THE EFFECT OF VOLUNATARY EXERCISE ON NEUROPATHIC PAIN

BY

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THE EFFECT OF VOLUNATARY EXERCISE ON NEUROPATHIC PAIN

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Abstract

Neuropathic pain results from damage to peripheral sensory axons, which can occur due to physical trauma (via nerve trauma) or metabolic diseases (via diabetes). Neuropathic pain can be a debilitating disease that often responds poorly from pharmacological interventions. The ability of exercise to reduce behavioral sensitivity associated with neuropathic pain in rodents has been reported; however, the exercise-induced mechanisms were not researched in this study. Increases in neurotrophic factors (e.g. glial cell line-derived neurotrophic factor [GDNF]) have been proposed as a possible mechanism for reducing neuropathic pain. Therefore, the purpose of this study is to determine whether voluntary exercise can attenuate or delayed mechanical sensitivity associated with neuropathic pain and assess GDNF levels as a possible mechanism. Two neuropathic pain models (SNI and streptozotocin [STZ]-induced diabetes [type 1 model]) were assessed. Following surgery and/or injections, animals in the exercised groups were housed in standard cages with voluntary running wheels while the sedentary group was housed without wheels. To quantify neuropathic pain mechanical allodynia and hyperalgesia were assessed. GDNF protein levels and mRNA expression were examined in the lumbar region of the spinal cord, dorsal root ganglion (DRG), sciatic nerve, and skeletal muscle. Our results showed that both models produced mechanical hypersensitivity. However, voluntary exercise significantly reduced mechanical hypersensitivity within 2 to 3 weeks. Within the spinal cord, the SNI surgery increased GDNF protein levels, whereas diabetes significantly decreased GDNF

levels. Voluntary exercise had no effect on GDNF levels after nerve injury; however, among diabetic animals voluntary exercise significantly increased GDNF protein levels in the spinal cord and sciatic nerve, mRNA expression in skeletal muscle, along with axonal transport in the sciatic nerve. Intrathecal injections of recombinant GDNF ameliorated mechanical sensitivity associated with diabetic neuropathy, therefore implicating the potential therapeutic actions of GDNF. In summary, voluntary exercise caused favorable changes in behavioral sensitivity in both nerve-injured and diabetic rodents. In diabetic rodents this reduction in mechanical sensitivity may be due to an increase in GDNF and axonal transport, whereas the exercise-induced mechanism after nerve injury remains unknown. This investigation supports the use of exercise to complement current treatment strategies aimed at decreasing neuropathic pain associated with peripheral nerve injury and diabetes.

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Chapter 1

Introduction

1.1 Pain

1.1.1 Epidemiology of pain

Pain, whether acute or chronic, is the most common reason for health care visits [1]. It has been estimated that pain related symptoms account for 1 in 6 visits to a healthcare provider costing upwards to $100 billion. Estimates are that 25 million Americans experience acute pain; with another 50 million suffering from chronic pain. Pain not only affects the patient but also their family, society, and the economy. It is estimated that 36 million Americans affected by pain miss nearly 4 billion work days resulting in an extensive loss of work and productivity at an estimated cost of $65 billion per year [2]. Despite its prevalence, currently there is no treatment to prevent the development or adequately control pain [3-5].

Pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” [6]. Pain is classified as either nociceptive or neuropathic. Nociceptive pain is elicited by a noxious stimulation (mechanical, chemical, or thermal) of the peripheral sensory nerves, whereas, neuropathic pain arises from injury or disease of neurons in the peripheral (PNS) or central nervous system (CNS) [7, 8]. The ability to experience pain has a protective role. It warns of any potential or actual tissue damage, which therefore elicits a reflexive response to minimize the chances of damage from occurring, however, neuropathic pain offers no known biological advantage [3].

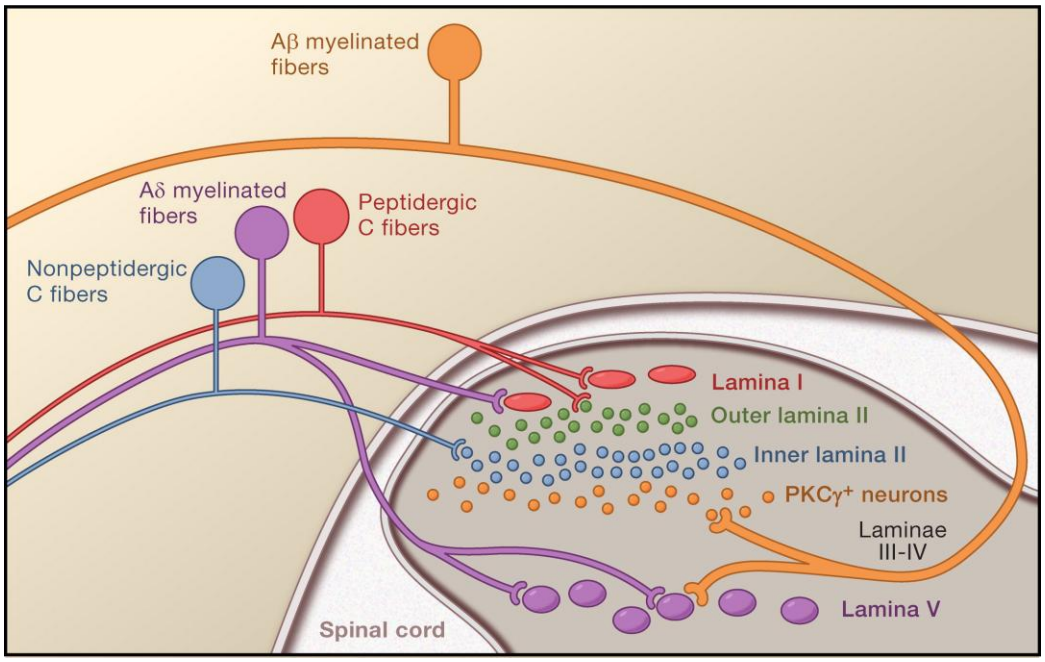
1.1.2 Nociceptors, the pain pathway, and mechanisms of pain

Nociceptors are primary sensory neurons that specialize in detecting intense thermal, mechanical, or chemical stimuli. There are two major classes of nociceptors: 1) A-delta (Aδ), which are medium diameter myelinated afferents that mediate acute, well localized “first” or fast pain, and 2) C-fibers, which are small diameter unmyelinated afferents that convey poorly localized, “second” or slow pain [9]. Another group of afferent fibers are A-beta (Aβ)-fibers. These fibers are large diameter myelinated and rapidly conducting that respond to innocuous stimulation (i.e. light touch). Nociceptors are unipolar neurons whose cells bodies are located in the dorsal root ganglia (DRG [for the body]) and trigeminal ganglion (for the face). These neurons give rise to a single axon that innervates peripheral target tissue and central axonal branch that enters the CNS to synapse on nociceptive second order neurons (interneurons) [9, 10]. Primary afferent nerve fibers project to the dorsal horn, which receives input from the periphery. Within the dorsal horn, the Aδ-fibers project to laminae I and V, C-fibers project to laminae I and II, and Aβ-fibers project deep within laminae III, IV, and V (Figure 1.1). Spinal cord neurons within lamina I generally respond to noxious stimuli, laminae III and IV non-noxious stimuli, and lamina V noxious and non-noxious stimuli [9]. Lamina II is comprised mostly of interneurons (both excitatory and inhibitory), which respond to nociceptive inputs and non-noxious stimuli.

Figure 1.1 Connections between primary afferent fibers and the spinal cord.

The unmyelinated, peptidergic C (red) and myelinated Aδ nociceptors (purple) terminate most superficially, synapsing upon large projection neurons (red) located in lamina I and interneurons (green) located in outer lamina II. The unmyelinated, non-peptidergic nociceptors (blue) target interneurons (blue) in the inner part of lamina II. By contrast, innocuous input carried by myelinated Aβ fibers (orange) terminates on interneurons in the ventral half of the inner lamina III/IV, whereas a second set of projection neurons within lamina V (purple) receive convergent input from Aδ and Aβ fibers [9]. Taken from Basbaum et al. (2009).

Figure 1.1 Connections between primary afferent fibers and the spinal cord.

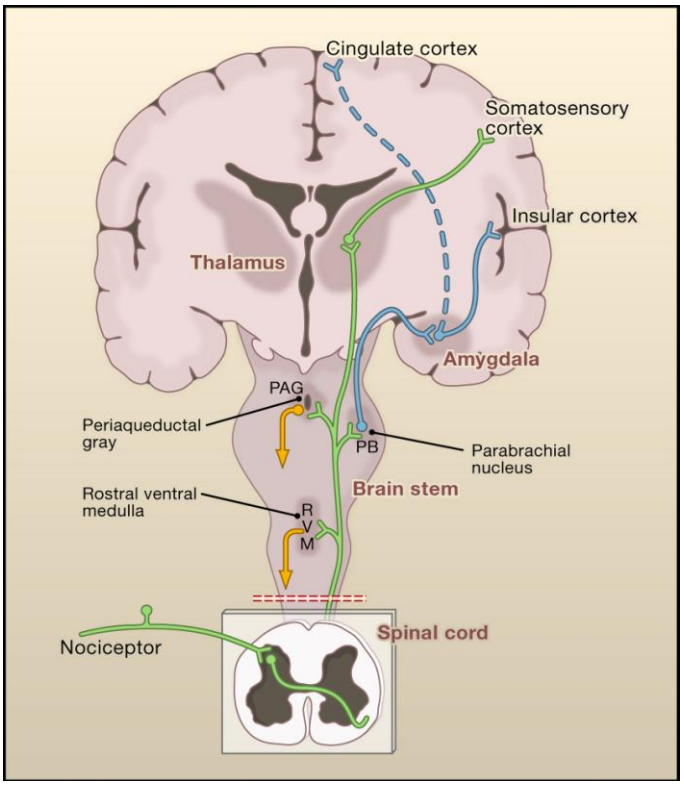


Nociceptors are excited only when stimulus intensities reach the noxious range [9]. After excitation, the peripheral terminal of the nociceptor transduces the external stimuli and initiates an action potential, which is conducted by the axon. The cell body in the DRG controls the identity and integrity of the neuron, which then relays this information to the central terminal (located in the dorsal horn), where it is transferred through the release of a variety of neurotransmitters, such as glutamate or substance P, to second order neurons at central synapses [10]. Axons of the second order neurons in the dorsal horn transfer this information across the anterior white commissure (midline of spinal cord) to the contralateral side of the spinal cord, where it ascends in the anterolateral white matter to the brainstem and thalamus. Within the thalamus, nociceptive information is encoded regarding to the type, intensity, and location of the pain. Next, the second order neurons synapse with third order neurons, within the thalamus, which then transmit the information to the somatosensory cortex where it is integrated and undergoes cognitive and emotional interpretation of the painful stimulus [2]. This sensory pathway is known as the spinothalamic pathway (Figure 1.2). The projection neurons that form this pathway originate predominantly in laminae I, II, and V of the spinal dorsal horn. This pathway relays information about pain and temperature.

The job of the nociceptive system is to enable and enforce protective behavioral responses such as withdrawal or avoidance of any painful stimuli. In case of an injury, the affected tissue becomes more vulnerable, therefore the

Figure 1.2 Anatomy of the pain pathway. Primary afferent nociceptors convey noxious information to projection neurons within the dorsal horn of the spinal cord. A subset of these projection neurons transmits information to the somatosensory cortex via the thalamus, providing information about the location and intensity of the painful stimulus. Other projection neurons engage the cingulate and insular cortices via connections in the brainstem (parabrachial nucleus) and amygdala, contributing to the affective component of the pain experience. This ascending information also accesses neurons of the rostral ventral medulla and midbrain periaqueductal gray to engage descending feedback systems that regulate the output from the spinal cord [9]. Taken from Basbaum et al. (2009).

Figure 1.2 Anatomy of the pain pathway.



nociceptive system adapts by locally lowering nociceptive thresholds and facilitating nociceptive responses. This adaptation enhances the vulnerability of the tissue, thereby ensuring adequate tissue protection. These behavior adaptations result in the development of allodynia (painful response to non-noxious stimuli) and hyperalgesia (exaggerate painful response to noxious stimuli). Allodynia and hyperalgesia are an appropriate shift in pain threshold to prevent further tissue damage. However, allodynia and hyperalgesia may persist long after the initial cause of injury, possibly due to dysfunction of parts of the PNS and CNS. Therefore, allodynia and/or hyperalgesia, becomes maladaptive rather than protective, and the pain is no longer a meaningful homeostatic factor or symptom of a disease but rather a disease on its own [6].

Aβ-fibers are thought to contribute to allodynia symptoms seen in pain patients. This is facilitated through several mechanisms: 1) Aβ-fibers sprout to deeper areas of laminae II, which is the area that C-fibers normally occupy; and 2) Aβ-fibers undergo a switch in their phenotype (due to inflammation) and begin synthesizing substance P [6, 11]. Nerve injury causes degeneration of central terminals of C-fibers in lamina II, thus depriving pain transmission neurons in the dorsal horn of nociceptive input. The degeneration of C-fibers triggers surviving Aβ-fibers in laminae III and IV to sprout into areas in lamina II, which are normally occupied by C-fibers and potentially establish contacts with deafferentated pain transmission neurons. Further compounding the situation, Aβ-fibers undergo a phenotypic transformation to a substance P-synthesizing mode, thereby

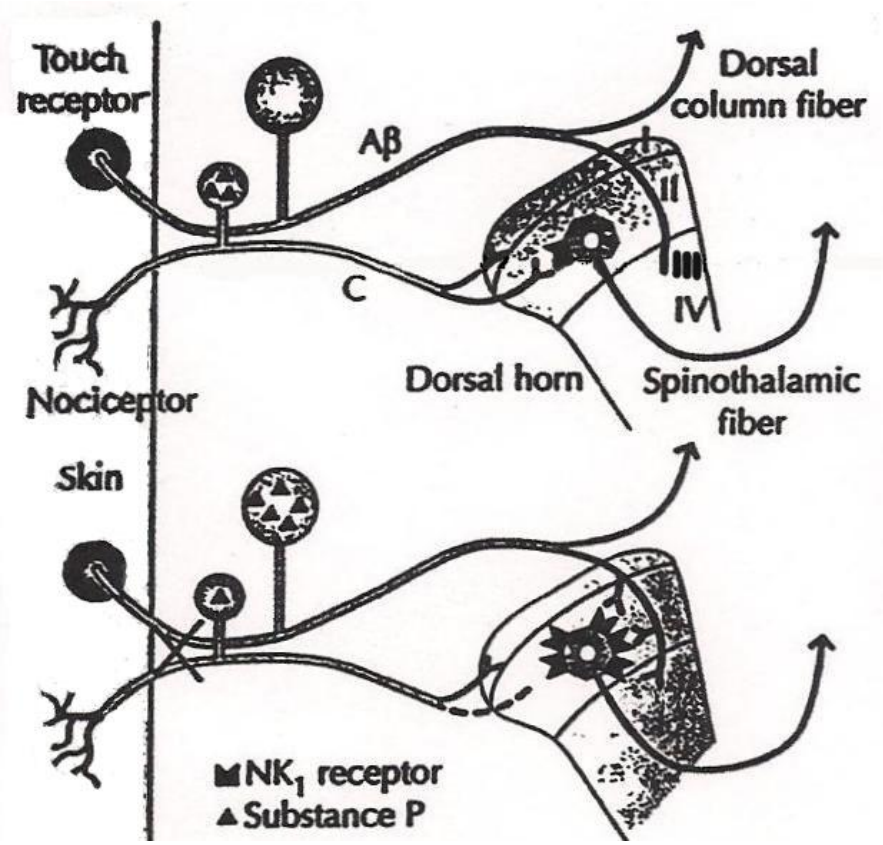
enhancing the responsiveness of spinal nociceptive neurons [6, 12] (Figure 1.3). Therefore, the reorganization and phenotypic changes associated with nerve injury allows non-noxious stimuli to reach lamina II (a pain transmission region of the spinal cord), resulting in light touch becoming transmissible as pain also known as mechanical allodynia [11].

Hyperalgesia can be classified as primary or secondary hyperalgesia. Primary hyperalgesia occurs at the site of the injury, and is mainly due to the sensitization of nociceptive nerve endings. This is typically reflected by an increase response to heat stimuli [13]. Secondary hyperalgesia occurs in the uninjured tissue surrounding the site of the injury. This form of hyperalgesia is not caused by the sensitization of the nociceptive nerve endings, but due to changes in the processing of sensory information in the CNS [6]. This form of hyperalgesia is characterized by an increased response to mechanical stimuli, rather than thermal [13]. Therefore, hyperalgesia can arise from both peripheral and/or central mechanisms (also known as peripheral and central sensitization).

Peripheral sensitization is produced when nociceptor terminals become exposed to products of tissue damage and/or inflammation [14]. Peripheral nerve injury provokes recruitment and activation of immune cells at the site of the nerve injury. Immediately after nerve injury, a release of inflammatory mediators by activated nociceptors or non-neural cells (mast cells, basophils, platelets, macrophages, neutrophils, endothelial cells, keratinocytes, and fibroblasts) that

Figure 1.3 Nerve injury-induced structural and neurochemical reorganization. Under normal conditions (top), myelinated Aβ-fibers terminate in lamina III/IV, whereas C-fibers terminate in lamina I/II. Substance P is expressed only in unmyelinated or thinly myelinated afferent fibers. After nerve injury (bottom), Aβ-fibers sprout into the lamina II region vacated by central terminals of C-fibers; large DRG neurons express substance P. Consequently, non-noxious stimuli can activate pain transmission pathways, resulting in mechanical allodynia in patients with neuropathic pain [12]. Taken from Taylor (2001).

Figure 1.3 Nerve injury-induced structural and neurochemical reorganization.



reside within or infiltrate into the injured area. Collectively, these factors release signaling molecules what are known as the “inflammatory soup” [9, 10, 15], such as neurotransmitters, neuropeptides (calcitonin gene-related peptide [CGRP], substance P, bradykinin), eicosinoids and related lipids (prostaglandins, thromboxans, leukotrienes, endocannabinoids), cytokines, chemokines, and extracellular proteases. Nociceptors express one or more cell-surface receptors capable of recognizing and responding to each of these proinflammatory or proalgesic agents, thus enhancing the excitability of the nerve fiber and heightening its sensitivity to temperature and touch [9]. Along with inflammatory responses, the Schwann cells release chemical signals that promote axonal growth (nerve growth factor [NGF]), which is retrogradely transported to the cell bodies of primary sensory neurons [16]. In the nucleus of the nociceptor, NGF promotes the increase of pronociceptive proteins (substance P, TRPV1, and the Nav1.8 voltage-gated sodium channel subunit) [17, 18]. Together, these changes enhance the excitability of the nociceptor and amplify the neurogenic inflammatory response.

Central sensitization is defined as “an increased responsiveness of nociceptive neurons in the CNS to their normal or subthreshold afferent input” [6]. When neurons in the dorsal horn of the spinal cord are subject to central sensitization, they may develop an increase in spontaneous activity, a reduction in the threshold for activation by peripheral stimuli, or an enlargement of their receptive fields [19]. The mechanisms for central sensitization include an

alteration in glutamatergic neurotransmission/ N-methyl-D-Aspartate (NMDA) receptor-mediated hypersensitivity and loss of tonic inhibitory control (discussed in Modulation of pain section) [9]. Glutamate is the main neurotransmitter in nociceptors. It binds to several receptors on postsynaptic neurons in the dorsal horn of the spinal cord, including ionotropic amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), NMDA, and glutamate receptor subtypes (mGlur). The NMDA receptor is one of the main triggers and effectors of central sensitization. Under resting conditions the NMDA receptor channel is blocked in a voltage-dependent manner by a magnesium (Mg^{2+}) ion sitting in the receptor pore. However, sustained release of glutamate and neuropeptides (substance P and CGRP) leads to sufficient membrane depolarization that forces Mg^{2+} to leave the NMDA receptor pore. Removal of Mg^{2+} allows entry of calcium into the neuron, which activates numerous intracellular pathways (phospholipase C/protein kinase C [PLC/PKC], phosphatidylinositoal-3-kinase [PI3K], and mitogen-activated protein kinase [MAPK] pathways) that contribute to central sensitization [19]. As injured tissue heals, the sensitizations induced by peripheral and central mechanisms are relatively short lasting and reversible. However, sensitization becomes pathological when the afferent fibers or central pathways become damaged, which can occur in pathological conditions such as diabetes, shingles, multiple sclerosis, etc., ultimately leading to neuropathic pain.

Many Aδ- and C-fibers display somatic sodium channels. These sodium channels include the sensory nerve specific (SNS, also known as PN3) and

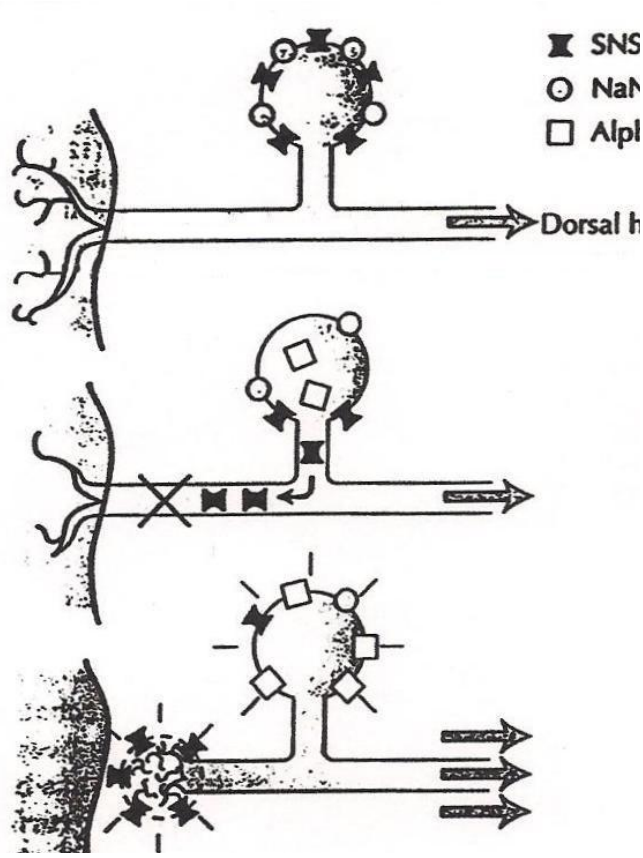
sensory nerve specific 2 (SNS2, also known as NaN) sodium channels, which are tetrodotoxin (TTX)-resistant sodium currents, and alpha-III sodium channels (TTX-sensitive sodium currents). Voltage-gated sodium channels generate and propagate action potentials in excitable cells. Nerve injury can result in an abnormal expression of peripheral sodium channels (Figure 1.4). Nerve injury produces a painful state due to an increase in excitation of sodium channels. After nerve injury there is a down regulation of the SNS and NaN sodium channel genes in the soma, and SNS is translocated to the site of the injury. After the down-regulation of SNS and NaN sodium channels, the expression of alpha-III sodium channel gene is upregulated in the DRG. These changes in sodium channel gene expression produces electrophysiological changes in the DRG neurons causing them to fire spontaneously or at inappropriate high frequencies [12, 20].

1.1.3 Modulation of pain

Spinal nociceptor neurons are under constant and powerful inhibitory control that processes sensory information in the dorsal horn for normal pain perception. The inhibitory system serves to maintain proper nociception by attenuating the responses of nociceptive neurons by maintaining proper response levels during nociception. This is obtained by, muting nociceptive neurons in the absence of noxious stimuli, preventing crosstalk between sensory modalities by separating labeled lines for nociceptive and non-nociceptive, and

Figure 1.4 Sodium channel-mediated ectopic activities. Under normal conditions (top), the SNS (hourglass shapes) and NaN (circles) are expressed on the cell membrane of DRG neurons. After nerve transaction (middle), the alpha-III sodium channel (squares) is expressed and SNS is translocated from the DRG soma to the site of injury and neuroma formation (marked by an X). As result (bottom), alpha-III accumulates at the DRG, and SNS accumulates at the neuroma (dashed circles), leading to ectopic activity at both sites (flare, denoted by short lines about the neuroma and cell body) [12]. Taken from Taylor (2001).

Figure 1.4 Sodium channel-mediated ectopic activities.



lastly, limiting the spread of excitatory stimuli to somatotopic areas of the central nervous system [6].

The brain has modulatory circuits whose main function is to regulate the perception of pain. As the ascending nociceptive information passes through the brainstem and reaches the brain, a network of brainstem structures and pathways that exert a modulatory effect on nociceptive transmission is activated [2]. The initial site of modulation is in the dorsal horn of the spinal cord. The descending modulatory effect is applied by inhibiting the release of neurotransmitters from the primary afferent fiber or by inhibiting the function of the neurotransmitter receptor on the postsynaptic neuron [2], thus controlling the transmission of nociceptive information to the higher regions of the brain. Descending control of spinal nociception arises from a number of supraspinal sites. Periaquaductal gray (PAG) has been established as a major component of the pain modulatory circuitry. PAG surrounds the cerebral aqueduct in the midbrain. It is heavily interconnected with the hypothalamus and limbic forebrain structures. The PAG projects to the rostral ventromedial-medulla (RVM), which in turn sends its output to terminals in laminae I, II, and V of the dorsal horn that are important in nociceptive function. The PAG-RVM system plays a pivotal role in organizing strategies for coping with intrinsic and extrinsic stressors, and is the central site of action of analgesic agents such as opioids, cyclooxygenase inhibitors, and cannabinoids [21]. This system acts as a negative feed-back loop for the control of the nociceptive input [5].

Nerve injury disrupts this endogenous pain inhibitory system by inducing changes in the spinal gamma-aminobutyric acid (GABA)-nergic and glycine systems. The major fast inhibitory neurotransmitters in dorsal horn of the spinal cord are GABA and glycine. GABA and glycine are coreleased at inhibitory synapses laminae I and II of the spinal cord [6]. Upon binding to its receptor, GABA activates chloride-permeable ion channels, which hyperpolarize neurons and impair the dendritic propagation of excitatory signals [22]. However, diminished GABA- and glycine-mediated inhibition in the dorsal horn of the spinal cord leads to pathologic pain in various states. For example, in animals with peripheral nerve injury (unilateral sciatic nerve ligation) that exhibited thermal hyperalgesia, spinal cord levels of GABA transporter protein (GAT-1) were reduced by 37% compared to controls [23]. Also, blocking the GABA and glycine receptors in the spinal cord can induce mechanical allodynia in uninjured animals [24]. Therefore, GABA and glycine play an important role in modulating nociceptive input in the dorsal horn of the spinal cord.

1.1.4 Management of pain

To date, there is no treatment that prevents the development of or sufficiently controls symptoms associated with neuropathic pain [3]. It has been estimated that 66% of patients with neuropathic pain do not experience sufficient pain relief [23]. The overall goal of pain management is to return the individual back to a productive life. There are two approaches used in order to obtain this goal: 1) pharmacological treatment strategy and 2) non-pharmacological

treatment strategy. There is a collection of drugs, for which randomized control clinical trials have shown efficacy in managing neuronal hyperexcitability in neuropathic pain. These include antidepressants, anticonvulsants, opioids, NMDA antagonists, non-steroidal anti-inflammatory drugs (NSAIDs), and topical. However, the efficacy of pharmacological treatment for neuropathic pain is still insufficient. As consequence, numerous non-pharmacological treatment strategies have been developed in order to reduce symptoms associated with neuropathic pain. For example, Figure 1.5 describes a survey of 22 non-pharmacological treatments for pain that was mailed to 732 practitioners, which were members of the International Association for the Study of Pain [25]. In this survey, practitioners were asked to list in order the non-pharmacological therapy treatment that they most frequently or referred their patients to participate in. Seventy-two percent of practitioners recommended the use of exercise to their patients in order to treat neuropathic pain symptoms. Of these practitioners, 80% believed that exercise should be part of legitimate medical practice. Surveys have shown that patients who participate in exercise to treat their neuropathic pain symptoms, rate exercise to be an effective non-pharmacological treatment. [26]. However, according to the survey conducted by Berman and Bausell (2000), only 59% of practitioners reported having sufficient knowledge to discuss the use of exercise as a therapy treatment with their patients. Therefore, knowledge of the effectiveness of exercise for treating neuropathic pain related symptoms is clearly warranted.

Figure 1.5 Non-pharmacological therapies. Proportion of respondents who A.) used non-pharmacological therapies in their practice or referred their patients to other providers, B.) reported sufficient knowledge to discuss therapies with patients, and C.) considered therapies a part of legitimate medical practice (n=362) [25]. Taken from Berman and Bausell (2000).

Figure 1.5 Non-pharmacological therapies.

	Proportion reporting use or patient referral	Proportion reporting enough knowledge to discuss with patient	Proportion considering therapy legitima medical practice
Counseling/psychotherapy	0.81	0.59	0.89
Electromagnetic applications: (TENS, magnets)	0.77	0.65	0.84
Exercise intervention	0.72	0.59	0.80
Acupuncture	0.69	0.70	0.84
Biofeedback	0.66	0.62	0.83
Behavioral medicine	0.62	0.57	0.78
Dietary prescription	0.59	0.33	0.71
Relaxation response techniques	0.50	0.43	0.61
Manipulation therapy (non-chiropractic)	0.46	0.49	0.52
Hypnotherapy	0.45	0.38	0.64
Chiropractic	0.40	0.44	0.55
Meditation	0.31	0.36	0.45
Prayer & spiritual direction	0.27	0.23	0.31
Herbal/botanical medicine	0.19	0.18	0.27
Tai Chi	0.18	0.17	0.21
Art therapy	0.14	0.14	0.31
Homeopathy	0.13	0.17	0.18
Megavitamins	0.13	0.18	0.16
Clinical ecology/environmental medicine	0.12	0.14	0.30
Neural therapy	0.11	0.11	0.13
Aromatherapy	0.04	0.09	0.06
Qi Gong	0.04	0.04	0.06

Neuropathic pain, often responds poorly to pharmacological interventions. As a non-pharmacological approach, several studies have investigated the effects aerobic exercise on symptoms associated with chronic or neuropathic pain. Exercise was shown to have beneficial actions on patients that suffered from chronic pain symptoms associated with fibromyalgia [27], chronic neck pain [28], chronic low back pain [29] and in animals experiencing peripheral neuropathic pain [30], spinal cord injury [31], and chronic muscle pain [32]. These studies have shown that acute and chronic aerobic exercise can improve pain thresholds with chronic and/or neuropathic pain, and in humans the reduction in symptom severity can last as long as 1 year after cessation of the exercise training. The analgesia experienced in these studies is believed to be exercise-induced.

Exercise-induced analgesia is poorly understood; however, there are several plausible mechanisms that may exist. Mechanisms involved include the opioid and non-opioid systems. As reviewed by Koltyn (2000), the release of endogenous opioids is the most commonly tested and proposed mechanism for exercise induced analgesia in healthy individuals. The same mechanism was reported to produce analgesic effects in chronic muscle pain after acute bouts of exercise. As reported by Bement and Sluka, exercise increased mechanical thresholds in rats with chronic muscle pain. However, these anti-allodynic effects of exercise were reversed with naloxone (an opioid antagonist), suggesting the activation of the endogenous opioid system even at this low intensity [33].

Endogenous opioids suppress calcium influx and the excitation and/or neurotransmitter release in many neuronal systems [34].

As for the non-opioid systems, neurotrophins have been examined for its potential involvement in analgesic responses that occur following exercise. Neurotrophins (brain derived neurotrophic factor [BDNF], NGF, and neurotrophin-3 [NT-3]) are a family of structurally and functionally related peptides that mediate potent survival and differentiation effects on a wide variety of neuronal populations in the CNS and PNS [35]. Extensive research has attributed neurotrophins playing an important role in modulating pain. Recently, numerous studies have shown that aerobic exercise, whether forced (via treadmill) or voluntary (via free-access running wheels), can increase neurotrophin expression [31, 36-42]. Aerobic exercise (via treadmill) has been shown to attenuate allodynia in spinal cord injured animals. This amelioration of allodynia was attributed to increases in BDNF in the lumbar region of the spinal cord and soleus muscle, which correlated with pain threshold levels. The modulatory effect seen in this study may be due to the ability for BDNF to increase β-endorphins in the PAG and dorsal spinal cord [43]. BDNF expressing neurons are present in all portions of the PAG and RVM-projecting PAG neurons [44]. Studies have shown that administration of BDNF into the PAG produces analgesia in rats [44, 45]. This is likely due to the downregulation of the tyrosine kinase receptor B (TrkB [contributes to pain facilitation]) signaling in the RVM neurons, which produces descending inhibition of nociceptive inputs potentially through the activation of

analgesic agents such as opioids (β-endorphins), thus inhibiting the transmission of pain [44, 46].

1.2 Diabetic neuropathy

1.2.1 Epidemiology of diabetic neuropathy

Neuropathy is a common secondary complication of diabetes. Currently, 20.8 million people in the United States are affected by diabetes, and by the year 2010 it is predicted to increase to approximately 220 million people worldwide. Recently, a population-based study reported that more than half of patients who have type 1 and 2 diabetes develop diabetic neuropathy (DN) [47]. Of these patients with DN, 15% to 30% suffer from painful diabetic neuropathy, whereas the remainder experiences a negative phenomenon, such as numbness [48]. Clinical symptoms associated with DN involve poor gait and balance associated with large sensory fibers, and abnormal cold and/or heat sensation associated with small sensory fibers. Chronic pain associated with diabetes is represented by hyperalgesia, allodynia, parethesias and spontaneous pain [49, 50]. Symptoms are described as prickling, tingling, “pins and needles”, burning, crawling, itching, abnormal sensation to temperature, and pain. Over time these symptoms may advance from the toes up the foot and leg and may involve the fingers and hands [51]. DN is associated with structural changes in the peripheral nerve that includes axonal atrophy, demyelination, loss of nerve fibers, and the blunted regeneration of the nerve fibers [52-54]. Nerve fibers maintenance in the periphery is balanced between nerve degeneration and regeneration under non-

neuropathic conditions, however if nerve degeneration exceeds nerve regeneration then fiber loss may progress. Therefore, the progressive nerve fiber loss in DN may be due to an impaired ability of the diabetic nerve to regenerate in response to the degenerative process [53].

