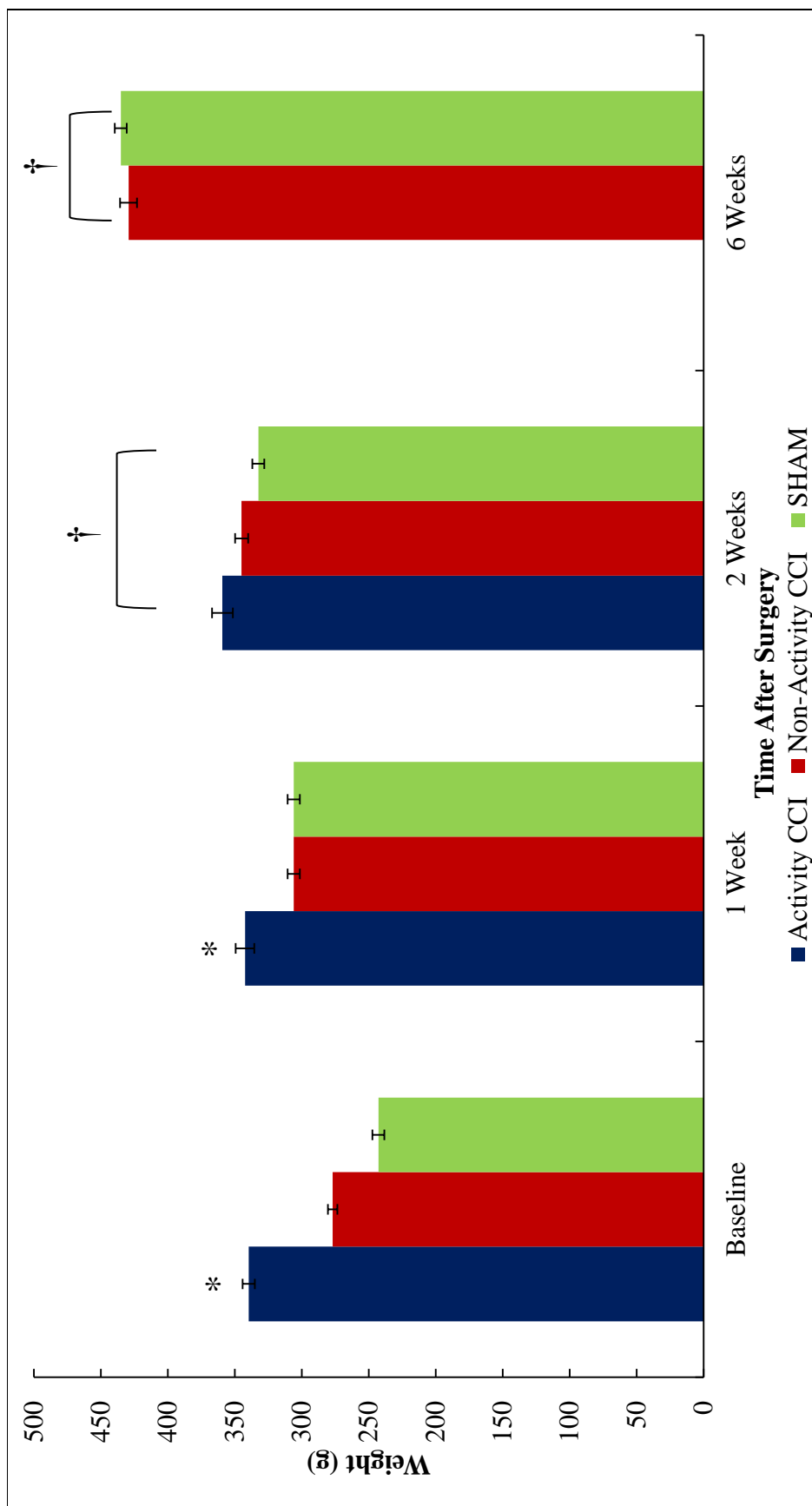
Figure 3.2. Weight gain is not altered by CCI or SHAM surgery in male, Sprague-Dawley rats. Data are Mean ± SEM for each group (n = 4-9). *p < 0.05 vs. Non-Activity CCI and SHAM. †p < 0.05 vs. Baseline. Note: Activity CCI rats euthanized 2 weeks after surgery.

Fasting values of FFA increased significantly following CCI ($F(1,10) = 18.6$, $p < 0.05$). FFA significantly increased between 2 and 6 weeks after CCI surgery ($t(4) = -3.35$, $p < 0.05$). FFA were not significantly different between CCI and SHAM rats at any time point ($p > 0.05$; see Figure 3.3). Weight did not significantly correlate with FFA levels at any time in CCI or SHAM rats ($p > 0.05$).
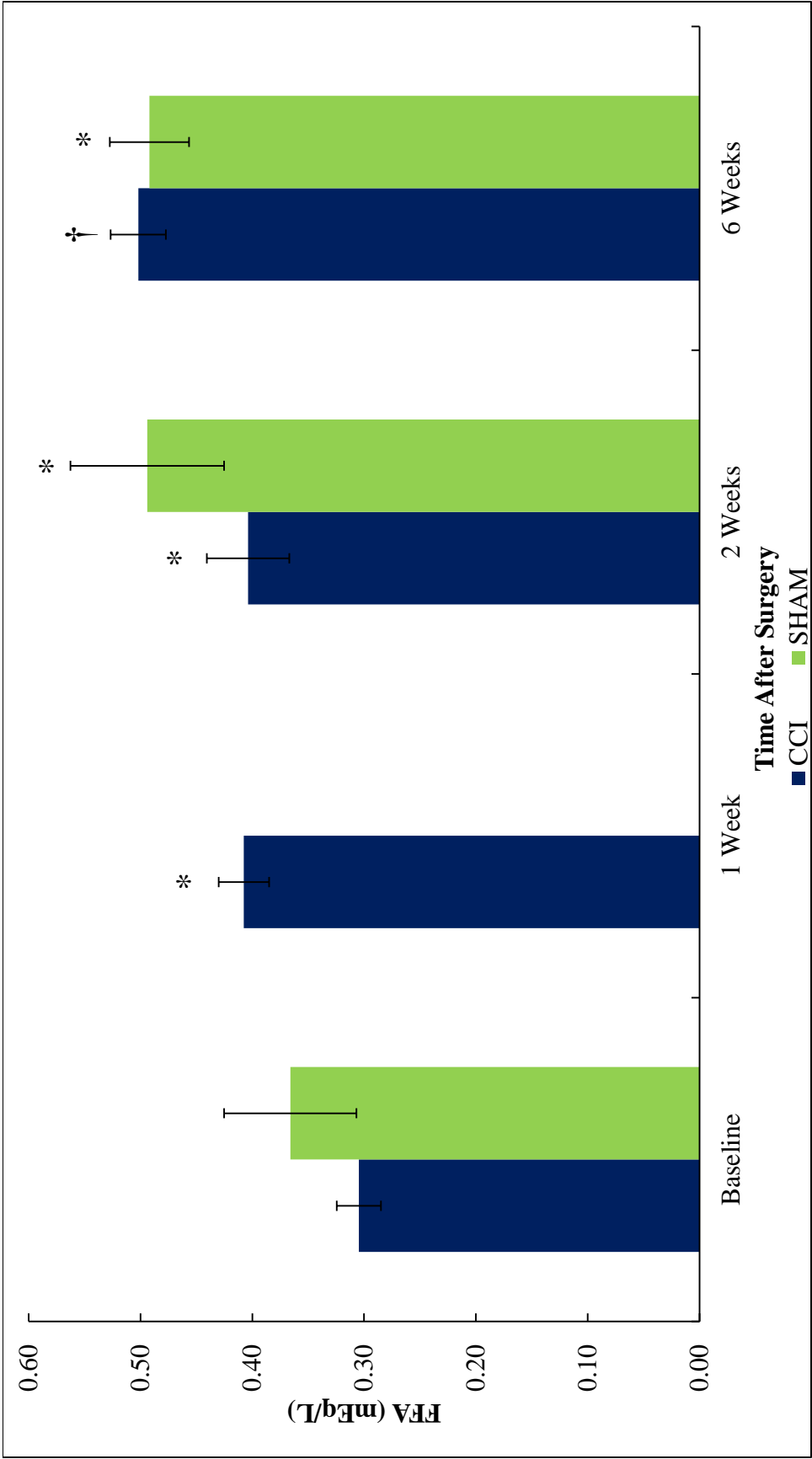
Figure 3.3. FFA increase in CCI and SHAM rats after surgery. Data are Mean ± SEM for each group (n = 5-8). *p < 0.05 vs. Baseline. †p < 0.05 vs. CCI 2 Weeks after surgery. Note: SHAM data were collected at Baseline, and 2 and 6 weeks after surgery.

Fasting insulin increased in CCI rats 6 weeks after surgery, but this trend was not significant (p = 0.1). Fasting insulin significantly decreased in SHAM rats 6 weeks after surgery (20.8 ± 1.7 vs. 11.7 ± 1.1 uU/ml; $F(1,4) = 15.8$; $p < 0.05$). Six weeks after surgery, fasting insulin was not significantly different between CCI and SHAM rats ($p > 0.05$, see Figure 3.4).

