A KETOGENIC DIET AND PHYSICAL ACTIVITY'S EFFECT ON PERIPHERAL NERVE FUNCTION

BY

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Abstract

Obesity and metabolic syndrome are a rising worldwide epidemic and have driven a subsequent increase in prediabetes and type 2 diabetes. We now know that patients who have prediabetes will begin to develop debilitating alterations in sensation due to early damage of peripheral sensory axons. There have been few therapies proposed to help patients developing metabolic syndrome induced painful neuropathies and those that have been developed often exhibit minimal benefit. While the mechanisms of prediabetes-induced peripheral neuropathy are still poorly understood, previous work has been able to show there is a significant benefit from physical activity that improve abnormal sensation and pain.

Early studies in this work demonstrate that exercise alters the metabolic status of peripheral neurons and can normalize heightened mechanical sensitivity induced by a high fat diet. Utilizing a genetic rat model of differing intrinsic aerobic levels, our results show that genetic differences that result in varied metabolism alters peripheral nerve function and sensation. Highlighting the importance of metabolism, our studies show that exercise increases the utilization of fat based fuels, providing a novel mechanism for improved sensation. We performed studies in mice utilizing a ketogenic diet that is high in fat and low in carbohydrates to push fat utilization in nerves. These studies revealed that consumption of a ketogenic improved abnormal peripheral sensation. Contrary to exercise or a control diet, a ketogenic diet was able to prevent and also reverse high fat diet induced mechanical allodynia. In addition to improved sensation, a ketogenic diet

increased axonal growth *in vitro* and *in vivo*. This exciting result of improved nerve growth has the potential to benefit millions of patients worldwide experiencing neuropathy and pain due to axonal degeneration.

Acknowledgments

I would like to thank my thesis committee, Dr. Doug Wright, Dr. Julie Christianson, Dr. Paige Geiger, Dr. John Thyfault, and Dr. Ken McCarson for serving on my committee and for all they have taught me throughout my time at the University of Kansas Medical Center. I am especially thankful for my advisor Dr. Wright, without his willingness to accept a student taking an enormous leap into the world of basic science and his continued strident efforts to help me succeed not just academically but to ensure I grow as both a student and in my future career, if not for him I would not be in the position I am today. I also want to thank all of the students who have had the willingness to help share with me their knowledge, without the help of the Christianson, Thyfault, and Geiger lab I would have never succeed as I have. Additionally, I would like to thank Al and Lila Self, for their foresight and generous donation to establish the Self Graduate Fellowship, without their funding my entire career would be a different path. If not for their philanthropy I would have been unable to continue my passion examining the utilization of exercise in new and emerging diseases. The training I received from their fellowship will benefit me going forward in more ways than I can begin to describe in a single page. Lastly, I would like to thank my amazing wife Christina for her unending support through the successes and failures I experienced on this wonderful journey obtaining my degree with many a long work filled years, without her support this would not have been possible.

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Chapter 1

Introduction

Twenty-five million Americans are encumbered by acute pain and over 50 million suffer from varying chronic pain syndromes, leading to a medical cost of over $635 billion a year [1]. This enormous health plight highlights the need to find novel interventions to reduce the burden of chronic pain. Generally speaking, chronic pain includes changes in peripheral tissues, such as the hands and feet, and the central nervous system, which often leads to even more debilitating and chronic effects disease progresses [2,3]. The perception of pain is a broad and complex mechanism to study, having multiple origins including nerve damage, metabolic disease, and numerous others. Each form of pain may be unique not only in its development but also in the treatments necessary to provide relief. Unfortunately, current therapies available for the treatment of these pain states are still associated with pain abatement and do not address underlying mechanisms driving the development of varying forms and levels of sensory discomfort [4].

Physical activity offers a wide array of benefits and is well documented to help in a myriad of diseases, however the specific mechanisms by which exercise exerts its benefits are poorly understood in the nervous system. The complexities of understanding how global cross organ communication and changes induce molecular changes to provide benefits in disease makes exercise research often hard to perform on a basic level. However, the clear benefits of exercise provide a strong rational to continue to study this complex intervention.

Nociceptive and neuropathic pain syndromes both receive physiological and behavioral benefits from exercise intervention, even though they are thought to have separate physiological characteristics. Nociceptive pain results from an expected noxious stimulus, while neuropathic pain occurs in the absence of a stimulus, or with a normally innocuous stimulus. The neuronal pathway of nociceptive pain starts with a noxious stimulus detected by a peripheral sensory peripheral terminal of an Aδ- or C-fiber. The electrical signal is then propagated up through spinal and thalamic pathways to terminate in an appropriate somatotopic region of the cortex [5]. In the case of neuropathic pain, adaptations occur in Schwann cells, satellite cells, the peripheral immune system, spinal microglia, and astrocytes that lead to the development of a painful syndrome when one would not normally exist [6]. Important areas to examine in these pain pathways are interneuronal interactions and the molecular and cellular changes that are initiated within them. This is an important aspect of any therapeutic target for pain due to the activity-dependent neuronal plasticity that occurs in the nervous system [7].

In response to new information about neuronal activity-dependent plasticity, a new and rapidly growing area within both pain research and neural physiology has begun to examine the effects of exercise on peripheral and central nervous system components. However, the scarcity of well-controlled basic research in this area hampers the utilization of exercise as a therapy for neuropathic and other chronic pain syndromes. While exercise intervention is growing quickly as a clinical therapeutic tool for many diseases, its use to reduce pain states is still relatively new and the research available leaves an incomplete picture of the molecular pathways affected. Continued research

therefore is vital to gain a better understanding of how exercise benefits the management of various pain syndromes and for the implementation of this therapeutic technique on a broader scale by physicians.

Sensory Pathways Sensitive to Exercise

A well-established effect of exercise is its firing of afferent sensory nerves from contracting muscles to the spinal cord. Signaling in sensory fibers of working muscles is increased during exercise and provides important feedback on the cardiovascular and respiratory systems during physical activity [8]. One example of afferent nerve activity affected by exercise is the exercise pressor reflex (EPR), which is responsible for the control of blood pressure and heart rate (HR) changes during physical activity through sympathetic nerve activation [9,10]. This reflex is partially mediated by the transient receptor potential vanilloid 1 (TRPV1) receptor, the sensory receptor respondent to capsaicin that is stimulated from temperature and pH level changes [11]. Similar to TRPV1, the acid sensing ion channel 3 (ASIC3) found on sensory nerve terminals in active skeletal muscle is involved in the regulation of arterial pressure through the EPR [12]. However, EPR is additionally modulated by sodium channel (Na_v) function; these channels in turn, may be modulated through reactive oxygen species levels in the dorsal root ganglion (DRG) [13]. The EPR sensory pathway intersects with known painful sensory pathways through the TRPV1, ASIC3 and sodium channel alterations; this cross talk can provide possible avenues by which exercises known benefits may also connect to painful sensory states.

Studies now demonstrate that exercise can induce molecular and cellular changes in DRG sensory neurons. The benefits of exercise can appear quickly as evidenced by improvements in regeneration after nerve injury following 3 - 7 days of exercise [14]. This effect may be related to increased production of a number of molecular signals, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), synapsin I (SNAP1), and growth associated protein 43 (GAP43) in sensory ganglia, thereby stimulating axonal growth [14,15]. Consistent with this idea, exercised animals display alterations in molecular mediators in their DRGs. Large DRG neurons undergo changes in mRNA expression that are associated with neuronal plasticity and apoptosis in response to prolonged exercise, including higher BDNF, NT3, SNAP1, and GAP43 mRNA levels compared to sedentary animals [16]. Exercise of animals that have been given a high fat diet suggests that exercise can reverse alterations in neurotrophin expression that are associated with a high fat diet, insulin resistance and pain [17]. For example, exercise of diabetic mice induces significant increases in GDNF protein in the spinal cord and sciatic nerve, along with axonal transport in the sciatic nerve (Wright, unpublished observations).

Our own studies suggest that exercise can induce important phenotypic changes can occur in peripheral terminals of epidermal axons [17]. A high fat diet increases the number of epidermal axons that express tropomyosin receptor kinase A (TrkA), the high affinity receptor for nerve growth factor (NGF). This phenotypic change in peripheral axons corresponds to an increase in pain thresholds of the mice. Importantly, however, continuous exercise reverses this phenotypic change and normalizes pain thresholds [17].

Finally, Schwann cell proliferation is increased following exercise and may play an important role in the increase in axonal regeneration necessary for appropriate response to peripheral nerve injury The benefits seen with peripheral nerve regeneration are significant enough to improve functional and morphological markers of nerve and motor function post exercise [18]. These studies bolster the idea that axonal regeneration responds positively to exercise.

Exercise's benefits are not only limited to the periphery, as they also display a substantial value to the central nervous system. The numerous benefits of exercise on both the peripheral and central sensory nervous system are highlighted in Figure 1. Centrally, the brain imparts bi-directional control of pain processing and pain modulation that alters the transmission and perception of pain and sensation [19]. The effects of physical activity on this system are grossly understudied and this important central modulation of pain and sensation would benefit by continued examination of the metabolic, inflammatory, and ionic changes within the CNS.

Although only a few studies have been published, regular physical exercise has been reported to prevent the development of chronic muscle pain and exercise induced muscle pain. Postulated mechanisms include reducing phosphorylation of the NR1 subunit of the N-methyl-D-aspartate (NMDA) receptor in the brainstem, modulating nociception and individual experiences [20]. An additional potential mechanism is the nitric oxide/cyclic GMP pathway as both aerobic and resistance exercise increase circulating

nitrate levels which in turn provides an antinociceptive benefit during physical activity [21-23].

Centrally, exercise increases the endogenous opioid content in brainstem regions important in pain modulation, suggesting that exercise-induced reversal of neuropathic pain may include an up-regulation of endogenous opioids [24]. This may be a key analgesic mechanism as patients with chronic pain display a reduced endogenous pain inhibition system and creating an imbalance between pain modulation systems [19]. This highlights another benefit of exercise whereby it can increase endogenous analgesic systems known to be critically important in modulating pain. However, the endogenous opioid system however has been disputed in its role in modulating internal antinociceptive effects during physical activity [25,26]. This group has instead suggested the endogenous endocannabinoid system is playing a prominent role in the antinociceptive benefits of exercise [27,28]. There is a definitive need to explore these endogenous systems that are sensitive to exercise and play a prominent role in anti-nociception.

The primary benefits of physical activity may have an additive effect when paired with pharmacological interventions. The osteoporosis drug risedronate combined with treadmill running decreases sensory nerve calcitonin gene-related peptide (CGRP) expression when compared to rats receiving only the drug [29]. In conclusion, while exercise is often thought of as a preventative intervention, there also appears to be a clear benefit after injury. The benefits of exercise affect the nervous system at multiple levels and multiple sites associated with sensory function. Emerging evidence is also revealing

the molecular pathways that seem sensitive to, including axonal growth, altered neurotrophin levels, and phenotypic changes in both the periphery and central components of the nervous system.

Figure 1: Overview of the positive changes induced by physical activity that benefit sensation.

Cooper et al. 2016, Emerging Relationships between Exercise, Sensory Nerves, and Neuropathic Pain *Frontiers in Neuroscience*

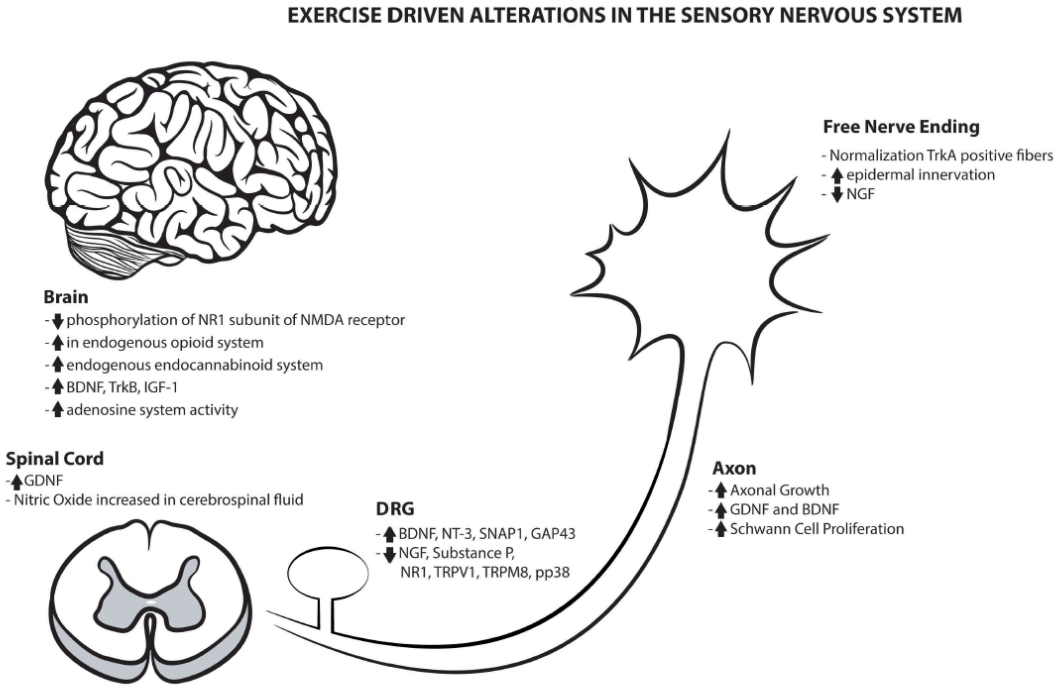

Figure 1.1 Exercise Driven Alterations in the Sensory Nervous System

Experimental Studies of Neuropathic Pain and Exercise

Many of these studies discussed above have centered on the ability of exercise to alleviate neuropathic pain associated with diabetes, as painful diabetic neuropathy (DPN) is an optimal condition in which exercise can have widespread benefits [30]. Table 1 highlights studies in rodents in which exercise was used as an intervention to treat pain. A delay in onset of diabetes-associated neuropathy with continuous exercise may be associated with changes in calcium channel function in the DRG allowing for an alteration in nociceptive signaling from the periphery [31]. Additional benefits such as increased motor nerve conduction velocities occur in diabetic patients, modifying or delaying the natural course of diabetic peripheral neuropath [32]. The mechanism by which exercise alleviates neuropathic pain is still unknown and may be the combination of many changes driven by physical activity. This may especially be true in instances where exercise has benefits in differing forms of neuropathy and/or allodynia.

TABLE 1 | Summary of various rodent studies addressing sensory dysfunction associated with pain.

Species	Mode of exercise	Pain model	Benefit	References
Rat	Forced running	Skin/muscle incision	↓ Substance P, TNF-α, IL-1β	Chen et al., 2013b, 2014
		Chronic muscle pain	↓ NR1 phosphorylation	Sluka et al., 2013
		Spinal cord injury and acidic saline	↓ Mechanical allodynia	Hutchinson et al., 2004; Sharma et al., 2010
		Sciatic nerve constriction	↓ Heat hyperalgesia and cold allodynia	Chen et al., 2012
		Sciatic nerve crush	↑- Schwann cell proliferation	Seo et al., 2009
		Lumbar spinal nerve ligation	↑- Endogenous opioids	Stagg et al., 2011
		Sciatic nerve cut	↓ NGF and BDNF	López-Álvarez et al., 2015
		Sciatic nerve cut	Normalized NKCC1 regulation	López-Álvarez et al., 2015
		Osteoporosis	↓ CGRP fibers in bone	Orita et al., 2010
		Paclitaxel-induced neuropathy	↑- Epidermal axon innervation	Park et al., 2015
		Streptozotocin	↑- HSP72	Chen et al., 2013a
		Streptozotocin	↓ TRPM8, TRPV1, and pp38	Yoon et al., 2015
		Acute antinociception	Activated endogenous cannabinoid system	Galdino et al., 2014b
		Acute antinociception	Activation of nitrous Oxide/cGMP pathway	Galdino et al., 2010a, 2015a
Rat	Swimming	Nerve constriction and inflammation	↓ Mechanical allodynia and heat hyperalgesia	Kuphal et al., 2007; Chen et al., 2012
		Streptozotocin	↓ TNF-alpha and IL-1β	Yoon et al., 2015
		CRPS type I	↑- Adenosine	Martins et al., 2013
Rat	Resistance exercise	Acute antinociception	Activated endogenous cannabinoid system	Galdino et al., 2014a
		Acute antinociception	Activated nitrous oxide/cGMP/KATP pathway	Galdino et al., 2015b
Mouse	Running wheel	High fat diet/pre-diabetes	↓ Mechanical allodynia	Groover et al., 2013
		High fat diet/pre-diabetes	↓ TrkA positive fibers	Groover et al., 2013
		High fat diet/pre-diabetes	↓ NGF, ↑- BDNF	Groover et al., 2013
		Nerve crush	↑- BDNF, NT3, GAP43, and SNAP1 (mRNA)	Molteni et al., 2004
Mouse	Treadmill	Sciatic nerve crush	↑- Nerve regeneration	Bobinski et al., 2011

A range of species, modes of exercises, pain model, and primary outcomes are provided.

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Table 1.1 Summary of various rodent studies addressing sensory dysfunction associated with pain

In a type I rodent model of DPN, running is able to reduce several modalities of allodynia, including mechanical, cold, and heat hyperalgesia. Key molecular markers such as TRPV1 (heat) and transient receptor potential cation channel subfamily M member 8 (TRPM8) are associated with allodynia[33]. Forced running leads to changes in these channels that is associated with pain reduction. Voluntary aerobic exercise reduces nociceptive symptoms to basal levels at early stages of diabetes such as pre-diabetes induced by a high-fat diet [17].

However, researchers and clinicians must be careful not to exceed the level of exercise at which activity is no longer a therapeutic tool. There is evidence that exercise can increase negative outcomes in some instances. An overzealous training protocol can lead to the loss of many benefits seen with physical activity due to the bodies self-protection through activation of glial cells in both motor and sensory systems [34].

Though diabetes is the most researched disease relative to exercise and neuropathic pain; physical activity is a useful therapeutic tool for a myriad of painful diseases. Paclitaxel-induced neuropathy frequently occurs in patients undergoing chemotherapy and induces a loss of sensation and sensory fiber loss in the skin. Treadmill exercise reduces sensory loss and increases epidermal nerve fiber density in paclitaxel-treated mice [35]. Additional results include the ability of exercise to decrease abnormal levels of detyrosinated tubulin in paclitaxel-treated nerves, highlighting important anti-neurotoxic effects of exercise [35]. Other studies have shown that moderate intensity exercise reduces hyperalgesia and increases in the neurotrophin, neurotrophin 3

(NT-3), which acts in an analgesic fashion in a number of different pain conditions [36]. In studies of spinal cord injury associated pain, treadmill training improves sensory function, ameliorated allodynia, and restored normal sensation after within 5 weeks of the spinal cord injury [37]. Physical activity not only improved pain scores, but also improved tissue health through increased phagocytic clearance in glial cells [38]. This finding suggests that rhythmic, weight-bearing exercise may be an effective intervention to counter spinal cord injury induced allodynia.

Varying modes and intensities of exercise have been tested to treat neuropathic pain, almost all of which have a positive effect [20,24,31,32,36,37,39]. However, not all forms and types of exercise provide the same type or degree of benefit, particularly related to the intensity of exercise [40]. For instance, treadmill running increases neurite outgrowth with low intensity but not high intensity exercise levels. However, studies have not rigorously investigated how varying modes of exercise impact a single model and a single sensory dysfunction. This is likely because researchers focus on a single exercise modality throughout their research study for consistency and control among experimental studies.

While aerobic exercise is the most commonly modality studied, even less common forms of exercise such as swimming provide a benefit to the nervous system. Swimming provides positive results as a therapy for induced nerve injury in rats, reducing both mechanical allodynia and thermal hyperalgesia [41]. In addition, swimming reduces pain hypersensitivity in a number of experimental models, including formalin

and nerve injury-induced animal models of persistent pain [39]. The mechanism by which swimming exercise reduces mechanical allodynia may involve endogenous adenosine and adenosine A_1 receptors [42]. It is reported that agonists to the adenosine A1 receptor reduce mechanical allodynia in a neuropathic pain model of diabetes, suggesting another possible mechanism in which exercise may reduce pain [43]. These positive results from swimming offer an attractive exercise for patients with neuropathic pain due to the reduced load on pain-affected extremities and problems with coordination many patients, especially in elderly patients. For this reason, additional research in swimming regimens is needed to understand benefits for neuropathic pain, as its utilization could be the best avenue for relief for many patients.

Just as exercise intensity and mode may be key factors in the benefits of physical activity, time of exercise onset and duration may prove to be important as well. Intense short-burst exercise significantly reduced mechanical allodynia in a chronic constriction injury model of neuropathic pain, resulting in better recovery of sensorimotor function [44]. The relationship of time between the onset of injury and the start of exercise is not clear, however, there are multiple studies that have reported positive results with exercise starting within one week of injury. Initiation of treadmill running 3 days after an induced injury had an immediate and long-lasting reduction in pain that was independent of the duration of exercise [45]. Exercise training beginning 5 days after injury was sufficient to prevent the development of neuropathic pain [46]. Also, exercise initiation 7 days after spinal nerve ligation was able to reduce thermal and tactile hypersensitivity [24]. These

studies suggest that there may be no need to wait for a certain amount of time to pass after injury before the introduction of exercise as a therapeutic aide.

The Relationship of Inflammation and Exercise

The immune and nervous systems interact substantially in chronic pain states via immune cells, glia and neurons that coordinate immune responses and the excitation of the pain pathway. Many of these interactions include the synthesis and release of inflammatory mediators and neurotransmitters [47]. When injured, damaged tissue will signal mast cell degranulation and pro-inflammatory cytokine release such as tumor necrosis factor alpha (TNF- α) and interleukin 1 beta (IL1-β). Additional actions include increased NGF signaling via TrkA that enhances substance P release and pain signal propagation in the spinal cord. In response to peripheral tissue injury, immune cells also synthesize and secrete anti-inflammatory cytokines (IL-10 and IL-6), pro-resolution lipid mediators and opioid peptides to suppress the pain from pro-inflammation cytokines [48,49]. Many of these pro-inflammatory signals are present acutely after exercise, however, chronic examination of these markers post-exercise often show a robust anti-inflammatory signaling cascade in response to these acute pro-inflammatory markers [50]. Overall, however, the chronic effects of exercise on inflammation are still viewed as beneficial to reduce inflammatory signaling in disease [51]. For this reason, exercise has been utilized as an intervention that can activate natural anti-inflammatory mechanisms that causes cells to secrete anti-inflammatory cytokines that suppress the pain induced by pro-inflammatory cytokines [52].

Numerous cytokines (interleukin 1 alpha (IL1- α), IL1-β, interleukin 2 (IL-2), interleukin 4 (Il-4), interleukin 6 (IL-6), interleukin 10 (IL-10), interferon gamma (IFN-γ), transforming growth factor beta (TGF-β), TNF-α) become active in the spinal cord and DRG during neuropathic pain [53-57]. Pro-inflammatory cytokines have been implicated in neuropathic and inflammatory nociceptive conditions in a wide array of research [58]. The most prominently studied inflammation marker, TNF-α, has been implicated as having a key role in both the peripheral and central mechanisms of sensitization to painful stimuli [59]. Due to their recurring presence during painful stimuli, inflammatory cytokines have been investigated for a mechanism by which exercise reduces allodynia [60].

A prominent benefit of both endurance and resistance exercise programs is their reduction of pro-inflammatory cytokines and increases in anti-inflammatory markers (Figure 2) [61]. The pro-inflammatory acute effects of exercise are proposed to cause a subsequent spike in anti-inflammatory cytokines that are long-lasting after completion of the exercise bout. Regular exercise has been found to decrease inflammatory markers in both young and older humans [62-65]. During and after exercise, increases in levels of IL-6 appear to drive a subsequent rise in levels of anti-inflammatory cytokines such as IL-10 and IL-1 receptor agonist (IL-1RA) [66]. IL-6 has been described as a myokine, a cytokine that is released from muscle fibers during contraction while exerting its effects on other organs [67]. When given as an intravenous infusion, IL-6 provide anti-inflammatory effects similar to a bout of exercise and suppressed pro-inflammatory cytokines such as TNF- α

suggesting that IL-6 levels are the cause of anti-inflammatory benefits seen from exercise [68].

Anti-inflammatory markers respond to the rise in IL-6 induced by exercise and have compounding effects that can cause a decrease in allodynia. Increases in IL-10 are able to decrease the expression of pro-inflammatory cytokines and, in-turn, increase the ability of T cells to provide inflammatory responses [69]. In mice that exercise via running wheels, T cells were increased in number and associated with a reduction in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines [70].

Figure 2: A) Overview of the inflammatory alterations in the sensory nervous system of rodents. Inflammatory cytokines are increased throughout the DRG, spinal cord, and peripheral tissues during numerous pain states, which is associated with the development of mechanical and thermal hypersensitivity.

B) Anti-inflammatory signaling observed in the sensory nerves of exercised rodents. Exercise's anti-inflammatory signals may reduce pro-inflammatory cytokines; while increasing heat shock proteins and T-cells leading to reduced mechanical and thermal hypersensitivity normally associated with inflammation.

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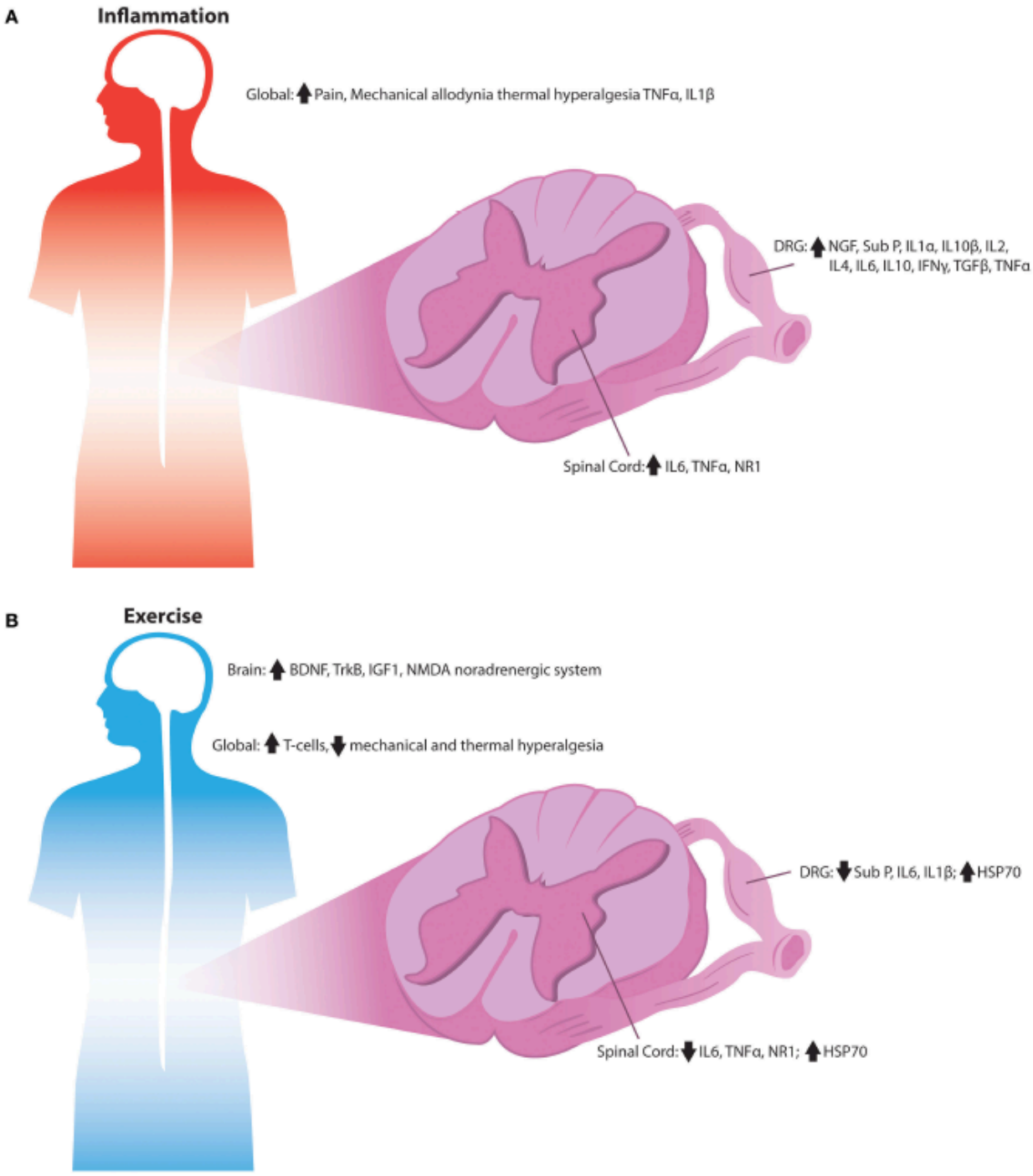

Figure 1.2 Signaling alterations associated with inflammation by exercise in the sensory nervous system

Research showing a decrease in allodynia and hyperalgesia due to exercise intervention suggests that the alterations in nociception are strongly influenced by the increased anti-inflammatory and decreased pro-inflammatory cytokines. For example, forced treadmill running reduces substance P, IL-6, TNF- α, NR1 and IL-1β levels after the development of mechanical allodynia induced by skin/muscle incision and retraction [71,72]. Swimming and treadmill exercise decrease mechanical allodynia, cold allodynia, and heat hyperalgesia while also decreasing TNF- α and IL1-β production [33,73]. Diabetes associated neuropathic pain is markedly reduced by progressive exercise training, possibly mediated by an increase of heat shock protein-72 (HSP72) without increases in TNF- α and IL-6 [74]. HSP72 is suggested to have a role in the inhibition of TNF- α and IL-6 as well as many other cytokines [75]. A greater expression of HSP72 and a reduction in diabetes-associated neuropathic pain, including thermal hyperalgesia and mechanical allodynia is seen after exercise [74]. HSP72 has an essential role in blocking inflammation and insulin resistance associated with a high-fat diet that can lead to type 2 diabetes [76]. Extended swimming reduces mechanical allodynia and thermal hyperalgesia in rats with formalin and nerve injury-induced inflammatory pain [39]. In conclusion, the interactions between release of pro-inflammatory cytokines and exercise's anti-inflammatory role via the up-regulation and release of anti-inflammatory myokines provides a mechanism that includes multiple sites and actions by which exercise can benefit overactive pain neurons.

