VOLUNTARY EXERCISE AND NEUROTROPHIN SIGNALING AFFECT THE DEVELOPMENT AND PRESENTATION OF PAINFUL NEUROPATHY

BY

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Abstract

Diabetic neuropathy (DN) is the most common and debilitating complication of type 1 and type 2 diabetes with approximately half of all patients developing neuropathy during the course of their lives. Additionally, patients with prediabetes also develop neuropathy, often presenting with painful symptoms, including burning and stinging sensations, as well as hyperalgesia and allodynia. Research suggests that altered neurotrophism may account for the development and maintenance of PDN, resulting in a dying back of peripheral neurons, leading to pain. Furthermore, patients suffering from painful diabetic neuropathy (PDN) have few therapeutic options, as pharmaceuticals are rarely effective and are only palliative in nature. However, recent research suggests that exercise may be beneficial in reducing PDN. The purpose of this work was to test the effects of obesity and a high-fat diet on the development of DN, to investigate how diabetes alters neurotrophins and to determine if voluntary exercise is capable of reducing PDN.

Initial studies used a model of obese, type 2 diabetes and investigated if voluntary exercise could reverse PDN. Diabetes resulted in mechanical allodynia, yet because these mice did not exercise, no benefit was gained; however, there was a significant correlation between physical activity and mechanical withdrawal thresholds. Additionally, we found that glial cell line-derived neurotrophic factor (GDNF) was decreased in the diabetic mice. These results suggest that diabetes does alter neurotrophin levels, which may lead to PDN. Next, using a high-fat diet to induce prediabetes, we found increased levels of nerve growth factor (NGF) protein, a neurotrophin known to mediate pain signaling, in the periphery, while exercise normalized these levels. Furthermore, prediabetes resulted in a switching of axonal phenotypes in the skin, increasing peptidergic nerve fibers, which was reversed with exercise. These results suggest that

increased NGF plays a critical role in mediating pain sensation in prediabetes and that exercise is capable of reversing this increase. Particularly, this study suggests that the ratio of peptidergic to nonpeptidergic axons may mediate the occurrence of PDN and may be more clinically significant than overall fiber density measures. Finally, we demonstrated that high-fat diet-induced PDN was reversed with a blocking antibody to NGF. In addition to decreases in mechanical withdrawal thresholds, anti-NGF treatment also normalized NGF levels within the DRG as well as normalizing epidermal innervation. Taken together, these studies demonstrate that exercise is capable of attenuating PDN, possibly through mediating NGF levels. It therefore appears that exercise and anti-NGF treatment are effective therapeutic strategies to prevent and reverse PDN.

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This work is dedicated to my parents, Rick and Brenda Groover.

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INTRODUCTION

Diabetes Mellitus

Diabetes mellitus is a chronic, multi-system metabolic disorder caused by both genetic and environmental factors and is characterized by hyperglycemia. Diabetes mellitus causes elevated blood glucose levels due to disrupted normal insulin signaling due to either loss of insulin production by the pancreas or loss of peripheral utilization of insulin. While disrupted insulin signaling is the cause of both types of diabetes mellitus, the etiology and clinical manifestations are specific to each type. Type 1 diabetes is an autoimmune disorder in which the insulin producing beta cells in the pancreas are destroyed and typically presents in young childhood. Type 1 diabetes mellitus accounts for approximately 5% of all diabetes diagnoses [1]. Type 2 diabetes is caused by insulin resistance in peripheral tissues and an overall insulin deficiency. Type 2 diabetes mellitus accounts for 90-95% of all diagnosed cases of diabetes and typically presents during middle or old age. Additionally, type 2 diabetes mellitus is associated with poor nutrition, lack of exercise, and obesity.

The World Health Organization (WHO) estimates that 347 million people worldwide have diabetes and it is predicted to become the seventh leading cause of death in the world by 2030. In the United States alone, the Centers for Disease Control and Prevention estimates 25.8 million adults and children are affected by diabetes. In fact, the prevalence of diabetes continues to increase with approximately 1.9 million new cases of diabetes diagnosed in people 20 years or older per year [1]. Diabetes is not only a health concern, but also a social and economic concern, as both type 1 and type 2 diabetes and the associated complications account for $116 billion in medical costs and an additional $58 billion in indirect costs annually [1].

Prediabetes

In comparison to type 1 and type 2 diabetes, a much larger portion of the world's population, including 79 million Americans, has prediabetes. Prediabetes is defined as blood glucose concentrations higher than normal levels, but not meeting the clinical definition of diabetes. Patients with prediabetes represent a high-risk population for developing diabetes. According to the World Health Organization (WHO), individuals at risk for developing diabetes have one or both prediabetic conditions: impaired fasting glucose (IFG) which is defined by fasting plasma glucose concentration greater than or equal to 6.1 (110mg/dl) and less than 7.0 mmol/L (126 mg/dl) and/or impaired glucose tolerance (IGT), defined by a fasting plasma glucose concentration of <6.1 mmol/L and a 2 hour post-load plasma glucose concentration between ≥7.8 (140 mg/dl) and <11.1 mmol/L (199 mg/dl) following a 75g oral glucose tolerance test [2]. The American Diabetes Association (ADA) uses the same thresholds for IGT, but the IFG is lowered to 5.6-6.9 mmol/L (100-125 mg/dl) [2-4]. In individuals with prediabetes, 5-10% develop diabetes annually with up to 70% eventually developing overt diabetes [5, 6].

Diabetic Neuropathy

Even with differing pathophysiologies, secondary complications, including heart disease, stroke, hypertension, nephropathy, retinopathy, and neuropathy occur in both type 1 and type 2 diabetes. Even though patients with prediabetes may not have the overt hyperglycemia like type 1 and type 2 diabetes, these patients are still at risk of developing complications. Of these complications, diabetic neuropathy is the most common and debilitating complication; some 60-70% of diabetic patients will develop some form of neuropathy [1]. Diabetic neuropathy can be classified as either a diffuse or focal neuropathy. Diffuse neuropathies are the most common and

are usually chronic and progressive in nature. Focal neuropathies are much less common and are generally acute in onset and are self-limiting, usually in about 6-8 weeks [7, 8]. Included in the diffuse neuropathies are distal symmetric sensorimotor polyneuropathy and diabetic autonomic neuropathy. Distal symmetric sensorimotor polyneuropathy is the most common and recognized form of diabetic neuropathy and from this point forward will be what is referred to as diabetic neuropathy (DN). Diabetic neuropathy is characterized by both sensory and motor deficits, with sensory dysfunction predominate.

Clinical Presentation

Although patients are susceptible to lesions in any nerve type, DN usually results in distal axonal degeneration in a dying-back, length dependent manner, manifesting at the distal extremities, beginning in the fingers and toes and eventually progressing proximally towards the trunk. This is termed a "stocking and glove" distribution and shows that it is the longest axons in the body that are affected first [9, 10]. Sural nerve biopsies from DN patients show loss of small, unmeylinated C-fibers, as well as small, myelinated Aδ fibers in the beginning stages of neuropathy, but then progress to large Aβ fiber dysfunction in later stages, with motor deficits occurring in severe, late stage diabetic neuropathy [10-13]. DN may present with combination of positive and negative symptoms. Positive symptoms include chronic pain, increased sensitivity to either mechanical or thermal stimuli, or parasthesias and is associated with small fiber degeneration [7]. Negative symptoms include loss of sensitivity to mechanical or thermal stimuli, chronic numbness, and altered or reduced proprioception and is more often associated with large fiber neuropathy [7]. In fact, negative symptoms are responsible for an increased risk of ulcers, foot infections, Charcot joints and amputations [14], accounting for 50-75% of all non-traumatic amputations [8]. As stated, patients may present with positive or negative symptoms. Perhaps

even more interesting, is that patients may present to the clinic with both symptoms: numbness in their feet and toes, yet very pronounced pain and sensitivity at the same time. While the majority of patients experience negative symptoms, or an insensate neuropathy, a portion of the diabetic population experiences disabling chronic pain. Prevalence rates of painful diabetic neuropathy vary, but most estimate that approximately 10-20% of the diabetic population experiences pain [15-17]. Additionally, as diabetic neuropathy progresses, motor function can also be affected, leading to limited mobility and causing impairments in walking, running, and even climbing stairs. Although motor modalities may be affected, this is only seen in advanced cases of diabetic neuropathy (for a review, see [18]).

Damage to neurons due to diabetic neuropathy are correlated to peripheral nerve pathology and clinical signs and symptoms. Initial diagnosis of DN relies on a physician's recognition of a combination of symptoms, followed by confirmatory testing. Following clinical evaluation, nerve injury can be further assessed through quantitative sensory testing, nerve conduction velocity (NCV) studies, and skin biopsies. Clinical evaluation is comprised of a variety of tests such as pinprick, vibration perception using a tuning fork, a 10g monofilament pressure test, and ankle reflexes to assess sensory modality dysfunction[19]. Nerve conduction velocity (NCV) studies are considered the "gold standard" for diagnosing DN and include sensory (SNCV) and motor (MNCV) electrophysiology. Nerve conduction velocity is measured using a compound action potential (CAP), which is the algebraic summation of all the action potentials produced by all the fibers that were fired by the stimulus. Nerve conduction velocity can be measured by the amplitude of the wave, the speed of the CAP, or a combination of both. Amplitude can be lowered due to loss of fibers within the nerve, or a loss of fibers reacting to the stimulus. The latency to the beginning of the CAP can also be altered and reflects changes in the

average firing speed. Alterations in NCV include a slowing of conduction velocity due to demyelination and loss of large myelinated fibers, as well as a decrease in nerve action potentials due to a loss of axons [10, 20, 21]. NCV studies are the most objective and noninvasive measures of nerve function, yet cannot be taken at face value for diagnosis alone. While NCV studies are useful for examining larger fiber dysfunction, skin biopsies and subsequent intraepidermal nerve fiber density (IENFD) measures are more appropriate for examining cutaneous small fiber loss.

Patients with prediabetes are susceptible to neuronal damage and diabetic neuropathy, much like patients with overt diabetes. While previously debated, it is now generally accepted that patients with prediabetes do develop DN. Clinically, 10-18% of patients in the clinic present with abnormal nerve conduction velocities [22, 23], signifying that the length-dependent nerve injury may predate the diagnosis of overt diabetes, suggesting neuropathy associated with prediabetes. Recent studies have established that patients can develop DN before the clinical diagnosis of prediabetes [24-26].

Neuropathy in individuals with prediabetes generally appears less severe than those with frank diabetes mellitus. Prediabetic neuropathy has been reported to have a slightly less distinct impairment in amplitude and conduction velocity in the sural nerve [26] as well a significantly improved amplitude in the peroneal nerve in prediabetes compared to diabetes [24]. Moreover, prediabetes appears to predominately affect sensory modalities, leaving motor modalities relatively unchanged [24, 27]. In addition to NCV studies showing sensory nerve involvement as compared to motor nerve involvement [24, 27], patients often exhibit sensory symptoms, including increased vibration perception and reduced temperature perception [27]. Due to the appearance of sensory symptoms rather than motor symptoms, it appears it is the small nerve fibers that are affected earliest and to a greater degree. Similar to overt diabetic neuropathy, this

is due to the absence of the protective myelin sheath covering the small fibers, yielding them more susceptible to glucose toxicity compared to larger, myelinated fibers [28]. It should be noted that because prediabetic neuropathy affects small fibers far more than large fibers, NCV studies are less accurate in prediabetes compared to diabetes. In fact, NCV studies may be normal in approximately one out of 10 patients with small fiber dysfunction [25]. In the setting of prediabetes, it is often times more useful to obtain a skin biopsy to quantify IENFD in order to confirm a dying back of cutaneous small fibers [26, 29].

Pathogenesis

The pathogenesis of the dying-back phenomenon of peripheral nerves in diabetic neuropathy is not completely clear; however, the small, unmyelinated neurons appear to be the most susceptible to hyperglycemia associated with both type 1 and type 2 diabetes, as well as prediabetes. It is well established that the duration of diabetes and glycated hemoglobin levels are associated with a high incidence of neuropathy [30, 31]. Additionally, the Diabetes Control and Complicatons Trials (DCCT) confirmed the beneficial effects of vigilant control of blood glucose levels on the incidence of chronic complications in 1441 type 1 diabetic patients [32], including successfully decreasing the incidence of neuropathy by 60% [32]. It is clear that chronic high blood glucose levels lead to peripheral nerve injury, though through many different metabolic pathways.

The anatomy and vascular supply of the peripheral nervous system both play a role in the vulnerability of neurons. The vascular supply to peripheral nerves is sparse and it is likely that blood flow is compromised following hyperglycemia [33], leaving the neurons vulnerable to ischemia. Malik *et al.* showed that patients who did not have overt neuropathy at the time of nerve biopsy, but displayed high-grade microangiopathic changes of endoneurial microvessels

later, developed blatant neuropathy, while patients without microvessel changes did not develop neuropathy [34]. Vascular endothelial growth factor A (VEGF-A) is a protein that stimulates vasculogenesis and angiogenesis, especially in embryonic development, and following exercise and injury. Importantly, VEGF-A is commonly misregulated in diabetic patients. Patients with proliferative retinopathy show increased vitreous and plasma levels of VEGF-A [35-37], while patients with diabetic neuropathy have shown decreased VEGF expression from skin biopsies [38]. Additionally, VEGF-A levels have been shown to be sensitive to ischemic agents, including oxygen, iron and glucose [39-42], and hyperglycemia has been shown to reduce VEGF-A RNA and protein levels [43, 44]. Furthermore, type 2 diabetes is associated with reduced VEGF signaling along with impaired angiogenesis [45]. This decreased VEGF expression in the skin could account for poor vascularization of peripheral nerves, leading to ischemia and poor neuron health. In accordance with this theory, plasmid VEGF-A/VEGF-C treatment for 6 months improved neuropathy symptom scores and pain scores in patients with diabetic neuropathy [46]. Moreover, clinical trials aimed at increasing peripheral circulation with VEGF-A have shown success in increasing circulation and decreasing neuropathy [47, 48]. Additionally, the peripheral nervous system lies outside of the blood-brain and blood-nerve barriers, leaving them more susceptible. Moreover, the neuronal cell body is relatively small in comparison to the extremely long distance of the axonal neuritis; consequently, distal axons are too weak to support themselves and must rely on the small cell body to transport nutrients, neurotrophic factors, as well as other signals. Damage to the neuronal cell body or the axons prevents proper cellular transport and thus the periphery cannot obtain the needed proteins necessary for support and maintenance [49].

It is believed that chronic hyperglycemia leads to peripheral nerve injury, though the mechanism remains elusive. Increased glucose flux through the polyol pathway has been the most extensively studied cause into the development of neuropathy. Early studies proposed the osmotic theory where increased polyol flux caused intracellular hyperosmoloarity by an accumulation of sorbitol into the cell cytoplasm, causing cell lysis [50, 51], yet no evidence of nerve edema or swollen cells have been found in diabetic nerve tissues [52]. However, more recently, using transgenic animals, the polyol pathway is a little clearer. Transgenic mice that overexpress human aldose reductase (AR), the enzyme responsible for reducing glucose to sorbitol, developed severe neuropathy when fed galactose, which is another AR substrate [53], evidence that even without hyperglycemia, increased polyol flux could cause peripheral nerve dysfunction much like diabetic animal models. When this study was then applied to streptozotocin (STZ)-induced diabetic animals, the STZ-treated animals developed much more severe NCV delays compared to non-transgenic STZ-treated controls, even though blood glucose levels were comparable [54]. While increased polyol flux is a part of the story, studies have shown that severe hyperglycemia can cause neuropathic changes, even in AR-deficient mice [55], and therefore, a pathway independent of the polyol pathway is critical. Additional avenues of study into the pathogenesis of diabetic neuropathy include advanced glycation end product (AGE) formation [56-58], oxidative stress [59, 60], protein kinase C (PKC) activation [61, 62], inflammation [63-65], and diminished cellular and trophic support [66-69]. Each of these mechanisms is currently being evaluated to determine the role and amount of significance each plays in the development of diabetic neuropathy. Despite years of extensive study into each proposed mechanism, there is still no panacea for diabetic neuropathy, and it will most likely

require a combination of treatments, targeting multiple pathways to begin to develop an effective treatment of this disease.

Rodent Models

There have been multiple rodent models developed to study diabetic neuropathy and the associated neuronal changes, as well as investigate the mechanisms underlying the disease, and to test possible therapies for the disease. Much of the research is investigating type 1 diabetes, and the most common model is using a drug known as streptozotocin (STZ). STZ is a naturally occurring chemical that is selectively toxic to the β-cells, located in the islets of Langerhans in the pancreas. STZ is transported by the GLUT2 receptor, which is highly expressed in the β-cells, where it alkylates the DNA in the cells, causing damage and cell death [70]. After injection, rodents display classic signs of diabetes, including polydipsia, polyuria, and hyperglycemia, and go on to develop secondary complications, including neuropathy [71].

While STZ is used most regularly to induce type 1 diabetes, models for type 2 diabetes are more diverse. Transgenic rodents are investigated most frequently, and include the leptin receptor mutation mouse (*db/db*) and the leptin deficient mouse (*ob/ob*), both on a C57Bl/6 background. Both mouse models result in insatiety, which leads to overeating and an obese, type 2 diabetic genetic model. Another model of type 2 diabetes, as well as prediabetes, is a high fat diet. A high-fat diet contains 54% kcal of fat, compared to a standard diet, which only contains 14% kcal from fat. Using a high fat model allows researchers to not alter the genetics of the mouse and instead allows for a closer resemblance to the American population. One significant point of investigation is to why one rodent model will present with positive symptoms and another model will present with negative symptoms. Rodent models of diabetes vary in their

presentation of diabetic neuropathy, much like human patients. The different presentations of diabetic neuropathy symptoms may offer understanding into the mechanisms and genetics underlying the development of one course of symptoms over another.

Streptozocin-induced diabetic rats typically develop mechanical, thermal, and chemical hyperalgesia, concurrent with impaired nerve conduction velocities [14, 72]. However, STZ-induced diabetic mice exhibit a variety of symptoms, with certain strains, such as the A/J strain developing mechanical hyperalgesia, the C57Bl/6 strain developing a mechanical hypoalgesia. Independent of neuropathy symptoms, both the A/J and C57Bl/6 strains develop sensory and motor NCV deficits and have decreased intraepidermal nerve fiber density [14, 72].

Type 2 diabetes models also vary in presentation of DN. Leptin deficient *ob/ob* mice develop mechanical hyperalgesia, thermal hypoalgesia, and deficits in NCV [73, 74]. Interestingly, leptin receptor null mutant *db/db* mice develop mechanical hypoalgesia, but no changes to thermal sensitivity [75, 76], though they also have slowed nerve conduction velocities. High-fat fed mice, a model more closely resembling prediabetes, exhibit mechanical hyperalgesia and thermal hypoalgesia [77, 78]. In a recent study, it was shown that a high-fat diet could in fact reverse the neuropathy phenotype in a STZ-induced C57Bl/6 mouse from a mechanical hypoalgesia to a mechanical hyperalgesia [78], highlighting the important role that diet may play in the development of diabetic neuropathy. It should be noted that while rodent strains do develop diabetic neuropathy, and associated neural deficits, no model fully emulates the human condition. Rodent models do not display the same fiber loss and neuronal degeneration to the same extent as seen in the human condition [14, 72, 79], which is most likely the result of shorter axons compared to humans.

Exercise

As stated previously, there is no definitive cure for diabetic neuropathy. Conventional treatments for peripheral neuropathy have primarily been palliative rather than curative, and symptom oriented rather than disease oriented. Even more, they have often been ineffective. At current, the best a patient can do to prevent and ameliorate painful neuropathy is to keep extremely tight control on their blood sugars. Current strategies for treatment of painful diabetic neuropathy include anticonvulsants, antidepressants, and opioids [15]. However, each of these drugs has limited success in the treatment of pain, and furthermore, each has significant side effect. An alternative approach is to use exercise, either alone or in combination with pharmacological intervention. It is widely accepted that moderate physical activity has general health benefits, including the prevention of cardiovascular disease, type 2 diabetes, osteoporosis, cancer, and depression [80-82]. Furthermore, it is also capable of providing analgesia, both in rodent models and human patients. Previous animal studies have shown that exercise is capable of treating chronic myalgia in rats via treadmill running [83], and swimming was able to reduce thermal and mechanical hyperalgesia in rat models of formalin-induced inflammatory pain [84] as well as partial peripheral nerve injury neuropathy [84, 85]. Numerous human studies have shown exercise-induced analgesia using a variety of noxious stimuli, including electrical, temperature, and pressure tests and analgesia following exercise appears to be most consistent with exercise performed at higher intensities (>60% of maximal aerobic capacity) [86-89].

In the setting of painful diabetic neuropathy, exercise is also beneficial, even though relatively few studies have been reported. In fact, physical activity has shown both protective [90, 91] and therapeutic [92] effects on DN. Studies evaluating the role of exercise in animal models of diabetic neuropathy show it can improve and maintain nerve function. Swimming

training was able to prevent motor dysfunction associated with STZ-induced diabetes [91]. Moreover, treadmill trained STZ-induced diabetic rats had a delay in the development of both mechanical and thermal hyperalgesia compared to sedentary STZ-induced rats [93]. Additionally, voluntary running wheel exercise improved mechanical thresholds at 6 weeks post-STZ in a mouse model of painful diabetic neuropathy (Unpublished data, Farmer 2010). Human studies have shown similar benefits for exercise in diabetic neuropathy. In a study by Balducci *et al.,* type 1 and type 2 patients without signs of neuropathy had a decreased incidence of motor and sensory neuropathy following 4 h/week brisk treadmill walking compared to sedentary patients, as measured by nerve conduction velocity and vibration perception threshold tests [90]. Furthermore, the Diabetes Prevention Program (DPP) found that lifestyle intervention that included a minimum of 150 minutes weekly of moderate intensity exercise of their choice, had a significant improvement in pain and epidermal nerve fiber density following one year of treatment [27]. In one of the most recent studies, Kluding *et al.* report the results of a small, unblended trial of exercise for painful diabetic neuropathy. Using aerobic and strength training exercises 3-4 days/week for 10 weeks, subjects had a 30% reduction in pain severity, a decrease in neuropathic symptoms based on the Michigan Neuropathy Screening Instrument (MNSI), and increased intraepidermal nerve fiber branching from a proximal skin biopsy [92]. These studies strongly suggest that exercise is effective in preventing and reversing symptoms of diabetic neuropathy, as well as painful symptoms, in rodent models and human patients.

The exact mechanism of exercise-induced analgesia is not completely understood and may involve multiple pathways to elicit pain relief. The most wide-spread research has focused on the endogenous opioid system. It is well known that endogenous opioids, like endorphins and enkephalins, are increased in both humans and rats following aerobic and anaerobic exercise [94-

96]. In fact, even a single bout of exercise can increase the production of endogenous opioids, leading to antinociception [97], while repeated exercise produces long-lasting antinociception [98-100] and increases in plasma and cerebrospinal fluid opioid concentrations [101, 102]. Furthermore, infusion of opioid antagonists naloxone and naltrexone has been shown to prevent exercise-induced analgesia [103].

As stated previously, diabetic neuropathy is thought to be, in part, a problem due to vascular insufficiency and peripheral ischemia. Exercise may be improving diabetic neuropathy by increasing peripheral blood flow via increased VEGF. Endurance training can induce marked changes in skeletal muscles, including an increase in capillarization, characterized by an increase in capillary density and capillary to fiber ratio [104-106]. This increase in capillary density and vascularization is thought to be triggered by VEGF. Previous studies have shown exercise-induced increases in VEGF mRNA in the skeletal muscle of both animals and humans [107, 108], and these increases in VEGF mRNA are associated with increases in VEGF protein content [109-111]. Interestingly, in one study, mice fed a high fat diet and treadmill trained showed significantly upregulated VEGF mRNA compared to normal chow and high fat sedentary counterparts [112]. These data suggest that exercise can act to upregulate VEGF mRNA, increase skeletal muscle VEGF protein, thereby increasing capillarization and improving peripheral blood flow. This may help prevent neural ischemia, and improve neuropathy.

Additionally, it is possible that neurotrophins play a major role in reducing pain behavior. Numerous studies have reported exercise-induced increases in neurotrophins including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophic factor 3 (NT-3) in both naïve and injured animals [113-117]. Injuries to both the central and peripheral nervous system can cause alterations in the expression of neurotrophins [118, 119], yet exercise

has been shown to restore neurotrophin levels [113, 114, 120, 121]. Glial cell line-derived neurotrophic factor (GDNF) has also been shown to be increased in an activity dependent manner in muscle in a spinal cord injury animal model, leading to better functional and behavioral recovery [122]. Finally, exercise can also cause changes to the synthesis and release of monoamine neurotransmitters, including norepinephrine, dopamine, and serotonin[123], which are capable of inhibiting descending nociception from the brain to the spinal cord.

Neurotrophins

Neurotrophic factors are a family of closely related proteins that were initially identified as survival factors for neurons, and are now known to control aspects of survival, development, and function of neurons in both the central and peripheral nervous systems. Neurotrophic factors are synthesized by peripheral tissues or neurons, often at a long distance from the neuronal cell body. During development, neurotrophic factors are retrogradely transported from the peripheral target, up the axon to the cell body [124]. The retrograde flow of neurons is essential for the life of the neuron in order to maintain its functional differentiated state [125]. Neurons that establish the retrograde flow survive and mature, while those that do not establish the connection undergo neuronal cell death.

The first neurotrophin to be discovered was nerve growth factor (NGF). It was initially discovered from an observation that sensory ganglia became hypertrophied when mouse sarcoma tissue was grafted onto a chick embryo [126]. NGF was discovered to be part of a larger family of related proteins that contain the following members: NGF, BDNF, NT-3, NT-4/5, and NT-6 (which exists only in teleost fish). Glial cell line-derived neurotrophic factor (GDNF) is in a different family of neurotrophins, the GDNF-family ligands (GFLs), which belongs to the

transforming growth factor-β superfamily. GDNF was originally purified from a rat glioma cell-line supernatant as a trophic factor for dopamine neurons, and only later discovered to have trophic effects on other neuronal subpopulations.

It should be noted that traditionally, the word "neurotrophin" refers to this specific family of proteins, while "neurotrophic factor" refers to the growth factors GDNF and ciliary neurotrophic factor (CNTF). In this manuscript, all growth factors, including NGF, BDNF, and GDNF will be referred to as "neurotrophins."

Neurotrophin Receptors and Signaling Cascades

Nerve growth factor and BDNF mediate their actions by binding to a corresponding family of receptor tyrosine kinases referred to as tropomyosin related kinase (Trks). Members of the trk gene family include trk A, which binds NGF with high affinity, trk B, which serves as a receptor from BDNF and NT-4/5, and trk C serves as the receptor for NT-3 [127]. In addition to the high affinity receptors, a low affinity receptor has been identified which binds all neurotrophins. p75 does not have a catalytic motif, yet it participates in binding each neurotrophin to its corresponding trk receptor. Activation of trk by their ligands results in dimerization of the receptor and phosphorylation of activation loop kinases [127]. Upon ligand binding to trk and subsequent phosphorylation, different signaling cascades are activated, including the ras-raf-MAPK, PI3K-Akt-GSKIII, PLCγ-DAG-PKC, and S6kinase pathways (**FIGURE 1).** Interestingly, if the p75 receptor is expressed in the absence of trk, neurotrophins can activate pathways downstream to p75 and actually promote apoptosis [128]. Furthermore, trk activation also leads to endocytosis of the ligand/receptor complex and subsequent retrograde transport to different membrane compartments, leading to transcriptional control over the

neuron. However, while the signaling cascades account for quick, acute responses, internalization of the receptor-neurotrophin complex and signaling is delayed, and may take hours or days.

Glial cell line-derived neurotrophic factor does not signal through trk and p75 receptors like the NGF family of neurotrophins. Instead, GDNF signals through the RET receptor tyrosine kinase and GDNF-family receptor-α receptor-1 (GFRα1) complex. GFRα1 is bound to the plasma membrane by a glycosyl phosphatidylinositol anchor. GFRα1does not have a cytosolic domain, yet is the binding site for GDNF. GDNF cannot signal through either RET or GFRα1 alone; they must be bound in a receptor complex. RET is a single-pass transmembrane protein with an intracellular tyrosine kinase domain. Once GDNF binds to GFRα1, the GDNF-GFRα1complex then binds to the extracellular domain of RET, leading to activation of the intracellular tyrosine kinase domain via autophosphorylation. Once phosphorylated, the tyrosine residues can activate various intracellular signaling proteins, including the ras-raf-MAPK, PI3K, and PLCγ pathways (**FIGURE 2)**, which regulate survival, differentiation, migration, chemotaxis, neurite outgrowth, and synaptic plasticity [129, 130].

Neurotrophin receptors are expressed in specific populations of neurons within the dorsal root ganglion (DRG). Approximately 40% of DRG cells express trkA [131-135] and those cells that do express trkA are principally small diameter. TrkA is expressed in the peptidergic subset of small-diameter cells, while few nonpeptidergic small-diameter DRG cells express trkA [132, 135]. Additionally, approximately 20% of larger, myelinated DRG cells express trkA. Furthermore, trkA-immunoreactivity show axons terminating in laminae I and IIo, very characteristic of nociceptive neurons. The nonpeptidergic subpopulation of small-diameter neurons, those which are GDNF responsive, account for approximately 34% of the cells in the

Figure 1: Neurotrophin Signaling: Members of the neurotrophin family signal through Trk and p75 receptors to activate downstream pathways. Neurotrophin binding leads to dimerization and autophosphorylation of the trk receptor. Downstream signaling cascades include the ras-raf-MAPK (mitogen-activated protein kinase) pathway, where MAPK translocates to the nucleus and acts to promote neuronal differentiation. Activation of the phosphatidylinositol 3-kinase (PI3K) pathway leads to neuronal growth and survival, while activation of phospholipase C-γ1 (PLC- γ1) results in promotion of synaptic plasticity. Original figure.