Figure 3.4. Fasting insulin increases in CCI rats after surgery but this trend was not statistically significant (p = 0.1). Fasting insulin decreases in SHAM rats after surgery. Data are Mean ± SEM (n = 5 per group). *p < 0.05 vs. SHAM Baseline.
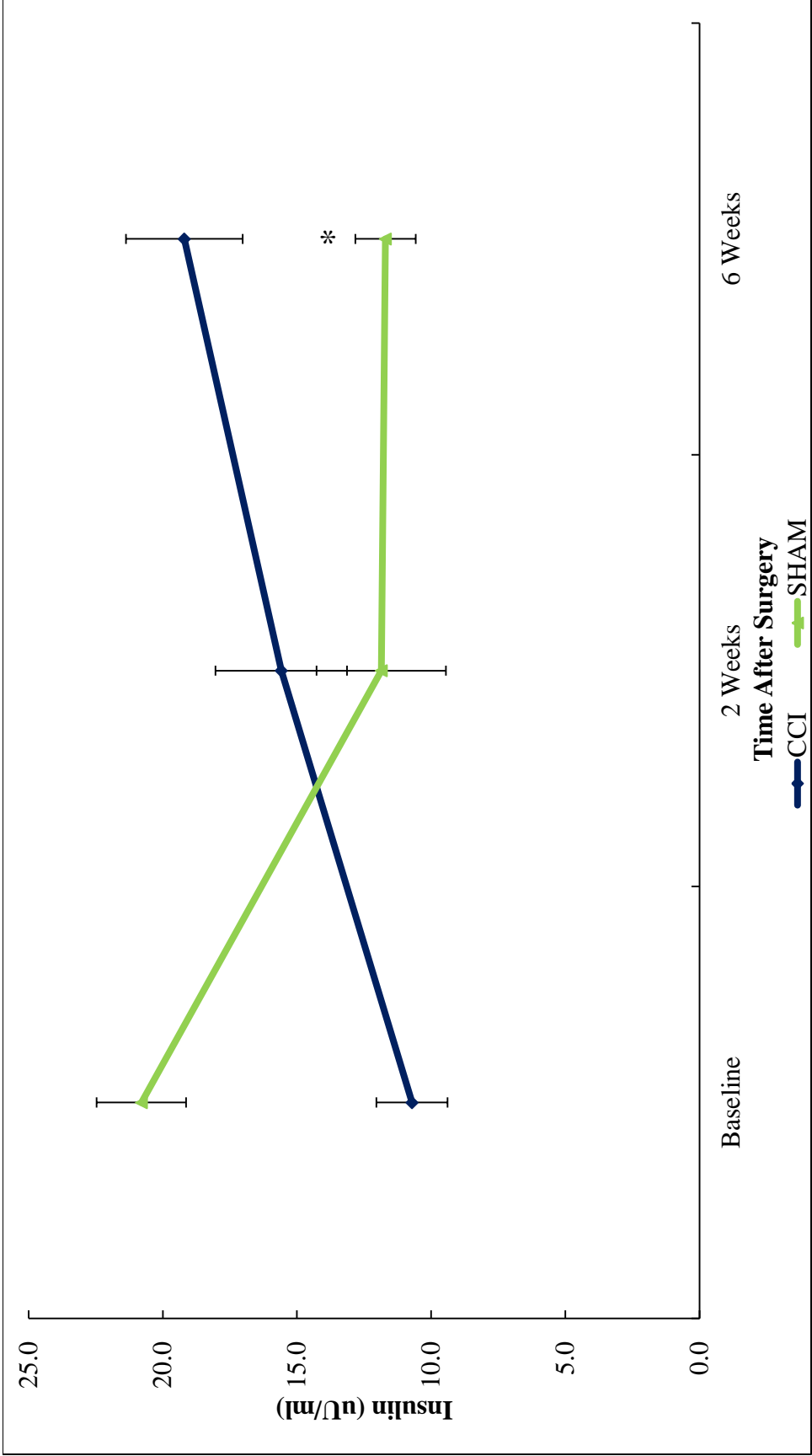
Fasting glucose did not increase in CCI or SHAM rats. In addition, glucose was not significantly different between CCI and SHAM rats (data not shown). Fasting glucose decreased in CCI rats 6 weeks after surgery (137.6 ± 8.2 vs. 115.8 ± 2.1 mg/dL; $p < 0.05$). Area above the curve was calculated for each ITT to account for inter-animal variability. Area above the curve did not change in CCI or SHAM rats. In a normal, non-insulin state, blood glucose is expected to decrease following an injection of insulin. With insulin resistance, glucose remains elevated (to a certain degree) despite introducing exogenous insulin. Three animals were removed from ITT analysis because their blood glucose increased after insulin injection prior to receiving CCI, indicating a failed test. It remains uncertain if these animals did in fact exhibit a state of insulin resistance. However, this is unlikely because the second and third ITT in these animals resembled a normal decline in blood glucose following insulin injection.

Spontaneous activity decreased in a sub-cohort of CCI rats ($n = 4$), but not significantly ($t(3) = 2.93$; $p = 0.06$; see Figure 3.5). Raw data were consolidated into 30 minute intervals and area under the curve for pre- and post-operative measurements was calculated for analysis.
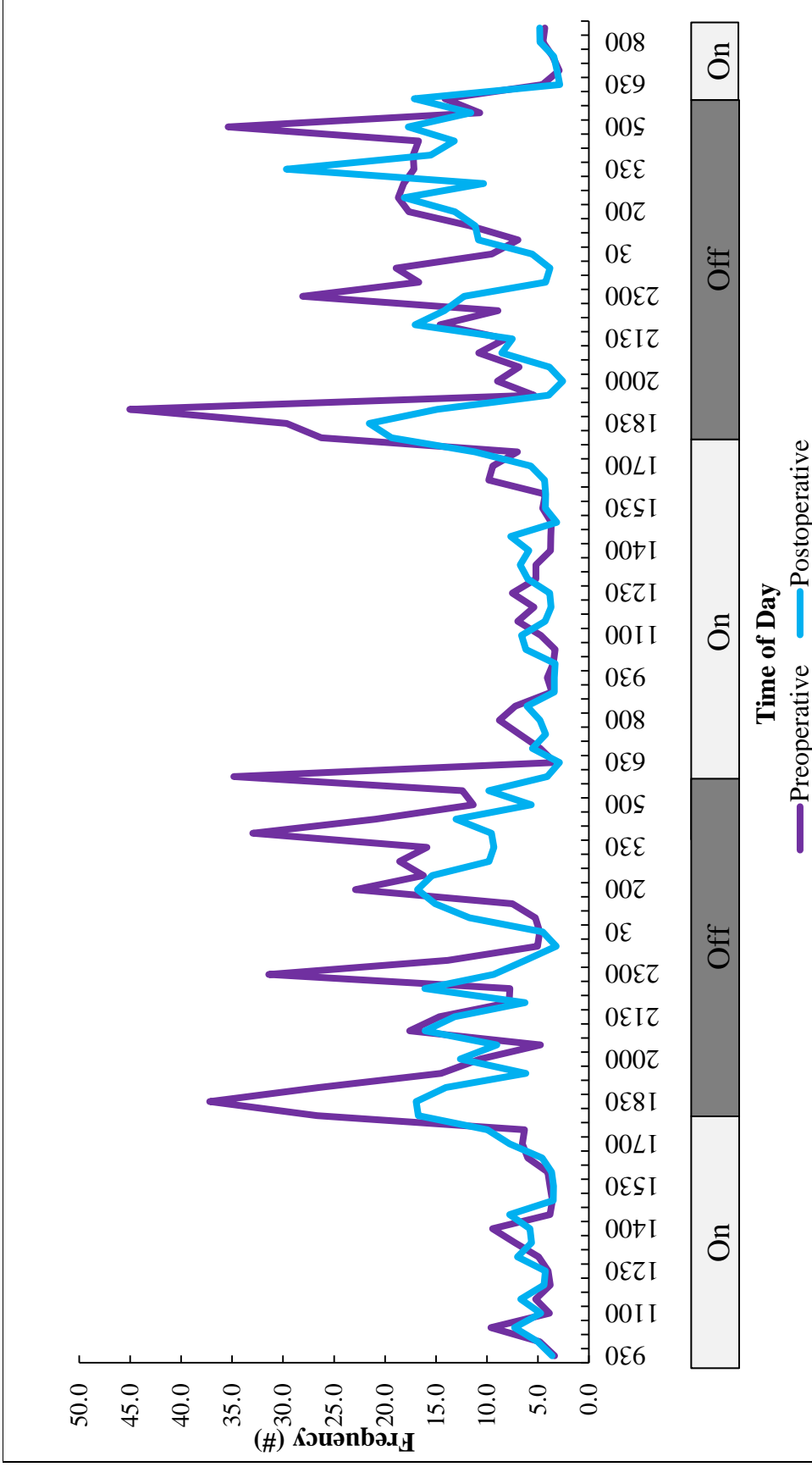
Figure 3.5. Spontaneous activity decreases in CCI rats 2 weeks after surgery, but only trended toward significance (p = 0.06). Data are Means (n = 4). Lights on at 0600 and off at 1800.

Rats with CCI-induced neuropathic pain developed muscle atrophy. CCI rats euthanized 2 and 6 weeks after surgery had smaller ($p < 0.05$) injured hindlimb soleus (SOL) and extensor digitorum longus (EDL) muscles compared to uninjured hindlimb SOL and EDL muscles. In addition, injured hindlimb SOL and EDL muscles were smaller ($p < 0.05$) in CCI compared to SHAM rats 6 weeks after surgery. Injured hindlimb SOL and EDL were not significantly different between CCI rats euthanized 2 and 6 weeks after surgery. Uninjured hindlimb muscles were not significantly different among CCI and SHAM rats (see Table 3.1).

Table 3.1

Skeletal muscle atrophy develops in injured hindlimbs of CCI rats

	Muscle			
	EDL M (SEM)		SOL M (SEM)	
	Injured	Uninjured	Injured	Uninjured
CCI 2 Weeks (n = 7)	18.2 (6.2)*	41.3 (5.8)	16.8 (9.4)*	34.0 (5.4)
CCI 6 Weeks (n = 5)	13.2 (3.6)*†	47.5 (5.2)	10.9 (7.1)*†	41.4 (4.1)
SHAM 6 Weeks (n = 5)	43.0 (2.9)	44.0 (2.2)	35.6 (4.1)	38.6 (2.7)

Units: mg/100g body weight. Data are Mean (SEM) for each group (n = 5-7). *p < 0.05 vs. Uninjured hindlimb. †p < 0.05 vs. Injured Hindlimb SHAM 6 Weeks. Note: 2 Weeks or 6 Weeks: CCI or SHAM rats euthanized 2 or 6 weeks after surgery; EDL: extensor digitorum longus; SOL: soleus.

In summary, rats with CCI-induced neuropathic pain developed thermal hyperalgesia. In addition, spontaneous activity decreased in rats with CCI-induced neuropathic pain, but this trend was not significant ($p = 0.06$). Changes in activity may have contributed to skeletal muscle atrophy in CCI rats. Increases in FFA were likely due to natural weight gain, which was unaffected by CCI. Insulin increased in CCI rats, but this trend was not significant ($p = 0.1$). Neither glucose nor insulin resistance increased following surgery in CCI or SHAM rats.

Discussion

Rats with CCI-induced neuropathic pain exhibited thermal hyperalgesia, a sign of neuropathic pain. Our data are consistent with findings that thermal hyperalgesia develops within two weeks of CCI surgery (Bennet & Xie, 1988; Jeong & Holden, 2009). Increased sensitivity (shorter latency) to a thermal stimulus continued up to 6 weeks after sciatic nerve ligation compared to SHAM surgery. Though not statistically significant, the increase in FWL between CCI rats tested 2 and 6 weeks after surgery rats may reflect a shift from hyper- to hypoalgesia which develops over time in this model of neuropathic pain (Bennet and Xie, 1988). Values for FWL are similar to data collected in our lab for both CCI and SHAM rats.

While the CCI model was effective at inducing thermal hyperalgesia, it did not contribute to altered weight gain or adverse metabolic outcomes. Sprague-Dawley rats are not predisposed to developing insulin resistance, which may have contributed to negative findings. However, "The Obese ZDF [zucker diabetic fatty] rat is a well-established animal model of non-insulin dependent diabetes mellitus [NIDDM] with a substantial body of work relating to a wide range of issues in NIDDM already reported"

(Corsetti, Sparks, Peterson, Smith, & Sparks, 2000, p. 239). More recent evidence has supported the use of this animal model in research pertaining to insulin resistance. Future research may determine the influence of neuropathic pain on phenotypic expression in an animal model predisposed to insulin resistance.

The development of neuropathic pain in CCI rats did not affect overall weight gain. The rate and amount of weight gained was similar to age and weight-matched SHAM rats in this study and aligns with information provided by the breeder (Charles River). Although not investigating neuropathic pain, Zhang et al. (2010) reported similar weight gain in uninjured male Sprague-Dawley rats fed standard rat chow.

In conjunction with weight gain, FFA levels increased regardless of surgery type. In the present study, increases in FFA were likely the result of natural growth and not a direct consequence of neuropathic pain. Barzilai and Rossetti (1995) found that FFA levels correspond with natural increases in weight and fat mass. Compared with Barzilai and Rossetti, rats in our study had higher FFA levels but also had more body mass. Increases in FFA were similar between CCI and SHAM rats, suggestive that CCI-induced neuropathic pain does not affect FFA.

