The Importance of Brain-Adipose Communication for Metabolic Homeostasis

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THE IMPORTANCE OF BRAIN-ADIPOSE COMMUNICATION FOR METABOLIC HOMEOSTASIS

By
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B.A. Boston University, 2003

A DISSERTATION
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (In Biomedical Science)

The Graduate School
The University of Maine
May 2019

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THE IMPORTANCE OF BRAIN-ADIPOSE COMMUNICATION FOR METABOLIC HOMEOSTASIS

By Magdalena Blaszkiewicz

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An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
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May 2019

Although the brain is the master regulator of all bodily functions, peripheral nerves provide a means by which the brain communicates to all tissues, regulates organ function, and maintains systemic homeostasis. Metabolic homeostasis relies on the maintenance of energy balance which is governed in large part by the brain and adipose tissue. Disruption of communication between these two organs can lead to a metabolic state of energy excess or insufficiency, i.e. energy imbalance. Energy balance is maintained when energy intake, governed by appetite, food intake and nutrient absorption, equates energy expenditure, which is influenced by physical activity, basal metabolic rate, and thermogenesis. Over the last few decades, surgical and chemical denervation studies have demonstrated how maintenance of brain-adipose communication through both sympathetic efferent and sensory afferent nerves helps regulate adipocyte size, cell number, lipolysis, and ‘browning’ of white adipose tissue (the process by which energy
storing adipose tissue becomes thermogenically active and expends energy). However, the factors that regulate peripheral nerve health and remodeling, defined to include neuropathy (nerve death) and plasticity (neurite outgrowth), within the adipose organ have not been well explored. Peripheral neuropathy is a condition of nerve die-back that begins in the skin and travels inwards. Here we have shown that underlying subcutaneous adipose tissues also display peripheral neuropathy with conditions that range from obesity and diabetes to aging and certain diets. Under all aforementioned conditions the tissue also exhibited a loss of locally produced neurotrophic factors, which we have now implicated in the mechanism of adipose neuropathy. Exercise is able to at least partially reverse adipose neuropathy, and is associated with an increase in local neurotrophic factor expression. Furthermore, AAV-mediated gene delivery of this neurotrophic factor was shown to increase innervation in subcutaneous adipose tissue in a murine model of diabetic peripheral neuropathy. Interestingly, exercise and cold stimulation are both able to increase gene and protein expression of pan-neuronal and synaptic markers in adipose tissue, suggesting increased innervation of the tissue. We have also demonstrated that in adipose tissue, a neurotrophic factor is produced by immune cells in the stromovascular fraction, under stimulation from noradrenergic signaling. Deletion of this neurotrophic factor from myeloid lineage immune cells leads to a striking and specific ‘genetic denervation’ of white adipose tissue only, sparing the spinal nerves, neuromuscular junction, brain, and brown adipose tissue. Therefore, we believe we have uncovered a novel mechanism for how adipose tissue metabolic health is regulated through remodeling of the tissue’s peripheral nerve network.
DEDICATION

For my parents, whose nurture and sacrifices afforded me the privileges of an education, the courage to fail, and the freedom to pursue the unknown. Thank you.

Lament on Morning

O’er glorified arcing sun
So persistent in your blinding rays
Intent on bringing all to light
That moonbeams enveloped
In a gentle haze
Morning dawns with old demands
That twilight endeavoured to forsake
Be gone! Oh wretched child of naïveté
And bring me fast to Hecate’s gate!
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Chapter 1:

INTRODUCTION

I. Energy balance

Energy, in the form of ATP, is the currency of the body. In most mammalian systems, this currency is mainly acquired through the intake of nutrients (food and drink) which are metabolized and stored as either glycogen, or as triglycerides in adipose depots throughout the body. When the body needs energy, these stored lipids are mobilized and undergo fatty acid oxidation to produce ATP. In a sense, this process can be viewed as ‘burning’ fuel to create energy. To maintain a state of energy balance and metabolic health, the brain works in concert with adipose tissues to store and expend energy. Energy homeostasis is the continuous harmonization between energy intake (EI) and energy expenditure (EE). EI is driven chiefly by the hypothalamus, the energy regulatory region of the brain that controls appetite by influencing food consumption. By contrast, EE is driven by multiple factors including individual basal metabolic rate (BMR) conferred by our cell’s mitochondrial activity, physical activity (exercise), and thermogenesis. Multiple organs of the body are involved in maintaining energy balance, including the gut, liver, adipose, and skeletal muscle. Communication between the brain and these peripheral organs is essential to maintain energy balance, and is orchestrated by both circulating/endocrine factors as well as the peripheral nervous system (PNS).