Exercise & Ketones

Exercise exerts a myriad of effects on the body; however, some of the most potent signaling effects of exercise are due to alterations in bioenergetics and fuel demands. The natural role of ketone bodies in both disease and exercise focuses around bioenergetics demand and fuel production. The production of ketone bodies occurs naturally in the liver following reduced carbohydrate consumption and/or the body recognizing reduced bioenergetic availability. Fat stores in the body are taken to the liver and through beta-oxidation are metabolized into acetoacetate, acetone, and beta-hydroxybutyrate. These ketone bodies an important fuel source as they can be transported to and burned by tissues which are unable/prefer not to utilize fat as a fuel source including the brain, heart, and skeletal muscle [77]. This metabolic function of an alternative fuel source is an evolutionary trait, which has been conserved primarily due to the brain's inability to utilize fat as a primary fuel source. Thus, the brain and other peripheral tissues must utilize fat fuel sources for survival in low energy or low carbohydrate states.

Ketogenesis begins when natural metabolic signals such as glucagon to insulin ratios or reduced hepatic glycogen content reach critical levels. These same natural signals of prolonged fasting, or reduced energy intake are also stimulated during and following exercise bouts [78]. When the natural energy demands of exercise exceed the carbohydrate based energy stores of skeletal muscle and other peripheral tissues, ketone production and utilization occurs as a prolonged fasting state, leading to down-stream signaling of reduced histone deacetylases, lactate, glycogen storage, and carbohydrate

utilization (Fig. 3). The study of ketone production and utilization during exercise focuses almost entirely on skeletal muscle, as it is the key peripheral tissue exerting a metabolic demand during exercise. For my own work the peripheral alterations in skeletal muscle should reasonably mirror alterations in peripheral nerves as well, which should rely and process responses to depletion in glycogen stores during high-energy demand periods[79]. There is evidence that ketone utilization would be higher in nerves than skeletal muscle, as the utilization of ketones is a requirement for the brain and excessive utilization in muscle would hinder this survival instinct [80,81].

While ketones will be drawn from the blood for an alternative fuel source during exercise, the post exercise absorption period can be 10x greater than during exercise in skeletal muscle [82]. The amount of ketones taken up following exercise is proportional to the intensity of the exercise and the depletion of glycogen in response to the exercise demands during exercise driving peripheral tissues to utilize ketones as an alternative fuel source post exercise until glycogen stores are restored [83]. Ketone metabolism itself is not necessary post exercise, as glucose can meet energy demands when not performing physical activity. However, if ketones were not used post exercise there would not be an inhibition the use of glucose allowing for increased glycogen store replenishment in skeletal muscle which is a key metabolic priority post exercise [84]. This highlights the strong nutritional demand control which exercise exerts on ketone production and uptake in peripheral tissues. The continued low glucose presence in the liver may continue to drive increased ketone utilization by other peripheral tissues besides skeletal muscle.

In addition to being a key nutritional metabolite during and following exercise, the ketone beta-hydroxybutyrate is an inhibitory signal for histone deacetytlases (HDACs)[85]. HDACs have numerous functions, however in relation to exercise, HDAC phosphorylation drives exercise responsive genes to increase, which highlights another crucial role for ketones and exercise in signaling [86]. The ability to inhibit HDACs can alter gene expression, which in turn can lead to signaling and tissues alterations for a wide array of effects. While the effects of ketone signaling on HDACs in response to exercise has still only been weakly linked, the effects of beta hydroxybutyrate on HDAC signaling in brain and nerves has appears to be very clear [85].

Figure 3: Overview of the downstream signaling effects of ketone bodies in peripheral tissues.

Evans et al. 2017 Metabolism of ketone bodies during exercise and training: physiological basis for exogenous supplementation *The Journal of Physiology*

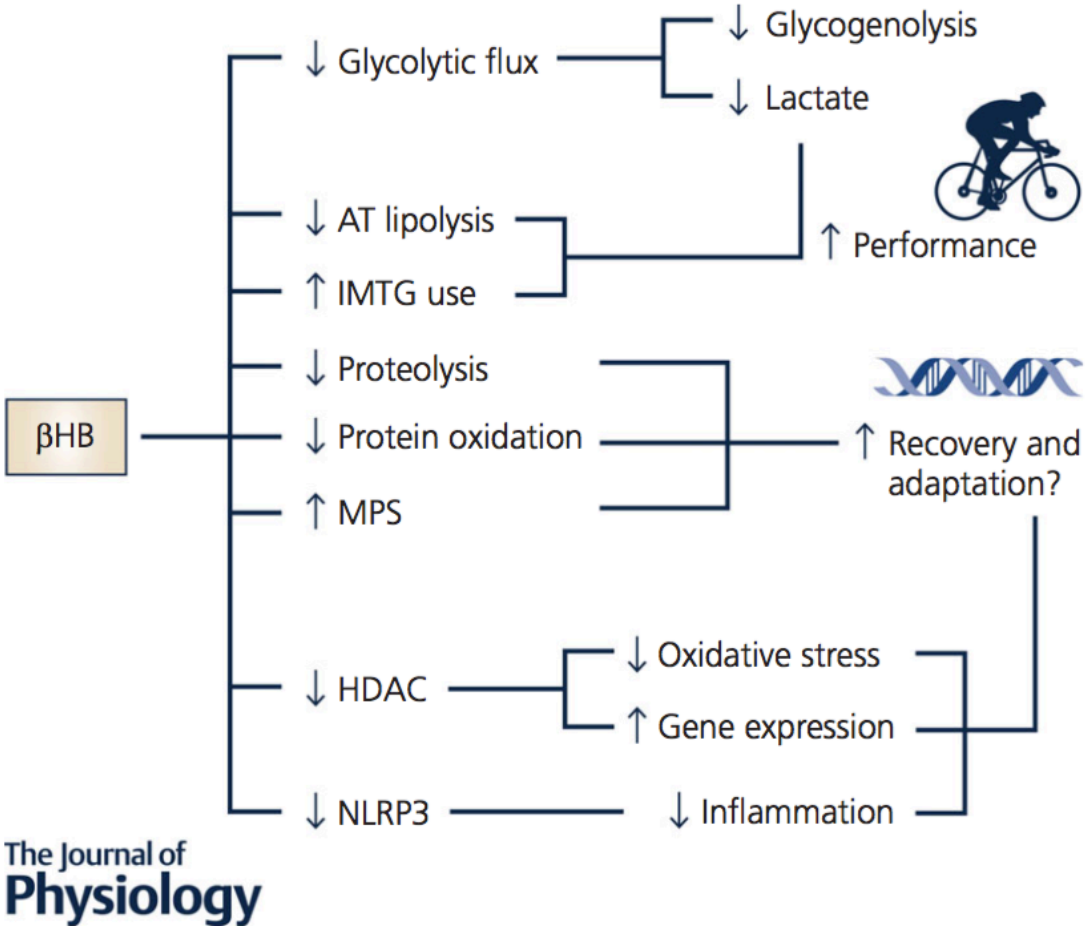

Figure 1.3 Ketone signaling effects

Ketogenic Diet & Nerves

A ketogenic diet has been researched and used to treat central nervous system diseases for hundreds of years. A ketogenic diet was ascribed to epilepsy by Hippocrates and even the Book of Matthew in the bible [77]. In more modern research, a ketogenic diet has been used to treat other nervous system diseases such as type 1 and type 2 diabetes, cardiovascular disease, glioblastoma, Alzheimer's disease, amyotrophic lateral sclerosis, concussion and traumatic brain injuries, and autism [87-95]. Traditionally, the role of the ketogenic diet in the nervous system was focused on fasting states when the brain required energy and was unable to consume carbohydrates. Ketogenesis primarily occurs in the liver and ketones are shuttled to the CNS for energy during low carbohydrate states, however recent research has also shown astrocytes can perform ketogenesis and produce ketones [96-98].

While the role of a ketogenic diet in disease has been heavily researched in the central nervous system, there is limited literature on the role of ketone metabolism on peripheral nerves. Metabolic improvements in liver and muscle in patients with metabolic syndrome indicate that a ketogenic may benefit peripheral nerves similar to other peripheral tissues. There has only been limited research examining the effects of a ketogenic diet on peripheral sensitivity and pain, and the mechanism by which a ketogenic diet may benefit the nerves is not yet understood.

In the sensory nervous system, recent investigations into the effects of a ketogenic diet on mechanical, thermal and locomotor alterations have offered conflicting results on

the benefits and disadvantages of a ketogenic diet [99-102]. One proposed mechanism for a reduction in pain is an increase in anti-inflammatory signaling induced by a ketogenic diet [103]. In the present research, this mechanism is also attractive as both a ketogenic diet and exercise are able to produce an anti-inflammatory signaling cascade allowing for a potential rescue of the chronic inflammation often observed with a high-fat diet [100].

Ketone metabolism increases peroxisome proliferator activated receptors in various tissues that results in reduced inflammatory states [104,105]. Ketone-based metabolism is a much better metabolic process as compared to glucose metabolism at a mitochondrial and cellular level as it produces less reactive oxygen species (ROS) [106]. In neurons, reductions in ROS due to caloric restriction/ketone signaling occurs in Complex I of the respiratory chain. In the brain, ketones inhibit reactive oxygen species production through an increase in NAD/NADH ratios during metabolism of beta-hydroxybutyrate as compared to glutamate [107]. This alteration in NAD/NADH ratio activates Sirt1, which inhibits apoptotic and inflammatory signaling and promotes increase mitochondrial biogenesis. There is also increases in uncoupling proteins (UCPs) that increase leakage of protons across the matrix and reduce the electrical gradient across the membrane and ROS production [108]. This improved mitochondrial respiration may combat mitochondrial changes associated with increased calcium signaling from neuronal injury and improve neuronal health and healing.

A final key anti-inflammatory mechanism is via potential increases in adenosine stimulated through a ketogenic diet. A ketogenic diet stimulates mitochondrial biogenesis

through Sirt1 signaling and in turn has shown increased ATP in the brain of animals on a ketogenic diet [109]. In neurons, low glucose levels induced by a ketogenic diet drives a release of ATP (produced through ketone metabolism in the neuron) released from cells and degraded to adenosine. Released ATP can be taken up by the cell again or signal through the adenosine receptor (A_1R). This increase in ATP production is also associated with an increase in neural adenosine levels and adenosine itself has shown anti-inflammatory affects, and is a known powerful modulator of pain [110]. Activation of A1R on immune cells reduces the production of pro-inflammatory cytokines and has shown anti-inflammatory benefits in neuronal tissues and disease [111]. Together these key metabolic pathways may all connect to mirror many powerful effects of exercise in the modulation and control of dietary induced pain.

Conclusion

The use of exercise as a therapeutic tool is a rapidly growing field in biomedical research. However, there is a dire need for increased research into understanding the role of exercise in sensory nerve disorders. The lack of understanding in the cellular pathways affected by exercise and the molecular changes that lead to benefits is a hindrance to the medical community. It is our expectation that certain types of pain may benefit from exercise, though different mechanisms driving the development of pain can vary. For instance, the benefits of exercise on painful diabetic neuropathy may be influenced by concurrent correction of metabolic abnormalities, while nerve damage associate pain may be associated with local, acute alterations in gene expression and inflammation. Both

exercise and a ketogenic diet are attractive interventions to study fat metabolism as both are able to push the body into a state of utilizing fat as a fuel source, thus increasing fat oxidation [112,113].

Chapter 2

Rats bred for low and high running capacity display alterations in peripheral tissues and nerves relevant to neuropathy and pain

Introduction

Physical activity and exercise affect a number of metabolic parameters, however, not all individuals experience the same benefits from exercise. A multitude of genetic factors influence actions of physical activity and exercise [114], and these benefits vary among different tissues and organs. Low capacity running (LCR) and high capacity (HCR) rats are an experimental rat model designed to examine the underlying mechanisms by which high and low aerobic capacity impact susceptibility for a variety of chronic disease conditions and lifespan. LCR and HCR rats are commonly used in physiological studies designed to understand mechanisms and genetics associated with exercise, activity and disease risk [115,116]. LCR and HCR rats are outbreed line generated from a founder population of male and female N:NIH stock rats based on intrinsic aerobic capacity assessed once at each generation by forced speed-ramped treadmill running until exhaustion, after which rats are kept in sedentary housing [117]. The metabolic differences between LCR and HCR rats have shed light on various aspects of activity and have proven useful in understanding how metabolic status affects disease. The LCR/HCR rat model has identified a genetic basis for the benefits of aerobic capacity and exercise on various organ systems including the heart, liver, skeletal muscle, lung, and brain [117-122].

There is growing interest about metabolic dysfunction and/or exercise impact the peripheral and central nervous system, particularly as it relates to neural disease [123-126]. It

is unclear how the genetic differences underlying intrinsic aerobic capacity between LCR and HCR rats alter the peripheral nervous system. Previous studies have reported that LCR rats have increased fat mass and pro-inflammatory signaling in adipose tissue, as well as alterations in cholinergic anti-inflammatory signaling [127,128]. Both are known to be important in peripheral nerve function and pain.

Here, we compared a number of pain- and neuropathy-relevant features of the peripheral nervous system in female LCR and HCR rats. The selective breeding of the LCR and HCR strains results in differences in peripheral nerve sensitivity, cutaneous innervation, and composition of the dermis and epidermis. The nature of the differences in female LCR rats is consistent with known alterations in the periphery associated with poor outcomes in pain and peripheral nerve disease in humans. These findings in LCR rats suggest they could serve as novel model to explore the genetic features important in pain and abnormal sensory function associated with low aerobic capacity, which is a known risk for developing obesity and type 2 diabetes.

Materials and Methods

LCR and HCR Rats

The development of high-/low-capacity rats (HCR/LCR) model displaying high and low intrinsic aerobic capacity has been previously described [122,129,130]. Female rats 40-50 weeks of age were dual housed on a 12-h light cycle on a control chow diet [8604 (14%kcal fat, 54% CHO, 32% protein, 3.9kcal/g), ENVIGO, Madison WI] throughout the period of analysis. All animal use was in accordance with NIH guidelines and conformed to protocols approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. All rats were examined at the University of Michigan prior to their shipment to the University of Kansas Medical Center for running distance, speed, time to exhaustion, and energy expenditure as previously described [129].

Body Composition

Body composition to assess fat mass was measured by MRI using the EchoMRI-100 (EchoMRI, Houston, TX). Fat mass and lean mass were analytically determined immediately prior to exsanguination. Animal weights were measured at the time of sacrifice allowing for both fat and lean mass to be reported relative to percent body weight.

Blood Chemistry

At the time of sacrifice, blood was drawn from the chest cavity by cardiac puncture and allowed to clot for 25 minutes at room temperature then placed on ice. All samples were then spun at 3,000g for 10 min at 4 °C and serum separated and frozen at -80 °C until subsequent analysis was performed. Blood serum analysis (total protein, albumin, globulin, sodium, potassium, chloride, CO_2, calcium, glucose, alkaline phosphatase, alanine aminotransferase, bilirubin, phosphorous, blood urea nitrogen, creatinine, cholesterol, triglycerides, and insulin) performed by Comparative Clinical Pathology Services LLC (Columbia, MO). All rats were removed from their food the previous night (12 hours prior to collection).

Behavioral Testing

Rats were acclimatized and tested for mechanical and thermal sensitivity as previously described [131]. Thermal sensitivity latencies from the six applications were used to calculate the mean latency per animal and mean latencies were combined to calculate group means. Mechanical sensitivity was measured twice (one week apart) 2 weeks prior to sacrifice, and the 2 testing days were averaged together to give one total mechanical sensitivity value.

Nerve Conduction Velocity Measurements

Rats were anesthetized with an IP injection of 50mg/ml phenobarbital sodium salt (Sigma, St. Louis, MO) and motor and sensory nerve conduction velocities were recorded. The left sciatic-tibial motor conduction system was stimulated proximally at the sciatic notch and distally at the ankle via bipolar electrodes with supramaximal stimuli (9.9mA) for 0.2 ms with low and high pass filters of 20 Hz and 10 Hz respectively. The latencies of the compound muscle action potentials were recorded via bipolar electrodes from the first interosseous muscle of the hind-paw and were measured from the stimulus artifact to the onset of the negative M-wave deflection. Motor nerve conduction velocity (MNCV) was calculated by subtracting the distal latency from the proximal latency and the result was divided into the distance between the stimulating and recording electrode. Hind limb sensory nerve conduction velocity (SNCV) was recorded in the digital nerve to the second toe by stimulating with a square-wave pulse of 2.4 mA for a duration of 1.0 ms utilizing a low and high-pass filter of 3Hz and 10Hz respectively. The sensory nerve action potential was recorded behind the medial malleolus. Ten responses were averaged to obtain the position of the negative peak. The maximal SNCV was calculated from the latency to the onset of the initial negative deflection and the distance between stimulating and recording electrodes.

Intraepidermal Nerve Fiber (IENF) and Langerhans Cell Measurements

Rats were exsanguinated following nerve conduction velocity measurements. Cutaneous tissue from the pad of the hind paw were collected, processed, and stained for IENFD as previously described [132]. IENF density (IENFD) was expressed as number of fibers per millimeter of epidermis from a total of 9 images per rat. The combined mean IENFD from each rat was used to calculate group means.

Langerhans cell measurements were performed using Langerin (E-17) (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA) to visualize Langerhans cells. Fluorescent images were collected using a Nikon Eclipse 90*i* microscope using a 10x objective. NIH image J software was used to measure each dermal region. Langerhans cell density was expressed as number of Langerhans cells per millimeter of epidermis from a total of 9 images per rat.

Mast Cell Quantification

Footpads were collected and processed as previously described for mast cell quantification [133]. After imaging a standardized region of interest was placed over all images using NIH image J software and all mast cells within the region of interest were counted manually.

Expression of mRNA Encoding Pain Genes

RNA was extracted and cDNA synthesized as previously described [134]. Primer sequences for ASIC3, CGRP, COMT, SCN9A, SCN10A, TrkA, TRPA1, TRPV1, and TRPV4 were created for rat sequences by Integrated DNA Technologies (Coralvile, IA). All reactions were performed in triplicate, and all mRNA levels were normalized to GAPDH. ΔΔCT values were used to calculate fold change and relative expression levels.

Statistical Analyses

Results are presented as means ± SEM. Data were analyzed using unpaired t-test and two-factor ANOVA with post hoc comparisons using Fisher's test of least square difference where appropriate. Statistical significance was set at $p < 0.05$ and analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA).

Results

LCR Rats Have Reduced Aerobic Capacity

LCR and HCR rats were examined for running distance, speed, time to exhaustion, and energy expenditure on a single occasion at the University of Michigan prior to their shipment to the University of Kansas Medical Center. Consistent with this model, LCR rats displayed significantly reduced running distance ($p<0.0001$), running speed ($p<0.0001$), time to exhaustion ($p<0.0001$), and energy expenditure ($p<0.0001$) compared to HCR rats (Figure 1a, b, c, d).

Figure 2.1. Rats classified as high capacity runners display increased abilities in all aspects of aerobic testing

Baseline testing of HCR and LCR rats shows increased (a) distance, (b) speed, (c) running time, and (d) energy expenditure in high capacity classified rats as compared to low capacity runners. (n=18 for both groups) All data are presented as mean ± SEM

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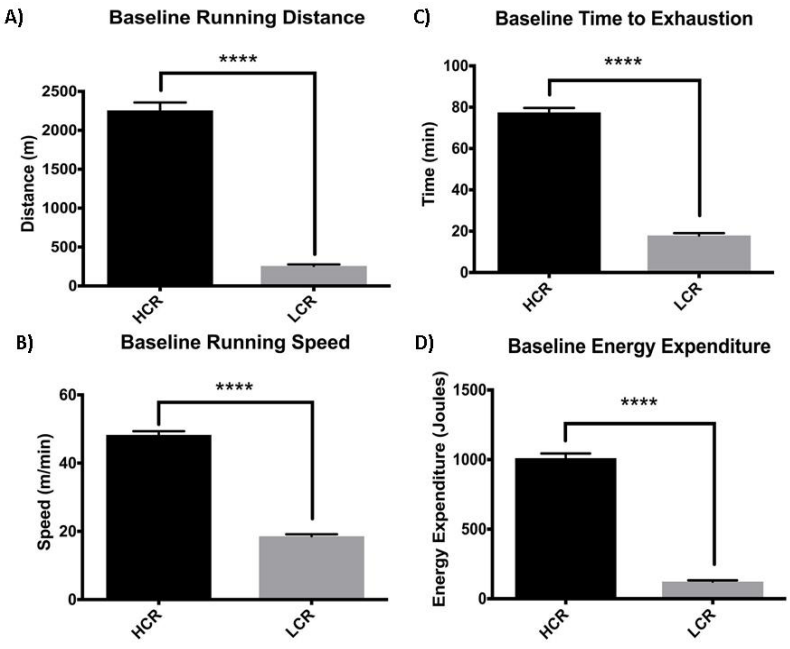

Figure 2.1 Aerobic Capacity of HCR/LCR Rats

LCR Rats Have More Fat Despite Weighing the Same as HCR Rats

Female LCR and HCR rats had equal body weights at the time of sacrifice (LCR, 248.3 ± 4.559; HCR, 246.6 ± 5.725), though the percent of body weight due to fat versus lean mass differed between groups (LCR, 8.44% ± 0.6589; HCR, 5.33% ± 0.4328). LCR rats had significantly more of their mass distributed as fat ($p = 0.0004$) and a lesser amount due to lean mass ($p = 0.003$).

LCR Rats Have Lower Mechanical Thresholds

Mechanical withdrawal thresholds of the hind paws were significantly reduced in LCR rats, as compared to their HCR counterparts ($p = 0.008$, Figure 2a). There were no differences in thermal latencies between LCR and HCR rats (Figure 2b). Conduction velocities for both motor and sensory neurons for both LCR and HCR appeared comparable with no differences were noted between LCR and HCR rats related to conduction velocities (Figure 2c & 2d).

Figure 2.2 Mechanical sensitivity is reduced in LCR females

(a) Mechanical sensitivity test displayed strain specific differences in basal sensitivity of LCR rats. (b) Thermal sensitivity testing showed no alterations in withdrawal latency between HCR and LCR groups. (c) Basal motor nerve conduction velocities show no strain differences between HCR and LCR rats. (d) Basal sensory nerve conduction velocities are unaltered between HCR and LCR rats. All data are presented as mean ± SEM; n=18 for all groups

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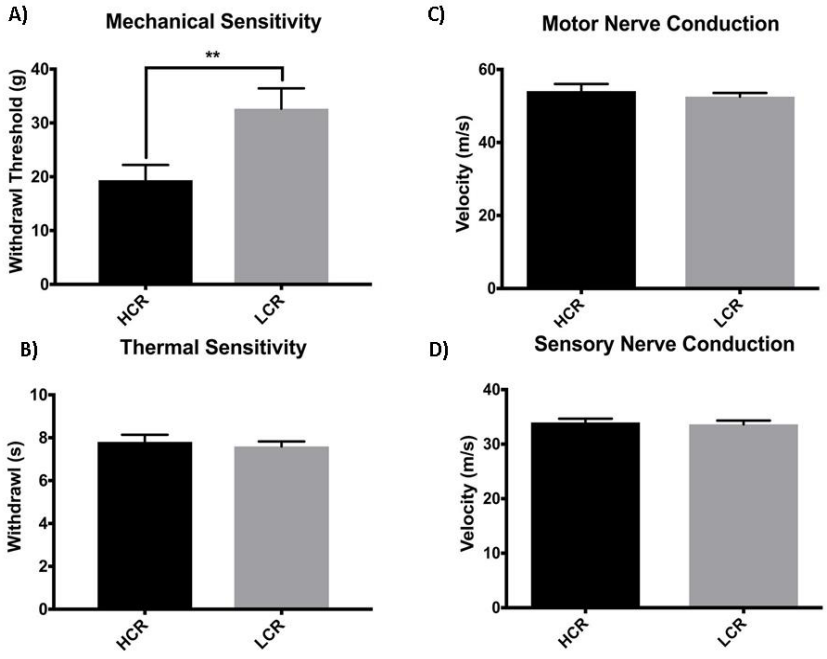

Figure 2.2 Mechanical Sensitivity in LCR Females

LCR Rats have Poor Indicators of Blood Chemistry

Analyses of a range of blood biomarkers are displayed in Table 1. LCR rats display increased potassium ($p = 0.005$), calcium ($p=0.027$), alkaline phosphatase ($p <0.0001$), and triglycerides ($p = 0.030$). All remaining blood serum markers (total protein, albumin, globulin, sodium, chloride, total CO_2, glucose, alkaline aminotransferase, gamma-glutamyltransferase, total bilirubin, phosphorus, blood urea nitrogen, creatine, cholesterol, and insulin) were not significantly different between LCR and HCR rats.

Table 2.1 LCR rats display few variances from HCR rats in serum markers

Serum blood analysis from 12 hour fasted HCR and LCR rats. LCR rats have statistically increased potassium, alkaline phosphatase, calcium, and triglycerides, while no other markers were statistically different between LCR and HCR rats.