Despite increases in FFA, no significant increases in fasting glucose or insulin were present in CCI rats. Fasting glucose was higher at all three time points compared to similar studies of insulin resistance (Chen et al., 2010; Ropelle et al., 2009; Sridhar et al., 2008). However, none of these studies employed rats with neuropathic pain. Both Sridhar et al. (2008) and Ropelle et al. (2009) used healthy male Wistar rats, in which glucose values were lower but insulin values were similar to our study. Chen et al. (2010) used uninjured male Sprague-Dawley rats, but reported higher insulin and glucose values

compared to our study. This was likely because Chen's rats were up to 6 weeks older than rats in our study.

Because no studies have examined the effects of neuropathic pain on metabolic outcomes such as glucose and insulin in rats, comparisons among studies are difficult. What can be concluded from our study is rats with CCI-induced pain did not demonstrate changes in insulin, although there was a trend towards significance ($p = 0.1$) with a small sample ($n = 5$). In addition, glucose did not increase in CCI rats, nor was it significantly different than SHAM rats. What remains interesting is the significant decrease in glucose following CCI surgery concurrent with an increase in insulin. This may represent a homeostatic mechanism for maintaining euglycemina in a painful state. In addition, higher insulin levels may have masked potential neuropathic pain-induced hyperglycemia/insulin resistance measured during ITT. Measuring insulin levels during ITT may have verified this hypothesis, and is a limitation of our study. What remains unclear is the inverse change in insulin values between CCI and SHAM rats despite comparable decreases in glucose.

Levels of glucose and insulin did not reflect a state of insulin resistance in either CCI or SHAM rats. Data collected during ITT support this conclusion. Normally, plasma glucose declines after exogenous insulin administration. In states of insulin resistance, glucose remains elevated following insulin injection. In our study, rats with CCI-induced neuropathic pain did not exhibit insulin resistance, as measured by ITT.

Spontaneous activity did not significantly decrease following CCI, but there was a trend toward significance. Regardless of this finding, we observed limping, guarding, and altered gait in our CCI rats, which likely reflect changes in overall animal

activity/movement (Bennett & Xie, 1988; Jaggi et al., 2009). However, while the overall mean activity did not decrease, small sample size and high within-group variability may have accounted for a lack of statistical significance ($p = 0.06$). Furthermore, the mechanism used for measuring spontaneous activity does not differentiate type of movement. For example, if the animal moves its head, the scale is disrupted and a recording is made. Therefore, overall spontaneous activity does not necessarily reflect recruitment or decreased weight bearing of the injured hindlimb muscles in CCI rats.

Decreases in spontaneous activity, though not significant, may have contributed to injured hindlimb soleus (SOL) and exetensor digitorum longus (EDL) muscle atrophy in CCI rats. However, no published reports confirm our findings in this rat model of neuropathic pain. These muscles were selected for our study based on distinct functional properties.

Skeletal muscle atrophy occurs in rats with induced nerve injury. In CCI rats, muscle atrophy of the SOL (Choe, Kim, An, Lee, & Heitkemper, 2011), gastrocnemius, and rectus femoris (Daemen et al., 1998) has been reported. Daemen et al. (1998) attributed muscle atrophy to motor denervation of the sciatic (ligated) and femoral (non-ligated) nerves, rather than hypokinesia. Our finding that EDL muscles are atrophied following CCI supports Daemen's conclusion. Following sciatic nerve crush injury, Beehler, Sleph, Benmassaoud, and Grover (2006) found atrophy of both the SOL and EDL muscles. Muscle atrophy is also present in animals with induced spinal cord injury (Hutchinson, Linderman, & Basso, 2001).

Skeletal muscle atrophy can also occur in the absence of nerve injury (hypokinesia). Muscle atrophy occurs in both the SOL and EDL using cast

immobilization in mice (Frimel et al., 2005), as well as tail cast suspension (Han, Zhu, Ma, & Du, 2007), space flight, and whole body suspension in rats (Musacchia, Steffen, Fell, & Dombrowski, 1990).

In our study, CCI rats had muscle atrophy of the injured hindlimb in both SOL and EDL. The underlying mechanisms attributable to these findings remain uncertain. While Daemen's argument that skeletal muscle atrophy in CCI rats is related to motor denervation is credible, it may not account for atrophy of the SOL observed in our CCI rats.

Rats in our pilot study demonstrated some decreased activity, which may have led to SOL muscle atrophy, which is supported by Daemen's conclusion. The fact that both SOL and EDL are atrophied in CCI rats suggests a combination of motor denervation and pain-related hypokinesia. However, a more important question that requires future study is the actual weight bearing/recruitment of injured hindlimb muscles to better elucidate the underlying mechanisms contributing to muscle atrophy in CCI rats.

Limitations of the present study include measurement timing and methodology. Short duration of data collection may have contributed to negative findings. In this study, fasting blood draws were timed to coincide with thermal hyperalgesia in the CCI model of neuropathic pain. In addition, the sequencing of ITT immediately following fasting blood draw was implemented to minimize isoflurane exposure. Despite the use of anesthesia, the stress of venipuncture and blood draw may have contributed to increased variance in response to exogenous insulin during ITT. Methodological limitations may have also affected the outcomes of the study.

Plasma hemolysis may have contributed to aberrant results for fasting insulin and glucose. The presence of hemolysis yields lower values for insulin (Cantrell, Hochholzer, & Frings, 1972; Cook, Glenn, & Armston, 2010), while artificially elevating (Morris et al., 2002) or unaffecting fasting plasma glucose (Ycel & Dalva, 1992). Anesthesia may have also contributed to changes in glucose and insulin, although this is unlikely. While isoflurane increases glucose in both humans (Diltoer & Camu, 1988) and dogs (Horber et al., 1990), glucose is unaffected in fasted rats under isoflurane anesthesia (Saha, Xia, Grondin, Engle, & Jakubowski, 2005).

The measure for insulin resistance, ITT, was chosen as a feasible and efficient method despite drawbacks such as human injection error and rate of insulin absorption by intraperitoneal injection. Alternatively, hyperinsulinemic euglycemic clamping is considered the gold standard for estimating insulin sensitivity/resistance in both humans and animals. However, this method requires extensive training, equipment, and time. Because the absence of insulin resistance was verified by fasting values of glucose and insulin, and ITT, future clamp studies are unnecessary to measure insulin resistance.

Conclusion

Rats with CCI-induced neuropathic pain exhibited thermal hyperalgesia, a trend toward decreased spontaneous activity, and skeletal muscle atrophy of the injured hindlimb. Increases in FFA were likely due to concurrent increases natural growth and not a consequence of neuropathic pain. Though neither glucose nor insulin increased in CCI rats, the brief time period between CCI surgery and subsequent blood draws, or a lack of predisposition towards insulin resistance in this animal model, may have

contributed to these findings. Future study will examine ways in which to prevent

decreases in activity and the development of muscle atrophy.

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Appendix

Protocol Timeline

Time After Surgery	Baseline				1 Week	
Day	0	2,3,4,5	6	7	14	17,18
Event	Animals Arrive	Activity Monitoring (n = 4)	All Animals: Fasting Insulin, Glucose, FFA. ITT	CCI/ SHAM Surgery	2 Week CCI: Fasting Insulin, Glucose, FFA. ITT	Activity Monitoring (n = 4)

Time After Surgery	2 Weeks			6 Weeks		
Day	21	22	24	49	50	52
Event	All Animals: Fasting Insulin, Glucose, FFA. ITT	2 Week CCI: THT	2 Week CCI: Muscle Dissection	6 Week CCI/ SHAM: Fasting Insulin, Glucose, FFA. ITT	6 Week CCI/ SHAM: THT	6 Week CCI/ SHAM: Muscle Dissection

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CHAPTER 4

Duloxetine Does Not Prevent Skeletal Muscle Atrophy or Activity Changes in Rats with CCI-induced Neuropathic Pain

Abstract

The study of neuropathic pain has predominantly focused on changes within the nervous system. Little attention has been devoted to the characterization of systemic changes that may accompany neuropathic pain. In addition, the ability to prevent changes in activity and muscle atrophy using duloxetine has not been explored. An initial pilot study using an established preclinical model of neuropathic pain, the chronic constriction injury (CCI), showed decreased spontaneous activity ($p = 0.06$) in male Sprague-Dawley rats, which may have contributed to skeletal muscle atrophy of the injured hindlimb. These findings led to the development of our current study.

Duloxetine hydrochloride (Cymbalta) is FDA-approved for the treatment of diabetic peripheral neuropathy, a form of neuropathic pain. In preclinical models of neuropathic pain, duloxetine attenuates thermal hyperalgesia and mechanical allodynia. The purpose of our study was to determine if repeated duloxetine administration prevents changes in activity and muscle atrophy by improving the processing of nociceptive input in CCI rats.

Male Sprague-Dawley rats were used in a pre-posttest experimental design. Animals were randomized to receive either CCI or sham surgery (SHAM). Following surgery, animals received daily injections of duloxetine (10 mg/kg) or normal saline.

Activity was scored before and after surgery. Nociceptive testing was conducted 6 and 24 hours after final injection. Skeletal muscles were dissected and weighed to determine muscle atrophy.

Rats with CCI-induced neuropathic pain developed muscle atrophy of the injured hindlimb and increased sensitivity to mechanical stimulus. CCI rats in this study did not demonstrate changes in activity or thermal hyperalgesia. Duloxetine did not prevent the development of muscle atrophy or mechanical allodynia. Duloxetine did not have an effect on activity or thermal hyperalgesia. Future studies will employ more stringent methods to better understand the underlying mechanisms associated with neuropathic pain-induced activity changes and muscle atrophy.

Key words: Neuropathic pain, duloxetine, muscle atrophy, mechanical allodynia, activity, thermal hyperalgesia.

Introduction

Neuropathic pain refers to "Pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" (Treede et al., 2008, pg. 1631). Neuropathic pain encompasses an array of pathological changes originating in both the peripheral and central nervous systems (PNS and CNS, respectively). Mechanical trauma, metabolic disease, neurotoxic chemicals, or infection can lead to neuropathic pain in the PNS. Central neuropathic pain can originate from other events such as spinal cord injury or multiple sclerosis (Costigan, Scholz, & Woolf, 2009). These traumatic events can lead to severe, long-lasting pain that can lead to the development of neuropathic pain through alterations in the processing of incoming noxious stimuli by the CNS.

In neuropathic pain, the transmission of noxious stimuli via specialized sensory neurons (C-fibers; Aδ-fibers), called primary afferent nociceptors, is altered, increasing the responsiveness of nociceptors to normal afferent input (Baron, 2009). As a result of increased nociceptor sensitivity, neuropathic pain is characterized by several manifestations, including thermal hyperalgesia and mechanical allodynia, an exaggerated response to a painful heat or normally non-painful stimulus, respectively (Niederberger, Khlein, & Geisslinger, 2008). These signs can last for years following initial injury in humans and can develop in less than two weeks in rats (Bennett & Xie, 1988).