Arguably the most important organs in maintaining metabolic health are adipose tissue and the brain. Mammalian adipose tissue comes in multiple forms and can serve
as both a site of energy storage as well as energy expenditure. The two classic types of adipose tissue are white adipose tissue (WAT) and brown adipose tissue (BAT), each morphologically specialized to either serve as a site of energy storage or energy expenditure, respectively. Lipid storage in WAT is more efficient than storage of protein and carbohydrates, as lipids store more kCal per gram than protein or carbohydrates and can be easily mobilized for energy. Storage of energy as lipids allows for maintained bodily functions during periods of food scarcity. This is an important adaptation in mammalian survival and evolution. WAT is evolutionarily While WAT serves predominately to store fuel, BAT serves as the organ for adaptive thermogenesis, a process that utilizes lipid fuels to drive heat production. To this end, adipocytes of WAT and BAT differ as they have evolved to fulfill their purpose. WAT adipocytes contain one large lipid droplet and little else, with all cellular machinery pressed against one side of the cellular membrane. On the other hand, BAT adipocytes are comprised of multiple small lipid droplets which increases surface area to allow ready release of fatty acids for fuel. This pattern of lipid droplet accumulation is termed multilocular. BAT adipocytes are also densely packed with mitochondria, from whence heat is dissipated, due to their unique expression of uncoupling protein 1 (UCP1).
Adaptive thermogenesis requires neuronal stimulation, and the action of catecholamine neurotransmitters such as norepinephrine (NE, also called noradrenaline). When activated by the sympathetic nervous system (SNS), BAT activates UCP1, which uncouples the mitochondrial electron transport chain and instead of producing ATP, the end product is the dissipation of the proton gradient as heat. Although UCP1 is a unique marker of BAT, it can also be induced in WAT under certain conditions such as cold stimulation (or noradrenergic stimulation). This process is known as ‘browning’ as the WAT begins to take on a BAT morphology, including the appearance of multilocular cells within the depot (Fig. 1). This process results in thermogenesis from WAT and fundamentally increases energy expenditure. There are also situations where WAT becomes thermogenically active, exhibits increased activity of UCP1, resulting in

Figure 1: Catecholamine induced browning of white adipose tissue (WAT). Sympathetic nerves release norepinephrine (NE) which binds to beta adrenergic receptors on adipocytes. This initiates a signaling cascade that results in the transcription of browning genes, including UCP1; and lipolysis, allowing free fatty acids (FFAs) to be taken up by mitochondria and enter the vasculature where they can be transported to BAT as further fuel for thermogenesis.
increased energy expenditure, but does not take on a multilocular morphology. These thermogenicaly active cells are paucilocular cells that look lite WAT adipocytes but can still burn calories.

The plasticity of certain WAT depots to undergo browning or UCP1-mediated thermogenesis is truly remarkable in its ability to shift from an energy storing organ to one that can expend energy, dependent on physiological or environmental needs of the body. Certain anatomical locations of WAT (in mouse) more readily undergo browning more than others (ie: subcutaneous, perirenal), and in humans the majority of BAT cells are the inducible/recruitable type that appear in WAT. Importantly, the ability of adipose tissue to respond appropriately to stimuli is dependent on proper brain-adipose communication.

Although we have known that brown fat thermogenesis in mammals is under neural control since the early 1960s [1-4] we are still striving to fully understand the mechanisms involved in maintaining adipose innervation, including demonstrating synaptic connections in cells of the adipose organ.

Considering the different physiological roles of BAT and WAT, their distinct gene expression profiles (ie: BAT highly expresses UCP1, WAT highly expresses leptin and adiponectin), and their differing developmental signatures [5], it is assumed that these organs would have different secretory profiles, which would affect nearby cells in a paracrine or autocrine manner. Current literature appears to support this notion [6, 7] We also know that some of these secreted molecules, known as adipokines, can affect distal organs through entering the circulation and acting in an endocrine manner [8] and that both BAT and WAT preferentially sequester certain lipids and have distinct lipid signaling
molecules [9, 10]. Partitioning of dietary fats between WAT, BAT and brain is the focus of the first research chapter of this thesis.

II. Brain-adipose communication

How does the brain communicate with adipose tissue? In similar fashion to most brain-organ communication, the endocrine system and the peripheral nervous system are essential for proper brain-adipose communication. Nerves of the sensory and autonomic nervous system transmit information from the periphery to the brain (afferent) and from the brain to the periphery (efferent), respectively.