1.2.2 Pathogenesis of diabetic neuropathy

In diabetic patients, the development and progression (distal to proximal direction and is axon length-dependent) of DN is thought to be mainly due to increased blood glucose concentrations (hyperglycemia). A persistent elevation in blood glucose leads to glucose toxicity [55]. Glucose toxicity contributes to β-cell dysfunction and the pathology of other complications (e.g. DN) associated with diabetes [55]. The pathogenesis of DN includes increased polyol pathway activation, advanced glycation end products (AGE), activation of PKC, poly(ADP-ribose) polymerase pathway (PARP), vascular insufficiency, and a reduction in neurotrophic factors (i.e. GDNF) [49, 50, 53]. These mechanisms lead to increases in oxidative stress and nerve dysfunction, which results in disturbances in the nerve degeneration/regeneration process.

In diabetes, hyperglycemia causes a 4-fold increase in neuronal glucose levels. If an elevation in blood glucose continues, then intercellular glucose metabolism leads to neuronal damage known as glucose neurotoxicity [56]. Axons are vulnerable to hyperglycemic damage due to their direct access to nerve blood supply [49]. Due to metabolic changes that occur in diabetes, C-

fibers have been proposed to be the most susceptible to damage because they are not protected with a myelin sheath. Damage to the C-fibers can lead to neuropathic pain due to mechanisms that were described earlier, such as sprouting and phenotypic changes of Aβ-fibers, alterations in sodium channels in the DRG, and increases in glutamate activity.

1.2.3 Management of diabetic neuropathy

Therapies for DN are divided into treatments that target the underlying pathogenic mechanisms and those aiming to relieve symptoms [49]. Pathogenic mechanism includes strict glycemic control through continuous glucose monitoring, whereas positive and negative symptoms associated with DN are treated with one or more medications [57]. Pharmacological therapies include treating the symptom itself or the cause of the DN in an attempt to reverse DN by diminishing the biochemical aberrations which induce neuronal damage [49]. However, the only proven method currently available to prevent DN and/or slow the progression of this disease is strict glycemic control [58]. Therefore, other therapy treatments need to be explored, such as exercise which has shown to be able to improve glycemic control [59].

Exercise has long been recognized as a part of therapy in the management of diabetes, yet many persons with diabetes fail to participate in basic physical activity due to secondary diabetic complications (i.e. DN). Recent studies have shown that exercise has beneficial effects on painful symptoms in

the setting of diabetes. In Type 1 and 2 diabetic patients who did not display any signs or symptoms of DN, aerobic exercise (brisk walking on a treadmill) reduced the onset of both motor (0% versus 17%) and sensory (7% versus 30%) neuropathy compared to sedentary diabetic patients [60]. As for patients with impaired glucose tolerance, lifestyle intervention (consisted of diet and exercise counseling) can also improve pain symptoms [61]. These improvements in pain thresholds may be due to the ability of exercise to increase myelin sheath thickness, which were seen in exercised diabetic animals that exhibited improvements in axonal and motor function [62]. These studies have shown that exercise can prevent the development DN and increase pain thresholds in patients with impaired glucose tolerance; however, the efficacy of exercise exerting a similar effect in diabetic patients with signs and symptoms with DN has yet to be explored.

1.3 Glial cell line-derived neurotrophic factor (GDNF)

GDNF is a neurotrophic factor that belongs to the transforming growth factor-β superfamily. GDNF specifically binds to GDNF family receptor α (GFRα), which then forms a complex with a receptor tyrosine kinase RET. RET can activate several intracellular signaling cascades (MAPK, PI3K, and phospholipase Cγ [PLC-γ]) (Figure 1.6), which regulate cell survival, differentiation, proliferation, migration, chemotaxis, branching morphogenesis, neurite outgrowth, and synaptic plasticity [63, 64]. GDNF has a trophic effect in the CNS, peripheral sensory, sympathetic, and spinal motor neurons [65].

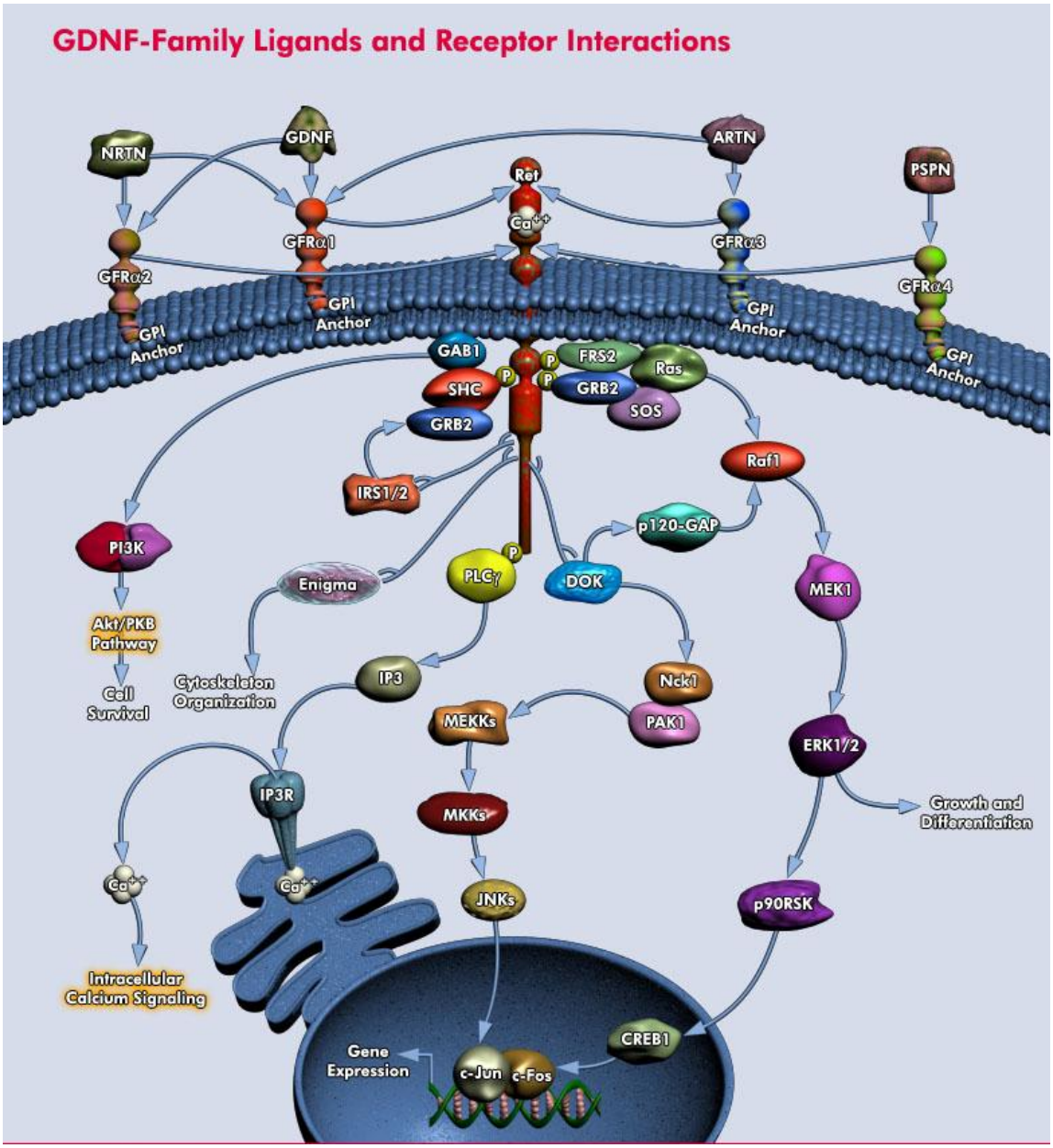
Varieties of tissues synthesize and secrete GDNF [63]. GDNF expression has been shown in various regions of the CNS [66, 67], PNS [66], and peripheral tissues [68, 69]. GDNF has been identified in the striatum, hippocampus, cortex, cerebellum, and spinal cord, Schwann cells, and DRG. It is believed that the Schwann cells are the major source of GDNF in the PNS [70], however it has also been shown to be produced by the glial satellite cells in the DRG neuron [71]. GDNF expression has also been identified in many peripheral non-neuronal tissues, such as the lung, liver, and ovary of adult rats [68] and the skeletal muscle of humans [69]. These findings provide evidence that GDNF is detectable in a number of nervous system structures and other non-neuronal tissues.

1.3.1 GDNF and pain

GDNF has been hypothesized to play an important role in the modulation of nociceptive signals especially during neuropathic pain states. However, the expression of GDNF in models of neuropathic pain has yielded contradictory results. Recent studies have shown that nerve injury (via chronic constrictive injury and spinal nerve ligation models) diminishes GDNF and RET expression in the sciatic nerve and DRG [72, 73]. Nagano et al. (2003) reported that in animals experiencing neuropathic pain, within 2 weeks GDNF and RET expression can be reduced by 40% in the DRG. On the contrary, studies have shown that transection of the sciatic nerve can result in increases in GDNF and its receptor GFRα-1 in the sciatic nerve and DRG. [74, 75]. After nerve injury, GDNF has been reported to increase 20-fold in the sciatic nerve [75] and these

Figure 1.6 GDNF-family ligands and receptor interactions. GDNF binds GFRα, which then forms a complex with RET; therefore, activating several intracellular signaling cascades, such as MAPK, PI3K, and PLC-γ. Taken from https://www1.qiagen.com/Geneglobe/PathwayView.aspx?pathwayID=197.

Figure 1.6 GDNF-family ligands and receptor interactions.



changes in expression can be affected for as long as 5 months [74]. Reasons as to contradictory results in GDNF expression after nerve injury remains unknown, however it may be due to the type of nerve injury model (chronic constrictive injury and spinal nerve ligation versus nerve axotomy) [73] or it may be associated with differences in activities of primary afferents between models [72]. However, these results suggests that GDNF is an important factor in the events following peripheral nerve injury, and the possible dysfunction of GDNF signaling in the nociceptive afferent system may contribute to the development and/or maintenance of neuropathic pain states.

GDNF has been shown to have potent analgesic effects in the state of neuropathic pain. Studies have reported that the administration of GDNF can ameliorate behavioral sensitivity associated with neuropathic pain [72, 76-79]. Possible mechanisms for attenuating behavioral sensitivity associated with neuropathic pain include promoting the survival of non-peptidergic nociceptive C-fiber neurons (isolectin B4 [IB4] binding), thereby decreasing Aβ-fiber sprouting [80], regulating sodium channels [77], and by regulating neuropeptides [76].

C-fiber nociceptors can be divided into two groups; 1) peptidergic (depends on NGF for survival), and 2) non-peptidergic (depends on GDNF for survival) [81]. Non-peptidergic C-fibers comprise of about 40% of the cells in the DRG. Nerve injury has been shown to reduce C-fibers by 50% [80]. This

reduction in C-fibers induces sprouting of myelinated sensory afferents (Aβ-fibers) into laminae I and II. However, administration of GDNF has been shown to rescue the reduction of C-fiber neurons in the DRG, thus preventing the sprouting of Aβ-fibers [80].

SNS and NAN sodium channels are expressed in 52% and 72% of IB4 binding C-fibers, respectively [82]. As described earlier, spontaneous activity of the nociceptor is due to the down-regulation of SNS and NaN sodium channels and up-regulation of alpha-III sodium channels [20]. In vitro and in vivo studies have demonstrated that administration of GDNF restores SNS and NaN sodium channel levels [77, 82] and suppress alpha-III sodium channel levels in the DRG [77]. This restoration of sodium channel function reduces ectopic discharges; therefore mechanical and thermal thresholds are reverted to normal levels in nerve injured animals [77].

A subpopulation of IB4 binding C-fiber neurons expresses the neuropeptide somatostatin. Somatostatin can inhibit the pronociceptive peptides, substance P and CGRP, from primary afferents [83]. Using the spared nerve injury (SNI) model, Adler et al. 2009, reduced mechanical allodynia by locally applying GDNF to the injured site of the sciatic nerve. They associated these reductions in allodynia to increases in prosomatostatin levels in the DRG. In vitro, they confirmed this assumption by showing that by exposing DRG neurons directly to GDNF, somatostatin increased nearly 3-fold, which lead to an

increased secretion of potassium. Potassium plays a key role in establishing the resting membrane potential and in the repolarization phase of action potentials in neurons. To date, GDNF is the only neurotrophic factor that has been found to stimulate somatostatin expression [76]. Together, these results suggest that GDNF may be a viable treatment for attenuating behavioral sensitivity associated with neuropathic pain.

1.3.2 GDNF and diabetic neuropathy

DN is associated with impaired neurotrophic support. Many studies have examined how diabetes alters neurotrophins (NGF, BDNF, NT-3, etc.) in the setting of DN; however, little is known about how diabetes affects GDNF production and its contribution to DN. Recent studies have reported that diabetes alters GDNF expression in central and peripheral tissues [84-87]. GDNF has been implicated to play an important role in the pathogenesis of DN. It is well know that diabetes induces alterations in electrophysiological functions that results in reductions in nerve conduction velocity. In diabetic rats with neuropathic symptoms (decreased nerve conduction velocity), GDNF is decreased. However, following GDNF gene delivery, these neuropathic symptoms associated with diabetes were alleviated [54]. This may be due to the restorative effects GDNF has on degenerating nerves. Earlier, it has been shown that administration of GDNF stimulated axon growth and branching in diabetic mice, thus improving innervations deficits caused by diabetes [84]. These results suggest that diabetes may alter GDNF expression and reductions in GDNF may

be associated with neuropathic pain, thus GDNF may play a role in attenuating behavioral sensitivity linked to DN.

1.3.3 GDNF and exercise

Numerous studies have reported that acute and chronic bouts of exercise increase neurotrophins (BDNF, NGF, and NT-3) in both injured and non-injured animals [31, 36-39, 41, 42]. These neurotrophins have reportedly been increased in various neuronal (spinal cord and brain) and non-neuronal (skeletal muscle) tissues. Few studies have assessed the effects exercise has on GDNF expression; however, studies have shown that acute and chronic exercise can alter GDNF expression in various neuronal and non-neuronal tissues [40, 88-90]. GDNF has been assessed in injured and non-injured animals and also Parkinson diseased animals following exercise. In non-injured rats, GDNF protein levels were assessed in the cervical region of the spinal cord [89] and in skeletal muscle (soleus, gastrocnemius, and pectoralis major) [90]. Forced exercise (via treadmill) increased GDNF protein levels in skeletal muscle, whereas, GDNF levels in the cervical region of the spinal cord remained unchanged. However, non-injured animals who were subjected to hindlimb unloading (suspension from hindlimb) exhibited significant decreases in GDNF levels of the soleus and gastrocnemius, suggesting that activity levels may modulate GDNF protein levels in skeletal muscle. In animals with spinal cord injury (transection of thoracic [T10]) and Parkinson disease (a neurodegenerative disease in the CNS), forced exercise (via motorized cycle apparatus and treadmill, respectively) increased

GDNF gene expression and protein levels, respectively, in the soleus muscle and striatum, respectively. In both animal models, exercised animals showed a better functional and behavioral recovery, which were attributed to increases in GDNF [40, 88]. Suggesting that increases of GDNF with exercise may exert a neuroprotective effect in the CNS. All the above reports demonstrated that forced exercise increases GDNF both centrally and peripherally, and these increases in GDNF may have beneficial effects on motor control; however further research is needed to determine whether the same can be seen in sensory mechanisms.

1.4 Axonal transport

Axonal transport is a neuronal mechanism by which various molecules are transported to maintain normal metabolism, physiological activity, and structural integrity [91]. The transfer of information between neurons is bi-directional (anterograde and retrograde transport). Anterograde transport maintains the structural and functional integrity of the axon, whereas retrograde transport serves as a feedback system to the cell body about the status quo pertaining to the peripheral parts of the axon [92]. Proteins can be internalized at the axon terminals (and be involved in retrograde transport) or at the somatodendritic membranes (and be involved in anterograde transport). After internalization, proteins are sorted into one of several subcellular pathways: 1) they may be recycled to the surface membrane, either as a ligand-receptor complex, or as a receptor only after dissociation of ligand and receptor; 2) upon internalization of trophic factors at the axon terminal, the ligand-receptor complex can be sorted

into a transport vehicle, vesicle, or endosome, for retrograde transport; 3) upon arrival at the cell body or after internalization at somatodendritic domains, proteins can be sorted to the Golgi system for anterograde transport; 4) upon internalization at the axon terminal and retrograde axonal delivery to the cell body, proteins may be directed to dendrites at the dendritic membranes to be packaged for release at synapses; and 5) instead of routing into recycling pathways, proteins may be targeted for degradation via endosome/lysosome or via proteasome pathways. Once the internalized the protein is then transported to the axon terminals (via anterograde transport) or to the somatodendritic domains (via retrograde transport) [93].

Retrograde transport for neurotrophic factors is achieved through endosomal trafficking. This comprises of the neurotrophic factor being taken up by receptor mediated endocytosis into clathrin-coated vesicles. These vesicles fuse with early endosomes in the axon terminal, which then travels retrogradely along axonal microtubules using dynein motor proteins. Upon reaching the cell body, the transport vesicles fuse with late endosomes and finally to lysosomes [94]. After internalization, proteins that are destined for anterograde axonal transport pass through the Golgi apparatus where they are then packaged into large dense core vesicles. Once the vesicle that contains the neurotrophic factor is properly packaged and loaded onto the anterograde machinery, kinesin motor proteins transport the neurotrophic factor to the axon terminus. After antero- and

retrograde axonal transport the protein can then be released from the neuron [93].

1.4.1 GDNF/ neurotrophin axonal transport and diabetes

Studies have reported that GDNF can be transported anterogradely [95, 96] and retrogradely [97] within neurons. Anterograde transport of GDNF has been reported in the sciatic nerve [95] and the hypoglossal nerve [96] and retrograde transport has been shown to occur in the motor neurons [97]. Studies have revealed that GDNF production is decreased in peripheral tissues of diabetic mice [54, 84]. Therefore, the availability of GDNF through axonal transport is important for the survival of motor and sensory neurons. GDNF axonal transport in diabetic animals has yet to be explored. However, several studies have shown that diabetes reduces axonal transport of neurotrophins (BDNF, NGF, and NT-3) in the sciatic [98-100] and cervical vagus [101] nerves. Reports have shown that diabetes reduces endogenous neurotrophin transport both anterogradely and retrogradely [98, 100] and this reduction can be up to 50% [99]. However the mechanisms of diabetes-induced diminished neurotrophin support are not known. Possible mechanisms for diabetes-induced reduction in the transport of neurotrophins include: 1) alterations in access to transportable neurotrophins (alteration in synthesis, storage, or processing); 2) alterations in receptors involved in recognition and transport (changes in synthesis, ligand binding, phosphorylation, internalization, etc.); and 3) general changes in the ability of the neuron to maintain machinery in transport [101].

1.4.2 Axonal transport and exercise

Earlier studies examined whether increases in neuronal activity would result in an increase in axonal transport by investigating the effects of acute [102] and chronic [91, 103, 104] exercise training (via treadmill [range 2 to 13 weeks]). In the sciatic nerve, acute and chronic exercise can increase protein transport up to 20% and 50%, respectively [102, 104], and this increase can occur in both intact and deafferented nerves [91]. These increases are due to the fact that exercise increases the transport velocity of proteins within the sciatic nerve. The reasoning behind this increase in protein transport and velocity may be due to that trained neurons are more responsive to alterations to their immediate neuronal environment. For example, exercise increases neuronal activity leading to changes at the nerve terminal. Consequently, the axonal transport of proteins are increased in order to compensate for the effects exercise has on the distal parts of the motoneurons, thus allowing repair and return to normal nerve function [91]. The results from these studies show that exercise (training by running) does affect axonal transport by increasing protein transport velocities, as well as the amount of protein transported.

1.5 Animal models of pain

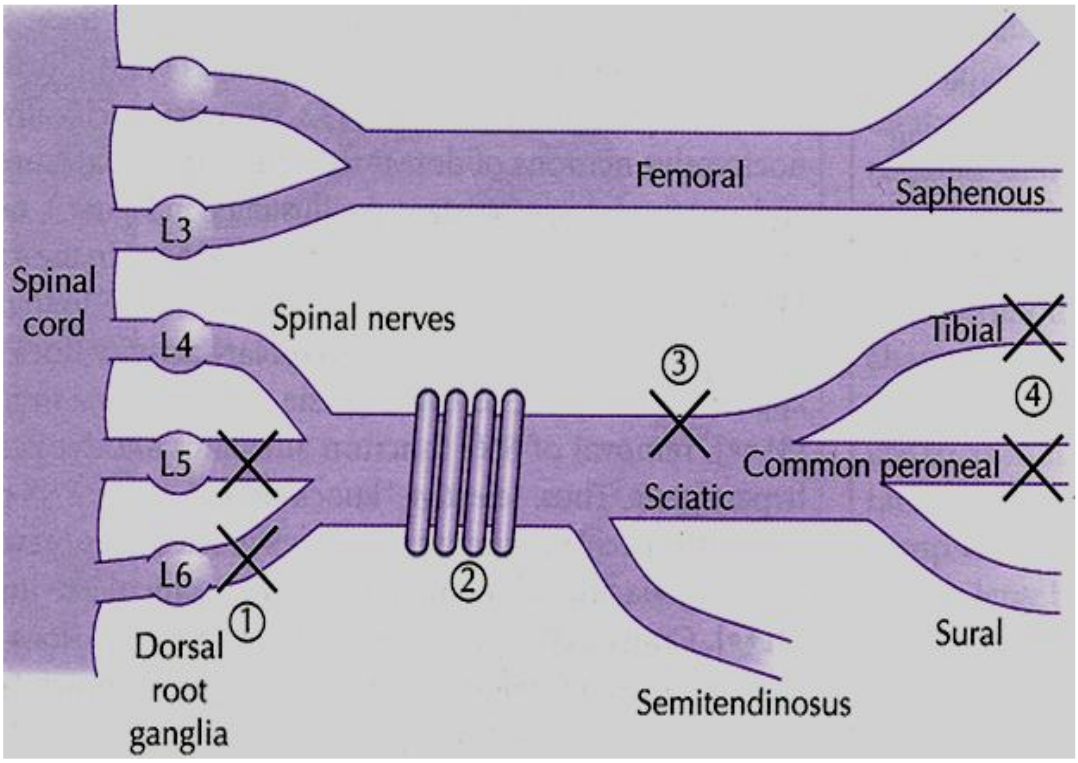
1.5.1 Neuropathic pain (peripheral nerve injury)

The understanding of clinical pain has been greatly accelerated due to the development of animal models that reproduces many elements of clinical pain syndromes. These models have led to a better understanding of the

pathophysiologic neural changes associated with neuropathic pain. Animal models of neuropathic pain that increase tactile and thermal sensitivity include: 1) spinal nerve ligation (SNL) [105], 2.) chronic constriction injury (CCI) [106], 3.) partial sciatic nerve ligation (PSNL) [107], and 4.) spared nerve injury (SNI) [108] (Figure 1.7). The most important criteria's for any animal model of neuropathic pain are the ease of the procedure and the ability to reproduce sensory deficits such as allodynia, hyperalgesia, and spontaneous pain over a sustained period [4]. However, the spinal nerve ligation, chronic constriction injury, and partial sciatic nerve ligation models produce several limitations such as the inability to separate injured from intact nerve fiber due to the fact that they co-exist in the same nerve trunk (CCI and PSNL), nerve regeneration may occur over the ligation site (SNL and PSNL), and the number of injured fibers are difficult to control, therefore producing injury may become difficult (CCI and PSNL) [109]. On the other hand, the SNI model is easy to perform and has been shown to produce profound and reliable pain behaviors that closely mimic many features of clinical neuropathic pain [108], therefore minimizing potential limitations described above [109]. The SNI model involves ligating and sectioning the tibial and common peroneal nerves, while sparing the sural nerve, which produces a long-lasting mechanical and thermal hypersensitivity. This model also allows the investigation of changes in both injured primary sensory neurons and neighboring intact sensory neurons and their contribution to the pathophysiology of neuropathic pain [110].

Figure 1.7 Animal models of neuropathic pain. 1.) spinal nerve ligation; 2.) chronic constriction injury; 3.) partial sciatic nerve ligation; and 4.) spared nerve injury [12]. Taken from Taylor (2001).

Figure 1.7 Animal models of neuropathic pain.



1.5.2 Diabetic neuropathy

The most common animal model of human diabetes is streptozotocin (STZ)-induced diabetes in rodents [111]. STZ is a glucose analogue that specifically damages pancreatic β cells [112]. Administration of STZ can rapidly produce characteristic signs of type 1 diabetes, such as an increase in food and water intake, failure to gain weight, and increased blood glucose levels. These animals can survive for periods of weeks to months depending on the severity of pancreatic damage induced, while insulin administration can keep animals alive for a year or more [113]. Studies have also shown that in rodents, STZ-induced diabetes can mimic clinical secondary complications such as painful DN [114, 115]. STZ-diabetic rats have been shown to develop sustained tactile allodynia, which can be measured via von Frey monofilament (technique similarly used in clinical practice) [116]. In diabetic patients the progression of painful diabetic neuropathy can take years, whereas in rodent it takes weeks. Thus, allowing investigators to assess diabetic complications in a more efficient time frame.

1.6 Proposed work

Currently, there is no treatment to prevent the development or adequately control neuropathic pain. As a result, there is an unmet clinical need and an ongoing challenge to develop more effective therapies. This can only be achieved if the relationship between the aetiology, mechanisms, and symptoms of neuropathic pain are understood [3]. Recent studies have demonstrated that aerobic exercise can reduce pain sensitivity in both human and animals

experiencing chronic and neuropathic pain. Recently, we have shown in an animal model forced aerobic exercise (via swimming) reduces symptoms associated with neuropathic pain [30]; however, the exercise-induced mechanisms were not researched in this study. In order to better translate these findings to humans, it needs to be determined whether voluntary exercise can produce similar effects on clinical problems, such as mechanical allodynia and hyperalgesia. Balducci et al. (2006) reported that exercise can adequately prevent the onset of painful DN in diabetic patients without signs or symptoms of DN. However, the ability of voluntary exercise to exert similar effects on diabetic patients with signs or symptoms of DN has yet to be explored. Therefore, to address this gap in knowledge we have designed a set of experiments that has allowed us to assess the effects of voluntary exercise on animal models of peripheral neuropathic pain (SNI model) and painful DN (STZ model), as well as the potential mechanism of exercise-induce analgesia.

Chapter 2

Effect of voluntary exercise on reducing mechanical sensitivity in nerve injured female rats

2.1 Abstract

Persistent pain, mainly neuropathic pain, can be a debilitating disease that often responds poorly from pharmacological interventions. The ability of exercise to reduce behavioral sensitivity associated with peripheral nerve injury in rodents has been reported; however, the exercise-induced mechanisms were not explored. The purpose of this study is to determine how peripheral nerve injury affects GDNF protein levels and whether voluntary exercise has any influence in order to attenuate or delay the behavioral sensitivity associated with neuropathic pain. Twenty-five female Sprague Dawley rats (200-250g) were used in this study. Sixteen rats were randomized into either the sedentary (Sed-Sham^25 [n=4]) or exercised (Ex-Sham^25 [n=4]) sham surgery group or sedentary (Sed-SNI^25 [n=4]) or exercised (Ex-SNI^25 [n=4]) spared nerve injury (SNI) surgery group, which participated in the 25 day duration study. The remaining 9 rats were randomized to either the sedentary (Sed-SNI^110 [n=4]) or exercised (Ex-SNI^110 [n=5]) SNI group, which participated in the 110 day duration study. Rats in the SNI groups had surgically-induced peripheral nerve injury surgery. Following recovery from surgery, rats in the exercised groups were housed in standard cages with voluntary running wheels while the sedentary group was housed without wheels. Mechanical allodynia and hyperalgesia were assessed via von Frey filaments and pin-prick test, respectively. GDNF protein levels were examined in the lumbar region of the spinal cord using immunoblot analysis. SNI surgery produced behavioral hypersensitivity in both sedentary and exercised groups. In both the 25 and 110 day study, voluntary exercise significantly

reduced mechanical allodynia and hyperalgesia after SNI. Twenty-five days post SNI surgery did not alter GDNF protein levels in the spinal cord; however, over time (110 days) GDNF levels significantly increased in the spinal cord of the SNI groups. The Sed-SNI^110 and Ex-SNI^110 groups exhibited a 2.3- and 1.6-fold increase in GDNF protein levels compared to the Sed-SNI^25 and Ex-SNI^25 groups, respectively. Voluntary exercise did not change GDNF protein levels in either exercised group. Voluntary exercise caused favorable changes in behavioral sensitivity following nerve-injury in female rats. This investigation supports the use of voluntary exercise to complement current treatment strategies aimed at decreasing neuropathic pain.

2.2 Introduction

Neuropathic pain is initiated or caused by a primary lesion or dysfunction in the structure and function to the nervous system [1, 12, 117, 118]. It is characterized by sensory abnormalities such as a painful response to a stimulus that does not normally provoke pain (allodynia) or an increased response to a painful stimulus (hyperalgesia) [4]. Peripheral neuropathic pain is the result of damage to the peripheral nervous system (via mechanical trauma, metabolic disease, etc.), which involves multiple pathophysiological changes to both the PNS and CNS [117]. Epidemiological studies have indicated that neuropathic pain affects ~5% of all adults, resulting in about 3.75 million Americans suffering from this debilitating disease [117-119]. Numerous pharmacological and non-pharmacological (e.g. aerobic exercise) therapy strategies have been developed to treat symptoms associated with neuropathic pain. Pharmacological therapy for neuropathic pain has had limited success [3, 4]; however, clinical studies have shown that exercise can reduce painful symptoms associated with fibromyalgia [27], chronic low back pain [29], and cancer treatment-related pain [120]. A survey by Warms et al., addressing the effectiveness of pharmacological and non-pharmacological therapies, reported that patients with a spinal cord injury rated regular exercise to be a more effective treatment for their neuropathic pain symptoms than any type of pain medication [26]. Thus, suggesting that exercise may be a viable therapy treatment to use in conjunction with other treatments aimed at reducing symptoms associated with neuropathic pain.

Recently, we have shown that forced exercise (via swimming) reduced behavioral sensitivity (cold allodynia and thermal hyperalgesia) associated with peripheral nerve injury (peripheral sciatic nerve ligation) [30]; however, the exercise-induced mechanisms were not researched in this study. One potential mechanism under investigation includes increases in neurotrophic support, which have been shown to increase with exercise [31, 36-42]. In an animal model of neuropathic pain, aerobic exercise attenuated spinal cord injury-induced mechanical allodynia by restoring neurotrophic factors to baseline levels in the spinal cord [31]. Exercise has also been shown to induce gene changes in the spinal cord that promote neuronal survival (phosphatidylinositoal-3-kinase [PI3K]) [39], which is activated by glial cell line-derived neurotrophic factor (GDNF). Thus, exercise can have beneficial physiological effects, and increases in neurotrophic factors (via exercise) may be an alternative mechanism for reducing neuropathic pain.

GDNF is important for modulating nociceptive signals during neuropathic pain states [72-75]. In the events following peripheral nerve injury, dysfunction of GDNF signaling in the nociceptive afferent system contributes to the development and/or maintenance of neuropathic pain. Aerobic exercise, a non-pharmacological intervention, has been shown to alter GDNF expression in various neuronal and non-neuronal tissues [40, 88-90]. As shown by Dupont-Versteegden et al. (2004), exercised animals exhibited an increase in functional recovery in spinal cord-injured rats, which were attributed to increases in GDNF

expression. Therefore, further studies are needed to determine whether the exercise-induced reductions in behavioral sensitivity exhibited in our peripheral nerve injured animals were also due to alterations in GDNF levels. To address this gap in knowledge, we evaluated changes in GDNF protein levels and associated sensitivity following exercise in the setting of neuropathic pain.

2.3 Methods

2.3.1 Rats

Twenty-five 8 week old female Sprague-Dawley rats (weight range, 200 to 250 g) were purchased from Charles Rivers Laboratories (Wilmington, MA) for both the 25 day and the 110 day study. The Institutional Animal Care and Use Committee of the University of Kansas Medical Center have approved the study protocol. One week prior to nerve injury surgery (acclimation period), rats randomized into the exercised groups were housed in cages with access to voluntary running wheels and rats randomized into the sedentary groups were housed in similar cages without running wheel access. During this acclimation period, rats in the exercised groups were allowed to participate in exercise voluntarily. Following the1 week acclimation period, rats within the sedentary and exercised groups underwent either the spared nerve injury (SNI) or sham surgery. Forty-eight hours after SNI or sham surgery, rats were returned to their respective cages. At the conclusion of each study, rats were sacrificed and the lumbar region of the spinal cord was harvested for GDNF protein analysis.

2.3.1.1 Short-term (25 day) study

Sixteen rats were randomized into either the sedentary (Sed-Sham^25 [n=4]) or exercised (Ex-Sham^25 [n=4]) sham surgery group or sedentary (Sed-SNI^25 [n=4]) or exercised (Ex-SNI^25 [n=4]) SNI surgery group, which participated in the 25 day duration study. Behavioral assessments (mechanical allodynia [via von Frey monofilaments] and hyperalgesia [via pinprick]) were conducted immediately prior to the sham or SNI surgery, day 14, and 25 post sham or SNI surgery.

2.3.1.2 Long-term (110 day) study

Nine rats were randomized to either the sedentary (Sed-SNI^110 [n=4]) or exercised (Ex-SNI^110 [n=5]) SNI group, which participated in the 110 day duration study. Behavioral assessments (mechanical allodynia [via von Frey monofilaments] and hyperalgesia [via pinprick]) were conducted immediately prior to the SNI surgery, and at day 5, 8, 12, 15, 19, 22, 26, 29, 33, 36, 40, 47, 50, 54, 57, 61, 64, 69, 89, 98, 103, and 110 after the SNI surgery.