Neuropathic pain, a form of chronic pain, affects an estimated 76.2 million people (U.S. Department of Health and Human Services, National Center for Health Statistics, 2006). Patients with neuropathic pain visit clinicians more frequently, have poorer health related quality of life, and report impaired sleep and activities of daily living (Hans, Masquelier, & De Cock, 2007; Taylor, 2006). Neuropathic pain also contributes to

changes in activity. Individuals with neuropathic pain report limitations in functional

ability (Eggermont, Bean, Guralnik, & Leveille, 2009; Rudy, Weiner, Lieber, Slaboda, &

Boston, 2007; Smith & Singleton, 2008; Toth, Lander, & Wiebe, 2009). De Sousa and

Frank (2007) found that pain limits several activities such as running, walking,

independently performing activities of daily living, and leisure activity. In our previous

pilot study, CCI rats exhibited a trend towards decreasing activity (p = 0.06) two weeks

after surgery, which may have contributed to skeletal muscle atrophy.

   Decreases in activity may contribute to skeletal muscle atrophy.   Our pilot

findings demonstrate significant skeletal muscle atrophy of the soleus (SOL) and

extensor digitorum longus (EDL) muscles following CCI surgery. However, no

published reports confirm our findings. These muscles were selected based on their

anatomical and physiological properties: SOL is involved in posture, while the EDL is a

non-load bearing muscle. Differences between these muscles may reflect differential

effects of neuropathic pain on muscle atrophy and activity.

   Skeletal muscle atrophy occurs in rats with induced nerve injury.   In CCI rats,

muscle atrophy of the SOL (Choe, Kim, An, Lee, & Heitkemper, 2011), gastrocnemius,

and rectus femoris (Daemen et al., 1998) has been reported. Following sciatic nerve

crush injury, Beehler, Sleph, Benmassaoud, and Grover (2006) reported atrophy of both

the SOL and EDL muscles. Muscle atrophy is also present in animals with induced

spinal cord injury (Hutchinson, Linderman, & Basso, 2001). Daement et al. (1998)

concludes that nerve injury contributes to non-load bearing (EDL) muscle atrophy via

motor denervation, while hypokinesia has a greater effect on postural muscle (SOL).

Skeletal muscle atrophy can also occur in the absence of nerve injury. Muscle atrophy occurs in both the SOL and EDL using cast immobilization in mice (Frimel, 2005), as well as tail cast suspension (Han, Zhu, Ma, & Du, 2007), space flight, and whole body suspension in rats (Musacchia, Steffen, Fell, & Dombrowski, 1990).

Whether skeletal muscle atrophy in CCI rats is primarily due to nerve injury/denervation or pain-related hypokinesia, CCI rats display behavioral perturbations that may contribute to decreased activity and skeletal muscle atrophy. CCI rats develop altered mobility, limping, favoring the unaffected side while sitting or standing, and exaggerated hindlimb guarding; in addition to thermal hyperalgesia and mechanical allodynia (Bennett & Xie, 1988), which likely occurs when bearing weight on the injured hindlimb.

While Daemen's argument that skeletal muscle atrophy in CCI rats is related to motor denervation is credible, it may not account for atrophy of the SOL observed in our CCI rats. Daemen et al. (1998) posit that SOL is more susceptible to hypokinesia. Rats in our pilot study demonstrated decreased activity, which may have led to SOL atrophy, supporting Daemen's argument. However, the fact that both SOL and EDL experienced muscle atrophy in our pilot study suggests a combination of motor denervation and pain-related hypokinesia. Although not approved for use in improving activity or the prevention of muscle atrophy, duloxetine hydrochloride is considered a first-line drug in the treatment of neuropathic pain.

Duloxetine hydrochloride (Cymbalta) is a selective serotonin and norepinephrine reuptake inhibitor approved for the treatment of diabetic peripheral neuropathic pain. In multiple double-blinded placebo controlled phase III clinical trials, duloxetine improved

24-hour average pain scores vs. placebo following 12 weeks of treatment, with efficacy beginning as early as 1 week. Participants in these studies also used less over the counter analgesics while taking duloxetine (Goldstein, Lu, Detke, Lee, & Iyengar, 2005; Raskin et al., 2005). Antinociceptive effects of duloxetine have also been demonstrated in pre-clinical models of nociceptive and neuropathic pain.

In CCI rats, duloxetine alleviates both thermal (Bomholt, Mikkelsen, & Blackburn Munro, 2005) and mechanical hyperalgesia (Bomholt et al., 2005; Piesla et al., 2009). Some have not found an effect of duloxetine on mechanical hyperalgesia/allodynia (Pedersen & Blackburn Munro, 2006), however, methodological differences may account for this discrepancy. Others have shown that using a place escape/avoidance paradigm, duloxetine attenuates aversive behavior (Pedersen & Blackburn Munro, 2006). Importantly, duloxetine does not affect general gait or motor performance (Bomholt et al., 2005; Piesla et al., 2009).

The mode of duloxetine administration may impact antinociceptive effect or duration. In rats with spinal nerve ligation, orally administered duloxetine (30 mg/kg) alleviates mechanical allodynia for up to 6 hours, with peak effects occurring 3 hours after administration (Iyengar, Webster, Hemrick Luecke, Xu, & Simmons, 2004). Duloxetine also increases tail flick latency 4 hours after oral administration (20 and 30 mg/kg). Conversely, intraperitoneal (IP) injected duloxetine (30 mg/kg) increases hind-paw latency on the hot plate test only up to 3 hours post-injection in CCI rats (Bomholt et al., 2005). Whether oral or IP, duloxetine administration has predominantly occurred immediately prior to (30-60 min) nociceptive testing (Bomholt et al., 2005; Pedersen & Blackburn Munro, 2006; Piesla et al., 2009).

Improvements in the processing of incoming sensory input, which may prevent activity changes and muscle atrophy, may reflect duloxetine mechanism of action. Hypothalamic stimulation activates descending serotonin and norepinephrine release in the dorsal horn and decreases acute pain (Holden & Naleway, 2001; Holden, Farah & Jeong, 2005). In addition, hypothalamic stimulation reduces CCI-induced neuropathic pain (Janean Holden, preliminary findings). Having serotonergic and noradrenergic effects, duloxetine improves the processing of nociceptive stimuli, albeit transiently, following treatment with a solitary administration. The effects of repeated duloxetine administration in rats with neuropathic pain have yet to be explored. Specifically, it is unknown whether repeated duloxetine administration prevents decreases in activity and muscle atrophy by improving the altered processing of nociceptive input in CCI rats.

The purpose of this study is to determine if repeated duloxetine administration prevents the development of decreased activity and muscle atrophy in rats with neuropathic pain. Should duloxetine improve the altered processing of nociceptive input in CCI rats, the use of non-denervated muscle may be improved and muscle atrophy in the affected hindlimb assuaged by increasing locomotion.

Methods

Animals

Male Sprague-Dawley rats (225-250g; Charles River) were used in a pre-posttest experimental design with controls. Rats were pair housed in a humidity and temperature controlled animal facility (12:12 light-dark cycle, lights on at 0700). Following a brief acclimation period, rats were randomized to one of four groups (n = 8 per group): CCI plus duloxetine (CCI+DUL), CCI plus normal saline (CCI+NS), SHAM plus duloxetine

(SHAM+DUL), or SHAM plus normal saline (SHAM+NS). To monitor potential duloxetine side effects without a surgical confound, an additional group of rats did not have surgery. Instead, this group only received daily duloxetine injections for two weeks (DUL; n =8). All procedures were approved by the University Committee for the Use and Care of Animals and meet Association for Assessment and Accreditation of Laboratory Animal Care standards.

Activity Monitoring

Activity monitoring was conducted prior to (BASELINE) and 2 weeks after surgery in a method adapted from Choe, Kim, An, Lee, & Heitkemper (2011). Rats were acclimated to activity cages (15 x 10 x 10 inches) for 20 minutes prior to video recording between 2000-2200 (lights off at 1900). Rats were video recorded in random order. Activity was scored every 15 seconds for 20 minutes (80 observations total) according to the following scale: 0 for inactivity/sleep, 1 for grooming, and 2 for activity (eating/drinking, locomotion, or rearing). Total scores were calculated from each video recording session with higher scores representing greater activity. All scoring was completed by the same investigator blinded to animal treatment.

Neuropathic Pain

Following BASELINE video recording, the CCI was used to induce neuropathic injury, as described in Bennet and Xie (1988). Briefly, under isoflurane anesthesia, an incision was made through the left thigh exposing the common sciatic nerve. Four chromic gut ligatures were tied around the nerve proximal to the trifurcation with sufficient tension to produce a leg twitch. The wound was closed with sutures and sterile skin clips. A single injection of buprenorphine (0.05 mg/kg) was given immediately after

CCI to minimize post-operative procedural pain. Age and weight matched SHAM rats received identical surgery without sciatic nerve ligation. No surgery was performed on DUL rats.

Duloxetine Administration

Rats received daily IP injections of either duloxetine (10 mg/kg) or an equivalent volume (1 ml/kg) of normal saline for two weeks after surgery. DUL rats received daily duloxetine injections for two weeks (10 mg/kg). Increasing the amount of duloxetine creates a dose dependent effect on mechanical allodynia (Iyengar et al., 2004) and thermal hyperalgesia (Bomholt et al., 2005) with higher doses inducing a greater effect. However, at 10 mg/kg, duloxetine effectively attenuates thermal hyperalgesia (Bomholt et al., 2005; Iyengar et al., 2004) and was chosen to produce the anticipated effect while minimizing potential side effects. IP administration occurred in the morning, six hours prior to nociceptive testing. Duloxetine injections were prepared as described by Ji (2008).

Nociceptive Testing

The primary aim of this study is to determine if duloxetine prevents muscle atrophy and decreased activity in rats with neuropathic pain. Nociceptive testing was conducted to determine if duloxetine improves the processing of incoming nociceptive input over time.

Nociceptive testing was conducted 2 weeks after surgery. Rats were tested 6 and 24 hours after final injection of DUL or NS to minimize antinociceptive effects in the immediate period (6 hours) following final injection. Rats were tested for mechanical

allodynia and thermal hyperalgesia. All testing was completed by the same investigator blinded to animal treatment.

Mechanical allodynia was measured using standard von Frey filaments (0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, 26.0 [grams]) in a manner adapted from (Ren, 1999; Susan Dorsey, personal communication). Rats were acclimated for 15 min in an inverted plexiglass cage with wire mesh floor with adequate access to hindlimb plantar surfaces. The cage was large enough for rats to turn around, but significantly restricted exploratory movement. Each filament used during testing was applied 5 times to each rat starting with the 4.0g filament. The filament was gently placed against the mid-plantar surface of the hind paw with enough force to slightly bend the filament. The filament was held for 5 seconds or until the animal removed its paw.

Testing began on the left side before applying the filament to the right side to allow time to pass before reapplying the stimulus. Rats were allowed to rest for 3-5 minutes between trials and between filaments. Brisk withdrawal of the hind paw while the filament was in contact with the paw or immediately upon removal of the filament from the paw was recorded as a positive response. Flaring of the toes while the filament was applied was not considered a positive response. If a particular filament did not elicit 3 or more positive responses out of 5 trials, testing continued with the next stiffest filament in the series and continued upward to identify the filament that elicited 3 out of 5 positive responses. If an animal did not respond to the largest filament, a value of 26.0 was recorded, indicating a lack of increased sensitivity to non-painful stimulus. If the animal responded to 3 or more trials, testing continued with the next smaller filament and continued downward until the filaments no longer elicited 3 or more responses.