Numerous studies over the past several decades have clearly demonstrated the importance of peripheral nerves in regulating BAT and WAT thermogenesis. In response to a cold environment, one that threatens thermal homeostasis, cold-sensing sensory nerves in the skin relay this environmental information to the brain. In turn, the brain especially the hypothalamus (the energy regulatory center), responds by increasing sympathetic outflow to adipose tissues, stimulating UCP1-mediated thermogenesis via release of NE [4, 11]. NE acts on beta-adrenergic receptors on adipocytes, which initiates an intracellular signaling cascade that ends in the transcription of lipolytic (in WAT) and thermogenic genes (in both WAT and BAT) and increases EE. Other modes of EE, such as diet induced thermogenesis, the post-prandial heat production that accounts for 5-15% of total daily EE, also requires intact innervation of adipose tissue [12].
Innervation of Adipose Tissues

**Innervation of Brown Adipose Tissue (BAT)**

The notion that neural innervation exists in adipose tissues is not a new one; from an anatomical and functional perspective, all tissues of the body should be innervated – an idea supported since at least the late 1800s. Cardiac tissue and blood vessels were found to be widely innervated, as well as pericardial white adipose tissue (WAT; which is a very ‘brown’ depot), in small rodents as early as the 1890s [13]. Direct evidence of innervation of brown adipose tissue (BAT; in mice and rats) was demonstrated in the mid-20th century [14]. Interestingly, even in the 1950s Sidman and Fawcett made a statement that would be equally relevant today. They wrote, “Relatively little attention has been paid in recent years to the influence of the nervous system on adipose tissue even though detailed experimental studies on this subject are to be found in the (literature).” They went on to cite a number of studies in the 1930s that presented evidence for the importance of adipose nerves; and themselves stated that mixed nerve types innervate BAT and that many nerve fibers were not associated with blood vessels but made direct contact with adipose cells [14]. Similarly timed investigations argued, with the support of fluorescent microscopy, that the sympathetic innervation found in BAT was restricted to the vasculature and was mainly vasoregulatory in function [15]; although, the existence of parenchymal nerve fibers in adipose tissue was at least becoming more accepted a year later [16].

Soon after, Bargmann et al. [17] in the late 1960s used electron microscopy to demonstrate (in mice, rats, and hedgehogs) that not only did sympathetic fibers envelope
BAT vasculature but that they also extended into the parenchymal space. Interestingly, as we describe below, this parenchymal innervation of adipose was rediscovered in recent years with whole depot imaging techniques. Bargmann et al. [17] were also able to see both non-myelinated and myelinated paravascular (their anatomical term) nerve bundles, but more importantly, they found that small unmyelinated axons were tightly associated with fat cells themselves. Interestingly, the axon terminals of these nerves, some of which were “embedded in invaginations” of the adipocyte surface, contained synaptic vesicles that the authors presumed to contain catecholamines [17]. This presumption was based on the mounting evidence for catecholamine release driving adaptive thermogenesis in BAT in multiple mammalian species [18-20], findings which also underscored that NE was essential for this process instead of the previously hypothesized epinephrine/adrenaline [21] [22].

Based on these studies, it became accepted that cold-induced thermogenesis caused an increase of NE release to BAT, which resulted in lipolysis and activation of uncoupling protein 1 (UCP1). However, if we look back to some of the original findings regarding adipose innervation [16], there may be another mechanism to consider. Early fluorescent microscopy imaging of BAT and WAT innervation argued for numerous nerve fibers surrounding the vasculature, predominantly around arteries and arterioles [15, 16]. Given this perspective, an alternative hypothesis could be that cold-induced thermogenesis does not result in an increase in the concentration of circulating NE, nor might it result in an increase in direct sympathetic drive to WAT and synaptically released NE. Instead there could be stimulation of vasodilation to tissues due to vasoregulatory innervation and no resulting change in the absolute catecholamine levels delivered to the
tissue per volume of blood, but instead an *increased volume of blood that would thereby deliver more catecholamine*. This idea could provide a vascular mechanism for how obese adipose tissues loses thermogenic capacity, given the vasculature damage that occurs due to chronic inflammation and the relative loss of vascular supply to adipocytes due to expanding adipose mass coupled with a lack of new angiogenesis, as reviewed by Stapleton, et al. [23].

These early microscopy studies could not benefit from the use of fluorescently labeled antibodies targeting markers of sympathetic innervation as they did not yet exist, thus instead they employed formaldehyde gas to form highly fluorescent isoquinoline derivatives formed from the presence of monoamines (including NE) [16]. The resulting observations indicated neurotransmitters in axon terminals of BAT and WAT nerves, and may have actually been a more specific result than what we get with antibody staining today, given the potential for off-target and non-specific binding of the antibodies. The downside of this formaldehyde gas technique was the inability to distinguish the subtype of monoamine they were fluorescing.