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	HCR-Fasted	LCR-Fasted
Total Protein (g/dl)	6.05	6.11
Albumin (g/dl)	3.40	3.43
Globulin (g/dl)	2.65	2.68
Sodium (mEq/L)	138.30	140.20
Potassium (mEq/L)	3.23	3.78**
Chloride (mEq/L)	98.74	99.52
Total CO2 (mEq/L)	25.70	27.80
Calcium (g/dl)	9.37	10.24*
Glucose (mg/dl)	180.00	169.50
Alkaline Phosphatase (U/L)	51.20	101.6****
Alanine aminotransferase (U/L)	58.20	75.60
Gamma-glutamyltransferase (U/L)	<3	<3
Total bilirubin (mg/dl)	0.11	0.10
Phosphorus (mg/dl)	5.01	4.69
Blood urea nitrogen (mg/dl)	18.40	19.50
Creatinine (mg/dl)	0.19	0.26
Cholesterol (mg/dl)	77.40	83.70
Triglycerides (mg/dl)	50.20	78.5*
Insulin (ng/ml)	1.05	2.17

* $p<0.05$
** $p<0.01$
*** $p<0.001$
**** $p<0.0001$

Table 2.1 Serum Markers in HCR/LCR Females

LCR Rats Have a Higher Density of Epidermal Axons

Total C-fiber axonal density was increased in the epidermal footpad of LCR rats (LCR = 23 fibers/mm^2; HCR =19 fibers/mm^2, p = 0.047, Figure 3c). The axonal subset of C-fibers, which express the tyrosine kinase receptor TrkA, and respond to NGF were significantly increased in LCR rats (LCR =14 fibers/mm^2; HCR = 11 fibers/mm^2, p=0.110, Figure 3d). The ratio of TrkA fibers to total PGP-9.5+ fibers was similar between the two groups (Figure 3e).

Figure 2.3. LCR rats display increased cutaneous innervation

Merged representative images of double immunofluorescent staining for the pan-neuronal markers PGP9.5 and TrkA. a) HCR b) LCR c) LCR animals show increased nerve fiber density in the hind paw skin. d) LCR rats display increased peptidergic nerve fiber density in the hind paw skin. e) The ratio of TrkA/PGP9.5 shows no difference between groups when accounting for total innervation. All data are presented as mean ± SEM; n=6 for all groups

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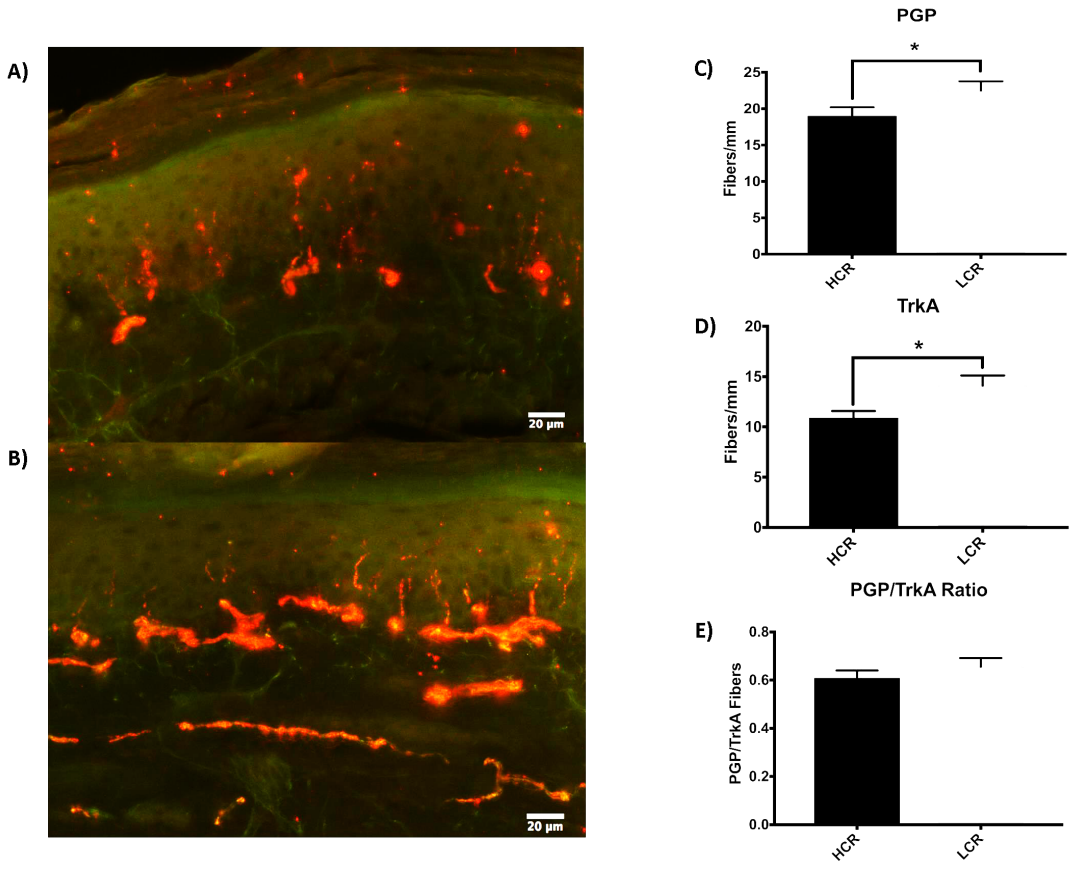

Figure 2.3 Cutaneous Innervation of HCR/LCR Rats

LCR Rats Have Increased Langerhans Cells and Mast Cells in Cutaneous Tissues

Quantification of the number of Langerhans cells in the epidermis revealed that LCR rats have significantly higher number of Langerhans cells compared to HCR rats ($p < 0.0001$, Figure 4c). In addition, the density of mast cells was increased in the dermis of LCR rat hind paws relative to HCR rats ($p = 0.024$, Figure 4f).

Figure 2.4. LCR rats display increased mast and langerhans cell density

Representative images of toluidine blue staining for mast cells. a) HCR b) LCR c) LCR rats show increased mast cell density in the hind paw skin. All data are presented as mean ± SEM; n=5 for all groups. Representative images of immunofluorescent staining for Langerin expression (E-17). d) HCR e) LCR f) LCR rats show increased Langerhans cell density in the hind paw skin. All data are presented as mean ± SEM; n=6 for all groups

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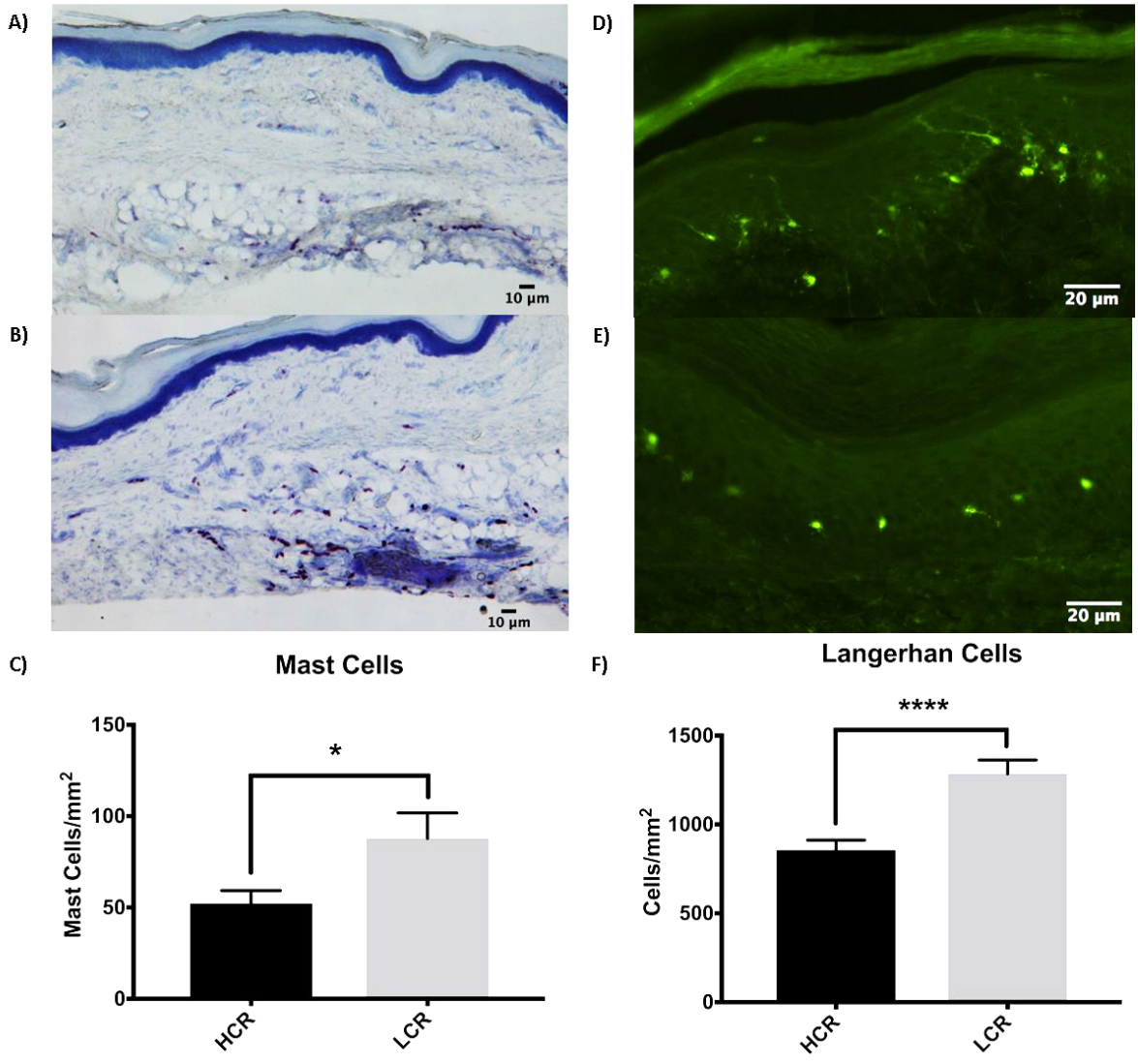

Figure 2.4 Mast Cell and Langerhans Cell Density

LCR and HCR Have Similar Expression of Pain Genes

Analysis of gene expression in the lumbar DRG of LCR and HCR rats revealed no significant strain-related differences in the levels of any of the mRNAs examined (ASIC3, CGRP, COMT, SCN9A, SCN10A, TrkA, TRPA1, TRPV1, and TRPV4).

Discussion

Metabolic syndrome is a medical epidemic in Western society that is characterized by a constellation of risk factors including insulin resistance, elevated triglycerides, hypertension, and central obesity. The metabolic syndrome is known to increase risk for type 2 diabetes and cardiovascular disease [135]. Numerous studies have linked both prediabetes and metabolic syndrome in animal models and human patients with the development of sensor motor polyneuropathy [136]. Preclinical modeling of polyneuropathy has been challenging and improved animal models encompassing the intricacies of human disease are vitally needed [137,138].

LCR and HCR rats reflect the complex nature of metabolic syndrome through extensive breeding for traits known to characterize both a healthy and poor metabolic state. The two strains can be divided based on their low and high health risk factors determined by their propensity to run greater or shorter distances based on their divergence in intrinsic aerobic capacity [117]. To date, only male LCR and HCR rats have been studied related to peripheral nerve function [115,116], despite a higher incidence of chronic pain conditions in female patients [139,140].

HCR rats displayed increased aerobic function and decreased fat mass consistent with prior characterization of the strain, while LCR rats displayed decreased aerobic function and increased fat mass [117,141]. Female LCR and HCR rats were not different in their overall weights. Prior studies have shown clear differences in body weight in female rats, though less pronounced compared to their male counterparts [142,143]. It is likely that

examination of aged female rats would have revealed differences in overall body weight. In the current study, body weight does not appear to be a confounding factor in our observations; differences in fat mass distribution is a more plausible modulator of peripheral nerve function rather than overall weight.

The present study is the first to examine the serum of female HCR and LCR rats across a number of metabolic markers. While potassium levels were statistically different, the differences were not physiologically relevant since values less than 6 mEq/L are considered normal [144]. Similarly, calcium levels fall between 9-11 mg/dl, and the values observed are not likely physiologically different between LCR and HCR rats [144]. Circulating triglycerides, although significantly higher in LCR rats, were still within reported ranges [144]. Alkaline phosphatase was also elevated in LCR rats however, levels remained below the maximum of reported ranges (maximum of 131 U/L) [144]. The serum analysis did not identify any differences that explain the anatomical and behavioral differences between LCR and HCR rats.

Cutaneous Mechanical Sensitivity

Female LCR and HCR rats do not demonstrate strain-related baseline differences in thermal sensitivity. Previous studies with male LCR and HCR rats demonstrated differences in thermal sensitivity between the strains with LCR mice having lower paw withdrawal latencies [115]. Conduction velocities of motor and sensory nerves are an

important clinical indicator of diabetic neuropathy and myopathy [145,146]. Here, no differences were observed between either sensory or motor nerve conduction velocity between LCR and HCR rats.

Male LCR and HCR rats do not demonstrate baseline mechanical sensitivity differences [116]. Female LCR and HCR rats demonstrated different baseline responses to mechanical withdrawal testing. This suggests, LCR rats have some level of insensitivity to mechanical stimuli in comparison to HCR rats. The perception of sensory input is likely altered in female LCR rats since the peripheral nervous system remains structurally intact with normal nerve conduction velocities. This baseline structural similarity between the two strains is important to establish, as the hallmarks of peripheral neuropathy are reduced conduction speeds, loss of myelination, and altered sensory sensitivity. This lack of structural differences leads us to hypothesize the differences in mechanical sensitivity are related to a chronic inflammatory state as a result of the metabolic syndrome occurring in LCR rats.

Models of type 2 diabetes presenting with common signs of metabolic syndrome such as increased body weight, fat mass, and blood glucose, often also show systemic inflammation and heightened sensitivity [147]. Previous work examining the role of inflammation in altered sensitivity shows that with profoundly increased inflammation there can be an alteration in both thermal and mechanical sensation [148,149]. Examination of painful areas has revealed that mast cells are a key marker of inflammatory changes in pain states and offer a key correlation between their density and resulting sensitivity [150].

Based on this information, we suggest the chronic inflammatory environment present in the LCR rat is a state that is primed to drive lower sensory thresholds and allodynia with alterations in diet, exercise, or disease. The LCR rat, given its baseline pro-inflammatory physiologic state, likely has compensatory central and peripheral modulators of allodynia that result in reduced sensitivity and higher thresholds. We hypothesize alterations to their physiologic state through diet, injury, or disease state will overwhelm their compensatory strategies to mitigate allodynia and promote sensory abnormalities. Hence, this rat model lends itself nicely to the study of polygenic features seen in patients with neuropathy but have been challenging for inbred or genetic animal models to accurately model.

Genetic differences underlying mechanical and thermal sensitivity have been well established by our laboratory and others [132,151]. Some have also implicated sex differences with the variability in sensory thresholds; despite this, majority of studies generally utilize male mice for studies [152,153]. Genetic approaches using inbred mouse strains have identified a number of mRNAs that are altered in their expression associated with pain genetics [151,154]. Based on this information we investigated a select number of known genes involved with nociceptive transmission in the LCR-HCR models. No strain differences were seen in the mRNAs studied that are known to have a role in nociception, which suggests at least in the expression of the genes studied, there is no underlying physiologic difference in nociceptor properties.

Intraepidermal Nerve Fiber Density

IENFD is a valuable tool to visualize sensory innervation of the epidermis by small unmyelinated C-fibers [155,156]. These C-fibers are responsible for transmitting mechanical, thermal, and noxious stimuli from the epidermis and are impacted by the metabolic status in animals and humans [157]. Reductions in IENFD correlate with small fiber damage and sensory neuropathies [158,159]. Previous work by our group and others has displayed that IENFD is altered by changes in diet and exercise [17,136,160]. Here, female LCR rats demonstrated a significant increase in the density of PGP-9.5+ intraepidermal nerve fibers compared to HCR rats. It should be reminded that alterations between groups are not due to physical activity effects on the skin, as these rats are not exercised after initial baseline examination. The LCR rats also had increased number of fibers expressing TrkA, the high affinity receptor for NGF. TrkA-positive axons play an important role in pain transmission [161,162]. However, the overall percentage of TrkA fibers was similar between groups. Studies have established allodynia and reduced mechanical thresholds are associated with increased TrkA fibers [17,163,164]. This increase in IENF and increase in TrkA+ fiber density may predict that LCR rats display a greater response to evoked allodynia and are at risk for longer duration of heightened sensitivity.

Langerhans Cells

Langerhans cells are transient dendritic antigen-presenting cells located in the epidermis. They play a critical role in the epidermal immune response and are also found within peripheral nerve bundles that innervate the plantar and palmar surfaces [165].

Langerhans cells function using mechanisms similar to macrophages and respond to similar signaling agents such as inflammatory cytokines and calcitonin gene-related peptide [166,167]. These cells are found in close proximity to regulatory T cells and effector memory T cells, again highlighting a strong antigen-response role in the epidermis [168]. Previous studies have demonstrated that Langerhans cells density correlates strongly with intraepidermal nerve fiber density and changes in mechanical sensitivity associated with allodynia [169,170]. In mice depleted of Langerhans cells using diphtheria toxin, mechanical sensitivity increased as a result of the loss of Langerhans cells [169]. Similar to this result, female HCR rats in this study display decreased epidermal Langerhans cells as compared to their LCR counterparts and decreased mechanical sensitivity thresholds.

Mast Cells

Similar to Langerhans cells, mast cells play a significant role in innate and adaptive immunity; however, they modulate neuropeptides involved in sensory processing and signaling [171,172]. Mast cells have a well-established neuroimmune response through the reaction to inflammatory signaling and subsequent synthesis and release of neurotrophins highlighting an important molecular marker of peripheral nerve inflammatory response [173,174]. Together these neurophysiological and molecular markers indicate peripheral nerve function and health status. Mast cell degranulation releases a number of molecules important in nociceptive signaling and degranulation is known to be an important contributor to pain [133,175]. Female LCR rats have significantly higher

numbers of mast cells in epidermis and dermis of the hind paw compared to female HCR. Again, this may be reflective of their inflammatory state, which could contribute to the development of sensation abnormalities.

In conclusion, female LCR and HCR rats demonstrated differences in mechanical, but not thermal sensitivity. The strains did not have altered gene expression for common markers, ion channels, and receptors of nociceptors. Female HCR and LCR rats had similar nerve conduction velocities and proportion of intraepidermal nerve fiber types in the hind paw. Female LCR rats show increased inflammatory cells within the peripheral nervous system that could increase allodynia in diseased states. In order to understand sex differences in allodynia and analgesia, female HCR and LCR were used to determine baseline characteristics of the peripheral nervous system in preparation for future studies investigating disease models affecting cutaneous sensation.

Chapter 3

Exercise alters metabolic parameters and inflammation to reduce diet-induced mechanical allodynia

Introduction

Diabetes is a rising epidemic throughout the United States and the world. As of 2014, 29.1 million people, or 9.3% of the population have diabetes [176]. A larger group emerging in the U.S. however, is patients with prediabetes, as 86 million adults are pre-diabetic and 9 out of 10 of these individuals are unaware that they have prediabetes [176]. A growing body of literature is discovering that patients with prediabetes begin to experience the onset of neuropathy that was traditionally thought to be a development of long-term diabetes [17,177,178]. While the neuropathy associated with prediabetes is less severe than neuropathy seen in overt diabetes patients [179], the painful symptoms are still a devastating complication for these patients.

A prominent model used to study painful neuropathy in diabetes is to utilize a high-fat diet to induce mechanical allodynia [17,180-182]. Our previous studies have demonstrated that exercise reduces, but does not fully restore diet-induced metabolic abnormalities [17]. Physical activity provides great benefits to medical and pharmaceutical treatments for individuals with neuropathy [183], and in many instances varying forms and intensities of exercise has been prescribed to alleviate the symptoms of a growing number of different types of neuropathic pain [31,32,36,37,39,184]. However, the mechanisms by which exercise exerts its beneficial actions remain poorly understood.

Poor diet and lifestyle are common factors connected with the development of both pre- and overt diabetes [185]. These often lead to an increase in fat mass, an increase in global inflammation and a myriad of problem often referred to as metabolic syndrome

affecting numerous tissues [186-189]. Mitochondrial alterations can affect metabolism in numerous tissues, though this topic has often been overlooked in the peripheral nervous tissues [190-193]. Numerous noxious pain sensors are activated/sensitized by inflammatory agents [149,194-197], many of which can be deactivated through the decrease of these inflammatory agents, possibly by exercise, which has a strong history of anti-inflammatory signaling even independent of a decrease in fat mass or weight [198,199].

Transient receptor potential vanilloid 1 (TRPV1) is one of the most prominently studied transient receptor potential (TRP) channels, and is often examined in its role of sensing chemical agents associated with inflammation and transmitting a noxious stimulus signal through the sensory nervous system [200,201]. TRPV1 translocation may require the binding of a synaptosomal protein complex involving synaptosomal-associated protein 23 kDa (SNAP23) & synaptosomal associated protein 25 kDa (SNAP25) based on the evidence that SNAP25 is implicated in the translocation of phosphorylated TRPV1 to the surface of the cell membrane [202]. During translocation and insertion into the membrane, TRPV1 activating signals can open another TRP channel, the transient receptor potential cation channel member A1 (TRPA1). This ability to activate the TRPA1 channel, known to drive mechanical sensitization, establishes a direct link between TRPV1 and our observed phenotype of mechanical allodynia from a high-fat diet [203].

In the current study, our results reveal that a high-fat diet alters molecular pathways involved in the development of inflammation and pain channels in the

periphery nerves. Effects of exercise have select actions, which is surprising in context of the many positive benefits on metabolic factors known to improve with exercise, and many of which would be predicted to influence pain pathways. However, we show that the alterations from exercise occur independent of alterations in body weight, fat mass, and traditional mitochondrial and metabolic signals, suggesting that exercise benefits sensory function in specific and unpredicted ways.

Experimental Procedure

Mice

Seven-week-old male C57/BL6 #027 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 chow diet (8604; Envigo, Madison Wisconsin; 14% kcals from fat, 32% protein, and 54% carbohydrate) or a high-fat diet (07011; Envigo; 54% kcals from vegetable shortening (hydrogenated) and corn oil fat, 21% protein and 24% carbohydrate). All mice were fed the standard diet through all baseline testing. After baseline behavioral testing was complete, mice were separated and the groups were given different diets. 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.

Voluntary Exercise

Following baseline testing, mice were separated into either sedentary controls or exercise groups. Exercise mice 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 mice were housed one or two per cage at the

suggestion of veterinary staff in the animal facility. Mice were given access to diet and running wheels simultaneously following baseline behavior testing and subsequent group assignment. Treatment groups are identified throughout the study as: chow-fed sedentary (CF-Sed), chow-fed exercise (CF-Ex), high-fat diet sedentary (HF-Sed), and high-fat diet exercise (HF-Ex).

Blood Measures

Animal weight and blood glucose (glucose diagnostic reagents; Sigma, St. Louis, MO) was measured weekly. At sacrifice, blood was drawn from the chest cavity and allowed to clot for 30 min on ice, then spun at 3,000g for 30 min at 4 °C and serum drawn off and frozen at -80 °C until bradykinin and β-hydroxybutyrate were analyzed from this sample. All mice were fasted three hours prior to blood collection for all blood chemistry panels.

Mechanical Sensitivity

Mice were allowed to acclimate to the testing equipment in two separate sessions prior to the initial baseline-testing day. Before each behavior test, mice were allowed to acclimate to the sound cushioned behavior testing room for 30 minutes followed by a 30-minute acclimation to the testing equipment. Mice were placed in individual clear plastic cages on a wire mesh table 55cm above the table. Von Frey monofilaments (0.07-4.0g)

were applied perpendicularly to the plantar surface of the hind paw until the filament bent. Testing began with the 0.6 g filament. If the mice 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 [204].

Body composition analysis

Body composition to assess water density and fat mass was measured by MRI using the EchoMRI-100 (EchoMRI, Houston, TX). Fat mass and lean mass were analytically determined by the instrument. Body composition was determined immediately before sacrifice at completion of 12 weeks of diet and exercise.

Metabolic Testing

A metabolic monitoring system (Promethion, Sable Systems Int., Las Vegas, NV) measuring oxygen consumption, carbon dioxide production, and a multi-dimensional infrared beam break system was employed to assess the total energy expenditure (TEE) and respiratory quotient (RQ) over a 48-hr period, 10 weeks after implementation of diet and exercise. Mice were singly housed in the metabolic chamber system and allowed to

acclimate to the chamber environment for two days prior to data collection. Data were analyzed as two 12-hour cycle averages (12 hours ambient light [07:00-19:00] and 12 hours of dark [19:00-07:00]) and were calculated per animal; these light and dark averages were then used to calculate group means.

mRNA Analysis

RNA was extracted from DRG using TRI Reagent (Sigma) and RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Sample concentration and purity were determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Complementary DNA (cDNA) was synthesized from total RNA using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using Sso Advanced Supermix Kit (Bio-Rad). A custom PCR plate was purchased from Bio-Rad to analyze the mRNAs for tumor necrosis factor (TNF-α), Interleukin 1 Beta (IL-1β), Interleukin 6 (IL-6), mitogen-activated protein kinase 14 (p38), mitogen-activated protein kinase 8 (JNK), nerve growth factor (NGF), synaptosomal-associated protein, 23kDa (SNAP23), syntaxin binding protein 4 (STXBP4), vesicle-associated membrane protein 2 (VAMP2), therefore these primer sequences are not available to publish. All reactions were performed in triplicate, and all mRNA levels were normalized to GAPDH. ΔΔCT values were used to calculate fold change and relative expression levels.

Western Blot Analysis

At the time of sacrifice, lumbar DRG 4-6 were flash frozen in liquid nitrogen and stored at -80 °C until processing. All 6 DRGs were sonicated in 100µl of ice-cold cell extraction buffer (Invitrogen, Carlsbad, CA) containing 55.5 µl/ml protease inhibitor cocktail (Sigma, St. Louis, MO) 200 mM Na_3VO_4 and 200 mMNaF and vortexed for 3-5 seconds every 10 min for 70 min while kept on ice for protein extraction. Following centrifugation at 7000 rpm at 4 °C for 15 min the supernatant was removed and the protein concentration of the supernatant was determined by Bradford assay (Bio-Rad, Hercules, CA) performed in triplicate. Samples were diluted in HES buffer (20 mmol/1 HEPES, 1 mmol/1 EDTA, 200 mol/1 sucrose, pH 7.4) and 5 x Laemmli buffer containing 100 mmol/1 dithiothreitol (Thermo Scientific) based on protein concentration to generate samples containing the same concentration of protein for analysis by Tris-glycine gels. Samples were heated in a boiling bath for 5 min. and stored at -20 °C until use. A second set of identical samples were made but not boiled for analysis of oxidative phosphorylation (OXPHOS) of electron transport complexes. Equal amounts of protein (35 µg) were loaded and separated on a 4-15% gradient Tris-glycine gel (Bio-Rad; 20mA/gel, 60 min) and then transferred to a nitrocellulose membrane (200 mA, 80 min, 4 °C). Membranes were blocked for 1-2 hrs at room temperature in Tris-buffered saline, 0.1% Tween 20 (TBST), and 5% nonfat dry milk or 1%BSA, followed by an overnight incubation with appropriate primary antibodies. Primary antibodies were diluted in TBST with 1% nonfat dry milk or 1-5% BSA of concentrations as listed at the end of this section. Following three brief washes with TBST, blots were incubated for 1 hr at room

temperature in TBST 1% nonfat dry milk supplemented with an appropriate HRP-conjugated secondary antibody at a concentration of 1: 10,000. Blots were then washed twice with TBST and once with TBS. Blots were then visualized using SuperSignal West Femto Enhanced Chemiluminescence reagent (Thermo Scientific, Rockford, IL). Membranes were exposed either to X-ray film or on a ChemiDoc XRS (Bio-Rad, Hercules, CA) and analyzed using Image J densitometry (for X-Ray film) or Image Lab 5.2 software (for ChemiDoc XRS) (Bio-Rad, Hercules, CA). Blots were then stripped and re-probed for β–actin or another target antibody. Blots were stripped for 15 min. at 55 °C in buffer containing 62.5 mmol/1 Tris-HCL, 2% SDSS, and 100 mmol/1 2-mercaptoethanol. Antibodies include Anti-Total OXPHOS (1:333, Abcam, Cambridge, MA); Anti-p38 MAPK and Anti-Phospho-p38 MAPK (1:1000, Cell Signaling, Danvers, MA); Anti-PPAR-α (1:500. Abcam, Cambridge, MA); Anti-Snap23 (1:1000, Abcam, Cambridge, MA); Anti-Snap25 (1:1000, Abcam, Cambridge, MA); and Anti-β-Actin (1:2500, Abcam, Cambridge, MA).