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Threshold was defined as the gram force of the smallest filament that elicited at least 3 withdrawal responses out of 5 trials. Lower thresholds (in grams) demonstrate increased sensitivity to non-painful stimulus or mechanical allodynia, a sign of neuropathic pain.

Changes in thermal nociceptive threshold (pain sensitivity) were measured using the tail flick analgesiometer (Ugo Basile, Collegevile, PA). Thermal hyperalgesic testing (THT) was conducted 30 minutes after mechanical allodynia testing. During this rest period, rats were returned to their home cages to minimize stress and potential nociceptor sensitization. Rats were acclimated to the testing procedure prior to data collection and were fully awake and gently restrained during testing. Foot withdrawal latency (FWL, sec) measures the reflexive withdrawal of the hindlimb in response to noxious thermal infrared heat stimulus. Both hindlimbs were tested 3 times and the data averaged to produce 1 FWL for each hindlimb. Shorter latencies demonstrate increased sensitivity to heat, or thermal hyperalgesia, a sign of neuropathic pain.

Muscle Atrophy

After THT, rats were euthanized and the extensor digitorum longus (EDL), soleus (SOL), and Gastrocnemius muscles were removed, weighed, frozen in liquid nitrogen, and stored at -70°C.

Statistical Analysis

Non-parametric tests were used for activity monitoring and von Frey analysis (Friedman Test and Kruskal-Wallis, respectively). One-way ANOVA with Bonferroni corrections was used for THT and muscle atrophy analyses.

Results

Three rats exhibited duloxetine side effects: abdominal bloating, diarrhea, decreased appetite and weight loss. Two were from the DUL group. One was from SHAM+DUL but did not perform differently than other rats in the group on any of outcome measures. No CCI+DUL rats developed side effects. Rats that received NS did not develop adverse effects.

All rats gained weight regardless of CCI or SHAM surgery ($F(1,35) = 241.8$, $p < 0.05$). Duloxetine did not affect weight gain in CCI or SHAM rats over the study duration (see Figure 4.1).
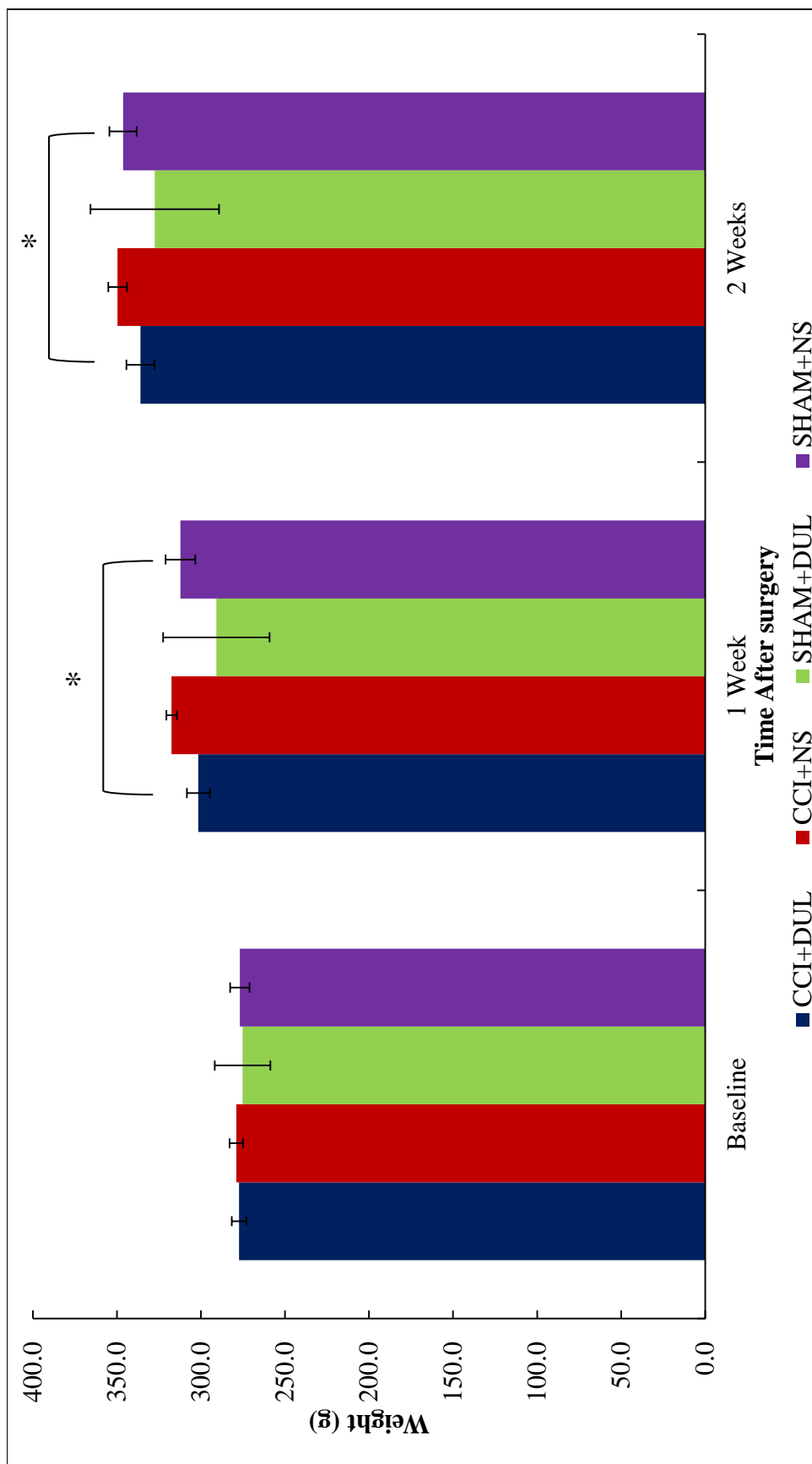
Figure 4.1. Weight gain is not altered by CCI or SHAM surgery in male, Sprague-Dawley rats and is unaffected by duloxetine treatment. Data are Mean ± SEM for each group (n = 8). *p < 0.05 vs. Baseline.

Rats with CCI-induced neuropathic pain developed muscle atrophy. Soleus, EDL, and Gastrocnemius muscles were significantly smaller in the injured vs. uninjured hindlimb in CCI rats ($p < 0.05$). Moreover CCI rats had significantly smaller injured hindlimb SOL, EDL, and Gastrocnemius muscles than SHAM (DUL and NS) and DUL rats ($p < 0.05$).

Duloxetine treatment did not prevent muscle atrophy in CCI rats. Injured hindlimb SOL, EDL, and Gastrocnemius muscles were not significantly different between CCI+DUL and CCI+NS rats ($p > 0.05$). Uninjured hindlimb muscle weights were not significantly different among groups ($p > 0.05$). DUL rats had smaller injured vs. uninjured SOL muscles ($p < 0.05$, see Table 4.1).

Table 4.1

Skeletal muscle atrophy is not prevented by duloxetine in CCI rats

Muscle

	EDL M (SEM)		SOL M (SEM)		GASTROCNEMIUS M (SEM)	
	Injured	Uninjured	Injured	Uninjured	Injured	Uninjured
CCI+DUL	22.6 (0.6)[†*]	42.1 (2.0)	15.2 (0.8)[†*]	41.2 (1.7)	187.5 (10.9)[†*]	543.3 (13.2)
CCI+NS	21.7 (0.9)[†*]	44.1 (1.2)	15.0 (1.3)[†*]	41.4 (1.7)	210.5 (13.9)[†*]	589.8 (8.8)
SHAM+DUL	37.8 (2.9)	40.8 (2.2)	37.5 (1.5)	37.8 (1.5)	528.6 (28.8)	538.4 (31.1)
SHAM+NS	37.3 (2.8)	40.7 (1.5)	35.1 (2.1)	36.7 (1.7)	560.2 (12.5)	561.5 (10.8)
DUL	41.3 (2.4)	41.3 (2.6)	36.1 (1.4)[†]	37.6 (1.3)	520.6 (26.9)	525.1 (30.5)

Units: mg/100g body weight. Data are Mean (SEM) for each group (n = 8). *p < 0.05 vs. SHAM (DUL and NS) and DUL. [†]p < 0.05 vs. Uninjured hindlimb. EDL: extensor digitorum longus; SOL: soleus.

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Activity was not significantly different among groups at BASELINE ($p > 0.05$). Two weeks after surgery, activity was not significantly different among rats with CCI-induced neuropathic pain compared to SHAM (DUL and NS) or DUL rats ($p > 0.05$). Duloxetine treatment did not affect activity in CCI or SHAM rats ($p > 0.05$; see Figure 4.2).
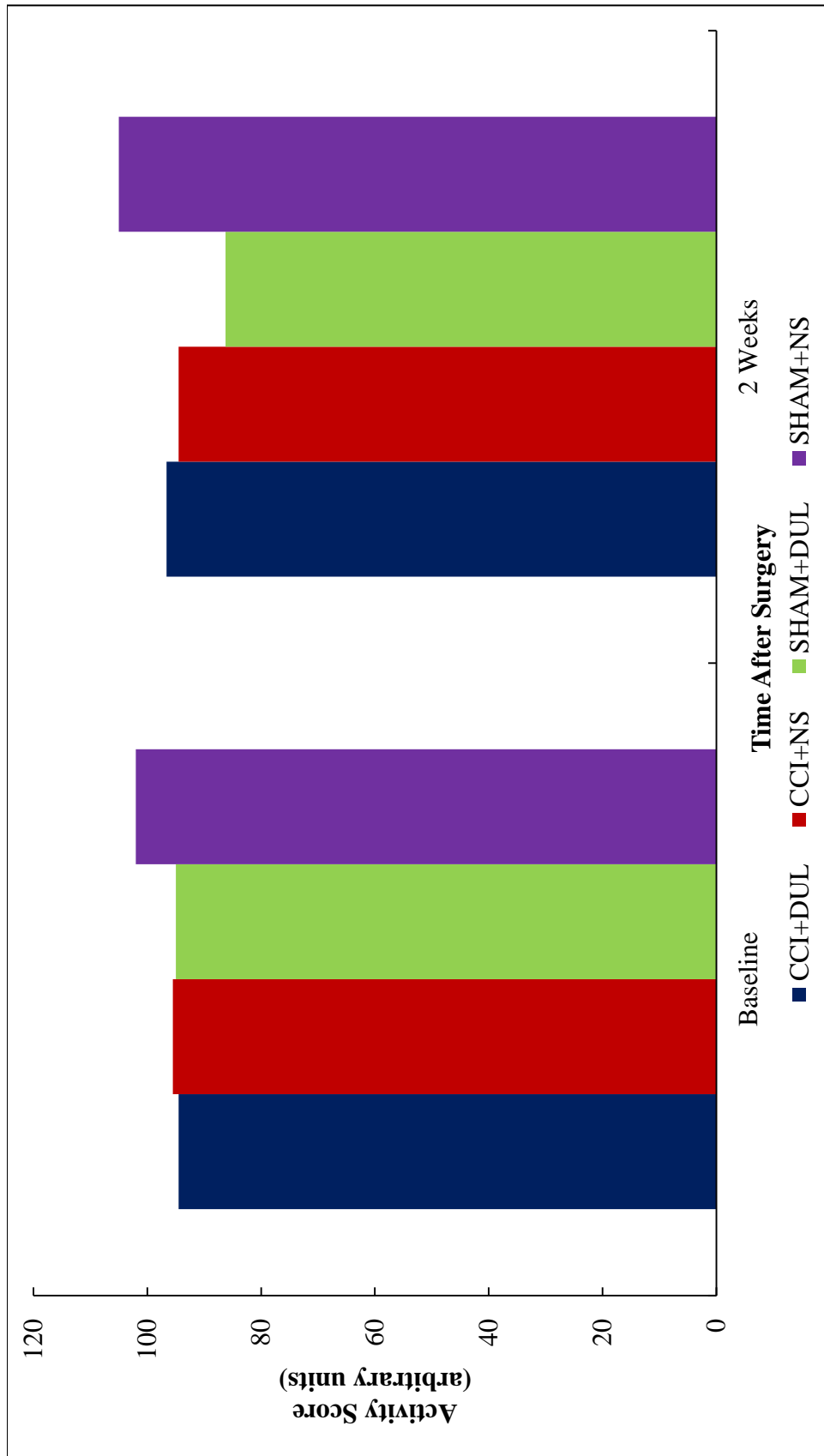
Figure 4.2 Rats with CCI-induced neuropathic pain did not develop changes in activity. Duloxetine treatment did not affect activity following CCI or SHAM surgery. Data for DUL (not shown), SHAM+DUL, and SHAM+NS were not significantly different than CCI rats. Ordinal data presented for each group (n = 8).