*Innervation of White Adipose Tissue (WAT)*

The observation of Bargmann et al. [17] that each adipocyte comes in contact with nerve fibers in the parenchyma is consistent with what we and others have recently reported in WAT [24-26]. Earlier studies using True Blue as a retrograde neural tracer (crystal implants in WAT) showed sensory innervation in inguinal subcutaneous WAT (i-scWAT), as evidenced by tracing back to the T13/L1-L3 dorsal root ganglia (DRG) [27]. The anterograde transneuronal viral tract tracer, the H129 strain of the herpes simplex
virus-1 (HSV-1), has also been used to trace sensory nerve projections from i-scWAT and intraperitoneal epididymal WAT (eWAT), through the same T13/L1 DRG in Siberian hamsters [28]. Together, these studies and others like them have indicated that adipose has bi-directional communication with the brain – through both afferent sensory fibers and efferent sympathetic fibers. However, the role of sensory innervation in WAT continued to be largely understudied in lieu of sympathetic nervous system (SNS) innervation studies in the intervening years. This made sense given the importance of NE for thermogenesis and lipolysis. More recently, new studies have revisited the function of adipose sensory nerves. Long-form leptin receptor (ObRb) was found on DRG neurons traced from i-scWAT, suggesting leptin could potentially communicate with sensory nerves in adipose via the DRG [29]. Furthermore, SNS-stimulated lipolysis, as well as intra-adipose injection of free fatty acids such as eicosanoidpentaenoic acid (EPA) and arachidonic acid (AA), could increase adipose afferent nerve activity [30]. This was capable of triggering BAT thermogenesis, an effect that was abolished with surgical denervation of i-scWAT [30].

Although early microscopy studies provided evidence of WAT innervation and hypothesized that these nerves were of SNS origin [16], irrefutable proof of SNS innervation of WAT came from the seminal studies of Youngstrom & Bartness, who demonstrated bidirectional innervation of WAT [31]. Using the retrograde fluorescent tract tracer FluoroGold injected into i-scWAT and eWAT, as well as the anterograde fluorescent tract tracer Dil, they determined that the SNS ganglia also at T13/L1-L3 innervated both fat pads. These studies further showed that there was innervation of the adipocytes themselves, and not just the blood vessels within a depot, as had been
previously demonstrated in the mid 1900s. The specificity of the tracing was confirmed by surgical denervation of the fat depots and by injecting the tracers directly into blood vessels. Furthermore, using pseudorabies virus (PRV) retrograde tracing from i-scWAT, which can only trace sympathetic neurons that are synaptically connected, they could create hierarchical connectome that mapped neuronal pathways from the tissue to the brain [32]. For the next 2 decades, the work of Timothy Bartness would continue to pioneer our understanding of adipose innervation in rodent models [33].

**Lessons from adipose denervation studies**

Multiple methods for and consequences of denervation of WAT and BAT have been reported in the literature, and have been described in detail in recent reviews [34, 35]. Of these, surgical denervation is considered the most effective at eliminating total neural input and output, as the nerve bundles innervating the tissue are physically resected. However, surgical denervation is generally non-specific to nerve type (as sensory and sympathetic nerve bundles tend to travel together), can be a technically challenging method, and can cause undesirable effects as nerves bundles that innervate adipose depots often innervate other tissues and can run along the vasculature. An alternative approach is chemical denervation. Chemical denervation can provide a selective and localized means of removing nerve supply to WAT depots. The first selective chemical denervation drug was 6-hydroxydopamine (6-OHDA) [36] which is taken up into NE storage vesicles leading to oxidative damage to the membrane and nerve degeneration, and thereby producing a reversible denervation of sympathetic
nerves while leaving sensory nerves intact [34]. Later, some chemical denervation studies using the chemical guanethidine had been used to produce chemical sympathectomy. This drug works by displacing NE from postganglionic sympathetic nerve endings, and decreases reuptake of NE by nerve terminals. When locally administered, it can deplete a tissue of NE, effectively depleting sympathetic tone. It has been used to provide direct evidence of the role of NE in WAT, as local injection of guanethidine to i-scWAT resulted in increased fat pad size due to adipocyte hyperplasia in Siberian hamsters [37]. This denervation experiment helped provide a mechanism by which the SNS regulates body mass, which is to regulate adipocyte dynamics and cell size. The current preferred sympathetic denervation drug is 6-OHDA, as subsequent preparations of guanethidine proved less reliable [35].

For chemical denervation of sensory nerves, capsaicin is typically used, which activates vanilloid receptors on unmyelinated and myelinated sensory nerves. The over-activation of the receptors causes an influx of calcium and sodium resulting in an excitotoxic effect [34]. The efficacy of capsaicin-mediated sensory denervation is validated by a reduction in calcitonin gene-related peptide (CGRP) and Substance P content, and although it is not as effective as surgical denervation, this method does appear to leave sympathetic efferent nerves intact [38].