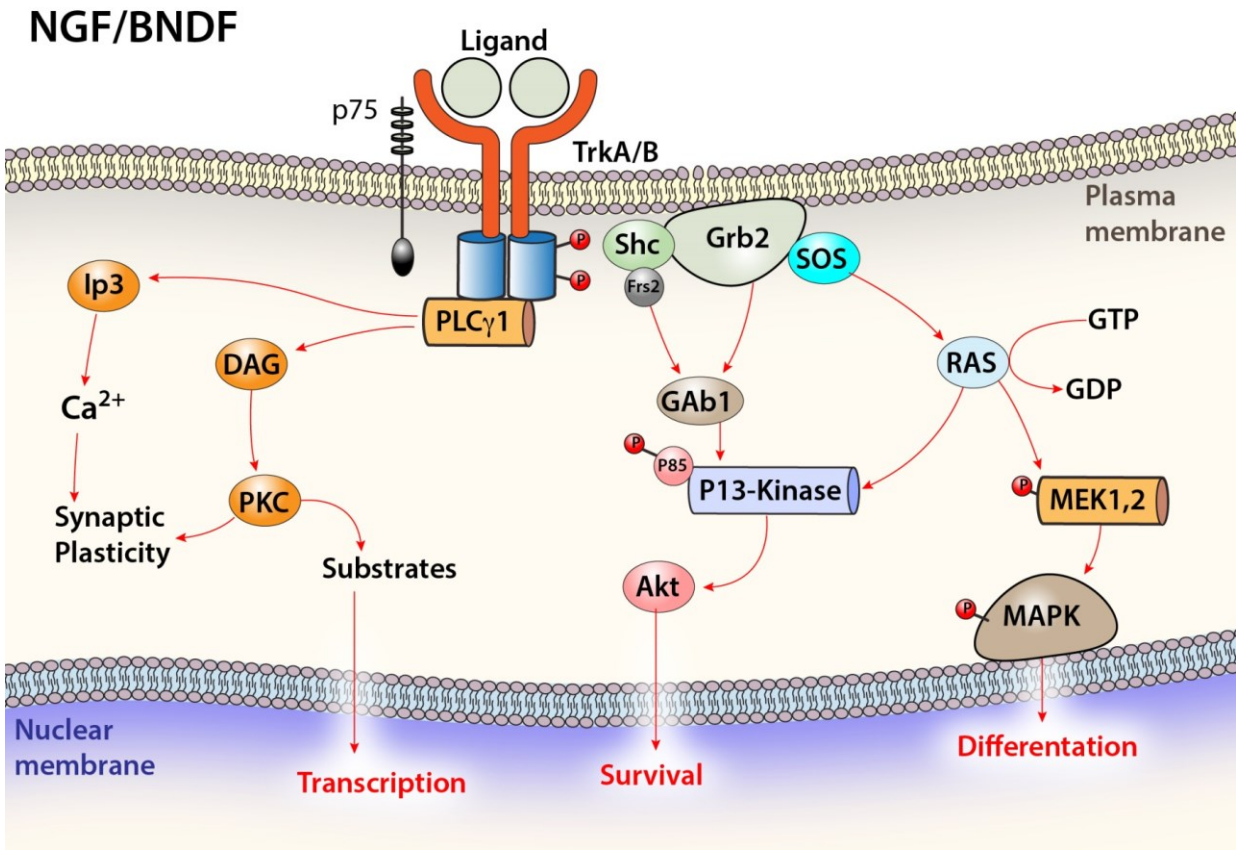
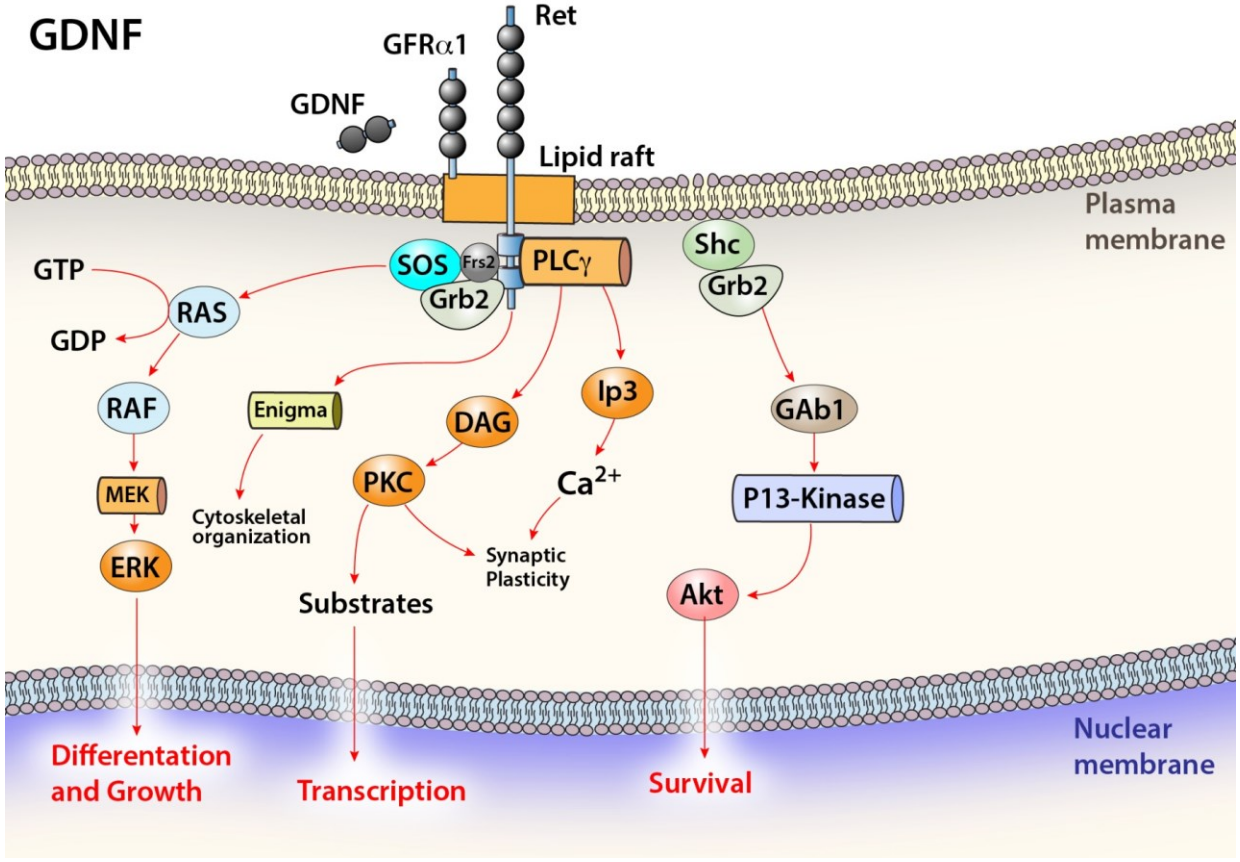

NGF/BNDF
Ligand
p75
TrkA/B
Plasma membrane
Shc
Grb2
SOS
Frs2
Ip3
PLCγ1
DAG
Ca2+
GTP
GDP
RAS
GAb1
P85
P13-Kinase
MEK1,2
PKC
Synaptic Plasticity
Substrates
Akt
MAPK
Nuclear membrane
Transcription
Survival
Differentation

Figure 2: GDNF Signaling: GDNF signals through the GFRα1-RET receptor complex. Upon GDNF binding, RET autophosphorylates at the tyrosine kinase domains and activates downstream signaling cascades. Similar to trk and p75 receptor activation, downstream signaling cascades include the ras-raf-MAPK (mitogen-activated protein kinase) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, and phospholipase C-γ1 (PLC- γ1) cascades. Resultant activation leads to cell growth and differentiation, cell survival and cytoskeletal reorganization.


GDNF
GFRα1
Ret
GDNF
Lipid raft
Plasma membrane
GTP
GDP
RAS
SOS
Frs2
Grb2
PLCγ
Shc
Grb2
RAF
Enigma
DAG
Ip3
GAb1
MEK
PKC
Ca2+
P13-Kinase
Cytoskeletal organization
Synaptic Plasticity
ERK
Substrates
Akt
Differentation and Growth
Transcription
Survival
Nuclear membrane

DRG [136], trkB is present in both small and large cell diameter within the DRG, with reports varying from 5 to 27% of lumbar DRG cells expressing trkB [131, 133, 134]. However, there does not seem to be a direct relationship between trkB expression in the DRG and functional subpopulations of DRG neurons. Roughly 20% of neurons express trkC within the DRG; however, in contrast to trkA, trkC appears to be almost solely located on large-diameter neurons, which are responsible for proprioception [131, 133, 134]. Almost all DRG neurons that express a trk receptor also express the p75 receptor, and p75 is not expressed independently of a trk receptor [136].

Neurotrophins and Pain

Neurotrophins have been implicated in pain in many different settings. Some neurotrophins are pronociceptive, some are antinociceptive, and some are implicated in both pro- and antinociceptive roles. The most well-known and studied neurotrophin in the setting of pain is NGF. In 1996, a study reported a genetic basis for congenital insensitivity to pain. Indo *et al* discovered that a mutation in the trkA receptor disrupted the normal signaling of NGF, and because of a lack of NGF signaling, there was no sensitivity to pain [137]. This study represents only a small aspect of the role that neurotrophins play in sensing pain.

Nerve Growth Factor

Nerve growth factor (NGF) is known to be a potent mediator of pain. Both rodent and human studies involving NGF administration demonstrate that NGF itself can cause hypersensitivity. It has been demonstrated that thermal and mechanical hyperalgesia is produced following systemic NGF administration in rats [138], while injections of NGF produce a thermal and mechanical hyperalgesia at the injection site in both rodents and humans [139, 140].

Additionally, in humans, an intravenous injection of low dose NGF results in myalgia, or deep muscle pain, which can last for days [141]. Furthermore, elevated NGF levels have been found in a variety of pain states in humans, including vasculitic neuropathy [142], chronic prostatitis [143], interstitial cystitis [144], fibromyalgia[145], and endometriosis [146].

Many of these effects appear to be a result of direct action on NGF on trkA nociceptors, since the effects can begin immediately. Direct action on nociceptors include increasing substance P and calcitonin gene-related peptide (CGRP), upregulating other receptors including TRPV1, P2X3 and ASICs [147], and upregulating $Na_v1.8$ ion channels [148, 149]. However, some of the effects are indirect and may involve NGF releasing algogens from a variety of peripheral cell types. Mast cells contain inflammatory mediators (i.e. serotonin and histamine) that are known to act on primary afferent nerve fibers [150]. Many mast cells express trkA receptors [151], and are thus activated by NGF binding. NGF can therefore result in mast cell degranulation and increase the proliferation of mast cells present in the tissue [151]. Furthermore, NGF can induce the expression of multiple cytokines in mast cells, including interleukin-3 (IL-3), IL-4, IL-10, TNF-α, and granulocyte macrophage colony-stimulating factor [152].

Neuropathic pain is associated with multiple neuropathies, with diabetic neuropathy the most common, but also may be associated with infections like HIV-induced neuropathy, drug treatments like cisplatin- and taxol-induced neuropathy and traumatic injury to either the peripheral nerves or spinal cord. In animal models of neuropathic pain, a large portion of sensory neurons undergo Wallerian degeneration and therefore lost contact with their normal peripheral targets. However, some axons within the same nerve remain intact and can traverse through the abnormal environment of the degenerating nerve. Because of this, there is a great interest in

neurotrophic factors and their neuroprotective effects. The central hypothesis is to replace or substitute endogenous neurotrophic factors for neurons that have lost connections from their normal target and to reestablish this connection. Many preclinical studies involving NGF have shown neuroprotective effects on small, nociceptive fibers as well as NGF reversing hypoalgesia [153-155]. However, the pronociceptive effects of NGF treatment are too great to be ignored. In phase II clinical trials, recombinant human NGF (rhNGF) was effective at ameliorating symptoms associated with diabetic neuropathy; however, there were painful side effects including myalgias and injection site hyperalgesia that were dose-limiting [156]. In a large-scale phase III clinical trial, rhNGF failed to confirm the earlier indications of efficacy after 48 weeks of treatment compared to placebo [157]. Following the phase III trial, further investigation into rhNGF was halted.

While many of these studies assessed the benefits of NGF treatment due to injured nerves and reduced target-derived neurotrophic support, there is increasing interest in reducing pain by blocking the actions of NGF. Several groups have tested the use of anti-NGF treatment in rodent models, with some success. Using a rat model of sciatic nerve constriction, Ramer *et al* showed decreased mechanical and thermal hyperalgesia[158], while Herzberg *et al* showed a delayed onset of hyperalgesia [119]. In fact, Pfizer has developed a drug for clinical use to block endogenous NGF. Tanezumab is a monoclonal antibody directed against nerve growth factor for the treatment of pain. Tanezumab is undergoing phase II clinical trials for the treatment of low back pain, osteoarthritis and interstitial cystitis [159-161]. While there has been some success in clinical trials for alleviating pain, many studies have been terminated due to safety concerns, including a study involving diabetic neuropathy [162].

Brain-Derived Neurotrophic Factor

Genetically modified mice have shown the important nature of BDNF in promoting survival during development of some sensory neurons, particularly in the trigeminal and nodose ganglia [163]. In fact, BDNF is critical for the development of sensory neurons involved in mechanoreception, like Meissner corpuscles and Pacinian corpuscles [164, 165]. Beyond this, however, BDNF appears to be an important modulator of pain.

Brain-derived neurotrophic factor is constitutively expressed by a subset of DRG neurons, which include small and medium-sized sensory neurons [133, 166]. It is dramatically upregulated in both small- and large-diameter neurons in models of inflammatory and neuropathic pain, respectively [167-169]. NGF is known to upregulate BDNF, and in the inflammatory pain states, it appears that increases in NGF in the periphery results in increases of BDNF as well. In models of neuropathic pain, BDNF has been found to be dysregulated; however, the dysfunction is lesion-specific. For example, in partial nerve injury and sciatic-constriction models, some small-diameter neurons that are left intact following injury display increases in BDNF expression, thought to be the result of increased target-derived trophic support [170]. However, following axotomy, BDNF mRNA expression is decreased in small-diameter neurons, but increased in medium to large DRG neurons [168]. BDNF is located in large dense-core vesicles in the primary afferent terminals located in the spinal cord [171], much like the vesicles that contain substance P. BDNF has been shown to be released following neuronal activity from these vesicles. For example, BDNF has been shown to be released from these vesicles following activation of nociceptive C-fibers with capsaicin [172].

Studies suggest BDNF may serve both a pro- and anti-nociceptive role, depending on the setting. As a pro-nociceptive factor, as stated before, BDNF is known to be upregulated in the DRG in conditions of peripheral inflammation. Intraplantar injections of BDNF causes transient thermal hyperalgesia for up to 5 hours [173]. While sequestering BDNF resulted in decreased nociceptive thresholds in models of inflammatory pain [174], it did not affect mechanical hyperalgesia resultant from capsaicin [175]. In rodent models of neuropathic pain, anti-BDNF antibodies reduced pain-related behaviors in DRG injured mice [176, 177] and rats [178]. Furthermore, in the same rat study, delivery of BDNF directly to the DRG of normal rats resulted in mechanical allodynia [178]. Additionally, BDNF-deficient mice exhibited downregulation of the ASIC2 sodium channels in medium- and large-diameter neurons, neurons that are necessary for normal mechanical signal transduction [179]. Importantly, visceral tissues express high levels of BDNF and its receptor trkB, more than in somatic tissues. BDNF is normally expressed at low levels in the pancreas, yet in chronic pancreatitis, it is highly expressed in the ducts and perineurium of nerves and BDNF content is correlated to the pain intensity in patients [180]. In contrast to the pronociceptive role BDNF has been shown to play, it also exhibits antinociceptive properties as well when delivered in higher doses and in much wider areas of the CNS. For example, when BDNF is intracerebroventricularly administered to the brain of rats, as well administration via BDNF-expressing grafts on the spinal cord of chronic constricted injury rats, pain-related behaviors decreased [181, 182]. In the setting of diabetic neuropathy, BDNF treatment was not able to improve sensory abnormalities associated with diabetes in a rodent model [72]; however, in a randomized, double-blind, placebo-controlled clinical trial, BDNF was able to improve cool detection threshold [183], suggesting some neuroprotective effects on a subset of thermal neurons.

Glial Cell Line-Derived Neurotrophic Factor

It is well established that GDNF is an important modulator in the nociceptive pathway. Much like BDNF, GDNF is contradictory in that it has both pro- and anti-nociceptive properties. GDNF has been shown to induce acute thermal hyperalgesia following intraplantar injections [184] and anti-GDNF antibodies have the ability to attenuate inflammatory hyperalgesia [185, 186]. Additionally, sciatic nerve transection can result in increased GDNF and GFRα-1 expression in both the sciatic nerve and DRG [187, 188]. However, much of the reporting has been on the anti-nociceptive properties of GDNF. In one of the most well-known and well-cited papers, Boucher *et al* demonstrated GDNF's ability to not only prevent, but also reverse sensory abnormalities associated with neuropathic pain. In this study, rats received either a partial sciatic ligation (PSL) or L5 spinal nerve ligation (SNL). Some rats received continuous concurrent intrathecal infusion of GDNF, NGF, or NT-3, and only continuous GDNF infusion was capable of preventing both mechanical and thermal hyperalgesia in both PSL and SNL treated rats [149]. Furthermore, delaying the infusion of GDNF until after hyperalgesia was well established in the rats, also resulted in the reversal of both mechanical and thermal hyperalgesia [149]. Another study using GDNF pellets implanted on the sciatic nerve of spared tibial nerve injury rats resulted in reversal of mechanical hyperalgesia [189]. And in the setting of diabetic neuropathy, daily intrathecal injections of GDNF were able to improve mechanical withdrawal thresholds (Farmer, K. Unpublished data, 2010).

Much like NGF, there are multiple working ideas on the mechanisms involved in the association of GDNF-induced analgesia. These include modulation of sodium channels and neuropeptides, as well as decreased Aβ-fiber sprouting and axonal regeneration into the spinal cord. Tetrodotoxin (TTX)-sensitive sodium channels can be blocked from ectopic activity in

damaged nerves, suggesting a role for these sodium channels in pain transmission [190]. L5 spinal nerve ligation can induce downregulation of two TTX-resistant sodium channels, SNS and NAN, and induce upregulation of alpha-III sodium channels in the L5 DRG [149, 191]. Treatment with GDNF suppressed alpha-III channel mRNA expression and partially restored SNS and NaN levels within the L5 DRG [149]. It therefore appears that ectopic activity in nociceptive fibers is TTX-sensitive, with TTX-sensitive alpha-III channels increasing with nerve injury and GDNF treatment prevents this upregulation.

Many IB4 binding neurons, those GDNF-sensitive, nonpeptidergic neurons, coexpress somatostatin. Somatostatin is released from the dorsal spinal cord in response to noxious stimuli and can inhibit the pronociceptive neuropeptides substance P and CGRP [192]. Adler *et al* investigated if somatostatin might contribute to the anti-allodynic mechanism of GDNF. Following intrathecal infusion of GDNF in a spared nerve injury model, after mechanical hyperalgesia was reversed, prosomatostatin was significantly increased in ganglia compared to control animals. This effect was not seen for other neuropeptides, including CGRP [189]. *In vitro* work confirmed these results; dissociated DRG neurons which were exposed to exogenous GDNF showed a threefold higher somatostatin content compared to NGF-treated neurons [189].

Two final mechanisms in which GDNF may exert analgesic effects are decreased Aβ sprouting and axonal regeneration. As discussed earlier, there are two subtypes of nociceptive C-fibers: peptidergic, which are NGF responsive, and nonpeptidergic, which bind IB4 and are GDNF responsive. Following sciatic nerve axotomy, the nonpeptidergic neuron population decreased from approximately 40% to <20% and intrathecal GDNF application was able to rescue the neuronal cell population within the DRG [193]. Myelinated A-fibers (mechanoreceptors) usually terminate within lamina I of the spinal cord with some terminations

in IIi lamina, but even fewer in lamina IIo. Following axotomy, terminations increased throughout lamina II and a more intense staining in lamina I. These changes were associated with increased Aβ-fiber sprouting [193, 194]. However, GDNF administration resulted in prevention of the Aβ fiber sprouting [193]. And finally, chronic intrathecal administration of GDNF following dorsal root injury can cause sensory axons to regrow back into the dorsal horn of the spinal cord and form functional synapses. These synapses result in a functional rescue of sensory nerve fibers [195]. All these results, taken together, show that GDNF is a powerful modulator of pain sensation, usually one in the process of antinociception, and could be used as a therapeutic target for chronic neuropathic pain. However, to this point, the only clinical trials testing GDNF have focused on Parkinson's disease and amyotrophic lateral sclerosis, not on painful neuropathies.

Inflammation

Obesity rates within the last 50 years have risen enormously. Within the United States, approximately 30% of the adult population is considered obese [196]. Increases in obesity rates have been linked to increased energy intake and reduced physical activity, and the rise in obesity is closely associated with prediabetes and type 2 diabetes, cardiovascular disease, neurodegeneration, biliary disease, and some cancers [197]. Importantly, inflammation is linked to not only obesity, but also insulin resistance and diabetes. Furthermore, this inflammatory component of diabetes has also been identified as a key mediator in the progression of diabetic complications, including nephropathy, retinopathy, and neuropathy [198]. Diabetes is associated with a state of chronic, low-grade inflammation which can be aggravated by hyperglycemia [199]. Moreover, chronic activation of intracellular proinflammatory pathways can lead to obesity-related insulin resistance and diabetes. Cytokines like interleukin (IL)-1β, IL-6, CCL2,

and TNFα can be released by both adipocytes and macrophages [200-202] and elevated levels of these proinflammatory cytokines have been identified in patients with insulin resistance and diabetes [203].

Adipose tissue is an important initiator in the inflammatory response to obesity. Inflammation associated with obesity is caused by the consumption of excess nutrients and adipocytes maintain the inflammation by actively secreting hormones, cytokines and chemokines. Adipocytes secrete adipokines, such as leptin and adiponectin [200-202] which promote insulin sensitivity. Additionally, adipose tissue from both obese humans and mice show increased numbers of macrophages [204, 205]. In fact, some reports suggest that greater than 40% of the total adipose tissue content from obese humans and rodents is macrophages, while only 10% in lean patients [206]. Adipose macrophages are a major source of proinflammatory cytokines. Macrophage activation sets up a feed-forward process where activation leads to release of chemokines and cytokines, which leads to further activation of macrophages and increased cytokine release, propagating the inflammatory state. The proinflammatory cytokines, IL-6, IL-1β, and TNFα elicit activation of the Jun N-terminal kinase (JNK), inhibitor of κB kinase (IKK)β, and other serine kinases which stimulate inflammatory pathway genes concomitantly [207], and these serine kinases can phosphorylate the insulin receptor substrate and interfere with normal insulin action, thus creating a state of insulin resistance. In as much as inflammation contributes to diabetes, chronic hyperglycemia affects multiple pathways including the aldose reduction, advanced glycation end product, reactive oxygen species and protein kinase C pathways [208]. Activation of each of these pathways may result in the activation of proinflammatory cytokines that can activate the immune system, leading to pancreas, adipose and vasculature damage, resulting in diabetes complications [209].

While inflammation is known to play a role in diabetes, little is known about its role in diabetic neuropathy, especially in the setting of neuropathic pain. Many cytokines and chemokines have been implicated in neuropathic pain, with studies finding inflammatory mediators increased in the tissues and serum of both human and rodent models of diabetes. Of these cytokines and chemokines, interleukins IL-1β, IL-6 and IL-8, as well as TNFα, have been investigated in neuropathic conditions. Animal studies using STZ to induce DN in mice have demonstrated increased IL-6 mRNA levels in the DRG and sciatic nerve compared to non-diabetic controls [210, 211]. In a clinical study investigating nondiabetic small-fiber neuropathy, researchers demonstrated a two-fold increase in IL-2 mRNA levels in peripheral blood compared to control subjects and in skin samples showed increases in the proinflammatory cytokines IL-6 and IL-8 [212]. Importantly, within neuropathic patients, investigators demonstrated that there were increases in gene expression of IL-1β, IL-6, and IL-8 mRNA in affected skin areas compared to unaffected skin areas [212]. More importantly however, in a clinical study investigating DN patients, sural nerve biopsies showed an increase in IL-6 protein expression [210], demonstrating that findings in rodent models are mirrored in human patients.

Tumor necrosis factor alpha (TNFα) is also a proinflammatory cytokine, much like the interleukins. TNFα has long been implicated in inflammatory and neuropathic pain conditions. Administration of TNFα in rodents can induce both thermal and mechanical hyperalgesia [213] as well as ectopic sensory neuron firing [214]. While TNFα is known to contribute to neuropathic pain, it has only recently been investigated in the role of diabetic neuropathy. Patients with diabetes show an increase in TNFα plasma protein content and an increase in mRNA levels compared to healthy controls [215-217]. Interestingly, increases in TNFα macrophage expression were correlated to pain intensity [215]. Animal studies have also

demonstrated an increase in circulating TNFα levels in type 1 diabetes models [218-221]. It appears that TNFα plays a vital role in the development of diabetic neuropathy due in part to a study where administration of TNFα into the sciatic nerve induced a reduction in motor nerve conduction velocity [64] and that TNFα-null diabetic mice fail to develop behavioral dysfunction and MNCV and SNCV slowing that is characteristic of DN [211]. Furthermore, using an anti-TNFα neutralizing antibody in STZ-injected diabetic rodents, serum TNFα levels and mRNA expression were returned to control levels, along with recovery of motor and sensory nerve conduction velocities, behavioral measures, as well as preventing epidermal nerve fiber loss [211].

As stated previously, NGF is known to play a significant role in mediating pain, especially inflammatory pain. The level of NGF has been shown to be elevated during inflammation [222, 223] and NGF administration produces hyperalgesia [138, 139] with anti-NGF attenuating inflammatory hyperalgesia [223-225]. Interestingly, increases in NGF levels can be induced to a substantial extent by IL-1β and TNF-α [226, 227]. When IL-1β is injected into the skin, it causes an increase in site specific NGF, while systemic administration of an IL-1-receptor antagonist is capable of reducing inflammatory hyperalgesia as well as the elevated levels of NGF [228]. TNF-α is an initiator of other cytokine cascades, including both IL-6 and IL-1β [229] when injected into the skin, results in cutaneous sensitivity and results in increased levels of IL-1β and NGF [230].

Finally, it has been suggested that cytokine levels may play a role in the development of painful versus insensate neuropathy. In animal studies, diabetic rodents exhibiting hypoalgesic behavior were found to have decreases in TNF-α DRG levels [231], while diabetic rodents displaying hyperalgesic behavior had increases in DRG TNF-α levels [211]. In support of these

findings, a clinical study involving patients with multiple neuropathy conditions, researchers determined that patients with painful neuropathy had greater levels of proinflammatory cytokines TNF-α and IL-2 compared to both control patients and those patients with painless neuropathy [217]. Notably, patients with painless neuropathy showed increased levels of the anti-inflammatory cytokine IL-10 compared to control patients [217]. Taken all together, these data imply that obesity and diabetes can result in a chronic low grade inflammation, leading to TNF-α and IL-1β proinflammatory cytokine release and downstream NGF release, resulting in peripheral pain.

Study Significance

Obesity, prediabetes and diabetes are major socio-economic issues in America today. Obesity, prediabetes and type 2 diabetes no longer affect only adults, but within the last decade, children are increasingly developing these metabolic disorders. Diabetic neuropathy, a complication from prediabetes and diabetes, is a physical and emotional burden, especially with many patients unable to obtain relief from their symptoms. Current treatment options for diabetic neuropathy, and more specifically painful neuropathy, are rarely fully effective and come with a multitude of side effects. Because the current treatment options are not fully effective, there remains a great need to improve upon and find new treatments to provide patients with not only symptomatic relief, but also reversal of the neuronal dying back phenotype.

Exercise has recently been shown to be effective in reducing pain sensitivity in both animal models and human patients with neuropathic pain. There are many avenues of investigation into the mechanism of exercise-induced analgesia, including opioid activation and modulation of neurotrophins. Diabetes is known to alter circulating levels of neurotrophins, as

well as cause a state of chronic low-grade inflammation. Meanwhile, exercise is known to alter dysfunctional neurotrophin levels, as well as reduce inflammation. It is therefore plausible to suggest that painful diabetic neuropathy might be the result of altered neurotrophin levels following inflammation and exercise is capable of restoring the neurotrophins. Yet most research in the field of diabetic neuropathy involves only type 1 and type 2 diabetes, with little research into prediabetes, though this field is increasing. The overall goal of this body of work is to investigate if voluntary running wheel exercise is capable of reversing behavioral aspects of diabetic neuropathy in an effort to improve clinical treatment of diabetic neuropathy in conjunction with current pharmacological treatment options. In particular, these studies were aimed at uncovering the mechanism of exercise-induced analgesia on diabetic and prediabetic neuropathy in hopes of providing new therapeutic targets, which include not only exercise, but the pathways which exercise activate to alleviate painful neuropathy.

The goal of the first study ("Ob/Ob Mice, A Model of Type 2 Diabetes, and the Effects of Voluntary Exercise") was to investigate how voluntary wheel running exercise affects the development of painful diabetic neuropathy in an obese, type 2 diabetic, *ob/ob* mouse model compared to sedentary diabetic controls. We discovered that *ob/ob* diabetic mice develop mechanical allodynia and will not voluntarily run, even when exposed early in life. We were able to show that because of the lack of exercise, the "exercised" obese animals did not gain any significant reversal in mechanical hypersensitivity. Additionally, we learned that sedentary diabetic mice displayed decreased GDNF protein levels in the hind paw skin compared to nondiabetic controls and this was not altered in "exercised" diabetic mice because there was no physical activity displayed by the "exercised" mice.