2.3.2 Spared nerve injury surgery

Peripheral SNI was induced following baseline assessments of behavior measurements. The SNI procedure was conducted in accordance to Decosterd and Woolf (2000). Under isoflurane anesthesia, the skin on the lateral surface of the left thigh of the rat was shaved and incised revealing the muscle. Using forceps, a section was made directly through the bicep femoris muscle exposing

the sciatic nerve and its three terminal branches: the sural, common peroneal and tibial nerves. Thereafter, the tibial and common peroneal nerves were tightly ligated with 4.0 silk threads followed by axotomy, 2mm distal to the ligation. Considerable care was taken to avoid any contact with the intact sural nerve or stretching any of these three terminal branches. Muscle and skin was closed using 4.0 silk sutures. Sham surgery procedures were performed in a similar manner; however it only involved exposure of the sciatic nerve and its branches [4, 108, 109]. Animals were allowed 48 hours for recovery before placement into their respective cages.

2.3.3 Voluntary exercise with free-access wheel running

Exercised rats were individually housed in their own cages containing an exercise wheel (Mini Mitter Co. Inc., a Respironics Company, Bend, OR) so that all access would be completely voluntary. Each wheel revolution was continuously recorded and summarized in 30 minute intervals with the Vital View Data Acquisition System (Mini Mitter Co. Inc.) throughout the duration of the study. Sedentary rats were individually housed in their own cages that did not contain an exercise wheel.

2.3.4 Behavioral measurements

Mechanical allodynia and hyperalgesia were assessed via von Frey monofilaments and pinprick stimuli, respectively. All measurements were conducted on the lateral side of the left paw. After randomization, baseline

behavioral testing was performed on all rats followed by sham or SNI surgery. After 48 hours of recovery, rats were returned to their respective cages and behavioral testing was conducted at designated time points outlined in each study protocol. Rats predominately exercised from 6 p.m. to 6 a.m., so all scheduled behavioral assessments were conducted at least 4 hours after 6 a.m. in order to minimize known potential acute analgesic effects that exercise may have produced. Prior to each assessment, rats were acclimated for 1 hour in the testing environment (an inverted Plexiglas box on top of an elevated one-quarter inch stainless steel mesh floor). The order of testing included mechanical allodynia, followed by a 30 min window before assessing mechanical hyperalgesia.

2.3.4.1 Mechanical allodynia

A standard set of Semmes-Weinstein von Frey hairs (Stoelting, Wood Dale, IL), were used to touch the lateral part of the plantar surface of the paw. The threshold was taken as the lowest force (grams) that induced a withdrawal response to one of five repetitive stimuli [108, 121]. An interval time of approximately 30 seconds was used between stimuli.

2.3.4.2 Mechanical hyperalgesia

The pinprick test was performed on the lateral part of the plantar surface of the paw. The paw was briefly touched with the point of a pin at an intensity to indent but not penetrate the skin. Using a stopwatch, duration (seconds) of paw

withdrawal was recorded with a minimal value of 0.5 seconds or until the animal ceased reaction to the stimuli [108, 121].

2.3.5 Vaginal smears

Responses to noxious stimuli have been reported to be influenced by the estrous cycle [122]. In order to ensure that our behavioral results were not skewed due to abnormal estrous cycling, daily vaginal smears were conducted. Briefly, the rat was gently held, the vagina was gently flushed once with saline and a drop of vaginal fluid was transferred to a clean, dry slide. Care was taken to not insert the dropper tip too deeply in the vagina, due to possibility of the rat becoming psuedopregnant. The sample was viewed using a standard power monocular microscope to view cells and to distinguish among the 4 phases: 1) diestrous, 2) proestrous, 3) estrous, and 4) metestrous [123].

2.3.6 Protein analysis by immunoblotting

GDNF protein levels were examined in the lumbar region of the spinal cord using immunoblot analysis. We selected the lumbar region because this is the area in which nociceptive afferent fibers, that mediate painful sensations, terminate. The lumbar region of the spinal cord of each animal was collected during animal dissection and immediately frozen in liquid nitrogen, to preserve protein integrity, and stored at -80˚C until use. Tissues were homogenized using lysis buffer containing 137mM NaCl, 20mM Tris-HCl, pH 8.0, 1% nonyl phenoxylpolyethoxylethanol (NP-40), 10% glycerol, 1mM

phenylmethanesulfonylfluoride (PMSF), 10µg/ml aprotinin, 1µg/ml leupeptin, and 0.5mM sodium vanadate. Tissue weight to buffer volume ratio was kept at 1:20 (e.g. 400µl buffer per 20mg tissue). In the lysis buffer, tissues were homogenized using a glass Teflon homogenizer, and then centrifuged at +4˚C, at 16,000g for 15 minutes. The supernatant was collected and total protein concentration was measured using BioRad protein assay reagent (BioRad Laboratories, Hercules, CA) in a 96 wellplate format, and analyzed with the microplate reader MRXII (Dynex Technologies, Chantilly, VA) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was used to separate proteins in the supernatants. Twelve percent polyacrylamide gels with 15 wells for protein loading were poured using electrophoresis grade reagents. Twenty micrograms of total protein per sample was loaded per well of a gel, and proteins were separated under ~150-200 V current at constant voltage (30 mA). The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane over night using sandwich tank transfer apparatus (Bio-Rad Laboratories). Membranes were blocked using a blocking buffer consisting of fresh 3% non-fat dry milk (BioRad Laboratories) suspended in phosphate buffered saline (PBS) with 0.1% Tween 20 (PBS-T) for 1 hour. Membranes were then incubated with primary antibody (GDNF rabbit-polyclonal [Santa Cruz Biotechnology, Santa Cruz, CA, catalog # sc-328; dilution 1:2,000]) diluted in the blocking buffer, for 1 hour. The membrane was then washed 3 times with PBS-T for 10 minutes each. The membrane was then incubated for 30 minutes with the secondary antibody (goat anti-rabbit [Santa

Cruz Biotechnology, catalog # sc-2004; dilution 1:2,000]) in the blocking buffer and then washed 3 times in PBS-T for 10 minutes. All membrane incubations and washes were done at room temperature on a shaker. Detection was achieved using enhanced chemiluminescent reagent (ECL; SuperSignalWestPico Chemiluminescent Substrate, catalog # 34080, Pierce, Rockford, IL]) according to manufacturer's recommendations, and exposure of membranes to X-ray film. The relative levels of protein were estimated by measuring mean pixel intensity of protein bands using Adobe Photoshop. Before the blocking step, all membranes were stained with Ponceau S solution to verify even loading, integrity of the extracted proteins and proper transfer [124]. If uneven loading of protein occurred, a single band on the Ponceau stained membrane was used for normalization. GDNF was detected as a single band migrating at a molecular weight of 36 kilodaltons (kDa). The specificity of the band was confirmed using a GDNF blocking peptide (Santa Cruz Biotechnology, catalog #sc-328P).

2.3.7 Data analysis

Group comparisons of body weight, mechanical allodynia and hyperalgesia, and distance exercised were made using one-way analysis of variance (ANOVA) with repeated measures. Post hoc analysis was conducted using the least significant difference (L-S-D) method when appropriate. ANOVA was used to analyze GDNF protein levels between groups. Pearson Product Correlation was used to determine the relationship between changes in

mechanical allodynia and hyperalgesia and weekly distance exercised over the course of each study along with the relationship between GDNF protein levels and total distance exercised throughout the 25 and 110 day studies. Statistical significance was set at $p \leq 0.05$. All statistical analysis was done using Statistical Package for the Social Sciences (SPSS) version 17. Data are presented as mean ± standard error of the mean (SEM).

2.4 Results

2.4.1 Animal characteristics

Throughout both studies (short- and long-term), all rats cycled normally, therefore testing did not disrupt the rats' estrous cycle. The rat's body weight is provided in Table 1. In the short-term study the Sed-Sham^25 group weighed significantly more than all other groups prior to surgery. Over the course of the 25 day study, all rats had significantly gained weight, however there were no differences between groups at day 25. Weight gains by the Sed-Sham^25, Sed-SNI^25, Ex-Sham^25, and Ex-SNI^25 groups increased by 30%, 33%, 28%, and 26%, respectively. Body weight for the rats in the 110 day study did not differ among groups at anytime. By day 25, the Sed-SNI^110 and Ex- SNI^110 groups weight significantly increased by 23% and 16% and by 61% and 56% at day 110, respectively.

Table 2.1 Body weight of rats in the short- (25 day) and long-term (110 day) study. Prior to surgery the Sed-Sham^25 group weighed significantly more than all other groups in the short-term study. After surgery, all groups gained body throughout their respective studies and there were no differences between groups. There were no differences in body weight between the SNI groups in the long-term study. Prior to nerve injury (PNI). * = Sed-Sham^25 vs. Ex-Sham^25, Sed-SNI^25, and Ex-SNI^25. $p<0.05$

Table 2.1 Body weight of rats in the short- (25 day) and long-term (110 day) study.

		Body Weight (grams)		
Study	Rat Group	PNI	Day 25	Day 110
25 day	Sedentary Sham (Sed-Sham^25)	222 ± 4*	291 ± 12	
	Exercised Sham (Ex-Sham^25)	205 ± 5	263 ± 10	
	Sedentary SNI (Sed-SNI^25)	204 ± 4	273 ± 3	
	Exercised SNI (Ex-SNI^25)	207 ± 4	260 ± 7	
110 day	Sedentary SNI (Sed-SNI^110)	222 ± 2	273 ± 10	357 ± 20
	Exercised SNI (Ex-SNI^110)	213 ± 6	248 ± 9	333 ± 15

2.4.2 Daily exercise distance

In the short-term study, the average daily exercise distance prior to surgery was 7.0±2.3 and 10.3±2.1 km/day, for the Ex-SNI^25 and Ex-Sham^25, respectively. Twenty-five days post surgery the Ex-SNI^25 and Ex-Sham^25 groups ran 6.1±2.9 and 12.9±4.5 km/day, respectively (Figure 2.1). Although the Ex-Sham^25 group exercised more daily, there were no significant differences in exercised distance between or within groups over the 25 day training period. In the long-term study, the exercised group exercised on average 8.7±2.1 km/day prior to the surgery, whereas, 110 days post surgery they exercised significantly less at 4.9±0.5 km/day (Figure 2.2).

2.4.3 Behavioral assessment (mechanical allodynia and hyperalgesia)

2.4.3.1 Short-term (25 days) study

Using an animal model of neuropathic pain, such as SNI, we evaluated the effects of long-term voluntary exercise on mechanical allodynia and hyperalgesia in rats following peripheral nerve injury. SNI surgery dramatically increased mechanical allodynia and hyperalgesia in both sedentary and exercised groups, which persisted throughout the remaining of the study (Figures 2.3 and 2.4). At day 14, there were no differences in mechanical allodynia between SNI groups; however, at day 14 the Ex-SNI^25 group exhibited a decrease in mechanical hyperalgesia compared to the Sed-SNI^25 group (1.71±0.4 vs. 2.71±0.3 seconds, respectively). Compared to the Sed-SNI^25 group, the Ex-SNI^25 group displayed a decrease in mechanical allodynia

Figure 2.1 Average daily exercise distance for the short-term (25 day) study. Prior to surgery the Ex-Sham^25 and Ex-SNI^25 groups exercised 10.3±2.1 and 7.0±2.3 km/day, respectively. Four weeks post surgery they exercised 6.1±2.9 and 12.9±4.5 km/day, respectively. Prior to nerve injury (PNI).

Figure 2.1 Average daily exercise distance for the short-term (25 day) study.

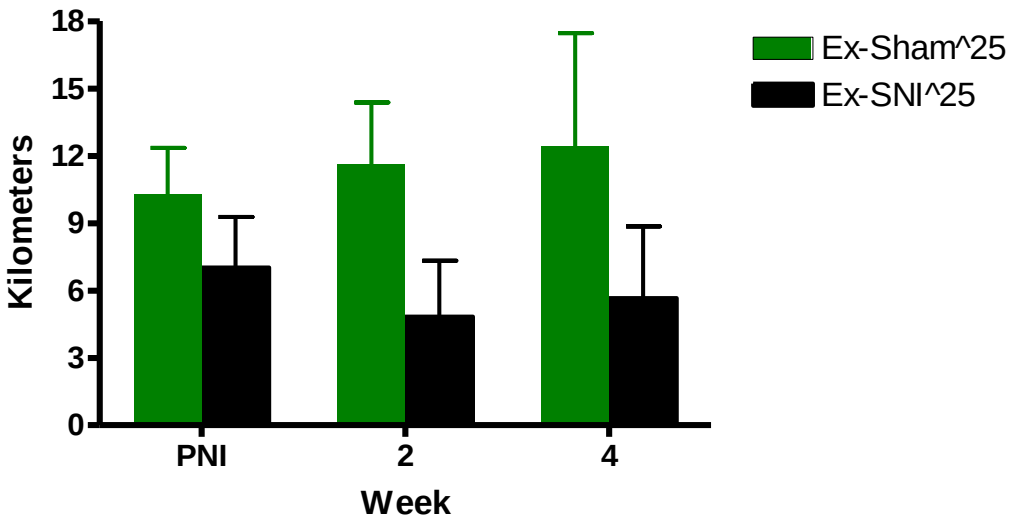


Figure 2.2 Average daily exercise distance for the long-term (110 day) study. Prior to nerve injury the Ex-SNI^110 exercised 8.7±2.1 km/ day; however, 110 days post surgery they exercised significantly less at 4.9±0.5 km/day; however, they continued to exercise despite the injury. *=Prior to nerve injury (PNI) vs. time point. $p<0.05$

Figure 2.2 Average daily exercise distance for the long-term (110 day) study.

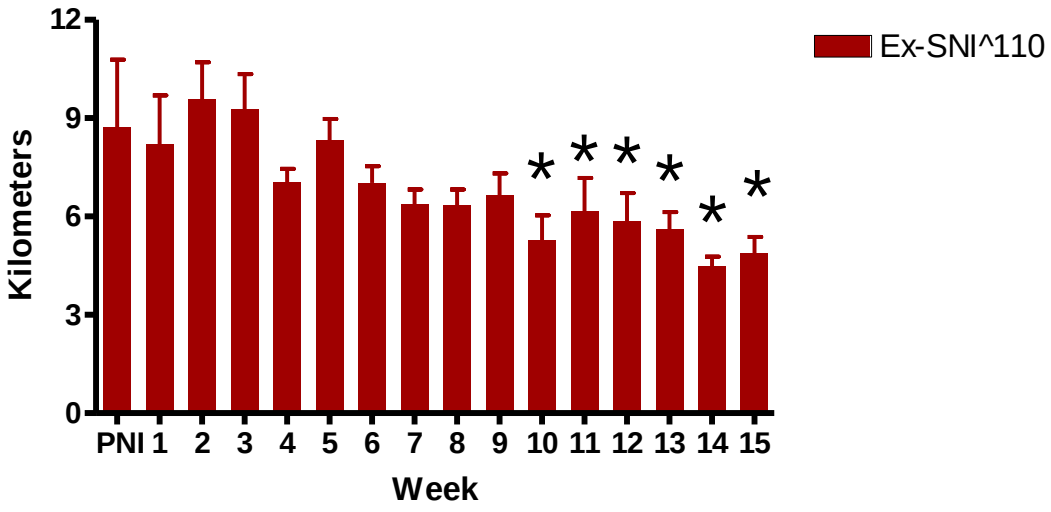


Figure 2.3 Mechanical allodynia for the short-term (25 day) study. Exercised nerve-injured rats displayed a reduction in mechanical allodynia on the lateral side of paw compared to their sedentary counterpart. *=Sed-Sham^25 vs.Sed-SNI^25, †=Ex-Sham^25 vs. Ex-SNI^25, and ‡=Sed-SNI^25 vs. Ex-SNI^25. $p<0.05$.

Figure 2.3 Mechanical allodynia for the short-term (25 day) study.

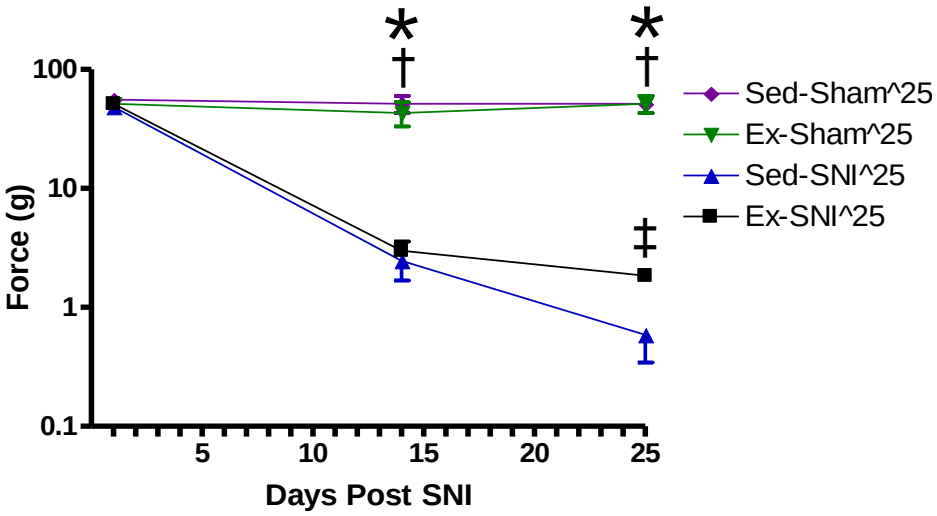
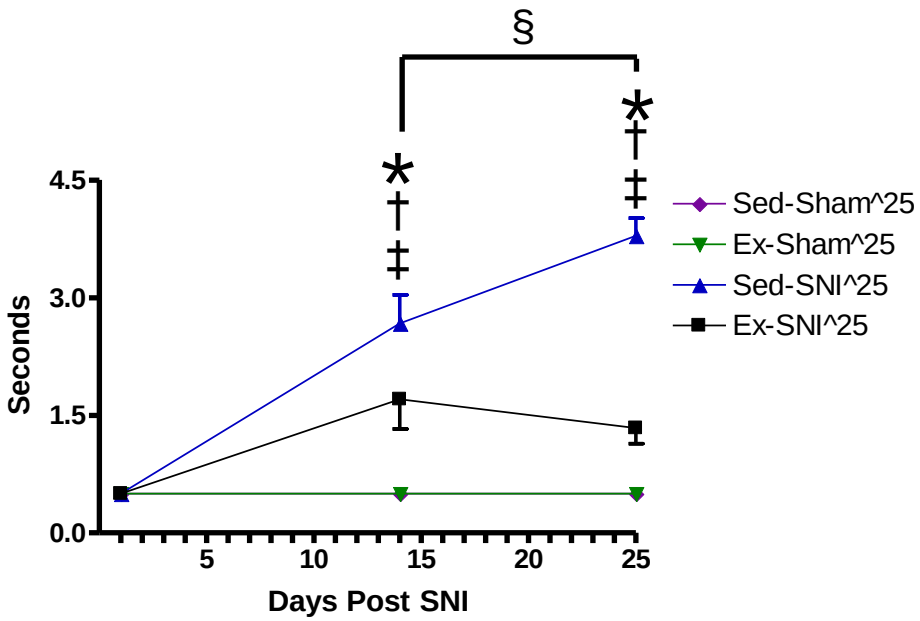


Figure 2.4 Mechanical hyperalgesia for the short-term (25 day) study. Exercised nerve-injured rats displayed a reduction in mechanical hyperalgesia on the lateral side of paw compared to their sedentary counterpart. Between group comparisons; *=Sed-Sham^25 vs.Sed-SNI^25, †=Ex-Sham^25 vs. Ex-SNI^25, and ‡=Sed-SNI^25 vs. Ex-SNI^25. Within group comparison of Sed-SNI^25; §=day 14 vs. day 25. $p<0.05$.

Figure 2.4 Mechanical hyperalgesia for the short-term (25 day) study.



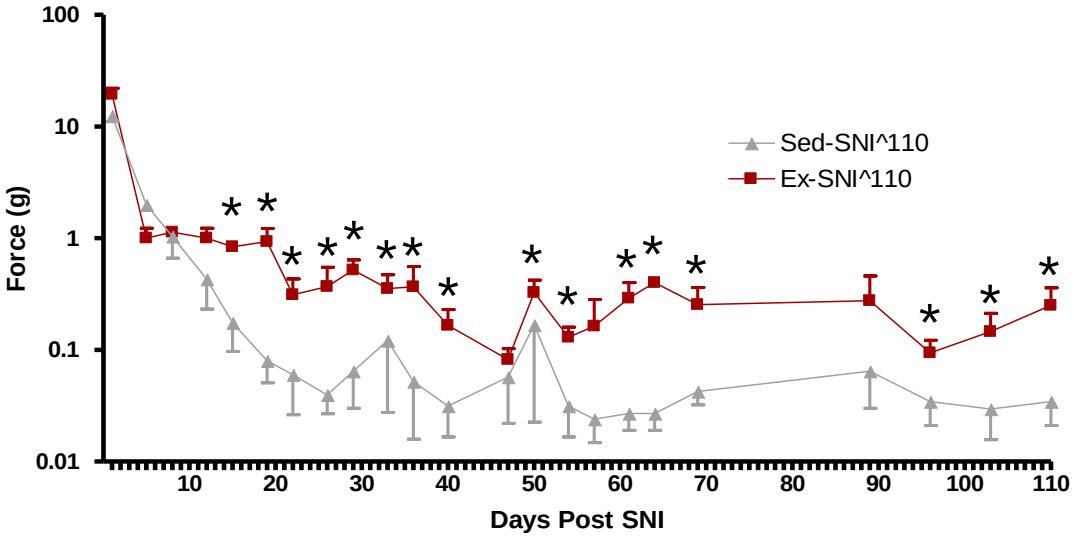
(0.5±0.2 vs. 1.8±0.1 grams, respectively) and hyperalgesia (3.7±0.2 vs. 1.3±0.2 seconds, respectively) after 25 days of voluntary exercise. Within group comparisons showed no changes in mechanical allodynia between day 14 and 25 after nerve injury in both sedentary and exercised SNI groups. However, Sed-SNI^25 group exhibited a significant increase in hyperalgesia at day 14, which continued to increase through day 25, whereas, exercise ceased any further increases at day 25. Sham (control) group displayed no significant changes in behavioral hypersensitivity over the 25 day training period. There was no correlation with weekly distance exercised and change in behavioral sensitivity (mechanical allodynia and hyperalgesia) over time among the exercised group with SNI.

2.4.3.2 Long-term (110 days) study

In the short-term study, rats with SNI experienced reductions in behavioral sensitivity at day 14, which continued through day 25. To ascertain whether the effect of exercise was long-lasting, we evaluated the effects of exercise training extended to 110 days (approximately, 15 weeks) on mechanical allodynia and hyperalgesia in rats with peripheral nerve injury and their sedentary counterpart. Sham sedentary and exercised groups were not used for this study due to the fact that there were no changes in behavioral sensitivity in the 25 day study. After SNI surgery both sedentary and exercised groups displayed a marked increase in mechanical allodynia and hyperalgesia which persisted throughout the 110 day period (Figure 2.5). Similar to the 25 day study, voluntary exercise resulted

Figure 2.5 Mechanical allodynia for the long-term (110 day) study. Exercised nerve-injured rats displayed a reduction in mechanical allodynia on the lateral side of paw compared to their sedentary counterpart. *$p<0.05$.

Figure 2.5 Mechanical allodynia for the long-term (110 day) study.



in a favorable change in pain behavior following nerve injury in these rats. The exercised group exhibited reductions in mechanical allodynia at 16 of the 22 time points assessed post nerve injury compared to the sedentary group. Similar to the 25 day study, rats in the exercised group exhibited beneficial reductions in mechanical hyperalgesia at day 22, and this trend remained throughout the study reaching statistical significance at day 110 (1.4±0.2 vs. 4.2±1.4 seconds, respectively) (Figure 2.6). Collectively, the 25 and 110 day studies support the use of voluntary exercise, which reduced behavioral symptoms following nerve injury in female rats. There was no correlation with weekly distance exercised and change in behavioral sensitivity (mechanical allodynia and hyperalgesia) over time among the exercised group with SNI.

2.4.4 GDNF protein level analysis

Observing the beneficial influence of exercise on behavioral sensitivity in rats with peripheral nerve injury, we sought to explore possible mechanisms involved and determined whether one of the essential neurotrophic growth factors, GDNF may be implicated. Immunoblotting was used to determine the effects voluntary exercise has on GDNF protein levels in the lumbar region spinal cord tissue. We found that 25 days post SNI surgery did not alter GDNF protein levels in the spinal cord compared to their respective control counterparts (Figure 2.7). However, over time (110 days) GDNF levels significantly increased in the spinal cord of the SNI groups. The Sed-SNI^110 and Ex-SNI^110 groups exhibited a 2.3- and 1.6-fold increase in GDNF protein levels compared to the

Figure 2.6 Mechanical hyperalgesia for the long-term (110 day) study. Exercised nerve-injured rats displayed a reduction in mechanical hyperalgesia on the lateral side of paw compared to their sedentary counterpart. *$p<0.05$.

Figure 2.6 Mechanical hyperalgesia for the long-term (110 day) study.

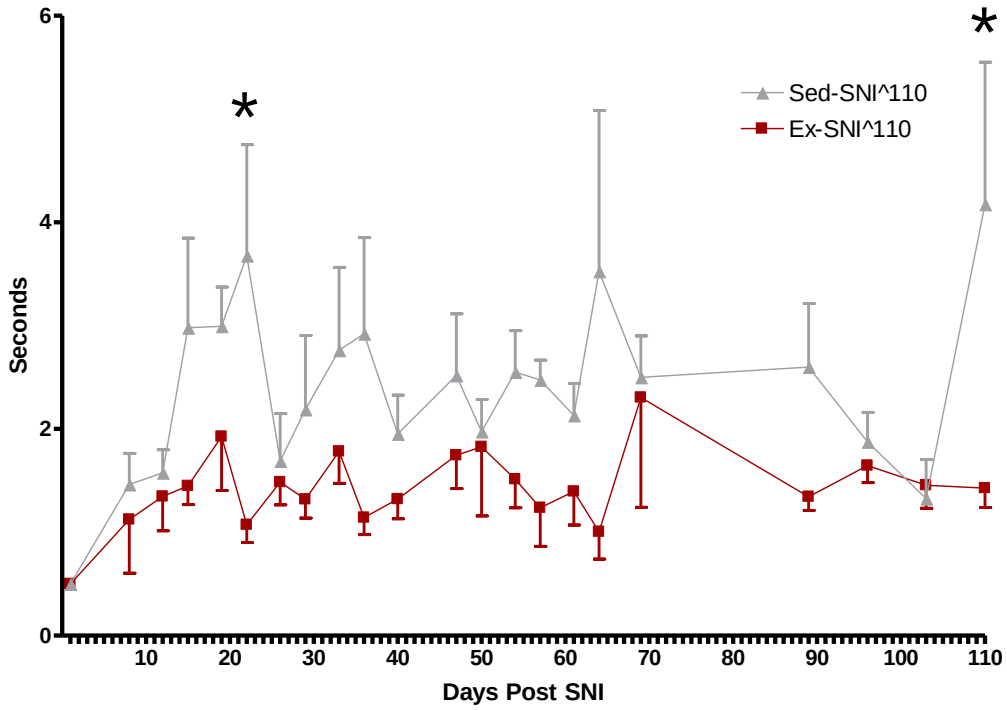
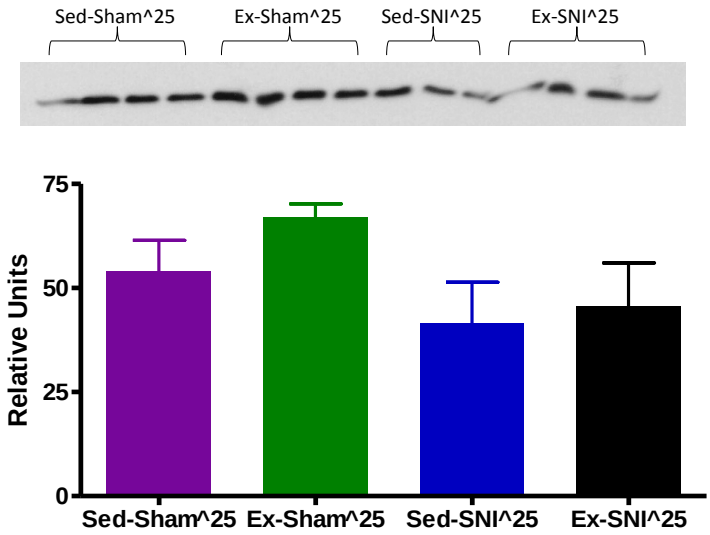


Figure 2.7 Immunoblot analysis of GDNF levels in the lumbar region of the spinal cord for the short-term (25 day) study. Upper panel shows representative immunoblot; lower panel shows histogram analysis of the immunoblot band intensity. 25 days post SNI surgery did not alter GDNF protein levels in the spinal cord compared to their respective control counterparts. *$p<0.05$.

Figure 2.7 Immunoblot analysis of GDNF levels in the lumbar region of the spinal cord for the short-term (25 day) study.



Sed-SNI^25 and Ex-SNI^25 groups, respectively (Figure 2.8). Twenty-five and 110 days of voluntary exercise did not change GDNF protein levels in either exercised group. Thus, peripheral nerve injury increased GDNF protein levels in the spinal cord of the SNI groups, whereas voluntary exercise had no effect. In both studies, there was no correlation with total distance exercised and change in GDNF protein levels at day 25 or 110 among the exercised group with SNI.

2.5 Discussion

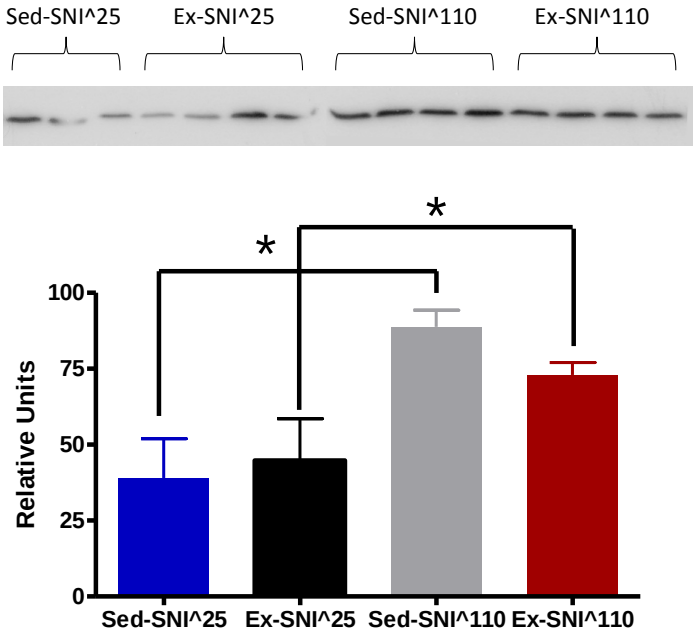
Our study is the first to report exercise-induced changes in mechanical hypersensitivity (mechanical allodynia and hyperalgesia) following nerve injury in female rats. We demonstrated that rats with peripheral nerve injury, which voluntarily exercised short (25 days) and long (110 days) terms, exhibited a statistical significant reduction in behavioral sensitivity. Alterations of GDNF levels in the lumbar region of the spinal cord were explored as a possible analgesic mechanism; however, voluntary exercise had no effect on GDNF. Our results support the hypothesis that long-term voluntary exercise reduces behavioral sensitivity in the setting of neuropathic pain.

2.5.1 Reduction of mechanical sensitivity by exercise

To make our current study more clinically relevant, our rats exercised voluntarily and behavioral sensitivity, such as mechanical allodynia and hyperalgesia, that neuropathic pain patients experience was assessed. In the current study, a model of neuropathic pain was adopted using the SNI model,

Figure 2.8 Immunoblot analysis of GDNF levels in the lumbar region of the spinal cord of the short- (25 day) and long-term (110 day) studies. Upper panel shows representative immunoblot; lower panel shows histogram analysis of the immunoblot band intensity. The sedentary and exercised groups in the long-term study exhibited a 2.3- and 1.6-fold increase in GDNF protein levels compared to their respective counterparts in the short-term study, respectively *$p<0.05$.

Figure 2.8 Immunoblot analysis of GDNF levels in the lumbar region of the spinal cord of the short- (25 day) and long-term (110 day) studies.



which produces profound and reliable pain behaviors that closely mimic many features of clinical neuropathic pain [108]. Similar to the results reported by Decosterd and Woolf (2000), the SNI surgery produced an immediate onset (day 5) of behavioral mechanical hypersensitivity, which persisted until the rats were sacrificed, suggesting it to be a valid animal model to replicate neuropathic pain. Previously, using the peripheral sciatic nerve ligation model we demonstrated that long-term exercise reduces behavioral sign of neuropathic pain after 18 to 25 days [30]. Parallel to our previous study, in both of the present studies we were able to reproduce reductions in mechanical allodynia and hyperalgesia within the first 3 weeks of exercise training, which lasted up to 15 weeks.

2.5.2 Mechanisms of exercise-induced analgesia

Many potential mechanisms contribute to the pathogenesis of neuropathic pain, which occurs through changes in both the peripheral and central nervous systems. Mechanisms may include the redistribution or changes in sodium channels in the nerve or the sprouting of Aβ-fibers from lamina III/IV to lamina II of the dorsal horn. However, GDNF has been shown to regulate sodium channels [77] and decrease Aβ-fibers sprouting by promoting the survival of non-peptidergic nociceptive C-fiber neurons (isolectin B4 [IB4] binding) [80], thus, ameliorating behavioral sensitivity associated with neuropathic pain [72, 76-79]. Therefore, GDNF has potent analgesic effects in the state of neuropathic pain.