**Denervation of BAT**

Some of the most elegant studies demonstrating the importance of neural control for adipose tissue metabolism and function involved surgical denervation of BAT. By removing the neural supply to the organ, observed metabolic perturbations were directly
linked to a lack of brain-adipose communication. Indirect evidence for sympathetic control of lipolysis had been demonstrated by studies showing an increased rate of NE turnover (NETO) in both BAT and WAT following cold exposure in rats [39]. Increased NETO in WAT has also been demonstrated with fasting in rats [40]. Denervation studies have added credence to these findings. Unilateral and bilateral denervation of intrascapular (iBAT) is technically less difficult than denervation of WAT depots since nerve bundles innervating iBAT are more easily visualized and anatomically defined (5 intercostal nerves that unilaterally innervate iBAT) [41] than those innervating WAT depots [35], and originate mainly from the stellate ganglion [42]. Bilateral denervation of iBAT resulted in greatly impaired thermogenesis and reduced overall energy expenditure, increased body fat mass [43], and ‘whitening’ of the tissue [44]. Furthermore, experiments with unilateral iBAT denervation showed decreased presence of tyrosine hydroxylase (TH) [44] and UCP1 [45] only in the denervated fat pad compared to the intact contralateral pad. In studies where sympathetic drive to BAT increased total energy expenditure, surgical denervation blunted the effect [44, 46]. This surgical denervation of BAT was done by severing the large nerve bundles transiting to BAT, as anatomically defined [41], while leaving nearby vasculature intact [47]. Furthermore, bilateral chemical sympathectomy of iBAT increased NETO in i-scWAT [47], demonstrating adipose tissue crosstalk with brain in the attempt to reestablish energy homeostasis. Chemical denervation of sensory innervation to iBAT also impaired thermogenesis [48], demonstrating the need for sensory feedback from iBAT for proper thermogenic function.
Denervation of WAT

There are metabolically relevant consequences of losing the nerve supply to WAT. Surgical denervation of WAT led to increased fat pad mass and white adipocyte proliferation and differentiation, as demonstrated in both rat [49] and Siberian hamsters [50-52]. The i-scWAT fad pad is innervated by multiple nerve bundles entering the tissue at multiple locations. Surgical denervation of i-scWAT was accomplished by tracking these nerves under 4x magnification to their terminal location and bisecting the nerves at that area [50]. Retroperitoneal WAT (rWAT) is perhaps more easily surgically denervated by lifting the kidney and cutting the three nerve bundles right before they enter the fat pad [51]. These experiments underscored the importance of innervation for regulating hypertrophy versus hyperplasia, as well as for controlling levels of lipolysis. However, surgical denervation could not reveal which nerves are most essential in maintaining proper body mass and metabolic health, as the technique denerves both sympathetic and sensory nerves and causes disturbance of vasculature. Furthermore, surgical denervation at the level of the ganglia would not work because the ganglia that innervate WAT is not as discrete as the one that innervates BAT, so denervation of other off-target tissues would occur leading to indirect effects on metabolism.

The Bartness lab and others have subsequently used chemical denervation to gain a deeper understanding of which nerves act in adipose depots and how. Chemical sympathectomy (with 6OHDA) of one fat pad increased total body fat and increased adipocyte number in the contralateral fat pad in rats and mice [53]. One interpretation of these data would be that sensory feedback from the denervated pad led to effects in the non-denervated pad via the central nervous system. However, treatment with
peripherally administered leptin could reduce fat pad size independent of innervation status [53], confirming that endocrine and neuronal effects mediate adiposity. In subsequent studies, 6-OHDA denervation of sympathetic nerves in one or both fat depots reduced the NE content and inhibited NETO in other WAT pads as well as in iBAT [54].

Over and over, sympathetic denervation of WAT resulted in increased depot mass characterized by an increase in cell number and a decrease in lipolysis, as recently reviewed [34]. On the other hand, sensory denervation of iWAT and eWAT increased fat pad mass via hypertrophy instead of hyperplasia, providing a means of differential control of WAT by sympathetic versus sensory nerves [38, 52]. The next step in our understanding must be to delineate how different neuropeptides and neurotransmitters exert these effects, and whether sensory nerve products potentially modulate the effects of NE from SNS nerves.

**III. Immune cells and adipose tissue**

**The stromal vascular fraction (SVF), and role of immune cells in adipose tissue**

Adipose tissue is a greatly heterogeneous organ that maintains a multitude of diverse cells types (Fig.2). The non-adipocyte cell fraction of adipose tissue is known as the stromal vascular fraction (SVF). The SVF contains multipotent stem cells (known as adipose-derived stem/stromal cells); various progenitor cells including endothelial precursor cells and preadipocytes (that can undergo white or brown adipogenesis); pericytes; and a wide diversity of immune cells. Adipose SVF immune cell populations include innate immune cells such as: several subtypes of monocyte-macrophages,
dendritic cells, mast cells, neutrophils, eosinophils, and innate lymphatic cells (ILCs); as well as adaptive immune cells, such as: several subsets of T cells, natural killer cells, and B cells. Although, many of these immune cells are known to play a role in adipose tissue health, this review focuses predominantly on monocytes/macrophages, as they represent the most abundant type of leukocyte in adipose tissue [55] and directly relate to the later chapters of the thesis.