Rats with CCI-induced neuropathic pain developed mechanical allodynia. CCI rats had lower thresholds of the injured hindlimb compared to SHAM rats 6 hours after last injection ($p < 0.05$). CCI rats had significantly lower threshold of the injured vs. uninjured hindlimb 6 and 24 hours after last injection ($p < 0.05$).

Duloxetine treatment did not prevent mechanical allodynia in rats with neuropathic pain. Injured hindlimb sensitivity to mechanical stimulus was not different between CCI+DUL and CCI+NS rats 6 or 24 hours after last injection ($p > 0.05$). Uninjured hindlimb thresholds were not significantly different among groups 6 or 24 hours after last injection, nor did they change over time (see Figure 4.3).
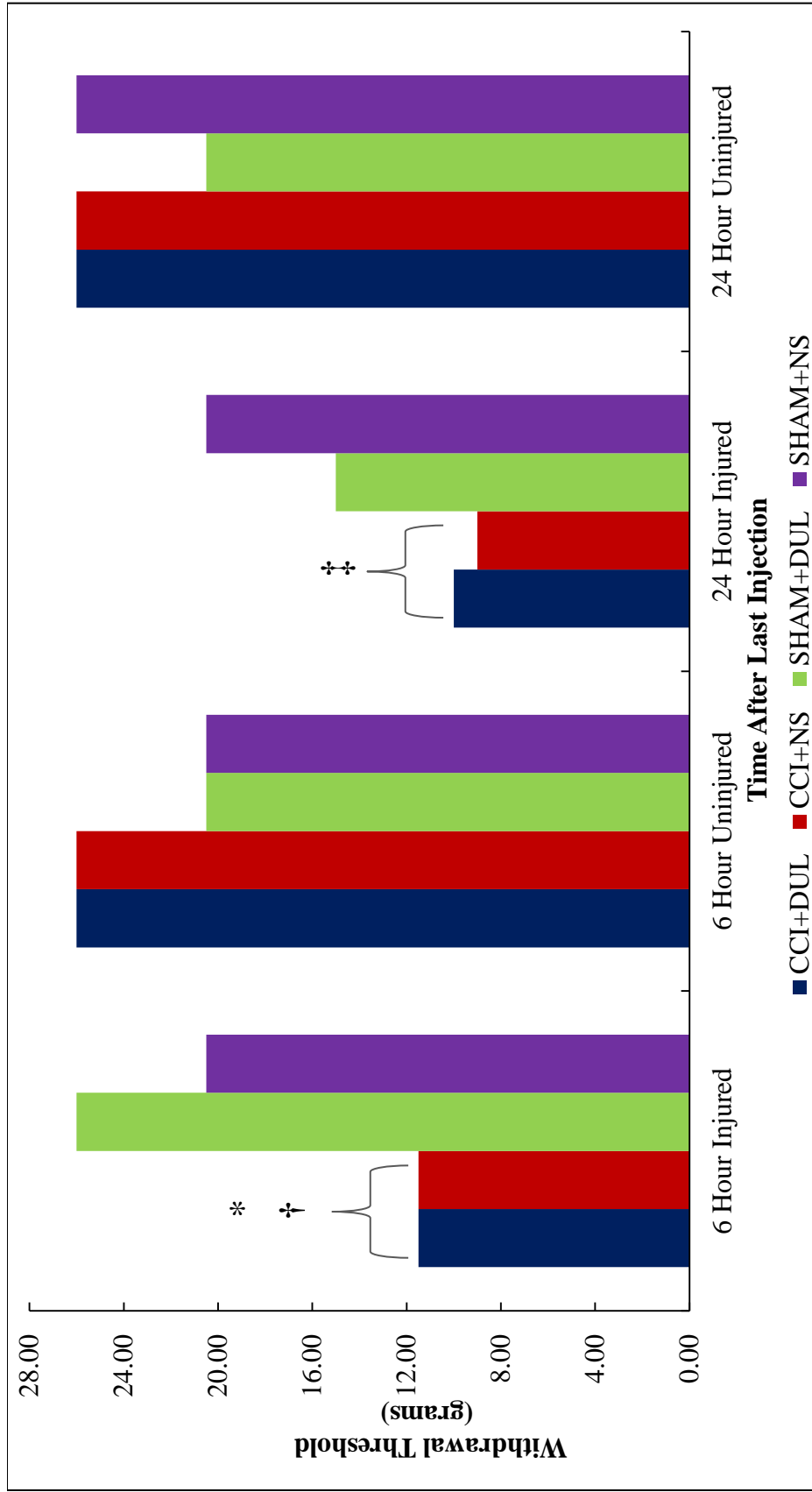
Figure 4.3. Rats with CCI-induced neuropathic pain developed increased sensitivity (decreased threshold) to a mechanical stimulus of the injured hindlimb. Duloxetine treatment did not affect tolerance to a mechanical stimulus 6 or 24 hours after last injection. Ordinal data are presented for each group (n = 8). *p < 0.05 vs. 6 Hour Uninjured CCI (DUL and NS). †p < 0.05 vs. 6 Hour Injured SHAM (DUL and NS). ‡p < 0.05 vs. 24 hour Uninjured CCI (DUL and NS). SHAM (DUL and NS) and DUL (data not shown) rats did not exhibit increased sensitivity to mechanical stimulus.

Rats receiving CCI surgery did not exhibit thermal hyperalgesia in this study. Foot withdrawal latencies (FWL) of the injured hindlimb were not significantly different among groups 6 or 24 hours after last injection ($p > 0.05$). Six hours after last injection, FWL was greater in the injured vs. uninjured hindlimb in CCI (DUL and NS) rats, but only trended towards significance ($p = 0.15$). On average, DUL and SHAM+NS animals had longer FWL than CCI and SHAM+DUL groups. Duloxetine did not significantly affect sensitivity to thermal stimulus (see Figure 4.4).
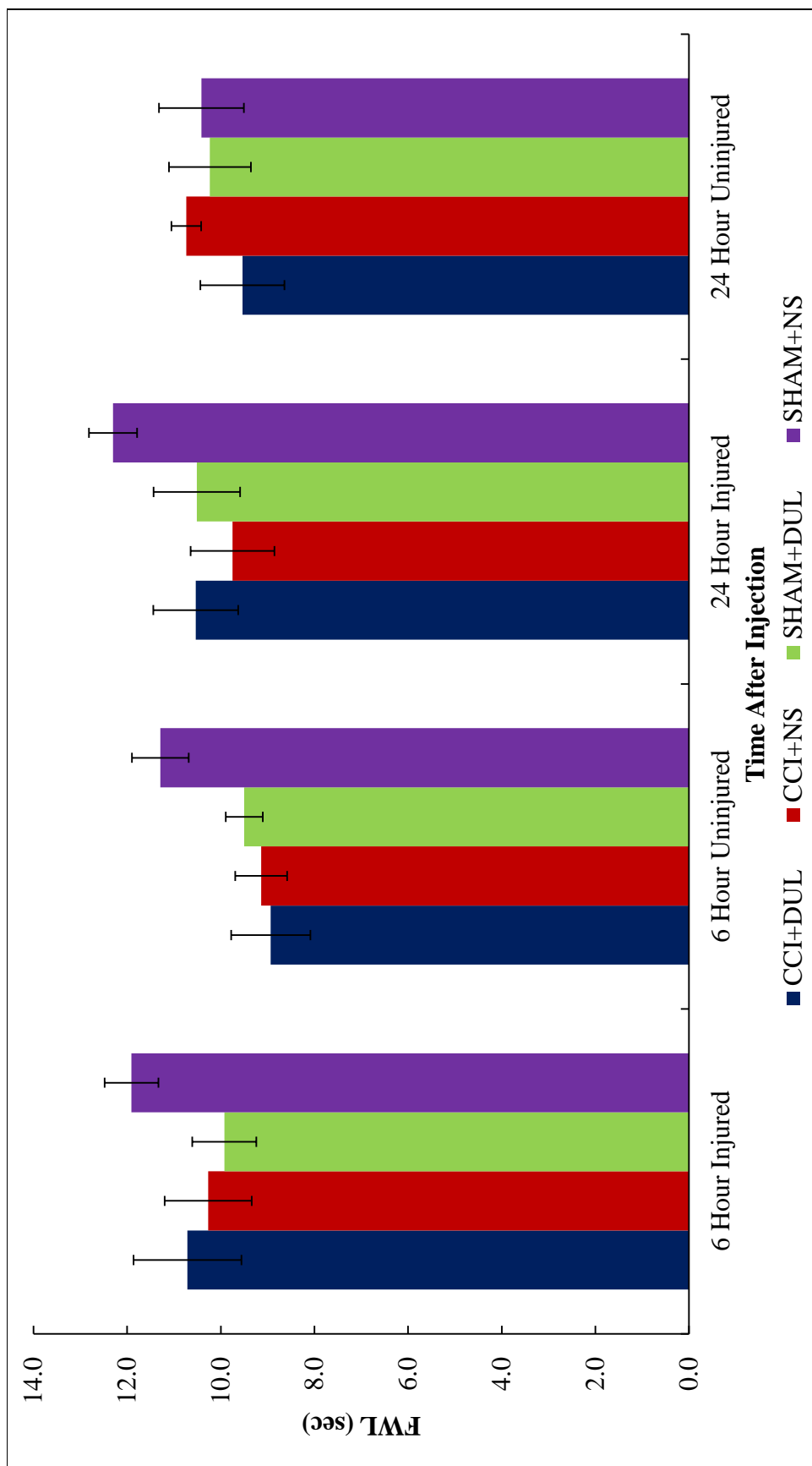
Figure 4.4. Rats with CCI-induced neuropathic pain did not exhibit thermal hyperalgesia. Duloxetine treatment did not affect sensitivity to thermal stimulus. Data are Mean (SEM) for each group (n = 8).