In the second study ("Exercise-Mediated Improvements In Painful Neuropathy Associated With Pre-Diabetes In Mice"), we investigated a high-fat diet model of prediabetes and the effect of exercise on metabolic parameters, behavioral sensitivities, and neurotrophin levels. In this study, we discovered that a high-fat diet alone can induce metabolic parameters of prediabetes as well as mechanical and visceral hypersensitivity. Exercise delayed but did not prevent the onset of many metabolic parameters and reversed the mechanical and visceral hypersensitivities. We also discovered that NGF levels, in conjunction with TrkA nerve fibers, were increased in high-fat sedentary animals, leading to allodynia, but exercise normalized both the NGF and TrkA nerve fiber counts in correlation with behavioral measures.

The goal of the final study ("Administration of Anti-Nerve Growth Factor Antibody Attenuates Mechanical Allodynia in High-Fat Diet Induced Pre-Diabetes") was to investigate a mechanism in which exercise could be exhibiting analgesic effects. In this final study, we used an anti-NGF antibody systemically to determine if preventing NGF signaling was analgesic. We discovered that animals fed a high-fat diet were again prediabetic and displayed mechanical hyperalgesia through 5 weeks of feeding. Additionally, treatment with anti-NGF resulted in a reversal of mechanical allodynia, as well as a normalization in TrkA nerve fiber density, which was increased in the high-fat fed control IgG injected group. This study gave a mechanism in which exercise could be analgesic; prediabetes causes an excess of NGF and TrkA fiber densities, resulting in diabetic neuropathy, and exercise acts to lower NGF levels and normalize nerve fiber densities.

Overall, this body of work significantly contributes to the literature regarding the treatment of painful diabetic neuropathy. Additionally, it is possible that these findings could change the way the clinic diagnoses painful versus insensate neuropathy by staining skin

biopsies for TrkA in addition to PGP9.5. Because treatment options for diabetic neuropathy are not fully effective and come with a wide range of side effects, our results confirm that prescribing exercise to those patients suffering from painful neuropathy could be highly beneficial in treating their pain. Additionally, our results could offer insight into a new treatment plan for patients, one that includes exercise and a drug for diminishing NGF signaling.

CHAPTER 2

Ob/Ob Mice, A Model of Type 2 Diabetes, and the Effects of Voluntary Exercise

Abstract

With a lack of effective therapies for the treatment of painful diabetic neuropathy, recent research suggests that exercise may be beneficial in not only remediating metabolic abnormalities associated with type 2 diabetes, including increased weight gain and blood glucose levels, but may also be effective in providing attenuation of painful neuropathy symptoms. Additionally, research suggests diabetic neuropathy may be the result of decreased neurotrophin signaling and alterations in the distribution of neurotrophins, both centrally and peripherally. We hypothesized that type 2 diabetes results in a downregulation of neurotrophins, specifically glial cell line-derived neurotrophic factor (GDNF), during periods of mechanical allodynia and that exercise is beneficial by increasing GDNF production. Here, we investigated if obese, diabetic *ob/ob* mice receive benefits from voluntary running wheel exercise. O*b/ob* mice were placed in cages with running wheels at 5 weeks of age, a time when *ob/ob* mice are just beginning to display signs of obesity. Additional *ob/ob* mice were placed in cages with no running wheels and serve as sedentary controls. Strain-matched non-diabetic mice were included to contain both exercised and sedentary groups. Both sedentary and exercised *ob/ob* mice develop diabetic neuropathy with symptoms similar to those of human type 2 diabetic patients, including increased weight gain, hyperglycemia, hyperinsulinemia and tactile mechanical allodynia. Our results suggest that obese, diabetic *ob/ob* mice do exercise, but at levels significantly below the non-obese control mice. Furthermore, our results show that diabetes results in a decrease in GDNF protein expression in the hind paw skin, but not in the dorsal root ganglion (DRG) or spinal cord. However, due to the lack of significant running distances, exercised *ob/ob* mice did not display any improvement in metabolic parameters, or mechanical withdrawal thresholds. There was also no significant increases in GDNF protein expression in the hind paw skin. These results suggest that diabetes does cause a reduction in neurotrophin production, which may be

responsible for the development of mechanical allodynia and without reaching some minimum amount of exercise, there is not enough beneficial effect to reverse metabolic and behavioral outcomes; it therefore appears that there may be an exercise threshold that must be met to obtain beneficial results.

Introduction

Type 2 diabetes is a growing concern for our society, with 95% of all diagnosed diabetes cases being type 2 [1]. Type 2 diabetes is associated with obesity, lack of physical exercise and impaired glucose uptake and utilization. Patients with type 2 diabetes are faced with the same complications associated with type 1 diabetes, which includes increased risk of heart disease and stroke, retinopathy, nephropathy, and neuropathy. Patients who have had diabetes for extended periods of time, as well as those patients who lack tight glucose control, are at increased risk of developing complications. The most common complication of diabetes is diabetic neuropathy (DN), specifically diabetic polyneuropathy, which results in symmetrical loss of nerve function beginning in the toes and progressing proximally, eventually affecting the hands and arms [7]. All sensory modalities can be affected, but recent studies show that the small, unmyelinated C-fibers and thinly myelinated fibers are the first nerve fibers injured. Injury of these fibers can lead to constant pain, tingling, and burning, as well as chronic numbness, oftentimes leading to foot ulcers and eventual amputation. One of the most distressing symptoms that patients suffer from is neuropathic pain and paresthesias [232], and these symptoms can last for years, drastically affecting quality of life [233]. Approximately 15% to 30% of patients suffer from painful DN, whereas the remaining patients experience loss of sensation [234]. Despite many patients suffering from painful neuropathy, the pain management for DN remains unsatisfactory. There is a clinical need for more effective strategies and therapeutic targets to meet the high demand of patients suffering from painful DN.

Painful DN is clinically hard to treat, with many patients not responding to traditional pharamacological approaches [235]. Interestingly, exercise has been recognized as an integral part of therapy for type 2 diabetic patients, often with the goal of improving metabolic risk

factors for the development of complications [236]. Additionally, exercise is prescribed to reduce hyperglycemia and body fat in type 2 patients as abdominal obesity is associated with insulin resistance, hyperinsulinemia, hyperglycemia, dyslipidemia, and hypertension [237]. While exercise is prescribed to improve metabolic aspects of diabetes, exercise has proven to be effective in reducing behavioral sensitivities associated with neuropathy in rodent models [84]. Furthermore, exercise has demonstrated the ability to prevent the onset of painful DN in patients without signs and symptoms of DN [90] and shown to improve self-reported pain scores [92].

One of the more prominent mechanisms believed to underlie diabetic neuropathy is growth factor deficiency [238]. It has recently been reported that diabetes can alter GDNF expression in various tissues [239, 240], and GDNF is known to play an integral role in nociceptive processing in neuropathic pain states [241-243]. Moreover, aerobic exercise can increase GDNF in the skeletal muscle of both healthy and spinal cord-injured animals. Likewise, GDNF treatment has established a role in analgesia, where both intrathecal and local administration of GDNF resulted in attenuation of nocifensive behaviors [149, 189]. In this study, we investigated whether exercise can attenuate painful diabetic neuropathy in a model of type 2 diabetes, the *ob/ob* mouse. We also examined the role of GDNF, which might play a role in attenuating behavioral measures.

Materials and Methods

Animals: 4 week-old *Ob/Ob* (B6.V-Lep^{ob}/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57Bl/6.J mice were used as nondiabetic control mice. The early age chosen was to try to establish a running paradigm before the severe obesity phenotype

manifested. All mice were maintained on a 12:12h light/dark cycle in the research support facility at the University of Kansas Medical Center. Mice were given *ad libitum* access to food and water. All animal use was in accordance with NIH guidelines and conformed to the principles specified by the University of Kansas Medical Center Animal Care and Use Protocol.

Metabolic Parameters: Body weight and blood glucose (glucose diagnostic reagents; Sigma, St. Louis, MO) levels were taken weekly for 9 weeks. Serum insulin (mouse insulin ELISA; Alpco, Salem, NH) was evaluated at the end of the study. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated with the following equation: (blood glucose (mg/dl) X (serum insulin (ng/mL))/405.

Voluntary Exercise: Beginning at 5 weeks of age, and following baseline testing, control and *ob/ob* mice were separated into either sedentary controls or exercise groups. Sedentary animals were housed two per cage (*ob/ob*, n=6, Sed-D; control, n=6, Sed-C). Exercise animals (*ob/ob*, n=5, Ex-D; control, n=6, Ex-C) were housed individually in cages designed to hold stainless-steel running wheels (Mini Mitter; Bend, OR) and given free access to run 24/7. VitalView (Mini Mitter) software measured total wheel revolutions for each mouse during the course of the study. Using wheel revolutions, running distance (in kilometers) was calculated using the equation:

$$Distance\ (km) = Total\ Wheel\ Revolutions \times 0.00036$$

Distance from each mouse was used to calculate the mean distance per animal per week, and mean distances were combined to calculate group means.

Mechanical Behavior Testing: Behavior was assessed weekly to distinguish signs of diabetic neuropathy. To test mechanical sensitivity, mice were allowed to acclimated to the

testing equipment in two individual sessions prior to the initial testing day. On the day of testing, mice were allowed to acclimate to the behavior testing room for 30 minutes, followed by a 30-minute acclimation to the testing equipment. Mice were placed in individual clear plastic cages (11x5x3.5cm) on a wire mesh table 55 cm above the table. von Frey monofilaments (find filaments used) were applied perpendicularly to the plantar surface of the hindpaw until the filament bent. Testing began with the 0.6 gram filament. If the animal withdrew its paw, the response was counted as a positive response, and the next lowest gram filament was applied to the hindpaw. If the animal did not withdraw their paw after a 5 second application, the next larger gram filament was applied. Filaments were applied until there was an initial change in response, followed by four additional filament applications. The 50% withdrawal threshold was calculated using the formula previously reported [244]:

$$50\%\ \text{g threshold} = (10[X_f + \kappa\delta])/10{,}000$$

Where X_f= value (in log units) of the final von Frey fiber used, κ= tabular value for the pattern of positive/negative responses and δ=mean difference (in log units) between stimuli.

Western Blotting: Immediately following sacrifice, L4-L6 spinal cord and dorsal root ganglion from both sides were dissected out, snap frozen in liquid nitrogen, and stored at -80° C. Frozen tissue samples were sonicated separately in Cell Extraction Buffer (Invitrogen; Carlsbad, CA) containing 55.55 ul/ml protease inhibitor cocktail, 200mM Na_3VO_4, and 200mM NaF. Following sonication, protein was extracted on ice for 30 minutes and vortexed every 10 minutes. Samples were then centrifuged at 12,000xg for 12 minutes. Following centrifugation, supernatant was measured with a Bradford assay (Bio-Rad, Hercules, CA). Samples were then boiled with Lane Marker Reducing Sample Buffer (Thermo Scientific, Waltham, MA) for 3 minutes. Equal amounts of protein were loaded per lane and the samples were separated on a 4-

20% gradient tris-glycine gel (Invitrogen) and then transferred to a nitrocellulose membrane. Membranes were then probed with the following primary antibodies: GDNF (1:2000, Santa Cruz Biotechnology; Santa Cruz, CA) and α-Tubulin (1:2000, Abcam; Cambridge, MA). Bands were visualized with anti-rabbit or anti-mouse HRP conjugate secondary antibodies (Santa Cruz) and ECL (Thermo Scientific Pierce; Waltham, MA). Densitometry with ImageJ (NIH) was then used to analyze each lane.

GDNF Protein Quantification: Immediately following sacrifice, hindpaw foot pads were removed, snap frozen in liquid nitrogen, and stored at -80° C. Frozen tissue samples were homogenized separately in lysis buffer (20mM Tris-HCL (pH 8.0), 137mM NaCl, 1% NP40, 1mM PMSF, 10% glycerol, 10 μg/mL aprotinin, 1μg/mL leupeptin, 0.5 mM sodium vanadate, and 4% Triton X-100), with homogenates centrifuged at 12,000xg for 15 minutes and supernatants collected. Total protein content was assayed using the Bradford methods (BioRad; Hercules, CA) and equal amounts of protein were loaded for each sample. GDNF protein was quantified using a commercially available ELISA kit (GDNF Emax ImmunoAssay System; Promega, Madison, WI). Briefly, a 96-well plate was coated with Anti-GDNF monoclonal antibody and incubated overnight at 4° C. Following a 1 hour blocking incubation, samples of mouse hindpaw homogenates were added at 30ug/well and incubated for 6 hours. The plate was then incubated with an anti-human GDNF polyclonal antibody overnight at 4° C, followed by incubation with anti-chicken IgY, HRP conjugate, and a color change was elicited with a TMB One solution followed by 1N hydrochloric acid to stop the reaction. The plate was read at 450nm.

Statistical Analysis: All data are presented as mean ± SEM. Data were analyzed using a two-factor ANOVA or repeated measures ANOVA. Post hoc analysis was run using Fisher's test

of Least Square Difference when appropriate. All statistics were run using SPSS Statistics 20. Statistical significance was defined as $p \leq 0.05$.

Results

Ob/Ob Mice Do Not Voluntarily Exercise Similar to Control Mice: Control and *ob/ob* mice were placed in voluntary running wheel cages at 5-weeks old. Initially, both groups of mice began to run, yet even within the first week, *ob/ob* mice ran statistically less than their control counterparts. Average daily distances can be seen in Figure 2.1. Over the course of the study, control mice ran significantly greater distances than *ob/ob* mice. Interestingly, both exercise groups decreased their physical activity over the course of the 9 week study, with Ex-C mice exhibiting a 48% decline and Ex-D mice exhibiting an 89% decline in average running distance.

Ob/Ob Mice Develop Type 2 Diabetes: Both Sed-D and Ex-D mice weigh significantly more than either of their control groups, beginning at baseline and continuing throughout the course of the 9-week study (Figure 2.2A). Sed-C mice gained an average of 44.7% body weight and Ex-C mice gained 30.1% body weight, while Sed-D mice gained an average of 105% body weight and Ex-D mice gaining 76.1% of their body weight. While Ex-D mice did not exercise equidistant to Ex-C mice, Ex-D mice did not gain as much weight over the course of the study, with significant differences beginning at 6 weeks and continuing for the length of the study.

Sed-D and Ex-D mice both had elevated blood glucose levels in comparison to their controls, yet Sed-D mice were only significantly different from Sed-C mice at week 1 (Figure 2.2B). Beginning at week 1 and continuing for the next 9 weeks, Ex-D mice had significantly elevated blood glucose levels compared to Ex-C mice and interestingly, Sed-D mice. Over the course of 9 weeks, Sed-C average glucose level was 142.7±8.5 mg/dL, Ex-C average glucose

was 139.5±7.9 mg/dL, Sed-D average glucose was 189.9±18.6 mg/dL and Ex-D average blood glucose was 256.6±47.4 mg/dL. Additionally, at the end of 9 weeks, both Sed-D and Ex-D mice showed significantly elevated serum insulin levels a common diagnostic measure for type 2 diabetes, in comparison to Sed-C and Ex-C groups, respectively (Figure 2.2C). Sed-C serum insulin levels measured 1.14±0.18 ng/mL, Ex-C insulin was 0.82±.13 ng/mL, Sed-D serum insulin was 37.21±8.87 ng/mL and Ex-D insulin levels were at 24.02±8.93 ng/mL. While exercise appeared to decrease serum insulin levels in the *ob/ob* mice, the decrease was not statistically significant. Finally, the HOMA-IR, a measure of insulin resistance, was calculated from week 9 fasting glucose and fasting serum insulin levels. Sed-D and Ex-D mice had significantly higher HOMA-IR as compared to Sed-C and Ex-C mice (Sed-C, 0.277±.0037 vs Sed-D, 16.206±4.125; Ex-C, 0.354±0.069 vs Ex-D, 10.792±1.308).

Ob/Ob Mice Develop Mechanical Allodynia: Cutaneous mechanical sensitivity was measured to determine if *ob/ob* mice develop nociceptive diabetic neuropathy. Baseline measurements showed no significant differences in withdrawal thresholds between any of the groups. By 6 weeks, Sed-D displayed a significantly lower withdrawal threshold compared to the Sed-C group (Figure 2.3A). This decreased withdrawal threshold continued through the rest of the study. Ex-D mice also developed mechanical allodynia, indicated by a lowered withdrawal threshold, but did not reach statistical significance until 7 and 9 weeks (Figure 2.3A). However, because Ex-D mice did not exercise to any great extent, if the mechanical withdrawal of the control mice and the *ob/ob* diabetic mice are combined, we again see a statistically significant lowered threshold, indicating a neuropathy associated with allodynia (Figure 2.3B).

Figure 2.1: ***Average Weekly Running Distance:*** The average weekly distance run by both control and diabetic animals are presented in table **(A)** and graph (**B**) form. The datum presented in the table illustrates that diabetic *ob/ob* mice did not voluntarily exercise, sometimes not reaching one kilometer running in a week's time span. All data are presented at mean ± SEM. ***P*<0.01, ****P*<0.001,*****P*<0.0001.

A

Average Weekly Distance Ran (km)									
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**	**Week 7**	**Week 8**	**Week 9**
Control	6.66±0.50	7.23±0.56	4.88±0.87	4.00±0.92	4.16±1.00	4.52±0.59	3.47±0.61	3.34±0.61	3.49±0.32
Ob/Ob	1.11±0.56	0.53±0.32	0.33±0.21	0.25±0.15	0.20±0.12	0.19±0.11	0.15±0.09	0.10±0.06	0.12±0.09

B

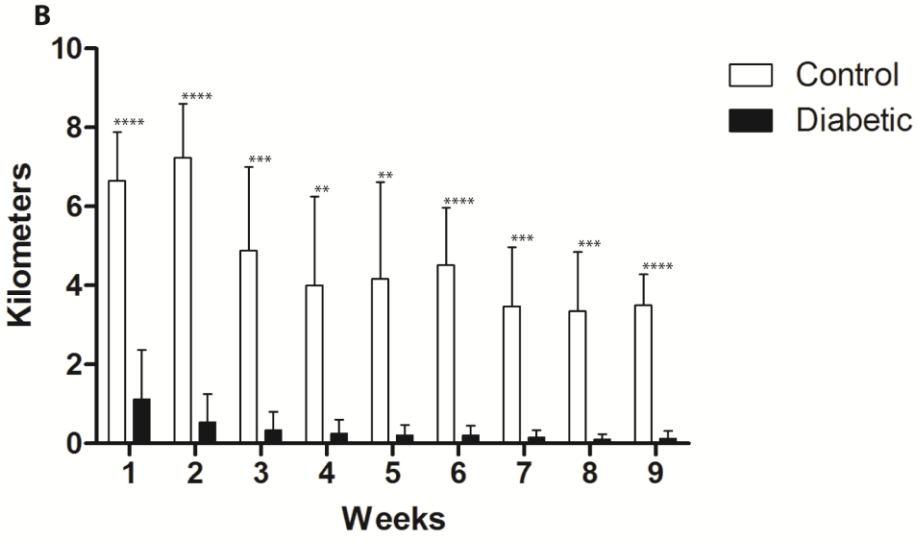


Figure 2.2: ***Ob/Ob Mice Develop Classical Type 2 Diabetes Symptoms:*** (A) The *ob/ob* genotype causes increased weight gain in both the Sed-D (n=6) and Ex-D (n=5) groups in comparison to their controls (Sed-C, n=6; Ex-C, n=6). (B) Glucose levels are elevated in both Sed-D and Ex-D mice, yet Ex-D display significantly increased glucose levels even in comparison to Sed-D mice. (C) Serum insulin levels are dramatically increased in both the sedentary and exercise diabetic mouse groups at the end of 9 weeks. (D) HOMA-IR, a measure of insulin resistance, in significantly increased in both Sed-D and Ex-D groups compared to Sed-C and Ex-C groups, respectively. All data are presented is presented as mean ± SEM. *Sed-C vs Sed-D; #Ex-C vs Ex-D; +Sed-D vs Ex-D. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

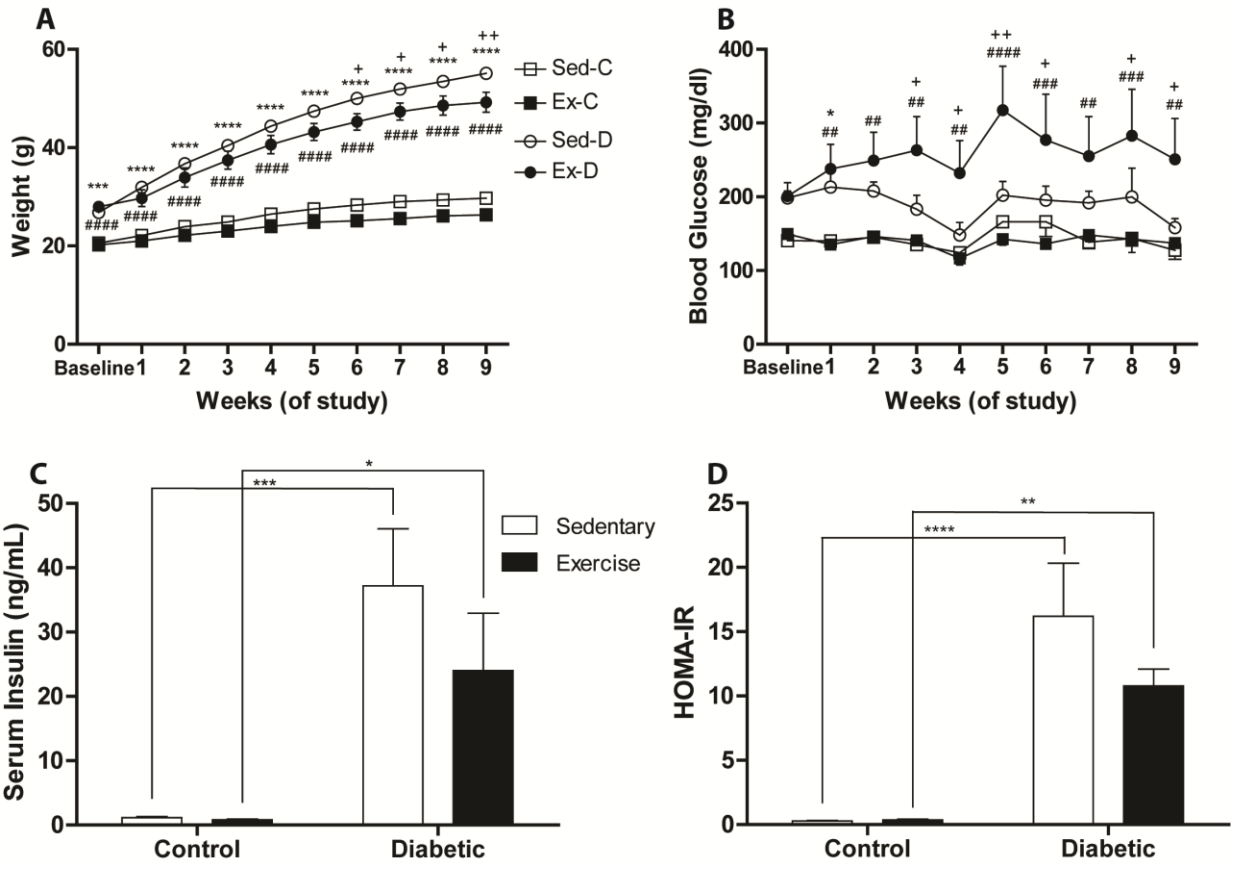
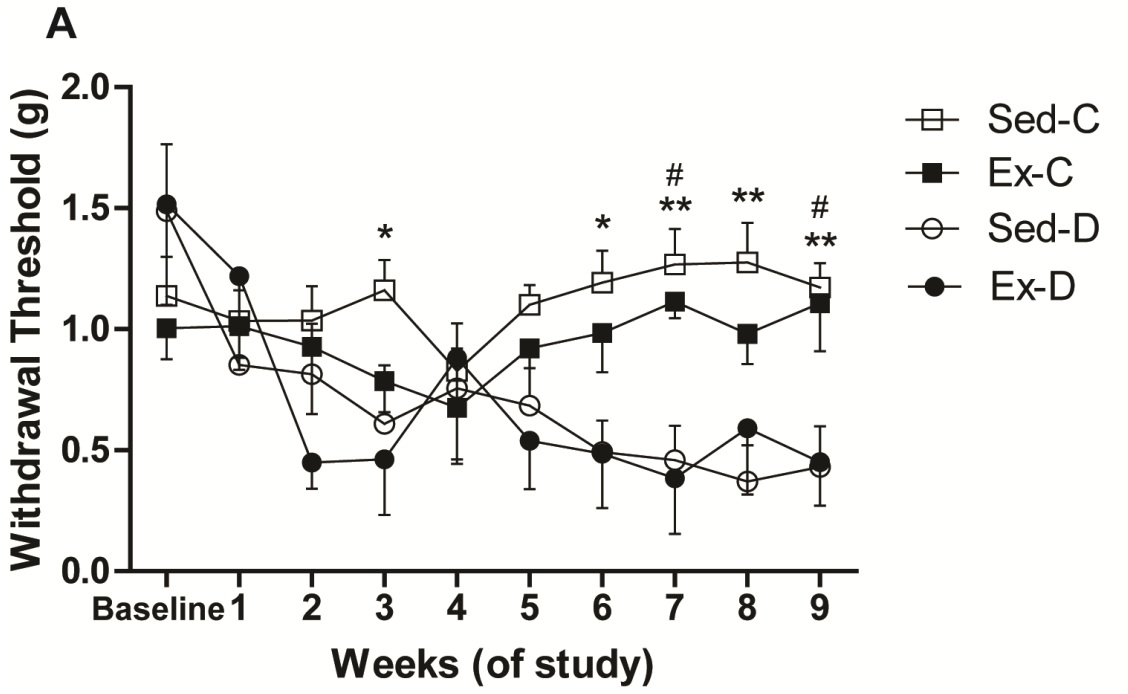
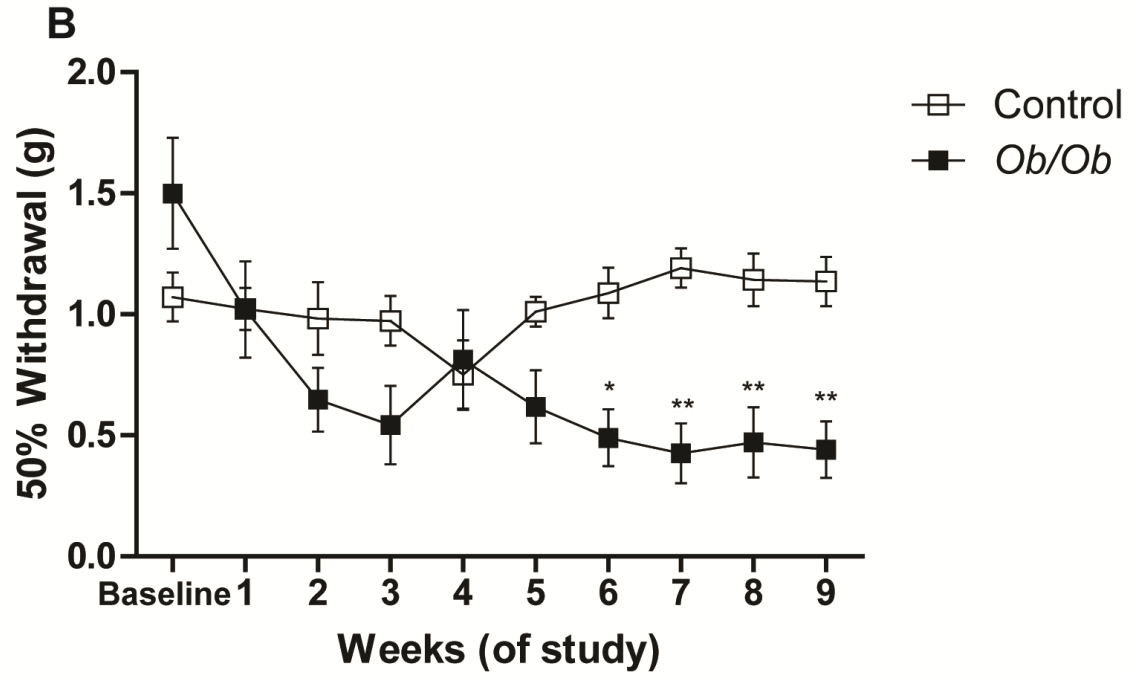

A
Weight (g)
Weeks (of study)
Baseline
Sed-C
Ex-C
Sed-D
Ex-D
B
Blood Glucose (mg/dl)
Weeks (of study)
C
Serum Insulin (ng/mL)
Sedentary
Exercise
Control
Diabetic
D
HOMA-IR
Control
Diabetic

Figure 2.3: ***Ob/Ob, Type 2 Diabetic Mice Develop Mechanical Allodynia:*** Both sedentary and exercise diabetic animals develop mechanical allodynia when assessed using von Frey monofilament testing. (A) Sed-D develop mechanical allodynia beginning at 6 weeks and continuing on through the end of the study. Ex-D mice display mechanical allodynia at week 7 and week 9. (B) When behavior is grouped into control and *ob/ob* groups, there is a significant development of mechanical allodynia beginning at 6 weeks running to the end of the study. Mice were grouped together due to no change in behavior of control mice and because *ob/ob* mice did not run to a great extent. Data are presented at mean ± SEM. *Sed-C vs Sed-D; #Ex-C vs Ex-D; +Sed-D vs Ex-D. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.


A
Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
Baseline 1 2 3 4 5 6 7 8 9
Weeks (of study)
Sed-C
Ex-C
Sed-D
Ex-D
*
*
#
**
**
#
**


B
50% Withdrawal (g)
2.0
1.5
1.0
0.5
0.0
Baseline 1 2 3 4 5 6 7 8 9
Weeks (of study)
Control
Ob/Ob
*
**
**
**

Mechanical Sensitivity is Correlated with Physical Activity in Diabetic Mice: While *ob/ob* mice as a group did not voluntarily exercise to any great extent, individual mice did run different distances. In Ex-D mice, there is a significant correlation between individual running distances and individual withdrawal thresholds (R^2=0.798, *P*=0.041), where greater running distances is correlated with increased mechanical withdrawal thresholds (Figure 2.4A). In Ex-C mice, there is not a statistically significant correlation between running distance and mechanical withdrawal threshold (Figure 2.4B).