**Role of macrophages and monocytes in adipose tissue**

Macrophages and their undifferentiated precursors, monocytes, are critical leukocytes that function in both the innate and adaptive immune response. The role of macrophages in adipose tissue has been well studied for the past 15 years, yet new information regarding this complex relationship is constantly arising. Historically, these adipose resident macrophages have been delineated into two types, pro-inflammatory or M1 type and anti-inflammatory or M2 type macrophages. In this simplified scheme, M1 macrophages are responsible for initiating the healing process during acute inflammation that occurs during tissue injury, by releasing pro-inflammatory cytokines to recruit phagocytic macrophages to an injured site. When a tissue is injured unhealthy cells will undergo apoptosis, cells undergoing apoptosis secrete cytokines that recruit phagocytic cells to the injury site in order to clear up the debris and allow for cell renewal/tissue repair to happen. These phagocytic macrophages further secrete pro-inflammatory cytokines to recruit monocytes and macrophages to injured tissue. In adipose tissue, dead or dying adipocytes become surrounded by macrophages which from “crown-like” structures around the cell as they phagocytose it. Once cellular debris has been cleared, the secretory profile of these immune cells switches from a pro-inflammatory to an anti-
inflammatory one. This polarizes macrophages from an M1 phenotype to an M2 phenotype. M2 macrophages are now responsible for initialing the regeneration program, by secreting the appropriate growth factors to aid in cell renewal and tissue repair. However, under unhealthy conditions macrophages contribute to a chronic state of inflammation. When tissue injury is constantly present, as in states of obesity where cells continuously become hypoxic and die, M1 macrophages maintain a pro-inflammatory cytokine secretome in order to keep recruiting cells and handle the debris clearance. This results in an imbalance of M1 to M2 macrophages and a reduced efficiency in regeneration of healthy tissue. In adipose tissue, this chronic low-grade inflammation has been linked to contributing to insulin resistance (IR), and the development of metabolic syndrome, as reviewed by [56]. Another consequence of obesity and the influx of macrophages is the development of macrophage foam cells. Foam cells are formed as macrophages clearing debris from dying adipocytes (formation of crown like structures) accumulate excess free fatty acids [55] and cholesterol [57]. This accumulation of lipids results in a foamy cytosol, hence the term foam cell, and has been suggested to alter macrophage metabolism resulting in a pro-inflammatory shifted secretory profile that contributes to IR and atherosclerotic lesions in humans and mice [57, 58].

Many studies have proposed manipulating the M1 to M2 ratio of adipose resident macrophages in order to alleviate states of chronic inflammation and reestablish a healthy adipose organ. However, success limited by our understanding of the complexity of macrophage phenotypes. Genetics contributes to the ability of preventing a chronic inflammatory response—certain mouse lines such as the Balb-c strain, are more skewed towards an anti-inflammatory immune cell phenotype. These mice have less chronic
adipose inflammation even under obese conditions and are protected from developing insulin resistance and diabetes. Sex differences also play a role in immune response. Females (up to a certain age) appear to be more resistant to developing chronic low-grade inflammation in adipose tissue and IR. This has been attributed to the protective role of estrogen which may be preventing hypertrophy of adipose tissue even on a high fat diet [59].

Although much of the obesity field is lagging in understanding or acceptance of the diversity of macrophage phenotypes, and the extent to which these various cell phenotypes contribute to energy balance, a slow change in perception is occurring. For example, a new source of crosstalk between BAT and WAT, appears to be mediated by immune cells. Recently, Cereijo et al. [60] discovered a BAT secreted chemokine, CXCL14, which triggers macrophage polarization and mediates browning in WAT. This batokine is secreted by BAT in response to thermogenic stimulation, which we know is a direct result of sympathetic activation, and provides us with another example of the layered mechanisms of neural, vascular, and immune system interplay in regulating adipose tissue.

**Neuro-immune interactions**

It is well appreciated that immune cells, especially macrophages play a critical role in energy balance and metabolic health. The study of neuro-immune interactions is a burgeoning field currently gaining much traction. Microglia are glial cell that act as macrophages in the brain and spinal cord. As part of the innate immune system, these
cells play a critical role in immune defense and maintain homeostasis through control of cell differentiation, proliferation and formation of synaptic connection, in the CNS [61-65]. As is the case with all macrophages, microglia actively respond to traumatic brain injury and initiate a healing/repair program, which reestablishes CNS homeostasis. Microglia also play a role in learning-associated synaptic plasticity [66]. It is believed that synaptic plasticity is mediated by the synthesis and release of brain derived neurotrophic factor (BDNF) by microglia, as reviewed by [67]. On the other hand, microglia can play a role in chronic inflammation of the CNS [67], similarity to how tissue resident macrophages are at the crux of chronic inflammation in adipose tissue under obese conditions.