Discussion

Duloxetine is a selective serotonin and norepinephrine reuptake inhibitor used in the treatment of neuropathic pain. In preclinical models, duloxetine attenuates thermal hyperalgesia and mechanical allodynia following a single treatment. The purpose of our study was to determine if repeated duloxetine administration prevents changes in activity and muscle atrophy by improving the processing of nociceptive input in CCI rats.

Muscle atrophy was present in CCI rats. Current findings are consistent with our previous study, in which CCI rats developed smaller SOL and EDL muscles in injured vs. uninjured hindlimbs. Choe et al. (2011) also reported a similar degree of gastrocnemius and SOL muscle atrophy in CCI rats. The presence of muscle atrophy and mechanical allodynia suggest the presence of neuropathic pain, despite the absence of thermal hyperalgesia in the current study.

Duloxetine treatment did not prevent the development of muscle atrophy in CCI rats. Duloxetine is not intended for maintaining muscle mass. It was hypothesized, however, that if duloxetine improved the processing of nociceptive input in CCI rats, decreases in activity and muscle weight might be prevented.

Muscle atrophy occurs with decreased weight-bearing or induced nerve injury. Muscle atrophy occurs in both SOL and EDL using cast immobilization in mice (Frimel, 2005), as well as tail cast suspension (Han et al., 2007), space flight, and whole body suspension in rats (Musacchia et al., 1990). When muscles are not recruited for movement or posture (i.e. muscle contraction), muscle atrophy can develop.

Muscle atrophy also occurs after nerve injury. Daemen et al. (1998) reported atrophy of the gastrocnemius and rectus femoris muscles in CCI rats. Following sciatic

nerve crush injury, Beehler et al. (2006) reported atrophy of both the SOL and EDL muscles. Muscle atrophy is also present in rats with induced spinal cord injury (Hutchinson et al., 2001). What remains uncertain are the respective contributions of decreased activity and nerve injury on muscle atrophy.

The muscles selected for our study were based on distinct functional properties. While Daemen et al. (1998) conclude that EDL muscle atrophy is the result of motor denervation, they attribute SOL atrophy to hypokinesia. Our study does not fully support this conclusion, as both SOL and EDL were atrophied in CCI animals without decreases in activity. Though our activity measure did not confirm findings from our initial study, the underlying mechanisms contributing to neuropathic pain-associated muscle atrophy warrant further study.

Rats with CCI-induced neuropathic pain did not develop changes in activity two weeks after surgery. In addition, duloxetine treatment did not affect activity in CCI or SHAM rats. In our first study, CCI rats had developed a trend towards decreased activity two weeks after surgery ($p = 0.06$). The absence of activity changes in this study does not confirm our initial findings. Apart from our initial study, there have been no published reports describing activity changes in CCI rats apart from Choe et al. (2011), who found decreases in activity following CCI surgery.

There are several possible explanations for our findings. First, the method used in the current study for activity monitoring provides a general representation over a limited period of time. In our previous study, rats were monitored a much longer time (up to 48 hours) using a gravimetric scales method. In their study, Choe et al. collected data for 5 minutes per day over 14 days (2011). The method used in the current study did not allow

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for sufficient data collection with which to make sufficient conclusions regarding activity changes in CCI rats using this method. Ultimately, our study likely does not accurately reflect overall activity and is a significant limitation.

Our results may have also been negative because the activity measure was not discriminate enough to differentiate activity with such a limited scoring system. Using a total score to improve between group variability failed to detect significant differences among groups. More specifically, this method does not measure the degree and/or frequency with which the injured hindlimb is recruited or used to bear weight. Finally, duloxetine side effects may have negatively affected activity. Duloxetine has not been shown to affect motor performance (Bomholt et al., 2005; Piesla et al., 2009). However, while overt duloxetine side effects did not affect all groups equally, an underlying effect may have masked activity levels. In future studies we will collect more data over longer periods of time with less subjective measures. In addition, attention will be paid to the actual weight bearing of injured hindlimb muscles in CCI rats.

Rats with CCI-induced neuropathic pain exhibited mechanical allodynia in response to von Frey stimulation. CCI rats had significantly lower withdrawal threshold on the injured than the uninjured hindlimb 6 hours after last injection. Duloxetine treatment did not increase tolerance to mechanical stimulus in CCI rats.

Duloxetine has been approved for the treatment of major depressive disorder, diabetic neuropathy (Dugan and Fuller, 2004), and generalized anxiety disorder (Khan and Macaluso, 2009). However, no studies show duloxetine as a preventative treatment for neuropathic pain. In this regard, our study confirms what is known about duloxetine.

The effects of single duloxetine administration on mechanical allodynia are mixed. While several authors have found that duloxetine increases threshold to von Frey filaments (Iyengar et al., 2004; Jones, Peters, & Shannon, 2005), others have failed to show an effect (Bomholt et al., 2005; Pedersen & Blackburn Munro, 2006). Our findings are consistent with the latter studies, which used the same type of rat strain and pain model. However, our study examined the effects of repeated duloxetine injections in contrast to single drug administration in the studies above.

Rats that received CCI did not exhibit thermal hyperalgesia in this study. In addition, duloxetine did not have an effect on thermal hyperalgesia. While sensitivity to thermal stimulus was lower (longer FWL) in the injured compared to the uninjured hindlimbs of CCI rats 6 hours after last injection, this difference was not significant between CCI+DUL and CC+NS. SHAM+NS and DUL did tend to have longer FWL of the injured hindlimb than other groups, but SHAM+DUL did not mimic this trend.

Our findings are not consistent with literature pertaining to single duloxetine administration on thermal hyperalgesia. Acute duloxetine administration attenuates thermal hyperalgesia in preclinical models of pain (Bomholt et al., 2005; Iyengar et al., 2004; Jones et al., 2005). However, only Bomholt et al. (2005) used the CCI model with significant effects only up to 180 minutes after IP injection. Despite different models, duloxetine effects on thermal hyperalgesia have only been seen up to 4 hours after injection (Iyengar et al., 2004).

Our study examined the ability of repeated duloxetine administration to improve nociceptive processing over time rather than short-term effects. For this reason, time between last injection and nociceptive testing was designed to negate short-term drug

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effects. That we did not see an effect at 6 hours supports previously published literature that acute effects of the drug diminish within a brief period of time, even if administered daily for two weeks. It is important to note that mechanical allodynia, a measure that relates more closely to muscle activity and weight bearing, was present in these rats despite changes in thermal sensitivity.

Testing only at 6 and 24 hours after the last duloxetine injection may not have allowed us to capture differences to thermal responses in male rats. However, conducting nociceptive testing in the immediate period following final duloxetine injection would have provided comparisons with previous work and confirm the short-term effects of duloxetine in these animals. Future studies will plan for more frequent nociceptive testing, both in the immediate and extended time period after duloxetine injection.

One reason why we did not see differences in thermal testing may relate to the use of males in the present study. Based on observations from our lab, male CCI rats have significantly shorter FWL of the injured hindlimb than male SHAM. However, this difference between male rats was smaller than the difference between female CCI and SHAM rats. The use of females may have improved the ability to detect significant differences between CCI and SHAM rats in the present study.

Another limitation of this study is related to our inability to test for duloxetine serum levels in rats. As such, we do not know whether every animal received the same levels of duloxetine, and this limitation may have affected the study outcome. Although duloxetine pharmacokinetics have not been described in animals, human studies have documented consistent findings.

Duloxetine levels are predominantly measured using chromatographic methods (Anderson, Reed, Lintemot, Kegler, DeQuintas, Sandberg et al., 2006; Lantz, Gillespie, Rash, Kuo, Skinner, Kuan, et al., 2003; Ma, Zhang, Li, Cen, Xu, Wang et al., 2007; Sharma, Goldberg, & Cerimele, 2000; Skinner Kuan, Pan, Sathirakul, Knadler, Gonzalez, et al., 2003;). These methods have demonstrated reliability, with inter- intra coefficients of variation <10% (Ma, et al., 2007); although duloxetine metabolism differences between subjects may increase variability (Sharma et al., 2000). Even with various measurement techniques, duloxetine pharmacokinetics are consistent in humans.

In humans, duloxetine pharmacokinetics appear to follow a dose dependent trend, with increasing doses corresponding to increased maximum plasma concentration (range 22.7-69.6 ng/ml) and half-life (range 13-16 hours) (Ma et al., 2007). Time to maximum plasma concentration (~6 hours) and average drug half-life (range 9.9-16.0 hours) are relatively consistent across studies (Lantz, et al., 2003; Ma, et al., 2007; Skinner, et al., 2003). While these studies support the presence of duloxetine following oral administration in humans, extrapolation to injected duloxetine in animals is difficult.

Injection of duloxetine may provide more reliable serum levels than oral administration, but there is the risk that the injected duloxetine did not get deposited in a site where it was usable. Only 3 animals developed obvious side effects, one of which was in the testing group (SHAM+DUL). While side effects of duloxetine are mild to moderate (Westanmo, Gayken & Haight, 2005), well tolerated in humans, and diminish within 8 weeks (Dugan and Fuller, 2004), the lack of side effects does not necessarily indicate insufficient serum levels in our study. Future studies require a determination of

serum levels to assure adequate dosing, and dose response testing to determine the optimum amount of duloxetine for neuropathic conditions.

Conclusion

Duloxetine is an effective, first-line treatment for individuals with neuropathic pain. Muscle atrophy and mechanical allodynia developed in rats with CCI-induced neuropathic pain, but were not prevented by duloxetine treatment. Despite the absence of thermal hyperalgesia and decreased activity, methodological issues contributed to these findings. Future study is necessary to elucidate the underlying mechanism contributing to neuropathic pain-associated muscle atrophy. In addition, lack of information on duloxetine pharmacokinetics and antinociceptive effects in animals warrants further study.

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CHAPTER 5

Conclusion

Neuropathic pain is significant health problem. In addition to the altered processing of sensory input that produces painful responses, neuropathic pain has significant effects on activity and overall quality of life. The study of neuropathic pain has predominantly focused on changes within the nervous system. Advances in the treatment of neuropathic pain have also received increasing attention. However, the effects of neuropathic pain on muscle functioning have yet to be described. In addition, the ability of specific treatments to prevent changes in activity and muscle atrophy by modulating the processing of neuropathic input has not been studied. The purpose of this research was to explore the metabolic, activity, and musculoskeletal changes that may accompany neuropathic pain, as well as the ability to prevent these changes by improving the processing of nociceptive input.

In an initial study, the relationships between neuropathic pain and muscle functioning were explored by describing basic metabolic, activity, and musculoskeletal changes in an established model of neuropathic pain, the chronic constriction injury (CCI, Bennett & Xie, 1988). Rats with CCI-induced neuropathic pain had decreased activity, although this trend was not statistically significant (p = 0.06), and may have contributed to atrophy of injured hindlimb muscles. However, CCI rats did not exhibit maladaptations in metabolic indices: glucose or insulin resistance. CCI rats did not

demonstrate changes in insulin, although there was a trend increase towards significance (p = 0.1). Levels of fasting free fatty acids (FFA), which associate with insulin resistance, were elevated in CCI rats, but were likely due to natural weight gain. The findings from our first study led to a subsequent study in which duloxetine was repeatedly administered to determine if short-term improvements in the processing of nociceptive input prevented decreases in activity and muscle atrophy in CCI rats.