Increases in endogenous neurotrophins in the lumbar region of the spinal cord have been attributed to reducing mechanical allodynia in a model of central neuropathic pain involving spinal cord injury [31]. To gain insight into possible neurotrophic factor involvement in neuropathic pain, we assessed GDNF protein levels in the lumbar region of the spinal cord. Studies exploring the expression of GDNF in models of neuropathic pain have yielded contradictory results [72-75]. Nerve injury, via chronic constrictive injury and spinal nerve ligation, reduces GDNF expression in the dorsal root ganglion and sciatic nerve as early as 7 and 14 days post surgery, respectively. Whereas, nerve transaction has shown to increase GDNF expression in the sciatic nerve 48 hours after denervation. This increase in GDNF expression can remain at high levels for at least 5 months after injury [75]. Contrary to these results, measured GDNF protein levels in the spinal cord were not altered 25 days after peripheral nerve injury in our study. However, over time (110 days) GDNF protein levels increased compared to levels at day 25 regardless of exercise intervention. We were unable to determine whether this increase in GDNF levels was due to the nerve injury or age because there were no sham control groups to compare to at this time period. Earlier studies demonstrated that GDNF can be affect by peripheral nerve injury as soon as 48 hours after nerve injury [75]. Therefore, a time course study should be explored to look at GDNF levels at earlier time points, which were described in earlier studies [72-75], in order to determine whether GDNF may have a role in development and/or maintenance of neuropathic pain induced by SNI.

Aerobic exercise has been shown to increase GDNF expression in neuronal (striatum) and non-neuronal (skeletal muscle) tissues [40, 88, 90]. In the spinal cord, initially, 25 days of voluntary exercise did not increase GDNF levels in either the sham or SNI group in our study. Parallel to the sedentary group, the exercised group exhibited an increase in GDNF protein levels at day 110 following nerve injury; however there were no difference in GDNF levels between the sedentary and exercised groups at day 110. Therefore, a mechanism responsible for the exercise-induced reductions in behavioral sensitivity exhibited in rats experiencing neuropathic pain is not likely to involve the GDNF pathway and remains to be elucidated. Suggested mechanisms include the activation of the endogenous opioid system [32, 125] or increases in endogenous neurotrophins [31], which are the most commonly tested hypothesis in human and animal research. Endogenous opioids and neurotrophins act as a central modulator of pain [34, 126], thereby reducing symptoms associated with neuropathic pain.

The SNI model induces robust behavioral modifications, which leads to sensory and motor deficits [108]. These deficits caused by nerve injuries can be compensated by the reinnervation of denervated targets by regeneration of injured axons [127]. Exercise has been shown to increase regeneration of sensory [128] and motor neurons [129], which correlates with the distance the animals exercised [128]. Van Meeteren et al. (1997) demonstrated that exercise can significantly improve sensory and motor function during the early phase of

nerve regeneration (within 2 to 3 weeks), which persisted throughout the remainder of the study (150 days) [130]. These findings are similar to the time period that our animals exhibited reduction in mechanical sensitivity. Molteni et al. (2004) reported that exercised-induced axonal regeneration was consistent with the rise in neurotrophins (BDNF and NT-3). They believe that activation of the neurotrophin signal transduction pathways during exercise may play a critical role promoting axonal growth [128]. Thus, enhanced nerve regeneration due to increases in neurotrophins, which have been shown to increase with exercise [31, 36-42], may be another potential mechanism for the exercise-induced reductions in mechanical sensitivity.

2.5.3 Clinical implications

The overall goal of pain management is returning the individual to a productive life. Patients typically receive a multimodal approach of pharmacological and non-pharmacological interventions for pain management [131]. As reported, pharmacological interventions are not always effective in managing the patient's neuropathic pain [3, 4]. The use of non-pharmacological interventions, such as exercise is gaining momentum. In a survey, 72% of practitioners, which were members of the International Association for the Study of Pain, recommend the use of exercise to their patients in order to treat neuropathic pain symptoms. Of these practitioners, 80% believed that exercise should be part of legitimate medical practice [25]. Our results support the idea that long-term exercise can be considered to complement current treatment

strategies aimed at attenuating neuropathic pain. However, according to the survey by Berman and Bausell (2000), only 59% of practitioners reported having sufficient knowledge to discuss the use of exercise as a therapy treatment with their patients. Therefore, knowledge of the effectiveness of exercise for treating neuropathic pain related symptoms is clearly warranted.

2.5.4 Conclusion

A 30% reduction in pain has been shown to be clinically important [131, 132]. Given the diversity of neuropathic pain mechanisms, patient responses, and diseases, no single pharmacological intervention is effective; therefore treatments must be individualized and managed with a multidisciplinary approach. When considering the strategy for managing neuropathic pain, treatments with the lowest risk for adverse effects should be considered first. Evidence supporting exercise for treating neuropathic pain is limited; however given its presumed safety, exercise should be considered whenever appropriate [131].

Results obtained from our study, demonstrate the ability of voluntary exercise to attenuate the severity of behavioral hypersensitivity to mechanical stimuli following nerve injury in female rats. Voluntary exercise did not alter GDNF protein levels in the spinal cord, therefore other mechanisms may be involved in reducing mechanical allodynia and hyperalgesia exhibited by the exercised rats. The present study will set the foundation for future studies

examining how exercise affects behavioral sensitivity in other persistent pain models (e.g. diabetic neuropathy), with the goal of potentially limiting the dependency on medication for relieving symptoms associated with neuropathic pain.

Chapter 3

Effect of voluntary exercise on mechanical allodynia and glial cell line-derived neurotrophic factor (GDNF) levels in female diabetic rats

3.1 Abstract

Diabetic neuropathy (DN) is the most common type of neuropathy worldwide. Growth factor deficiency (e.g. glial cell-derived neurotrophic factor [GDNF]) is one of the main aetiologies of DN. Aerobic exercise has been shown to reduce behavioral sensitivity by increasing neurotrophin production. However, the ability of exercise to reduce complications of DN (e.g. mechanical allodynia) and alter GDNF levels has yet to be explored. The purpose of this study is to determine how diabetes affects GDNF levels and whether voluntary exercise has any influence in order to attenuate or delay the behavioral sensitivity associated with DN. Sixteen Sprague Dawley rats were rats were randomized into one of four groups (4 rats per group): 1) sedentary non-diabetic control (Sed-C), 2.) exercised non-diabetic control (Ex-C), 3) sedentary diabetic (Sed-D), and 4) exercised diabetic (Ex-D). The rats in the diabetic groups received streptozotocin (STZ)-injections to induce diabetes followed by insulin pellet implantation. Both exercised groups had access to voluntary running wheels. To quantify DN, mechanical allodynia (assessed via von Frey filaments) was measured prior to STZ-injections and twice a week for 8 weeks following injections. GDNF protein levels were examined in the lumbar region of the spinal cord using immunoblot analysis. After 8 weeks of voluntary exercise, the Ex-D (7.4g) group exhibited significantly higher mechanical thresholds compared to Sed-D (2.9g), suggesting a favorable response to exercise. There were no changes in GDNF expression between the sedentary groups; however 8 weeks of exercise increased GDNF protein expression in both exercised groups compared to their sedentary

counterparts. GDNF protein in the dorsal or ventral spinal cord of exercised diabetic rats revealed that the greatest increases in GDNF protein occurred in the ventral horn. Voluntary exercise decreased mechanical allodynia and this reduction may be due to an increase in GDNF, which may be exerting its analgesic effects in the ventral horn of the lumbar region of the spinal cord. Thus, this study supports the use of voluntary exercise to complement current treatment strategies aimed at decreasing DN.

3.2 Introduction

Peripheral neuropathy is a common complication associated with diabetes, with more than half of patients who have Type 1 and 2 diabetes experiencing neuropathic complications [47]. Of these patients, 15% to 30% suffer from painful diabetic neuropathy, whereas the remainder experiences a negative phenomenon, such as numbness [48]. Diabetic neuropathy (DN) is associated with structural changes in the peripheral nerve that includes axonal atrophy, demyelination, loss of nerve fibers, and the blunted regeneration of the nerve fibers [52, 53]. Clinical symptoms associated with DN involve poor gait and balance associated with large sensory fibers, and abnormal cold and/or heat sensation associated with small sensory fibers. Chronic pain associated with diabetes is represented by hyperalgesia, allodynia, parethesias and spontaneous pain [49, 50]. Despite its frequency and considerable disability, the management for DN remains unsatisfactory. As a result, there is an unmet clinical need and an ongoing challenge to develop more effective therapies [133].

Persistent pain caused by DN often responds poorly to pharmacological interventions. As a non-pharmacological approach, we have recently shown that forced exercise (via swimming) reduces behavioral sensitivity (cold allodynia and thermal hyperalgesia) in an animal model of neuropathic pain (peripheral sciatic nerve ligation) [30]. Exercise has long been recognized as a part of therapy in the management of diabetes, yet 31% of type 2 diabetic patients fail to participate in basic physical activity [134] possibly due to secondary diabetic complications (i.e.

DN). The ability of exercise to prevent the onset of painful diabetic neuropathy in individuals without signs and symptoms of DN has been reported [60]; however, the efficacy of exercise exerting a similar effect in individuals with signs and symptoms of DN has yet to be explored.

Growth factor deficiency is one of the main aetiologies of DN [53]. Recent studies have reported that diabetes alters the expression of GDNF expression in various central and peripheral tissues [86, 135]. GDNF is well known to play an important role in the modulation of nociceptive signals especially during neuropathic pain states [72, 73, 78]. In addition, studies have shown that aerobic exercise can increase GDNF in skeletal muscle of healthy and spinal-injured animals [88, 90]. In spinal-injured rats, it was postulated that the release of GDNF from exercised muscles may be involved in spinal cord plasticity following injury and increased GDNF in the periphery can be transported centrally to exert its neurotrophic actions on spinal cord cells [88].

Here, experiments were performed in diabetic rats to test whether exercise can attenuate painful sensory symptoms associated with diabetes. Our results suggest that exercise can exert beneficial actions on painful DN, and these actions may be mediated by exercise-induced increases in GDNF.

3.3 Methods

3.3.1 Animals

Sixteen 8 week old Sprague-Dawley female rats with initial body weight of 200-250 grams were purchased from Charles River (Wilmington, MA). Rats were randomized into one of four groups (4 rats per group): 1) sedentary non-diabetic control (Sed-C), 2.) exercised non-diabetic control (Ex-C), 3) sedentary diabetic (Sed-D), and 4) exercised diabetic (Ex-D). After randomization, control rats were injected with 0.4 ml of sodium citrate buffer and diabetic rats were made diabetic by a single injection of streptozotocin (STZ), a toxin that destroys pancreatic beta islets, thus causing hyperglycemia and diabetes. This is a widely used model of type 1 diabetes [116], which mimics many chronic complications observed in human diabetic, such as DN [111]. Mechanical allodynia (via von Frey monofilaments) was assessed prior to injections and twice a week for 8 weeks. After injections all rats in the exercised groups were placed in cages with access to voluntary running wheels and sedentary rats were placed in cages without running wheel access. Eight weeks after injections peak volume of oxygen (VO_{2peak}) was measured to assess cardiorespiratory fitness. After the 8 week intervention rats were over-anesthetized with Nembutal and the lumbar region of the spinal cord was harvested for GDNF protein analysis.

3.3.2 Behavioral measurements: mechanical allodynia

After randomization, baseline assessments were performed on all rats followed by STZ- or vehicle-injections. Rats predominately exercised from 6 p.m.

to 6 a.m., so all scheduled behavioral assessments were conducted at least 4 hours after 6 a.m. in order to minimize known potential acute analgesic effects that exercise may have produced. Prior to each assessment, rats were acclimated for 1 hour in the testing environment (an inverted Plexiglas box on top of an elevated one-quarter inch stainless steel mesh floor). A standard set of Semmes-Weinstein von Frey hairs (Stoelting, Wood Dale, IL), which are a series of nylon monofilaments of increasing stiffness that apply defined levels of force (grams) as they are pressed to the point where they bend, was applied in sequence to the plantar surface of the left hind paw. Starting with the filament that possesses a buckling weight of 2.0 g, a positive response is recorded after lifting of the paw and the next lightest filament is used for the next measurement. Absence of a response after 5 seconds, the next filament of increasing weight was used. This procedure continued until four measurements had been made after an initial change in the behavior or until five consecutive negative (given the score of 15 g) or four positive (score of 0.25 g) scores occur [136].

3.3.3 Induction of diabetes

All rats assigned to the diabetic groups were made diabetic by a single intraperitoneal (i.p.) injection of STZ (50 mg/kg body weight, freshly dissolved in 10mM sodium citrate, pH 4.5, with 0.9% NaCl), whereas the control rats were injected with 0.4 ml of sodium citrate buffer. Typically, 2 days later, diabetes was confirmed by measuring glucose concentration in a blood sample obtained by a

tail prick using a glucometer (Accu-Check Active; Rosche Diagnostics, Indianapolis, IN).

3.3.4 Insulin treatment

Diabetic rats were treated with low dose of insulin to maintain body weight with persistent hyperglycemia. One week after STZ-injections rats in the diabetic groups were implanted with a 2.5-mm segment of an insulin capsule (cut down from the 7.0-mm length [results in persistent hyperglycemia] Linplant sustained release insulin capsule; Linshin, Scarborough, Ont. Canada) placed subcutaneous at the scruff of the neck. Under isoflurane anesthesia, the scruff of the neck was shaved and the skin was pierced with a 16 gauge disposable hypodermic needle and withdrawn. The trocar was then pushed through the skin's orifice just created by the needle. The implant was inserted into the proximal end of the trocar. Using the stylet the implant was pushed until it exits from the distal end of the inserted trocar. Blood glucose levels were tested weekly after implantation to ensure that the rat remains hyperglycemic, and body weight was monitored weekly. If the body weight for the hyperglycemic animal drops below 20% of starting weight, a new 2.5-mm segment of capsule was inserted.

3.3.5 Vaginal smears

Estrous cycle was assessed to determine whether our rats were menstruating normally throughout the experiment. To monitor the rat's estrous

cycle, daily vaginal smears were performed. Briefly, the rats were grasped from behind its head. With a medicine dropper containing two drops of saline, the vagina will be gently flushed once and a drop of vaginal fluid was transferred to a clean, dry slide. Care was taken to not insert the dropper tip too deeply in the vagina, due to possibility of the rat becoming psuedopregnant. The slide was placed under a low power monocular microscope to view cells. The estrous cycle of a rat consists of 4 phases: 1) diestrous, 2) proestrous, 3) estrous, and 4) metestrous. The diestrous smear consisted of mainly leucocytes and some epithelial cells. The number of nucleated epithelial cells increases during proestrous and early estrous; leucocytes disappear. In full estrous, the smear consisted entirely of large cornified epithelial cells. Leucocytes appear again in metestrous, together with some remaining cornified epithelial cells [123].

3.3.6 Voluntary exercise with free-access wheel running

Exercised rats were individually housed in their own cage containing an exercise wheel (Mini Mitter Co. Inc., a Respironics Company, Bend, OR), thus, all exercise was voluntary. Each wheel revolution was continuously recorded and summarized in 30 minute intervals with the Vital View Data Acquisition System (Mini Mitter Co. Inc.) throughout the duration of the study. Sedentary rats were individually housed in their own cage that does not contain an exercise wheel; however they were exposed to a noise from running wheels as the cages with wheels were housed in the same room.

3.3.7 Calorimetry for VO_{2peak} measurements

Cardiorespiratory capacity was measured using an indirect calorimetry after 8 weeks of exercise training. Indirect calorimetries were carried out using an open circuit small animal calorimeter (Eco-Oxymax) attached to an enclosed modular treadmill (both from Columbus Instruments, Columbus, OH). Each trial measured oxygen consumption (VO_2 ml/kg/hr), respiratory exchange ratio (RER), and heat production (kcal/hr) during the resting and exercise phases. Fresh ambient air was drawn through the chamber at a flow rate of 2 L/min during the resting phase (first 10 intervals) and at 3.5 L/min during the exercise phase (remainder of test). The treadmill was started at a speed of 10 meters/min, 0 º grades. Every five intervals the speed was increase by 5 meters/min. The test stopped when the animal was no longer able to run.

3.3.8 Protein analysis

GDNF protein levels were examined in the lumbar region of the spinal cord using immunoblot analysis. We selected the lumbar region because this is the area in which nociceptive afferent fibers that mediate painful sensations terminate. The lumbar region of the spinal cord of each animal was collected during animal dissection and immediately frozen in liquid nitrogen, to preserve protein integrity, and stored at -80˚C until use. Tissues were homogenized using lysis buffer containing 137mM NaCl, 20mM Tris-HCl, pH 8.0, 1% nonyl phenoxylpolyethoxylethanol (NP-40), 10% glycerol, 1mM phenylmethanesulfonylfluoride (PMSF), 10µg/ml aprotinin, 1µg/ml leupeptin, and

0.5mM sodium vanadate. Tissue weight to buffer volume ratio was kept at 1:20 (e.g. 400µl buffer per 20mg tissue). In the lysis buffer, tissues were homogenized using a glass Teflon homogenizer, and then centrifuged at +4˚C, at 16,000g for 15 minutes. The supernatant was collected and total protein concentration was measured using BioRad protein assay reagent (BioRad Laboratories, Hercules, CA) in a 96 wellplate format, and analyzed with the microplate reader MRXII (Dynex Technologies, Chantilly, VA) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was used to separate proteins in the supernatants. Fourteen percent polyacrylamide gels with 15 wells for protein loading were poured using electrophoresis grade reagents. Twenty micrograms of total protein per sample was loaded per well of a gel, and proteins were separated under ~150-200 V current at constant voltage (30 mA). The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane over night using sandwich tank transfer apparatus (Bio-Rad Laboratories). Membranes were blocked using a blocking buffer consisting of fresh 3% non-fat dry milk (BioRad Laboratories) suspended in phosphate buffered saline (PBS) with 0.1% Tween 20 (PBS-T) for 1 hour. Membranes were then incubated with primary antibody (GDNF rabbit-polyclonal [Santa Cruz Biotechnology, Santa Cruz, CA, catalog # sc-328; dilution 1:2,000]) diluted in the blocking buffer, for 1 hour. The membrane was then washed 3 times with PBS-T for 10 minutes each. The membrane was then incubated for 30 minutes with the secondary antibody (goat anti-rabbit [Santa Cruz Biotechnology, catalog # sc-2004; dilution 1:2,000]) in the blocking buffer

and then washed 3 times in PBS-T for 10 minutes. All membrane incubations and washes were done at room temperature on a shaker. Detection was achieved using enhanced chemiluminescent reagent (ECL; SuperSignalWestPico Chemiluminescent Substrate, catalog # 34080, Pierce, Rockford, IL]) according to manufacturer's recommendations, and exposure of membranes to X-ray film. The relative levels of protein were estimated by measuring mean pixel intensity of protein bands using Adobe Photoshop. Before the blocking step, all membranes were stained with Ponceau S solution to verify even loading, integrity of the extracted proteins and proper transfer [124]. If uneven loading of protein occurred, a single band on the Ponceau stained membrane was used for normalization. GDNF was detected as a single band migrating at a molecular weight of 36 kilodaltons (kDa). The specificity of the band was confirmed using a GDNF blocking peptide (Santa Cruz Biotechnology, catalog #sc-328P).

3.3.9 Data analysis

For protein analysis and VO_{2peak} an analysis of variance (ANOVA) with a least significant difference (L-S-D) post hoc analysis was conducted for identification of significant differences between groups. Mechanical allodynia and daily exercise distance were analyzed using repeated measures ANOVA with a LSD post hoc analysis conducted for identification of significant differences within and between time and groups. Independent samples t-test was used to analyze

GDNF protein levels within groups. Significance was set at 0.05 ($p<.05$). Data are presented as mean ± standard error of the mean (SEM).

3.4 Results

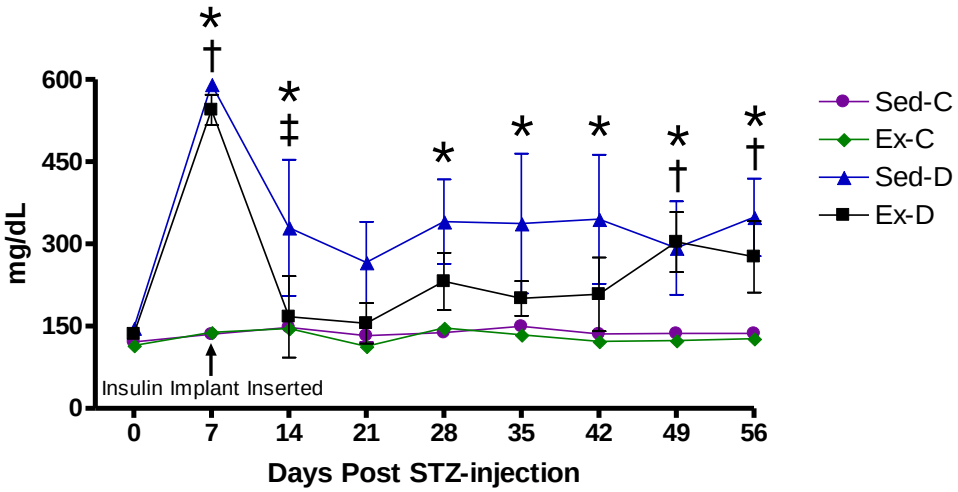
3.4.1 Animal characteristics

Blood glucose for the sedentary and exercised non-diabetic groups did not change over the 8 week experiment (136±8 vs. 127±4 mg/dL, respectively, at day 56). After STZ-injections the diabetic groups had marked increase in blood glucose compared to their respective control group, which persisted throughout the 8 week study. Exercised diabetic animals exhibited an improvement in blood glucose at day 14 (1 week after inserting the insulin pellet) compared to their sedentary counterpart, which diminished during the remainder of the study (276±65 vs. 348±70 mg/dL, respectively, at day 56). After insertion of the insulin pellet the, Ex-D group's blood glucose was similar to the Ex-C group; however by day 49 the Ex-D group's blood glucose had returned to hyperglycemic levels where it remained until the end of the study (Figure 3.1).

Over the 8 week study, all groups exhibited an increase in body weight. The Sed-C, Ex-C, Sed-D, and Ex-D groups showed a 63.7%, 55.1%, 23.6%, and 40.9% increase in body weight over the 8 week experiment, respectively. There were no differences in body weight between the diabetic groups (Sed-D vs. Ex-D [278±13 vs. 300±14 grams, at day 56]) and exercised groups (Ex-C vs. Ex-D [297±15 vs. 300±14 grams, at day 56]) throughout the study. However, the body

Figure 3.1 Blood glucose levels in rats throughout the 8 week training program. Insulin pellet was inserted at day 7 post STZ-injection. STZ-injections markedly increase in blood glucose in the diabetic groups compared to their respective control groups, which persisted throughout the 8 week study. Exercised diabetic animals exhibited an improvement in blood glucose at day 14 (1 week after inserting the insulin pellet) compared to their sedentary counterpart, which diminished during the remainder of the study (276±65 vs. 348±70 mg/dL], respectively, at day 56). *=Sed-C vs. Sed-D, †=Ex-C vs. Ex-D, and ‡=Sed-D vs. Ex-D. $p<0.05$

Figure 3.1 Blood glucose levels in rats throughout the 8 week training program.



weight of the Sed-C (352±27 grams [at day 56]) group was significantly greater during the majority of the study compared to the Ex-C (297±15 grams [at day 56]) and Sed-D (278±13 grams [at day 56]) groups (Figure 3.2).

3.4.2 Maximal metabolic measurements

To quantify the effect of exercise, VO_{2peak} was evaluated. As shown in Figure 3.3, at the end of 8 weeks, cardiorespiratory capacity did not significantly differ between the Ex-D and Sed-D groups; however, the Ex-D group demonstrated favorable trend (3196 vs. 2890 ml/kg/hr, respectively). Ex-C significantly differed from Sed-C (3389 vs. 2758 ml/kg/hr, respectively) possibly because the Ex-C group on average exercised more than Ex-D group, which reached significance during week 7 (Figure 3.4).

3.4.3 Behavioral assessments

We evaluated the effects of habitual exercise on mechanical allodynia in diabetic rats that developed mechanical allodynia and their respective controls (Figure 3.5). After STZ-injections both diabetic groups exhibited an increase in mechanical allodynia. The Sed-D group continued to exhibit an increase in mechanical allodynia throughout the remainder of the 8 week study. Exercise caused a favorable change in pain behavior following STZ-induced diabetes in these rats. At day 56 mechanical allodynia was significantly less in the Ex-D compared to the Sed-D group (7.4±1.7 vs. 2.9±0.6 grams, respectively). Among non-diabetic groups, the Sed-C group exhibited greater levels in mechanical

Figure 3.2 Body weight of rats throughout the 8 week training program. Insulin pellet was inserted at day 7 post STZ-injection. The Sed-C, Ex-C, Sed-D, and Ex-D groups showed a 63.7%, 55.1%, 23.6%, and 40.9% increase in body weight over the 8 week experiment, respectively. Body weight of the Sed-C (352±27 grams [at day 56]) group was significantly greater during the majority of the study compared to the Ex-C (297±15 grams [at day 56]) and Sed-D (278±13 grams [at day 56]) groups. *=Sed-C vs. Sed-D, †=Sed-C vs. Ex-C. $p<0.05$

Figure 3.2 Body weight of rats throughout the 8 week training program.

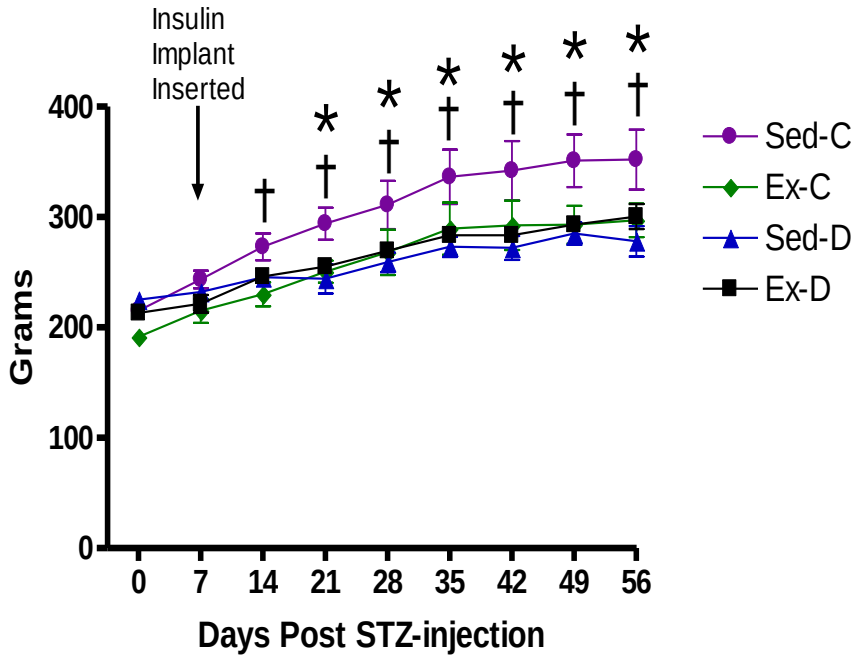


Figure 3.3 Peak volume of oxygen uptake (VO_2 [an index of cardiorespiratory fitness]) in exercised and sedentary rats at 8 weeks. At 8 weeks, Ex-C significantly differed from Sed-C (3389 vs. 2758 ml/kg/hr, respectively), while the Ex-D group demonstrated a similar trend compared to the Sed-D group (3196 vs. 2890 ml/kg/hr, respectively)* $p<0.05$

Figure 3.3 Peak volume of oxygen uptake.

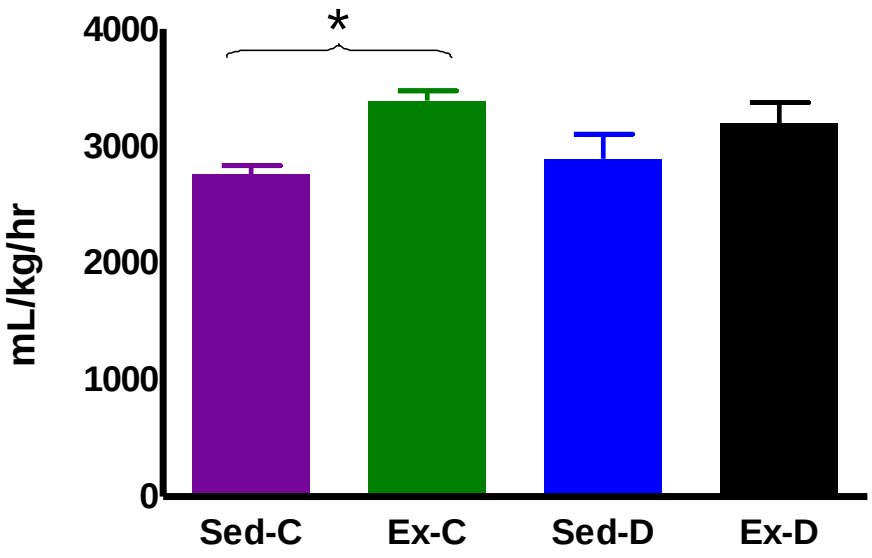


Figure 3.4 Average daily exercise distance throughout the 8 week training program. During week 8 the Ex-C and Ex-D groups exercised 10.2±1.7 and 4.8±1.5 km/day, respectively. * $p<0.05$

Figure 3.4 Average daily exercise distance throughout the 8 week training program.

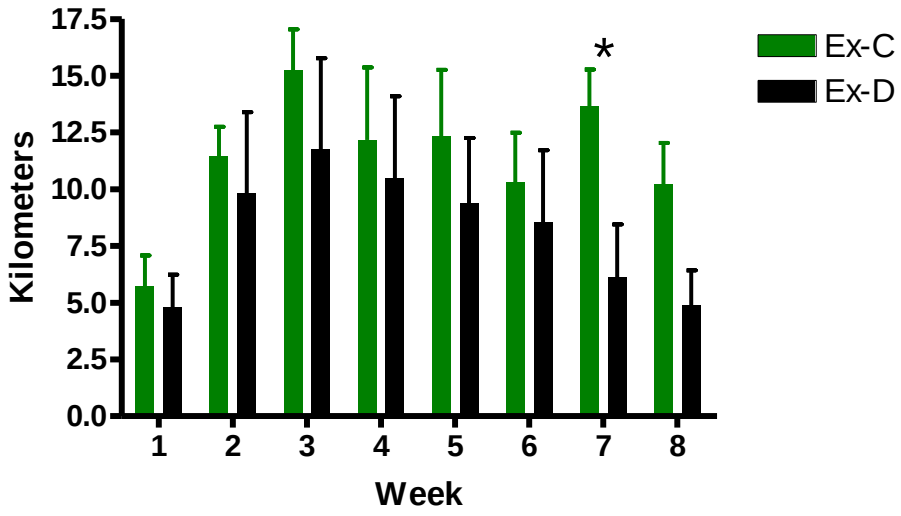
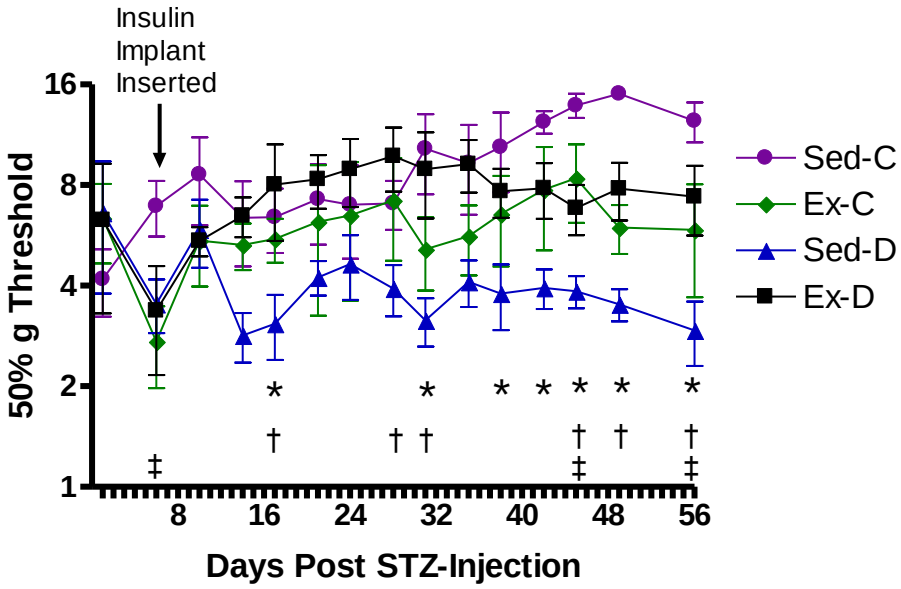


Figure 3.5 Mechanical allodynia assessed using a von Frey monofilament test 56 days post STZ-injection. Insulin pellet was inserted at day 7 post STZ-injection. At day 56 von Frey (force [g]) assessments were significantly different between the Ex-D and Sed-D group (7.4±1.7 vs. 2.9±0.6, respectively). *=Sed-C vs. Sed-D, †=Sed-D vs. Ex-D, and ‡=Sed-C vs. Ex-C. $p<0.05$

Figure 3.5 Mechanical allodynia assessed using a von Frey monofilament test 56 days post STZ-injection.



thresholds compared to the Ex-C group at day 56 (12.5±1.7 vs. 5.9±2.1 grams, respectively). However, 8 weeks of exercise in STZ-induced diabetic rats resulted in decreases in mechanical allodynia.

3.4.4 Protein analysis

Protein analysis was used to determine the effects voluntary exercise has on GDNF (via immunoblotting) protein levels in the lumbar region in rat spinal cord and dorsal and ventral horn of spinal cord tissue.