GDNF Protein Levels Are Altered in the Periphery by Diabetes: We next wanted to investigate if diabetes can alter GDNF protein expression in neural and non-neuronal tissues and if exercise had any role in regulating GDNF protein expression. Our western blot data demonstrates that diabetes did not alter GDNF protein expression in either the dorsal root ganglia (Figure 2.5A) or the spinal cord (Figure 2.5B). We then investigated GDNF protein levels via an ELISA in the footpad skin. Our results demonstrate that GDNF protein is decreased in Sed-D hindpaw skin compared to Sed-C skin (Figure 2.6), with concentrations of 47.5±19.7 pg/mL and 107.1±15.5 pg/mL, respectively (*P*=0.024). However, there were no significant differences between Ex-C and Ex-D mouse groups (81.9±20.8 vs 65.7±9.3 pg/mL, *P*=0.533) or between Sed-D and Ex-D groups (47.5±19.7 vs 65.7±9.3 pg/mL, *P*=0.484).

Discussion

Using o*b/ob* mice as a model of type 2 diabetes associated with obesity, we demonstrate that these mice develop cutaneous mechanical allodynia and decreased GDNF protein levels in the hindpaw skin. However, our results also reveal that the small amount of exercise in the *ob/ob* diabetic mice was not capable of reversing either the mechanical allodynia or the GDNF protein

Figure 2.4: ***Running Distance Positively Correlates with Mechanical Withdrawal Threshold:*** (A) Ex-D mouse mechanical withdrawal thresholds are positively correlated with the distance ran. Some Ex-D mice ran more than the average mouse, and those animals display higher withdrawal thresholds (n=5). (B) There is no correlation in the Ex-C group with distance ran versus withdrawal thresholds (n=6).

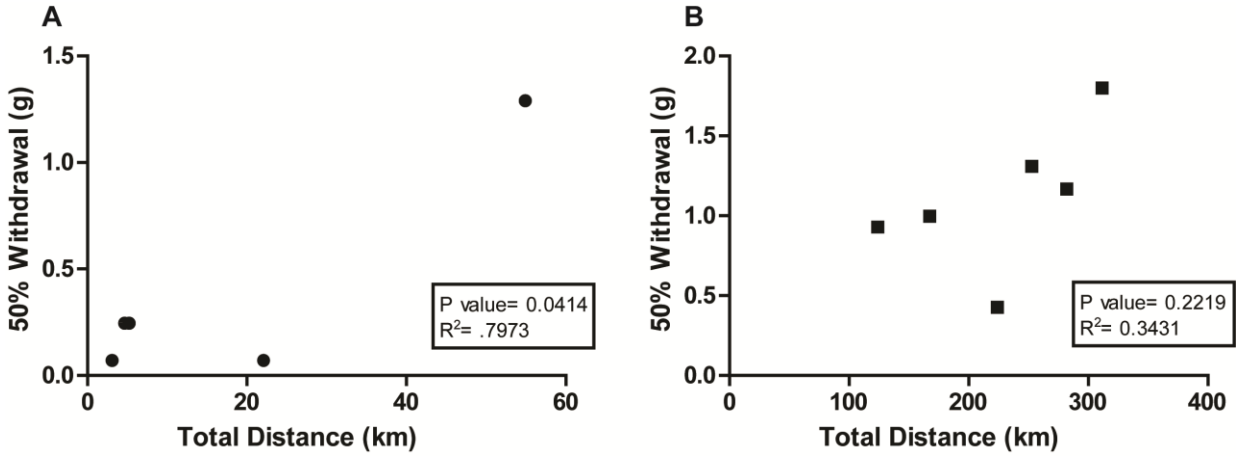

A
50% Withdrawal (g)
1.5
1.0
0.5
0.0
0
20
40
60
Total Distance (km)
P value= 0.0414
R2= .7973
B
50% Withdrawal (g)
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
Total Distance (km)
P value= 0.2219
R2= 0.3431

Figure 2.5: ***Representative Western Blots of GDNF in the DRG and Spinal Cord*****:** Protein was harvested from dorsal root ganglia and L4-L6 spinal cord from Sed-C (n=6), Ex-C (n=6), Sed-D (n=6) and Ex-D mice (n=5). (A) Western blot data from the harvested DRG show that diabetes had no effect on GDNF protein. Additionally, exercise did not change the GDNF content either. (B) Similar to the DRG, spinal cord protein extracts did not show any alteration in GDNF protein content following either diabetes or exercise.

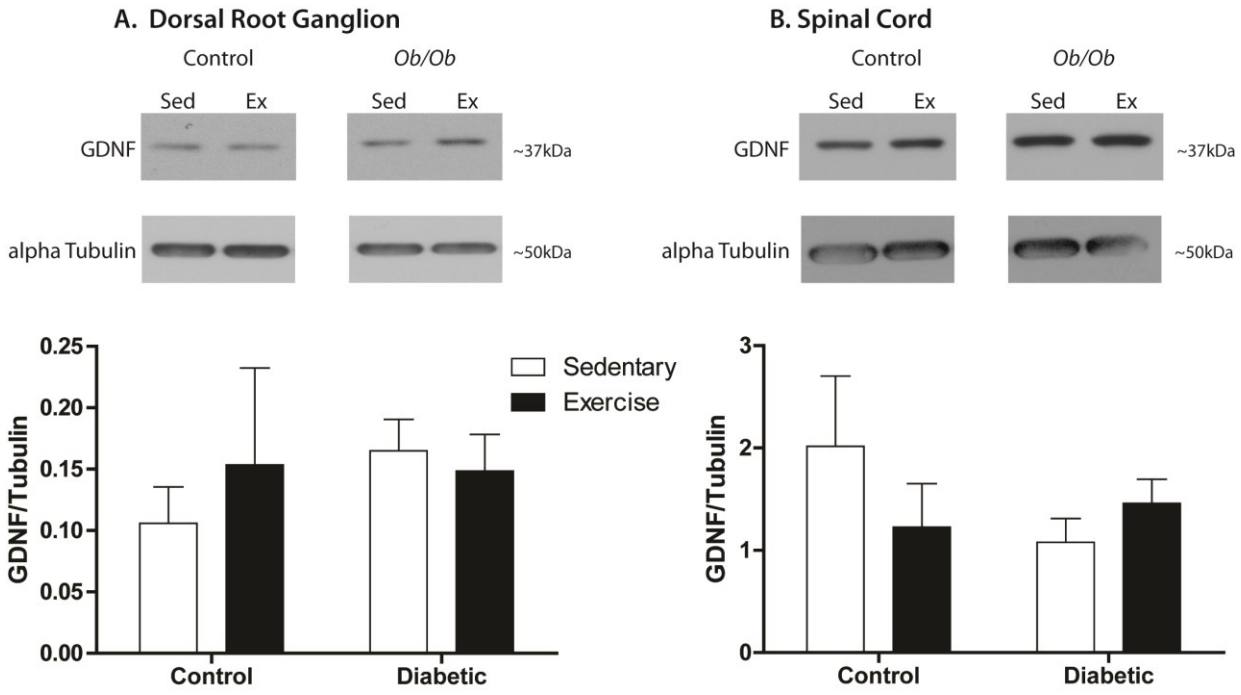
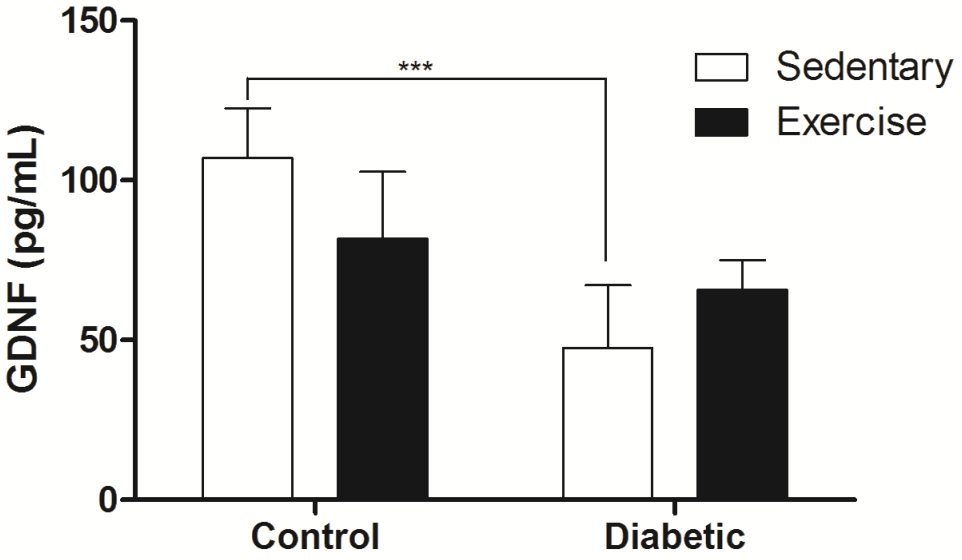

A. Dorsal Root Ganglion
Control
Ob/Ob
Sed
Ex
Sed
Ex
GDNF
~37kDa
alpha Tubulin
~50kDa
B. Spinal Cord
Control
Ob/Ob
Sed
Ex
Sed
Ex
GDNF
~37kDa
alpha Tubulin
~50kDa
GDNF/Tubulin
0.25
0.20
0.15
0.10
0.05
0.00
Sedentary
Exercise
Control
Diabetic
GDNF/Tubulin
3
2
1
0
Control
Diabetic

Figure 2.6: ***GDNF Protein Levels are Decreased in the Hindpaw Skin Due to Diabetes*****:** After harvesting protein from the hindpaw skin of each mouse, an Enzyme-Linked Immunosorbent Assay (ELISA) for GDNF was ran to assess total GDNF content. GDNF content was decreased in the skin of Sed-D mice after 9 weeks. Although it appears that GDNF content was decreased in the Ex-D group as well, there were no significant differences from the Ex-C group. Data are presented at means ± SEM where n=5-6 per group. ***$P<0.001$.


150
100
50
0
GDNF (pg/mL)
***
Sedentary
Exercise
Control
Diabetic

deficits. These findings demonstrate that there is a threshold of exercise needed to elicit beneficial results associated with neuropathy and neurotrophin deficits.

Control C57Bl/6 mice run voluntarily when given free access to running wheels. Our results show that *ob/ob* mice do not exercise to the same extent and run significantly fewer kilometers compared to control mice, even when started at a young age. The most likely explanation for the lack of exercise in the *ob/ob* mouse strain involves the reward systems in the brain. Food, particularly those high in sugar and fat, are potent rewards [245]. Dopamine and leptin are both implicated in the rewarding effects of food; food has been shown to result in dopamine release in the brain [246] and patients with leptin deficiency (like *ob/ob* mice) show activation of dopamine release to visual food stimuli alone [247]. Additionally, exercise is known to activate the reward centers of the brain, including dopamine [248-250]. Interestingly however, it has been shown that decreases in striatal dopamine 2 receptors (D2R) are linked to compulsive food intake in obese rodents [251] and that decreases in D2R-related signaling may reduce the sensitivity to natural rewards, like exercise [252]. It is therefore likely that the decreased leptin in the *ob/ob* mice leads to overeating, which causes an overactive reward response to food, which is diminishing the exercise reward. This is the most plausible explanation for the lack of exercise in the *ob/ob* mouse strain and the reason that our mice did not run in the experiment.

Leptin-deficient *ob/ob* mice develop increased weight gain, hyperinsulinemia, elevated blood glucose levels and are insulin resistant. Due to the lack of exercise in the current study, none of these metabolic parameters were improved in the Ex-D group compared to the Sed-D group. In fact, in the current study, exercised diabetic mice had significantly elevated blood glucose levels compared to both exercise control mice, as well as sedentary diabetic mice. One

reason for the increase in blood glucose levels in Ex-D animals may be due to increased glycogen stores in the muscle, adipose and liver. The small amount of exercise may increase GLUT4 transport to the membrane resulting in increased muscle uptake of glucose [253] and therefore glycogen stores. It is plausible that Ex-D mice have greater glycogen stores to pull from when in the fasting state and therefore maintain significantly elevated blood glucose levels compared to Sed-D mice. It should be noted however, that even though Sed-D mice do not have markedly increased blood glucose levels, by study's end, they are severely insulin resistant, another hallmark of type 2 diabetes.

Our results are consistent with previous studies, including the first to characterize the *ob/ob* mouse as a model of diabetic neuropathy, with our mice developing cutaneous mechanical allodynia beginning at 6 weeks of study, or at 11 weeks old [74]. While we did not investigate into great depth the extent of diabetic neuropathy in the current study, the *ob/ob* mouse line has been reported to show characteristic diabetic neuropathy symptoms seen in human patients. *Ob/Ob* mice develop motor and sensory nerve conduction velocity deficits in the sciatic nerve at 11 weeks old, the same time in which we see changes in mechanical sensitivity. In addition to mechanical sensitivity, diabetic *ob/ob* mice develop thermal hypoalgesia, or a loss of thermal sensation, at the same time. Another characteristic sign of diabetic neuropathy is loss of intraepidermal nerve fibers (IENF), where the *ob/ob* mouse line has been reported to result in a 78% reduction in IENF density compared to nondiabetic controls [74]. All of these combined characteristics result in a mouse model of type 2 diabetes with peripheral neuropathy, including cutaneous hypersensitivity, as confirmed by our study.

An important aspect of the current study was to investigate if exercise was capable of reversing painful diabetic neuropathy in a type 2 diabetes model. Based on our results, we cannot

conclude that exercise is effective in reversing or alleviating cutaneous allodynia, since the Ex-D group did not exercise to any appreciable extent. It would be interesting to examine the other symptoms of neuropathy to establish if any exercise at all is capable of reversing neuropathy or if there is an "exercise threshold" which must be met to see reversal of neuropathy symptoms. Low intensity bouts of exercise have been shown to reverse chronic myalgias in rats with treadmill walking [83], and bouts of lower intensity exercise with treadmill walking were also able to reverse nocifensive behaviors via nerve ligation, albeit slower than with high intensity exercise [103]. Importantly in our study, when individual mouse running distance is plotted against withdrawal threshold there is a positive correlation. One shortcoming of this observation, however, is the small study group size. More data need to be collected on this to determine if there is in fact a positive correlation to running distance and cutaneous sensitivity reversal. Yet even with a small study group, these data seem to suggest that there is an "exercise threshold" that needs to be met to elicit positive results, and this threshold might not be unattainable to many patients.

Another central question in the current study is whether diabetes can change neurotrophin expression and if exercise can ameliorate said changes. One common etiology of diabetic neuropathy is loss of neurotrophic support [69]. Lowered GDNF levels have been shown to correlate with diabetic neuropathy symptoms in type 1 rats, and GDNF gene replacement is capable of alleviating the neuropathy [254]. While we observed no changes in neural tissues, our results are consistent with findings that GDNF is decreased in the skin in diabetic patients [255]. However, our data did not support a recovery in GDNF levels following exercise, most likely due to a minimal exercise regimen by the Ex-D group. GDNF is known to be a potent analgesic in neuropathic pain states [149, 189, 241] whose analgesic mechanisms may include survival of

nonpeptidergic C-fibers and decrease of Aβ-fiber sprouting [193], regulating sodium channel and neuropeptide expression [149, 189]. Therefore, loss of GDNF in the periphery may result in decreased trophic support and loss of C-fibers, leading to excess fiber sprouting and dysregulation of sodium channels and neuropeptides, leading to mechanical allodynia. The effects of exercise may reverse the decrease in GDNF in the periphery, but the current study needs larger study groups to be able to examine the effects of exercise. Since it is known that exercise can increase GDNF levels [256-258], it is likely that in a large enough sample size, exercise could not only reverse the GDNF protein decreases following diabetes, but also reverse the cutaneous mechanical allodynia.

The current study demonstrates that obese, type 2 diabetic *ob/ob* mice develop diabetic neuropathy beginning around 11 weeks old. The data also suggest that a possible mechanism of the cutaneous mechanical allodynia is due to decreases in GDNF protein levels in the hind paw skin, possibly resulting in neuronal dysfunction. Further studies need to be completed to increase the study subject number, as well as to investigate the role that exercise might play in reversing allodynia and GDNF dysfunction. This study lays the foundation for examining if exercise is capable of reversing not only metabolic abnormalities in obese, type 2 diabetic patients, but if it is also capable of alleviating painful neuropathy symptoms by increasing GDNF protein levels in the periphery.

CHAPTER 3

Exercise-Mediated Improvements In Painful Neuropathy Associated With Pre-Diabetes In Mice

Abstract

Recent research suggests that exercise can be effective in reducing hyperalgesia in animals and humans with neuropathic pain. To investigate mechanisms in which exercise may improve hyperalgesia associated with prediabetes, C57Bl/6 mice were fed either standard chow or a high-fat diet for 12 weeks and were provided access to running wheels (exercised) or without access (sedentary). The high-fat diet induced a number of prediabetic symptoms, including increased weight, blood glucose, and insulin levels. Exercise reduced but did not restore these metabolic abnormalities to normal levels. In addition, mice fed a high-fat diet developed significant cutaneous and visceral hyperalgesia, similar to mice that develop neuropathy associated with diabetes. Finally, a high-fat diet significantly modulated neurotrophin protein expression in peripheral tissues and altered the composition of epidermal innervation. Over time, exercise normalized behavioral hypersensitivity, neurotrophin levels, and epidermal innervation. These results confirm that elevated hypersensitivity and associated neuropathic changes can be induced by a high-fat diet and exercise may alleviate these neuropathic symptoms. These findings suggest that exercise intervention could significantly improve aspects of neuropathy and pain associated with obesity and diabetes. Additionally, this work could potentially help clinicians determine those patients that will develop painful versus insensate neuropathy using intraepidermal nerve fiber quantification.

Introduction

Diabetic neuropathy (DN) occurs in up to 60-70% of diabetes patients. Distal symmetric DN is the most common neuropathy associated with diabetes and may present with either positive (pain, burning or tingling), or negative symptoms (numbness or altered proprioception) [1]. Neuropathy symptoms can precede diagnosis of diabetes and may develop in the initial stages of glucose dysregulation, or prediabetes [259, 260], with prediabetes being defined as impaired fasting glucose and/or impaired glucose tolerance [261]. While neuropathy associated with prediabetes is usually less severe than neuropathy in overt diabetic patients [24], it is still a devastating complication of the disease. Unfortunately, painful symptoms are the predominant feature in prediabetes patients. Current treatment options for patients with painful diabetic neuropathy (PDN) are rarely effective and less than 30% of patients achieve satisfactory pain relief [262].

Recent studies have demonstrated that cutaneous nerve growth factor (NGF) is increased in hindpaw skin of rodent models of type 1 and type 2 diabetes [263, 264]. It has been suggested that increased NGF may play a significant role in the development of PDN. Additionally, NGF is known to be critical in the development and maintenance of chronic pain, especially inflammatory pain [138, 265]. In fact, cutaneous injections of NGF result in thermal and mechanical hyperalgesia in both animals and humans [140]. Besides NGF, additional neurotrophins that play a role in pain sensation include brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). Like NGF, BDNF is believed to play a role in the development and maintenance of pain states, as BDNF is upregulated in the dorsal root ganglion (DRG) in inflammatory conditions and in models of neuropathic pain [266]. Furthermore, delivery of antibodies against BDNF reduced pain-related behaviors in rat [178]

and mouse [177]. While NGF and BDNF are important for the development and maintenance of pain, GDNF is believed to play an antinociceptive role. Previous studies have shown that administration of exogenous GDNF results in analgesia in various models of neuropathic pain [149, 189].

Exercise training has long been suggested to reduce pain and improve functional outcomes [84, 103]. In fact, a recent study demonstrated that aerobic and strength training had positive effects on diabetic peripheral neuropathy patients, improving pain and other neuropathic symptoms [92]. Furthermore, studies have shown exercise increases neurotrophin expression [113, 267, 268] and increased neurotrophins can promote neuronal health [114, 269, 270]. Additionally, these exercise-induced neurotrophin alterations are associated with analgesia [120]. Previous studies have begun to investigate potential therapeutic roles of neurotrophins in diabetic neuropathy using type 1 and type 2 models [68, 264, 271]. In the current study, we investigated how a high-fat diet alters metabolic and neural features normally associated with DN, as well as how exercise modulates these neuropathy phenotypes. We demonstrate that a high-fat diet induces mechanical allodynia and visceral hyperalgesia, and that exercise reverses these behavioral changes. The exercise-induced modulation of behavior was associated with normalization of neurotrophin levels and epidermal fiber density.

Materials and Methods

Animals and Diet: Seven week-old male C57Bl/6 mice were purchased from Charles River (Wilmington, Mass) and maintained on a 12:12h light/dark cycle in the research support facility at the University of Kansas Medical Center. All mice were given *ad libitum* access to

food and water and were fed either a standard diet (8604; Harlan Teklad, Madison, Wisconsin; 14% kcals from fat, 32% protein, and 54% carbohydrate) or a high-fat diet (07011; Harlan Teklad; 54% kcals from lard and corn oil fat, 21% protein, and 24% carbohydrate). All animals were fed the standard diet through all baseline tests. After baseline tests, the animals were separated according to diet. All animal use was in accordance with NIH guidelines and conformed to the principles specified in a protocol approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

Energy Intake: Daily food intake was measured by monitoring the weight of the remaining food after an initial food bolus. New food boluses were given every 3-4 days. Energy intake was calculated by:

$$\text{Intake per day} = \frac{\text{(Initial Weight of Food/Final Weight of Food)}}{\text{\# of Days Between Feedings}}$$

Standard diet energy was calculated by multiplying the intake per day by 3.0kcal/g, while high-fat diet energy was calculated by multiplying intake per day by 4.9kcal/g. The combined mean energy intake from each mouse was used to calculate the group means.

Voluntary Exercise: Following baseline testing, animals were separated into either sedentary controls or exercise groups. Exercise animals were housed individually in cages designed to hold stainless-steel running wheels (Mini Mitter; Bend, OR) and given free access to run 24/7. VitalView (Mini Mitter) software measured total wheel revolutions for each mouse during the course of the study. Sedentary animals were housed one to two per cage at the suggestion of the veterinarians in the animal facility. Mice were started on diet and running wheels simultaneously following baseline behavior testing. Treatment groups are identified

throughout the study as: standard diet sedentary (Std-Sed), standard diet exercise (Std-Ex), high-fat diet sedentary (HF-Sed) and high-fat diet exercise (HF-Ex).

Blood Chemistry: Animal weight, blood glucose (glucose diagnostic reagents; Sigma, St. Louis, MO), and serum insulin (mouse insulin ELISA; Alpco, Salem, NH) were measured biweekly. Hemoglobin A1c levels (A1CNow+; Bayer, Sunnyvale, CA) were measured at 0, 6, and 12 weeks following high-fat diet and exercise initiation. All mice were fasted three hours prior to blood collection for all blood chemistry panels, with the exception of the glucose tolerance test.

After 12 weeks, an intraperitoneal glucose tolerance test (IPGTT) was performed after a 6 hour fast. Animals were given an intraperitoneal (IP) injection of glucose at 2g glucose/kg body weight. Blood glucose levels were measured via tail clip immediately before glucose injection and 15, 30, 60 and 120 minutes thereafter.

Behavior Testing: Behavior testing to assess signs of diabetic neuropathy was carried out at baseline and biweekly time points. For all behavioral tests, animals were allowed to acclimate to the testing equipment in two separate sessions prior to the initial testing day. Before each behavior test, animals were allowed to acclimate to the behavior testing room for 30 minutes followed by a 30-minute acclimation to the testing equipment.

Mechanical Sensitivity: Mice were placed in individual clear plastic cages on a wire mesh table 55 cm above the table. von Frey monofilaments (0.07-4.0 g) were applied perpendicularly to the plantar surface of the hindpaw until the filament bent. Testing began with the 0.6 g filament. If the animals withdrew their paw, it was counted as a positive withdrawal and the next lowest filament was applied. If the animal did not respond, the next larger filament was applied. Filaments were applied until there was an initial change in response followed by four additional

filament applications. The 50% withdrawal threshold was calculated using the formula from the up-down method previously described [244].

Thermal Sensitivity: Mice were placed in individual clear plastic cages on a thermal analgesiometer and a 4.0 V radiant heat source was applied twice to each hind paw for a total of four tests. Time elapsed for each animal to withdraw the hind paw was counted as withdrawal latency (sec). Latencies from four applications were used to calculate the mean latency per animal and mean latencies were combined to calculate group means.

Visceral Sensitivity: At 12 weeks, electrode implantation and colorectal distension (CRD) were performed as described previously [272]. The electromyographic (EMG) activity of the abdominal musculature was amplified, filtered and recorded with Spike 2 software (Cambridge Electronic Design, Cambridge, UK) during each applied pressure: 15, 30, 45, 60, and 75mmHg, in triplicate for 20 s with a 4-minute rest period in between. CRD responses were quantified by measuring the area under the curve for the entire distension period divided by the duration of the distension and expressed as a percent of baseline activity (10s prior to distension).

Nerve Conduction Velocity: At 12 weeks, immediately before sacrifice, animals were anesthetized with an IP injection of Avertin (1.25% v/v tribromoethanol [Sigma-Aldrich], 2.5% tert-amyl alcohol [Sigma-Aldrich], dH_2O; 200 µL/10 g body weight) and motor and sensory nerve conduction velocities were recorded as previously described [78].

Intraepidermal Nerve Fiber (IENF) Measurement: Immediately following nerve conduction velocity measurements, animals were sacrificed at 12 weeks. Right hind foot pads were collected, immersed for one hour in Zamboni's fixative (3% paraformaldehyde, 15% picric acid in 0.1M phosphate buffer [PBS, pH 7.4]), rinsed overnight in 1% PBS, immersed in 30%

sucrose in PBS overnight, cryoembedded in mounting media (OCT Compound; Sakura Finetek, Torrance, CA), and sectioned on a cryostat (Leica CM 1950; Leica Biosystems, Richmond, IL) at 30μm before immunohistochemistry.

After a 5-minute thaw at room temperature, sections were incubated with blocking solution (1.5% normal donkey serum, 0.5% porcine gelatin, 0.5% Triton X-100) at room temperature for 6 hours. Slides were then incubated overnight at 4° C in primary antibody diluted in blocking solution. Slides were then washed 2x10 minutes in PBST, followed by a 1-hour incubation with antibodies conjugated to different fluorophores. Sections were then washed 2x10 minutes in PBS, rinsed in deionized distilled water and coverslipped.

Intraepidermal nerve fiber quantification was performed using rabbit anti-PGP9.5 (1:400; Chemicon, Temecula, CA) to visualize all intraepidermal fibers and rat anti-Trk A (1:250; R&D Systems, Minneapolis, MN) to visualize peptidergic fiber types. AlexaFluor 488 and AlexaFluor 555 (1:2000; Molecular Probes, Eugene, OR) were used as fluorophore conjugated secondary antibodies. Fluorescent images were collected using a Nikon Eclipse 90*i* microscope using a 40x objective. NIH Image J software was used to measure each epidermal region. IENF density (IENFD) was expressed as number of fibers per millimeter of epidermis from a total of 9 images per mouse. The combined mean IENF density from each mouse was used to calculate the group means.

Growth Factor Quantification: Immediately following sacrifice, L4-L6 spinal cord, all dorsal root ganglion, sciatic nerve, gastrocnemius, and hindpaw skin were dissected out, snap frozen in liquid nitrogen, and stored at -80° C. Frozen tissue samples were homogenized separately in lysis buffer (20mM Tris-HCL (pH 8.0), 137mM NaCl, 1% NP40, 1mM PMSF, 10% glycerol, 10 μg/mL aprotinin, 1μg/mL leupeptin, 0.5 mM sodium vanadate, and 4% Triton

X-100), homogenates centrifuged and supernatants collected. Total protein concentration of each sample was measured using a protein assay based on the Bradford method (Bio-Rad protein reagent; Hercules, CA). Each protein was quantified using an ELISA kit (BDNF, GDNF, and NGF Emax ImmunoAssay Systems; Promega, Madison, WI) following the manufacturer's instructions. Equal amounts of each protein were loaded to quantify levels of BDNF, GDNF, and NGF.

Statistical Analysis: All data are presented as mean ± SEM. Data were analyzed using a two-factor ANOVA or repeated measures ANOVA with post hoc comparisons analyzed using Fisher's test of least square difference where appropriate. All statistics were run using SPSS Statistics 20. Statistical significance was defined as $p \leq 0.05$.

Results

Running Distances in Exercise-Grouped Mice: Std-Ex and HF-Ex mice began running the day following baseline behavior measures. Average daily distances per 2 weeks can be seen in Table 3.1. High-fat fed mice averaged significantly greater distances than standard diet fed mice through 6 weeks. Interestingly, both exercise groups decreased physical activity over the course of the study, with Std-Ex mice exhibiting a 56% decline and HF-Ex mice exhibiting a 70% decline in average distance ran over the course of 12 weeks.