Statistical Analyses

Results are presented as means ± SEM. Data was analyzed using a two-factor ANOVA with post hoc comparisons analyzed using Fisher's test of least square difference where appropriate. Statistical significance was set at $P < 0.05$ and analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA).

Results

By 6 weeks, both high-fat fed groups had decreases in withdrawal thresholds compared to chow-fed exercised and sedentary (HF-Sed, p=0.072; HF-Ex, p=0.068) mice, although thresholds of high-fat fed mice were only statistically significantly decreased relative to chow-fed exercised mice (HF-Ex, p = 0.049; HF-Sed, p = 0.051). At the conclusion of the study, high-fat sedentary mice maintained decreased thresholds relative to the chow-fed sedentary controls (p = 0.018), whereas thresholds of high fat exercised mice were rescued and fell between chow sedentary mice and high fat sedentary mice (p = 0.008) (Fig. 1).

Figure 3.1: Mechanical thresholds were reduced in both HF-Ex and HF-Sed mice compared to control mice after 6 weeks of a high-fat diet. HF-Ex mice return to baseline levels at 12 weeks of physical activity. (n=8 for all groups) All data presented as mean ± SEM *: CF-Sed vs HF-Sed #: CF-Ex vs HF-Sed ^: HF-Sed vs HF-Ex °: CF-Ex vs HF-Ex.

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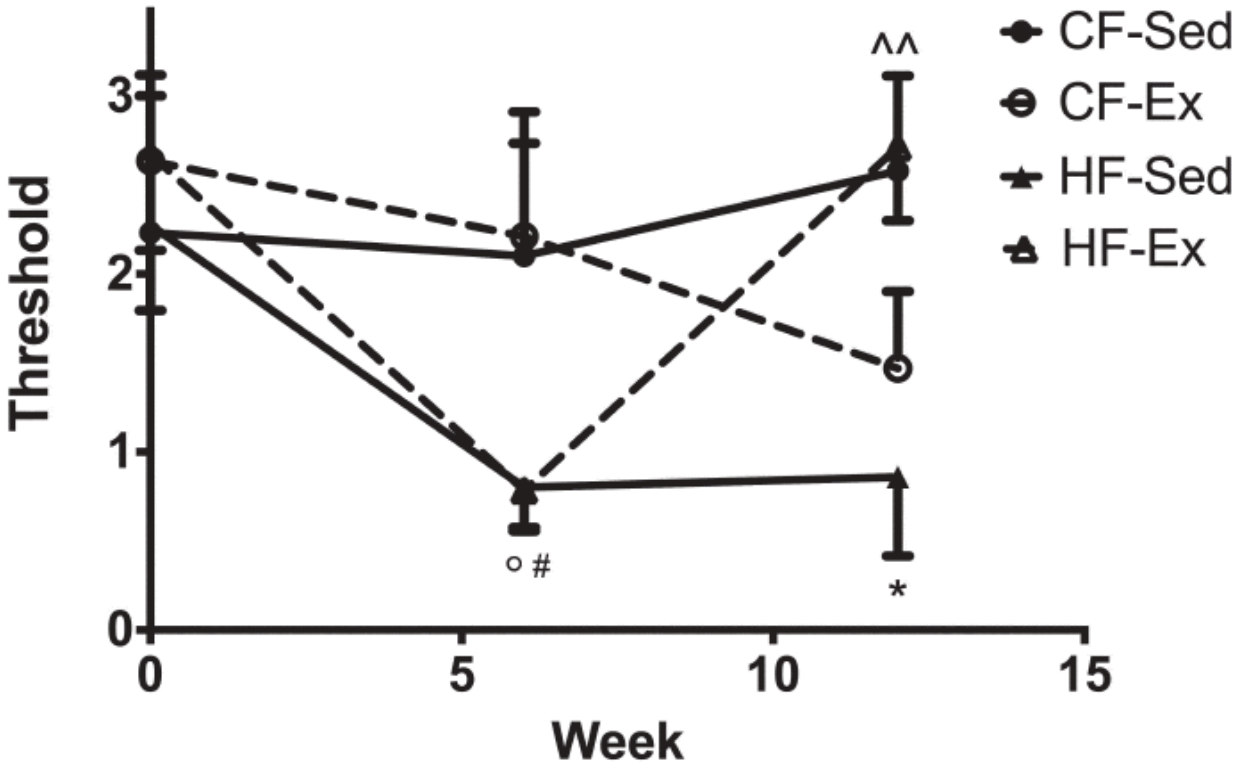

Figure 3.1 High Fat Diet-Induced Mechanical Allodynia

Figure 3.2: (A) A high-fat diet causes weight gain in both HF-Sed and HF-Ex mice compared to their standard diet controls (n=8 for all groups). (B) A high-fat diet slightly increases blood glucose levels in both exercised and sedentary mice, though hyperglycemia does not develop in either cohort. (C) A high-fat diet causes fat mass gains in both HF-Sed and HF-Ex mice compared to their standard controls (n=4 for all groups).(D) High-fat sedentary mice show increased ketones in the blood compared to both chow-fed and high-fat fed mice. A main effect of exercise in reducing the level of blood ketones is present ($p < 0.05$). All data presented as mean ± SEM. *:CF-Sed vs HF-Sed #: CF-Ex vs HF-Sed +: CF-Sed vs HF-Ex ^: HF-Sed vs HF-Ex °: CF-Ex vs HF-Ex

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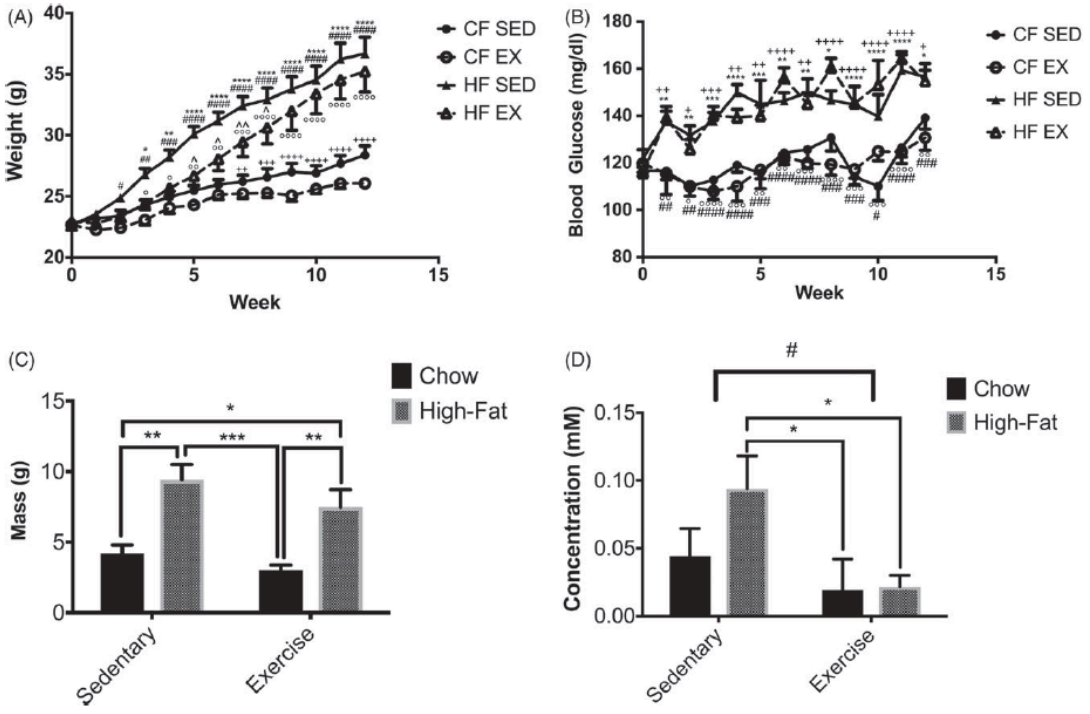

Figure 3.2 Metabolic Measures Altered by Diet and Exercise

High-fat sedentary and high-fat exercised mice gained more weight compared to both chow-fed groups (Fig. 2a). Additionally, measurements of body weight due to changes in fat vs. lean mass revealed a main effect of diet on fat mass ($p < 0.0001$, Fig. 2b). Both high-fat sedentary and high-fat exercise mice gained significantly more fat mass compared to chow-fed groups (CF-Sed vs. HF-Sed, $p = 0.001$; CF-Sed vs. HF-Ex, $p = 0.025$; CF-Ex vs. HF-Sed, $p = 0.0002$; CF-Ex vs. HF-Ex, $p = 0.005$, Fig. 2b). No alterations in lean mass were noted between any groups.

High-fat sedentary and high-fat fed exercised mice had elevated blood glucose levels compared to chow-fed mice two weeks after diet and exercise initiation (Fig. 2c). Both high-fat diet groups' blood glucose levels remained below 250 mg/dl.

Serum bradykinin was not altered by diet or exercise when compared to all groups or by main effects. Serum β–hydroxybutyrate displayed a main effect of exercise ($p = 0.021$) and was significantly elevated in high fat sedentary mice compared to both exercised groups (HF-Sed vs. CF-Ex, $p = 0.016$; HF-Sed vs. HF-Ex, $p = 0.0119$), though not different compared to chow-fed sedentary (HF-Sed vs. CF-Sed, $p= 0.081$, Fig. 2d).

Figure 3.3: (A) Dark cycle respiratory quotient (RQ) has a main effect of diet as seen with high-fat fed mice as compared to chow-fed controls ($p < 0.0001$). (B) A decreased RQ corresponding with diet is still present ($p < 0.0001$) during light cycle hours though the difference in RQ is less significant. (C) Light cycle total energy expenditure (TEE) is unaltered by diet or exercise (D) Dark cycle TEE is increased with exercise in chow-fed mice but not high-fat fed mice as compared to chow-fed controls. A high-fat diet did increase TEE in sedentary mice as compared to their chow fed counterpart. (n=4 for all groups) All data presented as mean ± SEM.

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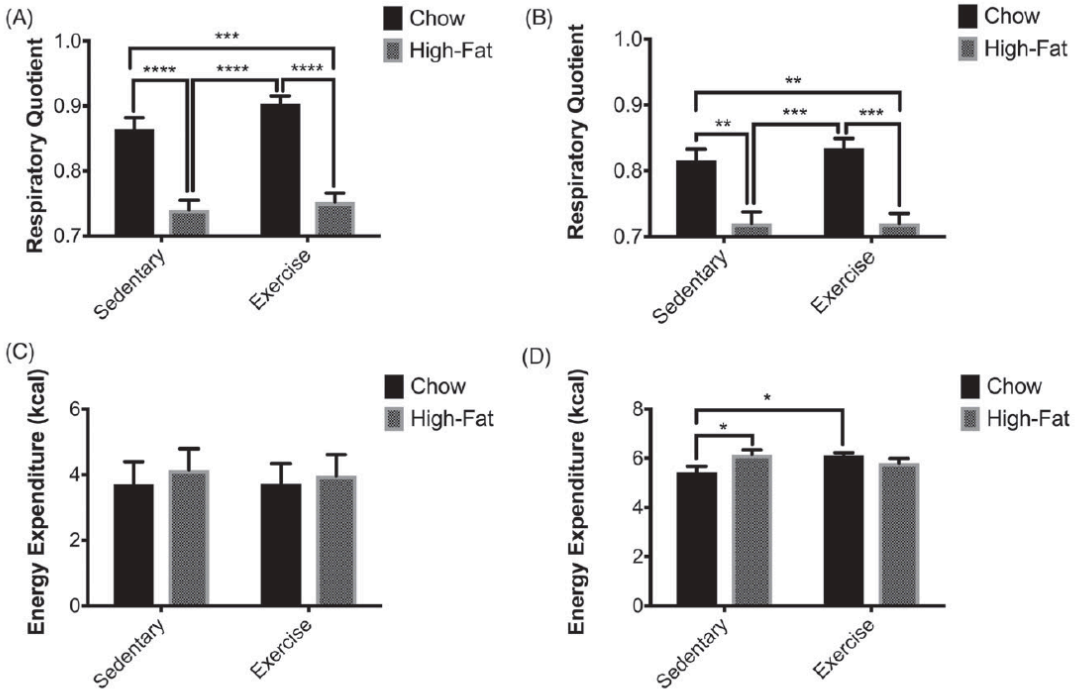

Figure 3.3 Respiratory Quotient and Energy Expenditure

Regardless of exercise training, mice on a high-fat diet had a reduced RQ (dark cycle $p<0.0001$; light cycle $p < 0.0001$); while mice fed a normal chow diet had an RQ of ~0.8 (Figs. 3a, b). Analysis of fuel source utilization in relation to dark-light cycles displayed significant differences (CF-Sed vs. HF-Sed, $p <0.0001$; CF-Sed vs. HF-Ex, $p = 0.0002$; CF-Ex vs. HF-Sed, $p <0.0001$; CF-Ex vs. HF-Ex, $p <0.0001$). The light cycle showed similar albeit less significant alterations (CF-Sed vs. HF-Sed, $p = 0.001$; CF-Sed vs. HF-Ex, $p = 0.0013$; CF-Ex vs. HF-Sed, $p = 0.0003$; CF-Ex vs. HF-Ex, $p = 0.0003$).

Analysis of energy expenditure was divided into 12-hour dark and light cycle quantifications and revealed no differences between any groups (Fig. 3c). Dark cycle analysis displays increased energy expenditure of both high-fat sedentary ($p = 0.0120$) and chow-fed exercised mice ($p = 0.0254$) compared to chow-fed sedentary mice (Fig. 3d).

The expression of mRNA encoding TFAM, DGKB, BDH1, HSP60 and HIF1-α was unaltered by diet or exercise. HSP70 mRNA levels were increased by exercise compared to sedentary mice (CF-Sed vs. HF-Ex, $p = 0.014$; HF-Sed vs. CF-Ex, $p = 0.014$; HF-Sed vs. HF-Ex $p = 0.003$, Fig. 4a). TrkA mRNA was reduced in high fat fed exercised mice compared to chow-fed sedentary mice ($p = 0.024$). Diet or exercise did not alter the expression of mRNAs encoding CGRP and NGF (Fig. 4b).

Exercise significantly decreased IL-1β ($p = 0.011$) and IL-6 ($p = 0.032$) levels in high fat-fed exercised mice compared to high-fat sedentary mice. IL-6 levels were elevated relative to chow-fed sedentary mice in chow-fed exercised ($p = 0.007$) and high-

fat sedentary mice ($p = 0.002$, Fig. 4c). TNF-α was not significantly reduced in high-fat fed exercised mice relative to high fat sedentary mice ($p = 0.053$). In addition, p38 was decreased in high-fat fed exercised mice relative to chow-fed sedentary mice ($p = 0.032$) and high-fat sedentary mice ($p = 0.0376$). JNK was significantly decreased in high-fat fed exercised mice relative to high fat sedentary mice ($p = 0.039$).

TRPV1 mRNA expression was increased in chow-fed exercised mice relative to chow-fed sedentary ($p = 0.037$) and high-fat exercised mice ($p = 0.026$, Fig. 4d). AKAP150 was significantly increased relative to chow-fed sedentary ($p = 0.030$) and high-fat exercised mice ($p = 0.012$). TRPA1 was increased in high-fat fed sedentary mice compared to chow-fed sedentary ($p = 0.049$) and high-fat exercised mice ($p = 0.040$). mRNAs encoding SNAP23 ($p = 0.035$), SNAP25 ($p = 0.009$), and Stxbp4 ($p = 0.033$) were increased in high-fat fed sedentary mice compared to high-fat fed exercised mice. VAMP2 mRNA was slightly decreased in high-fat fed exercised mice compared to high-fat sedentary ($p = 0.084$). VAMP2 was statistically different in high-fat exercised mice compared to chow-fed sedentary mice ($p = 0.017$, Fig. 4e).

Figure 3.4: (A) Exercise increases gene expression of HSP70, but not HSP60, relative to sedentary controls. (B) Exercise reduces gene expression of inflammatory cytokines (TNF- α, IL-1β, IL-6, p38, and JNK) induced with a high-fat diet and sedentary environment. (C) Traditional pain related genes are unaltered by diet or exercise, except TrkA, which is reduced in HF-Ex mice relative to CF-Sed controls. (D) Exercise reduces gene expression of synaptosomal complex components (SNAP23, SNAP25, STXBP4) in high-fat diet fed mice. (E) A high-fat diet increases gene expression of AKAP150 (the kinase for TRPV1) and TRPA1 relative to chow-fed controls, and exercise reduces gene expression back to levels not different from chow fed controls. (n=4 for all groups) All data presented as mean ± SEM.

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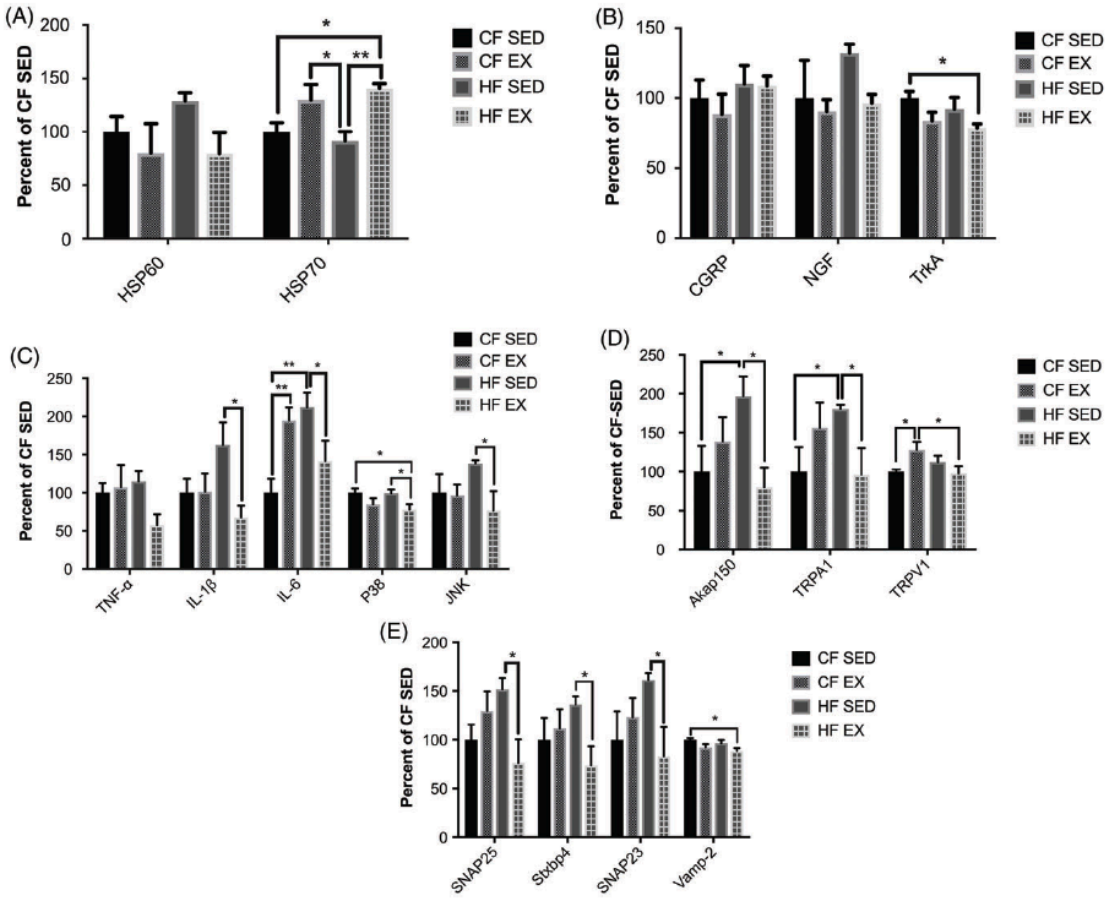

Figure 3.4 Diet and Exercise Induced Gene Expression Changes

Western blot analyses revealed no changes in phosphorylation of p38 protein in the DRG due to diet or exercise. There were no significant alterations in any of the electron complexes analyzed between groups (Fig. 5a, 5b, 5c, 5d). PGC1-α, phosphorylated and total AMPK protein levels were not different across groups. Analysis of SNAP23 and SNAP25 protein levels also revealed no differences in response to diet or exercise.

Analysis of PPAR-α protein expression revealed increases in high-fat sedentary and high-fat exercised mice compared to chow-fed sedentary and chow-fed exercised mice (HF-Sed vs. CF-Sed, $p = 0.0188$; HF-Sed vs. CF-Ex $p = 0.0184$; HF-Ex vs. CF-Sed $p = 0.0076$; HF-Ex vs. CF, $p = 0.0079$, Fig. 5e).

Figure 3.5: (A, B, C, D) Electron complex's II, III, IV, and V are unaltered by either diet or exercise in the DRG of mice (E) The main effect of a high-fat diet increases PPAR-α in both exercise and sedentary mice as compared to their chow-fed counterparts ($p < 0.001$). (CF-Sed n=6, CF-Ex n=5, HF-Sed n=7, HF-Ex n=8) All data presented as mean ± SEM.

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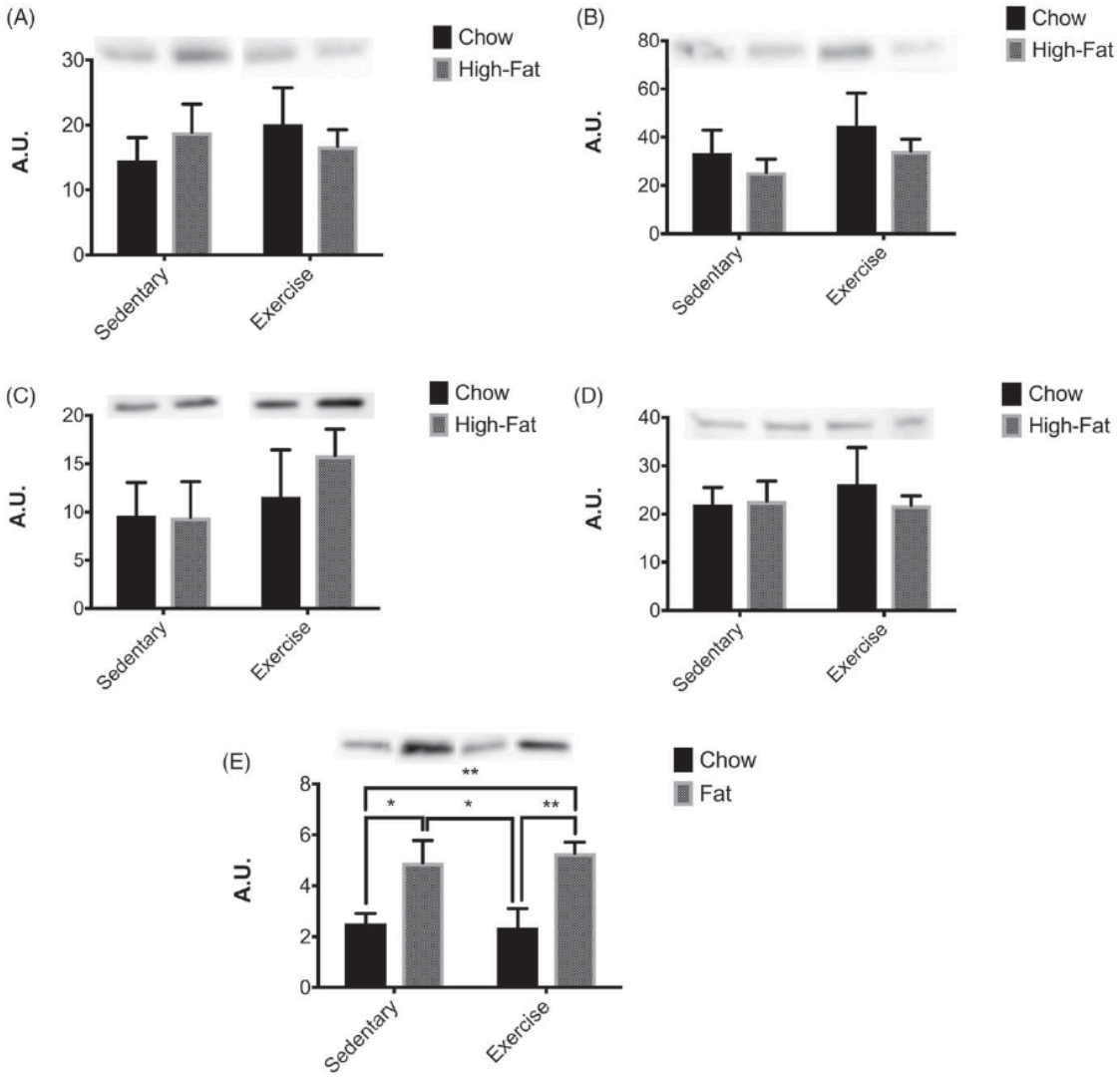

Figure 3.5 Western Blot Analysis of Mitochondrial Complexes

Discussion

Our results demonstrate that mice fed a high-fat diet develop mechanical allodynia [17,180]. Mice with access to running wheels have a slow attenuation of mechanical allodynia after 6 weeks of exercise. We investigated potential molecular mediators that may explain how exercise benefits allodynia caused by a high-fat diet and revealed that exercise can correct sensory impairments without inducing changes in several key metabolic parameters.

In previous studies using high-fat diets, sedentary mice increase fat storage and weight gain, while exercised mice fail to gain weight and increase fat mass similar to sedentary mice [205]. Exercised mice fed a high-fat diet gained weight similar to high-fat fed sedentary mice. It is plausible to suggest these metabolic changes are due to exercise-induced increases in muscle mass as opposed to fat. Our results suggest changes other than fat mass reduction may be important in influencing allodynia based on the result that high-fat fed exercised mice did not differ in lean and fat mass compared to sedentary high-fat fed mice.

Since fat mass and weight changes may not explain the benefits associated with exercise, differences in utilization of fat as an energy source could be important. All our metabolic measures were obtained in cages where mice did not have access to running wheels to avoid acute metabolic changes associated with exercise. High-fat diets induce whole body lipid oxidation as evidenced by respiratory quotients (RQ) that approach a ratio of 0.7 (VCO_2/VO_2) [206]. Exercised and a high-fat fed sedentary mice appeared to

utilize fats as a primary fuel source, suggesting that exercise does not lead to greater utilization of fat as an energy source. This is supported by our findings that sedentary high-fat fed mice had a greater resting energy expenditure compared to high-fat exercised mice. The sedentary high-fat fed mice had the highest fat pad mass, the greatest weight gains, and burned the most energy, indicating resting energy output may not be a key factor in the amount and utilization of fat related to allodynia. One caveat is that the current study performed the calorimetric analysis in caging without running wheels, which could be argued to dampen the effects of 10 prior weeks of training.

We found increased blood levels of β-hydroxybutyrate in sedentary high-fat fed mice and that exercise significantly reduced β-hydroxybutyrate levels. Normally when utilizing fat as a key fuel source, ketone levels are elevated [207]. Exercised high-fat fed mice may process fat more efficiently, leading to a decrease in ketones. We analyzed PPAR-α, a fatty acid-activated transcription factor increased during ketogenesis[208]. While PPAR-α protein levels were increased by the high-fat diet, exercise was unable restore elevated levels of PPAR-α in the DRG. The role of PPAR-α in DRG neurons is complex, as it has been shown to play a role in both analgesia and pain [209].