3.4.4.1 Whole lumbar region of the spinal cord

Eight weeks of diabetes did not affect GDNF proteins levels in the spinal cord compared to its sedentary control (Figure 3.6). However, voluntary exercise increased GDNF protein levels among both exercised groups. The Ex-D and Ex-C group displayed a 3.2- and 1.5-fold increase in GDNF protein levels compared to their sedentary counterparts, respectively. Thus, diabetes did not cause changes in GDNF protein levels, while exercise training increased its level in both diabetic and control groups.

3.4.4.2 Dorsal and ventral horn of spinal cord

To assess where GDNF is exerting its analgesic effects in the spinal cord, we examined protein levels in the dorsal and ventral horn of the lumbar region of the spinal cord. There were no changes in GDNF protein levels in the dorsal horn among either control or diabetic groups (Figure 3.7). However, diabetes

Figure 3.6 Immunoblot analysis of GDNF levels in the lumbar region of the spinal cord of non-diabetic and diabetic rats. Upper panel shows representative immunoblot; lower panel shows histogram analysis of the immunoblot. A.) sedentary groups, B.) control groups, and C.) diabetic groups. There were no differences between either sedentary groups. Both exercised groups demonstrated an increase in GDNF levels compared to their sedentary counterparts after 8 weeks of voluntary exercise.* $p<0.05$

Figure 3.6 Immunoblot analysis of GDNF levels in the lumbar region of the spinal cord of non-diabetic and diabetic rats.

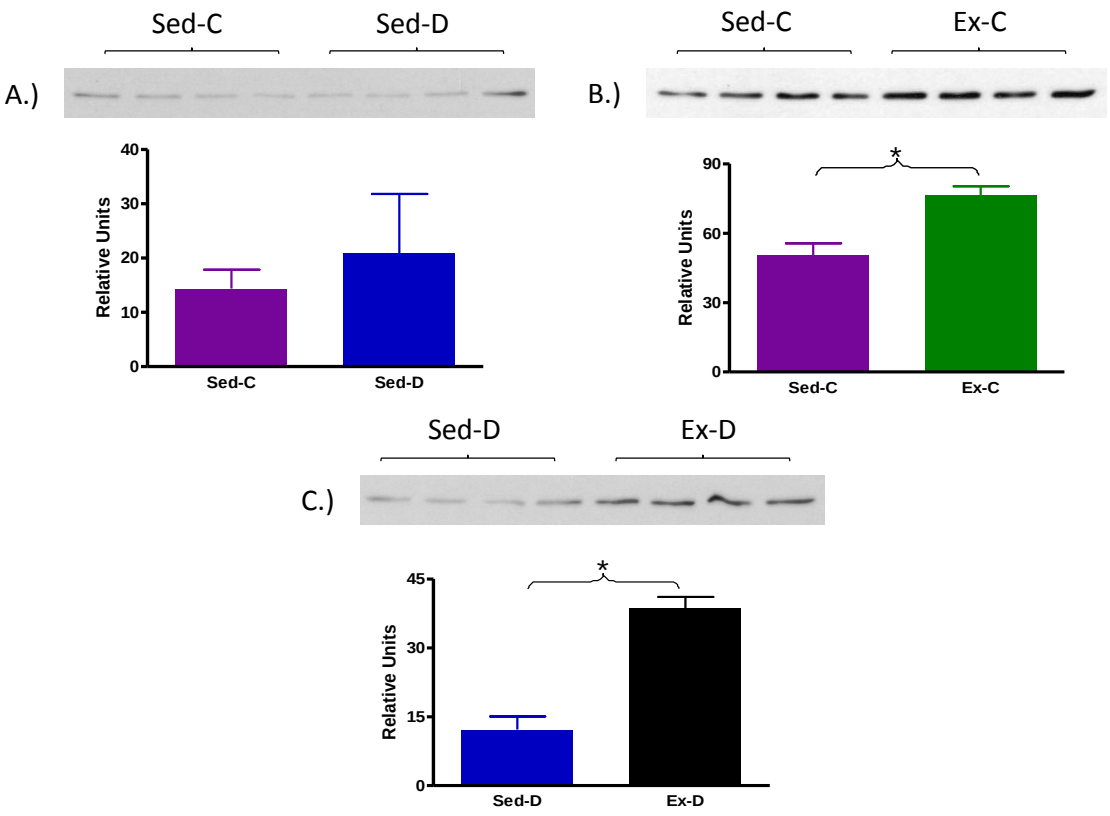
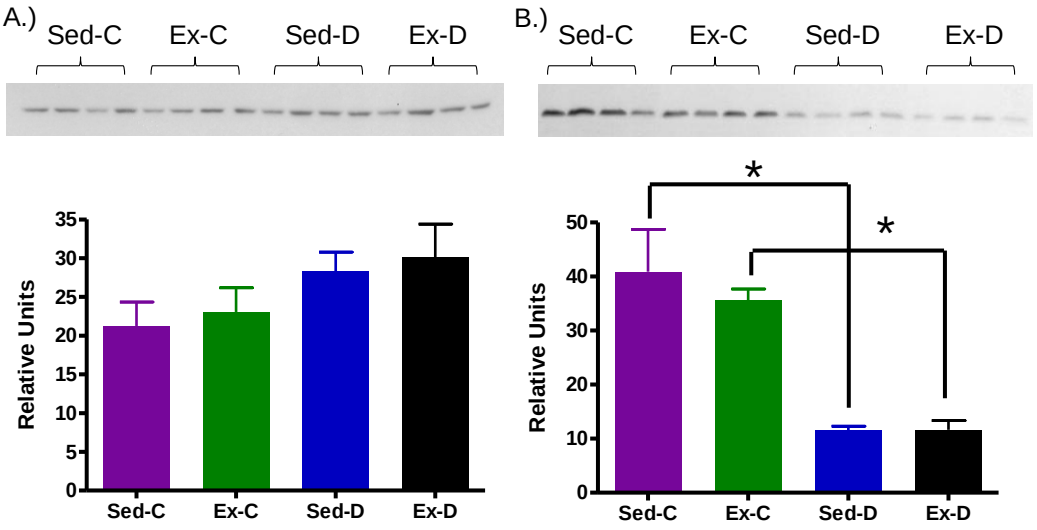


Figure 3.7 Dorsal horn and ventral horn. Immunoblot analysis of GDNF levels in the dorsal horn of the lumbar region of the spinal cord of non-diabetic and diabetic rats. Upper panel shows representative immunoblot; lower panel shows histogram analysis of the immunoblot. A.) dorsal horn and B.) ventral horn. There were no differences in GDNF levels in the dorsal horn between groups. Diabetes significantly decreased GDNF levels in the ventral horn of both diabetic groups compared to their respective controls. * $p<0.05$

Figure 3.7 Dorsal horn and ventral horn.



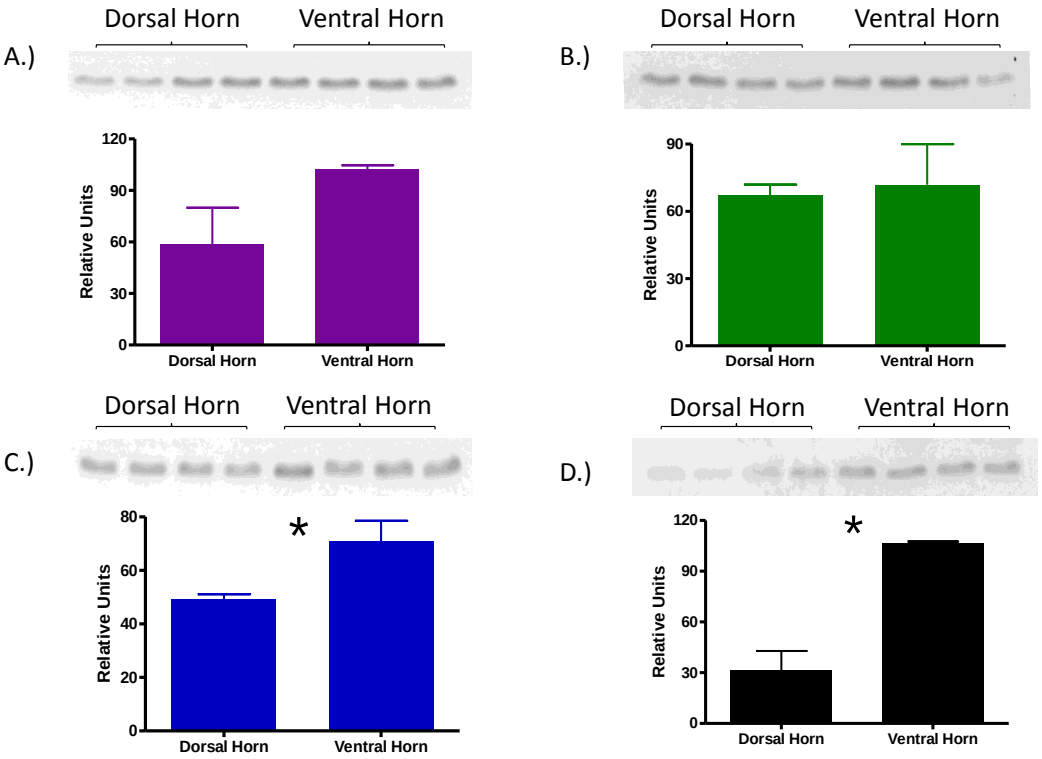
significantly decreased GDNF protein levels in the ventral horn. The Sed-D and Ex-D groups exhibited a 3.5- and 3.0-fold decrease in GDNF levels in the ventral horn compared to respective controls, respectively. Exercise had no effect on GDNF protein levels in the dorsal or ventral horn in either the diabetic or control group. Within group comparisons showed that GDNF levels did not differ between dorsal and ventral horn of the control groups (Figure 3.8). However, in both diabetic groups GDNF proteins levels were significantly higher in the ventral horn compared to the dorsal horn. GDNF protein levels in the ventral horn of the Sed-D and Ex-D groups' was 1.4- and 3.4-fold greater compared to levels in the dorsal horn. These results suggest that the GDNF is may be exerting its analgesic effects in the ventral horn of the lumbar region of the spinal cord.

3.5 Discussion

Painful diabetic neuropathy is a prominent clinical problem with few treatment options. Here, we have tested the ability of voluntary exercise to improve pain indices in diabetic rats. Our results suggest that voluntary exercise significantly improves mechanical hypersensitivity in diabetic rats. Moreover, our studies suggest that these analgesic actions may be mediated by exercise-induced increases in neurotrophic factor levels. Voluntary exercise significantly increased GDNF protein levels in the spinal cord. This study strongly suggest that exercise has beneficial actions on alleviating pain in diabetic patients and provide a potential mechanism by which exercise changes neurotrophic factor levels that are beneficial to neural pain circuitry.

Figure 3.8 Dorsal horn versus ventral horn. Immunoblot analysis of GDNF levels in the dorsal and ventral horn of the lumbar region of the spinal cord of non-diabetic and diabetic rats. Upper panel shows representative immunoblot; lower panel shows histogram analysis of the immunoblot. A.) Sed-C, B.) Ex-C, C.) Sed-D, and D.) Ex-D. GDNF levels did not differ between the ventral horn versus the dorsal horn among both control groups. Diabetes significantly increased GDNF levels in the ventral horn compared to the dorsal horn among the sedentary and exercised diabetic groups (1.4- and 3.4-fold increase, respectively). * $p<0.05$

Figure 3.8 Dorsal horn versus ventral horn.



3.5.1 Exercise reduces diabetes-induced mechanical allodynia

Painful diabetic neuropathy is primarily associated with burning, spontaneous pain in the extremities, although mechanical allodynia is also generally present. To date, best approach to study painful neuropathy in rodents is assessment of mechanical sensitivity using monofilaments. This is in large part due to the inability to assess or detect spontaneous pain in rodents. Sprague Dawley rats are well known as a model of diabetes-induced mechanical allodynia [116]. In the current study, diabetic Sprague Dawley rats develop significant mechanical allodynia within 3 weeks of diabetes onset, which persisted throughout the 8 week study.

An important focus of this study was to test whether voluntary exercise could modify the increased pain sensitivity of diabetic rats. In diabetic patients without any signs of symptoms of DN, exercise can delay the development of motor and sensory neuropathy [60]. Currently, there is limited knowledge about the effectiveness exercise has on attenuating neuropathy associate with DN. To gap this knowledge we have shown that, long-term voluntary exercise reduces mechanical allodynia in diabetic rats. Diabetic rats showed transient development of mechanical allodynia; however 17-days of exercise returned mechanical thresholds to baseline levels in the diabetic group, which persisted throughout the 8 week experiment. Although not tested, it would be interesting to determine if exercise interventions were terminated whether diabetic rodents would again develop mechanical allodynia. It is important to point out that our results only

address pain associated with diabetes. We have shown that the administration of GDNF can improve mechanical and inflammatory (formalin) behavioral sensitivity in diabetic mice with insensate DN [137]. Whether exercise can improve insensate neuropathy in non-painful models remains unknown. It will be interesting to determine which various aspects of diabetic neuropathy can be modified by exercise.

3.5.2 Mechanisms of exercise-induced analgesia

Understanding the molecular mechanisms by which exercise exerts actions on peripheral nerve function remains poorly understood. Putative mechanisms likely include involvement of both opioid and non-opioid systems. Exercise-induced release of endogenous opioids is the most commonly tested and proposed mechanism for exercise induced analgesia in healthy individuals [125]. In an animal model of neuropathic pain via spinal cord injury, the amelioration of mechanical allodynia has been attributed to an increase in neurotrophins in the lumbar region of the spinal cord and soleus muscle [31].

Deprivation of neurotrophic factor support contributes to the pathogenesis of DN [54]. Liu et al. (2009) demonstrated that neuropathic symptoms in diabetic rats correlated with the levels of GDNF expression. As GDNF expression in the sciatic nerve diminished neuropathic symptoms increased; however, after GDNF gene delivery, these neuropathic symptoms were alleviated. GDNF is important for regulating cell survival, differentiation, proliferation, migration, chemotaxis,

branching morphogenesis, neurite outgrowth, and synaptic plasticity [63, 64] and is also known to have potent analgesic effects in neuropathic pain states [72, 76-79]. Therefore, as a possible mechanism for reducing mechanical allodynia in diabetic rats, we assessed GDNF protein levels in the lumbar region of the spinal cord. Voluntary exercise dramatically increased GDNF protein levels in lumbar spinal cord of diabetic rats. Western blots of GDNF protein in the dorsal and ventral spinal cord of exercised diabetic rats revealed that the greatest increases in GDNF protein occurred in the ventral horn, suggesting that motoneurons may play an important role in modulating mechanical allodynia in diabetic rats. Increases in mechanical allodynia exhibited by the sedentary diabetic group cannot be attributed to alterations in neurotrophic support; however increases in GDNF via voluntary exercise may have a therapeutic effect. Possible mechanisms for these analgesic actions by GDNF include promoting the survival of non-peptidergic nociceptive C-fiber neurons (isolectin B4 [IB4] binding), therefore decreasing Aβ-fiber sprouting [80], regulating sodium channel expression [77], and by regulating neuropeptides expression [76]. Thus, this study demonstrated that exercise causes a favorable change in mechanical allodynia in female rats with diabetes. These results strongly argue that GDNF may be a viable treatment for attenuating acute and chronic pain states associated with peripheral nerve damage.

3.5.3 Clinical implications

Exercise has long been used to manage diabetes. Exercise has been shown to lower glucose levels and improve insulin action. In diabetic patients with less extreme glucose levels, exercise can improve insulin sensitivity and glucose disposal. Also, aerobic exercise can improve cardiorespiratory fitness (VO_{2max}), thus reducing the risk of developing cardiovascular diseases, including coronary heart disease, stroke, peripheral vascular disease, and congestive heart failure [138]. Besides the general health benefits associated with regular exercise, many diabetic patients do not regularly exercise due to symptoms associated with DN. Numerous primary therapies have been attempted in DN, with variable results. Management primarily relies on the pharmacological treatment of symptoms associated with DN (e.g. neuropathic pain) [139]. Currently, there is no form of therapy that has been identified to provide unequivocal, safe, and effective stabilization or reversal of the DN [51]. However, our results support the idea that long-term exercise can be used to complement current treatment strategies aimed at decreasing neuropathic pain in patients with DN, which may aid in decreasing the dependency of medication.

3.5.4 Conclusion

Results obtained from our study, demonstrates the influence voluntary exercise has on attenuating the severity of behavioral hypersensitivity to mechanical stimuli associated with DN in female rats. The significance of this research has given insight to possible mechanism by which exercise has an

effect on reducing mechanical allodynia in diabetic females. The present study has set the foundation for future studies examining the production and transportation of GDNF along with the therapeutic effects it may have. The mechanism by which exercise attenuates DN is poorly understood and more research is clearly warranted.

Chapter 4

Effect of voluntary exercise on mechanical allodynia and GDNF production and transport in male diabetic mice

4.1 Abstract

The mechanisms that lead to painful symptoms associated with DN remain elusive. Growth factor deficiency (e.g. GDNF) is one of the main aetiologies of DN, which may be due to disturbances in axonal transport. Studies have shown that exercise can increase GDNF and axonal transport of proteins, however, the ability of exercise to increase transport of GDNF to areas in need of trophic support, which may reduce behavioral sensitivity associated with DN, has yet to be explored. Therefore, the purpose of this study is to determine how diabetes affects GDNF production and transport and whether voluntary exercise has any influence in order to attenuate or delay mechanical sensitivity associated with DN. Inbred A/J mice were randomized into a sedentary (Sed-C) and exercised (Ex-C) non-diabetic control group or a sedentary (Sed-D) and exercised (Ex-D) diabetic group. The diabetic groups received streptozotocin (STZ)-injections to induce diabetes. The exercised mice were housed in standard cages with access to voluntary running wheels. To quantify DN, mechanical allodynia (assessed via von Frey filaments) was measured prior to STZ-injections and once a week for 6 weeks following injections. GDNF protein levels and mRNA expression were analyzed in the lumbar region of the spinal cord, dorsal root ganglion, sciatic nerve, and skeletal muscle. GDNF axonal transport was assessed in the sciatic nerve using the double ligation technique. At diabetes duration of 6 weeks, the diabetic animals exercised about 3 Km/day. After 6 weeks, diabetes significantly increased mechanical allodynia, however, 6 weeks of voluntary exercise alleviated mechanical allodynia in the Ex-D group

compared to the Sed-D group (2.8g vs. 1.6g, respectively). Six weeks of diabetes significantly decreased GDNF levels in the spinal cord, whereas voluntary exercise increase GDNF protein levels in the spinal cord and sciatic nerve along with mRNA expression in skeletal muscle compared to it sedentary counterpart. Two weeks of voluntary exercise increased GDNF protein levels in the sciatic nerve, which is when mechanical thresholds in the Ex-D group returned to baseline levels. Voluntary exercise increased axonal transport in the exercised diabetic group, particularly in an anterograde direction. Measurements of the degree of exercise (total distance) significantly correlated with the amount of GDNF accumulated in the proximal segment of the sciatic nerve, suggesting that the amount of exercise may regulate levels of GDNF production and transport from motoneurons. To determine the potential therapeutic actions GDNF may have, intrathecal injections of recombinant GDNF were administered for two weeks. Similar to the 6 week study, intrathecal injections of recombinant GDNF ameliorated mechanical sensitivity associated with DN. Voluntary exercise decreased mechanical allodynia and this reduction may be due to an increase in GDNF and axonal transport of this neurotrophic factor to areas in need of trophic support. Thus this study supports the use of voluntary exercise to complement current treatment strategies aimed at decreasing DN.

4.2 Introduction

Under normal conditions, nerve fibers maintenance in the periphery is balanced between nerve degeneration and regeneration; however diabetes impairs the ability of the diabetic nerve to regenerate in response to the degenerative process. Therefore, structural changes in the peripheral nerve such as axonal atrophy, demyelination, loss of nerve fibers, and the blunted regeneration of the nerve fibers may occur, ultimately leading to DN [52-54]. The decreased nerve regenerative capacity in diabetes has been associated with impaired neurotrophic support, which may reflect reductions in the availability of neurotrophic factors. It is believed that neurotrophic factor deficiency (e.g. GDNF) in diabetes may be due to disturbances in axonal transport, which is critical for maintaining normal metabolism, physiological activity, and structural integrity of the nerve [53, 91].

GDNF is a potent trophic factor that influences the development, survival, and maintenance of neurons in the central and peripheral nervous systems [54]. Recently, it has been shown that GDNF production is decreased in peripheral tissues of diabetic rats [54]. Therefore, the availability of GDNF through axonal transport for the survival of motor and sensory neurons is diminished. Studies have revealed that GDNF can be transported both anterogradely [95, 96] and retrogradely [97] within neurons. Several studies have shown that diabetes reduces axonal transport of neurotrophins (BDNF, NGF, and NT-3) [98-101], However GDNF axonal transport in diabetic animals has yet to be explored.

Earlier studies have shown that exercise can increase axonal transport of enzymes and proteins up to 20% and 50%, respectively, which can occur in both intact and deafferented nerves [91, 102-104]. Exercise-induced increases in axonal transport are due to increases in transport velocity in the neuron. It is believed that exercise increases neuronal activity leading to changes in the nerve terminal, therefore, axonal transport of proteins is increased in order to compensate for the effects exercise has on the distal parts of the neurons. As a result, the neuron is allowed to repair itself in order to return to normal nerve function [91].

The diabetic nerve is unable to maintain the normal degeneration/regeneration process, which leads to positive (parethesia as described as prickling, tingling, “pins and needles”, burning, crawling, itching, abnormal sensation to temperature, and pain) or negative (numbness, injury insensitivity) sensory symptoms. Exercise studies have alluded to that trained neurons are more responsive to alterations to their immediate neuronal environment. Therefore, experiments were performed to determine whether diabetic mice with mechanical allodynia display alterations in GDNF production and/or disruptions in and axonal transport, and whether voluntary exercise has any influence on these outcomes. Our results suggest that exercise can exert beneficial actions on painful DN, and these actions may be mediated by exercise-induced increases in GDNF production and transport.

4.3 Methods

4.3.1 Animals

Ninety-seven male A/J mice (8 week old) were purchased from Jackson Laboratories. Mice were housed in the animal facilities at the University of Kansas Medical Center (KUMC) where they were exposed to a 12 hour light/dark cycle and were given food and water ad libitum. All animal use protocols were approved by the Institutional Animal Care and Use Committee of the KUMC.

4.3.1.1 6 week study

Thirty-two mice were involved in a 6 week study. Mice were randomized into one of 4 groups: 1) sedentary non-diabetic control (Sed-C [n=7]), 2) exercised non-diabetic control (Ex-C [n=7]), 3) sedentary diabetic (Sed-D [n=11]), and 4) exercised diabetic (Ex-D [n=7]). Following randomization, all mice in the diabetic groups received two intraperitoneal (i.p.) injections of STZ (freshly dissolved in 10mM sodium citrate, pH 4.5, with 0.9% NaCl, [Sigma, St. Louis, MO]) on consecutive days (85 [day 1] and 65 [day 2] mg/kg body weight) to induce diabetes, where as the control animals were injected with 0.4 ml of sodium citrate buffer, pH 4.5. Mice were fasted 3 hours before and 3 hours after each injection. After injections, the exercised mice were housed in cages with access to voluntary running wheels while the sedentary groups were housed in cages without wheels. Weight and glucose levels were monitored weekly. Behavioral sensitivity to mechanical allodynia (assessed via von Frey filaments) was measured prior to injections and once a week for 6 weeks. All behavioral

assessments were performed on the plantar surface of the left hind paw. After the end of the 6 week experiment, mice were over-anesthetized with Avertin. Protein and mRNA analysis of the lumbar region of the spinal cord, dorsal root ganglia, sciatic nerve, and skeletal muscle (gastrocnemius) were conducted following the 6 week study.

4.3.1.2 GDNF intrathecal injection study

Thirty-five mice participated in a 2 week study. Mice were randomized into one of 5 groups (7 mice per group): 1) sedentary non-diabetic control (Sed-C), 2) exercised non-diabetic control (Ex-C), 3) sedentary diabetic (Sed-D), and 4) exercised diabetic (Ex-D), and 5) sedentary diabetic with GDNF intrathecal injections (Sed-DWG). Induction of diabetes, animal housing, and behavioral testing were the same to that of the 6 week study. After induction of diabetes, mice in the Sed-DWG group received daily intrathecal injections of human recombinant GDNF for 2 weeks. After the end of the 2 week experiment, mice were sacrificed and GDNF protein levels were analyzed in the lumbar region of the spinal cord and sciatic nerve.

4.3.1.3 Double ligation study

Twenty-nine mice were randomly assigned to 5 groups. Groups' consisted of a sedentary (Sed-C [n=7]) and exercised (Ex-C [n=7]) non-diabetic control group, a sedentary (Sed-D [n=7]) and exercised (Ex-D [n=7]) diabetic group, and a sedentary non-diabetic sham group (Sed-Sham [n=1]). Induction of diabetes

and animal housing were the same to that of the 6 week study. Six weeks after injections, double ligation of the sciatic nerve was used to measure GDNF transport. The double ligation technique was used to measure GDNF levels obtained in specific regions of sciatic nerve (distal, middle or in-between the ligation, and proximal). This technique was used for the following reasons: 1) it interrupts axonal flow, thus allowing us to visualize and quantify the transported substance; 2) it provides clear separation of proximal and distal segments of the ligated nerve, demonstrating retrograde and anterograde transport of substances by accumulation in these segments; 3) accumulation of some substances in the proximal segment of the ligated nerve fibers provides evidence for anterograde, whereas accumulation in the distal segment suggests retrograde transport; and 4) the increase and accumulation of the same substance in the middle segment provides information about local synthesis of the substance [140].

4.3.2 Running methods

Exercised mice were individually housed in their own cage containing an exercise wheel (Mini Mitter Co. Inc., a Respironics Company, Bend, OR), thus, all exercise was voluntary. Each wheel revolution was continuously recorded and summarized in 30 minute intervals with the Vital View Data Acquisition System (Mini Mitter Co. Inc.) throughout the duration of the study. Sedentary rats were individually housed in their own cage that did not contain an exercise wheel.

4.3.3 Behavioral analysis: mechanical allodynia

Mice primarily exercised from 6pm to 6am, to avoid acute exercise effects all behavioral assessments were done 4 hours after they ceased running. Mice were placed under a transparent plastic dome on an elevated wire grid. A standard set of Semmes-Weinstein von Frey hairs (Stoelting, Wood Dale, IL), which are a series of nylon monofilaments of increasing stiffness that apply defined levels of force (grams) as they are pressed to the point where they bend, were applied in sequence to the plantar surface of the left hind paw. Starting with the filament that possesses a buckling weight 0.15g, a positive response is recorded after lifting of the paw and the next lightest filament is used for the next measurement. Absence of a response after 5 seconds, the next filament of increasing weight was used. This procedure continued until four measurements were made after an initial change in the behavior or until five consecutive negative (given the score of 6g) or four positive (score of 0g) scores occur. All behavioral assessments were performed on the plantar surface of the left hind paw. Behavioral assessments were performed once a week for 2 or 6 weeks [141].

4.3.4 Induction of diabetes

Following randomization, diabetes was induced by two intraperitoneal (i.p.) injections of STZ (freshly dissolved in 10mM sodium citrate, pH 4.5, with 0.9% NaCl, [Sigma, St. Louis, MO]) on consecutive days (85 [day 1] and 65 [day 2] mg/kg body weight), where as the control mice were injected with 0.4 ml of

sodium citrate buffer, pH 4.5. Mice were fasted 3 hours before and 3 hours after each injection. Mice weight and blood glucose (using glucose diagnostic reagents; Sigma) were measured 1 week post STZ-injection and once a week throughout the study. Mice were included in the diabetic group if their blood glucose levels were >16.0 mmol/L at every measure.

4.3.5 Intrathecal injections

After induction of diabetes, mice were injected intrathecally once per day with human recombinant GDNF. GDNF was dissolved in aritificial cerebrospinal fluid at a concentration of 20nM. Intrathecal injections (50 µl, 1µg GDNF) were performed according to Christianson et al. (2003) between the L6 and S1 vertebrae with a 27-guage needle. Intrathecal delivery of GDNF was used to provide the best opportunity to deliver GDNF to the spinal cord and to avoid problems associated with poor retrograde transport of GDNF from the periphery.

4.3.6 Double ligation technique

Under Avertin anesthesia, the skin on the lateral surface of the left thigh was incised revealing the muscle. Using forceps, a section was made directly through the bicep femoris muscle exposing the sciatic nerve. Ligations consisting of two 4.0 silk ligatures were tied tightly around the sciatic nerve about 3mm apart at the mid-thigh. Muscle and skin were closed using 4.0 silk sutures. Sham surgery procedures were performed as above, however it only involved exposure of the sciatic nerve without any ligations. The double ligatures were applied for

18-20hrs, as suggested by several studies [95, 96, 100, 140]. The mice were over-anesthetized using Avertin, and the left sciatic nerve was exposed and intermediately frozen with tissue tech and placed at -80˚C until further analysis.

4.3.7 Protein analysis

GDNF protein levels were examined in the lumbar region of the spinal cord, DRG, sciatic nerve, and skeletal muscle (gastrocnemius) using immunoblot analysis. The harvested tissues of the mice were collected during dissection and immediately frozen in liquid nitrogen, to preserve protein integrity, and stored at -80˚C until use. Tissues were homogenized using lysis buffer containing 137mM NaCl, 20mM Tris-HCl, pH 8.0, 1% nonyl phenoxylpolyethoxylethanol (NP-40), 10% glycerol, 1mM phenylmethanesulfonylfluoride (PMSF), 10µg/ml aprotinin, 1µg/ml leupeptin, and 0.5mM sodium vanadate. Tissue weight to buffer volume ratio was kept at 1:20 (e.g. 400µl buffer per 20mg tissue) for the spinal cord and skeletal muscle and at 1:50 for the DRG and sciatic nerve. In the lysis buffer, tissues were homogenized using a glass Teflon homogenizer, and then centrifuged at +4˚C, at 16,000g for 15 minutes. The supernatant was collected and total protein concentration was measured using BioRad protein assay reagent (BioRad Laboratories, Hercules, CA) in a 96 wellplate format, and analyzed with the microplate reader MRXII (Dynex Technologies, Chantilly, VA) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was used to separate proteins in the supernatants. Fourteen percent polyacrylamide gels with 15 wells for protein

loading were poured using electrophoresis grade reagents. Twenty (spinal cord), 50 (DRG and sciatic nerve), and 100 (skeletal muscle) micrograms of total protein per sample was loaded per well of a gel, and proteins were separated under ~150-200 V current at constant voltage (30 mA). The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane over night using sandwich tank transfer apparatus (Bio-Rad Laboratories). Membranes were blocked using a blocking buffer consisting of fresh 3% non-fat dry milk (BioRad Laboratories) suspended in phosphate buffered saline (PBS) with 0.1% Tween 20 (PBS-T) for 1 hour. Membranes were then incubated with primary antibody (GDNF rabbit-polyclonal [Santa Cruz Biotechnology, Santa Cruz, CA, catalog # sc-328; dilution 1:2,000]) diluted in the blocking buffer, for 1 hour. The membrane was then washed 3 times with PBS-T for 10 minutes each. The membrane was then incubated for 30 minutes with the secondary antibody (goat anti-rabbit [Santa Cruz Biotechnology, catalog # sc-2004; dilution 1:2,000]) in the blocking buffer and then washed 3 times in PBS-T for 10 minutes. All membrane incubations and washes were done at room temperature on a shaker. Detection was achieved using enhanced chemiluminescent reagent (ECL; SuperSignalWestPico Chemiluminescent Substrate, catalog # 34080, Pierce, Rockford, IL]) according to manufacturer's recommendations, and exposure of membranes to X-ray film. The relative levels of protein were estimated by measuring mean pixel intensity of protein bands using Adobe Photoshop. Before the blocking step, all membranes were stained with Ponceau S solution to verify even loading, integrity of the extracted proteins and proper transfer [124]. If

uneven loading of protein occurred, a single band on the Ponceau stained membrane was used for normalization. GDNF was detected as a single band migrating at a molecular weight of 36 kilodaltons (kDa). The specificity of the band was confirmed using a GDNF blocking peptide (Santa Cruz Biotechnology, catalog #sc-328P).

4.3.8 mRNA analysis

The expression of GDNF was examined in the lumbar region of the spinal cord, DRG neuron, and skeletal muscle (gastrocnemius) using real time reverse transcription-polymerase chain reaction (RT-PCR) technique. At week 6 all mice were over-anesthetized using a single injection of Avertin, and tissues were dissected. Tissues were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) using a glass Teflon homogenizer. With use of Rneasy Mini Kit (Qiagen Sciences, Maryland, USA) total RNA will be extracted according to the manufacturer's instructions. RT reaction was performed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) in order to convert mRNA to cDNA. The resulting cDNA was subjected to PCR amplification using Taqman Gene Expression Assay (Applied Biosystem, Foster City, CA, #Mm00599849_m1) as per manufacturer's protocol, and MyiQ Single Color Real Time PCR Detection System machine (Bio-Rad Laboratories). The size of the PCR products was checked by 2% agarose gel-electrophoresis and staining with ethidium bromide. GDNF gene expression was determined relative to the housekeeping gene, GAPDH (Applied Biosystem, #Mm99999915_g1).