A High-fat Diet Induces Prediabetes: HF-Sed mice gained significantly more weight throughout the study than all other groups, beginning at two weeks (Figure 3.1A). HF-Ex mice gained excess weight compared to Std-Ex mice; however, HF-Ex mice weighed significantly less than HF-Sed mice throughout the study. While HF-fed mice gained more weight than Std diet

Table 3.1: ***Average Daily Distances:*** Average daily distances on running wheels by both exercise groups through 12 weeks of study. Datum is presented as mean±SEM. Standard diet, n=9; High-fat diet, n=13. *=$p<0.05$, **=$p<0.01$

Average Daily Distance (km)						
	Week 2	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
Standard Diet	8.71±1.13	7.14±1.01	4.96±0.36	4.36±0.51	4.37±0.44	3.85±0.38
High-fat Diet	11.48±0.97**	9.15±0.79*	6.88±0.31*	5.88±0.50	4.28±0.45	3.49±0.41

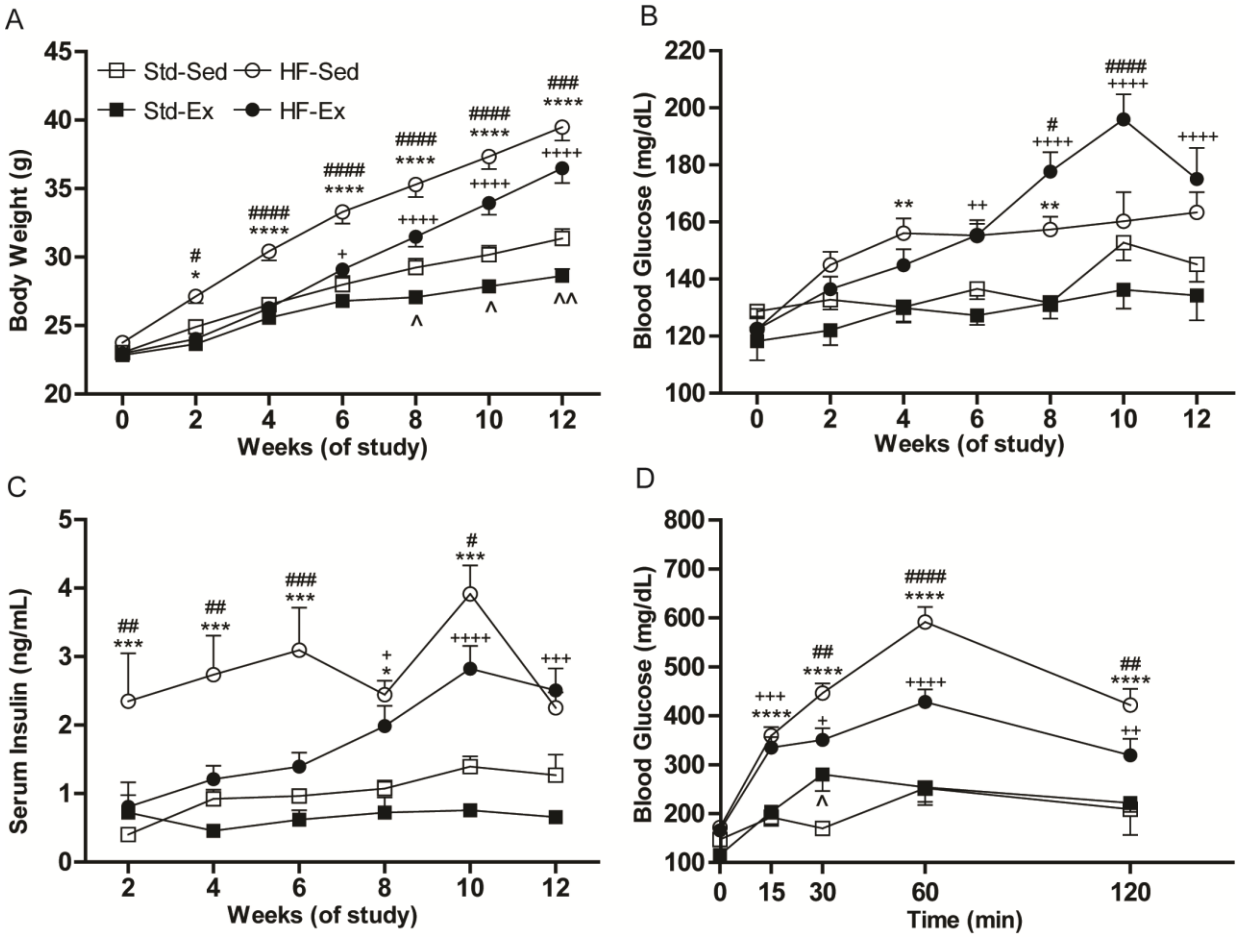
mice, energy intake between the groups was not significantly different (Std-Sed 2.48±0.40, n=7; Std-Ex 3.53±0.43, n=12; HF-Sed 2.59±0.35, n=12; HF-Ex 3.24±0.35, n=16 kcal/day).

HF-Sed and HF-Ex mice had significantly elevated blood glucose levels compared to their Std-fed counterparts, beginning at 4 and 6 weeks, respectively (Figure 3.1B); however their blood glucose levels remained below the threshold of diabetes throughout the study (250mg/dl, which is based upon the consensus of the field). Furthermore, hemoglobin A1c levels measured at 12 weeks were not significantly different between groups (Std-Sed 4.50%±0.11 [26mmol/mol], Std-Ex 4.48%±0.09 [25mmol/mol], HF-Sed 4.44%±0.11 [25mmol/mol], HF-Ex 4.64% ±0.08 [27mmol/mol]).

HF-Sed mice displayed significantly elevated insulin levels compared to all other groups, as early as two weeks post-diet change, whereas the onset of significantly elevated serum insulin in HF-Ex mice was not observed until 8 weeks (Figure 3.1C). An IPGTT was also performed at 12 weeks, and both groups of HF-fed mice displayed significantly increased blood glucose levels over the course of the experiment (Figure 3.1D). While blood glucose levels of HF-Ex mice were significantly higher than those of Std-Ex mice, they were also significantly lower than those of the HF-Sed group (Figure 3.1D). Collectively, these data suggest that mice fed a high-fat diet develop a condition similar to prediabetes beginning two weeks following diet intervention that was partially attenuated by exercise.

High-fat Diet Induced-Hypersensitivity is Attenuated by Exercise: Cutaneous sensitivity was assessed by determining mechanical and thermal withdrawal thresholds. At baseline, there were no significant differences between mice in either mechanical or thermal thresholds. By

Figure 3.1: ***A High-Fat Diet Induces Symptoms of Prediabetes:*** **(A)** A high-fat diet causes increased weight gain in both the HF-Sed (n=21) and HF-Ex (n=24) compared to their standard diet controls (Std-Sed, n=14; Std-Ex, n=16). **(B)** Glucose levels are slightly elevated in high-fat fed animals, though overt hyperglycemia does not develop in either sedentary or exercise mice. **(C)** High-fat sedentary mice develop early hyperinsulinemia which continues throughout the study. High-fat exercise mice develop hyperinsulinemia, but not until 8 weeks. **(D)** A glucose tolerance test performed at 12 weeks shows significantly elevated blood glucose levels in both HF-Sed and HF-Ex mice. All data are presented as mean±SEM. *Std-Sed vs HF-Sed; +Std-Ex vs HF-Ex; #HF-Sed vs HF-Ex; ^Std-Sed vs Std-Ex. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.


A
Std-Sed
HF-Sed
Std-Ex
HF-Ex
Body Weight (g)
Weeks (of study)
B
Blood Glucose (mg/dL)
Weeks (of study)
C
Serum Insulin (ng/mL)
Weeks (of study)
D
Blood Glucose (mg/dL)
Time (min)

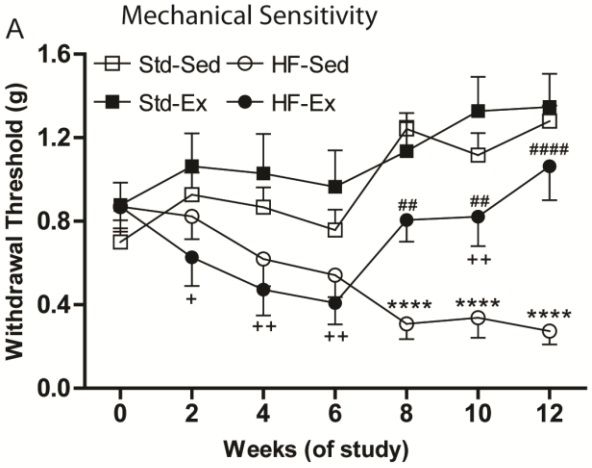
week 2, the HF-Ex mice had a significantly lower mechanical withdrawal threshold compared to the Std-Ex group (Figure 3.2A). By week 4, HF-Ex mice had significantly lower withdrawal thresholds than either Std diet group. In addition, the HF-Sed group was also significantly more sensitive than the Std-Ex group at this time point. Interestingly, at 8 weeks, HF-Ex withdrawal thresholds returned to baseline levels and were significantly increased compared to the HF-Sed mice, yet at 10 weeks, were still significantly lower than Std-Ex mice (Figure 3.2A). By the end of 12 weeks, the mechanical thresholds of HF-Ex mice were no longer significantly different from either Std-Sed or Std-Ex mice, but were significantly higher than HF-Sed mice (Figure 3.2A). No effect of diet or exercise was observed on hindpaw thermal sensitivity (Figure 3.2B).

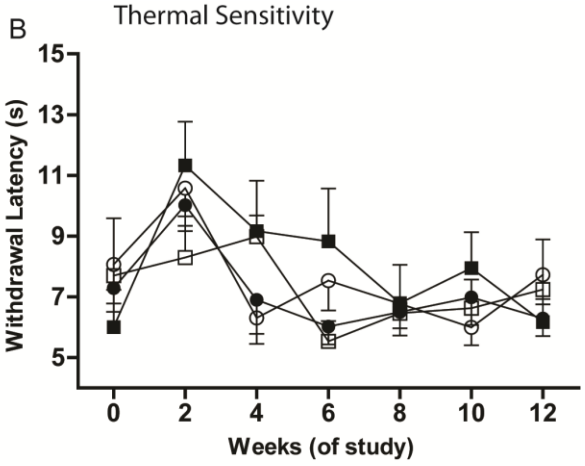
In addition to assessing changes in hindpaw sensitivity, we measured visceromotor response (VMR) during colorectal distension at 12 weeks to determine whether HF diet influenced visceral hypersensitivity. All four groups of mice displayed an increased VMR with increasing intraballoon pressure (Figure 3.2C). Although an overall significant effect of diet-exercise was not observed ($p=0.0954$), posthoc analysis revealed that HF-Sed mice had a significantly higher VMR than both HF-Ex and Std-Sed mice at the two highest intraballoon pressures applied (Figure 3.2C).

Nerve Conduction Velocity and Intraepidermal Innervation: Neither a high-fat diet nor exercise, alone or in combination, altered motor or sensory nerve conduction velocities following 12 weeks of study (Figures 3.3A-B).

To determine if nerve fiber densities were altered by diet or exercise after 12 weeks, epidermal innervation of the hindpaw skin was examined using PGP9.5 antibody, a pan-neuronal marker, and a TrkA antibody, the high affinity receptor for NGF. Intraepidermal nerve fibers

Figure 3.2: ***A High-Fat Diet Induces Mechanical and Visceral Hypersensitivity and Is Reversed by Exercise:*** **(A)** von Frey mechanical sensitivity testing showed allodynia in both HF-Ex (n=13) and HF-Sed (n=13) compared to control (Std-Ex, n=10; Std-Sed, n=10) animals. **(B)** Thermal withdrawal latencies were unchanged based on diet or exercise. Std-Sed (n=4), Std-Ex (n=6), HF-Sed (n=8) HF-Ex (n=11). **(C)** Visceral hypersensitivity is significantly increased in HF-Sed (n=3) animals at the two highest pressues, but is normal in HF-Ex (n=8) animals compared to controls (Std-Sed, n=4; Std-Ex, n=3). All data are presented as mean±SEM. *Std-Sed vs HF-Sed; +Std-Ex vs HF-Ex; #HF-Sed vs HF-Ex; ^Std-Sed vs Std-Ex. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.


A
Mechanical Sensitivity
Std-Sed
HF-Sed
Std-Ex
HF-Ex
Withdrawal Threshold (g)
1.6
1.2
0.8
0.4
0.0
0
2
4
6
8
10
12
Weeks (of study)
+
++
++
##
##
++
####
****
****
****


B
Thermal Sensitivity
Withdrawal Latency (s)
15
13
11
9
7
5
0
2
4
6
8
10
12
Weeks (of study)

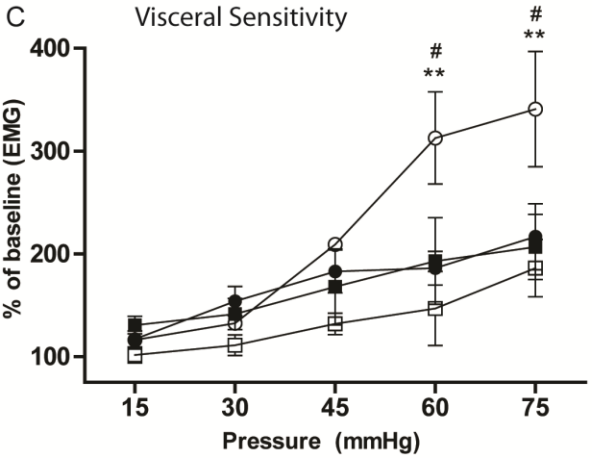
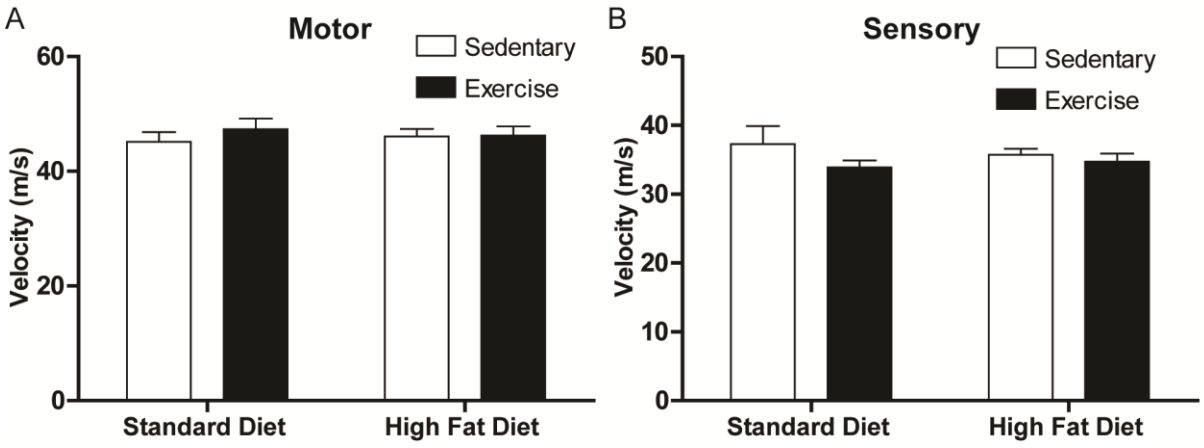

C
Visceral Sensitivity
% of baseline (EMG)
400
300
200
100
15
30
45
60
75
Pressure (mmHg)
#
**
#
**

Figure 3.3: ***Nerve Conduction Velocities Are Not Altered:*** **(A)** Motor and **(B)** sensory nerve conduction velocities are not altered by either a high-fat diet or exercise. All data are presented as mean±SEM. Std-Sed (n=4), Std-Ex (n=6), HF-Sed (n=8), HF-Ex (n=11).


A
Motor
Sedentary
Exercise
Velocity (m/s)
60
40
20
0
Standard Diet
High Fat Diet
B
Sensory
Sedentary
Exercise
Velocity (m/s)
50
40
30
20
10
0
Standard Diet
High Fat Diet

were either TrkA-positive (Figure 3.4, arrows) or TrkA-negative (Figure 3.4, arrowheads) and colocalization allowed for quantification of the intraepidermal nerve fiber phenotype. Representative images in Fig. 4 illustrate the presence of both PGP9.5 and TrkA in all groups including Std-Sed (Figure 3.4A-C), Std-Ex (Figure 3.4D-F), HF-Std (Figure 3.4G-I), and HF-Ex (Figure 3.4J-L). Quantification of IENFD showed that neither a high-fat diet nor exercise significantly altered the number of PGP9.5-immunopositive fibers (Figure 3.5A). However, HF-Sed mice had a 1.36-fold increase in the number of TrkA positive fibers compared to Std-Sed mice (Figure 3.5B). HF-Ex mice did not display this same increase in TrkA fiber density. In addition, the fiber density of HF-Ex TrkA was not significantly different from Std-Ex, but was 1.39-fold lower than HF-Sed TrkA (Figure 3.5B). To confirm that the increase in TrkA fiber counts was not due to an overall increase in total fibers, we calculated the percentages of TrkA-positive/PGP9.5-positive fibers in each animal and normalized the data to the means of Std-Sed for each group (Figure 3.5C). We found a 1.4-fold increase in the ratio of HF-Sed mice to Std-Sed mice compared to only a 1.1-fold increase in the HF-Ex mice.

Neurotrophins Expression is Altered by Both Diet and Exercise: In the current study, we wanted to investigate if a high-fat diet, exercise, or a combination of both can alter neurotrophin protein expression in neural and non-neuronal tissues. Our results demonstrate a 1.4-fold increase in GDNF levels in both the sciatic nerve (Figure 3.6A) and spinal cord (Figure 3.6B) of Std-Ex compared to Std-Sed mice. Additionally, GDNF was increased 1.5-fold in the sciatic nerve of HF-Sed mice compared to the Std-Sed mice (Fig. 6A). In the DRG, HF-Ex animals appeared to have a decreased concentration of GDNF in comparison to HF-Sed animals, yet it was not significant (p=0.0501; Figure 3.6C). In non-neuronal tissues, the gastrocnemius muscle and hind paw

Figure 3.4 (A-L): ***Intraepidermal Nerve Fiber Phenotype is Altered by a High-Fat Diet:*** Increased TrkA receptor expression in the hind paw skin of high-fat mice. Representative images of double immunofluorescent staining for the pan-neuronal marker PGP9.5 (**[A]** Std-Sed; **[D]** Std-Ex; **[G]** HF-Sed; **[J]** HF-Ex), NGF receptor TrkA (**[B]** Std-Sed; **[E]** Std-Ex; **[H]** HF-Sed; **[K]** HF-Ex) or merged images (**[C]** Std-Sed; **[F]** Std-Ex; **[I]** HF-Sed; **[L]** HF-Ex). Arrowheads indicate TrkA-/PGP9.5+ neurons; Arrows indicate TrkA+/PGP9.5 neurons. Scale bar equals 50μm. All data are presented as mean±SEM, n=6 animals/group. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.

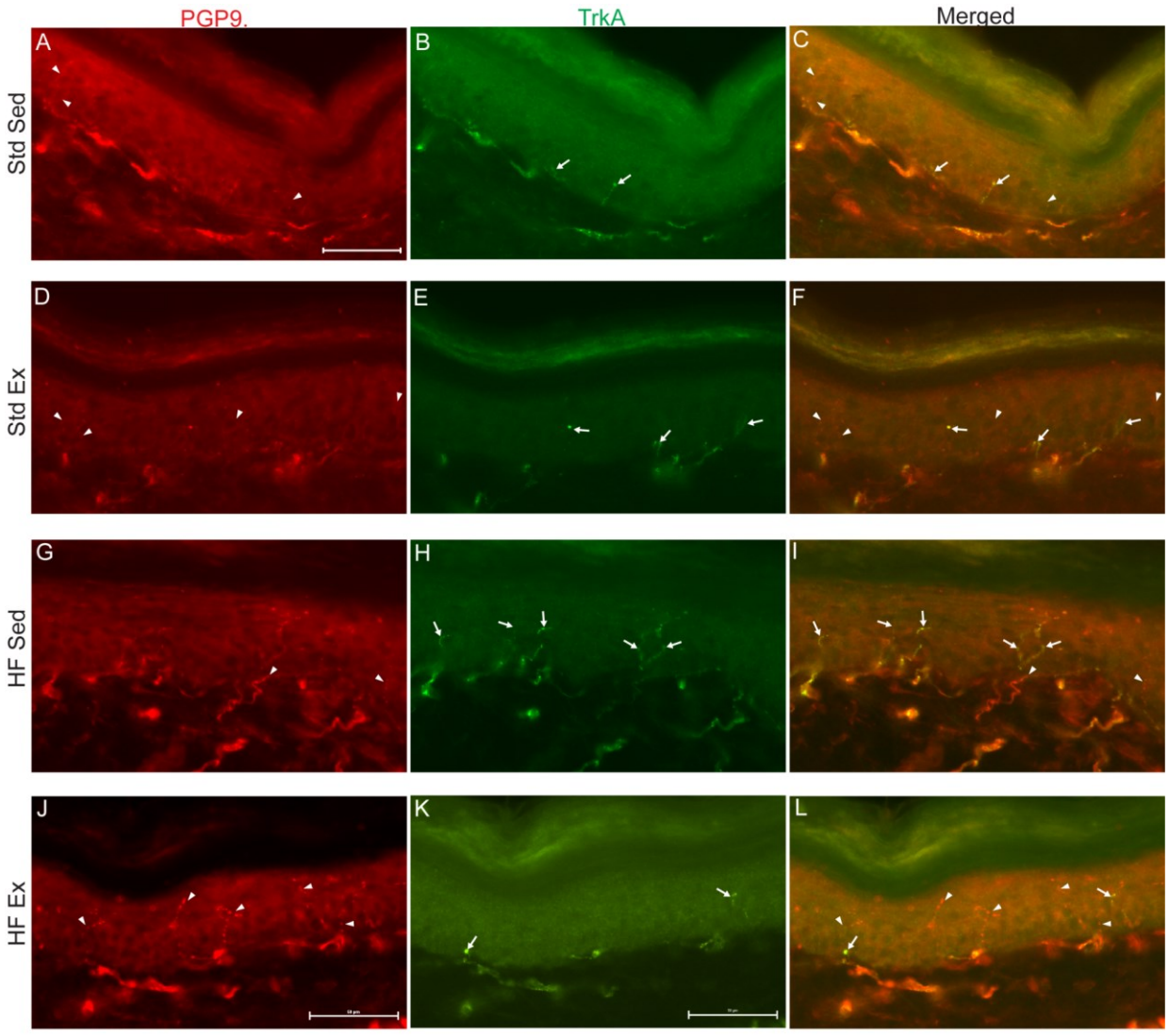

PGP9.
TrkA
Merged
Std Sed
Std Ex
HF Sed
HF Ex
A
B
C
D
E
F
G
H
I
J
K
L

Figure 3.5: ***Intraepidermal Nerve Fiber Quantification:*** IENF quantification for PGP9.5 **(A)** shows no change in overall fiber count due to diet or exercise. However, quatification of TrkA+ neurons **(B)** show an increase in HF-Sed mice and a recovery in HF-Ex mice. Additionally, the ratio of TrkA/PGP9.5 shows HF-Sed mice retain this increase and reversal due to exercise **(C)**. Scale bar equals 50μm. Datum is presented as mean±SEM, n=6 animals/group. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.

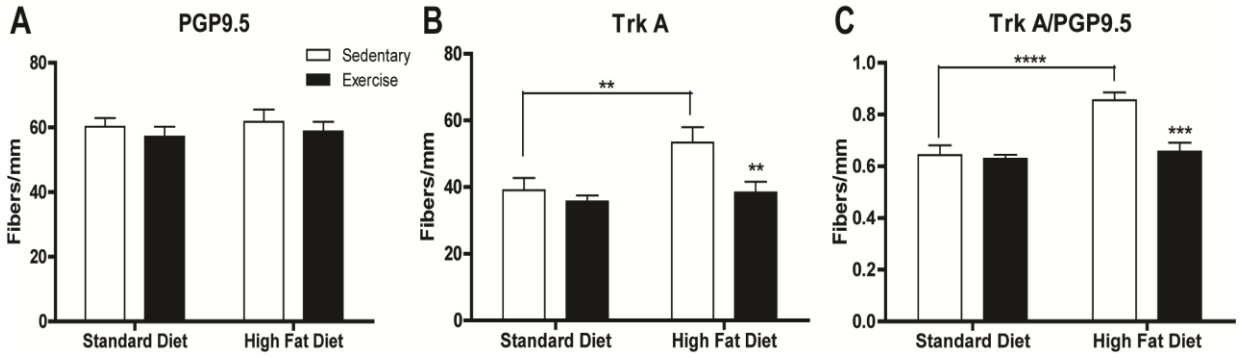
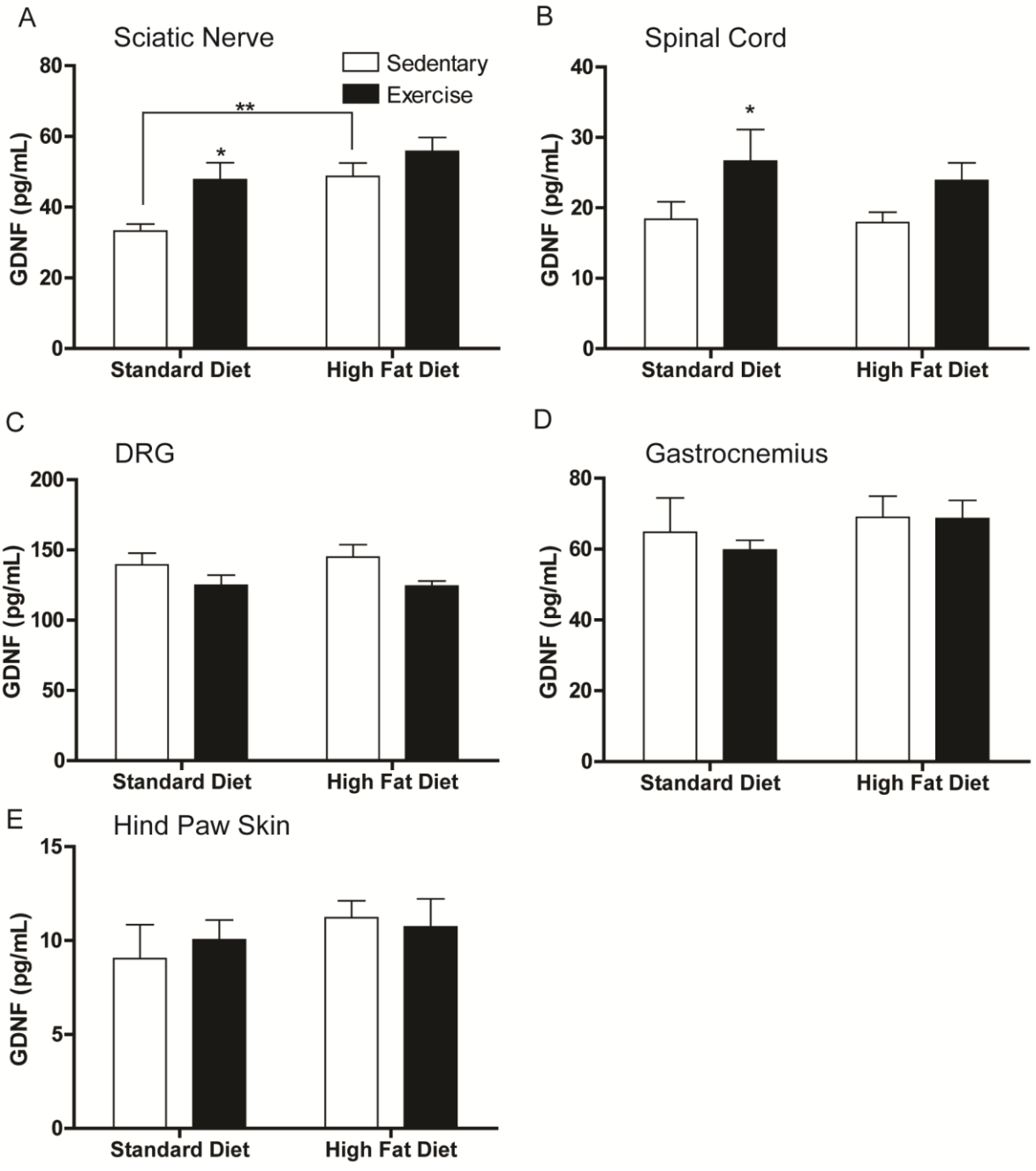

A
PGP9.5
Sedentary
Exercise
Fibers/mm
80
60
40
20
0
Standard Diet
High Fat Diet
B
Trk A
**
**
Fibers/mm
80
60
40
20
0
Standard Diet
High Fat Diet
C
Trk A/PGP9.5
****
***
Fibers/mm
1.0
0.8
0.6
0.4
0.2
0.0
Standard Diet
High Fat Diet

Figure 3.6: ***GDNF Protein Content in Neural and Non-Neuronal Tissues:*** Quantification of glial cell-line derived neurotrophic factor (GDNF) in **(A)** sciatic nerve, **(B)** spinal cord, **(C)** dorsal root ganglia (DRG), **(D)** gastrocnemius, and **(E)** hindpaw skin. The only changes in GDNF content was seen in the spinal cord **(A)** where exercise in the standard diet group was increased and in the sciatic nerve **(C)** where GDNF was increased in both Std-Ex and HF-Sed mice compared to control mice. Data are presented as means±SEM where n=9-11 per group per tissue. *=$p<0.05$, **=$p<0.01$.


A
Sciatic Nerve
Sedentary
Exercise
GDNF (pg/mL)
80
60
40
20
0
**
*
Standard Diet
High Fat Diet
B
Spinal Cord
GDNF (pg/mL)
40
30
20
10
0
*
Standard Diet
High Fat Diet
C
DRG
GDNF (pg/mL)
200
150
100
50
0
Standard Diet
High Fat Diet
D
Gastrocnemius
GDNF (pg/mL)
80
60
40
20
0
Standard Diet
High Fat Diet
E
Hind Paw Skin
GDNF (pg/mL)
15
10
5
0
Standard Diet
High Fat Diet

footpad, there were no significant alterations in GDNF levels in any of the animal cohorts (Figures 3.6D,E respectively).

In contrast to GDNF, NGF protein expression was mostly altered in the non-neuronal tissues as opposed to the neural tissues. In the neural compartments, there were no changes in the sciatic nerve (Figure 3.7A) as was seen with GDNF protein, yet NGF levels were increased in HF-Sed mice versus Std-Sed mice in the DRG (Figure 3.7B). Peripherally, NGF was increased in HF-Sed mice compared to Std-Sed mice in both gastrocnemius muscle (Figure 3.7C) and hind paw footpad (Figure 3.7D) (4.6- and 2.1-fold increase, respectively). Additionally, our results show that exercise significantly decreased NGF levels 2.2-fold in the footpad of high-fat fed mice. The protein values were below the detection limit of the NGF ELISA for the spinal cord.