Our results suggest the benefits of exercise are not mediated via increased fat metabolism alone, since high fat exercised mice have increased fat pad mass and burn fats as a primary fuel source, while expending less resting energy than high-fat fed sedentary mice. Exercised mice fed a high-fat diet displayed improved ketone processing and similar utilization of fat, while at the same time displaying decreased expression of

mediators associated with inflammation. This suggests that exercise exerts benefits by affecting inflammatory pathways that influence the peripheral nervous system compared to the whole-body fat storage and utilization.

The reduction of inflammatory mRNAs (TNF-α, IL-1β, IL-6, p38, JNK) in high-fat exercised mice as compared to sedentary high-fat mice is consistent with exercise's anti-inflammatory role. Based on the exercise-induced changes in inflammatory gene expression, we hypothesized that this could lead to changes in TRP ion channels associated with pain [210]. Our results revealed that TRPV1 mRNA was unaltered by diet or exercise. A kinase involved in the translocation and insertion of TRPV1 into membranes, AKAP150, was significantly decreased in high-fat exercised mice. Modification of TRPV1 trafficking and translocation are consistent with alterations in the synaptosomal complex. SNAP23/25, STXBP4, and VAMP2 were all decreased in high-fat diet exercised mice. These results point to a possible mechanism related to TRP channel trafficking and membrane insertion.

TRPA1 was also examined as it often co-localizes with TRPV1 and is co-activated through TRPV1 signaling [203,211]. TRPA1 mRNA was elevated in high-fat sedentary mice and decreased in high-fat exercised mice. Changes in TRPA1 levels provide a strong link to the mechanical allodynia that occurs in response to a high-fat diet, and also suggests that future studies of both TRPV1 and TRPA1 are needed to fully understand the role of diet and exercise on sensory function.

Chapter 4

Positive Impacts of Ketogenic Diet on Metabolic Syndrome-Induced Mechanical Allodynia in Mice

Introduction

Obesity and metabolic syndrome are a growing international epidemic, and have been strongly linked to the development of chronic pain [212]. The consumption of high fats and carbohydrates leads to metabolic alterations and changes in sensory function in mice similar to changes that occur in human patients, which include obesity, elevated blood glucose, insulin resistance, and mechanical allodynia [17,180,182]. Physical activity and exercise can improve many of these symptoms, including reversing mechanical allodynia induced by a high fat diet [17]; however, the mechanisms by which exercise leads to alterations in metabolic and sensory nerve function is poorly understood. The present study aimed to examine if a ketogenic diet has similar beneficial effects on obesity, chronic pain, and sensory nerve function as compared to exercise intervention.

High fat, low carbohydrate diets that lead to increased ketone production popularly named 'ketogenic' diets are a rapidly emerging intervention for a wide array of clinical diseases [103,213,214]. Historically, ketogenic diets were first utilized in the treatment of epilepsy after it was noted that patients who fasted had reduced seizure occurrence [214]. Recently, ketogenic diet intervention has been tested as a treatment for several nervous system diseases, including glioblastoma, Alzheimer's disease, amyotrophic lateral sclerosis, traumatic brain injuries, and autism [87-95]. Relevant to peripheral nerve function, to date, a limited number of studies have examined how a ketogenic diet impacts mechanical and thermal sensation [99-102].

Previous research has demonstrated that exercise and a high fat diet create distinctive metabolic phenotypes both systemically and in the peripheral nervous system [215]. Both exercise and ketogenic diets are attractive approaches for therapeutic intervention to treat obesity and metabolic syndrome as both increase fat oxidation [112,113]. Additionally, both exercise and ketogenic diet can stimulate anti-inflammatory signaling cascades and reduce chronic inflammation that occurs in response to a high fat diet [100]. A dietary intervention may also be a more attractive clinical intervention, as numerous patients are often unable or unwilling to perform physical activity.

Here, we examine several parameters of peripheral nervous system function from mice fed a ketogenic diet. We have previously reported that mice fed a high fat and carbohydrate-rich diet develop symptoms related to painful peripheral neuropathy in prediabetes including mechanical allodynia [17,180,215]. In the current study, we provide evidence that despite consuming very high levels of fat, mice fed a ketogenic diet fail to develop mechanical allodynia, opposed to mice fed a high fat diet. Additionally, a ketogenic diet can reverse high fat diet-induced mechanical allodynia independent of other obesity-related measures. Together, these results suggest that a ketogenic diet may be an effective intervention for prediabetic and diabetic peripheral neuropathy, and may provide a promising intervention to modify nociception.

Experimental Procedure

Diet and Mice

Seven-week-old male C57/BL6 #027 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 in corn cob bedded, forced air cages. All mice were given ad libitum access to food and water and were fed either a standard chow diet (8604; Envigo, Madison Wisconsin; 14% kcals from fat, 32% protein, and 54% carbohydrate), a high fat diet (07011; Envigo; 54% kcals from vegetable shortening (hydrogenated) and corn oil fat, 21% protein and 24% carbohydrate), or a ketogenic diet (96355; Envigo; 90.5% kcals from vegetable shortening (hydrogenated) and corn oil fat, 9.2% protein, and 0.3% carbohydrate). Standard diet energy was calculated as previously described [17].

Two cohorts of mice were utilized to examine the effects of dietary and exercise interventions. The first cohort was fed a control diet through all baseline testing. After baseline behavioral testing was complete, mice were separated to provide all groups with equal baseline values for behavioral and metabolic measures; and the groups were given different diets and exercise intervention (control fed-sedentary, high fat-sedentary, high fat-exercise, or ketogenic). The second cohort henceforth referred to as 'obesity interventions' mice were all fed a high fat diet for eight weeks and through baseline testing. After baseline behavioral testing (at week 8), mice were separated into similar groups as in cohort 1 (control fed-sedentary, high fat sedentary, high fat-exercise, or

ketogenic). All mice were 8 weeks of age at the start of diet and exercise. A timeline of diet and exercise intervention for both cohorts is displayed in Figure 1. All studies were in accordance with NIH guidelines and conformed to protocols approved by the institutional Animal Care and Use Committee.

Figure 4.1: a) All mice remained on a control diet until baseline testing was completed, then mice were changed to experimental diets and exercise for 12 weeks. b) Obesity Interventions cohort timeline: all mice remained on a high fat diet for 8 weeks, at which point baseline testing was completed, groups where then formed and mice were changed to experimental diets and exercise for 12 additional weeks.

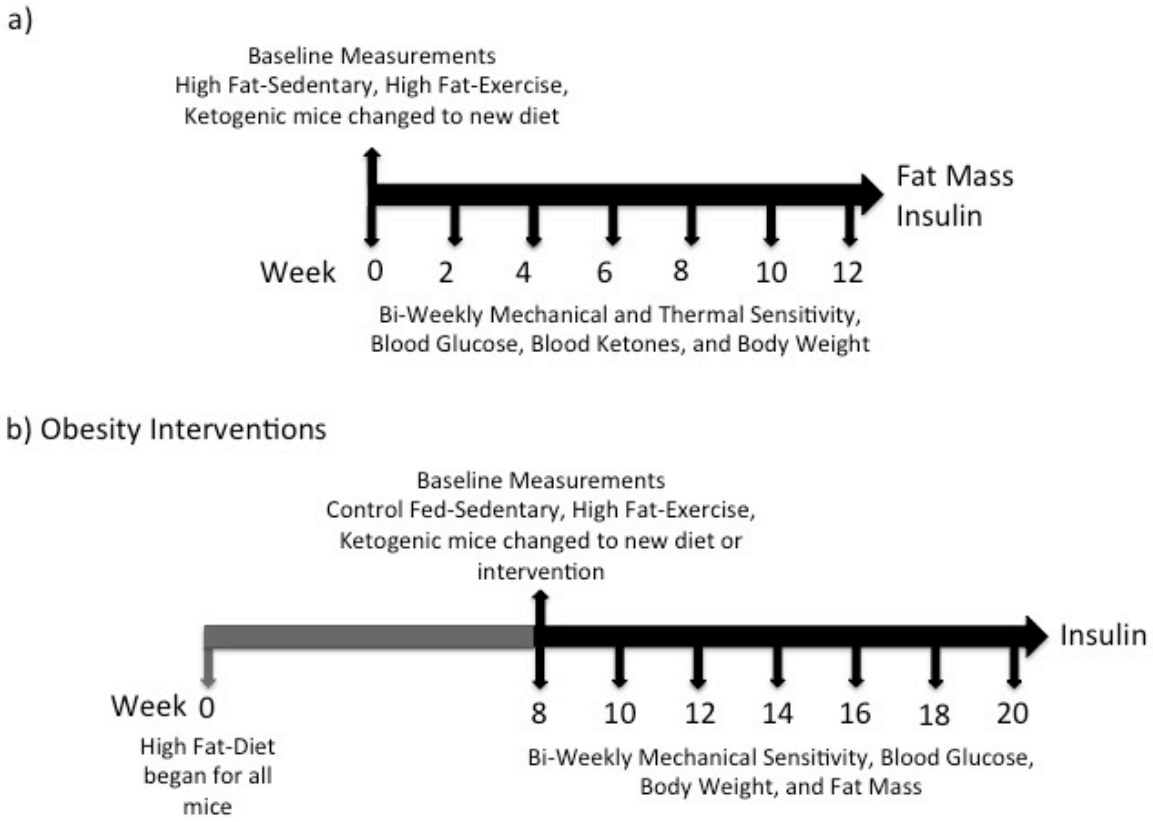

Figure 4.1 Experimental Timeline

Blood Measurements

Ketogenic-fed and obesity intervention groups of mice underwent assessments for weight and blood glucose (glucose diagnostic reagents; Sigma, St. Louis, MO) weekly after a 3 hour fast [17]. Additionally, at the time of sacrifice following a 3 hour fast, blood was drawn from the chest cavity and allowed to clot for 30 min on ice, spun at 3,000g for 30 min at 4 °C and serum drawn off and frozen at -80°C until insulin was analyzed by ELISA (Alpco; Salem, NH). Blood ketones (β-Ketone blood test strips; Precision Xtra; Abbott Laboratories; Chicago, IL) were measured at baseline, week 1, 4, 8, and at sacrifice following a 3 hour fast.

After 4 weeks, an intraperitoneal glucose tolerance test (IPGTT) was performed after a 6 hour fast. Animals were given 1g glucose/kg body weight. Blood glucose levels were measured via tail clip immediately before glucose injection, and 15, 30, 60, and 120 minutes thereafter.

Body Composition

Body composition to assess fat mass was measured using the EchoMRI-100 (EchoMRI, Houston, TX). The body composition of the first cohort of mice was determined immediately before sacrifice. Mice in the obesity intervention groups had body composition determined biweekly from baseline testing until sacrifice.

Behavior Testing

Mechanical sensitivity was assessed using Von Frey monofilaments as previously described [17]. Thermal thresholds were assessed by placing mice in individual clear plastic cages on a Hargreaves's apparatus and a 4.0 V radiant heat source was applied three times to the hind paw as previously described [17]. Sensory behavioral assessments were carried out at baseline and biweekly for all mice for 12 weeks. Cohort 1 mice (dietary intervention) were examined for both mechanical and thermal sensitivity, while mice in the obesity intervention groups were only examined for mechanical sensitivity.

RNAseq

Total RNA for the first cohort of mice was extracted with TruSeq Stranded mRNA Library Prep kit (Illumina, CA). Six biological replicates were used for each dietary and exercise intervention group, for a total of 24 samples. Pair-end sequencing was performed using the Illumina platform with read length of 100 bp and an insert size of 160 bp. FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used to access sequence quality, with manual inspection of the results before proceeding to subsequent steps. The raw gene count data was obtained using RSEM[216] using the default parameters. Within RSEM, we used the bowtie2[217] as mapping tool and the mouse mm10 genome assembly as the reference genome.

The Bioconductor package "edgeR"[218] was used for preprocessing the raw gene count data. Gene count data were normalized by library size and genes with non/low transcription were removed. With regard to the latter, genes with expression level >1 cpm

in at least 2 out of the 24 samples were retained, resulting in a total of ~16,000 genes that were considered for subsequent statistical analyses.

Transcriptional changes in pain-associated genes were then compared across the different dietary and exercise intervention groups. First, a list of 25 pain-associated genes was manually selected. The normalized and log-transformed expression of those genes were then used in principal component analysis (PCA) to gain insight into the potential drivers of variation in the expression of pain-associated genes. To visualize the PCA results, scatterplots of the first, second, and third principal components, which collectively explained nearly 75% of the variation across the 25 selected pain-associated, were generated. Finally, to examine the association between the top principal components and dietary and exercise intervention group, a series of ANOVA models were fit that modeled the each of top 3 principal components as the response, against the different dietary and exercise intervention groups. For these analyses, statistical significance was defined as $p < 0.05$.

Statistical Analysis

All data is presented as mean ± SEM. Data was analyzed using a one-way ANOVA, two-factor ANOVA, or repeated measures ANOVA with post hoc comparisons analyzed using Fisher's test of least square difference where appropriate. Statistical significance was defined as $p < 0.05$ and all statistics were run using either GraphPad

Prism 7.0 (GraphPad Software Inc., La Jolla, CA) or the R statistical programming language (version 3.4).

Results

Obesity Measures

High fat-sedentary mice weighed significantly more than all other groups from week 2 until the completion of the study. High fat-exercise and ketogenic mice also gained significantly more weight than control-fed sedentary mice across the final 6 weeks of the study, though both groups remained below high fat-sedentary mice weights (Fig. 2a). High fat-exercise and ketogenic mice consumed the greatest amount of energy (Fig. 2b). High fat-sedentary mice displayed a significant increase in fat mass as a percent of their body weight compared to all other groups (high fat-sedentary vs. control-fed sedentary $p<0.0001$; high fat-sedentary vs. high fat-exercise $p<0.0001$; high fat-sedentary vs. ketogenic $p=0.0028$). Additionally, high fat-exercise and ketogenic mice have increased fat mass as compared to control-fed sedentary mice (high fat-exercise vs. control-fed sedentary $p<0.0001$; ketogenic vs. control-fed sedentary $p<0.0001$), though less than high fat-sedentary. Though there was no difference in body weight, ketogenic mice had greater fat mass than high fat-exercised mice ($p=0.0420$) (Fig. 2c). Blood ketones were consistently elevated in ketogenic diet-fed mice compared to all other groups, which peaked at 2 weeks on the ketogenic diet (2d).

Figure 4.2. (a) A high fat diet causes weight gain relative to all groups, while exercise or a ketogenic diet displays weight gain relative to standard diet controls (n=18 for all groups). (b) High fat exercised and ketogenic fed mice display increased energy intake relative to sedentary controls (n=18 for all groups) (c) A high fat diet causes fat mass gains relative to all groups, while exercise or a ketogenic diet display fat mass gains relative to standard controls, and a ketogenic diet displays greater fat mass as compared to HF-Ex mice (n=18 for all groups) (d) Ketogenic fed mice experience increased serum ketone levels after 1 week with a decrease over time while still remaining slightly elevated compare to all other groups All data presented as mean ± SEM *:CF-Sed vs. HF-Sed #: Keto vs. HF-Sed ^: HF-Sed vs. HF-Ex +: HF-Ex vs. CF-Sed °: Keto vs. CF-Sed $: Keto vs. HF-Ex * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

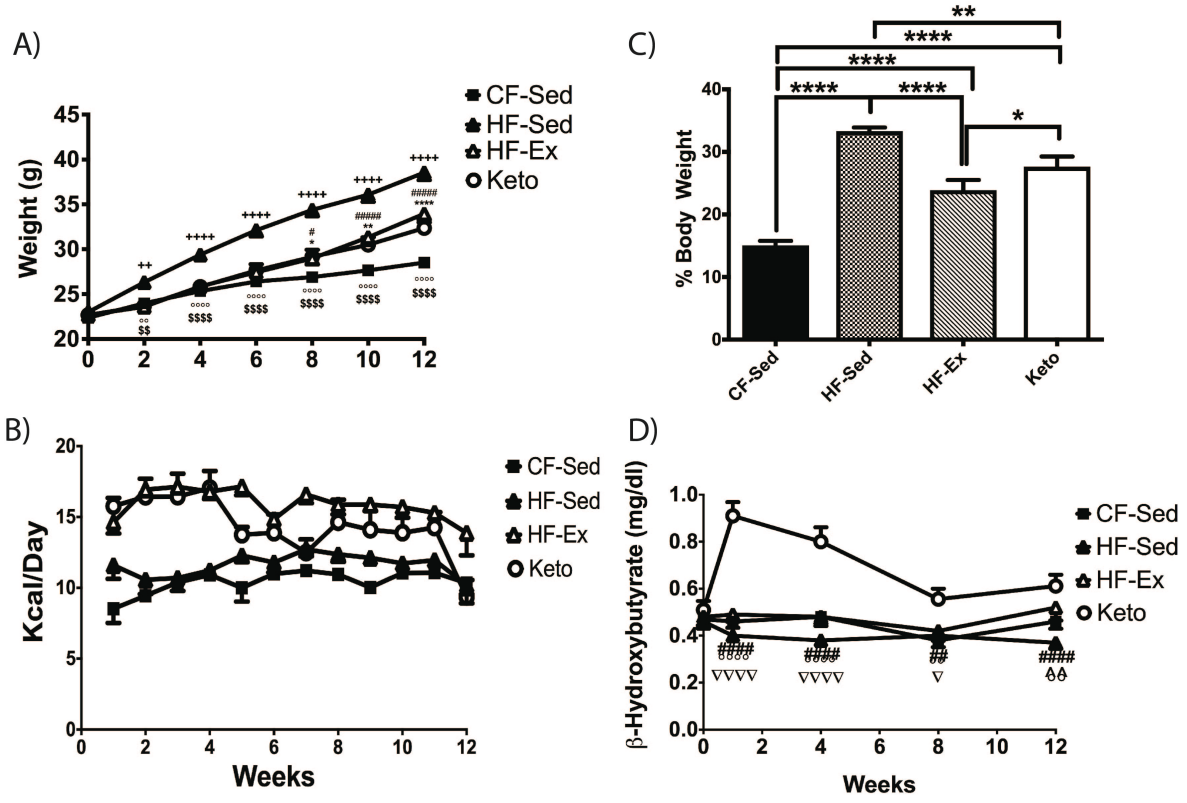

Figure 4.2 Metabolic Alterations Observed with a Ketogenic Diet

Blood Measures of Prediabetes

High fat-sedentary, high fat-exercise, and ketogenic mice had mildly elevated blood glucose levels compared to control-fed sedentary counterparts beginning after 2 weeks of dietary intervention for high fat-sedentary and ketogenic mice and 4 weeks for high fat-exercise mice (Fig. 3a). Fasting insulin was significantly elevated in high fat-fed mice (high fat-sedentary vs. control-fed sedentary $p<0.001$; high fat-sedentary vs. ketogenic $p<0.0001$; high fat-exercise vs. control-fed sedentary $p<0.0001$; high fat-exercise vs. ketogenic $p<0.0001$), but was not altered in ketogenic fed mice compared to control-fed sedentary mice (Fig. 3c). High fat-sedentary mice displayed significantly increased blood glucose levels during IPGTT at 60 and 120 minutes compared to control-fed mice (Fig 3b). HOMA-IR, a measure of beta cell function and insulin resistance, was significantly increased in high fat-fed mice but was unaltered in ketogenic fed mice (high fat-sedentary vs. control-fed sedentary $p<0.001$; high fat-sedentary vs. ketogenic $p<0.0001$; high fat-exercise vs. control-fed sedentary $p<0.0001$; high fat-exercise vs. ketogenic $p<0.0001$) (Fig 3d).

Changes in Sensory Thresholds

After six weeks, high fat-sedentary and high fat-exercise mice displayed decreased thresholds in mechanical sensitivity, though only high fat-sedentary mice were statistically different (high fat-sedentary vs. control-fed sedentary $p=0.0268$; high fat-

exercise vs. control-fed sedentary p=0.0565; high fat-sedentary vs. ketogenic p=0.0482; high fat-exercise vs. ketogenic p =0.094). After 8 weeks, mechanical thresholds in high fat-exercise mice began to return towards baseline threshold levels, while high fat-sedentary mice still exhibited heightened sensitivity (high fat-sedentary vs. control-fed sedentary p =0.0421; high fat-sedentary vs. ketogenic p=0.0161). After 12 weeks, high fat-sedentary mice continued to display mechanical allodynia, while high fat-exercise returned to baseline mechanical thresholds (high fat-sedentary vs. ketogenic p=0.0136; high fat-sedentary vs. control-fed sedentary p=0.0702; high fat-sedentary vs. high fat-exercise p=0.0658) (Fig. 5a). Throughout the course of the study, no groups displayed any changes in thermal sensitivity (Fig. 5b).

Figure 4.3. (a) A high fat diet in both exercised and sedentary mice, or a ketogenic diet, slightly increase blood glucose levels relative to control mice, through hyperglycemia does not develop in any group (n=18 for all groups). (b) A high fat diet in sedentary mice reduces IPGTT relative to control mice, while ketogenic animals display no significant alteration from controls (n=5 for all groups). (c) After 12 weeks, a high fat diet increases serum insulin levels as compared to both ketogenic and control diet animals. (d) A high fat diet regardless of exercise increases HOMA-IR values as compared to both control and ketogenic-fed animals. All data presented as mean ± SEM *: CF-Sed vs. HF-Sed #: Keto vs. HF-Sed ^: HF-Sed vs. HF-Ex +: HF-Ex vs. CF-Sed °: Keto vs. CF-Sed $: Keto vs. HF-Ex * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

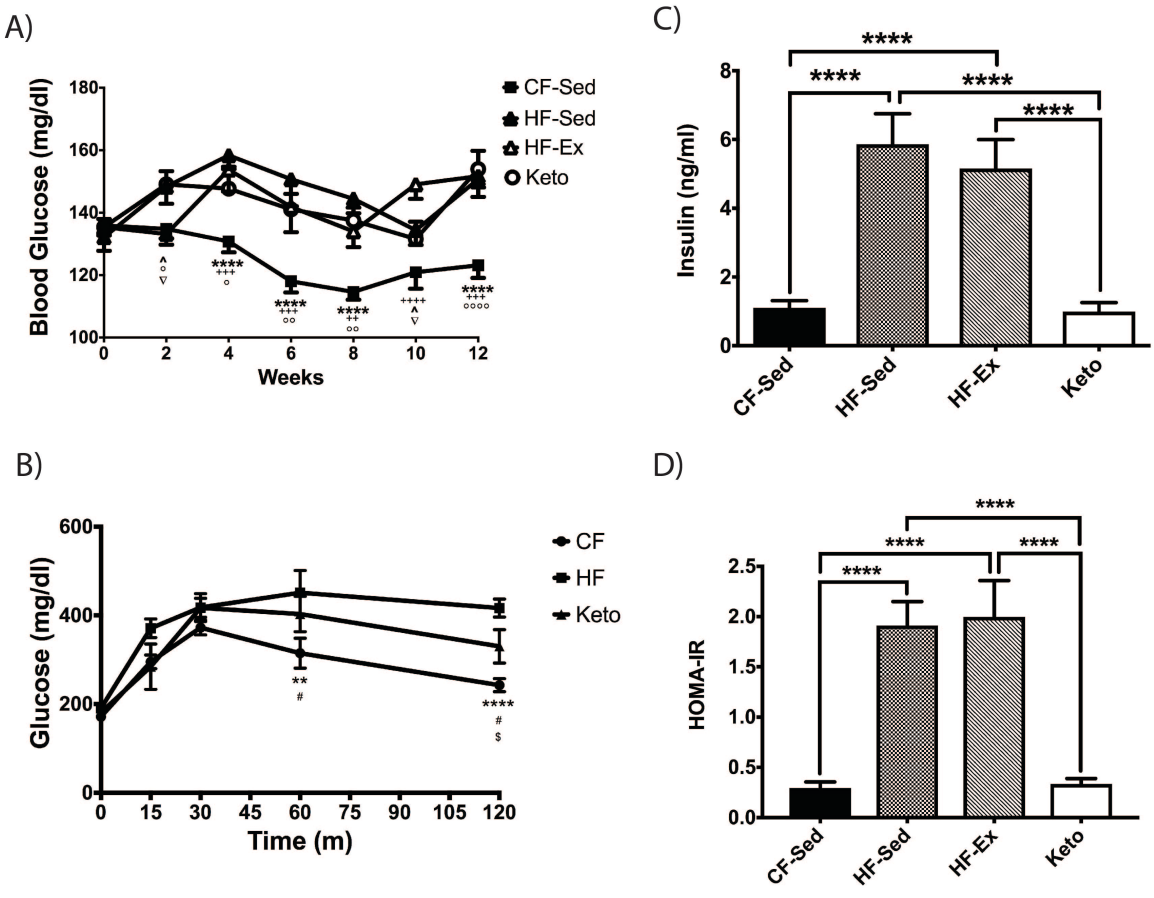

Figure 4.3 Obesity Intervention Metabolic Alterations

Obesity Interventions

Intervention Obesity Measures

All groups were initially started on a high fat diet at 8 weeks of age. Following 8 weeks of being fed a high fat diet, these mice were then divided into different groups based on their diet and exercise intervention. High fat-sedentary and ketogenic mice weighed significantly more than all other groups from week 2 until the completion of the study. High fat-exercise mice also gained significantly more weight than mice switched to a control-fed diet (Fig. 4a). Control-fed sedentary mice were the only group to display a decrease in body weight from baseline measures. Ketogenic diet-fed mice in the obesity intervention consumed the greatest amount of energy, mirroring their increased body weight compared to other groups (Fig. 4b). Elevated fat mass mirrored bodyweight alterations, as high fat-sedentary and ketogenic mice displayed significant increases in fat mass as a percent of their body weight compared to all other groups. High fat-exercise mice maintained their fat mass below high fat-sedentary and ketogenic mice, yet above control-fed sedentary mice (Fig. 4c). Mice switched to a control-fed sedentary diet had significantly less fat mass as compared to all other groups.

Intervention Blood Measures of Prediabetes

Only mice switched to a control-fed sedentary diet displayed a decrease in blood glucose levels (Fig. 4d). Fasting insulin levels at sacrifice were significantly reduced in control-fed sedentary mice compared to high fat-sedentary (control-fed sedentary vs. high

fat-sedentary $p<0.05$) and high fat-exercised (control-fed sedentary vs. high fat-exercise $p<0.01$) mice. Mice switched to a ketogenic diet had insulin levels between control-fed sedentary and high fat-sedentary mice, although not significantly different (Fig. 4e). Only mice switched to a control-fed sedentary diet showed a decrease in HOMA-IR as compared to high fat-sedentary ($p=0.05$) and high fat-exercise ($p<0.01$) mice (Fig. 4f).