4.3.9 Immunohistochemistry

Following nerve ligation, GDNF protein levels were assessed in the sciatic nerve of mice. The double ligated nerve was sectioned on a cryostat at 7μm and placed on microscope slides. Slides were encircled with a hydrophobic barrier using a Pap Pen (Research Products International, Mt. Prospect, IL), which were then treated with a blocking solution (0.5% porcine gelatin, 1.5% normal donkey serum, 0.5% Triton-X in Superblock Buffer [Pierce, Rockford, IL]) for 1 hour at room temperature. Sections were then incubated overnight at +4˚C with primary antibody (GDNF rabbit-polyclonal [Santa Cruz Biotechnology, #sc-328; diluted 1:100]) that was diluted in blocking solution. Sections were washed 2 x 10 minutes with PBS-T followed by incubation for 1 hour with fluorochrome-conjugated secondary antibody (donkey anti-rabbit Alexa 555; 1:2,000; Molecular Probes, Eugene, OR) diluted in PBS-T and Superblock (1:1 ratio). Following incubation, sections were washed in PBS for 2 x 10 minutes. After washing, slides were cover slipped and stored at +4˚C until viewing [142]. The relative levels of GDNF were detected by measuring threshold levels in the sciatic nerve using ImageJ. Minimum and maximum threshold levels were set at 20 and 50 pixels, respectively. Each segment (distal, middle, and proximal) of the sciatic nerve was photographed using a Nikon Eclipse E800 fluorescent microscope at 20x magnification. Using ImageJ percent area of GDNF coverage was obtained by measuring area accumulated with GDNF divided total area of the segment of the sciatic nerve.

4.3.10 Data analysis

Data analyses were conducted for each variable. One-way analysis of variance (ANOVA) with repeated measures was applied for between groups comparisons for daily distance exercised, blood glucose, body weight, and mechanical allodynia. When appropriate, *Post hoc* analyses were conducted with a least significant difference (L-S-D) method. Independent samples t-test was used to analyze GDNF protein levels and mRNA expression. The Pfaffl method was used to calculate the relative expression ratio (fold differences) of GDNF expression compared to the Sed-C group [143]. Pearson Product Correlation was used to determine the relationship between daily exercise distance and changes in behavioral sensitivity, relationship between total distance exercised and GDNF levels at week 2 and 6, and the relationship between total distance exercised and GDNF axonal transport at week 6. Statistical significance was set at $p \leq 0.05$. Data are represented as mean ± standard error of the mean (SEM).

4.4 Results

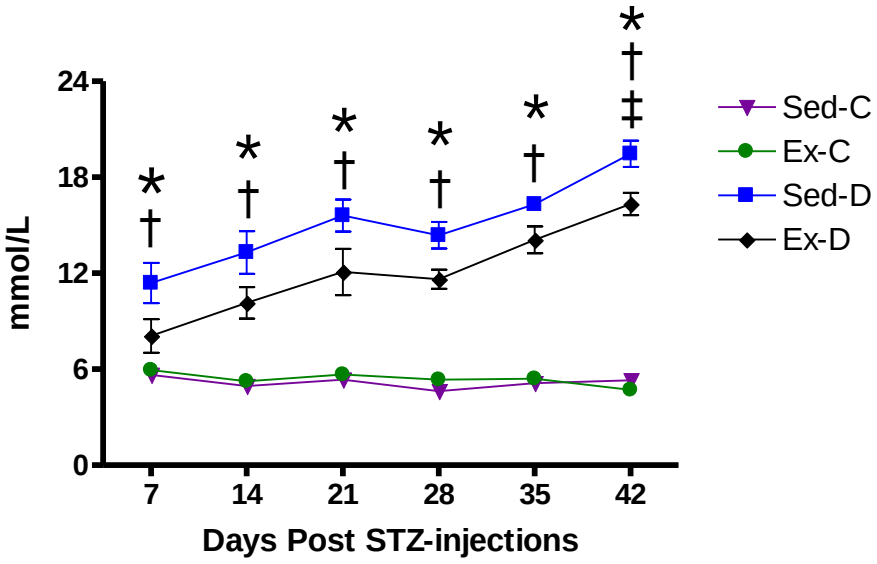
4.4.1 6 week study

4.4.1.1 Animal characteristics

Blood glucose for the non-diabetic groups did not change over the 6 week study (Figure 4.1). After STZ-injections the diabetic groups had marked increase in blood glucose compared to their respective controls. During the first 5 weeks voluntary exercise did not have an effect on blood glucose in the diabetic animals. However, there was a significant difference between the diabetic

Figure 4.1 Blood glucose levels in mice throughout the 6 week study. STZ-injections markedly increase in blood glucose of the diabetic groups compared to their respective controls. Voluntary exercise significantly improved blood glucose levels of the exercised diabetic group compared to the sedentary diabetic group at week 6 (19.5±0.8 vs. 16.3±0.7 mmol/L, respectively). *=Sed-C vs. Sed-D, †=Ex-C vs. Ex-D, and ‡=Sed-D vs. Ex-D. $p<0.05$

Figure 4.1 Blood glucose levels in mice throughout the 6 week study.



sedentary and diabetic exercise group at week 6 (19.5±0.8 vs. 16.3±0.7 mmol/L, respectively).

Over the 6 week study the Sed-C group exhibited a 13.5% increase in body weight (Figure 4.2). Six weeks of voluntary exercise prevented any changes in body weight in the Ex-C group (0.9%), which was significantly less than their sedentary counterpart. Both diabetic groups exhibited significant reductions in body weight compared to their respective controls. The diabetic sedentary and exercised groups displayed a 13.6% and 16.7% reduction in body weight over the 6 week experiment, respectively, however voluntary exercise further reduces body weight among the diabetic mice.

4.4.1.2 Exercise data

Mice were given unlimited access to voluntary running wheels for 6 weeks. At week 6 the Ex-C and Ex-D mice exercised on average 3.6±0.4 and 3.5±0.4 km/day, respectively (Figure 4.3). There were no significant differences in distance ran between the exercised non-diabetic and diabetic groups throughout the 6 week training period.

4.4.1.3 Behavioral assessments: mechanical allodynia

One week after STZ-injections, both diabetic groups exhibited an increase in mechanical allodynia; suggesting this inbred mouse strain develops a painful neuropathy. At week 6, the sedentary diabetic group displayed a significantly

Figure 4.2 Body weight of the mice throughout the 6 week study. The Sed-C and Ex-C group exhibited a 13.5% and 0.9% increase in body weight. The Sed-D and Ex-D groups displayed a 13.6% and 16.7% reduction in body weight over the 6 week experiment, respectively, which was significantly less than their respective control. *=Sed-C vs. Sed-D, †=Ex-C vs. Ex-D, ‡=Sed-D vs. Ex-D, and §=Sed-C vs. Ex-C. $p<0.05$

Figure 4.2 Body weight of the mice throughout the 6 week study.

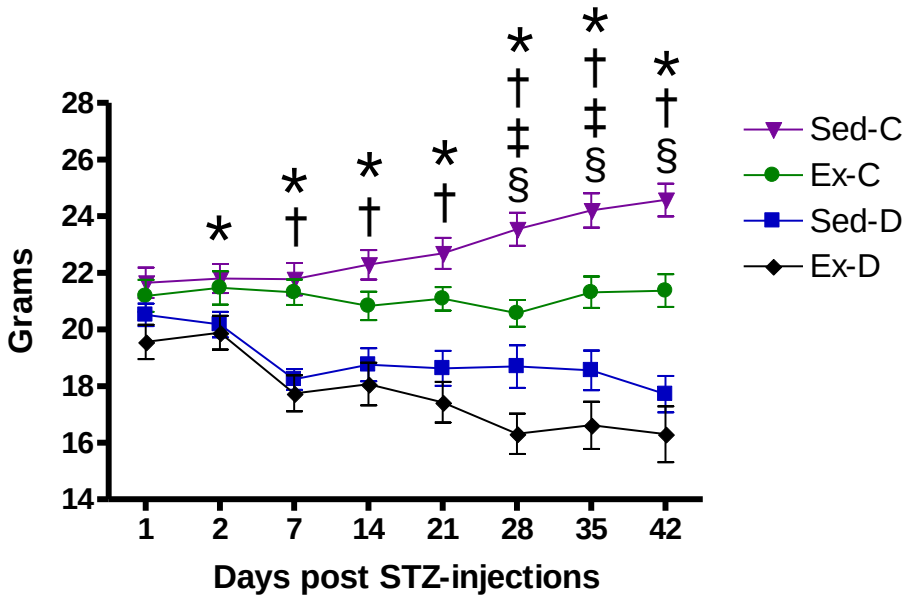
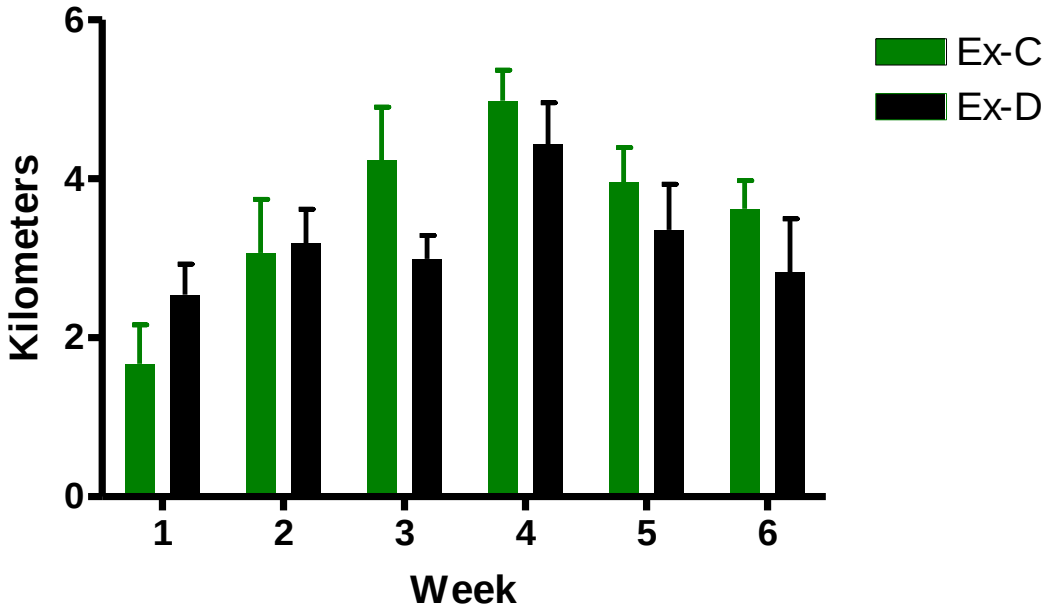


Figure 4.3 Average daily exercise distance throughout the 6-wk study. During week 6 the Ex-C and Ex-D group exercised 3.6±0.4 and 3.5±0.4 km/day, respectively.

Figure 4.3 Average daily exercise distance throughout the 6-wk study.



higher mechanical allodynia compared to sedentary non-diabetic control group (1.6±0.3 vs. 2.8±0.5 grams, respectively). However, 6 weeks of voluntary exercise attenuated mechanical allodynia in the diabetic exercised group compared to the sedentary diabetic group (3.3±0.3 vs. 1.6±0.3 grams, respectively). There were no changes in behavioral sensitivity among the non-diabetic groups (Figure 4.4). There were no correlations with daily exercised distance and changes in mechanical allodynia or GDNF proteins levels in the spinal cord, DRG, sciatic nerve, and skeletal muscle.

4.4.1.4 GDNF protein analysis

Protein analysis was used to determine the effects voluntary exercise has on GDNF protein levels in the lumbar region of the spinal cord tissue (Figure 4.5), DRG (Figure 4.6), sciatic nerve (Figure 4.7), and skeletal muscle (gastrocnemius [Figure 4.8]). Six weeks of diabetes decreased GDNF protein levels in spinal cord of the sedentary diabetic group by 4.7-fold compared to sedentary non-diabetic control group. However, voluntary exercise significantly increased GDNF levels in the exercised diabetic group by 3.1-fold compared to the sedentary diabetic group. Exercise did not alter GDNF protein levels in the non-diabetic group and there were no significant differences between either exercised groups. In the DRG, the sedentary diabetic group exhibited a marked 1.9-fold increase of GDNF compared to the sedentary non-diabetic control group; however voluntary exercise had no effect on GDNF levels among the diabetic

Figure 4.4 Mechanical allodynia assessed using a von Frey monofilament test 6 weeks post STZ-injection. Diabetes significantly increased mechanical allodynia compared to it sedentary counterpart (1.6±0.3 vs. 2.8±0.5 grams, respectively). At week 6 exercise favorably increased pain thresholds in diabetic mice compared to their sedentary counterpart (3.3±0.3 vs. 1.6±0.3 grams, respectively). *=Sed-C vs. Sed-D and †=Sed-D vs. Ex-D. $p<0.05$

Figure 4.4 Mechanical allodynia assessed using a von Frey monofilament test 6 weeks post STZ-injection.

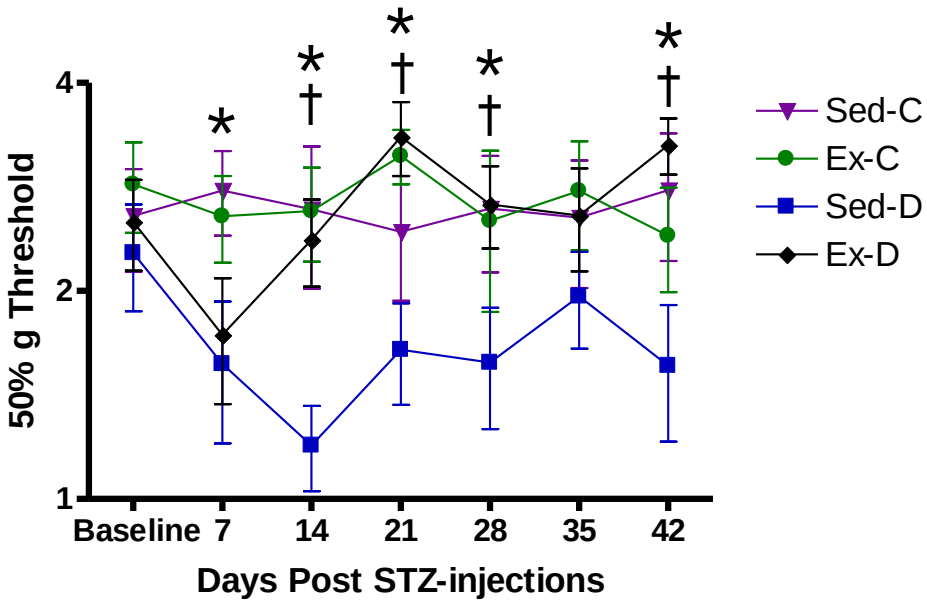


Figure 4.5 Immunoblot analysis of GDNF levels in the lumbar region of the spinal cord 6 weeks post STZ-injection. A.) Sed-C versus Sed-D. B.) Sed-C versus Ex-C. C.) Sed-D versus Ex-D. D.) Ex-C versus Ex-D; Upper panel shows representative immunoblot; Lower panel shows histogram analysis of the immunoblot. Diabetes significantly decreased GDNF, whereas, the Ex-D group demonstrated an increase in GDNF levels compared to Sed-D after 6 weeks of voluntary exercise. * $p<0.05$

Figure 4.5 Immunoblot analysis of GDNF levels in the lumbar region of the spinal cord 6 weeks post STZ-injection.

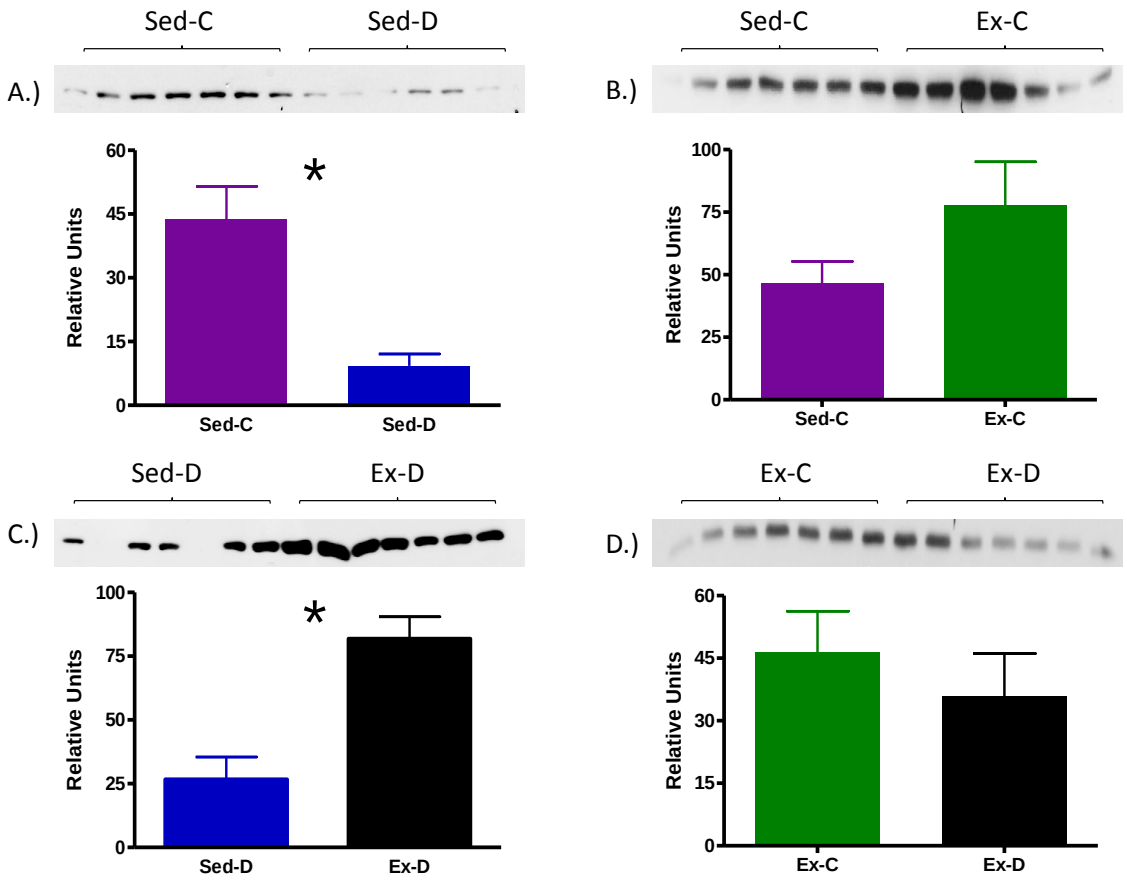


Figure 4.6 Immunoblot analyses of GDNF levels in the DRG 6 weeks post STZ-injection. A.) Sed-C versus Sed-D. B.) Sed-C versus Ex-C. C.) Sed-D versus Ex-D. D.) Ex-C versus Ex-D; Upper panel shows representative immunoblot; Lower panel shows histogram analysis of the immunoblot. Diabetes significantly increased GDNF levels. Voluntary exercise increased GDNF levels among the control group, however, exercise did not alter GDNF levels among the diabetic group. * $p<0.05$

Figure 4.6 Immunoblot analyses of GDNF levels in the DRG 6 weeks post STZ-injection.

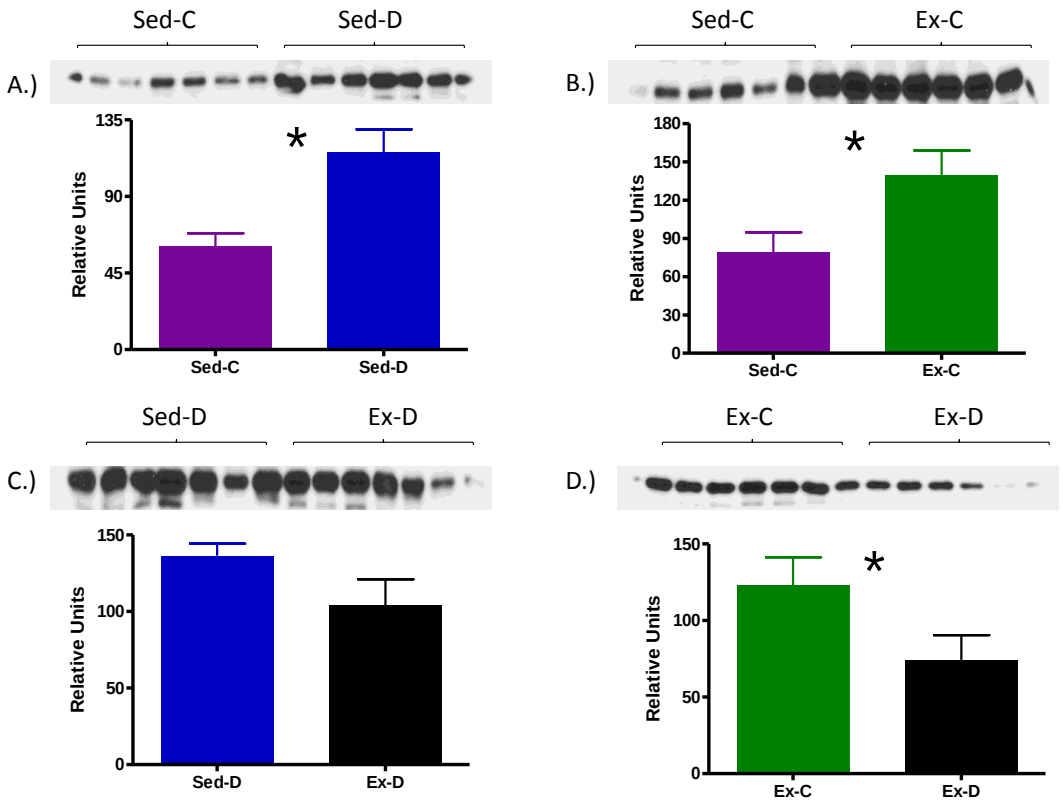


Figure 4.7 Immunoblot analysis of GDNF levels in the sciatic nerve 6 weeks post STZ-injection. A.) Sed-C versus Sed-D. B.) Sed-C versus Ex-C. C.) Sed-D versus Ex-D. D.) Ex-C versus Ex-D; Upper panel shows representative immunoblot; Lower panel shows histogram analysis of the immunoblot. Diabetes had no effect of GDNF levels; however, voluntary exercise significantly increased GDNF levels among both exercised groups compared to their sedentary counterparts. * $p<0.05$

Figure 4.7 Immunoblot analysis of GDNF levels in the sciatic nerve 6 weeks post STZ-injection.

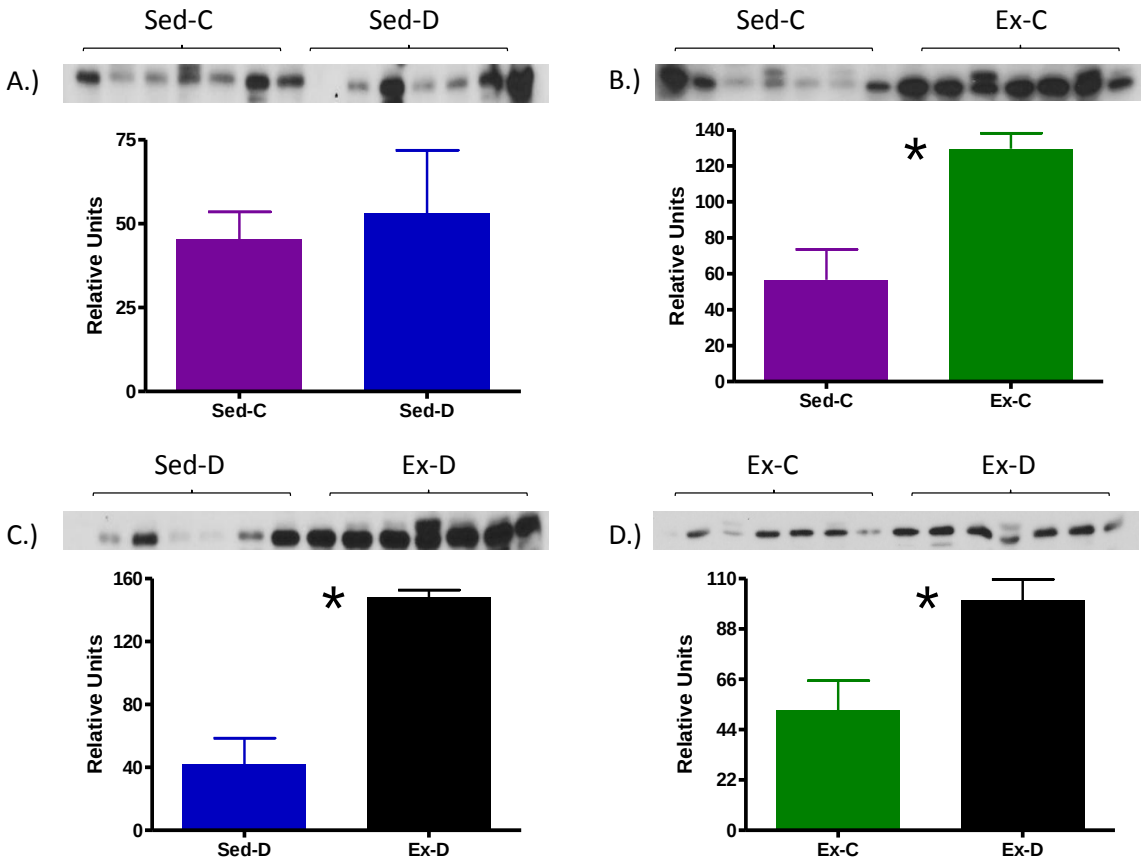
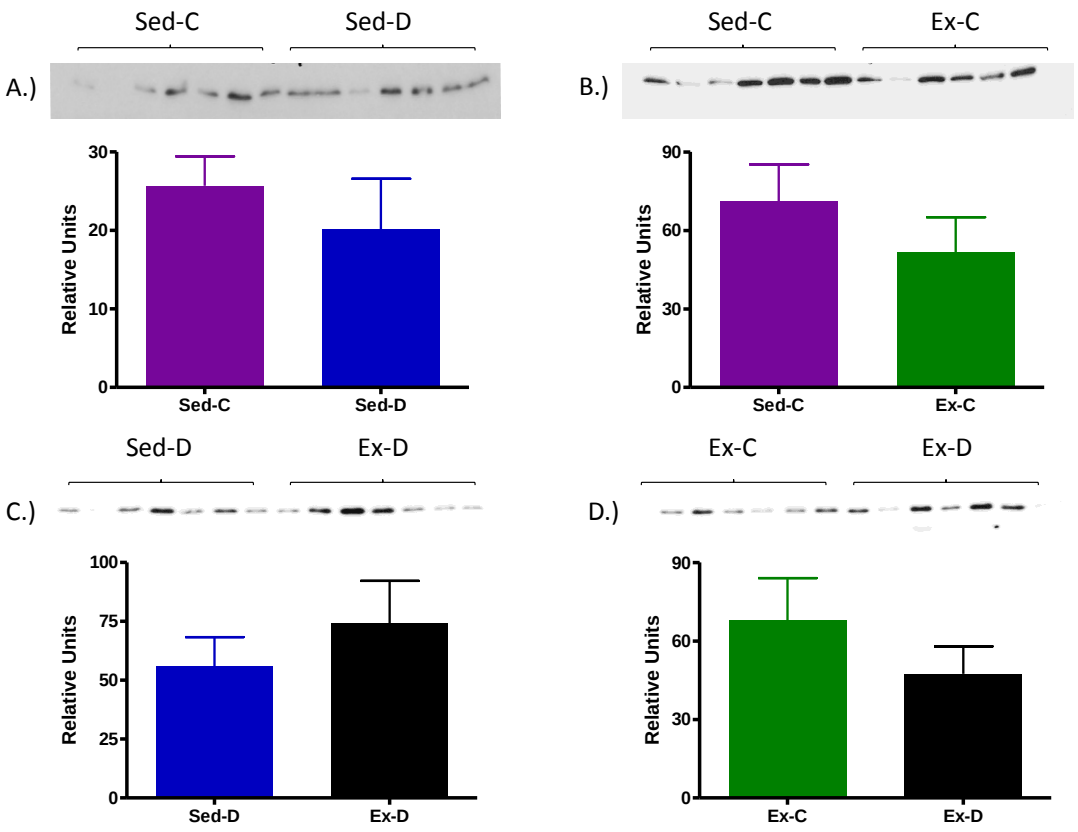


Figure 4.8 Immunoblot analysis of GDNF levels in the gastrocnemius skeletal muscle 6 weeks post STZ-injection. A.) Sed-C versus Sed-D. B.) Sed-C versus Ex-C. C.) Sed-D versus Ex-D. D.) Ex-C versus Ex-D; Upper panel shows representative immunoblot; lower panel shows histogram analysis of the immunoblot. There were no differences between either groups.

Figure 4.8 Immunoblot analysis of GDNF levels in the gastrocnemius skeletal muscle 6 weeks post STZ-injection.



group. Six weeks of voluntary exercise increased GDNF levels in the exercised non-diabetic control group by 1.7-fold compared to the sedentary non-diabetic control and exercised diabetic group. Diabetes did not alter GDNF in the sciatic nerve among the sedentary groups. However, voluntary exercise robustly increased GDNF levels in the exercised non-diabetic and diabetic groups by 2.2 and 3.5-fold compared to their sedentary counterparts, respectively. The exercised diabetic group also showed a 1.9-fold increase in GDNF compared to the exercised non-diabetic control group. Diabetes and exercise did not alter GDNF protein levels in skeletal muscle. There were no correlations with total distance exercised and GDNF proteins levels in the spinal cord, DRG, sciatic nerve, and skeletal muscle.

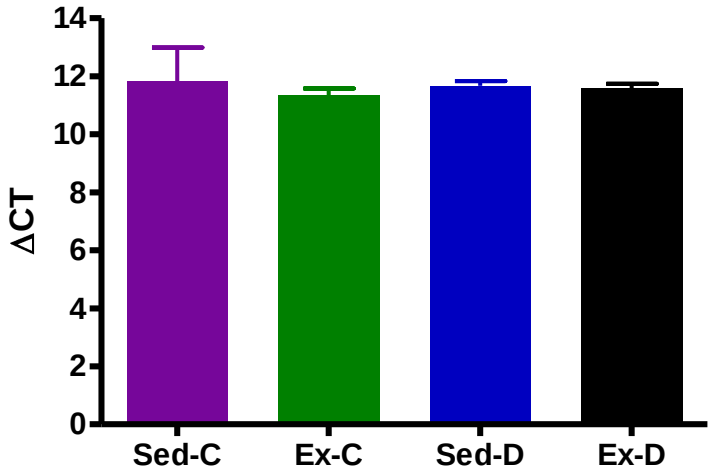
4.4.1.5 GDNF mRNA analysis

RT-PCR was used to determine the effects voluntary exercise has on GDNF mRNA expression in the lumbar region of the spinal cord tissue (Figure 4.9), DRG (Figure 4.10), and skeletal muscle (gastrocnemius [Figure 4.11]). RT-PCR was not assessed in the sciatic nerve due to the inability to obtain enough RNA to perform the assay. Voluntary exercise favorably increased GDNF mRNA expression in gastrocnemius muscles of diabetic mice. The Ex-D group exhibited a significant 4.3-fold increase in GDNF expression compared to the Sed-D group. Changes in GDNF expression were not detected in the gastrocnemius muscle among the non-diabetic control groups. Changes in GDNF expression due to diabetes and exercise in the spinal cord or DRG were not detected.

Figure 4.9 mRNA analysis of GDNF levels in the lumbar region of the spinal cord 6 weeks post STZ-injection. A.) Change in cycle threshold (delta [Δ] CT) and B.) Fold change in GDNF expression compared to the Sed-C group. Changes in GDNF expression between groups were not detected.

Figure 4.9 mRNA analysis of GDNF levels in the lumbar region of the spinal cord 6 weeks post STZ-injection.

A.)



B.)

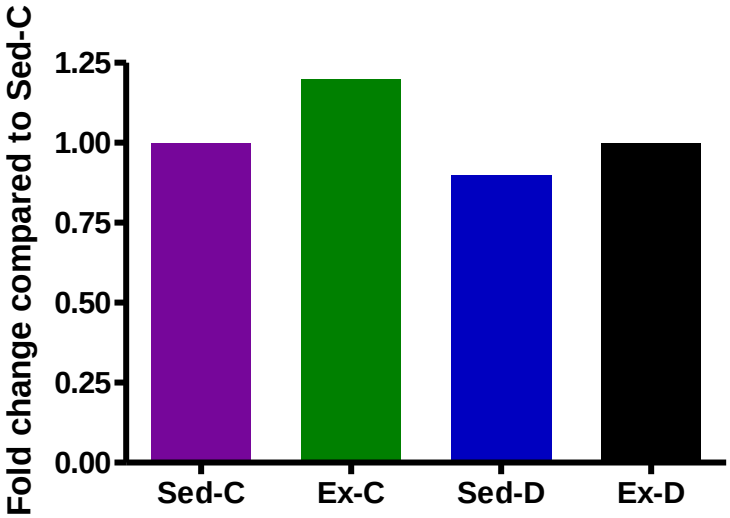
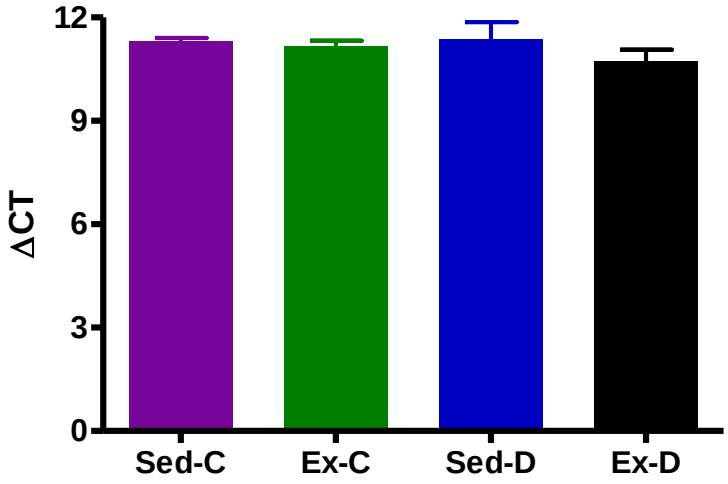


Figure 4.10 mRNA analysis of GDNF levels in the DRG 6 weeks post STZ-injection. A.) Change in cycle threshold (delta [Δ] CT) and B.) Fold change in GDNF expression compared to the Sed-C group. Changes in GDNF expression between groups were not detected.

Figure 4.10 mRNA analysis of GDNF levels in the DRG 6 weeks post STZ-injection.

A.)



B.)