Finally, we investigated changes in BDNF protein expression in non-neuronal and neural tissues. In all tissues except sciatic nerve (Figure 3.8B-E), we saw no alterations to BDNF levels associated with a high-fat diet or with exercise. However, in the sciatic nerve (Figure 3.8A), significant increases in BDNF levels were evident following exercise in both the standard diet and high-fat diet groups. While BDNF was increased following exercise, a high-fat diet in combination with exercise appeared to slightly restore BDNF expression to control levels.

Discussion

Using a high-fat diet to induce prediabetes, we demonstrate that C57Bl/6 mice develop cutaneous and visceral hyperalgesia, alterations in neurotrophin protein levels, and changes in the composition of epidermal innervation. Our results reveal that voluntary exercise can reverse cutaneous and visceral hypersensitivity, as well as normalize peripheral NGF levels and

Figure 3.7: ***NGF Protein is Upregulated in Non-Neuronal Tissues by a High-Fat Diet:*** Quantification of nerve growth factor (NGF) in **(A)** sciatic nerve, **(B)** DRG, **(C)** gastrocnemius, and **(D)** hindpaw skin. NGF levels were below the limit of detection in the spinal cord. A high-fat diet caused an increase in NGF in the DRG (A), gastrocnemius muscle (B), and hindpaw skin. While it appears that exercise decreases NGF closer to baseline levels in high-fat diet mice in the DRG and gastrocnemius, the only place where there was a significant decline in NGF content from HF-Sed to HF-Ex was in the hindpaw skin. Data are presented as means±SEM where n=6-11 per group per tissue. *=$p<0.05$, **=$p<0.01$.

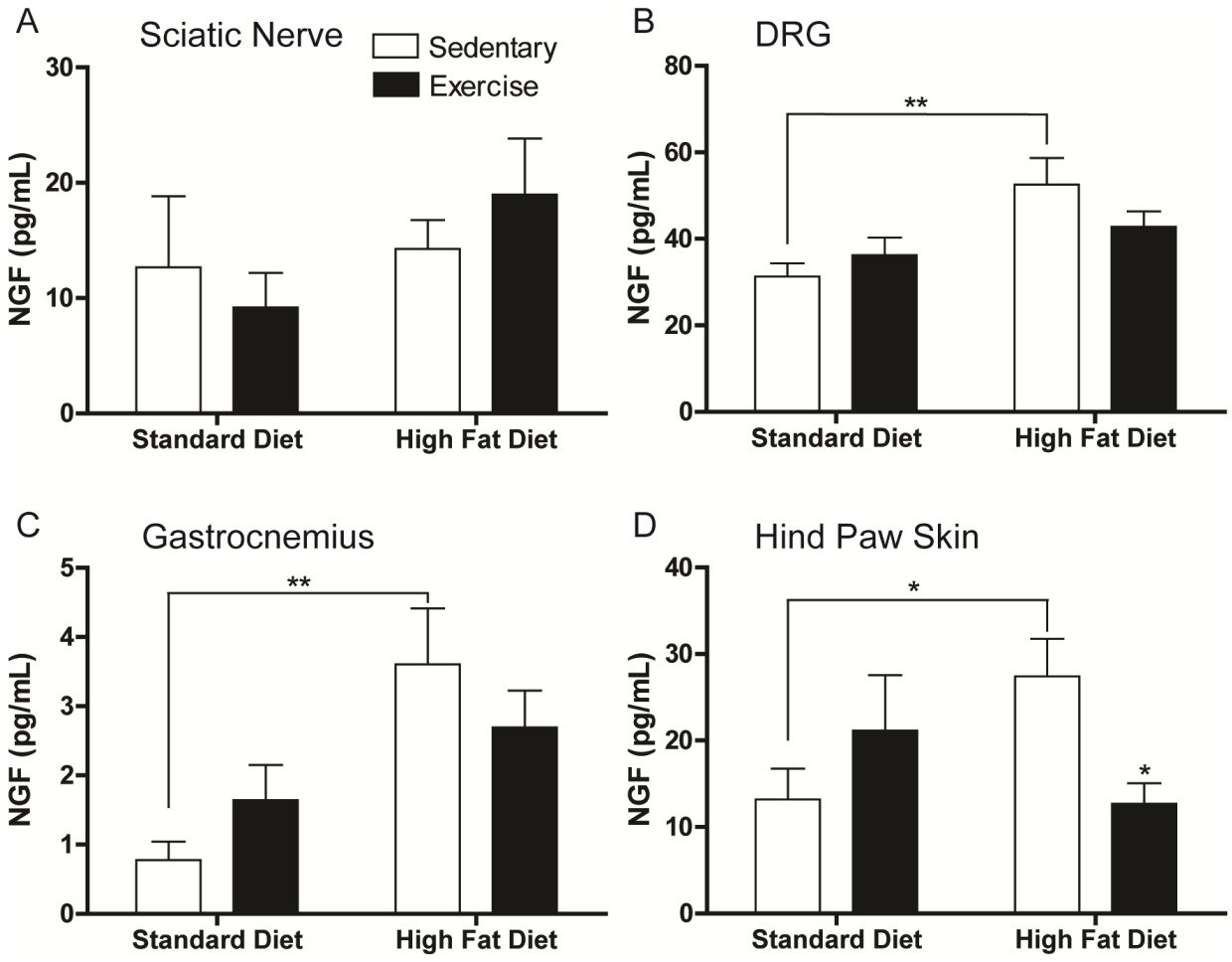
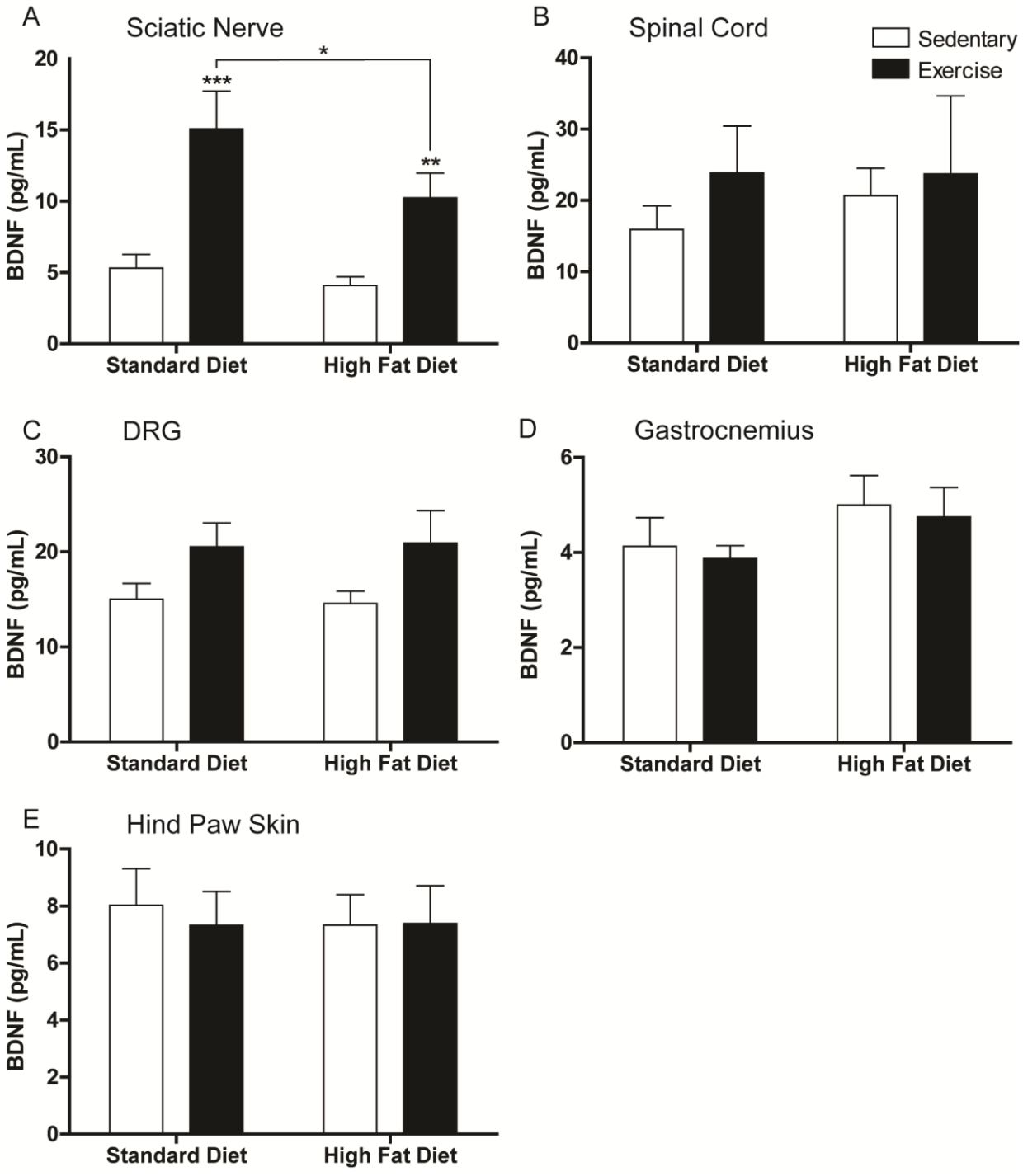

A
Sciatic Nerve
Sedentary
Exercise
NGF (pg/mL)
30
20
10
0
Standard Diet
High Fat Diet
B
DRG
**
NGF (pg/mL)
80
60
40
20
0
Standard Diet
High Fat Diet
C
Gastrocnemius
**
NGF (pg/mL)
5
4
3
2
1
0
Standard Diet
High Fat Diet
D
Hind Paw Skin
*
*
NGF (pg/mL)
40
30
20
10
0
Standard Diet
High Fat Diet

Figure 3.8: ***BDNF Protein Alteration is Restricted to the Sciatic Nerve:*** Brain derived neurotrophic factor (BDNF) protein quantification in **(A)** sciatic nerve, **(B)** spinal cord, **(C)** dorsal root ganglia (DRG), **(D)** gastrocnemius, and **(E)** hindpaw skin. BDNF protein expression was altered only in the sciatic nerve **(C)** where exercise significantly increased expression in both exercise groups. HF-Ex mice had significantly decreased BDNF expression compared to Std-Ex mice. Other tissues did not display any alterations in BDNF protein expression. Data are presented as means±SEM where n=8-11 per group per tissue.


A
Sciatic Nerve
BDNF (pg/mL)
20
15
10
5
0
***
*
**
Standard Diet
High Fat Diet
B
Spinal Cord
Sedentary
Exercise
BDNF (pg/mL)
40
30
20
10
0
Standard Diet
High Fat Diet
C
DRG
BDNF (pg/mL)
30
20
10
0
Standard Diet
High Fat Diet
D
Gastrocnemius
BDNF (pg/mL)
6
4
2
0
Standard Diet
High Fat Diet
E
Hind Paw Skin
BDNF (pg/mL)
10
8
6
4
2
0
Standard Diet
High Fat Diet

epidermal innervation. These findings provide evidence of how neurotrophin levels and epidermal innervation can change in a setting of painful prediabetes, and how exercise may be beneficial in correcting these changes.

Mice fed a high-fat diet develop significant weight gain, mild hyperglycemia, hyperinsulinemia, and abnormal responsiveness to glucose. Exercise delayed several metabolic features, including weight gain, hyperinsulinemia and glucose intolerance. Exercise was unable to prevent or reverse the metabolic features, suggesting that exercise-mediated improvements in sensory symptoms may occur independently from metabolic status. Interestingly, both exercise groups decreased their activity over the 12-week study, and the reason(s) why mice decline their running wheel activity remains unclear. Moreover, as HF-Ex mice decreased exercise levels, their prediabetic-related metabolic parameters worsen. Furthermore, while many of the metabolic parameters worsen by 8 weeks, HF-Ex mice are still exercising far more than their HF-Sed counterparts, suggesting that the high-fat diet may overwhelm the ability of exercise to keep metabolic parameters in check.

Our studies agree with previous reports that a high-fat diet induces prediabetic-like symptoms and alters nociceptive behaviors in rodents [77, 78]. However, our results differ from other studies in that no alterations in thermal sensitivity or nerve conduction velocities were observed [77, 273] and may be due to the younger age of our mice and/or the reduced length of our study. Notably, we demonstrate that in addition to cutaneous hypersensitivity, high-fat fed mice also display visceral hypersensitivity. Visceral hypersensitivity has been reported previously in streptozotocin-treated rats, and our studies extend this finding to mice fed a high-fat diet [274]. These findings are relevant, as diabetic patients with autonomic neuropathy suffer from gastroparesis, limited esophageal motility and incontinence [275], and visceral pain [276].

Our data suggest that hypersensitivity of visceral organs may reflect problems associated with visceral afferent nociception. Importantly, exercised mice displayed normalized cutaneous mechanical and visceral sensitivity, consistent with a role for exercise to normalize abnormal sensation [84].

In the current study, mice fed a high-fat diet did not develop reductions in total epidermal innervation. These findings agree with previous reports that prediabetic obese mice do not have altered intraepidermal innervation following 16 weeks of dietary intervention [77]. Additionally, the role of intraepidermal nerve fibers in human prediabetic patients related to pain status need additional study, as conflicting results have been reported [29, 277].

However, while total epidermal innervation was not reduced in high-fat fed mice, there were significant increases in TrkA-positive axons. High-fat fed mice displayed an increase in TrkA axons compared to mice on the standard diet. These findings are important because of the integral role of NGF in pain. TrkA is the high affinity receptor for NGF, and TrkA-expressing fibers are small, peptidergic fibers that express key nociceptive neuropeptides [278]. Our findings of increased epidermal TrkA fibers in concert with mechanical allodynia in mice fed a high-fat diet are consistent with studies in type 2 mice that report increases in both PGP9.5 and TrkA epidermal fibers in mice displaying mechanical allodynia [264, 279]. This finding is also supported by a complimentary study in which type 1 diabetic mice developed mechanical hypoalgesia when peptidergic epidermal fibers were selectively lost [280]. These findings highlight the strong relationship between peptidergic fiber innervation and nociceptive input.

In addition to NGF-responsive axons, remaining epidermal fibers are nonpeptidergic and sensitive to GDNF. While peptidergic neurons convey nociceptive signals, nonpeptidergic

neurons may have more complex functions, including analgesia. Both local [189] and intrathecal [149] administration of GDNF can reduce mechanical and thermal sensitivities. All cutaneous axons arise developmentally as NGF-sensitive fibers, but divide postnatally to form peptidergic and nonpeptidergic axonal populations [281]. An increase in TrkA-expressing fibers could arise from *de novo* TrkA expression in nonpeptidergic fibers, leading to a change in axonal phenotype. It should be noted that nonpeptidergic fibers were not identifiably labeled, so we cannot conclusively state that nonpeptidergic fibers were lost at the expense of peptidergic fibers. It should also be noted that, although not quantified, TrkA did not appear to be increased in keratinocytes of high-fat animals, as reported in human keratinocytes in pre-diabetic states [282, 283]. Our findings that a high-fat diet can increase the TrkA expressing fibers may suggest that the balance of fibers, i.e. peptidergic versus nonpeptidergic, could be key in regulating cutaneous nociceptive thresholds. Our results also suggest that exercise can correct the phenotypic change and abnormal behavioral sensitivity. Together, these findings suggest that alterations in subsets of epidermal axons may be sufficient to regulate cutaneous sensation and could be a key step that initiates pain or sensory loss in diabetes. Alterations in axonal phenotypes as an underlying mechanism could help explain settings where sensory symptoms are evident in patients with normal IENF levels.

In the current study, GDNF protein levels were increased by exercise in the spinal cord and sciatic nerve of standard diet-fed mice. GDNF is known to be a potent analgesic in several rodent pain models [149, 284, 285], including type 1 diabetes (Wright, unpublished observations). It is not clear why GDNF is elevated in these neural compartments following exercise and it will be important to determine the consequences of elevated GDNF, particularly as axonal phenotypes change.

Nerve growth factor has seemingly opposing actions, acting as both pro- and antinociceptive. Nerve growth factor has been reported to be effective in reducing allodynia in models of chronic constriction injury (CCI) [286] and diabetic neuropathy [153] as well as neuropathic pain in patients [287]. In contrast, NGF can elicit hyperalgesia [138] and modulate inflammatory states [230, 288]. Here, NGF protein concentration was significantly increased in high-fat fed mice in the DRG, gastrocnemius muscle and hind paw skin. While we observed normalized NGF protein content in the DRG and gastrocnemius in HF-Ex animals, these changes were not significant. However, this decrease of NGF in hindpaw skin from HF-Sed animals to HF-Ex animals was significant. This datum suggests that a high-fat diet causes an increase in NGF content and that exercise reverses this abnormality. Thus, the increased NGF content may be related to diet-induced inflammation in the periphery that is responsive to exercise intervention.

Obesity can induce an insulin-resistant state and prediabetes. Furthermore, high-fat-diet-induced obese mice display signs of chronic low-grade inflammation [289, 290], including increased proinflammatory macrophages that secrete cytokines such as tumor necrosis factor α (TNF-α), macrophage migration inhibitory factor (MIF), interleukin-1β (Il-1β) and Il-6 [291]. Increases in proinflammatory cytokines can directly lead to lowered insulin sensitivity [290]. Additionally, IL-1β and TNF-α can increase NGF release from cultured fibroblasts [226] and sciatic nerve [227]. Since a high-fat diet is known to induce prediabetes and increase inflammation, elevated levels of NGF may be associated with these inflammatory changes. Our results reveal that HF-Sed mice have elevated NGF in the gastrocnemius muscle and hind paw skin, while HF-Ex mice have levels similar to control mice. Interestingly, along with the increase in NGF protein seen in the hindpaw skin in the HF-Sed group, our study revealed an increase in

TrkA-expressing epidermal axons. Previous groups have shown that overexpression of NGF can lead to hyperinnvervation in the bladder [292], can cause Aδ fibers to become responsive to substance P [293], and can promote the sprouting of TrkA-expressing nociceptors, resulting in hyperinnervation of the skin [294]. Taken together, our results suggest that prediabetes is associated with increased levels of NGF, and possibly peripheral inflammation, leading to an increase in epidermal axons bearing a nociceptive TrkA phenotype.

Another key finding in this study is that mice that exercised had normal levels of NGF in the skin, and normal ratios of TrkA- and non-TrkA-positive axons. Here, exercise may act to decrease elevated NGF associated with the high-fat diet, leading to normalized TrkA-positive axonal innervation. Exercise has anti-inflammatory actions [295] and physical activity can increase systemic anti-inflammatory cytokines. Following exercise, Il-6 is increased by up to 100-fold and then followed by an increase in Il-1 receptor antagonist (Il-1ra) and Il-10, all cytokines with anti-inflammatory effects [295]. We suggest that a while a high-fat diet may induce systemic inflammation and increase NGF, exercise may counteract rising inflammation and normalize NGF levels.

The current study demonstrates that mice fed a high-fat diet develop prediabetes symptoms, visceral hypersensitivity and mechanical allodynia. The high-fat diet also changes the composition of peptidergic and nonpeptidergic phenotypes of epidermal axons, while not changing the overall number of epidermal fibers. These findings suggest that alterations in cutaneous sensitivity may be related axonal phenotypes innervating the epidermis. In addition, this study demonstrates that exercise can correct many of the abnormalities, including visceral hypersensitivity and mechanical allodynia.

CHAPTER 4

Administration of Anti-Nerve Growth Factor Antibody Attenuates Mechanical Allodynia in High-Fat Diet Induced Pre-Diabetes

Abstract

Research suggests that nerve growth factor (NGF) may play an important role in the development and maintenance of diabetic neuropathy. In the current study, C57Bl/6 mice were fed either standard chow or a high-fat diet for 8 weeks. A high-fat diet induced symptoms of prediabetes, including increased weight gain and blood glucose levels. Additionally, mice fed a high-fat diet developed substantial mechanical allodynia, similar to symptoms of overt diabetic neuropathy. After 8 weeks of high-fat feeding, the phenotype of epidermal axons was changed, with a significant increase in peptidergic neurons while not affecting the overall fiber density, as well as increasing NGF protein expression within the dorsal root ganglia (DRG). After the establishment of mechanical allodynia, high-fat fed mice were treated with an anti-NGF antibody for two weeks. Anti-NGF treatment caused a normalization of withdrawal thresholds at 6 and 7 weeks, but returned to allodynic levels at 8 weeks. Intraepidermal nerve fiber density and phenotype were normalized in anti-NGF treated high-fat mice, along with NGF protein expression levels within the DRG. These results confirm that increased hypersensitivity and neuropathic changes, including epidermal innervation and neurotrophin expression, can be induced by a high-fat diet and that blocking NGF signaling may attenuate these changes. Furthermore, this study highlights the importance NGF plays in the development and maintenance of diabetic neuropathy and could be a potential therapeutic target for treating painful diabetic neuropathy.

Introduction

Diabetic neuropathy is a common complication in diabetic and prediabetic patients, with approximately 60-70% experiencing symptoms. The most common type of neuropathy is distal symmetric polyneuropathy and may present with either positive (pain or burning) or negative (numbness or altered proprioception) symptoms. Estimates vary, but approximately 50% of all patients with diabetic neuropathy and type 2 diabetes experience painful diabetic neuropathy (PDN) and approximately 33% of patients with prediabetes are seen clinically for painful neuropathy-like symptoms [296]. Prediabetes is defined as having impaired fasting glucose and/or impaired glucose tolerance [261] and is closely related with central obesity and dyslipidemia. In fact, dyslipidemia has been identified as being a major independent risk factor for increasing a patient's chances of developing neuropathy [297, 298]. While neuropathy associated with prediabetes is not as severe as those with overt diabetes, painful symptoms are the predominate feature. Furthermore, treatment options for PDN are seldom effective and come with significant side effects [262].

Nociceptive neurons can be divided into two groups: peptidergic and nonpeptidergic fibers. Peptidergic neurons contain neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P, substances which help mediate nociceptive signaling. Peptidergic neurons are nerve growth factor (NGF) sensitive and require NGF during development for cell growth, differentiation and cell survival [299]. These neurons express the tropomyosin-related kinase A (TrkA) high affinity receptor for NGF, and upon NGF binding to the receptor, it initiates signaling cascades and is internalized and transported in a retrograde manner to the cell body within the dorsal root ganglion (DRG) [300]. NGF is produced by target tissues in the periphery, including keratinocytes in the skin and fibroblasts [301, 302] and plays an important

role in generating neuropathic pain ([265] for a review). Alternatively, nonpeptidergic neurons do not contain CGRP or substance P, are glial cell line-derived neurotrophic factor (GDNF) sensitive, and express the receptors RET and GFRα1. These neurons also play a role in nociceptive signaling, though may be more antinociceptive than pronociceptive [149, 189].

The role of NGF in inflammatory and neuropathic pain is well established, with studies showing increased expression in osteoarthritis [303, 304], chronic pancreatitis [143], endometriosis [146], chronic constriction injury and diabetic neuropathy [264, 279]. In a previous study in our laboratory, NGF levels were increased in the hind paw skin of prediabetic mice with cutaneous allodynia, but was found to be normalized in rodents that exercised and had normal withdrawal thresholds [305]. We therefore wanted to investigate if the increase in nerve growth factor, and the subsequent normalization following exercise, was a consequence of or the driving force behind the cutaneous sensitivity. Previous studies have demonstrated the effectiveness of anti-NGF treatment in treating pain, including sciatic nerve constriction [119, 158], spinal nerve ligation [170] and bone cancer pain [306]. In the current study, we investigated how a blocking antibody to NGF would affect painful prediabetic neuropathy, NGF protein levels and TrkA-positive intraepidermal innervation. We demonstrate that a high fat model of prediabetes induces mechanical allodynia and that anti-NGF treatment is capable of reversing this behavioral outcome, and this change is associated with a normalization of NGF in the dorsal root ganglia and TrkA-positive innervation.

Materials and Methods

Animals and Diet: Male C57Bl/6 mice were purchased from Charles River (Wilmington, Mass) at seven weeks of age. The mice were maintained on a 12:12h light/dark cycle housed in

the research support facility at the University of Kansas Medical Center. Mice were given *ad libitum* access to food and water and were fed either a high fat diet (07011; Harlan Teklad; 54% kcals from lard and corn oil fat, 21% protein, and 24% carbohydrate) or standard chow diet (8604; Harlan Teklad, Madison, Wisconsin; 14% kcals from fat, 32% protein, and 54%). Prior to any baseline testing, all animals received the standard chow. Following baseline tests, animals were divided into two groups according to diet. All animal use was in accordance with NIH guidelines and conformed to the principles specified in a protocol approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

Energy Intake: Daily food intake was measured by monitoring the weight of the remaining food after an initial food bolus. New food boluses were given every 3-4 days. Energy intake was calculated using the formula:

$$\text{Intake per day} = \frac{\text{(Initial Weight of Food/Final Weight of Food)}}{\text{\# of Days Between Feedings}}$$

Standard chow energy was calculated by multiplying the intake per day by 3.0kcal/g, while high-fat diet energy was calculated by multiplying intake per day by 4.9kcal/g. The combined mean energy intake from each mouse was used to calculate the group means.

Blood Chemistry: Both animal weight and blood glucose (glucose diagnostic reagents; Sigma, St. Louis, MO) were measured weekly. All mice were fasted three hours prior to blood collection for all blood glucose testing.

Behavior Testing: Behavior testing to assess signs of diabetic neuropathy was carried out at baseline and weekly thereafter for 8 weeks. For both behavioral tests, animals were allowed to acclimate to the testing equipment in two sessions prior to the initial testing day. Prior to each

behavior test, mice acclimated to the behavior testing room for 30 minutes followed by a 30-minute acclimation to the testing equipment. Behavioral tests were done on different days each week so as not to stress the animals.

Mechanical Sensitivity: Mice were placed in individual clear plastic cages on a wire mesh table 55 cm above the table. von Frey monofilaments (0.07-4.0 g) were applied to the plantar surface of the hindpaw in a perpendicular manner until the filament bent. Testing began with the 0.6 g filament. Once the filament bent, if the mouse reacted to the filament and withdrew the paw, it was counted as a positive response and the next lowest filament was used. If the animal did not respond to the filament, the next larger filament was applied. Filaments were applied until there was an initial change in response followed by four more filament applications. The 50% withdrawal threshold was calculated using the formula from the up-down method previously described by Chaplan *et al.* [244].

Thermal Sensitivity: Mice were placed in individual clear plastic cages on a thermal analgesiometer and a 4.0 V radiant heat source was applied twice to each hind paw for a total of four tests. Left and right paws were tested alternately, allowing more time for normalization. The time it took for each animal to withdraw their hindpaw was measured and counted as withdrawal latency (sec). Latencies from four applications were used to calculate the mean latency per animal and mean latencies were combined to calculate group means.

Anti-NGF Antibody Treatment: Mice were not separated into treatment groups until after week 5 behavioral tests were completed and mechanical allodynia was firmly established. To inhibit NGF action, following week 5 blood glucose, we administered anti-NGF antibody (10mg/kg, mouse monoclonal antibody clone AS21; Exalpha Biologicals, Maynard, MA) or control IgG intraperitoneally once a week for two weeks, weeks 5 and 6, both times following

behavioral tests and blood glucose collection. The four groups are as follows: standard chow + IgG control (Std-IgG, n=9), standard chow + anti-NGF (Std-NGF, n=8), high fat diet + IgG control (HF-IgG, n=9), and high fat diet + anti-NGF (HF-NGF, n=8).

Intraepidermal Nerve Fiber (IENF) Measurement: Animals were sacrificed at 8 weeks. Animals were sacrificed using isoflurane and cervical dislocation. Immediately following sacrifice, right hind foot pads were collected and immersed for one hour in Zamboni's fixative (3% paraformaldehyde, 15% picric acid in 0.1M phosphate buffer [PBS, pH 7.4]). Footpads were then rinsed overnight in 1% PBS, immersed in 30% sucrose in PBS until tissue floated, cryoembedded in mounting media (OCT Compound; Sakura Finetek, Torrance, CA), sectioned on a cryostat (Leica CM 1950; Leica Biosystems, Richmond, IL) at 30μm and stored at -20˚C until immunohistochemistry was completed.

Slides were thawed for 5 minutes at room temperature, then sections were incubated with blocking solution (1.5% normal donkey serum, 0.5% porcine gelatin, 0.5% Triton X-100) at room temperature for 6 hours. Slides were then incubated overnight at 4° C in primary antibody diluted in blocking solution. Slides were then washed 2x10 minutes in PBST, followed by a 1-hour incubation with antibodies conjugated to different fluorophores. Sections were then washed 2x10 minutes in PBS, rinsed in deionized distilled water and coverslipped.

IENF quantification was performed using rat anti-Trk A (1:250; R&D Systems, Minneapolis, MN) to visualize peptidergic fiber types and rabbit anti-PGP9.5 (1:400; Chemicon, Temecula, CA) to visualize all intraepidermal fibers, both peptidergic and nonpeptidergic. AlexaFluor 488 and AlexaFluor 555 (1:2000; Molecular Probes, Eugene, OR) were used as fluorophore conjugated secondary antibodies, respectively. Fibers were counted live with a blinded observer. Fluorescent images were collected using a Nikon Eclipse 90*i* microscope using

a 40x objective. NIH Image J software was used to measure each epidermal region. IENF density (IENFD) was expressed as number of fibers per millimeter of epidermis from a total of 9 images per mouse. The combined mean IENFD from each mouse was used to calculate the group means.