Figure 4.4. (a) A high fat diet in both exercised and sedentary mice, or a ketogenic diet, slightly increase blood glucose levels relative to control mice, through hyperglycemia does not develop in any group (n=18 for all groups) (b) Following 8 weeks of a HF diet, mice switched to a ketogenic diet still consume the most energy per day (c) Following 8 weeks of a HF diet, mice given access to a voluntary running wheel or a control diet display a reduction in fat mass (n=10 for all groups) d) Following 8 weeks of a HF diet, only a CF-Sed intervention reduced blood glucose levels over the next 12 weeks (n=10 for all groups) (e) CF-Sed rescue cohort animals experience a rescue of insulin levels, while Keto animals show neither an increase from CF-Sed animals nor a decrease from either HF diet group. (f) Following 8 weeks of a HF diet, only CF-Sed intervention experience a rescue of HOMA-IR, while Keto animas show neither an increase from CF-Sed nor a decrease from HF diet groups. All data presented as mean ± SEM #: HF-Sed vs. Keto ^: HF-Sed vs. HF-Ex °: CF-Sed vs. Keto ∇: HF-Ex vs. Keto * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

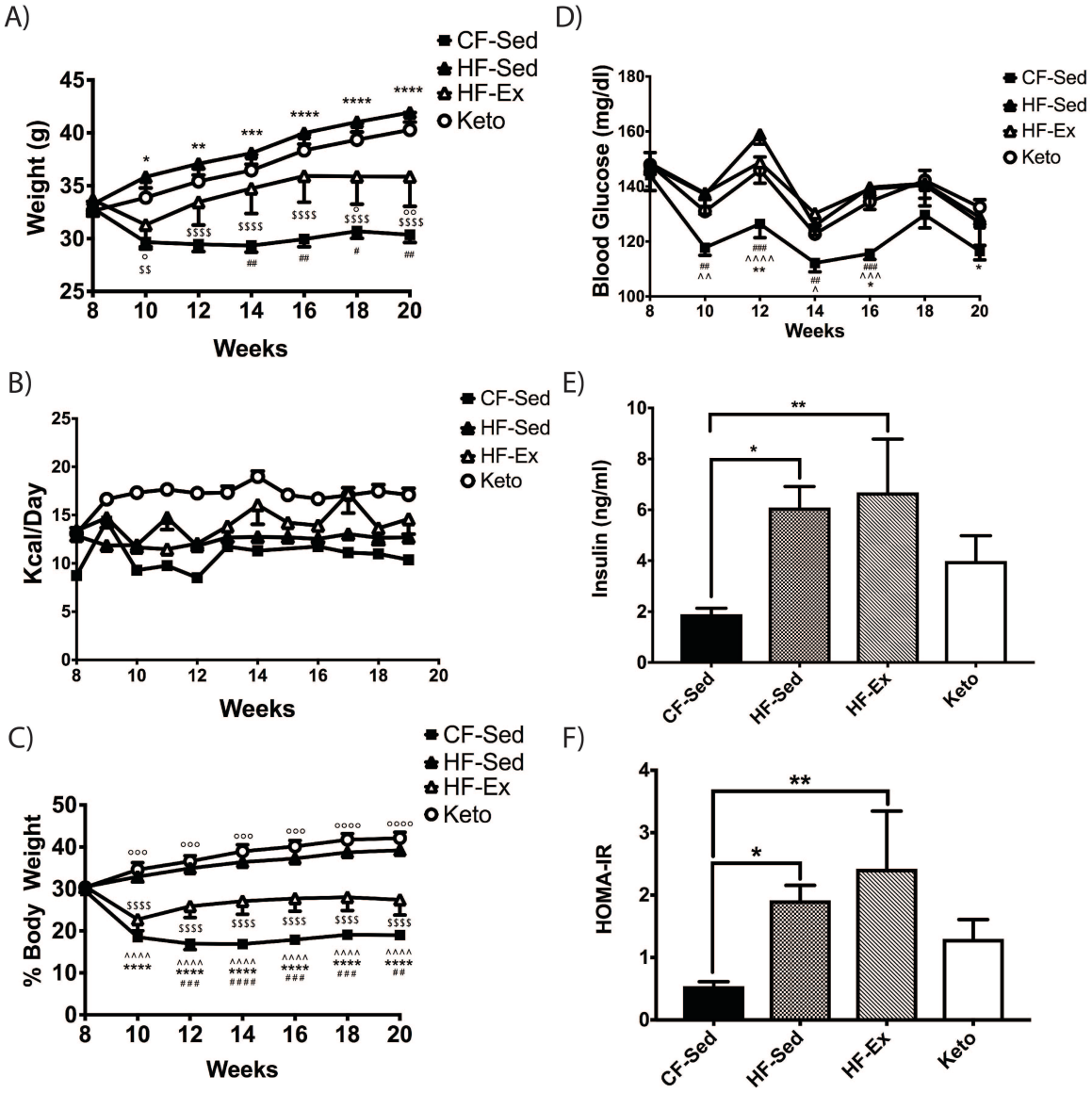

Figure 4.4 Behavioral Alterations of a Ketogenic Diet

Intervention Changes in Sensory Thresholds

Following 8 weeks of a high fat diet, all groups displayed mechanical allodynia. However, 4 weeks after dietary intervention, only ketogenic diet-fed mice displayed a significant increase in mechanical thresholds to near an expected standard baseline value for hind paw sensation, which was maintained until sacrifice (ketogenic vs. high fat-sedentary $p=0.0033$; ketogenic vs. control-fed sedentary $p=0.0119$; ketogenic vs. high fat-exercise $p=0.0426$) (Fig. 5c). No other groups displayed changes in their mechanical sensitivity following a interventional change in their diet, including control diet-fed mice.

Figure 4.5. (a) Von Frey mechanical sensitivity examination shows sensitivity in HF-Sed animals compared to control animals beginning at 4 weeks, and compared to Keto animals beginning at 6 weeks (b) Hargreaves's thermal sensitivity examination displayed no alterations in sensitivity regardless of diet or exercise. (c) Only ketogenic animals experience a reduction in mechanical sensitivity to levels following 8 weeks of a HF-Sed diet. All data presented as mean ± SEM *: CF-Sed vs. HF-Sed #: Keto vs. HF-Sed * $: Keto vs. HF-Ex * $p<0.05$; ** $p<0.01$; *** $p<0.001$

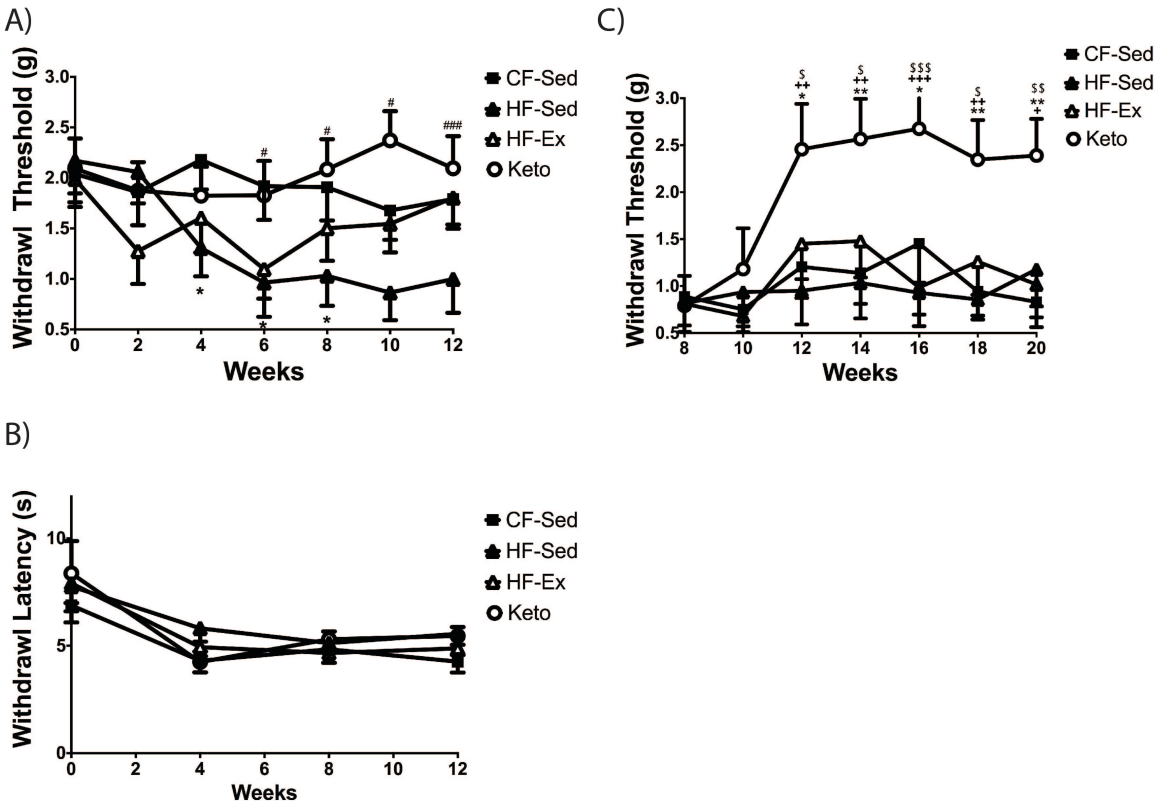

Figure 4.5 Behavioral Alterations of a Ketogenic Diet

Alteration in Pain Associated Genes

A principal components analysis (PCA) was applied to a preselected set of 25 pain-associated genes to understand whether variation in expression of pain-associated genes was related to dietary and exercise intervention. Collectively, the top 3 principal components explained 72.6% of variation across the 25 pain-associated genes, with 36.5%, 22.2%, and 13.9% of variation explained by the first, second, and third principal component (PC), respectively. Associations between both the first and third PC and dietary and exercise intervention group were observed (p = 0.061 and p = 0.006 for PC1 and PC3, respectively), with the first PC reaching only borderline statistical significance. High fat-exercised animals displayed a unique profile compared to all other groups based on PC1 and PC3, as indicated in Fig. 6a. To understand which pain-related genes were responsible for the unique profile exhibited by high fat-exercised mice, we conducted a differential expression analysis comparing gene expression between high fat-exercised mice and all other dietary and exercise intervention groups. Asic3 (p = 9.68E-6), Ntrk1 (p =0.001), and Prkaca (p = 3.09E-4) were significantly down regulated, whereas P2rx3 (p = 0.011), Scn10a (p = 0.015), Ret (p = 0.013), Scn11a (p = 0.009), and Prkca (p = 0.011) were significantly upregulated in high fat-exercised mice (Fig. 6b).

Figure 4.6. (a) Genes were grouped into components that statistically describe the greatest variation between groups, and subsequent principal component analysis (PCA) of these groups of pain-associated genes displays a unique profile for high fat-exercised animals as compared to all other groups. (b) Heat map of pain associated genes following 12 weeks of diet and exercise interventions. Red represents increased mRNA expression, and green represents decreased gene expression. Each vertical lane represents gene changes for a single mouse. Genes significantly different between groups highlighted in yellow dashed boxes (genes utilized for PC1 top yellow box, genes utilized for PC3 bottom yellow box).

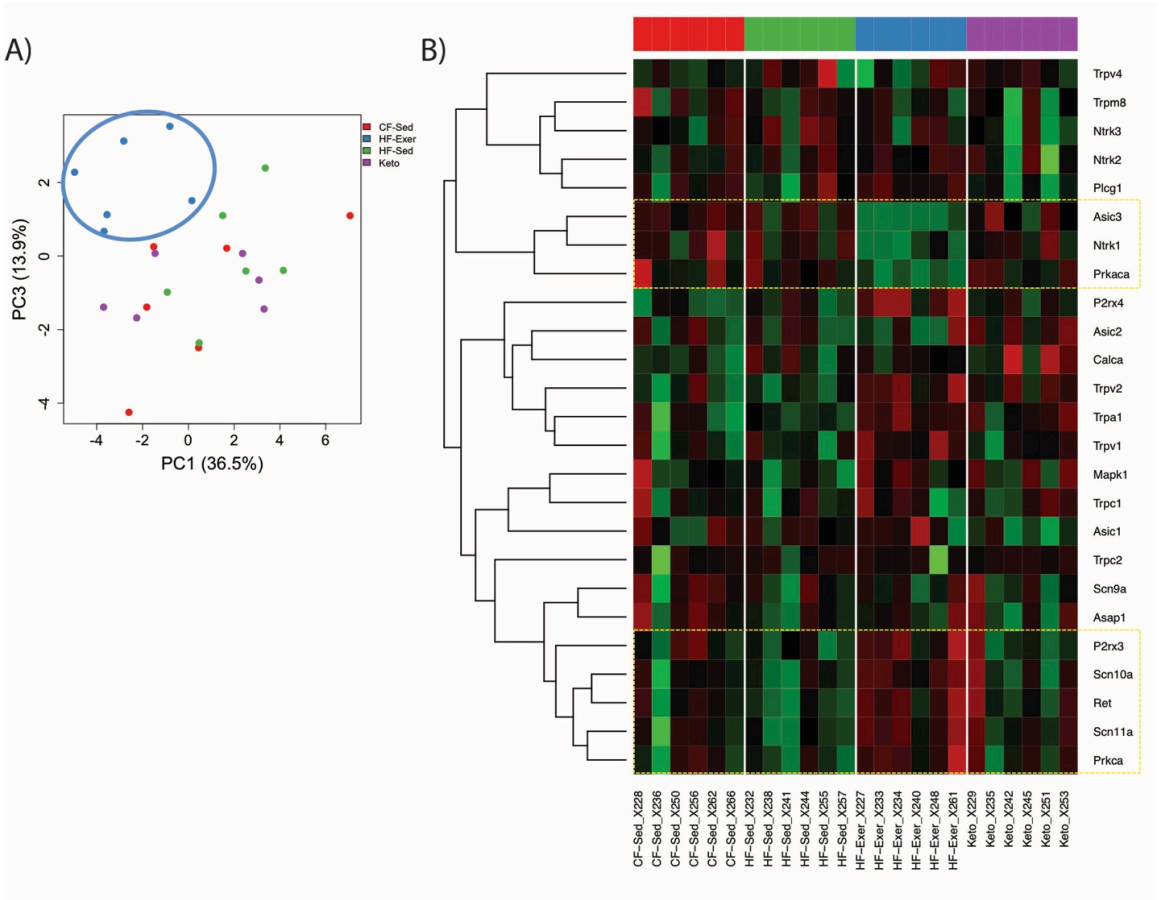

Figure 4.6 Ketogenic Mice Display A Different Pain Gene Profile Than High Fat Exercise Mice

Discussion

There exists increasing evidence of the association in the rise of metabolic syndrome and obesity with the development of various pain syndromes [212]. This has driven an emergence of research to combat these changes in sensation driven by these disorders [17,180,182,215]. Our results reaffirm that a high fat diet leads to numerous metabolic changes representative of prediabetes, including mechanical allodynia. In addition, we have shown that compared to a high fat diet, consumption of a ketogenic diet, does not lead to the development of mechanical allodynia. Similarly, a ketogenic diet is associated with lower insulin levels. Importantly, the consumption of a ketogenic diet reversed pre-existing mechanical allodynia related to the long-term consumption of a high fat diet independent of changes in other metabolic parameters. As an interesting comparison, a control diet, lead to restoration of improved metabolic outcomes but failed to reverse established mechanical allodynia. This data begins to identify complex relationships between biomarkers of metabolic status and peripheral nerve and nociceptive function, and suggests that these relationships may be independently altered by either exercise or dietary interventions. Importantly, these results suggest for the first time that a ketogenic diet may improve nociception associated with obesity and prediabetes that may be translatable to clinical interventions to address a number of painful conditions.

Ketogenic Diet and Metabolic Changes

Previous studies have demonstrated that a high fat diet can serve as a valuable approach to model obesity, increased fat mass, and insulin resistance [17,180,215]. Here, mice fed a high fat diet predictably developed increased body weight and fat deposition, mildly increased blood glucose and increased insulin levels. Exercise was able to prevent increases in body weight and fat deposition despite any changes in elevated blood glucose and insulin levels. Thus, exercise was able to blunt several but not all metabolic changes associated with a high fat diet consistent with previous studies [215]. Similar to exercise, a ketogenic diet also resulted in comparatively reduced body weight and fat deposition but was unable to lower blood glucose. One important distinction is that the ketogenic diet did not lead to hyperinsulinemia similar to a high fat diet or exercised mice on a high fat diet, consistent with previous studies [219]. A role for insulin and insulin resistance in sensory function, nociception and neuropathy is becoming increasingly important. Our results are consistent with the idea that insulin levels may be a key modulator of peripheral nerve function and that insulin resistance has a negative impact on nociception [220-224].

Another important aspect of this study analyzed whether a ketogenic diet could be used as an intervention to improve poor metabolic control and pre-existing nociceptive changes induced by a high fat diet. As noted above, a high fat diet leads to symptoms resembling prediabetes and only a change to the control diet was able to return mice to normal levels of metabolic biomarkers. Surprisingly, mice fed a ketogenic diet continued

to increase their body weight, fat deposition, blood glucose and insulin similar to sedentary mice fed a high fat diet. Contrary to our expectations, exercise initiated eight weeks after consuming a high fat diet was unable to correct metabolic maladies. High fat-exercise mice saw no reduction in insulin levels; however previous work did show high fat-exercise mice do show improved glucose tolerance [17]. Though exercise blunted increases in some metabolic measures, its overall effect exhibited maintenance of the starting metabolic status without improvements to normal levels. Together, these results are somewhat surprising as neither exercise nor a ketogenic diet led to significant improvements in metabolic parameters when used as an intervention after consuming a high fat diet for eight weeks.

Sensory Changes Accompanying a High Fat or Ketogenic Diet

The impact of a ketogenic diet on sensation and neuropathy has received little attention. Previous research reported changes in thermal sensitivity in both juvenile and adult rats when fed a ketogenic diet [100,101]. In the current study, mice never displayed changes in thermal sensitivity in response to a high fat or a ketogenic diet, suggesting that there may be important species differences involved in changes associated with changes. In contrast, mechanosensation was quite sensitive to dietary and metabolic changes. Similar to previous studies, a high fat diet led to the development of mechanical allodynia [17,215]. Importantly, high-fat diet induced allodynia was corrected by exercise [17,215]. We show for the first time that a ketogenic diet does not lead to the development of

mechanical allodynia. This suggests that despite the elevated fat content in both the ketogenic and high fat diet, the amount and utilization of the fat appears to be important. In concordance, our experiments to intervene with a ketogenic diet after consuming a high fat diet for 8 weeks led to remarkable improvements in mechanosensation compared to mice fed a high fat diet with or without exercise or even using a control diet intervention. Interestingly, mice switched to a control diet had a reversal in all metabolic parameters, but failed to reduce the high fat diet induced mechanical allodynia. We postulate that the failure by a control diet to improve mechanosensation could be due to metabolic memory of peripheral pathways. If true, it is plausible to suggest that a ketogenic diet may be able to overcome or reset metabolic memory in sensory components that convey mechanosensation, and lead to a reduction in allodynia or pain associated with prediabetes.

Genetic Alterations With A High-Fat Diet and Exercise

A high fat diet and exercise drives numerous alterations in peripheral nerves, including the development of mechanical sensitivity [17,124,180,182,215]. Analysis of lumbar DRG mRNA expression of selected pain-associated genes revealed that high fat-fed exercised animals developed unique changes in mRNA expression in genes that associated with pain. Moreover, this analysis revealed that ketogenic and high fat-exercised animals do not show the same patterns in mRNA expression changes. The finding that high fat-exercised mice have altered mRNA expression of these genes

suggest that exercise intervention may have a more direct impact on sensory neuron function through changes in specific genes important to nociception. Down-regulation of key genes associated with ASIC3 signaling suggests there may be anti-inflammatory signaling occurring at the level of the DRG. It also suggests that the powerful effect of the ketogenic diet may utilize other mechanisms to provide benefits on nociception. These may include reduced peripheral inflammation from non-neuronal tissues, modulation of central inhibitory systems, and alterations in glutamate levels or signaling, which are postulated actions of a ketogenic diet associated with epilepsy treatment [225]. It is clear that the effects of a ketogenic diet merit further investigation, including the breadth of its effect on other forms of nociceptive, nociplastic and neuropathic pain.

Conclusion

Our current research reveals that a ketogenic diet provides significant benefits to the peripheral nervous system. A ketogenic diet did lead to obesity and increased fat deposition, but not hyperinsulinemia or mechanical allodynia. Exercise can also provide benefits, but it appears that these benefits may be through unique mechanisms and lead to specific changes in gene expression in the DRG. The ability of a ketogenic diet to not only prevent, but to also reverse mechanical allodynia highlights the exciting potential for a dietary intervention to impact patients experiencing metabolic syndrome-associated pain.

Chapter 5

Effects of a ketogenic diet on axonal metabolism and growth

Introduction

The growing epidemic of obesity and diabetes has led to a dramatic increase in various pain syndromes and a personal as well as economic burden. A common complication associated with diabetes and metabolic syndrome is a loss of small fibers in the skin and increased pain [226,227]. With the development of metabolic syndrome, some have proposed that there is a loss of axonal regenerative capacity leading to a loss of small fibers that in diabetes [228]. The current work examined metabolic, sensory, and molecular benefits on peripheral nerves from consuming a ketogenic diet. Previous examination of the effects of a ketogenic diet has primarily focused on the central nervous system and has shown to offer significant benefits to neurons. Caloric restriction increases ketone metabolism and can be a pro-growth signaling agent in the hippocampus and dentate gyrus [229,230]. However, the mechanism and function of increased neuronal growth is still under investigation.

The regulation of axonal growth and epidermal innervation has been studied by others intensely as the ability to innervate skin and re-grow nerves would be a profound therapeutic tool for not only diabetes, but also numerous other neuropathic diseases. One important signaling mechanism regulating nerve growth is the presence of the neurotrophins nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and glial derived neurotrohpic factor (GDNF)[231]. During development, expression of neurotrophins at target tissues is a key signal for axonal growth and innervation[232]. The ketone beta-hydroxybutyrate is now reported to stimulate signal neurotrophin production

through HDAC inhibition[108,233]. Neurotrophins can provide analgesic relief to numerous nociceptive states, and the stimulation of neurotrophin expression by ketone bodies is consistent with newly reported analgesic benefits of a ketogenic diet [234-237].

The metabolism of ketones has been studied in numerous peripheral tissues, but a role for ketone metabolism in the peripheral nervous system is not clear. Studies have shown that metabolism of ketones in peripheral tissues increases peroxisome proliferator activated receptors, which in turn reduce inflammatory states in disease [104,105]. Analysis of the molecular alterations of ketone metabolism also revealed benefits to mitochondrial metabolism that improve anti-inflammatory signaling and may identify important mechanisms associated with ketone metabolism.

Ketone-based metabolism produces less reactive oxygen species (ROS) compared to glucose metabolism at a mitochondrial and cellular level [106]. In neurons, reductions in ROS due to caloric restriction/ketone signaling may occur due to alterations in Complex I of the respiratory chain. In addition, ketones inhibit ROS production through an increase in NAD/NADH ratios while metabolizing beta-hydroxybutyrate as compared to glutamate[107]. This alteration in NAD/NADH ratio activates Sirt1, which inhibits apoptotic and inflammatory signaling while promoting increase mitochondrial biogenesis[107]. Improved mitochondrial respiration may offset detrimental mitochondrial changes associated with increased calcium signaling from neuronal injury to improve neuronal health and healing.

The present study investigates effects of ketone metabolism in peripheral nerves and its potential to alter nerve metabolism in settings of obesity and diabetes. Here, we address potential benefits of a ketogenic diet on peripheral axon growth, and compare them with consumption of a detrimental high fat diet or potential benefits of exercise. In summary, mice fed a ketogenic diet display improved axonal growth that may be stimulated by ketones. Additionally, mice fed a ketogenic diet show adaptations in mitochondria to a fat based fuel source, with reduced ROS production. This adaptation identifies an important mechanism that may explain anti-inflammatory associated benefits of a ketogenic diet on peripheral nerves.

Experimental Procedure

Diet and Mice

Seven-week-old male C57/BL6 #027 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 chow diet (8604; Envigo, Madison Wisconsin; 14% kcals from fat, 32% protein, and 54% carbohydrate), a high-fat diet (07011; Envigo; 54% kcals from vegetable shortening (hydrogenated) and corn oil fat, 21% protein and 24% carbohydrate), or a ketogenic diet (96355; Envigo; 90.5% kcals from vegetable shortening (hydrogenated) and corn oil fat, 9.2% protein, and 0.3% carbohydrate). Two cohorts of mice were utilized to examine the effects of dietary and exercise interventions on peripheral nerves. The first cohort contained mice fed the

standard diet through all baseline testing. After baseline behavioral testing was complete, mice were separated and the groups were given different diets to form the following groups: control-fed, high fat diet-sedentary, high fat diet-exercised, and ketogenic diet. The second cohort henceforth referred to as 'obesity intervention cohort' mice were all fed a high-fat diet for eight weeks and through baseline testing. After baseline behavioral testing was complete, mice were separated and the groups were given different diets or intervention. All mice were 8 weeks of age at the start of diet and exercise. A timeline of diet and exercise intervention for both cohorts is the same as Chapter 4, Figure 4.1. 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.

Intraepidermal Nerve Fiber Density (IENFD)

Footpads were collected and processed from both cohorts of mice as previously described for intraepidermal nerve fiber density [17].

Neurite Outgrowth

Lumbar DRGs 4-6 neurons were harvested and dissociated to a single cell suspension as previously described [238]. Mice were fed the control, high-fat, or ketogenic diet described above for four weeks prior to DRG dissection. Upon plating, all mice were given F-12 media with 10mM glucose for 4 days. Additional culture experiments utilized chow-fed mice in which DRG neurons were plated and were given F12 media custom

supplemented with varying levels of glucose (0mM, 5mM, 10mM) and/or (R)-(-)-3–hydroxybutyric acid (5mM or 10mM) (Sigma). Following the 4 days in culture, neurons were fixed with 4% paraformaldehyde for 10 minutes. Immunohistochemistry was performed with SMI-312 (Covance, Emeryville, CA), a pan-axonal marker, to visualize neurites and counterstained with nuclear marker, Hoechst 33342 (Invitrogen). Coverslips were mounted on slides and imaged. Neurite outgrowth area was quantified using Image J. A stereological grid was superimposed on images of the cultures, and the number of neurites crossing exactly through intersections of the grid was counted, as was the number of neuronal cell bodies producing neurites. Three regions of interest were imaged per coverslip, and three coverslips per group were analyzed for each animal and the neurite area per neuron was calculated according to the following equation [239]:

$$\frac{\left(\frac{\boldsymbol{neurite\ intersections}}{\boldsymbol{total\ grid\ intersections}}\right) \times \boldsymbol{total\ grid\ area}}{\boldsymbol{neurons\ extending\ neurites}} = \boldsymbol{neurite\ area}\ (\mu\boldsymbol{m^2})\boldsymbol{per\ neuron}$$

ELISA's

Following sacrifice, L4-L6 DRG and sciatic nerve were dissected out, snap frozen in liquid nitrogen, and stored at -80C until analysis. Tissue samples were homogenized and quantified for protein as previously described [17]. Proteins for BDNF (Abnova, Taipei City, Taiwan) and GDNF (Abcam, Cambridge UK) were quantified using an ELISA kit per the manufacturer's instructions.

OROBOROS

Mitochondrial respiration and ROS production was assessed using high-resolution respirometry and fluorescence (Oroboros Oxygraph-2k; Oroboros Instruments; Innsbruck, Austria). DRG or SN were permealized with 10μl digitonin and initially placed in respiration chambers in respiration media (MiR05; sucrose, 110 mM; KMES, 60 mM; EGTA, 0.5 mM; MgCl2, 3 mM; KH2PO4, 10 mM; HEPES, 20 mM; Glucose, 20mM; adjusted to pH 7.1 with KOH at 37C; and 1 g/L fatty acid free BSA) for assessment of basal respiration (Basal). Oxygen flux was measured by addition of palmitoyl-CoA (2mM) and malate (.8M) to the chambers in the absence of ADP (GM-State 2) for assessment of State 2 respiration. Oxidative phosphorylation (OXPHOS) with electron flux through complex I was then quantified by addition of ADP (.5M) for assessment of State 3 respiration. Maximal ADP respiration with electron flux through both complex I and complex II was assessed by the addition of succinate (1M) (Succinate: State 3-Complex I+II).