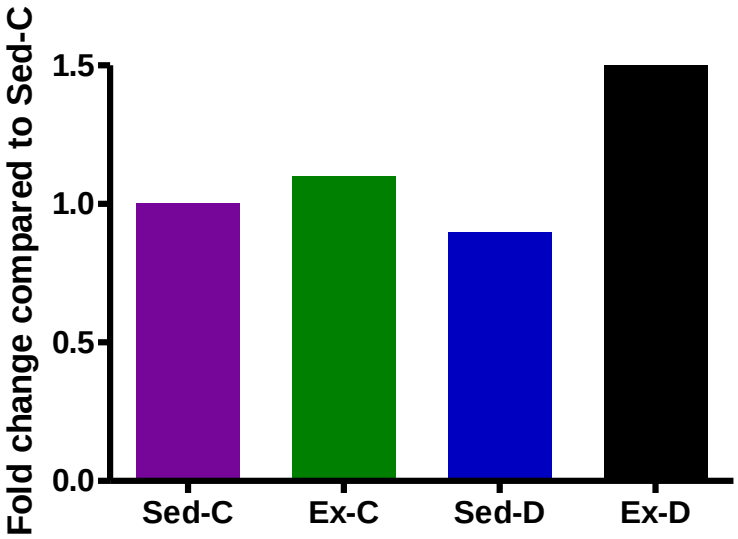
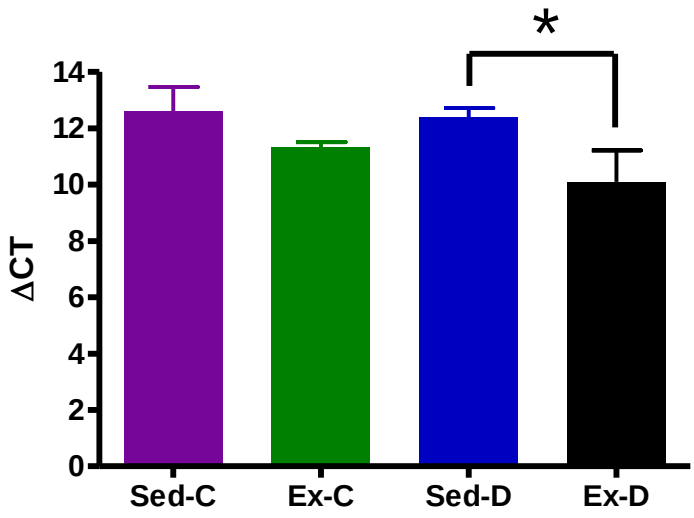


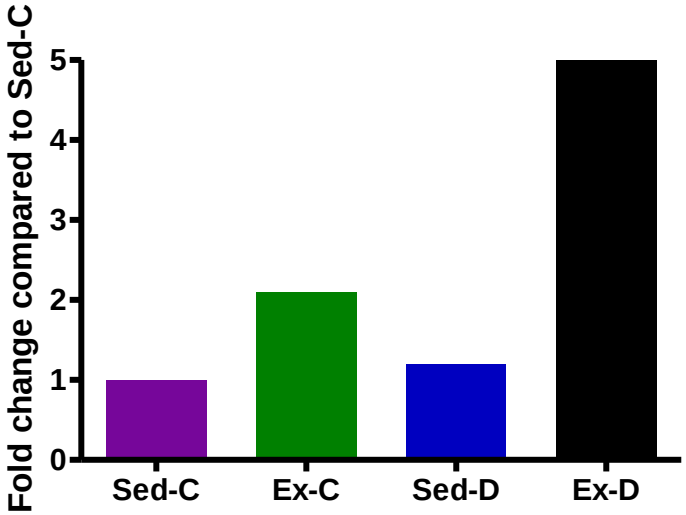
Figure 4.11 mRNA analysis of GDNF levels in the gastrocnemius skeletal muscle 6 weeks post STZ-injection. A.) Change in cycle threshold (delta [Δ] CT) and B.) Fold change in GDNF expression compared to the Sed-C group. Voluntary exercise significantly increased GDNF expression of the exercised diabetic group compared to its sedentary counterpart. *$p<0.05$

Figure 4.11 mRNA analysis of GDNF levels in the gastrocnemius skeletal muscle 6 weeks post STZ-injection.

A.)



B.)



4.4.2 GDNF intrathecal injection study

4.4.2.1 Animal characteristics

After STZ-injections, all diabetic groups exhibited a marked increase in plasma glucose compared to their respective controls, which persisted throughout the study (Figure 4.12). There were no significant differences in blood glucose levels between the Sed-D group and Sed-DWG (23.9 vs. 19.8 mmol/L, respectively). Similar to the 6 week study, 2 weeks of voluntary exercise did not affect blood glucose levels compared to its sedentary diabetic counterpart (24.0 vs. 23.9 mmol/L, respectively). Blood glucose for the sedentary non-diabetic control group did not change over the 2 week experiment and exercise had no effect on plasma glucose levels in the non-diabetic group (4.6 vs. 4.8 mmol/L, respectively).

Two weeks after STZ-injections the Sed-D, Sed-DWG, and Ex-D groups exhibited a 19.3%, 20.0%, and 23.8% reduction in body weight, respectively (Figure 4.13). At week 2, the Sed-C group showed a 3.1% increase in body weight, which was significant greater compared to their sedentary counterparts. Two weeks of voluntary exercise maintained basal body weight levels in the Ex-C group. There were no differences in body at week 2 between non-diabetic groups.

Figure 4.12 Blood glucose levels in mice throughout the 2 week study. All diabetic groups exhibited a marked increase in plasma glucose compared to their respective controls. *=Sed-C vs. Sed-D, †=Ex-C vs. Ex-D, and ‡=Sed-C vs. Sed-DWG. $p<0.05$

Figure 4.12 Blood glucose levels in mice throughout the 2 week study.

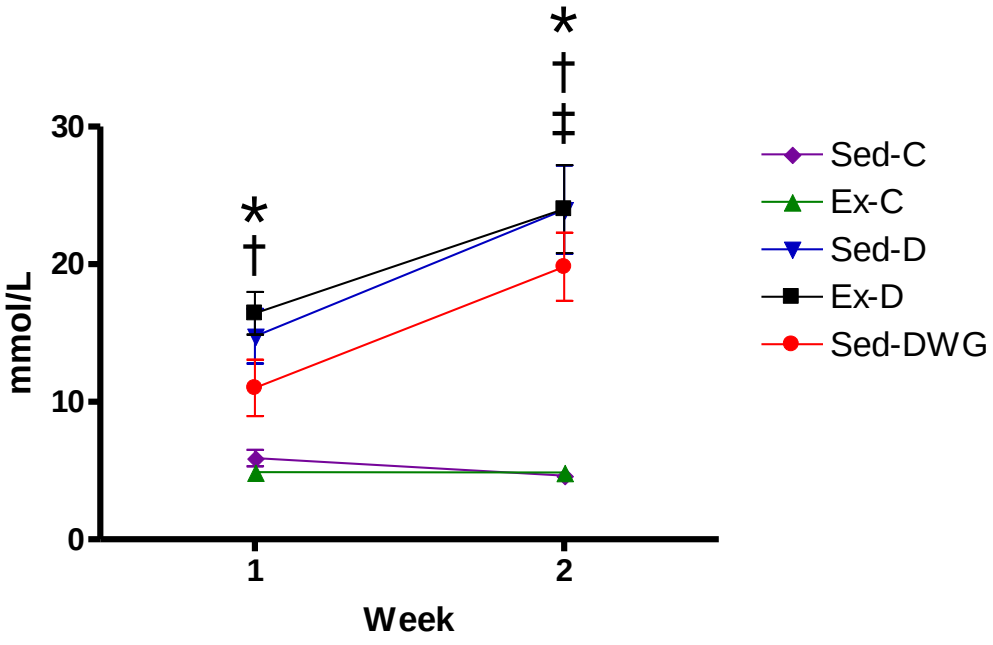
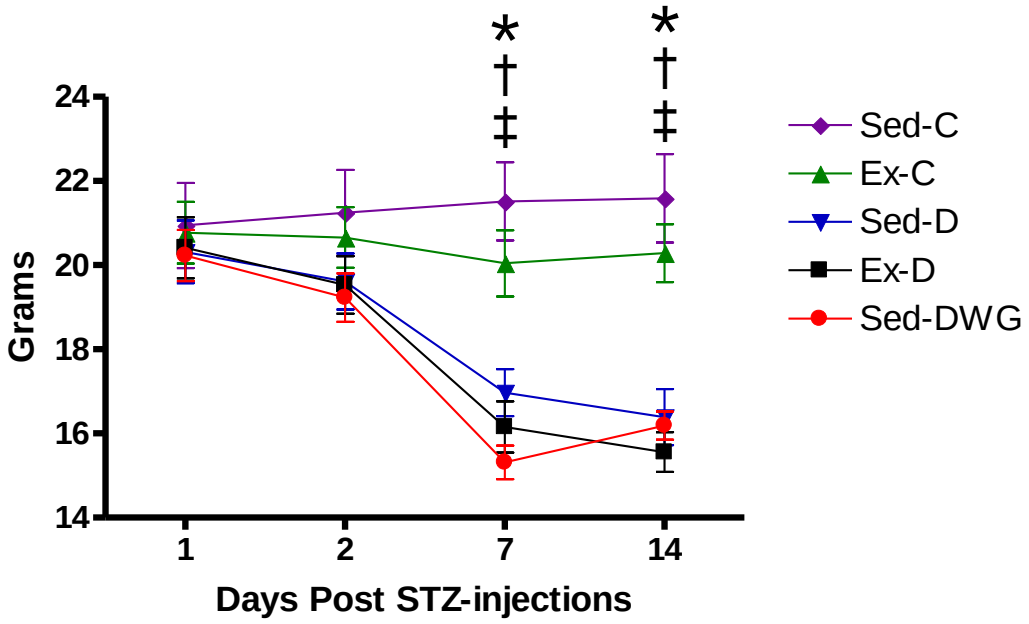


Figure 4.13 Body weight of mice throughout the 2 week study. At week 2, the Sed-C group showed a 3.1% increase in body weight, whereas, the Sed-D, Sed-DWG, and Ex-D groups exhibited a 19.3%, 20.0%, and 23.8% reduction in body weight, respectively. *=Sed-C vs. Sed-D, †=Ex-C vs. Ex-D, and ‡=Sed-C vs. Sed-DWG. $p<0.05$

Figure 4.13 Body weight of mice throughout the 2 week study.



4.4.2.2 Exercise data

Similar to the 6 week study all mice were given unlimited access to voluntary running wheels for 2 weeks. At week 2 the Ex-C and Ex-D mice exercised on average 1.8±0.6 and 1.7±0.6 km/day, respectively (Figure 4.14). There were no significant differences in distance exercised between the non-diabetic and diabetic exercise groups throughout the 2 week training period.

4.4.2.3 Behavioral assessments: mechanical allodynia

Similar to the 6 week study, diabetes increased mechanical allodynia by week 1 in the sedentary and exercised groups (Figure 4.15). Two weeks of voluntary exercise attenuated mechanical allodynia in the diabetic group compared to it sedentary counterpart (2.8±0.5 vs. 1.6±0.3 grams, respectively). Similar to voluntary exercise, 2 weeks of intrathecal injections of GDNF ameliorated changes in mechanical allodynia among the diabetic sedentary group (Sed-DWG), thus suggesting a potential therapeutic effect GDNF may exhibit. Again exercise did not alter mechanical allodynia in the non-diabetic control groups and there were no differences between exercised groups. Similar to the 6 week study there were no correlations with daily exercised distance and changes in mechanical allodynia.

4.4.2.4 GDNF protein analysis

The 6 week study showed increases in protein levels of GDNF in the lumbar region of the spinal cord and sciatic nerve. Therefore, immunoblot

Figure 4.14 Average daily exercised distance throughout the 2 week study. During week 2 the Ex-C and Ex-D groups exercised 1.8±0.6 and 1.7±0.6 km/day, respectively.

Figure 4.14 Average daily exercised distance throughout the 2 week study.

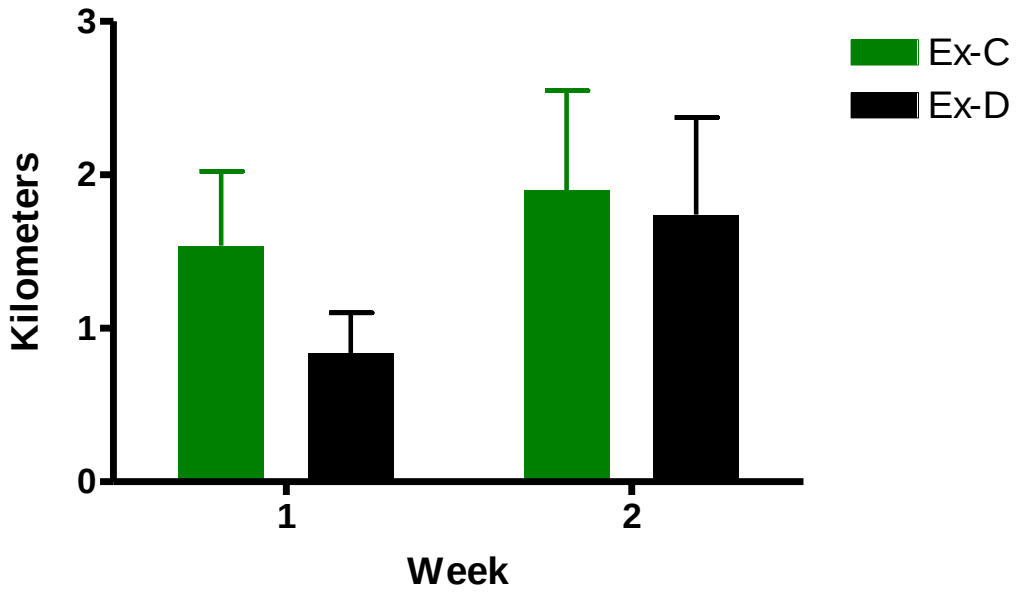
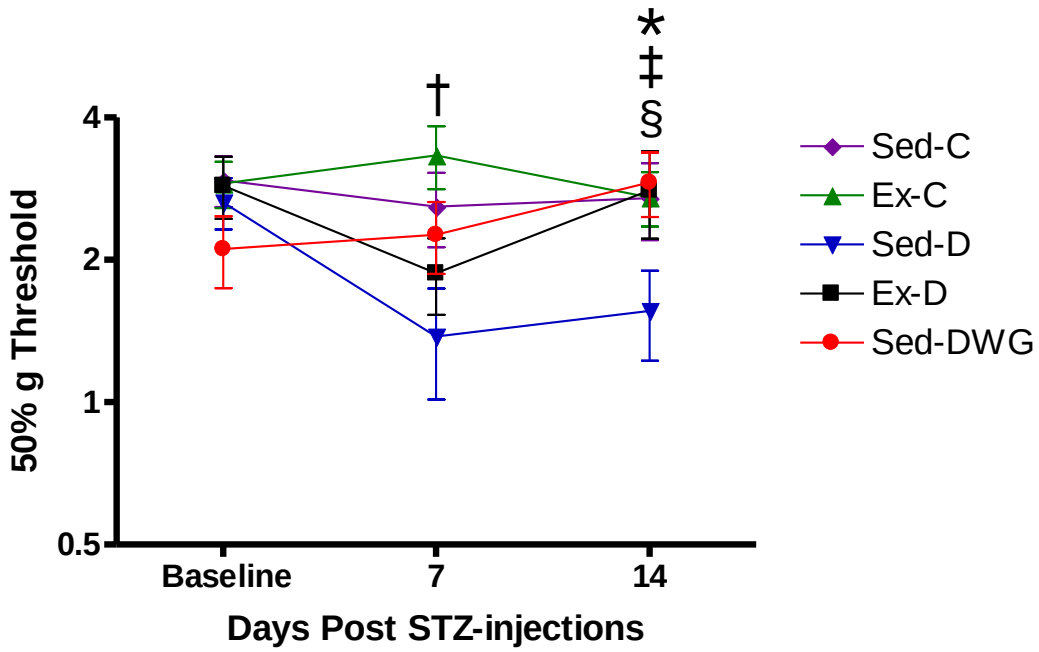


Figure 4.15 Mechanical allodynia assessed using a von Frey monofilament test 2 weeks post STZ-injection. 2 weeks of GDNF administration attenuated the development of behavioral sensitivity associated with DN. *=Sed-C vs. Sed-D, †=Ex-C vs. Ex-D, ‡=Sed-D vs. Ex-D, and §Sed-D vs. Sed-DWG. $p<0.05$

Figure 4.15 Mechanical allodynia assessed using a von Frey monofilament test 2 weeks post STZ-injection.



analysis was used to determine whether the reduction in mechanical allodynia, which is seen after 2 weeks of voluntary exercise were due to an increase in GDNF in the lumbar region of the spinal cord and sciatic nerve. Two weeks of diabetes and voluntary exercise did not alter GDNF protein levels in spinal cord (Figure 4.16). However, there is a trend showing an increase in GDNF protein levels in the exercised diabetic group compared to the diabetic sedentary group. GDNF protein levels were 1.7-fold higher in the Sed-C group compared to the Ex-C group. There were no significant differences between either exercised groups. In the sciatic nerve there were no differences in GDNF protein levels when comparing the sedentary (Sed-C vs. Sed-D), control (Sed-C vs. Ex-C), and exercised groups (Ex-C vs. Ex-D). However, exercise did significantly increased GDNF proteins levels in the sciatic nerve of the exercised diabetic group compare to its sedentary counterpart (Figure 4.17). Therefore, the attenuation of mechanical allodynia exhibited by the diabetic exercised group at day 14 may be due to increases in GDNF protein levels in the sciatic nerve. Total distance exercised at week 2 did not correlate with GDNF protein levels in the spinal cord or sciatic nerve.

4.4.3 Double ligation study

4.4.3.1 Animal characteristics

Six weeks after STZ-injection the diabetic groups had a significantly higher blood glucose levels compared to their respective controls (Figure 4.18). There were no differences between diabetic groups. At week 6, voluntary exercise did

Figure 4.16 Immunoblot analysis of GDNF levels in the lumbar region of the spinal cord 2 weeks post STZ-injection. A.) Sed-C versus Sed-D. B.) Sed-C versus Ex-C. C.) Sed-D versus Ex-D. D.) Ex-C versus Ex-D; Upper panel shows representative immunoblot; lower panel shows histogram analysis of the immunoblot. 2 weeks of diabetes and voluntary exercise had no effect on GDNF levels. * $p<0.05$

Figure 4.16 Immunoblot analysis of GDNF levels in the lumbar region of the spinal cord 2 weeks post STZ-injection.

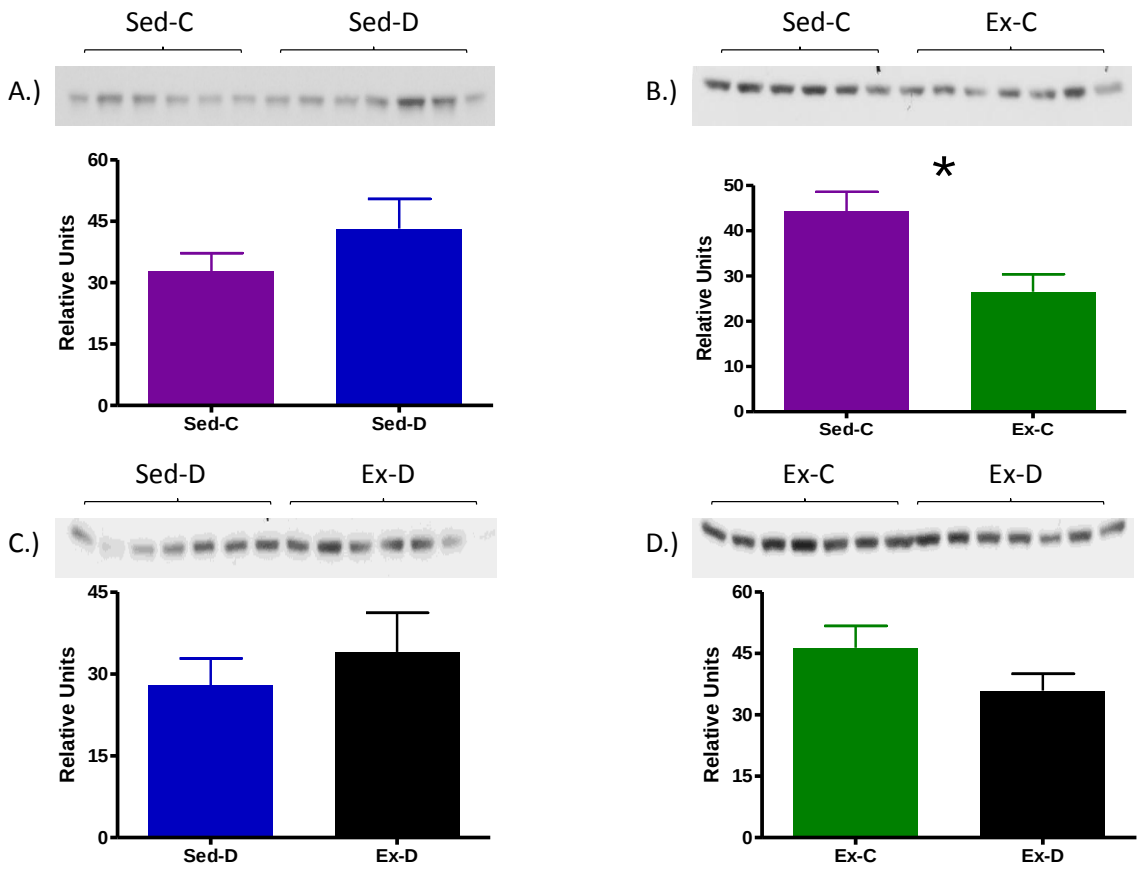


Figure 4.17 Immunoblot analysis of GDNF levels in the sciatic nerve 2 weeks post STZ-injection. A.) Sed-C versus Sed-D. B.) Sed-C versus Ex-C. C.) Sed-D versus Ex-D. D.) Ex-C versus Ex-D; Upper panel shows representative immunoblot; lower panel shows histogram analysis of the immunoblot. Voluntary exercise significantly increased GDNF levels in the exercised diabetic group compared to the sedentary diabetic group. * $p<0.05$

Figure 4.17 Immunoblot analysis of GDNF levels in the sciatic nerve 2 weeks post STZ-injection.

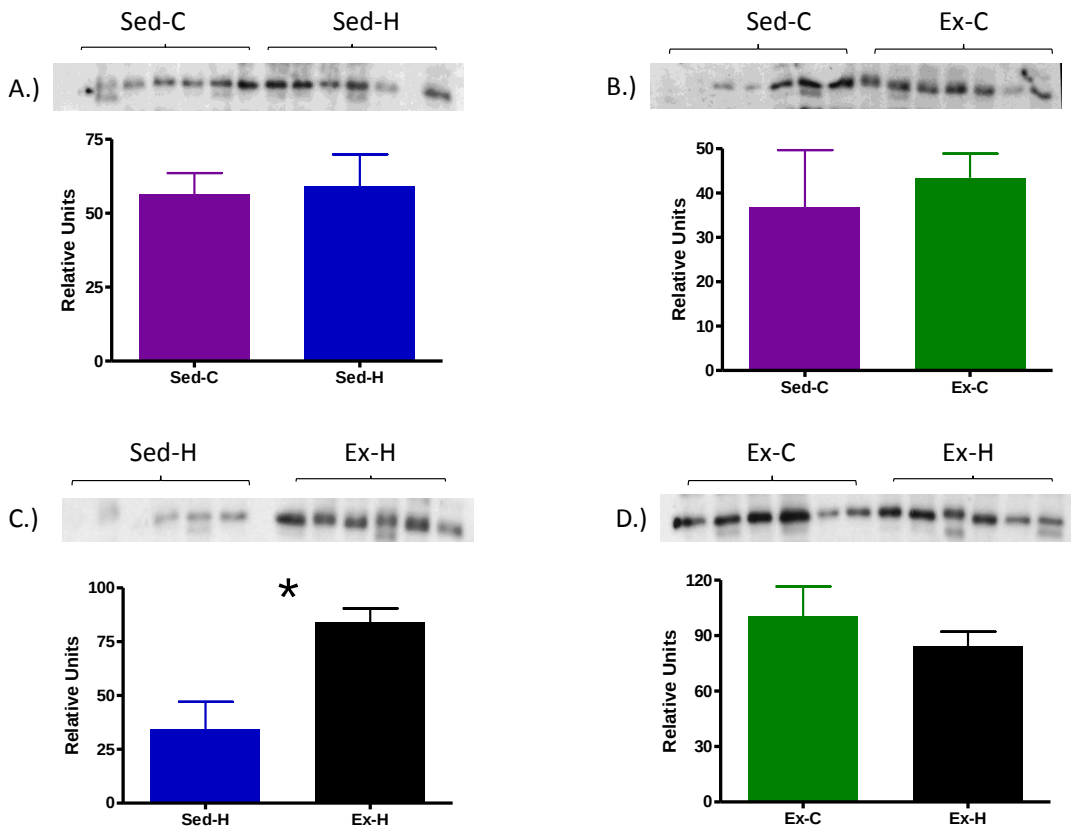
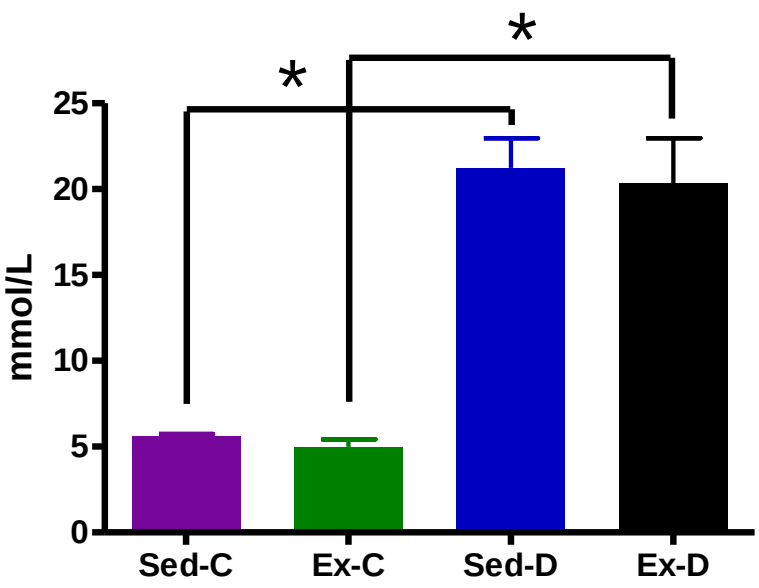


Figure 4.18 Blood glucose levels in the mice at the end of 6 weeks in the double ligation study. STZ-injection markedly increased blood glucose levels among the diabetic groups compared to their respective controls. Voluntary exercise had no effect on blood glucose levels throughout the 6 week study. *$p<0.05$

Figure 4.18 Blood glucose levels in the mice at the end of 6 weeks in the double ligation study.



not alter glucose levels among the control or diabetic groups (Ex-C [4.9 mmol/L] vs. Sed-C [5.5 mmol/L] and Sed-D [21.2 mmol/L] vs. Ex-D [20.3 mmol/L]).

Similar to the prior 6 week study the diabetes significantly decreased the body weight compared to their respective control group (Figure 4.19). The Ex-D group exhibited greater weight losses compared to the Sed-D group (17.2g vs. 18.4g), although it was not significant. Among the control groups, the Sed-C group exhibited a 9% increase in body weight, whereas as voluntary exercise prevented an increase in body weight (21.3g [at day 0] to 21.9g [at day 43]).

4.4.3.2 Exercise data

There were no significant differences in daily exercised distance between the non-diabetic and diabetic exercise groups throughout the first 5 weeks of the training period. However, at week 6 the Ex-C exercised significantly more than the Ex-D group (5.6±0.9 vs. 2.8±1.1 km/day, respectively, [Figure 4.20]).

4.4.3.3 GDNF transportation

GDNF axonal transport was assessed in the sciatic nerve using the double ligation technique. With this technique we are able to assess whether GDNF is being transported anterogradely (accumulation on proximal segment of nerve), retrogradely (accumulation on distal segment of nerve, or produced locally (accumulation in the middle segment of nerve). GDNF is present in an intact sciatic nerve (Figure 4.21). Table 1 lists areas that GDNF accumulated in

Figure 4.19 Body weight of the mice at the end of 6 weeks in the double ligation study. 6 weeks of diabetes duration significantly decreased the body weight of the diabetic groups compared to their respective control group *p<0.05

Figure 4.19 Body weight of the mice at the end of 6 weeks in the double ligation study.

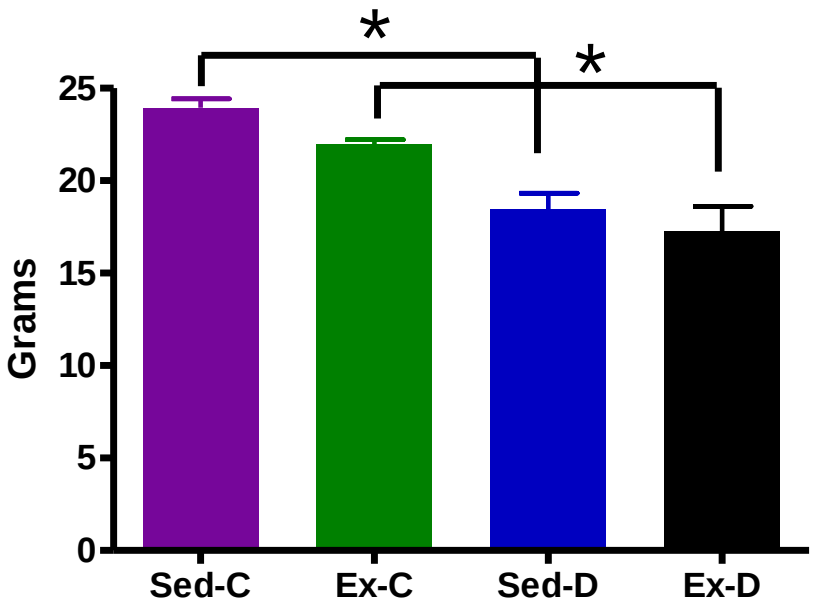


Figure 4.20 Average daily exercise distance throughout the 6 week double ligation study. During week 6 the Ex-C and Ex-D groups exercised 5.6±0.9 and 2.8±1.1 km/day, respectively. * $p<0.05$

Figure 4.20 Average daily exercise distance throughout the 6 week double ligation study.

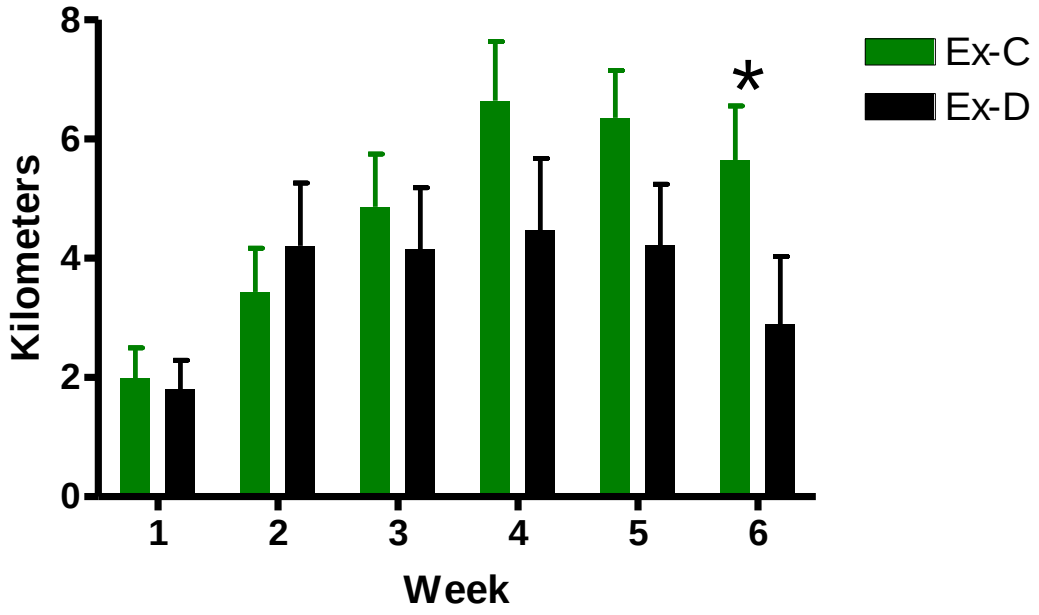
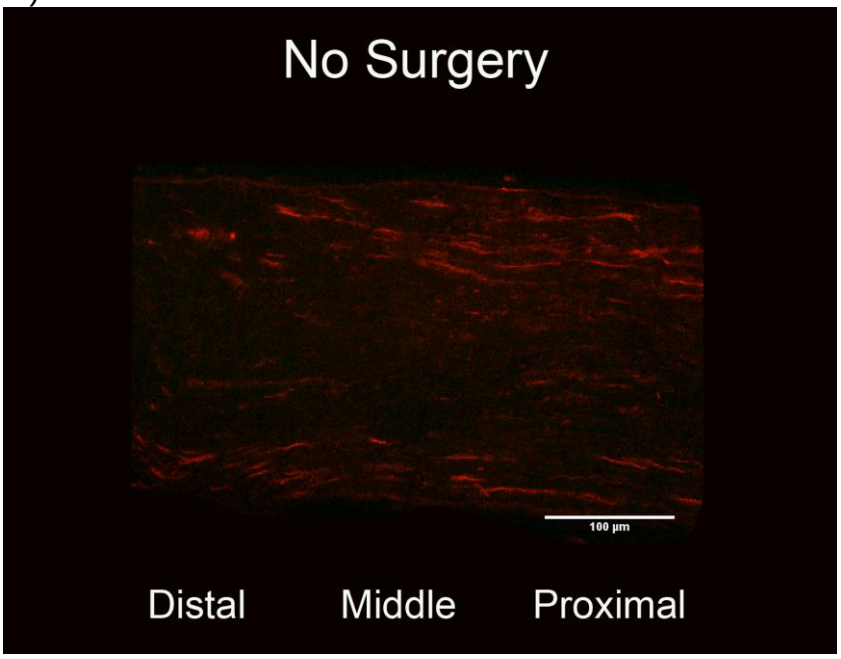


Figure 4.21 Immunohistochemical localization of GDNF in the distal, middle, and proximal areas of the sciatic nerve; sedentary non-diabetic control. A.) no surgery and B.) sham surgery. GDNF is detectable in the axons of the sciatic nerve. The sham surgery slightly increased GDNF levels in the sciatic nerve. Bar = 100μm.