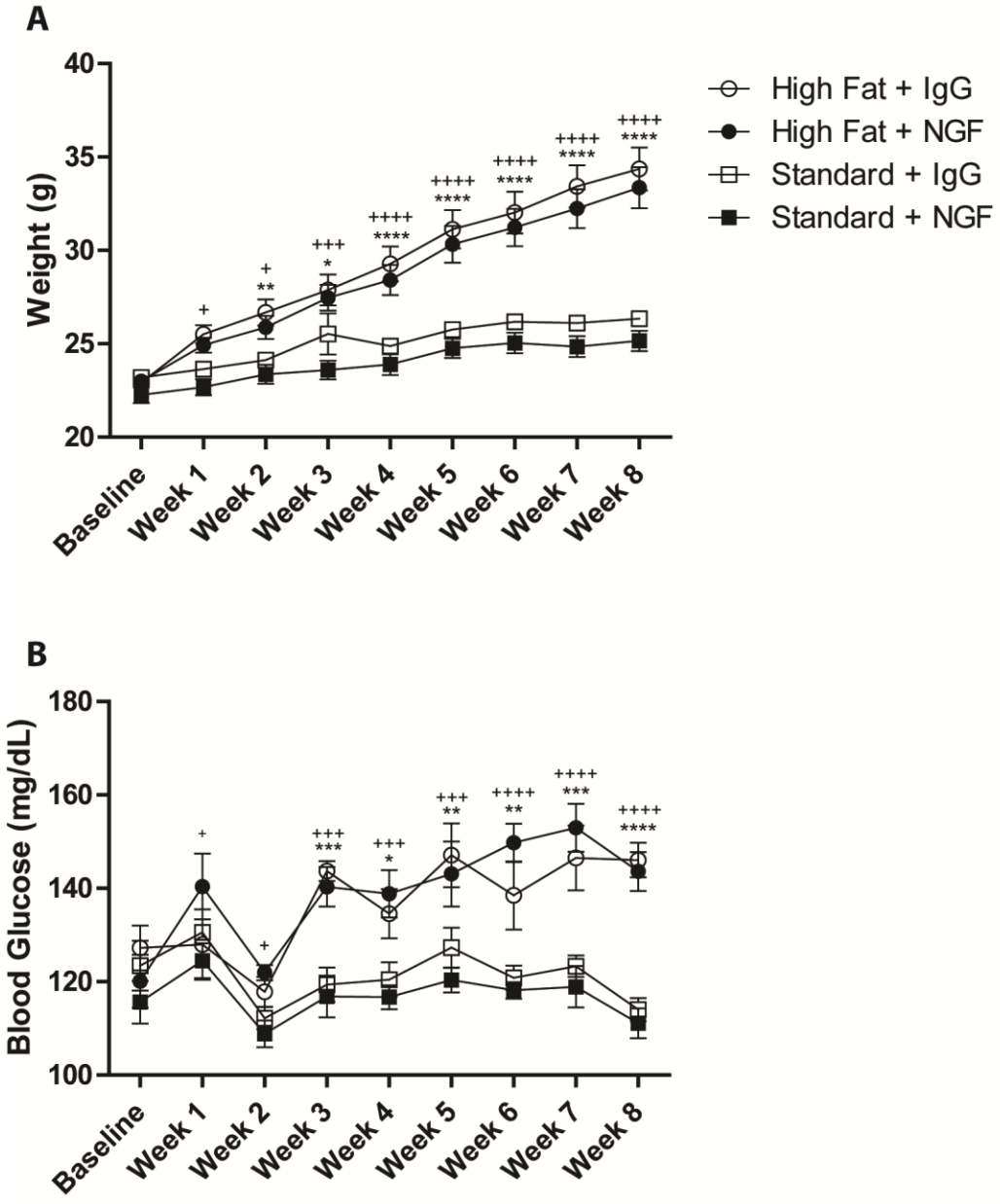
Growth Factor Quantification: Immediately following sacrifice, all dorsal root ganglia, both sciatic nerves, the right gastrocnemius, and the left hindpaw skin were dissected out, snap frozen in liquid nitrogen, and stored at -80° C. Frozen tissue samples were homogenized separately in lysis buffer (20mM Tris-HCL (pH 8.0), 137mM NaCl, 1% NP40, 1mM PMSF, 10% glycerol, 10 μg/mL aprotinin, 1μg/mL leupeptin, 0.5 mM sodium vanadate, and 4% Triton X-100), homogenates centrifuged and supernatants collected. Total protein concentration of each sample was measured using a protein assay based on the Bradford method (Bio-Rad protein reagent; Hercules, CA). Levels of each protein were quantified using a commercially available ELISA kit (NGF Emax ImmunoAssay Systems; Promega, Madison, WI) following the manufacturer's instructions. Briefly, a 96-well plate was coated with anti-NGF polyclonal antibody and incubated overnight at 4˚C. The following day, the plate was blocked for nonspecific binding and the tissue sample homogenates were added and incubated for 6 hours at room temperature. After washing the plate, the plate was then incubated overnight with an anti-NGF monoclonal antibody. After incubating the next morning with an anti-rat IgG, HRP conjugate, the plate was color activated and read using a spectrometer measuring at 450nm. Equal amounts of each protein for each tissue was loaded to measure total NGF levels.

Statistical Analysis: All data are presented as mean ± SEM. Data were analyzed using a two-factor ANOVA or repeated measures ANOVA with post hoc comparisons analyzed using Fisher's test of least square difference where appropriate. All statistics were run using SPSS Statistics 20. Statistical significance was defined as $p \leq 0.05$.

Results

A High-Fat Diet Induces Prediabetes: In order to establish that mice fed a high-fat diet develop prediabetes, we took weight and glucose measures weekly. Both HF-IgG and HF-NGF mouse groups gained significantly more weight than their standard diet counterparts (Figure 4.1A). Weight gain was established early, with HF-IgG mice reaching statistical significance at 2 weeks post-diet change, and HF-NGF mice reaching statistically significant numbers beginning at week 1. Treatment with anti-NGF antibody did not alter body weight, as seen in weeks 6 through 8, where HF-NGF animals continued gaining weight similar to HF-IgG mice (Figure 4.1A). In fact, over the course of the 8 weeks of study, Std-IgG mice gained 13.6% body weight and Std-NGF gained 13.0% body weight, while HF-IgG mice gained 50.0% body weight and HF-NGF mice gained 45.1% body weight. This observation is not surprising given the energy consumption data obtained over the course of the study. On average, Std-IgG mice consumed 2.02±0.03 kcal/day, Std-NGF mice consumed 1.93±0.04 kcal/day, HF-IgG mice ate on average 2.58±0.05 kcal/day and HF-NGF mice consumed 2.50±0.03 kcal/day (Std-IgG vs HF-IgG $P<0.0001$; Std-NGF vs HF-NGF $P<0.0001$). Another characteristic of prediabetes is increased fasting blood glucose, so we also investigated blood glucose levels weekly. HF-IgG and HF-NGF mice had significantly elevated blood glucose compared with their Std-fed controls, beginning at 1 week for HF-NGF animals and week 3 for HF-IgG mice (Figure 4.1B). While their blood glucose levels were significantly elevated, they did not reach the threshold of being classified with overt diabetes (250mg/dL) at any point during the study. Their elevated blood glucose, taken in conjunction with the increased weight gain, suggests a prediabetic like state beginning around 2 weeks. Anti-NGF treatment affected neither weight gain nor blood glucose.

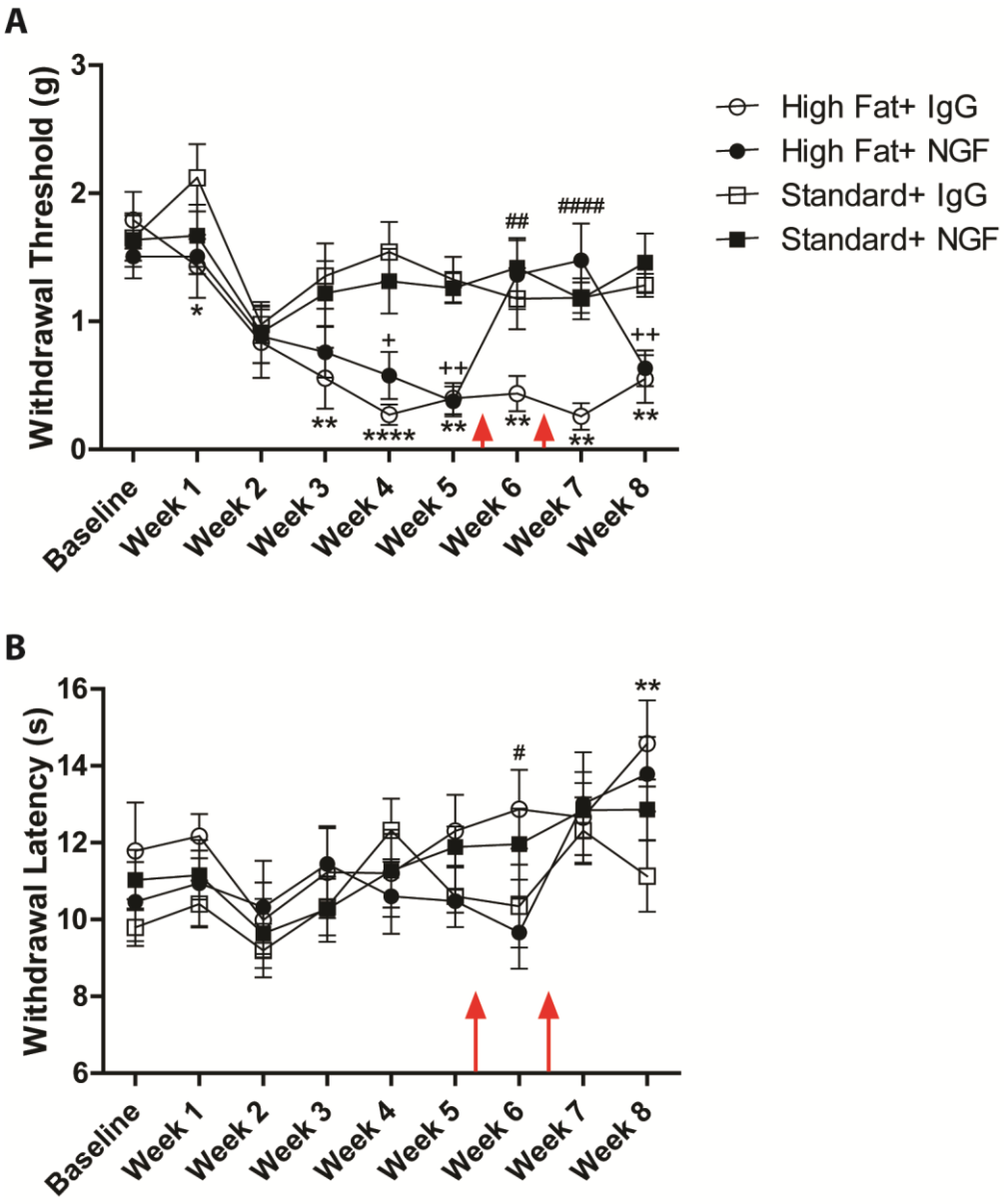
Figure 4.1: ***A High Fat Diet Increases Weight Gain and Blood Glucose:*** A high fat diet induces symptoms of prediabetes, including increased weight gain and increased blood glucose levels. (A) Both HF-IgG (n=9) and HF-NGF (n=8) groups of mice demonstrated significant increases in weight gain beginning 1 week after starting a high fat diet, compared to controls (Std-IgG, n=9; Std-NGF, n=8). Anti-NGF did not affect weight gain. (B) Glucose levels were significantly elevated in high fat fed animals beginning 1 week and 3 weeks for HF-NGF and HF-IgG, respectively and continued throughout the 8 weeks of study. All data are presented as mean ± SEM. *Std-IgG vs HF-IgG; +Std-NGF vs HF-NGF. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.


A
Weight (g)
40
35
30
25
20
Baseline
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Week 7
Week 8
High Fat + IgG
High Fat + NGF
Standard + IgG
Standard + NGF
B
Blood Glucose (mg/dL)
180
160
140
120
100

High-Fat Diet Induced Mechanical Allodynia is Attenuated by Anti-NGF Treatment: Patients suffering from prediabetic neuropathy often exhibit painful neuropathy-like symptoms. Cutaneous sensitivity was evaluated by investigating both mechanical and thermal withdrawal thresholds. At baseline, there were no significant differences in either mechanical or thermal sensitivities. However, beginning at 3 weeks, there was a significant decrease in mechanical withdrawal threshold in HF-IgG mice compared to Std-IgG controls which persisted through the rest of the study (Figure 4.2A). In addition, the HF-NGF mice also developed mechanical allodynia beginning at 4 weeks, and persisting through week 5 in comparison to Std-NGF controls. Because mechanical allodynia had been firmly established in both high fat-fed groups, anti-NGF antibody was administered following the week 5 behavior measurements. At 6 weeks, following anti-NGF treatment, there was a reversal of mechanical withdrawal thresholds to control levels, which persisted into week 7, after a second anti-NGF injection in week 6. During this two week time period, HF-NGF mice were not significantly different from Std-NGF mice, but were different from the HF-IgG. However, after anti-NGF administration was suspended, HF-NGF animals returned to a state of mechanical allodynia and had significantly decreased withdrawal thresholds compared to Std-NGF controls. In the context of mechanical sensitivity, it appears that inhibiting NGF signaling can reverse allodynia. However, in the context of thermal sensitivity, other than two isolated events, there were no significant differences in either HF-IgG or HF-NGF mice compared to their standard diet-fed controls (Figure 4.2B).

Intraepidermal Nerve Fiber Phenotype is Changed by Prediabetes: Since IENF density is often altered in diabetes, we investigated if IENF is changed in a model of prediabetes. Epidermal innervation of the hind paw skin was evaluated at 8 weeks post diet intervention and 2 weeks following the last anti-NGF antibody treatment. PGP9.5, an pan-neuronal marker, was

Figure 4.2: ***High Fat Diet-Induced Mechanical Allodynia is Reversed by Anti-NGF Treatment:*** (A) Mechanical withdrawal thresholds, assessed using von Frey monofilaments, were significantly reduced in both HF-IgG and HF-NGF mice through 5 weeks of study compared to control groups. Administration of anti-NGF resulted in reversal of mechanical allodynia in HF-NGF mice at weeks 6 and 7. (B)Thermal sensitivities were not affected by the high fat diet or anti-NGF treatment during the course of the study. Red arrows signify administration of anti-NGF antibody, which was given after week 5 behavior testing. All data are presented as mean ± SEM. *Std-IgG vs HF-IgG; +Std-NGF vs HF-NGF; #HF-IgG vs HF-NGF. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.


A
Withdrawal Threshold (g)
3
2
1
0
High Fat+ IgG
High Fat+ NGF
Standard+ IgG
Standard+ NGF
Baseline
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Week 7
Week 8
B
Withdrawal Latency (s)
16
14
12
10
8
6

used to determine total fiber density within the skin. Additionally, based on previous studies from our laboratory that showed an alteration in peptidergic, NGF sensitive fibers, a TrkA antibody was used to count the peptidergic fiber expressing cutaneous neurons. Intraepidermal nerve fibers were either TrkA-positive (Figure 4.3, arrows) or TrkA-negative (Figure 4.3, arrowheads) and colocalization allowed for quantification of the intraepidermal nerve fiber phenotype. Representative images show the presence of both PGP9.5 and TrkA in all groups, including Std-IgG (Figure 4.3A-C), Std-NGF (Figure 4.3D-F), HF-IgG (Figure 4.3G-I) and HF-NGF (Figure4.3J-L). Quantification showed that neither a high-fat diet nor treatment with anti-NGF significantly altered the number of PGP9.5-immunopositive fibers (Figure 4.4A). However, HF-IgG mice showed a significant 1.38-fold increase in TrkA positive fibers compared to Std-IgG mice (Figure 4.4B). Interestingly, HF-NGF mice did not display this same increase as their control IgG-treated high fat-fed counterparts. Furthermore, while HF-NGF mice did not display a significant change in TrkA fiber expression compared to Std-NGF mice, HF-NGF mice did have a 1.43-fold decrease in TrkA positive fibers compared to HF-IgG mice (Figure 4.4B). In order to confirm that the increase seen in TrkA positive fibers was not caused by an overall increase in fiber number, we calculated the percentages of TrkA-positive/PGP9.5-positive fibers in each group and normalized the data to the mean of the Std-IgG control group for each group (Figure 4.4C). We discovered a 1.38-fold increase in the ratio of HF-IgG mice to Std-IgG compared with only a 0.97-fold change in HF-NGF mice.

NGF Neurotrophin Expression is Altered by a High-Fat Diet: In addition to examining the intraepidermal phenotype changes caused by a high-fat diet, we also wanted to investigate if a high-fat diet could alter neurotrophin expression in neural and non-neuronal tissues. Because

Figure 4.3: ***Total Nerve Innervation is Not Altered by A High Fat Diet, yet TrkA Innervation Is:*** Representative images of double immunofluorescent staining for the pan-neuronal marker PGP9.5 ([A] Std-IgG; [D] Std-NGF; [G] HF-IgG; [J] HF-NGF), the NGF receptor TrkA ([B] Std-IgG; [E] Std-NGF; [H] HF-IgG; [K] HF-NGF) and merged images ([C] Std-IgG; [F] Std-NGF; [I] HF-IgG; [L] HF-NGF). Arrows indicate TrkA+/PGP9.5+ nerve fibers; Arrowheads indicate TrkA-/PGP9.5+ nerve fibers. Scale bar in (A) represents 10μm for each image. N=6 animals/group.

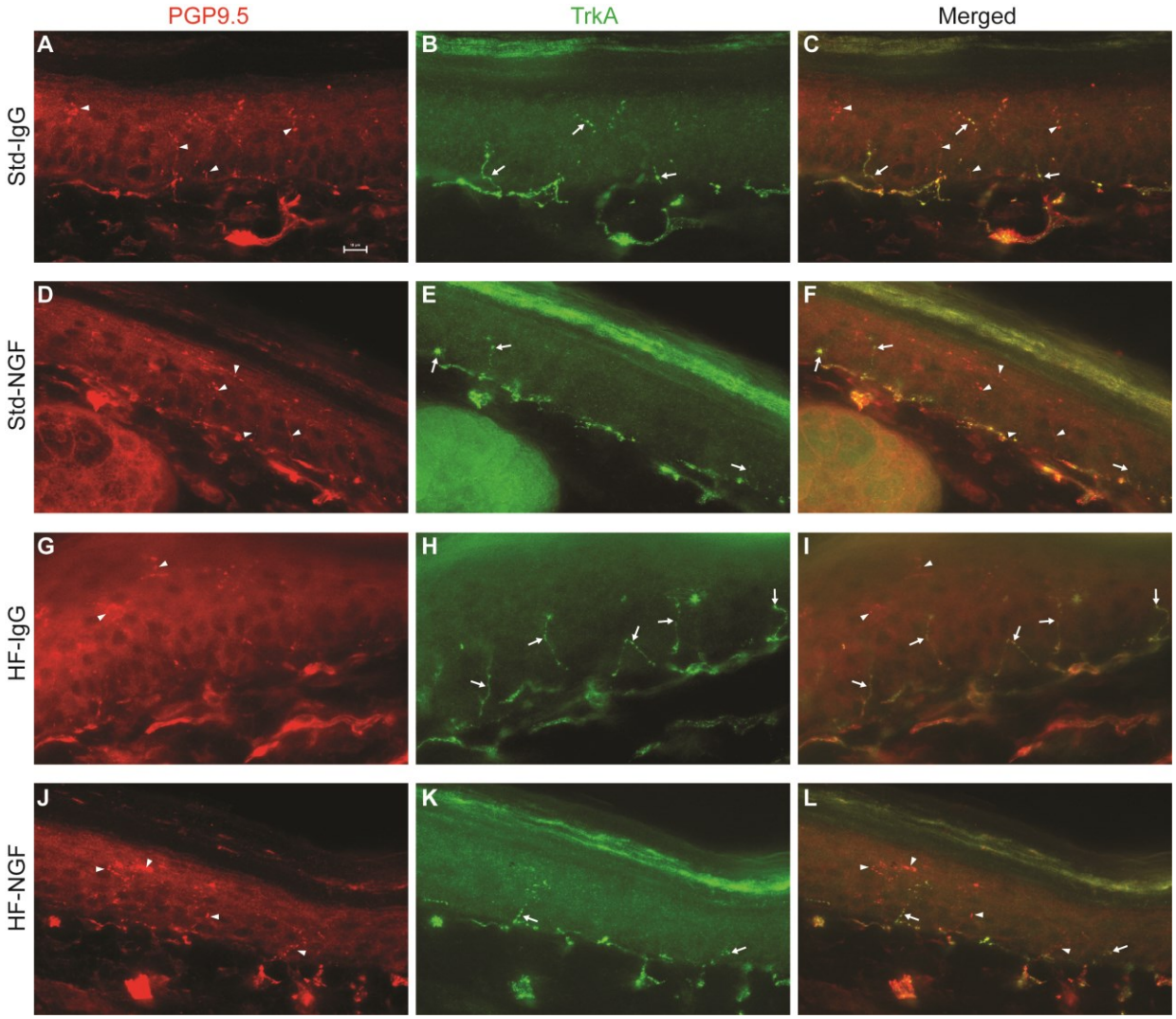
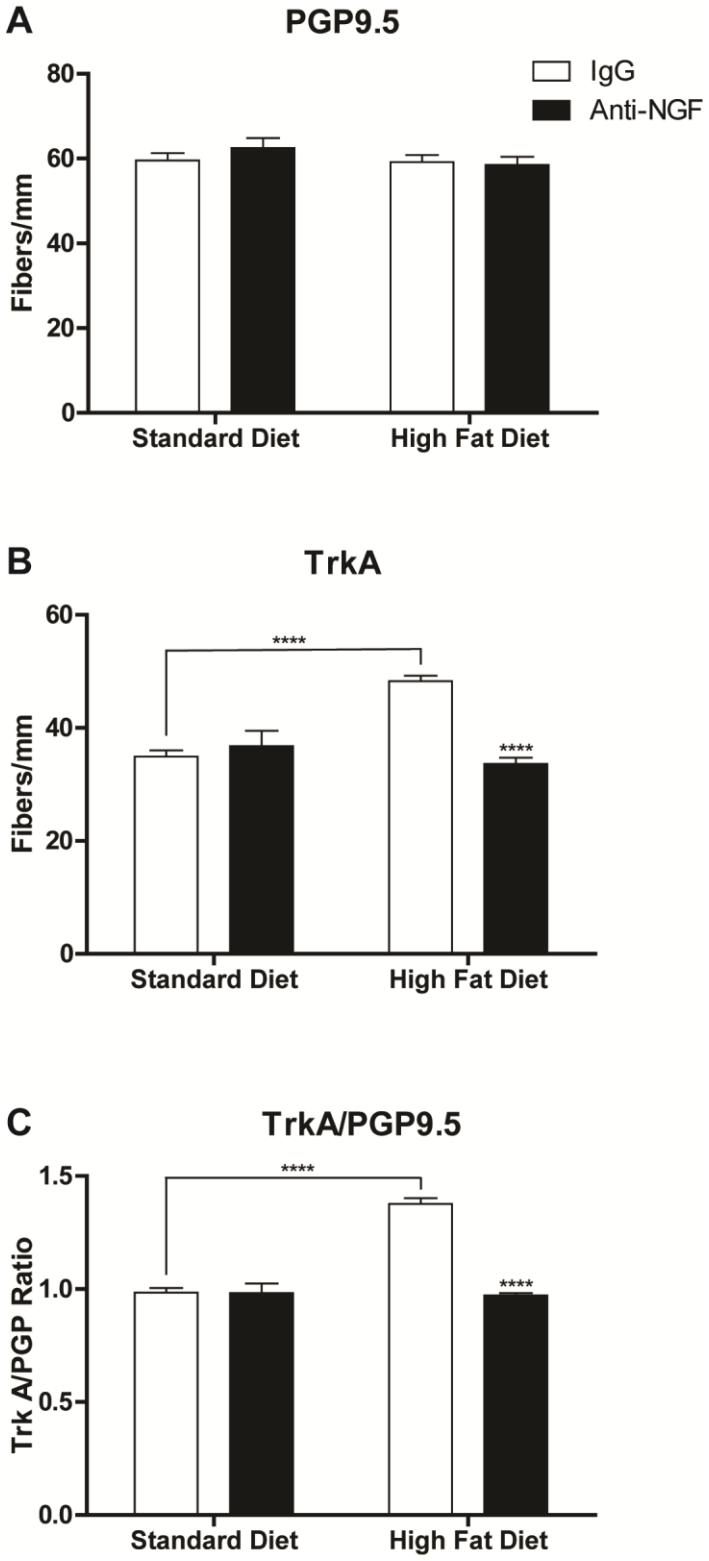

PGP9.5
TrkA
Merged
Std-IgG
Std-NGF
HF-IgG
HF-NGF
A
B
C
D
E
F
G
H
I
J
K
L

Figure 4.4: ***Quantification of Intraepidermal Nerve Fiber Density Reveals Alterations in TrkA expression:*** (A) PGP9.5 expressing fibers were not altered after 8 weeks on a high fat diet or following two weeks of anti-NGF treatment. (B) TrkA-positive neurons were increased in HF-IgG, yet anti-NGF treatment decreased TrkA-positive neurons to control levels. (C) TrkA/PGP9.5 ratio maintains the increase in HF-IgG animals and the recovery in HF-NGF animals. Data are presented as mean ± SEM, n=6 animals/group. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.


A
PGP9.5
IgG
Anti-NGF
Fibers/mm
80
60
40
20
0
Standard Diet
High Fat Diet
B
TrkA
Fibers/mm
60
40
20
0
****
****
Standard Diet
High Fat Diet
C
TrkA/PGP9.5
Trk A/PGP Ratio
1.5
1.0
0.5
0.0
****
****
Standard Diet
High Fat Diet

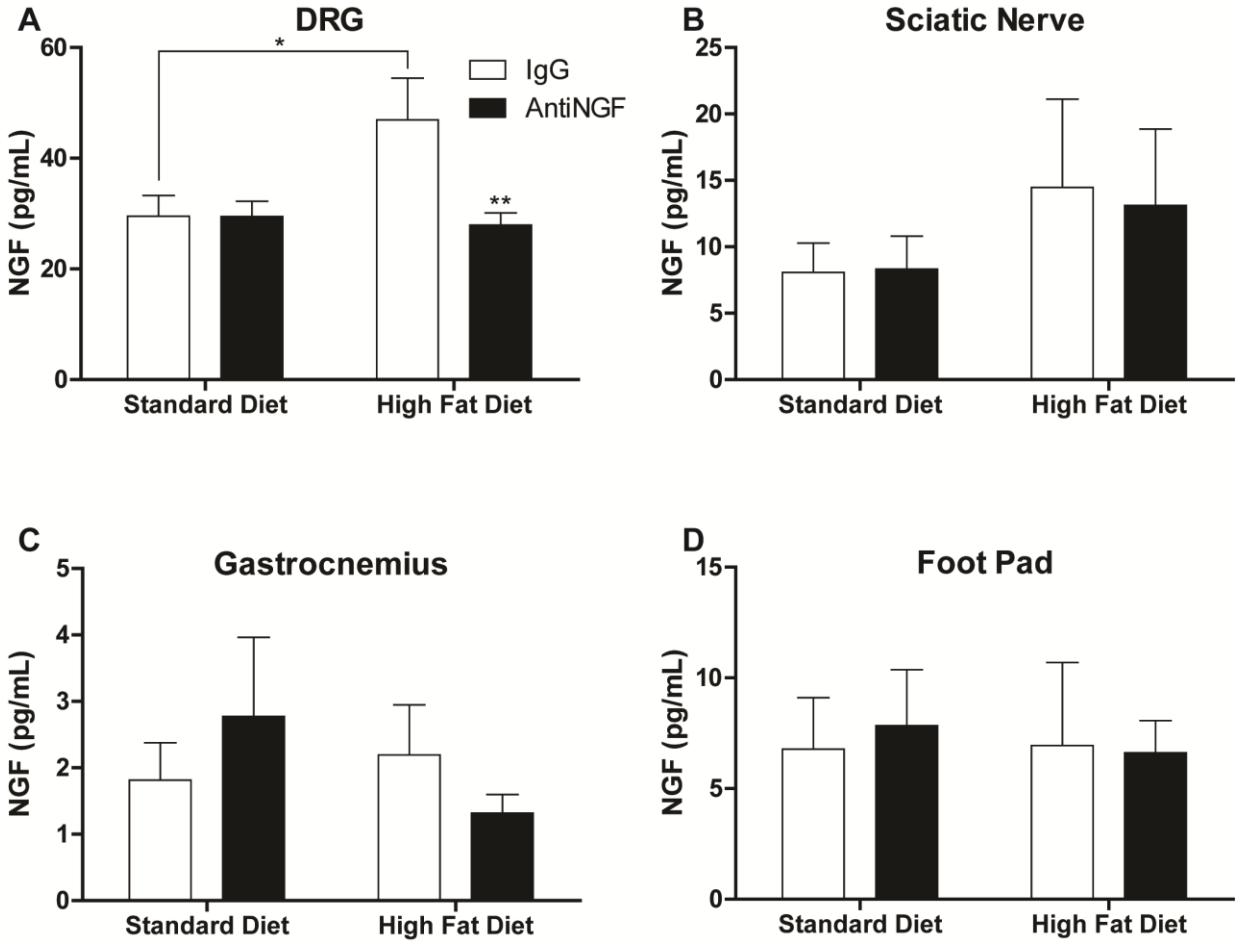
we examined TrkA fibers within the skin, it follows that we examined NGF, the ligand for TrkA, in these tissues. Our results show a 1.59-fold increase in NGF protein expression in the dorsal root ganglion of the HF-IgG mice compared to Std-IgG mice (Figure 4.5A). However, HF-NGF mice did not show this same increase in NGF protein expression and instead showed a significant 1.68-fold decrease in NGF protein expression compared to HF-IgG mice (Figure 4.5A). In looking at the sciatic nerve, we saw no changes in NGF protein expression due to either a high-fat diet or anti-NGF treatment (Figure 4.5B). We then wanted to look at non-neuronal tissues, including the gastrocnemius muscle and the hind paw skin. Our results demonstrate no statistically significant change in nerve growth factor protein expression in the gastrocnemius muscle (Figure 4.5C) and no change in expression in the hind paw skin (Figure 4.5D).