RNAseq

Total RNA for the first cohort of mice was extracted with TruSeq Stranded mRNA Library Prep kit (Illumina, CA). Six biological replicates were used for each dietary and exercise intervention group, for a total of 24 samples. Pair-end sequencing was performed using the Illumina platform with read length of 100 bp and an insert size of 160 bp. FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used to access sequence quality, with manual inspection of the results before proceeding to subsequent steps. The raw gene count data was obtained using RSEM[216] using the default

parameters. Within RSEM, we used the bowtie2[217] as mapping tool and the mouse mm10 genome assembly as the reference genome.

The Bioconductor package "edgeR"[218] was used for preprocessing the raw gene count data. Gene count data were normalized by library size and genes with non/low transcription were removed. With regard to the latter, genes with expression level >1 cpm in at least 2 out of the 24 samples were retained, resulting in a total of ~16,000 genes that were considered for subsequent statistical analyses. The resulting genes were than used to perform KEGG pathway analysis to examine all genes associated with known pathways. Axonal guidance and mitochondrial oxidative phosphorylation pathways were examined for differences between ketogenic and control-fed mice. These alterations shown before are relative gene expression differences, and have not been analyzed for statistically significant alterations but are instead utilized to show full complete pathway changes.

Statistical Analyses

All data is presented as mean ± SEM. Data was analyzed using a one-way ANOVA, two-factor ANOVA, or repeated measures ANOVA with post hoc comparisons analyzed using Fisher's test of least square difference where appropriate. Statistical significance was defined as $p < 0.05$ and all statistics were run using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA).

Results

Intraepidermal Nerve Fiber Density

Nerve fiber densities were altered by both diet and exercise, though in opposing fashions. Nerve fiber density is decreased by exercise (CF-Sed vs. HF-Ex $p=0.0002$; HF-Sed vs. HF-Ex $p=0.0017$; Keto vs. HF-Ex $p<0.0001$) and increased with a ketogenic diet (CF-Sed vs. Keto $p=0.0496$; HF-Sed vs. Keto $p=0.0225$; HF-Ex vs. Keto $p<0.0001$) (Fig. 1c).

Obesity Intervention Cohort: Mice placed on a ketogenic diet display an increase in nerve fiber density compared to all groups (Keto vs. CF-Sed $p=0.0158$; Keto vs. HF-Sed $p=0.0033$; Keto vs. HF-Ex $p<0.0001$). HF-Ex mice display decreased nerve fiber density relative to CF-Sed mice as seen in the initial cohort ($p=0.0004$) (Fig. 1d).

Figure 5.1. (a) Epidermal innervation of ketogenic mice following 12 weeks of diet (b) Epidermal innervation of animals placed on ketogenic diet following 8 weeks of a high fat diet c) Ketogenic diet-fed mice display increase epidermal nerve fiber density as compared to all other groups, while high fat-exercised mice display decrease nerve fiber density as compared to all groups (d) Obesity intervention cohort ketogenic-fed mice again display increased epidermal nerve fiber density as compared to all other groups, while high fat-exercised mice again display decreased nerve fiber density as compared to control-fed mice. All data presented as mean ± SEM * $p<0.05$; ** $p<0.01$; *** $p<0.001$, **** $p<0.0001$

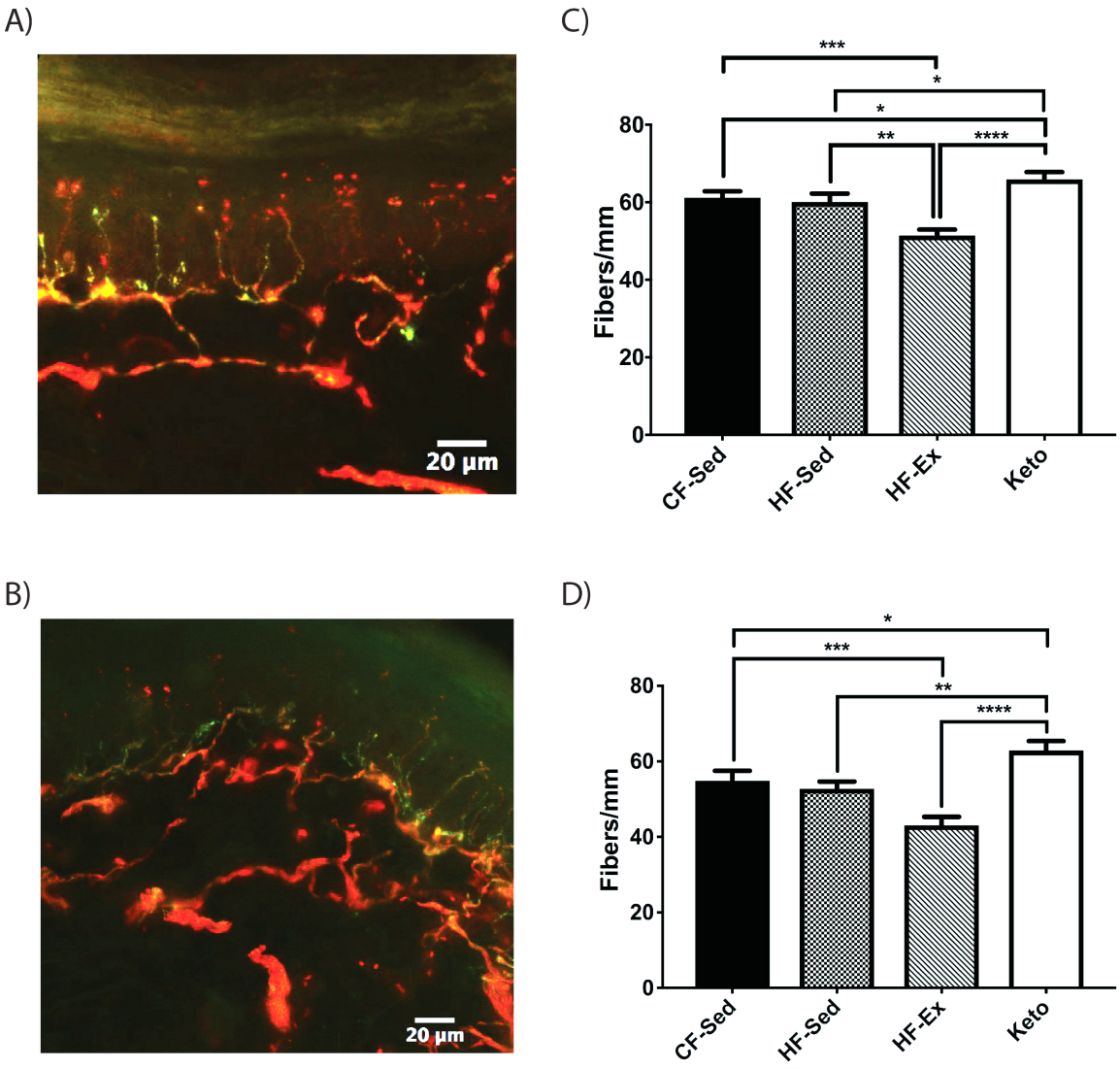

Figure 5.1 IENFD Alterations by Diet and Exercise

Neurite Outgrowth

Following four weeks of dietary intervention, primary cultured neurons from mice fed a ketogenic diet and grown in 10mM glucose F12 media displayed increased neurite outgrowth compared to a control ($p=0.0197$) or high fat ($p=0.001$) diet (Fig. 2c). A follow up examination of neurons from control diet-fed mice displayed increased neurite outgrowth when grown in media containing 5mM Glucose and 5mM Ketones ($p=0.001$) and 10mM Ketone and 0mM Glucose ($p=0.0343$) as compared to standard 10mM Glucose F12 media (Fig. 2d).

Figure 5.2. (a) Neurite outgrowth of primary DRG cultured in 10mM glucose F12 media from mice fed a ketogenic diet for four weeks prior to sacrifice (b) Neurite outgrowth from primary DRG cultured in 5mM glucose 5mM ketone (c). Mice fed a ketogenic diet one month prior to culture display improved neurite outgrowth as compared to mice fed a control or high fat diet when all placed in 10mM F12 media (d) DRG cultured in F12 media supplemented with 5mM ketones and 5mM glucose or 10mM ketones display improved neurite outgrowth as compared to traditional 10mM glucose media. All data presented as mean ± SEM * $p<0.05$; *** $p<0.001$

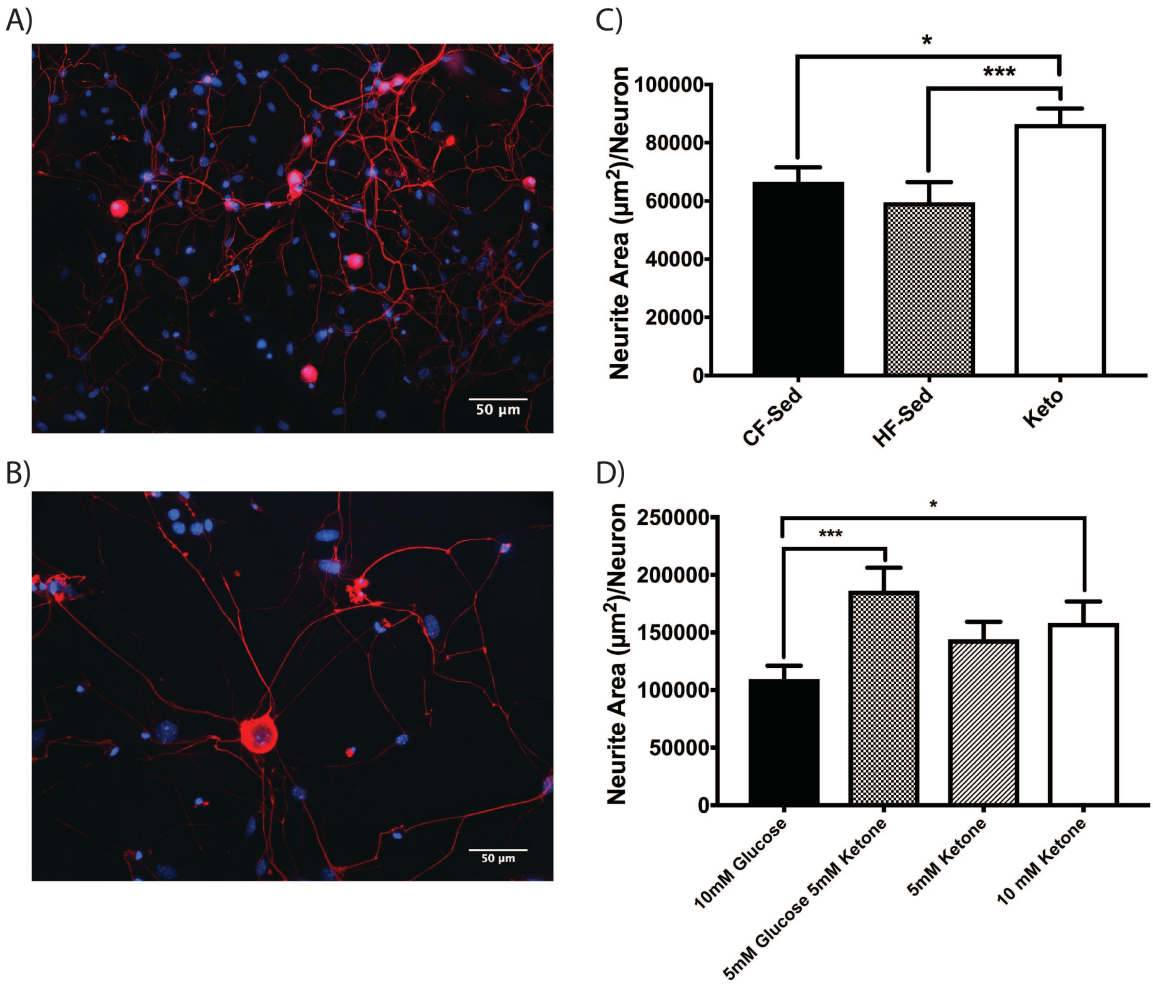

Figure 5.2 Ketones Increase Neurite Outgrowth

ELISA's

DRG harvested in vivo following 12 weeks of dietary or exercise protocols display no differences in GDNF or BDNF protein levels (Fig. 3). Sciatic nerves from high fat-sedentary mice show significantly increased GDNF protein levels compared to control-fed ($p=0.034$) and ketogenic-fed ($p<0.0001$) mice; while high fat-exercise mice display increased GDNF protein levels compared to ketogenic-fed mice ($p=0.0145$) (Fig. 3).

Figure 5.3. (a) GDNF protein levels from DRG of mice following 12 weeks of diet or exercise intervention (n=8 for all groups) (b) GDNF protein levels from sciatic nerves of mice following 12 weeks of diet or exercise intervention (n=8 for all groups) (c) BDNF protein levels from DRG of mice following 12 weeks of diet or exercise intervention (n=7 for all groups) (d) BDNF protein levels from sciatic nerves of mice following 12 weeks of diet or exercise intervention (n=7 for all groups). All data presented as mean ± SEM * $p<0.05$; *** $p<0.001$

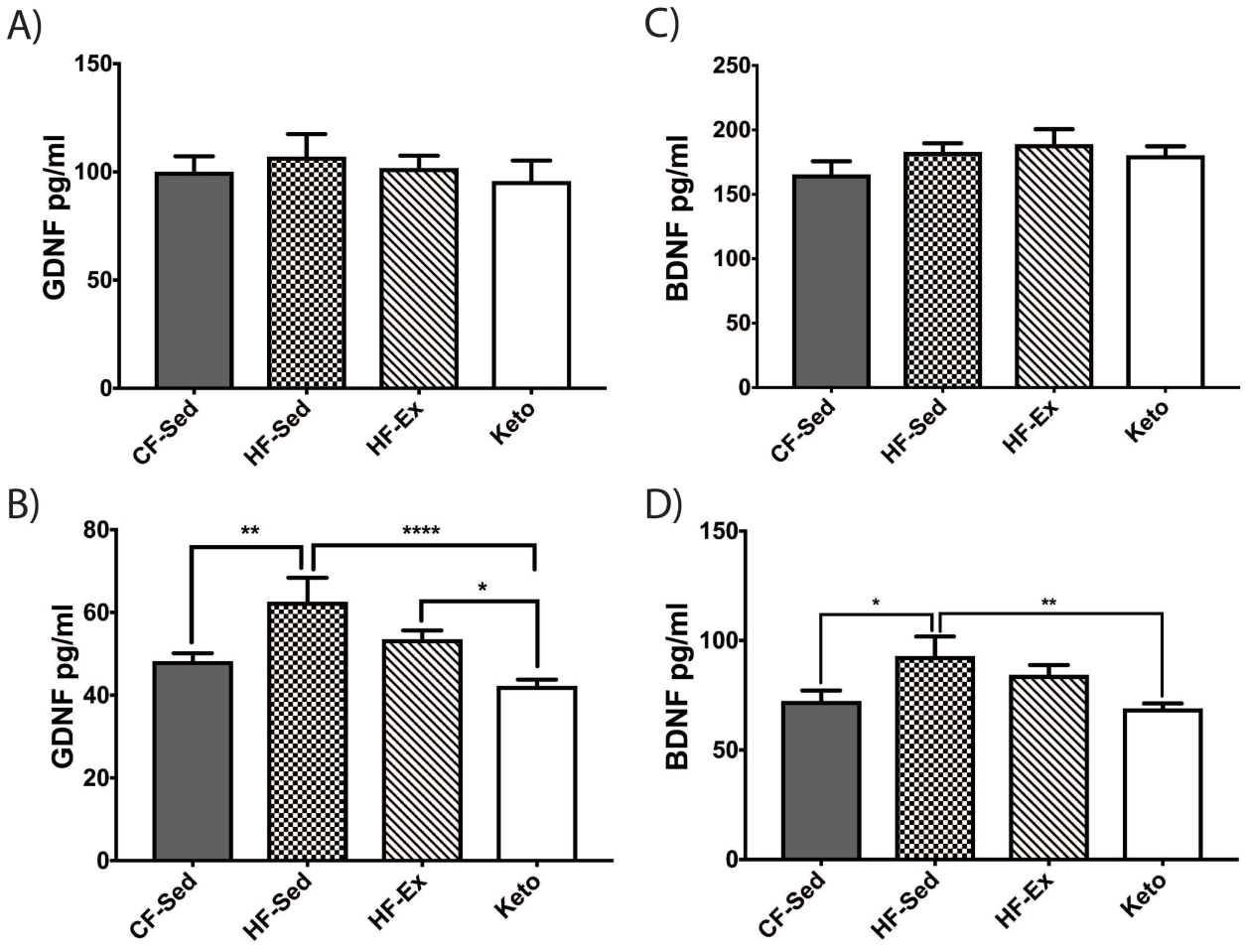

Figure 5.3 Dietary and Exercise Effects on Neurotrophin Protein Level in Mouse DRG and Sciatic Nerve

A Ketogenic Diet Alters mRNA Expression of Genes Important in Axon Growth

RNAseq was used to analyze expression patterns of known mRNAs associated with axon growth and/or inhibition in lumbar DRGs from ketogenic-fed mice following 12 weeks on the diet. This analysis reveals a trend of increased expression of axonal outgrowth-associated genes (NFAT, PLCγ, UNC5, EphA, Par3, Par6, SSH, BMP7, WNT signaling), as well as apparent decreases in mRNA encoding axon inhibitory signals compared to control-fed mice (CXCR4, Cdc42, Rac, Cofilin, CRMP, TRPC, CAamKII, Ptch1) (Fig. 4).

Additional mRNA analysis revealed that a ketogenic diet resulted in increased mRNA expression from the NADH dehydrogenase (Complex I), cytochrome c reductase (Complex III) cytochrome c oxidase (Complex IV) compared to control-fed mice (Fig. 5). Complex I associated genes: Ndufs, Ndufa, Ndufb, Ndufc; Complex III associated genes: ISP, QCR6, 7, 8, 9; Complex IV associated genes: COX4, 5a, 5b, 6c, 7a, 7b, 7b

Figure 5.4. Relative mRNA expression of axonal guidance pathway genes in DRG of mice fed a ketogenic diet to control-fed mice. Within each gene in the below figure, all 6 animals from the ketogenic-fed mice are represented by colored bars for their expression relative to control mice. Green indicates a decrease in relative expression, red represents an increase in relative expression, and gray represents no difference from control-fed mice (n=6 mice for all groups).

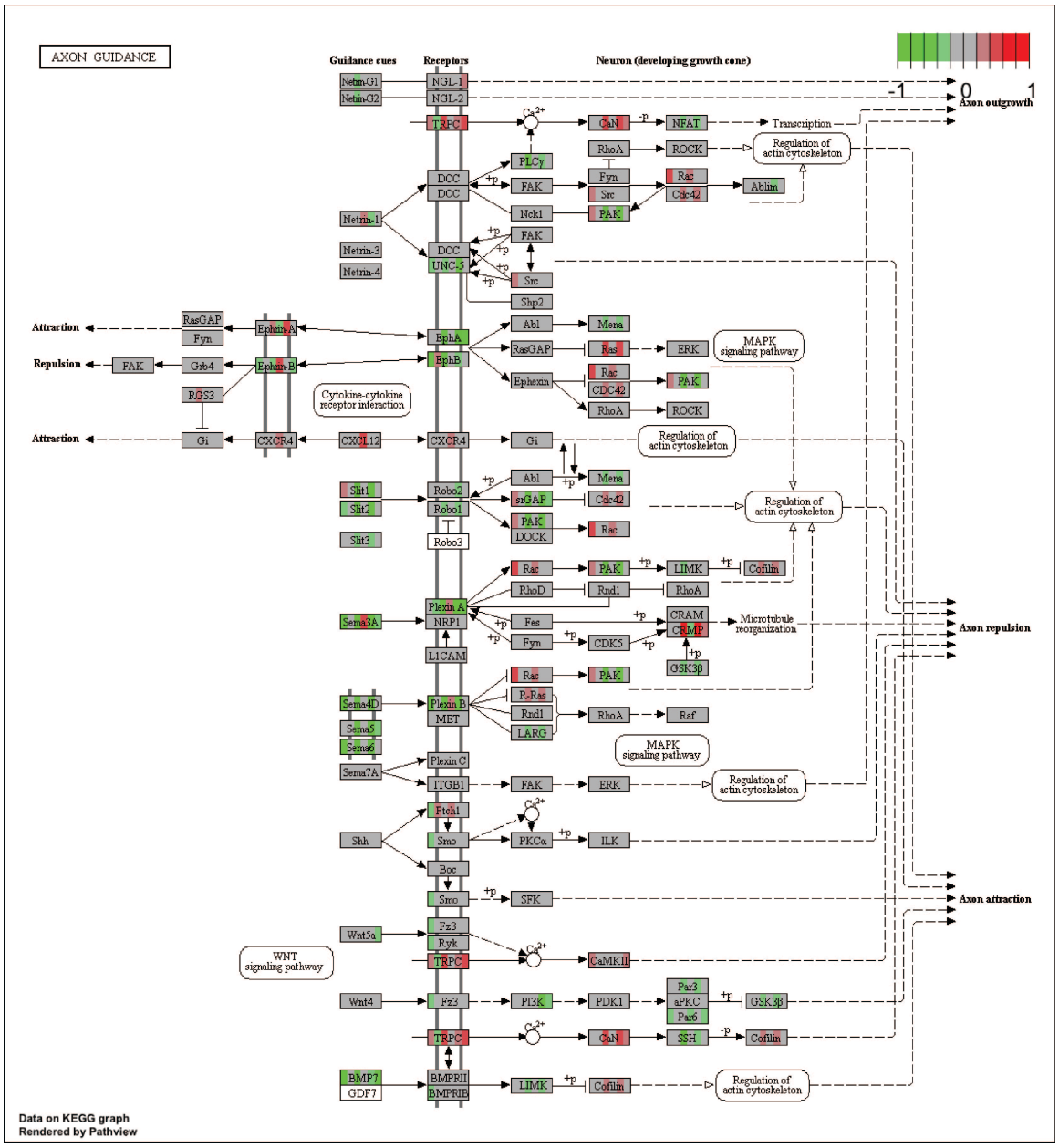

Figure 5.4 Expression of Axonal Guidance Associated Genes

Figure 5.5. Relative gene expression of oxidative phosphorylation complex genes in DRG of mice fed a ketogenic diet to control-fed mice. Within each gene in the below figure, all 6 animals from the ketogenic-fed mice are represented by colored bars for their expression relative to control mice. Green indicates a decrease in relative expression, red represents an increase in relative expression, and gray represents no difference from control-fed mice (n=6 for all groups).

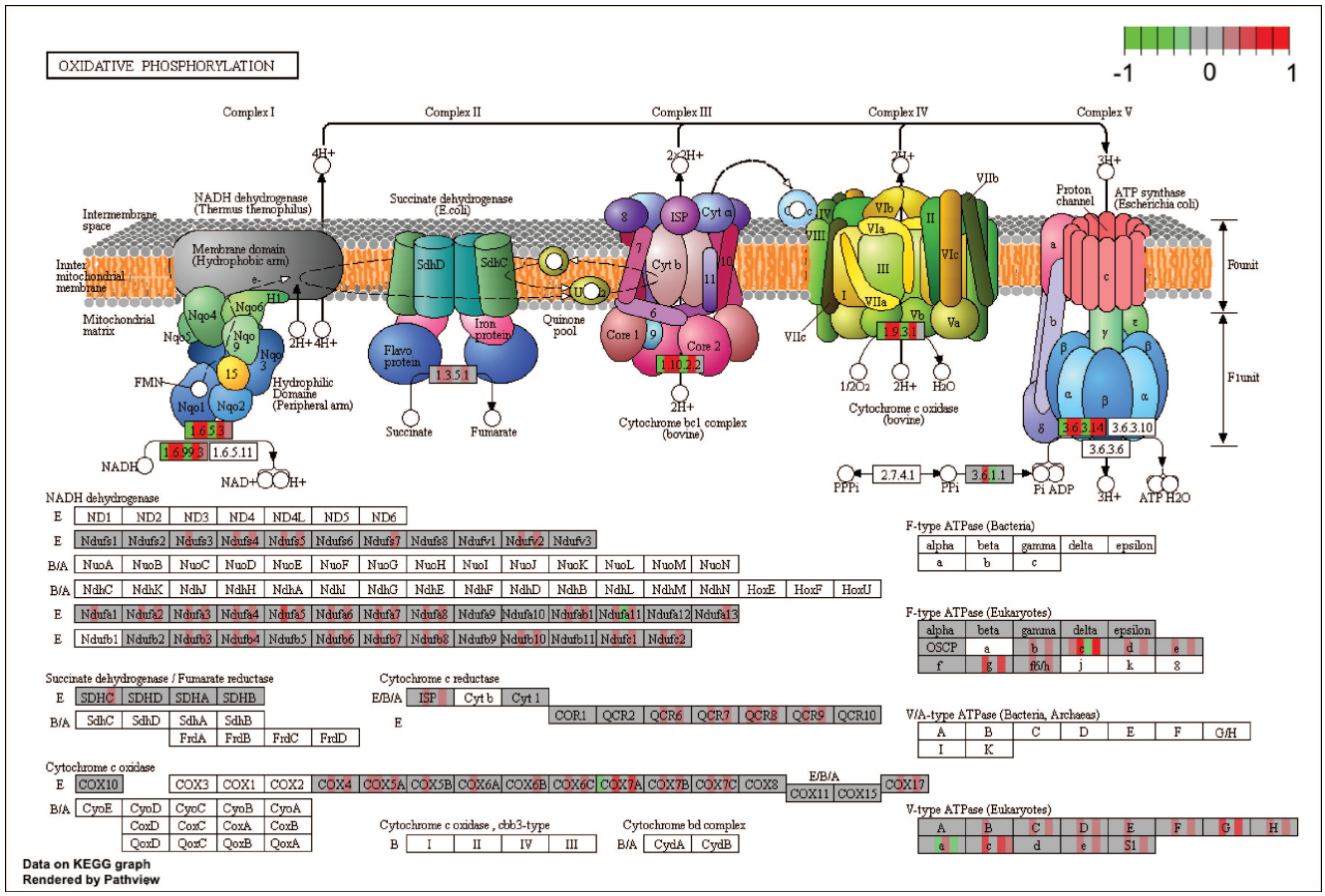

Figure 5.5 Gene Expression of Oxidative Phosphorylation Complexes

Mitochondrial Respiration of Fat Metabolism in DRG

DRG neurons display no differences in basal or maximal respiration of palmitoyl-CoA following 10 weeks among a high-fat, ketogenic, or control diet (Fig. 6a & 6b). DRG also show no change in H2O2 flux, a measure of ROS production, during metabolism of palmitoyl-CoA regardless of diet (Fig. 6c & 6d).

Mitochondrial Respiration of Fat Metabolism in Sciatic Nerve

Sciatic nerves from control-fed mice have elevated basal ($p=0.03$) and maximal ($p=0.02$) oxidative respiration from palmitoyl-CoA as compared to high fat-fed mice (Fig. 7a & 7b). Sciatic nerves from ketogenic-fed mice have decreased H2O2 flux during maximal respiration ($p=0.019$), a sign of reduced ROS production when metabolizing palmitoyl-CoA compared to control-fed mice (7c & 7d).

Figure 5.6. (a) Basal ADP dependent (State III) respiratory rate of DRG following 10 weeks of diet (b) Maximal respiratory rate of DRG (c) State III rate of ROS production in DRG (d) Maximal rate of ROS production in DRG (n=8 for all groups).