Figure 4.21 Immunohistochemical localization of GDNF in the distal, middle, and roximal areas of the sciatic nerve.

A.)



B.)

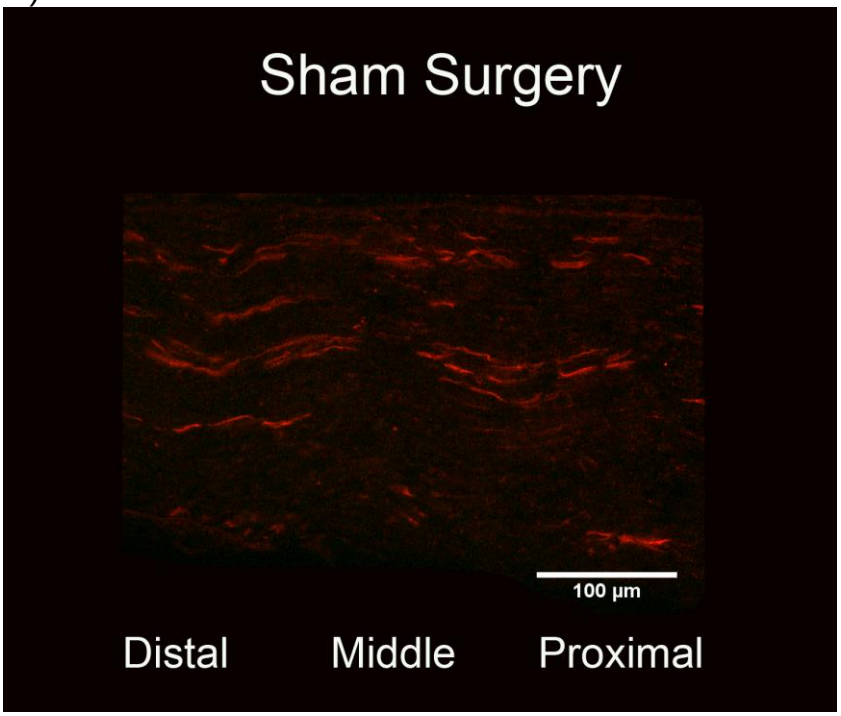


Table 4.1 Accumulation of GDNF in an intact sciatic nerve, and in the distal, middle and proximal segments of the sciatic nerve after double ligation surgery. The sham surgery slightly increased GDNF levels in the sciatic nerve. All groups exhibited an increase in accumulation of GDNF in all three segments of the sciatic nerve. The Sed-C group exhibited a greater increase in the middle segment; however there was more GDNF accumulation in the proximal segment compared to the distal segment. The remainder groups had a greater accumulation in the proximal segment compared to the middle and distal segments.

Table 4.1 Accumulation of GDNF in an intact sciatic nerve, and in the distal, middle and proximal segments of the sciatic nerve after double ligation surgery.

	Percent Area (Mean ± SEM)		
	Distal	**Middle**	**Proximal**
Sed-C	**3.9 ± 0.8**	**11.0 ± 6.2**	**7.3 ± 3.2**
Ex-C	**6.9 ± 1.9**	**9.3 ± 1.6**	**14.2 ± 2.9**
Sed-D	**7.0 ± 2.8**	**9.5 ± 3.1**	**12.3 ± 3.5**
Ex-D	**8.9 ± 3.2**	**12.6 ± 2.6**	**17.3 ± 3.2**
Sed-Control (no surgery)		**1.0 ± 0.5**	
Sed-Control Sham (nerve exposed)		**1.9 ± 0.3**	

the sciatic nerve. Although not significant the sham surgery slightly increased the amount of GDNF present in the sciatic nerve. After surgery, all groups exhibited an increase in accumulation of GDNF in all three segments of the sciatic nerve. The Sed-C group exhibited a greater increase in the middle segment; however there was more GDNF accumulation in the proximal segment compared to the distal segment. The remainder groups had a greater accumulation in the proximal segment compared to the middle and distal segments (Figure 4.22). The relationship between distance exercised and GDNF accumulation in the proximal segment was analyzed. In diabetic mice, total distance exercised significantly correlated with the amount of GDNF accumulated in the proximal segment of the double ligated sciatic nerve ($r=.890$, $p=.018$), illustrating that anterograde transport of GDNF increased with the amount of exercise the mice participated in (Figure 4.23). Exercise increased the amount of GDNF accumulation compared to their respective controls, suggesting that exercise has a favorable effect on increasing GDNF axonal transport.

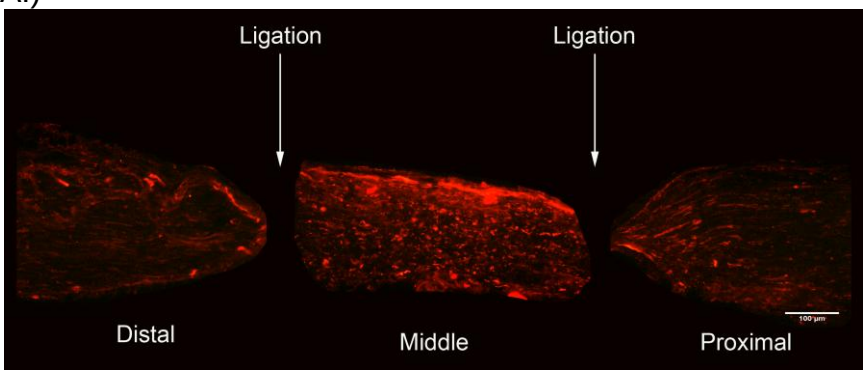
4.5 Discussion

Painful DN is a prominent clinical problem with few treatment options. Here, we have tested the ability of voluntary exercise to improve pain indices in diabetic mice. Our results suggest that voluntary exercise significantly improves mechanical hypersensitivity in diabetic mice. Moreover, our studies suggest that these analgesic actions may be mediated by exercise-induced increases in neurotrophic factor levels. Voluntary exercise significantly increased GDNF

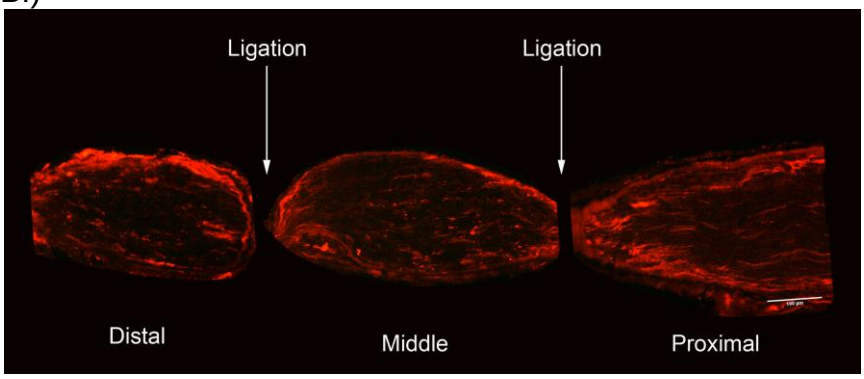
Figure 4.22 GDNF axonal transport in the sciatic nerve. Immunohistochemical localization of GDNF in the distal, middle, and proximal areas of the sciatic nerve of the double ligation study. A.) sedentary non-diabetic control, B.) exercised non-diabetic control, C.) sedentary diabetic, D.) exercised diabetic. Each segment of the sciatic nerve is a representative of the entire group. Bar = 100μm. All groups exhibited an increase in accumulation of GDNF in all three segments of the sciatic nerve. The Sed-C group exhibited a greater increase in the middle segment; however there was more GDNF accumulation in the proximal segment compared to the distal segment. The remainder groups had a greater accumulation in the proximal segment compared to the middle and distal segments.

Figure 4.22 GDNF axonal transport in the sciatic nerve.

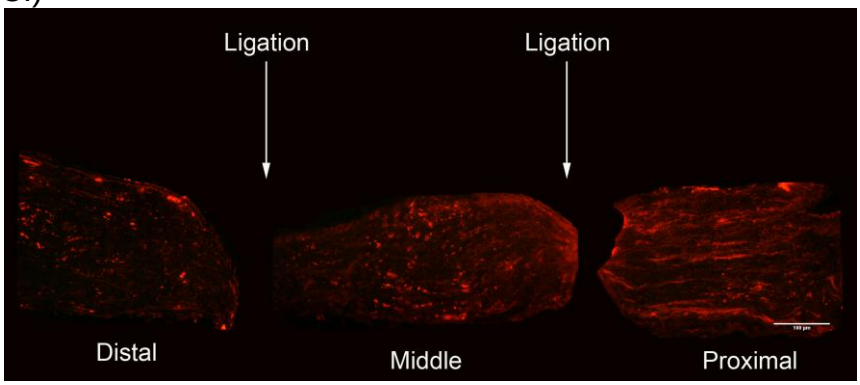
A.)



B.)



C.)



D.)

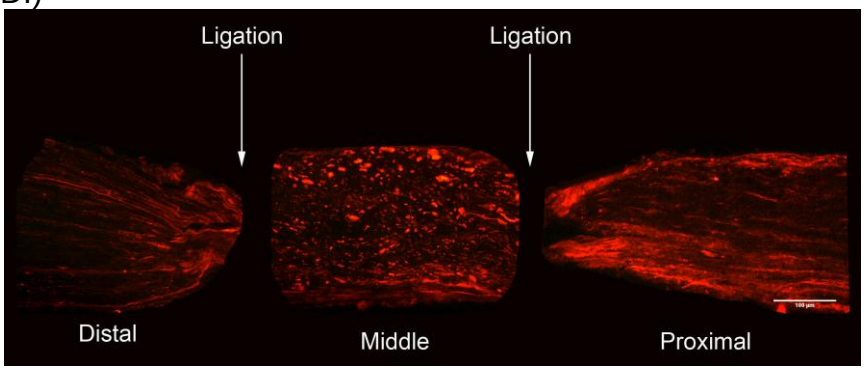
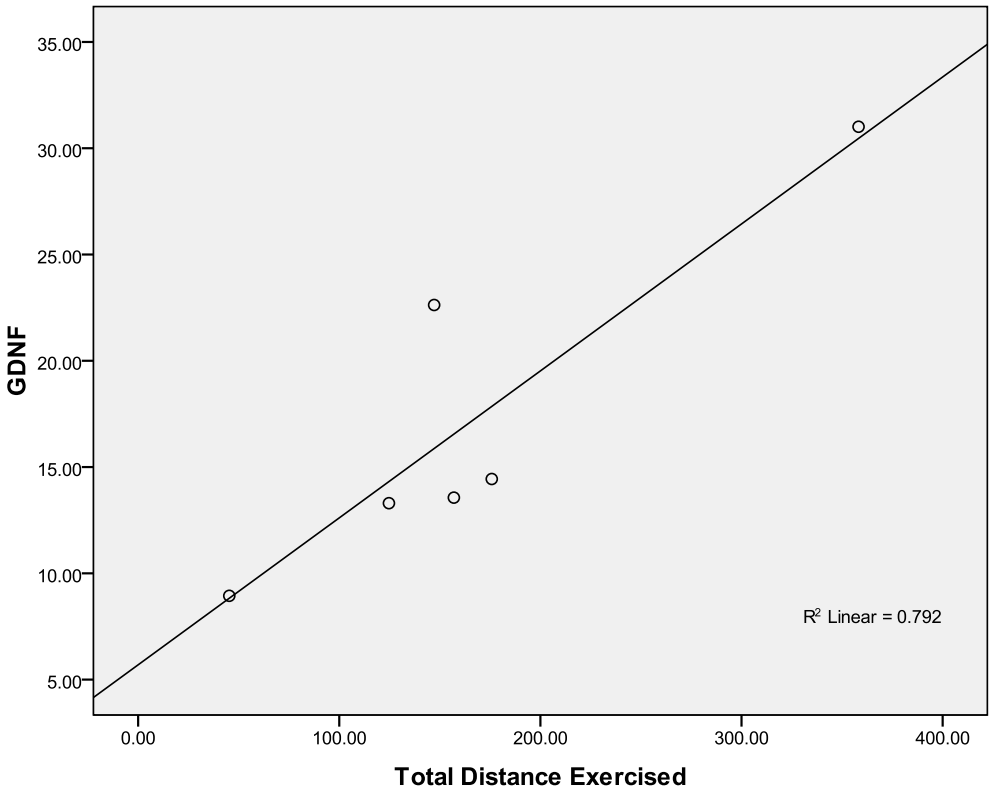


Figure 4.23 Plot of the levels of GDNF vs. total distance exercised in proximal side of the sciatic nerve after double ligation surgery. In diabetic mice, total distance exercised significantly correlated with the amount of GDNF accumulated in the proximal segment of the double ligated sciatic nerve (r=.890, p=.018), illustrating that anterograde transport of GDNF increased with the amount of exercise the mice participated in

Figure 4.23 Plot of the levels of GDNF vs. total distance exercised in proximal side of the sciatic nerve after double ligation surgery.



protein levels in the spinal cord and sciatic nerve, mRNA levels in skeletal muscle of exercised diabetic mice, and direct application of GDNF to the spinal cord and sensory neurons improved pain indices similar to exercise. These studies strongly suggest that exercise has beneficial actions on alleviating pain in diabetic patients and provide a potential mechanism by which exercise changes neurotrophic factor levels that are beneficial to neural pain circuitry.

4.5.1 Mechanical allodynia in mice

Sprague Dawley rats are well known as a model of diabetes-induced mechanical allodynia [116], but this is the first report of A/J inbred diabetic mice displaying a similar behavioral change. As early as one week after diabetes onset, sedentary diabetic mice exhibited a 31% increase in mechanical allodynia, which persisted throughout the duration of the studies. Other mouse strains have been reported to develop mechanical hypersensitivity, including leptin null mutant (*ob/ob*) mice that resemble Type 2 diabetes [144, 145]. However, other inbred mouse strains such as C57Bl/6 mice display mechanical insensitivity [84, 146]. Importantly, these differences in diabetes-induced behavioral sensitivity among different inbred strains may provide improved models to study the range of neuropathic changes present in humans with diabetic neuropathy.

4.5.2 Exercise reduces mechanical allodynia

Our results from the current study add to the view that exercised-induced alterations in pain sensitivity are sensitive to neurotrophic factor levels, which

was illustrated in our rat study. Experiments in the current study support this view, as 2 weeks of voluntary exercise increased GDNF levels in the sciatic nerve of diabetic mice, which is where the initial behavioral differences were seen, and intrathecal delivery of GDNF during the emergence of mechanical allodynia in diabetic mice restored mechanical sensitivity to normal levels. Collectively, these results strongly argue that GDNF may be a viable treatment for attenuating acute and chronic pain states associated with peripheral nerve damage. Although our results support the view that GDNF has potent therapeutic actions in painful diabetic neuropathy, our studies do not provide clear insight as to whether GDNF depletion in diabetes is involved in the initiation and maintenance of mechanical allodynia.

4.5.3 Exercise and axonal transport

An important issue related to the ability of exercise to increase GDNF levels is the site at which this occurs. GDNF is synthesized and secreted by a number of tissues [63] including various regions of the central nervous system [66, 67], peripheral nervous system [66], and peripheral tissues [68, 69]. To gain some perspective about the source(s) of GDNF in the spinal cord and sciatic nerve, we performed nerve ligation studies to determine if GDNF protein was transported retrogradely from the gastrocnemius muscle. Our results suggest that exercise-induced changes in GDNF increase both anterograde and retrograde levels of transported GDNF, with the greatest changes occurring in an anterograde direction. These findings are in agreement with previous studies that

report anterograde transport of GDNF in the sciatic nerve and hypoglossal nerve [95, 96] and retrograde transport in the sciatic nerve [97]. Western blots of GDNF protein in the dorsal or ventral spinal cord of exercised rats revealed that the greatest increases in GDNF protein occurred in the ventral horn, suggesting that motoneurons may play an important role in accumulating and/or synthesizing GDNF in response to exercise. We believe that this anterograde axonal transport is due to an increase accumulation of GDNF protein in the ventral horn, which was illustrated in our diabetic exercise rats. It is believed that the anterograde axonal transport of GDNF in the peripheral nerve may be released at the axon terminals in the skin and muscle, therefore possibly serving as a neuromodulator [95].

Based on these findings, it is plausible to suggest that GDNF may act as an important signaling molecule that is responsive to exercise and serves to modulate central neural signaling and peripheral tissue function. Exercised-induced increases in GDNF occurred in skeletal, which may influence spinal circuitry via retrogradely transport and released within the spinal cord. Exercise then increases GDNF levels in neural tissues that are transported anterogradely to influence peripheral tissues. It is possible that these mechanisms represent endogenous systems that enable communication between sensory/motor neurons and peripheral tissues that respond to exercise. Finally, we have demonstrated in the current study that this potential mechanism may be

heightened in the disease setting of diabetes in which neurotrophic signaling may be compromised.

4.5.4 Clinical implications

Understanding the effects diabetes has on neurotrophic support and function in neurons is critical for understanding and alleviating secondary complications associated with painful DN. Currently, long-term glycemic control is the only treatment recommended to treat degenerative DN [113]. Recently, it has been shown that voluntary exercise enhances axonal regeneration of sensory neurons [128]. In diabetic animals, exercise prevents myelin damage, therefore protecting and attenuating electorphysiological deficits that occur in the setting of DN [62]. Our study demonstrated that voluntary exercise and recombinant GDNF can alleviate mechanical allodynia in diabetic mice. Exercise-induced changes in mechanical allodynia are believed to be due increasing GDNF levels and the availability of this trophic factor through axonal transport. Our results support the use of voluntary exercise and recombinant GDNF to complement other therapy treatments used to attenuate neuropathic pain associated with DN.

Exercise is considered an important component for treating diabetes, however, it may exacerbate diabetic complications or may produce consequences due to existing complications [138]. Before the start of any regular physical activity, the potential risks (i.e. foot ulcers, cardiovascular function, etc.) needs to be assessed in order to minimize any complications the diabetic patient

may experience. Therefore, clinicians and patients must work together to maximize exercise-induced benefits while minimizing the adverse events in order to obtain a healthier lifestyle and improve the patients quality of life [138].

4.5.5 Conclusion

Results obtained from our study, demonstrates the beneficial effects voluntary exercise has on reducing mechanical hypersensitivity in diabetic mice. The significance of this study has shown the therapeutic effects GDNF has on reducing mechanical allodynia associated with DN. Therefore, GDNF and molecules in the GDNF signaling pathway are potential therapeutic targets for reducing behavioral sensitivity associated with painful DN.

Chapter 5

Discussion and conclusion

5.1 Summary and findings

Among the most debilitating types of chronic pain is peripheral neuropathic pain [147], which results from damage to peripheral sensory axons [11]. Damage to these axons can occur due to physical trauma, such as nerve trauma (via surgery, compression, etc.), or metabolic diseases, such as diabetes [11]. These physical and metabolic insults lead to multiple pathophysiological changes in both the PNS and CNS [117]. Neuropathic pain is challenging to manage. Many pharmacological treatments are available to treat neuropathic pain; however, only 40% to 60% of patients obtain partial relief of their pain [148]. Therefore, the identification of efficacious non-pharmacologic treatments for neuropathic pain, and the development of strategies to prevent neuropathic pain are needed to advance the management of this debilitating disease [148].

Recently, we have shown that forced exercise (via swimming) reduced behavioral sensitivity associated with peripheral nerve injury [30]; however, the exercise-induced mechanisms were not researched in this study. Numerous mechanisms are responsible for peripheral neuropathic pain. In the events following peripheral nerve injury, dysfunction of GDNF signaling in the nociceptive afferent system contributes to the development and/or maintenance of neuropathic pain [72-75]. Several studies have shown that exercise can alter GDNF expression in various neuronal and non-neuronal tissues [40, 88-90]. Therefore, experiments were conducted in order to determine whether the

beneficial effects exercise had on behavioral sensitivity in nerve injured rats were due to changes in GDNF levels.

Our results shown in Chapter 2 demonstrated the beneficial effects voluntary exercise has on mechanical sensitivity associated with neuropathic pain. Both SNI groups developed a marked hypersensitivity to mechanical stimulation of the lateral surface of the hindpaw 5 days after surgery. Within the first 3 weeks of training, exercise reduced mechanical allodynia and hyperalgesia, which lasted up to 110 days. GDNF protein levels in the spinal cord increased following nerve injury; however there were no difference in GDNF levels between the sedentary and exercised groups at day 110. Therefore, a mechanism responsible for the exercise-induced reductions in behavioral sensitivity exhibited in rats experiencing neuropathic pain is not likely to involve the GDNF pathway and remains to be elucidated. However, this conclusion is difficult to propose due to the fact that there were not age matched controls in order to determine whether the increases in GDNF levels, which were exhibited at day 110, were due to the SNI surgery or age itself.

Several plausible mechanisms exist for analgesia following exercise. These mechanisms involve the opioid and non-opioid systems. The most commonly tested mechanisms has been exercise-induced release of endogenous opioids at either peripheral, spinal, or central sites capable of modulating pain [149]. As reviewed by Koltyn (2000), data regarding the

exercise-induced analgesia via the opioid system in non-pathological conditions are mixed. However, exercise-induced release of endogenous opioids is believed to be involved in attenuating behavioral sensitivity associated with chronic muscle pain [32]. Non-opioid systems that reduce pain are believed to involve the serotonergic pathway, neurotrophin availability, and increases in blood pressure. Serotonin modulates pain sensitivity through the descending pain pathways [150], through the release of GABA [151]. Recently, Mazzardo-Martins et al. (2010) showed that high-intensity extended swimming reduced behavioral sensitivity in mice. This reduction in nociception was attenuated by pretreatment with rho-chlorophenylalanine methyl ester (PCPA, an inhibitor of serotonin synthesis), therefore implicating the involvement of serotonergic pathway in exercise-induced analgesia [152]. Numerous studies have shown that aerobic exercise can increase neurotrophin expression [31, 36-42]. Neurotrophins have been shown to induce analgesia by modulation of the glutamatergic transmission and neuropeptide release through both opioid and serotonergic mechanisms [126]. Therefore, it is believed that increases in neurotrophins with exercise may have analgesic effects. Elevation in blood pressure is believed to be a potential mechanism in reducing pain following exercise. As reviewed by Koltyn and Umeda (2006), there may be an interaction between exercise, hypoalgesia, and blood pressure. During exercise baroreceptors are activated due to increases in systolic blood pressure [153]. Research indicates that activation of baroreceptors results in CNS inhibition and that stimulation of the baroreceptor are associated with decreases in pain perception [154]. Therefore, one or more of these

mechanisms may be responsible for the reductions in mechanical sensitivity seen in the exercised SNI rats.

Peripheral neuropathic pain can result from axonal damage by metabolic diseases, such as diabetes. Type 1diabetes is considered to be a chronic immune mediated disease characterized by a selective loss of insulin producing β-cells in the pancreatic islets of genetically susceptible subjects [155]. This disease imposes a substantial burden on the nervous system, which results in peripheral nerve damage that leads to DN [51]. Numerous mechanisms are involved in the pathogenesis of DN, which includes reductions in neurotrophic support (i.e. GDNF) [156]. Liu et al. (2009) demonstrated that neuropathic symptoms in diabetic rats are negatively correlated with the levels of GDNF expression. As GDNF expression in the sciatic nerve diminished, neuropathic symptoms increased; however, after GDNF gene delivery, these neuropathic symptoms were alleviated [54]. Aerobic exercise has been shown to increase GDNF in skeletal muscle [88, 90], which is innervated by both sensory and motor neurons via the sciatic nerve. Therefore, a series of experiments were conducted in order to determine how diabetes affects GDNF protein levels and mRNA expression and whether voluntary exercise has any influence in order to attenuate or delay the behavioral sensitivity associated with DN.

Our results shown in Chapter 3 and 4 demonstrated that voluntary exercise ameliorates mechanical allodynia associated with DN in both rats and

mice. One week after STZ-injections, the diabetic groups in both species exhibited an increase in mechanical sensitivity. Similar to the SNI study, voluntary exercise attenuated mechanical allodynia in both rats and mice within the first 3 weeks post STZ-injections, which persisted throughout the remaining of the studies. Diabetes duration of 6 weeks significantly reduced GDNF protein levels in the spinal cord of mice, however, 8 weeks did not in rats. Six and 8 weeks of voluntary exercise significantly increased GDNF protein levels in the spinal cord of diabetic animals in both mice and rats, respectively. In diabetic rats, this increase was greater in the ventral horn of the spinal cord. Among mice, voluntary exercise robustly increased GDNF protein levels and mRNA expression in the sciatic nerve and gastrocnemius muscle compared to their sedentary counterparts, respectively. Among both species, voluntary exercise increased mechanical thresholds around the 2 week time period (day 14 for mice and day 17 for rats). Our results showed that diabetes did not reduce GDNF protein levels in the spinal cord and sciatic nerve two weeks post STZ-injection in mice. However, 2 weeks of voluntary exercise increased GDNF levels in the sciatic nerve of diabetic mice, which is where the initial behavioral differences were seen.

Mechanisms that may mediate GDNF expression include mechanical activity, such as exercise. It has been shown that mechanical activity, in the form of stretch, positively stimulates trophic factor production in vascular and bladder smooth muscle cells [157]. It is believed that increased production and release of

growth factors are activated by the protein kinase C signaling pathways through an influx of calcium via voltage-gated calcium channels signaling pathways [90, 158]. Adenosine monophosphate-activated protein kinase (AMPK) may also mediate GDNF production. AMPK regulates skeletal muscle transcription and phenotype [159]. Exercise or muscle contraction is the most potent activator of AMPK in skeletal muscle. Work loads of 60% are sufficient enough to increase AMPK activity [160]. In skeletal muscle AMPK acutely increases ATP production. In ischemia states, such as diabetes, ATP is broken down into Adenosine [161]. Yamagata et al. (2007) demonstrated that adenosine induces and regulates the expression of GDNF. Therefore, many factors may play a role in exercise-induce gene expression of GDNF. However, it remains unknown how exercise increases GDNF expression in skeletal muscle and future studies are needed.

To further investigate the role GDNF plays in attenuating mechanical sensitivity associated with DN, diabetic mice received intrathecal injections of GDNF for 2 weeks. Our study showed that administration of GDNF for 2 weeks prevented the onset of mechanical sensitivity in diabetic mice. Therefore, our results support the view that GDNF has potent therapeutic actions in painful DN; however, our studies do not provide clear insight as to whether GDNF depletion in diabetes is involved in the initiation and maintenance of mechanical allodynia.

It is believed that neurotrophic factor deficiency in diabetes may be due to disturbances in axonal transport, which is critical for maintaining normal metabolism, physiological activity, and structural integrity of the nerve [53, 91].

GDNF is synthesized and secreted by a number of neuronal and non-neuronal tissues [63, 66-69]. To gain some perspective about the source(s) of GDNF in the spinal cord and sciatic nerve, we performed nerve ligation studies to determine if GDNF protein was transported retrogradely from the gastrocnemius muscle. Our results suggest that exercise-induced changes in GDNF are due to increases in both anterograde and retrograde levels of transported GDNF, with the greatest changes occurring in an anterograde direction. Collectively, these results suggest that exercise-induced increases in GDNF expression in the gastrocnemius muscle may influence spinal circuitry via retrogradely transport and release within the spinal cord. Increased accumulation of GDNF protein levels in the ventral horn, which was illustrated in our diabetic exercise rats, leads to anterograde axonal transport of GDNF in the sciatic nerve where it is released at the axon terminals in the skin and muscle where it could possibly serving as a neuromodulator. Therefore, it is possible that these mechanisms represent endogenous systems that enable communication between sensory/motor neurons and peripheral tissues that respond to exercise.

GDNF has been shown to be a target-derived trophic factor, in which GDNF may be obtained from the target and transported retrogradely to the cell body (neurotrophic hypothesis), where it can exert its trophic effects [162]. In our study, we demonstrated that voluntary exercise increases GDNF expression in the gastrocnemius muscle and retrograde axonal transport. Therefore, another possible mechanism for reducing in mechanical allodynia in our exercised

diabetic animals may be due to retrograde axonal transport of GDNF from the gastrocnemius muscle to the dorsal horn where it inhibit pronociceptive peptides [76].

Microglia, are a major immune cell population in the CNS [163]. They represent 5% to 10% of glia in the CNS and are considered resident macrophages [147]. Microglia, are inactive in healthy adult CNS, and become activated in acute and chronic neurodegenerative diseases [163]. After CNS injury, microglia become activated and they produce cytotoxic inflammatory mediators, such as nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor-a (TNF-a), and interleukin-1 beta (IL-1b). It is believed that this action plays a crucial role in triggering secondary tissue damage, which in turn leads to the progression of CNS diseases. Alternatively, activated microglia also play a role in phagocytotic activities, such as removing neural cell fragments and myelin debris, via the production of neurotrophic factors. This is beneficial to neuronal survival in the damaged CNS [163]. It has been established that microglia express GDNF receptor complex GFRα and Ret [164, 165]. Chang et al. (2006) demonstrated that GDNF positively regulates microglial activities, such as phagocytosis and the upregulation of adhesion molecules via the MAPK signaling pathway. Therefore, GDNF administration may have a protective effect on neurons by modulating microglial activities and increasing adhesion molecules to aid in axonal regeneration in order to return the neuron to its normal function. Collectively, there are many potential mechanisms that may exist on

how GDNF regulates nociceptive inputs both peripherally and centrally. Whether, pain modulation via GDNF occurs in the periphery or in the spinal cord remains to be seen, as a result, more research is clearly warranted.

5.2 Clinical implications

Treating symptoms associated with neuropathic pain continues to be a clinical challenge due to the variability and complexity of the disease. Presently, therapeutic options are largely limited to pharmacological treatments [119], which results in limited success [3]. Therefore, we explored the use of voluntary exercise as potential non-pharmacological therapy to treat neuropathic pain symptoms. Collectively, our results support the use of voluntary exercise to complement current therapy treatments used to attenuate behavioral sensitivity associated with neuropathic pain. Evidence supporting exercise for treating neuropathic pain is limited; however given its presumed safety, exercise should be considered whenever appropriate [131]. Given the diversity of neuropathic pain mechanisms, patient responses, and other potential disease that may affect the patient's ability to exercise, such as cardiovascular disease, treatments must be individualized.

Exercise has long been recognized as a part of therapy in the management of diabetes. While exercise may be a cost effective treatment, it may exacerbate complications or produce consequences due to existing complications associate with diabetes [138]. Prior to starting an exercise program

patients with diabetes should be evaluated in order to prevent exacerbation of macro and micro vascular complications. Diabetic patients experiencing peripheral neuropathy should only consider exercises they can tolerate without intensifying their painful symptoms. Most importantly, all patients should monitor their blood glucose levels before, during, and after all bouts of exercise. For diabetic patients, the overall benefits of exercise are clearly significant, such as preventing and reducing painful DN [60, 61]. Therefore, clinicians and patients must work together to maximize exercise-induced benefits while minimizing the adverse events in order to obtain a healthier lifestyle and improve the patients overall quality of life [138].

5.3 Future directions

As described above exercise-induced analgesia involves several mechanisms. In the SNI study, future studies would explore the potential exercise-induced analgesic mechanisms described above. In diabetic animals one possible mechanism involves increases in GDNF both centrally and peripherally. To further investigate the role GDNF plays in attenuating mechanical allodynia in diabetic animals, a GDNF knockout model should to be explored. Homozygous GDNF mice ($GDNF^{-/-}$) with the deletion of the GDNF gene die with 1 to 1.5 days after birth, however the heterozygous GDNF mice ($GDNF^{+/-}$) with the deletion of one allele of the GDNF gene have been shown to survive at least for 14 weeks [166, 167]. Similar to the wild type mice ($GDNF^{+/+}$), $GDNF^{+/-}$ mice displayed normal neuronal innervations of the intestine [168], along

with no deficits in facial motor neurons, dopaminergic neurons and fiber density, and noradrenergic neurons [169]. Therefore, GDNF$^{+/-}$ mice are a useful model for studying the effects of endogenous GDNF [167]. With this animal model we may be able to determine whether exercise-induced increases in GDNF is a mechanism involved in reducing mechanical allodynia in diabetic animals or whether other exercise-induced mechanism described above are involved.

The goal of our research is to enhance therapy treatments in humans by being able to translate our bench top findings to bedside practices. Our studies showed that the mechanical sensitivity and GDNF levels did not correlate with the total amount of exercise the animals participated in. To make our findings translational we need to determine the duration of exercise our animals need to participate in order to attenuate their mechanical sensitivity. Humans must exercise at a workload of greater than 70% of their maximal aerobic capacity [125]. Therefore, experiments are needed exploring whether exercising animals at a workload greater than 70% is sufficient enough to induce analgesia along with the mechanism that may be involved.

5.4 Conclusion

The body of work presented in this dissertation extends the current knowledge of the current pain literature by examining the use of voluntary exercise as a non-pharmacological therapy for treating neuropathic pain. The findings of our studies demonstrate the beneficial effects exercise has on

attenuating neuropathic pain. Our studies allude to potential mechanisms that result in analgesia following extended exercise. Future animal and human studies are greatly needed in order to determine the biological mechanisms that integrate exercise and pain, and future clinical studies are needed to determine the importance of timing and intensity of exercise in terms of injury onset and therapeutic effectiveness for neuropathic pain [30].

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