**Neuroimmune interactions in adipose tissue**

Although the role of immune cells, especially macrophages, in adipose tissue has been and continues to be an active area of study, neuroimmune interactions in adipose tissue remain largely obscure. It had been suggested that BAT and WAT macrophages synthesize catecholamines in response to cold [68]. Although Nguyen et al. argued that cold-induced adaptive thermogenesis requires alternatively activated (M2, anti-inflammatory) macrophages and did demonstrate that preventing this macrophage polarization resulted in an impaired thermogenic response, these findings were later challenged to suggest a different explanation for the observed relationship between macrophages and catecholamines. In 2017, Fischer et al. used a mouse model with genetic deletion of TH (the rate limiting enzyme in catecholamine production) in hematopoietic cells [69]. By this method, they refuted the findings that alternatively activated macrophages synthesize catecholamines. In their study, not only did deletion of TH in hematopoietic cells have no effect on energy expenditure, RNA sequencing on
macrophages from various adipose tissues revealed that no macrophage population tested contained transcripts for TH.

The debate between these two sets of findings may have found its resolution with some recent publications. Studies using zebrafish have demonstrated a role for macrophage signaling over long distances between non-immune cells during tissue developmental remodeling, whereby macrophages transported airineme vesicles between two different cell types [70]. Furthermore, buried in literature from the 1970s, was evidence that mouse peritoneal macrophages accumulated NE in vitro [71]. These studies allow for the possibility of macrophages transporting other materials such as NE within adipose tissues.

Soon after, Pirzgalska, et al. presented data that supported similar macrophage behavior but pertinent to neuroimmune interactions in adipose tissue [72]. They recently described a distinct macrophage population that associates in a specific manner with SNS nerves of i-scWAT. The appropriately named sympathetic neuron-associated macrophages (SAMs), can be found interacting with SNS fibers within WAT and are not only morphologically distinct from adipose tissue resident macrophages (ATMs), but exhibit a gene expression pattern distinct from adipose and other tissue macrophages [72], including expression of genes related to synaptic signaling, cell-cell adhesion, and neuron development. Unlike the circular morphology of ATMs, SAMs wrap around SNS fibers and exhibit an extended shape with long dendritic like projections. Like observations made by Nguyen, et al. SAMs contained significant amounts of intracellular NE, but lacked the requisite enzymes for NE synthesis, as previously reported for macrophages [69, 73]. These macrophages were of the Cx3cr1+ lineage and exhibited a
pro-inflammatory state more similar to classically activated than M2 type macrophages, however, their most distinguishing feature was the expression of solute carrier family 6 member 2 (Slc6a2; a known NE transporter), as well as monoamine oxidase A (MAOA), an enzyme that degrades NE. Although other macrophages have been shown to express MAOA [72], only SNS fiber associating SAMs expressed Slc6a2 [72]. The authors proposed, quite believably, that SAMs were acting as an NE sink, taking up excess NE after SNS stimulation and degrading it. The transport of NE by SAMs was not explored. They also showed that SAMs are recruited to WAT in obesity (both diet induced and genetic models) and may be contributing to the adipocyte hypertrophy through over-degradation of NE. They went on to show that ablation of Slc6a2 from SAMs in obese mice lead to an obesity rescue, through reestablishment of NE levels that served to increase lipolysis and energy expending processes such as browning of WAT. SAMs (with analogous molecular machinery) were also identified in the SNS tissue of humans [72]. SAMs have been shown to associate with other neuronal tissues, such as the SCG and thoracic chains, and are also present in BAT [72]. However, their abundance in BAT is much lower than WAT, and their role in BAT may not be as metabolically relevant as in is in WAT, but this remains to be seen.

In a parallel study, Camell et al. presented findings of ATMs in aged mice that degrade NE and contribute to age-related lipolysis impairment in visceral adipose tissue (VAT) [74]. ATMs from 24-month-old mice showed increased expression of MAOA and other catecholamine degrading enzymes in a NOD-, LRR- and pyrin domain-containing (NLRP)3 inflammasome dependent manner, compared to young 3 month old mice [74]. Furthermore, Camell, et al. independently showed that certain ATMs closely associated
with TH+ nerves in VAT [74]; providing the likelihood that what they called nerve associated macrophages (NAMs) may be the same cells as SAMs. Although Camell, *et al.* used a LysM-Cre:mTmG reporter mouse model to visualize their ATMs, which is a broader myeloid marker than the Cx3cr1+ model Pirzgalska *et al.* used, they did not investigate whether NAMs expressed Slc6a2 (SAM marker) even though the morphology of their NAMs is consistent with that of SAMs.