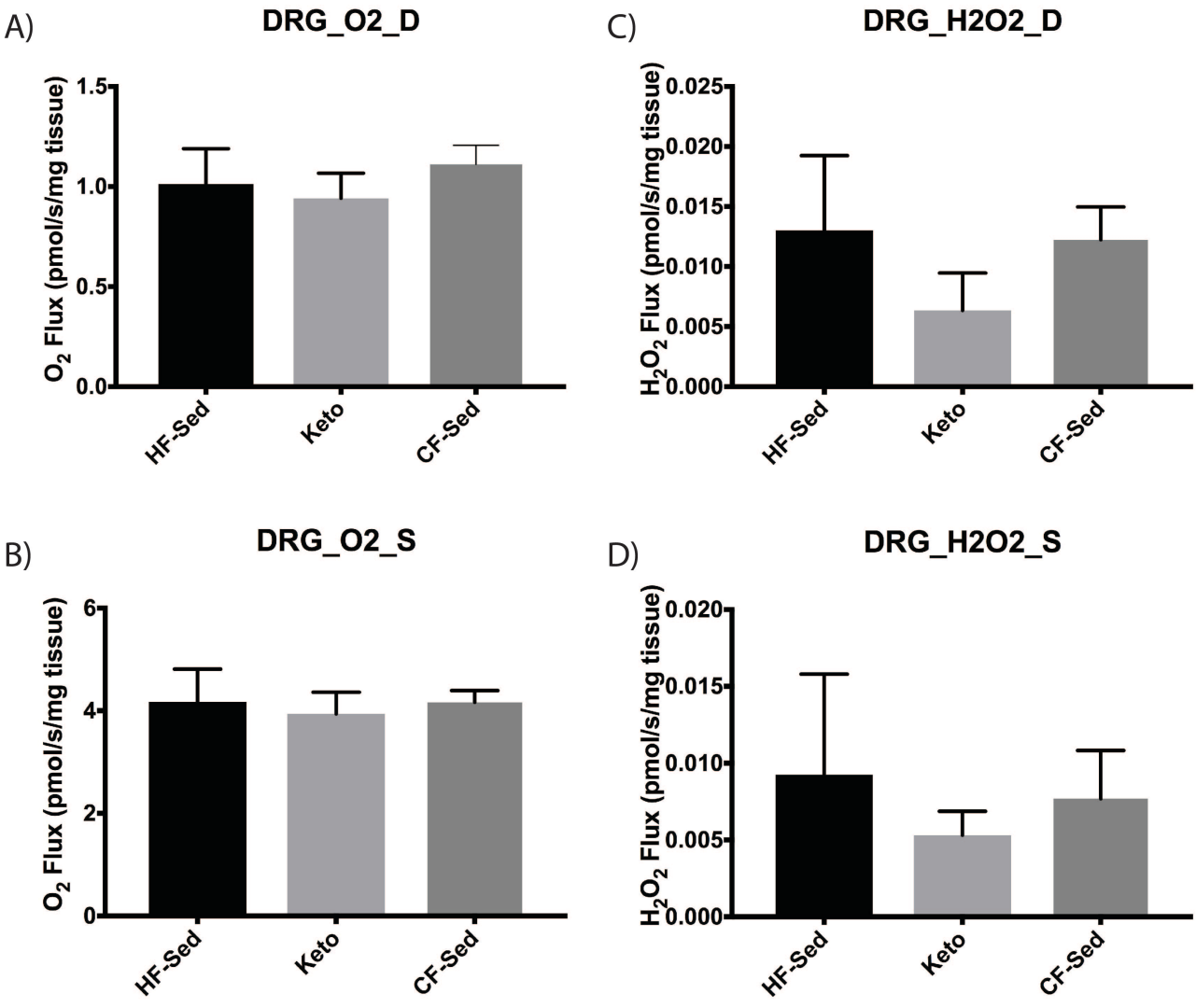

Figure 5.6 Oxidative Respiration and ROS production in DRG

Figure 5.7. (a) Basal ADP dependent (State III) respiratory rate of SN following 10 weeks of diet (b) Maximal respiratory rate of SN (c) State III rate of ROS production in SN (d) Maximal rate of ROS production in SN (n=8 for all groups).

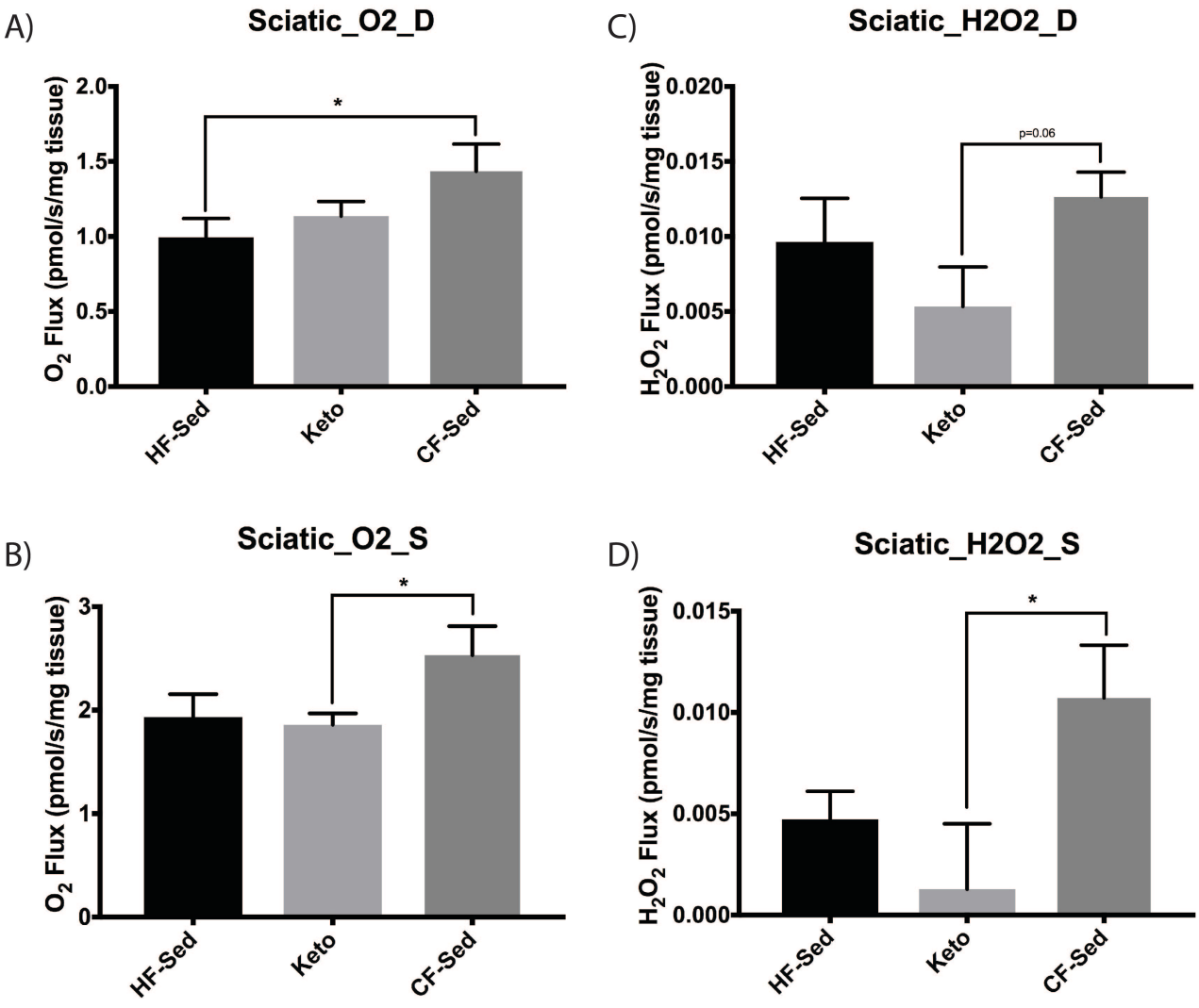

Figure 5.7 Oxidative Respiration and ROS production in Sciatic Nerve

Discussion

The growing epidemic of obesity and metabolic syndrome is coupled with an increase in distal symmetric sensorimotor polyneuropathy (DSPN), one of the most common forms of peripheral neuropathy. DSPN is classically characterized by a dying back phenotype in which the distal tip of the axon retreats or degenerates. This axonal degeneration is commonly accompanied with pain and/or numbness in the extremities [240]. To this point there have been limited therapies to help intervene or rescue this dying back neuropathy[241,242]. Neurite outgrowth has been show to be increased in mice lacking the acetylcholine receptor, and M_1R null mice given streptozotocin to induce diabetic neuropathy did not see a reduction in neuronal innervation[241]. The only other intervention to improve diabetic neuropathy increased Hsp70 in sensory neurons to improve insulin and IGF1 signaling providing protection against neurodegeneration in diabetic phenotypes[243]. The work presented here shows that a ketogenic diet can increase cutaneous innervation, even in mice with obesity and metabolic syndrome. One proposed mechanism of metabolic dysfunction leading to a dying back neuropathy is an increase in mitochondrial dysfunction and increased oxidative stress [244]. Here we also show that mitochondria in DRGs from mice fed a ketogenic diet adapt to fat based fuel sources with reduced oxidative stress compared to mice fed a traditional diet. Together these results provide a potential new intervention using a ketogenic diet for conditions associated with axonal degeneration.

Nerve Density & Outgrowth

A common complication associated with diabetes and metabolic syndrome is a loss of small fibers in the skin and increased pain [226,227]. With the development of metabolic syndrome, some have proposed that there is a loss of axonal regenerative capacity leading to a loss of small fibers that is a common complication of diabetes [228]. Currently, there are few approaches that have attempted to prevent or rescue this loss of innervation associated with neuropathy [241,242,245,246]. The present results may offer extremely attractive new interventions to prevent a loss of innervation and perhaps rescue and promote an increase in nerve growth.

Analysis of IENF density in mice fed different diets revealed that a ketogenic diet resulted in a 10% increase in IENF density compared to mice fed a high fat diet. This was also true in mice that received a ketogenic diet as an intervention from established obesity. Further, *in vitro* experiments revealed that DRG axon growth was enhanced in mice that were fed a ketogenic diet for 4 weeks. It is interesting that high fat-exercised mice displayed a decrease in IENFD despite having a reduction in mechanical allodynia, perhaps again highlighting disparate mechanisms by which exercise and a ketogenic diet reduce mechanical allodynia, as seen in Chapter 4. Additional *in vitro* experiments in mice fed a control diet revealed that DRG neurons supplemented with either only ketones, or ketones and glucose displayed increased axonal growth compared to DRG neurons in media containing only glucose. From these results, we hypothesize that a

ketogenic diet and ketone bodies themselves may alter metabolism that promotes axon integrity and/or axon growth.

Recent evidence suggests that ketone bodies can stimulate neurotrophin production via inhibition of HDAC[108]. Based on the extensive evidence that neurotrophins are powerful stimulators of axon growth[231,232], we examined whether the expression of two neurotrophins, BDNF and GDNF was altered in the DRG or sciatic mice within our study. Our results revealed that GDNF and BDNF levels were reduced in the sciatic nerves of ketogenic fed mice with no alterations in the DRG for any dietary group. This could suggest that the ketogenic-fed mice experience improved shuttling and use of the neurotrophins from the distal end of the axon. Compartmentalization of neurotrophins and the timing of these changes are likely complex, and further study is needed to elucidate how ketone bodies and neurotrophin signaling may be linked when consuming a ketogenic diet. However, increases in neurotrophin levels could suggest increased pro-growth signaling is responsible for the improved IENFD observed in mice fed a ketogenic diet.

After completing RNAseq analysis of genes relevant to axonal guidance pathway signaling for the entire know pathway, the pathway picture suggests that a ketogenic diet alters axonal guidance related gene expression. The genes represented in the pathway analysis were not required to be individually statistically significant; instead, they were denoted as changes by individual mice relative to controls. The trend of genes suggest the key signaling of the axonal pathways may not only be an increase in outgrowth signaling

but also suggest a reduction in the axonal repulsion signals that can arise. The gene expression changes that signal attraction while reducing repulsion signaling could be the reason for such significant INEFD changes in both prevention and recovery models as seen in Fig 1. Moreover, this may offer signaling with the ability to respond to counter axonal repulsion and death signaling during disease states.

Mitochondrial Function & Respiration

One potential mechanism underlying poor peripheral nerve function may be the of abnormal mitochondrial function and increase oxidative stress [244]. This theory is based on the idea that in mitochondrial metabolism, an increase in ROS leads to increased lipid peroxidation and impaired mitochondrial function in the axon. Collectively, this may lead to axonal degeneration and death [247]. The primary alteration observed in mitochondrial oxidation besides increased ROS production was a reduction of genes associated with the enzymatic activity of Complex's I and IV [247].

Our present research suggests that consumption of a ketogenic diet may benefit peripheral nerves by improving mitochondrial function. RNAseq analysis of changes in mRNA expression of genes important in oxidative phosphorylation pathways displayed increased gene expression of NADH Dehydrogenase (Complex I) genes, as well as a decrease in cytochrome C oxidase (Complex IV) genes in ketogenic-fed mice relative to controls. This suggests that contrary to control diet-fed mice, axonal growth in ketogenic diet-fed mice may increase genes associated with complex I and IV. This is important

since it has been reported that mitochondrial function and gene expression is negatively altered in the sensory neurons of animal models of type 1 diabetes[220].

Ketones inhibit ROS production through an increase in NAD/NADH ratios, and our results are consistent with this view [107]. In our study, the decrease in mitochondrial H_2O_2 flux in sciatic nerves during steady state and maximal respiration shows ketogenic-fed animals have a direct decrease in ROS production when metabolizing fats. This key metabolic improvement may provide the key adaptation which neurons adapt to metabolizing fats by a ketogenic diet. The changes in mitochondrial function were only observed in the sciatic nerve, suggesting that more peripherally-located mitochondria are more susceptible to metabolic dysfunction than those in the neuronal cell body. This also agrees with literature that the more distal nerves are the most susceptible to metabolic dysfunction when modeling diabetes[248].

Conclusion

The present work highlights the important impact of dietary changes on peripheral axon growth, gene expression, and mitochondrial function. We have shown that a ketogenic diet promotes peripheral axon growth and improves mitochondrial function. Here, we provide evidence that peripheral neurons can use a fat based fuel source and can adapt to this fuel source. The ability to use dietary interventions to improve axonal growth and function in settings of axon degeneration is an important step forward for a myriad of neuropathies, including DSPN.

Chapter 6

Conclusions

Summary and Findings

Obesity has risen to a global epidemic and with this rise has come a subsequent increase in the rates of cardiovascular disease, metabolic syndrome, and diabetes. This rapid increase in obesity-related disease has also driven increases in biomedical research to combat these common diseases. However, this research is often reserved to selected tissues such as skeletal muscle, cardiac tissue, and the liver. Research limited to these traditional tissues has resulted in insufficient knowledge on other peripheral organs that are affected by obesity. This dissertation focused on the peripheral nervous system and how obesity, metabolic syndrome, diet, and exercise affect sensation and pain. Our goal was to conduct experiments to examine mechanical allodynia, metabolic syndrome, inflammation, and mitochondrial reactive oxygen species production in an effort to understand the effects of altered metabolism on obesity related alterations such as diabetic neuropathy.

Initial work in this dissertation examined the effect of genetic regulation of aerobic capacity using a model of genetic variance in metabolic syndrome. Our analysis of female HCR and LCR rats displayed profound changes in female LCR rats associated with fat mass, inflammatory signals, and mechanosensation. These findings are consistent with the view that the peripheral nervous system is susceptible to metabolic changes known to be important in human disease. Next, studies examined the role of a high fat diet and exercise on metabolic syndrome-induced mechanical allodynia. Mice fed a high fat diet and allowed to exercise developed significant changes in inflammation, mechanosensation, and fat metabolism. Exercised impacted fat metabolism in mice by

leading to decreased circulating ketones. This work led to the discovery that a ketogenic diet is extremely beneficial to peripheral nerve sensation and growth. Thus, the final studies in this dissertation examined the effect of an extremely high fat, low carbohydrate 'ketogenic' diet on the function, sensitivity, and growth of peripheral nerves. Together the present results show that the peripheral nervous system is altered by metabolic insults, and in turn adaptable to physical activity and dietary changes. Significantly, our results reveal that a ketogenic diet is beneficial to mitochondrial function, mechanosensation, and axonal growth in peripheral nerves. Together, these studies highlight a potential new intervention for millions of individuals experiencing neuropathic symptoms associated with obesity and metabolic syndrome.

Chapter 2

This chapter utilized low capacity running (LCR) and high capacity (HCR) rats to examine the underlying mechanisms by which high and low aerobic capacity impacts peripheral nerves. LCR and HCR rats are commonly used in physiological studies designed to understand mechanisms and genetics associated with exercise, activity and disease risk. We compared a number of pain- and neuropathy-relevant features of the peripheral nervous system in female LCR and HCR rats on a control diet to understand baseline alterations due to only inbred aerobic capacity.

The LCR and HCR rats allow for a unique model of inherit genetic variance in respiratory fitness, which also manifest as increased metabolic syndrome induced symptoms later in life. Our study found that inbred variance in aerobic function in female

rats is associated with alterations in fat mass and mechanosensation. In LCR female rats, clear differences were evident in increased fat mass and increased inflammatory cells in the skin. Obesity, coupled with an increased presence of inflammatory cells suggests that low aerobic capacity in LCR rats can heighten inflammatory responses in the peripheral nervous system. This peripheral nerve inflammatory response can then affect mechanosensation by perhaps priming the system for future insults. It was interesting to observe the relationship between body weight, fat mass, and sensory changes. This revealed the novel but important finding that peripheral nerves have altered function that are independent of whole body changes in weight and fat mass.

Chapter 3

In this chapter, mice were placed on a control or high fat diet and allowed access to either a sedentary or exercise wheel environment for 12 weeks. High fat-sedentary and high fat-exercised mice developed mechanical allodynia and metabolic syndrome over 6 weeks. High fat-exercised mice however, experienced a return to basal levels of mechanosensation by week 12. High-fat sedentary animals experienced elevated fat mass and displayed alterations in inflammatory mediators in the peripheral nerves. Our results revealed that a high fat diet increases expression of pro-inflammatory mRNAs in DRGs and these changes in gene expression could be reduced with physical activity. These changes were coupled with improvements in mechanosensation and provide additional evidence that inflammatory pathways in the peripheral nervous system are important in mechanical allodynia.

We found that the benefits of exercise on the nervous system are not mediated via increased fat metabolism alone, since high fat exercised mice have increased fat pad mass and burn fats as a primary fuel source, while expending less resting energy than high-fat fed sedentary mice. Often exercise is thought to increase metabolism, decrease obesity and fat mass, thereby decreasing proinflammatory signaling[61]. However, our results differ from this view based on decreased pro-inflammatory signaling in the peripheral nerves without any improvement in obesity or fat mass.

Lastly, mice fed a high-fat diet but allowed to exercise displayed improved ketone processing and similar utilization of fat compared to high fat-fed sedentary animals. Increase ketone utilization and decreased expression of mediators associated with inflammation led to questions of whether a ketogenic diet could impact peripheral nerves and mechanosensation in Chapters 4 & 5. Results in Chapter 3 suggests that there are molecular alterations that occur in peripheral nerves due to altered fat metabolism/increased utilization of ketones. In turn, this alteration in metabolism may play a key role in the improvement in mechanosensation observed with physical activity.

Chapter 4

This chapter examined mice placed on a control, high fat, high fat with exercise, or ketogenic diet for 12 weeks. Follow up studies examined these same dietary groups following 8 weeks of a high fat diet to induce obesity and metabolic syndrome. These studies suggested that the peripheral nervous system is sensitive to metabolic insults but may not mirror changes in whole body metabolism. These results are consistent with the

hypothesis that benefits of exercise are through altered peripheral nerve metabolism. A ketogenic diet led to obesity and increased fat deposition, but did not impact hyperinsulinemia or lead to mechanical allodynia similar to a high fat diet. Another unique alteration with a ketogenic diet is that the gene expression patterns of pain-associated genes were different from high fat-exercised animals. A high fat diet leads to mechanical allodynia; whereas both exercise and a ketogenic diet result in normal mechanosensation. Importantly however, only a ketogenic diet could reverse mechanical allodynia that was previously induced by a high fat diet. These differences in pain reduction and gene expression profiles suggest that a ketogenic diet has actions that are unique from those mediated by exercise.

We also examined the ability of dietary and exercise therapies to correct obesity as a result of a high fat diet. One interesting result from this study was that once C57Bl/6 mice became obese from consuming a high fat, interventions using either a control diet or exercise failed to return mice to normal weight or metabolic biomarkers. In addition, the ability of a ketogenic diet to reverse mechanical allodynia highlights the exciting potential for a dietary intervention for patients with metabolic syndrome-associated pain. Perhaps increased ketones through dietary intervention provided a greater benefit to nociceptive sensations, and our work lays the groundwork for further work on ketone metabolism and pain. The cellular and tissue-wide mechanisms impacted by a ketogenic diet, including anti-inflammatory and neurotrophic signaling, will require further study.

Chapter 5

In this chapter, studies examined the role of a ketogenic diet and ketones in altering peripheral nerve metabolism (mitochondrial respiration and ROS production) and axonal growth. Examination of skin from mice fed a ketogenic diet display increased innervation even after the development of obesity and metabolic syndrome. To understand the cause of increased skin innervation, studies utilized a ketogenic diet and primary cultures of DRG neurons in the presence of ketone molecules and in response to consumption of a ketogenic diet. Additional work examined respiration in mitochondria from DRG neurons and sciatic nerve when using fat a primary source of fuel.

Increased IENF density and improved in vitro neuronal outgrowth with a ketogenic diet or ketone supplementation is a groundbreaking observation. Ketone supplementation to cultured neurons displayed significantly better axonal growth as compared to glucose-based fuel sources. Additionally, animals fed a ketogenic diet for 1 month prior to primary cell culture, or control diet fed mice cultured in the presence of ketone bodies displayed significantly increased axonal outgrowth. Overall, the improvement in axonal growth and function from a ketogenic diet offers a new therapeutic intervention for a myriad of neuropathies, including DSPN.

DRG and sciatic nerves were analyzed in response a fat based fuel source to explore the role of fat metabolism in neurons. Mice fed a ketogenic diet displayed improved mitochondrial function when metabolizing fats as evident by reduced ROS production in the sciatic nerves compared to control-fed mice. This key metabolic

adaptation to a fat as fuel may provide significant functional benefits to peripheral axons and suggests that peripheral nerves can adapt to fat as a fuel source.

Clinical Implications

The use of physical exercise and as a therapeutic treatment to specifically address pain is a relatively new and developing field. The goal of metabolic alterations to benefit painful neuropathies by diet and exercise is an even newer field. The majority of research on exercise for peripheral pain syndromes in human subjects is associated with diabetic or pre-diabetic neuropathic pain. The few studies that have utilized physical activity are displayed in Table 6.1 Importantly, however, it has been reported by numerous groups that exercise can be performed safely in patients with type 2 diabetic neuropathies and exercise intervention produces a marked improvement in certain neurological disorders [249,250]. Historically, clinicians may have been reluctant to encourage exercise in patients with diabetic neuropathy due to the risk of possible adverse outcomes such as foot ulcers in insensate feet or increased pain. However, the results of the present work clearly show there is a new and exciting potential that may achieve some of the healthy benefits of exercise through the use of a ketogenic diet.

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TABLE 2 | Summary of human studies addressing sensory dysfunction associated with pain.

Mode of exercise	Pain model	Benefit	References
Aerobic exercise	Diabetic neuropathy	↑- Motor conduction velocity	Balducci et al., 2006
	DPN and metabolic syndrome	↑-Epidermal innervation	Kluding et al., 2012; Singleton et al., 2015
	DPN	↓ Pain ratings	Kluding et al., 2012
Vibration Platform	DPN	↓ Pain levels and improved gait	Hong et al., 2013
	DPN	↓ Neuropathic pain scale	Kessler and Hong, 2013
	DPN	↓ Pain ratings	Kessler and Hong, 2013

Modes of exercises, pain model, and primary outcomes are provided.

Table 6.1 Summary of human studies addressing sensory dysfunction associated with pain

Aerobic exercise is an often-studied modality in clinical programs for people with diabetes. Diabetics have experienced a benefit in both motor and sensory neuropathy measures. Aerobic exercise reduces the development of diabetic neuropathy [32], as well as increasing the intraepidermal nerve fiber density (IENFD) and visual analog scale (VAS) pain measure in people with diabetes [228,249,251-253]. Similarly, exercise induced improvement in metabolic syndrome patients saw an increase in cutaneous IENFD even though these patients were non-diabetic [228]. The exciting finding that a ketogenic diet increases axon growth offers a potential to translate a new therapy into the human population suffering from an axonal degeneration. Physical exercise when paired with diet counseling has already been shown to result in partial cutaneous re-innervation in pre-diabetic individuals [252]. The potential to pair exercise with a ketogenic diet may offer an attractive new therapy for patients.

Throughout the studies we performed, we noted effects of exercise and a ketogenic diet on inflammatory signaling. The potential to reduce inflammatory responses in peripheral nerves is an extremely attractive translational finding, as there are a myriad of peripheral diseases associated with heightened inflammatory responses. Some common neuropathic inflammatory conditions such as Guillain-Barré Syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIPD), and multifocal motor neuropathy may benefit from a ketogenic diet. The integration of both acute (GBS) and chronic (CIPD) inflammatory conditions in peripheral neuropathies can make the combat of inflammatory conditions often difficult needing to have short term and long term acting mechanisms. However, the benefit of the ketogenic diet appear to occur quite

quickly (~1-3 weeks) even when used as an intervention to correct a neuropathic symptom. Long term utilization of a ketogenic diet may be difficult in a human population, though it has been utilized for >6 months in humans to combat CIPD already [254].

While there are numerous potential benefits from a ketogenic diet for patients, such as being able to help those unable or willing to exercise, the most significant benefit may be increased axonal growth. One of the most common forms of peripheral neuropathy is distal symmetric sensorimotor polyneuropathy (DSPN). DSPN involves a dying back phenotype where the most distal part of the axon retreats with accompanying pain and/or numbness in the extremities [240]. DSPN is often associated with metabolic diseases such as diabetes, hypothyroidism, vitamin deficiencies and chronic liver disease. Our present work on metabolic syndrome and the ketogenic diet is an extremely attractive intervention. This potential dietary tool to rescue axonal loss that affects millions of patients in the US may provide the first therapeutic intervention of its kind to combat this growing epidemic.

Future Directions

The above work presents some attractive mechanisms to combat numerous ailments beyond the peripheral nerve disease. However, this is still much to be understood about the effects of not only a ketogenic diet, but also metabolic syndrome in diseases and in nervous tissue itself. Following these studies, future work should examine the molecular adaptations, which occur in peripheral nerves in an effort to deduce the crucial steps in ketone signaling. Based on the current results, I hypothesize that the most productive avenues are anti-inflammatory pathways, improved mitochondrial function and decreased ROS production. Additionally, analgesia could be mediated by increased A1R activation and anti-inflammatory actions. Finally, future research should continue to identify cellular mechanisms used by the ketone β-hydroxybutyrate to stimulate axonal growth.

In addition to providing an inflammatory response, adenosine and its receptor can provide analgesic relief through its direct signaling in sensory nerves. As described in Chapter 1, adenosine increases with a ketogenic diet, and is involved in analgesic benefits during exercise as well. Future work should examine the role of adenosine in the analgesic benefit of a ketogenic diet in metabolic and obesity-induced pain. Experiments should examine the production of ATP in sensory neurons with a ketogenic diet. Increased energy output in combination with increase adenosine levels may be important as an analgesic mechanism.

Increased ATP production likely would result from improved mitochondrial respiration. Therefore, future work should look to examine mitochondrial respiration and expand on the discovery of decreased ROS production from a fat fuel source in the DRG. Research in diabetic neuropathy has already recognized the importance of oxidative stress in the development of sensory dysfunction in peripheral neurons [255,256]. One attractive mechanism of future studies is the NRF2/ROS pathway, which displays potential overlap with ketogenic alterations. The utilization of a ketogenic diet may reduce ROS through an increase in NADH/NAD ratio, inducing antioxidant response elements (AREs) that are promoted by the transcription factor NRF2 [257]. This pathway has shown to alter by insulin signaling in pancreatic beta-cells and it is possible a ketogenic diet alters similar insulin signaling and ROS production in sensory neurons.

Chapter 7 References

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