In our second study, rats with CCI-induced neuropathic pain developed atrophy of injured hindlimb muscles. However, duloxetine treatment did not prevent the development of muscle atrophy in CCI rats. Evidence of muscle atrophy and mechanical allodynia in CCI rats supported the presence of neuropathic pain, despite the absence of thermal hyperalgesia in that study. In addition, CCI rats did not develop changes in activity, which did not confirm results from our initial study. This discrepancy, however, was likely due to methodological differences/limitations. Though not fully supportive of our hypotheses, the findings in these two small, exploratory studies advance nursing science and contribute to our understanding of the relationships between neuropathic pain and muscle changes.

Advances in nursing science are being made with the exploration of biological and pathophysiological processes (Rudy & Grady, 2005). "Basic science research done by nurse scientists is often distinct from other disciplines in that the inception of the hypothesis, the conclusions drawn, and the delineation of areas for further research are from a nursing perspective" (Garrett, 2000, pg. 228). From a holistic perspective, nursing research includes physiologic function, which complements psychosocial, spiritual, and environment factors, as part of the overall system (Holden, 1996; Witek-Janusek, 2004).

Animal models used in nursing research should be selected to achieve the outcomes of an appropriately designed study (Holden, 2011), with the ultimate goal of improving the health of whole persons.

Animals have been extensively used in nursing research (Page, 2004). While human research has many benefits, the use of animals in research also has advantages. With greater control over the environment, extraneous variables, and subject recruitment, an investigator can reduce unintended sources of variability when using animals, an important consideration especially when investigating mechanisms. In addition, specific models can be used to study psychosocial factors or symptoms using a variety of outcomes (Page, 2004).

The use of animals also includes limitations. When using an animal model, the research focus is often reduced to measure specific outcomes (Page, 2004). However, "all research must be somewhat reductionist, in that a research question focuses on some part of an individual or animal as it relates to the whole organism" (Holden, 1996, pg. 312). In human research, sources of internal and external validity are monitored, controlled, or accounted for, to conclude valid results with a high degree of confidence. The ability to control many extraneous factors may further reduce findings to a very narrow focus. As such, animal research is often seen as more reductionist than human work.

In addition to the reductionist criticism, obtaining adequate funding and resources create obstacles to animal research in nursing (Page, 2004). However, resources through the National Institutes of Nursing Research have improved the availability of funding for animal research in nursing (Rudy & Grady, 2005). Differences among species, including

humans, also raise concerns regarding generalizing of findings (Rodgers, Anderko, & Page, 2004). In our studies, similarities in pain physiology between humans and rats supported use of the CCI model in rats. Although direct bedside application of our findings is inappropriate, our research contributes to the body of knowledge to be used in future (nursing) research, and is foundational for further clinical studies.

The ethical treatment of animals may also be viewed as an argument against animal research (Rodgers, Anderko, & Page, 2004). Humans must be informed of the risks, benefits, objectives, and procedures of a study, as well as the freedom to withdraw. Animals, however, do not have these privileges and additional planning and care must be taken to ensure their humane treatment, overseen by rigorous accrediting organizations (Institute for Laboratory Animal Research, 2010).

The CCI, a model of neuropathic pain, was selected for our studies. Because of similarities to human neuropathic pain conditions, the CCI model has been extensively used to study nervous system changes and response to pharmacologic agents due (De Vry, Kuhl, Franken-Kunkel, & Eckel, 2004; Jaggi, Jain, & Singh, 2009). In addition to reliably producing signs of neuropathic pain – thermal hyperalgesia and mechanical allodynia – this was a familiar model, one that has been used for previous studies in our lab.

Our current studies, however, aimed at examining the relationships between of neuropathic pain and muscle functioning by exploring basic metabolic, activity, and musculoskeletal changes in rats with CCI-induced neuropathic pain. Limited evidence supports a relationship between acute nociceptive pain and changes in metabolism (Greisen et al., 2001). Moreover, no published studies have examined the metabolic

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changes occurring with neuropathic pain. Our initial study was the first known investigation of metabolic changes in CCI rats.

Similar attention has been given to the relationship between activity and neuropathic pain. Human studies document the impact of pain on daily activities and overall quality of life (Eggermont, Bean, Guralnik, & Leveille, 2009; Rudy, Weiner, Lieber, Slaboda, & Boston, 2007; Smith, Torrance, Bennett, & Lee, 2007; Toth, Lander, & Wiebe, 2009). More specifically, activity is decreased in CCI rats (Choe, Kim, An, Lee, & Heitkemper, 2011). Our first study provides evidence to show a trend of decreased activity in CCI rats and confirms the presence of muscle atrophy in rats with CCI-induced neuropathic pain.

Several studies have examined the effects of CCI-induced neuropathic pain on muscle atrophy (Choe et al., 2011; Daemen et al., 1998). Other models of nerve injury have also shown muscle atrophy (Beehler, Sleph, Benmassaoud, & Grover, 2006; Hutchinson, Linderman, & Basso, 2001). Our studies confirm findings that muscle atrophy is present in animals with nerve injury. While various hypotheses purport different underlying mechanisms contributing to muscle atrophy in neuropathic pain, additional investigation in this area of study is needed.

Our subsequent study aimed at understanding the ability of a drug to relieve neuropathic pain and prevent altered muscle functioning and activity. Duloxetine has been shown to attenuate mechanical allodynia and thermal and mechanical hyperalgesia/allodynia (Bomholt, Mikkelsen, & Blackburn Munro, 2005; Iyengar, Webster, Hemrick Luecke, Xu, & Simmons, 2004; Piesla et al., 2009). However, these studies investigated the short-term effects of duloxetine following a single treatment.

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Our study built on this information to determine if short-term improvements in sensory information processing would prevent changes in activity and muscle atrophy. Ours was the first study to explore the use of duloxetine in this way.

There are several strengths to this research. Primarily, both studies confirm the presence of neuropathic pain induced by sciatic nerve ligation. CCI rats exhibited thermal hyperalgesia and mechanical allodynia following sciatic nerve ligation in our first and second study, respectively. Importantly, our work broadens the characterization of neuropathic pain in CCI rats, showing a trend towards decreased activity and muscle atrophy of the injured hindlimb.

Several limitations of our research should be highlighted. While the CCI model was effective at producing signs of neuropathic pain, it did not contribute to adverse metabolic changes. Sprague-Dawley rats are not predisposed to developing insulin resistance, which may have contributed to negative findings. A rat model predisposed to insulin resistance (Zucker) may be used in future research to determine the influence of neuropathic pain on phenotype expression. The study duration may have also failed to capture long-term changes. This is unlikely, as rats tested up to 6 weeks after CCI surgery did not exhibit metabolic changes.

Method selection may have also contributed to negative findings. In our first study, the insulin tolerance test (ITT) was used to measure insulin resistance. High variability among animals may have contributed to negative results. The hyperinsulinemic euglycemic clamp technique is an alternative method, but requires extensive equipment and training. Overall, the ITT was effective at detecting changes in

blood glucose during the test, albeit contradictory to our hypothesis. Negative findings related to activity in the second study were also likely due to methodological limitations.

Activity was measured using the gravimetric method in our first study. Our results indicated that rats with CCI-induced neuropathic pain had a trend of decreased activity following surgery ($p = 0.06$). This precise and reliable method allowed data collection over a long period of time, providing an accurate measurement of activity in this small sample of rats ($n = 4$). Our second study, however, involved a much larger sample ($n = 40$) and the logistical effort and financial cost of using the gravimetric method was not feasible. Instead, a direct behavioral observation method was used.

Direct observation is a simple, cost-efficient, yet reliable method for activity measurement (Choe et al., 2011). The method described in Choe was adapted for our study and rats were observed before and after surgery (CCI or SHAM). This procedure failed to provide sufficient data to detect significant differences and confirm findings from the initial study.   Furthermore, gravimetric and behavioral observation methods did not differentiate type of activity, specifically, the actual use or weight bearing of the injured hindlimb. Techniques such as the 'Cat Walk' system (Vrinten & Hamers, 2003) quantify the degree of hindlimb loading and will advance our understanding of activity/weight bearing in rats with CCI-induced neuropathic pain in future studies.

Different protocols were also used to measure thermal hyperalgesia between studies. In our first study, rats were lightly anesthetized (sodium pentobarbital, 35 mg/kg) and tested over a period of 45 minutes, with light sedation lasting up to 2 hours. In the second study, the use of light anesthesia could have potentially confounded the effects of duloxetine. Pentobarbital has been shown to depress spinal transmission of

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nociceptive information (Sandkuhler, Fu, Helmchen & Zimmerman, 1987). Though this depression may not have interfered with duloxetine, the use of pentobarbital-induced light anesthesia was not used in our second study.

Thermal testing is often done in unanesthatized animals (Berge, Garcia-Cabrera, & Hole, 1988; Isabel, Wright, & Henry, 1981; Martinez-Gomez, Cruz, Salas, Hudson, & Pacheco, 1994; Tjolsen, Lund, Berge, & Hole, 1989; Yeomans & Proudfit, 1994), but high variability in our study likely contributed to negative results.

An alternative hypothesis for our thermal testing results may relate to the use of males in the present study. Based on observations from our lab, the difference in foot withdrawal latency of the injured hindlimb between male CCI and SHAM rats was smaller than the difference between female CCI and SHAM rats. In addition, male SHAM display more hyperalgesia than female SHAM. The use of females may have improved the ability to detect significant differences between CCI and SHAM rats. However, the presence of mechanical allodynia and muscle atrophy support the presence of neuropathic pain in our second study, despite the absence of thermal hyperalgesia.

Finally, duloxetine failed to prevent changes in activity and muscle atrophy in rats with CCI-induced neuropathic pain. Current evidence supports a transient attenuation of thermal hyperalgesia and/or mechanical allodynia (Bomholt et al., 2005; Iyengar et al., 2004; Piesla et al., 2009), however, the timing between drug administration and nociceptive testing in our study was designed to minimize short-term duloxetine effects. A limitation of this approach was a failure to confirm the findings of previous work by testing rats within the immediate period following drug treatment.

Future work will build upon the findings of our research. Specifically, that rats with CCI-induced neuropathic pain develop muscle atrophy raises questions for further inquiry. Though not consistently demonstrated, CCI rats also had decreased activity, which may have contributed to muscle atrophy. Additional research with more robust methods will further characterize activity changes in CCI rats. Duloxetine treatment failed to prevent changes in activity and muscle atrophy, and the underlying mechanisms contributing to muscle atrophy in CCI rats remain unclear.

Some postulate that denervation contributes to non-load bearing muscle atrophy, while postural muscle is more susceptible to hypokinesia (Daemen et al., 1998). Our findings support this conclusion. In both studies, EDL muscles of the injured hindlimb were atrophied, supporting Daemen's conclusion. In addition, CCI rats demonstrated a trend of decreased activity in our first study, which may have contributed to SOL muscle atrophy. While the argument for denervation/hypokinesia is valid, our results bring into question the degree to which denervation and hypokinesia contribute to muscle atrophy. The additional issue of muscle loading/recruitment may be a more appropriate investigative strategy into the underlying mechanisms associated with muscle atrophy in neuropathic pain. Future study will seek to improve the characterization of activity changes and specific mechanisms contributing to muscle atrophy in rats with CCI-induced neuropathic pain.

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