Discussion

In the current study, we demonstrate that high-fat diet induced prediabetes causes mechanical allodynia which is reversed by administration of an anti-NGF antibody. Additionally, high-fat diet induced prediabetes results in increased TrkA-positive cutaneous innervation and increased NGF protein expression in the dorsal root ganglia, both of which are normalized in mice treated with anti-NGF. These findings provide further evidence for the role that nerve growth factor is integral in pain sensation and painful prediabetes, and shows that by reducing NGF signaling systemically, abnormal innervation and protein expression can be prevented and reverse painful neuropathy.

Our study confirms previous studies in that mice fed a high fat diet develop signs of prediabetes, including increased weight gain and elevated fasting blood glucose levels [77, 305].

Figure 4.5: ***NGF Protein is Increased in the Dorsal Root Ganglia of High Fat Fed Mice:*** Quantification of nerve growth factor (NGF) in (A) DRG, (B) sciatic nerve, (C) gastrocnemius and (D) footpad. HF-IgG mice displayed an upregulation of NGF protein within the dorsal root ganglia, while HF-NGF mice showed a recovery to control levels (A). There was no alteration in the sciatic nerve (B) nor in the peripheral tissues, the gastrocnemius (C) and hind paw foot pad (D). All tissues were loaded with equal total protein concentrations. Data are presented as mean ± SEM. Std-IgG, n=9; Std-NGF, n=8; HF-IgG, n=9; HF-NGF, n=8. **P*<0.05, ***P*<0.01.


A
DRG
NGF (pg/mL)
60
40
20
0
*
**
IgG
AntiNGF
Standard Diet
High Fat Diet
B
Sciatic Nerve
NGF (pg/mL)
25
20
15
10
5
0
Standard Diet
High Fat Diet
C
Gastrocnemius
NGF (pg/mL)
5
4
3
2
1
0
Standard Diet
High Fat Diet
D
Foot Pad
NGF (pg/mL)
15
10
5
0
Standard Diet
High Fat Diet

However, as we did not test other parameters in the current study, the full extent of prediabetes could not be determined. Based on previous findings by our laboratory, it can be safely assumed that mice fed a high fat develop an impaired glucose tolerance and hyperinsulinemia [78, 305], yet results for nerve conduction velocity differ, with other studies' results showing no change in either sensory or motor nerve conduction velocity at 12 weeks [305], and changes in motor nerve conduction at 8 weeks [78] and both motor and sensory nerve conduction abnormalities at 16 and 22 weeks [77]. Furthermore, based on these results, decreasing NGF signaling systemically does not alter any of the metabolic parameters, with HF-NGF mice exhibiting the same degree of weight gain and blood glucose as the HF-IgG mice.

The current study demonstrates that mice fed a high fat diet develop mechanical allodynia, or a painful response to a normally non-painful stimulus, and is in accordance with other previous studies [77, 78, 305, 307]. Additionally, studies using obese models of type 2 diabetes have also demonstrated that rodents develop increased cutaneous sensation [74, 264], indicating obesity and dyslipidemia may play a significant role in the development of painful neuropathy [26, 308]. Importantly, our study also demonstrated the important role that NGF could play in mediating pain in the setting of prediabetes. After the establishment of mechanical allodynia, systemic administration of an anti-NGF antibody was able to reverse the cutaneous sensitivity, indicating that obese prediabetic mice may have an excess of NGF protein, an excess of TrkA receptors or increased NGF signaling in both the periphery and central processes . In the setting of nerve and spinal cord injury, local expression of NGF in the DRG neurons is considered to be important in the contribution of pain [309, 310]. In the setting of diabetic neuropathy, NGF gene expression was shown to be increased in the sciatic nerve of STZ-induced diabetes [311], as well as in the spinal cord, DRG, and hind paw skin of obese *db/db* type 2 mice

[264] during periods of mechanical allodynia. Our study indicates that mice fed a high fat diet develop mechanical allodynia, but that NGF might be playing a critical role in the development of the pain, as blocking NGF signaling results in a normalization of mechanical withdrawal thresholds.

Moreover, we found a significant increase in the number of TrkA-positive neurons in the hind paw skin of HF-IgG animals, but no increase in high fat-fed mice treated with anti-NGF. While there was an increase in the TrkA positive neurons, there was no change in the overall density of neurons in any mouse group as measured with the pan-neuronal marker PGP9.5. These results are similar to other findings showing no alteration in IENF density resultant from prediabetes [77], as well as results from a previous study in our laboratory, showing 12 weeks of high fat feeding results in increased TrkA expression in the periphery which is reversed by exercise [305]. In fact, it was based on this study's findings that exercise can normalize IENF densities that the current study stemmed from. Increased TrkA expression in the periphery is an important finding, and supports our hypothesis that increased NGF can modify the phenotype of cutaneous neurons. Because we show an increase in TrkA expression, but no change in overall fiber density, we hypothesize that the increase in TrkA expression is due to a neuronal phenotype switching, and not axonal sprouting, though it should be noted that we did not directly look at nonpeptidergic fibers due to a lack of good antibodies. TrkA is the high affinity receptor for NGF and is located on small, unmyelinated peptidergic neurons which express and release nociceptive neuropeptides including CGRP and substance P. While not investigated in the current study, Cheng *et al.* discovered increased substance P immunoexpression in the *db/db* DRG, indicating an increase in peripheral NGF causes an increase in nociceptive substance P expression in the more centrally located processes.

Importantly our results demonstrate only an increase in TrkA expression in the skin and not in NGF protein expression as found in the ELISA. Groover *et al.* demonstrated that following 12 weeks of a high fat diet, both TrkA immunoexpression and NGF protein were increased significantly over standard-diet controls. However, in the current study, no significant changes were found in peripheral NGF protein expression, while HF-IgG mice displayed elevated NGF expression in the DRG. These changes highlight the importance of timing in this process. Our data imply that timing is critically important and that expression and distribution of nerve growth factor fluctuates over time. Importantly, the footpad NGF expression did not recapitulate our previous findings, yet there was an increase in NGF in the DRG. This demonstrates the important role that the dorsal root ganglia play in sequestering NGF, most likely in an attempt to prevent neuronal degeneration from injured axons. Additionally, increased NGF in the DRG could be indicative of sympathetic neuronal "baskets" surrounding the sensory neurons [312-316]. Sympathetic baskets have been implicated in neuropathic pain, with noradrenergic sympathetic fibers extensively sprouting into the dorsal root ganglion. Sympathetic baskets are integral in painful behaviors as electrophysiological studies have shown primary afferent excitation in injured dorsal root ganglia following sympathetic stimulation [317-319], thought to be mediated in part by α2 adrenoceptors [320]. Additionally, neuropathy-related behaviors, such as allodynia, are correlated with the density of sympathetic sprouting and the number of baskets formed within the DRG [316, 317, 321], and surgical sympathectomy at the spinal nerve level alleviates mechanical and thermal hypersensitivities [322]. Importantly, NGF is known to contribute to sympathetic sprouting[323]. In fact, overexpression of NGF in the skin also results in the formation of baskets within the DRG [324]. Furthermore, administration of anti-NGF resulted in attenuation of painful behavior in chronic sciatic constriction injury in rats

and prevented the formation of sympathetic baskets [325]. It will be important to investigate this phenomenon in future studies to see if sympathetic basket formation plays a role in high-fat diet-induced neuropathy.

In conclusion, we demonstrate that a high-fat diet induces prediabetic-like symptoms, including painful neuropathy, which is reversed by systemic administration of anti-NGF antibody. A high-fat diet also changes the phenotype of cutaneous axons to that of a more peptidergic-like population, while not affecting overall fiber density in the epidermis. Furthermore, administration of anti-NGF is capable of preventing neuronal phenotype switching, which may be responsible for the normalization of mechanical withdrawal thresholds. Further studies need to be completed looking at fiber composition at the time of anti-NGF administration, as well as NGF expression. These data highlight the importance NGF plays in pain-like behaviors and warrants further investigation as a treatment for painful diabetic neuropathy.

CHAPTER 5

Conclusions

Findings and Summary

Diabetic neuropathy is the most common and incapacitating complication of diabetes and prediabetes. While the majority of patients will develop some form of neuropathy, it is not clear why some patients develop painful symptoms and others insensate neuropathy. Approximately one in four people with diabetes are afflicted by painful neuropathic pain and recent research reports that patients with prediabetes most often develop painful neuropathy, even before a clinical diagnosis of overt diabetes. Chronic painful diabetic neuropathy impacts patient quality of life, including mood, sleep, work and interpersonal relationships [326]. Unfortunately, neuropathic pain is difficult to manage. While many pharmacological treatment options are available, approximately 40-60% of patients only obtain partial relief of their symptoms [235]. Additionally, treatment for painful neuropathy is only palliative in nature and does not treat the cause of pain. Therefore, it is imperative to identify other treatments for the relief of painful diabetic neuropathy.

The overall purpose of this study was to examine the progression of painful diabetic neuropathy in type 2 diabetes and prediabetes, examine the role of exercise in alleviating neuropathic pain and identify a potential mechanism of exercise-mediated improvements in diabetes and prediabetes. Studies in this dissertation evaluated the effects of obesity and a high-fat diet on measures commonly used to diagnose diabetic neuropathy, including mechanical, thermal and visceral behavioral sensitivity measures, nerve conduction velocities, and epidermal innervation. Additionally, these studies utilized running wheels to examine the effectiveness of voluntary exercise to prevent and reverse metabolic, behavioral, epidermal abnormalities. Finally, experiments were performed to assess abnormal neurotrophism as a potential mechanism for the development of diabetic neuropathy induced by a high-fat diet.

Low physical activity, poor diet and excess body weight are closely associated with the development of both type 2 diabetes and prediabetes. Closely associated with poor diet, obesity and diabetes, dyslipidemia consists of increased triglycerides and free fatty acids, decreased HDL levels with HDL dysfunction, and slightly increased LDL levels (for a review, see [327]). Furthermore, dyslipidemia has been recently identified as an independent factor in the development of diabetic neuropathy and may explain the development of diabetic neuropathy in the prediabetic patients who do no exhibit overt hyperglycemia [328]. In these studies, we identified the effects of a high-fat diet on the development and progression of diabetic neuropathy and show how a high-fat diet may induce pain.

Findings from this study confirm previous results that C57Bl/6 mice fed a high-fat diet develop prediabetes and painful neuropathy [77, 78]. These results are clinically relevant, as most prediabetic patients present with painful neuropathy. However, these results do signify the importance of diet and lipid profile on the development on painful neuropathy. STZ-induced diabetic C57Bl/6 mice normally develop an insensate neuropathy, demonstrating a reduced response to mechanical stimuli. Previous reports from our laboratory demonstrated that STZ-induced diabetic mice fed a high-fat diet in fact switched from an insensate neuropathy to a painful neuropathy [78], and these results further confirm the role that diet plays in modulating diabetic neuropathy. Many previous studies focused on mechanical and thermal sensitivities. Importantly, our results looked not only peripherally at cutaneous sensation, but we also investigated visceral sensitivity and discovered that a high-fat diet induces colorectal hypersensitivity. Few previous reports have demonstrated visceral hypersensitivity in STZ-induced type 1 diabetes rodent [274]. We are the first group to extend these findings to prediabetes. This finding is significant because in addition to patients developing peripheral

neuropathy, many diabetic patients also develop autonomic neuropathies which may result in gastroparesis, incontinence, and visceral pain. Our results suggest that in addition to peripheral afferent neuron dysfunction, as demonstrated by reduced mechanical withdrawal thresholds, visceral afferent nociception may also be impaired by prediabetes leading to visceral hypersensitivity. However, our results differ from other studies in which a high-fat diet has been shown to induce thermal hypoalgesia [77, 297]. Thermal paw withdrawal latencies were not affected by a high-fat diet at either 8- or 12-weeks in the current studies. It is possible that with longer studies, possibly 16 or 22 weeks, that we would see changes in thermal sensations. Another possible reason we did not observe changes in thermal latencies is the mechanism of heat testing. There are different thermal nociceptors that are activated at different heat thresholds beyond the threshold for pain perception (approximately 45˚C) [329, 330]. It is possible that the heat source and the rate are not testing the correct thermal nociceptors and therefore we are not observing changes. Additionally, the dichotomous relationship of mechanical allodynia and the "normal" thermal hypoalgesia demonstrates that the nerves conveying each sensation are differentially damaged in diabetes and prediabetes.

It is interesting to note that while mice fed a high-fat diet do develop prediabetes, the actual clinical diagnosis of diabetic neuropathy is probably not appropriate in the following studies. Clinical diagnosis of diabetic neuropathy includes a combination of symptoms, including not only quantitative sensory test, but also nerve conduction velocity studies and skin/nerve biopsies. Nerve conduction velocities are considered the gold standard of clinical diagnosis, yet we did not demonstrate any change in nerve conduction velocity in our high-fat diet induced-prediabetic mice. Moreover, we were also not able to detect changes in overall intraepidermal nerve fiber density. The most plausible explanation for the lack of changes in nerve conduction

velocity and epidermal innervation lie in the length of the study design. In another study using a high-fat diet to induce prediabetes, nerve conduction deficits were seen at 16- and 22-weeks post-diet intervention [77]. Therefore, longer presentation of prediabetes may be necessary to see overt changes in nerve conduction velocities.

Another significant finding in our studies was that a high-fat diet may be capable of changing the epidermal neuronal phenotype. Using immunohistochemistry and the pan-neuronal marker PGP9.5, we did not observe any loss in total epidermal innervation. These findings are in accordance with other previous studies in rodents [77], as well as clinical investigations in patients with metabolic syndrome [331], yet the apparent loss of epidermal innervation is still controversial [26, 331]. The finding that total innervation was not increased in prediabetic mice is significant because it suggests that there is no axonal sprouting occurring in the epidermis following nerve injury. Additionally, this datum suggests that total innervation density may not be as important as previously thought; however, alterations in phenotype of epidermal neurons may be. Significantly, our results show that high-fat fed mice, which display cutaneous, mechanical allodynia, have a significantly elevated proportion of peptidergic-to-nonpeptidergic nerve type. These results are consistent in findings of type 2 [264, 279] diabetes where peptidergic neuronal subtypes have been found to be increased in mice. Moreover, previous findings have shown a loss of peptidergic innervation in the skin in a model of type 1 diabetic insensate neuropathy, while showing no loss of total epidermal innervation [280]. There is a normal balance of peptidergic and nonpeptidergic neurons innervating the skin, and it is these fibers that are responsible for conveying nociceptive signals. These results suggest that an alteration in the subtypes of cutaneous axons may be sufficient to drive the development of either

painful or insensate neuropathy, and may also explain why some patients have normal intraepidermal nerve fiber densities while displaying sensory symptoms.

In the previous studies, nonpeptidergic innervation was not directly investigated. Future studies should employ the use of Mrg-D+ mice to investigate what happens to the nonpeptidergic neuron population and to determine if the change in epidermal innervation was due to a *de novo* expression of TrkA in nonpeptidergic fibers and a phenotype switching or due to axonal sprouting of existing TrkA positive neurons. Because the nonpeptidergic neurons are GFP-tagged in the MrgD+ mice, GFP-expressing fibers would be a direct measure of nonpeptidergic fibers. The A/J mouse line develops mechanical allodynia following STZ-induced diabetes, and increased peptidergic epidermal innervation would provide additional support to our hypothesis that ratio of neuron subsets helps drive painful neuropathy. Besides addressing diabetic neuropathy, it would be prudent to investigate other neuropathic pain models, including HIV-induced neuropathy, chemotherapy-induced peripheral neuropathy, and even idiopathic neuropathy and the innervation phenotype in each and how diet and lipid profiles affect different neuropathic pain conditions. Additionally, because a high-fat diet/dyslipidemia is capable of inducing behavioral and epidermal innervation changes, investigation into the effects of lipid lowering drugs could provide interesting insight.

As stated previously, low physical activity is a major contributor to the development of type 2 diabetes and prediabetes. Exercise has long been "prescribed" for these obese, diabetic patients in an effort to lose weight and help improve their metabolic parameters. In fact, one prospective study showed that walking at least two hours per week as associated with a decrease in the occurrence of premature death by 39%-54% due to any cause, and 34%-53% due to cardiovascular disease among diabetic patients [332]. However, exercise may be far more useful

than just improving metabolic parameters. Exercise has been shown to be useful in reversing pain conditions, including fibromyalgia, osteoarthritis, sciatica, and even diabetic neuropathy [92, 333-336]. However, exercise-induced mechanisms of pain relief remain elusive. In these studies, we investigated how exercise changes the development and progression of diabetic neuropathy and identified a possible mechanism.

Results from these studies confirm the use of exercise as a treatment option for painful diabetic neuropathy. Results from the anti-NGF treatment study indicate that physical activity is capable of ameliorating mechanical allodynia associated with a high-fat diet. Two weeks after beginning a high-fat diet, the high-fat fed mice exhibited an increase in mechanical sensitivity, and the sedentary mice had allodynia which persisted through 12 weeks. However, mice which had free access to running wheels began to show mechanical sensitivity improvement beginning at 8 weeks and which persisted through 12 weeks. These results are consistent with other studies where exercise reversed neuropathic pain-like behaviors in chronic constriction injury and spinal cord contusion models[85, 113, 337]. It is interesting to note that exercise did not prevent the onset of mechanical allodynia. Exercise began at the same time point as the dietary intervention in the second study, yet mechanical allodynia developed and was maintained through 6 weeks of study. Another point of interest is that exercise did not significantly improve any of the metabolic parameters associated with prediabetes. Often, improvements with exercise are believed to be associated with an overall improvement in wellbeing. However, our study demonstrates that exercise can improve behavioral outcomes without improving overall wellbeing, as high-fat fed exercised mice were still overweight, displayed hyperinsulinemia, increased blood glucose levels and impaired glucose intolerance. It would be sensible to measure lipids in these animals. While their overall metabolic parameters did not significantly

improve, perhaps the lipid panel was improved, which would further confirm that dyslipidemia is an important risk factor in the development and maintenance of diabetic neuropathy.

Another significant finding in our study was the visceral hypersensitivity was not present in the exercised group of animals. We cannot say at this time if exercise prevented or reversed the occurrence of visceral hypersensitivity, as only a terminal measurement was obtained. Future studies should investigate visceral sensitivity early on in the study and continue in order to determine if exercise prevents or reverses the hypersensitivity. These results are significant because autonomic dysfunction is a real and devastating complication of diabetes, which can include ortostatic hypotension, gastroparesis, hypoglycemic unawareness, resting tachycardia, and silent myocardial ischemia (for a review, refer to [338]). Our results show peripheral and visceral sensory dysfunction can be ameliorated by exercise, and further may support the notion that exercise can ameliorate autonomic dysfunction, with special care to those affected with cardiovascular exercise intolerance.

In addition to attenuating nocifensive behaviors, exercise normalized the increased TrkA fiber count in the hindpaw skin. Increased TrkA expression in the epidermis has been shown to be increased in painful neuropathy, as previously stated. It is therefore significant to find that the increase in TrkA fibers is normalized following exercise and correlates with mechanical withdrawal thresholds. We are one of the first groups to show that exercise can normalize the phenotype of epidermal neurons following prediabetes. The findings from our studies demonstrate that a high-fat diet causes an increase in peptidergic nerve fibers at 12 and 8 weeks, respectively, with chapter 3 showing a reversal in behavior beginning around 8 weeks. It is plausible that nerve phenotype switching begins around 8 weeks, and that part of the role of exercise is to prevent this nerve switching and therefore prevents the ongoing mechanical

allodynia. Additionally, it is important that our results showed no change in overall nerve fiber density due to either prediabetes or exercise. Reduced epidermal nerve fiber density is believed to contribute significantly to the development of neuropathic pain, mostly due to the dying back of neurons. In one study, diet and exercise intervention was used on prediabetic patients and following 1 year of intervention, intraepidermal nerve fiber density was increased significantly and correlated with improvement in pain using the visual analog scale (VAS) [27]. However, in this study, epidermal phenotype was not evaluated, only total innervation. Conversely in our study on prediabetic mice we show that prediabetes does not cause this dying back phenomenon, even in the presence of pain, and that exercise alone did not increase the nerve fiber density as shown by exercise controls. Again, while total innervation may be important in pain signaling, we hypothesize that it is the balance of peptidergic to nonpeptidergic neurons that make the biggest difference and that exercise is capable of maintaining this balance.

Exercise has been purported to be analgesic in a number of different ways. The most commonly tested mechanisms involve the release of endogenous opioids at central, spinal and peripheral sites [98, 101]. However, most opioid-induced analgesia following exercise is of a short duration, not long lasting, as seen in our results. Other common mechanisms associated with exercise-induced analgesia involve the descending serotonergic pathways and neurotrophin availability. Serotonin is known to play a role in descending inhibition of pain processing. Rivot *et al.* were the first group to report analgesia was induced by electrical stimulation of the rostral ventromedial medulla and was accompanied by serotonin release in the spinal cord [339]. Additionally, one study demonstrated the role of serotonin in exercise-induced analgesia by showing a reduction in abdominal contraction following IP acetic acid injection was attenuated by pretreatment with an inhibitor of serotonin synthesis [340]. One other common mechanism is

the alteration of neurotrophin synthesis and expression. Many studies have shown increases in production of neurotrophins following exercise in both naïve and injured animals, and exercise after central and peripheral nervous system damage can restore neurotrophin levels [113, 114, 120, 121]. These restorations in neurotrophin levels are important in the setting of diabetic neuropathy because decreased neurotrophic support is believed to be a key mechanism of pain. Our results in Chapter 2 support this claim, as type 2 diabetic mice displayed decreased GDNF expression in the hindpaw skin. Yet, we could not identify an increase in GDNF expression due to the low bouts of exercise. However, in the setting of prediabetes, we did not see these same decreases in GDNF expression in any tissue examined. It therefore appears that prediabetic neuropathy is not the result of decreased neurotrophic support like overt diabetes, but may in fact be a result of overtropism of NGF.

Our results are the first to demonstrate an increase of NGF in the DRG at 8 weeks and in the footpad at 12 weeks in prediabetes, along with an increase in TrkA expression in the epidermis. These results are consistent with other findings in type 2 diabetes [264, 279], but the first to show a similar result in prediabetes. We demonstrated that a high-fat diet caused significant increases in NGF that were normalized by exercise. As NGF is a significant player in nociceptive signaling, these results are important. In order to further investigate the role of NGF in producing mechanical allodynia, high-fat fed mice were given an anti-NGF antibody, which was also capable of reversing allodynia and normalizing NGF protein expression. Interestingly, the distribution of increased NGF protein levels were not consistent, at 8 weeks we see increases within the DRG while at 12 weeks we see increases in the epidermis, as well as the gastrocnemius. These results highlight the importance of timing and possibly a role in axonal transport. It is possible that at the earlier time point, 8 weeks, there is damage to the neuron

leading to overcompensation in NGF production and shunting to the DRG in order to help the neuron survive. However, at later time points, the overproduction of NGF in the periphery can no longer be shunted to the DRG due to axonal transport dysfunction, leading to an increase in peripheral NGF.

These increases in different tissues may result in different mechanisms of nociception, which could work separately or in concert. Increases in NGF in the periphery, along with increased TrkA may cause a chronic nerve firing, resulting in peripheral sensitization. This could account for both immediate and long term signaling. NGF binding to TrkA results in local PI3K activation [341], and internalization of the NGF/TrkA complex, which is retrogradely transported back to the cell body to activate CREB and Erk-5 signaling [342]. Increased NGF in the periphery could also contribute to excessive mast cell degranulation, release of pro-inflammatory cytokines, and peripheral pain [151, 152]. Centrally, increased NGF and NGF signaling is most likely causing an increase in the neuropeptides CGRP and substance P. CGRP and substance P are released from peptidergic neurons and mediate nociceptive signaling. While we did not examine neuropeptide expression, Cheng *et al.* showed increased gene expression in these neuropeptides along with increased NGF expression during the time of mechanical allodynia in type 2 diabetic mice [264]. Increased substance P enhance nociception by activating the neurokinin 1 receptor on secondary sensory neurons within the spinal cord; yet a neurokinin 1 antagonist failed to show improvement in painful diabetic neuropathy [343], implying increased neuropeptides are not the only mechanism for enhanced nociception.

Clinical Implications

Treatment of painful diabetic neuropathy remains a significant problem today. Therapeutic options include opioids, antidepressants, and anticonvulsants, all which come with a wide reaching range of side effects and which have limited success in alleviating pain. Our results highlight the use of voluntary exercise as a non-pharmacological treatment for painful diabetic neuropathy. Used alone or in combination with other therapies, there is good evidence for the alleviation of pain in diabetic and prediabetic patients. Clinical evidence supports the use of exercise in diabetic neuropathy. Besides addressing pain concerns, exercise can improve upon the overall health of the individual, improving glycemic control and weight management. Additionally, exercise can be considered more than a palliative treatment option for pain, as exercise has been shown to improve intraepidermal innervation in those patients with epidermal loss [27, 92]. It is important to note however, that before beginning any exercise program, patients need to consult with their physicians. Patients with pain may also experience numbness, balance problems and gait abnormalities, which may complicate any exercise and which could lead to falls and other injuries.

Another important finding in our study that can be directly related to the clinic is the alteration of epidermal nerve phenotype. IENF density is a common clinical component to diagnosing diabetic neuropathy. Many patients, however, have pain yet do not exhibit loss of epidermal innervation. Our results suggest that clinicians should look at more than just overall epidermal innervation, but examine the phenotype of the innervating axons. Our results indicate that epidermal phenotype may be of better predictive value than overall epidermal innervation. If these changes in epidermal phenotype begin early in disease progression, early identification could help to provide better therapeutic targets to curb the progression of diabetic neuropathy.

We suggest that clinicians should not only examine total epidermal innervation using PGP9.5 as a pan-neuronal marker, but also quantify TrkA axons in the hopes of better diagnosing and treating diabetic neuropathy.

Finally, our results support the further investigation and use of anti-NGF in the treatment of diabetic neuropathy. Interestingly, the anti-NGF human monoclonal antibody tanezumab, by Pfizer has been investigated in multiple painful settings. It was used in the treatment of osteoarthritis, chronic low back pain and even painful diabetic neuropathy. However, these clinical trial investigations were halted in 2010 due to reported cases of osteonecrosis in patients with osteoarthritis. However, in 2012, an independent adjudication committee determined that only two of 87 cases were treatment-induced osteonecrosis and the U.S Food and Drug Administration (FDA) then approved the continued development of the drug as long as certain safety procedures were followed. Early studies, however, did show analgesic effects from tanezumab compared to placebo in interstitial cystitis and chronic low back pain [344, 345] . The study involving diabetic neuropathy patients was orignially terminated due to safety concerns, but due to the approval from the FDA to continue testing, diabetic neuropathy may have a new drug target for the treatment of pain. However, long term treatment may not be the best course of action and later stages of neuropathy have shown reductions in NGF levels. Anti-NGF treatment in patients with low NGF levels in the first place may suffer from progressive fiber loss, complete loss of feeling and perhaps even motor dysfunction.

Future Directions

There are many directions to take this study in the future. Inflammation has been previously examined in diabetes and now it is accepted that in type 2 diabetes, there is a chronic,

low-grade inflammation (for a review, see [290]). IL-1β and TNFα are intimately associated to nerve growth factor, whereby both pro-inflammatory cytokines can increase synthesis and release of NGF. One interesting study would be to administer an anti-inflammatory agent over the course of the study to see if there is an increase in NGF and TrkA expression in the hindpaw. If pro-inflammatory mediators could be prevented from signaling, perhaps the feed-forward cycle between NGF, mast cell degranulation, and inflammation could be stopped. It would also be interesting to look at primary cell cultures of prediabetic DRG to investigate if inflammatory mediators such as IL-1β and TNFα could induce an overexpression of NGF and increase TrkA expression on the neurites. We believe that the inflammatory status of obese type 2 and prediabetic mice play a significant role in the upregulation of NGF and that exercise may be acting as an anti-inflammatory mechanism. Previous literature has shown alterations in inflammatory mediators in diabetes, but the upregulation of these mediators needs to be more fully examined in the prediabetic, high-fat fed model. It would also be a good experiment to have conditional NGF knockout mice where the expression of NGF in the epidermis alone could be prevented, rather than a systemic anti-NGF administration in the current study. Perhaps a knockout of NGF in the epidermis could prevent TrkA upregulation in the axons and prevent painful neuropathy.

Exercise caused a significant reversal in withdrawal thresholds. It would be an interesting study to examine an "exercise threshold" and prevent the mice from running at will. A preliminary study in our lab has shown that limited voluntary running did still attenuate the allodynia, though no controls were assessed and the sample size was very limited (Unpublished data, Cooper 2013). Finding beneficial effects in limited exercise would be greatly valuable to patients with limited mobility.

The anti-NGF treatment study needs to be evaluated further. We investigated changes in NGF levels and IENFD a weeks after the last anti-NGF administration. This study needs to evaluate these changes at different time points, including 5 weeks, before the administration of anti-NGF, and at 6 and 7 weeks to identify changes that are occurring during treatment. It would also be a good study to administer anti-NGF at the beginning of the study in order to prevent the occurrence of painful neuropathy in the first place and then identify neuronal changes within the epidermis and perhaps basket formation in the DRG. Finally, administration of anti-NGF antibodies should be given at later time points, around 12 weeks, when we know that epidermal innervation phenotype has changed and examine if treatment is still able to reverse phenotype switching and alleviate nocifensive behaviors.

Conclusions

This body of work extends the current body of work in the field of exercise that exercise is capable of attenuating neuropathic pain, specifically painful diabetic neuropathy caused by a high-fat diet and dyslipidemia. Additionally, this work suggests a mechanism in which painful neuropathy can arise, the axonal phenotype switching to that of a more peptidergic population and that exercise can prevent and or reverse the switching. Our studies also provide further evidence that NGF is critical in the nociceptive pathways and that by reducing NGF signaling, we may be able to effectively alleviate patients' pain. This body of work strongly suggests that exercise, along with anti-NGF treatment could be a viable treatment option for those with painful neuropathy and may do more than just treat the pain, but perhaps prevent and reverse the axonal damage leading to neuropathy in the first place.

CHAPTER 6

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