Mutations in another type of Cx3cr1+ macrophage that do not appear to be SAMs have been linked to decreased BAT innervation and subsequent loss of homeostatic energy expenditure [75]. Mice with Mecp2 (methyl-CpG-binding protein 2) deficiency in a subset of BAT resident Cx3cr1+ macrophages, exhibited lower expression of UCP1, a paucity of BAT tissue, and developed obesity after 3-4 months of age [75]. Interestingly these findings were BAT-specific and no changes were observed in WAT. Furthermore, tamoxifen inducible Cx3cr1cre:Mecp2fl/y mice were created to restrict Mecp2 deficiency from macrophage precursor cells (including brain microglia) and allowed for the mutation to be induced at a stage when tissue macrophages were mature. These animals were shown to respond to acute cold challenge, which appeared to rescue the BAT impairment [75]. Mecp2 is an ubiquitously expressed nuclear transcription regulator [76] that maintains mature neurons and synaptic connectivity [77]. Mecp2 deficient macrophages showed an up-regulation of PlexinA4, which is known to signal through semaphorins to guide axonal growth [78, 79]. Wolf *et al.* thus argued that overexpression of PlexinA4 in Mecp2 deficient macrophages inhibits axonal outgrowth in BAT, thus diminishing its function and ability to maintain homeostatic thermogenesis. They did confirm the presence of Sema6A+ neurons in BAT tissue, which supports their working model, as
PlexinA4 reverse-signals through Sema6a to inhibit axonal outgrowth [79]. However, the question remains as to what overcomes this axonal inhibition with cold exposure, since acute cold exposure appeared to rescue the impaired phenotype. Cold exposure increases NE content in adipose tissue. Macrophages along with other immune cells express adrenergic receptors that bind NE [80], and have been shown to produce neurotrophic factors such as NGF [81] and brain derived neurotrophic factor (BDNF) [82] in human peripheral blood and the human brain [82] suggesting another possible mechanism in regulation of axonal plasticity may exist in adipose tissue whereby immune cells are synaptically wired and release neurotrophic factor to nearby nerves.

In most studies mentioned above, except Nguyen, et al., it was clear that Cx3cr1+ immune cells are implicated in neuro-immune interaction in adipose tissue. These findings suggest that multiple subsets of Cx3cr1+ macrophages work in concert to maintain energy homeostasis though interactions with the neural innervation of adipose depots. There are many more studies necessary to fully understand the diversity of these immune cells, and what neuroimmune role Cx3cr1- macrophages may play in adipose tissue, if any. One issue that may have been addressed by the data described above is that definitions such as ‘classically activated’ or ‘alternatively activated’ (ie: M1 or M2) macrophages are not sufficient labels when describing the diverse populations of macrophages in adipose tissue. This has been well-understood by immunologists for many years, and the adipose field is now catching up to this idea.
**Figure 2. Tissue architecture and neuroimmune cell types in white (WAT) and brown adipose tissue (BAT) in the basal state.** Both WAT and BAT are comprised of lipid-laden mature adipocytes, where white adipocytes have one large (or unilocular) lipid droplet and brown adipocytes have many small (or multilocular) lipid droplets.
droplets. The stromal vascular fraction (SVF) is the non-adipocyte cell fraction of the tissue and contains preadipocytes (and stem/progenitor cells that will undergo white or brown adipogenesis), and a milieu of immune cells. Immune cell populations include innate immune cells such as: several subtypes of monocyte-macrophages, dendritic cells, mast cells, neutrophils, eosinophils, and innate lymphatic cells (ILCs); as well as adaptive immune cells such as: several subsets of T cells, natural killer cells, and B cells. Neurovasculature of WAT and BAT includes blood vessels, lymphatic vessels, and a dense nerve supply of both sensory and sympathetic fiber types, although it is currently unclear if one tissue has a greater extent of innervation than another, or if their nerve plasticity (such as with cold or exercise) differs between tissues or depots. Some nerves innervate the vasculature itself, while other nerves innervate the parenchyma of the tissue. It is currently unclear which cell types are directly innervated and receive synaptic input. Some nerve fibers are myelinated and others are unmyelinated (it has been suggested that the majority of TH+ nerve fibers in BAT are thin and unmyelinated [83]), and sensory nerve products such as CGRP, Substance P, and NPY [38, 83-85] have been detected in both tissues. Although NPY may also be released from vasculature associated sympathetic nerves as well [86], and should not be considered by itself a marker of sensory innervation. Sympathetic nerves release NE. Additional neurotransmitters and neuropeptides may also be active in WAT and BAT. The presence of Schwann cells in both BAT and WAT further underscores the presence of myelinated nerves [87]. Interestingly, Schwann cells, as a myelinated glial cell type, can behave similarly to an immune cell. Other immune cells, that are also present in adipose tissue, are well documented for neuroimmune functions, including monocyte/macrophage subtypes, as reviewed previously [88, 89]. Monocytes, as well as T and B cells can produce BDNF in human peripheral blood and in human inflammatory brain lesions [82]. Human CD4 + T cell clones (from peripheral blood) were shown to produce and release NGF in vitro [81]. In addition, eosinophils from human peripheral blood can produce NGF, NT3 and BDNF neurotrophic factors upon immunologic stimulus [90, 91], and mast cells contain mRNA for NGF and may be another source of neurotrophic factor [92]. It is currently unclear if these immune cell types play similar roles in adipose tissue, or if other tissue-resident immune cells have neuroimmune roles. Differences between BAT and WAT immune cells include: more SAMs in white than brown [72], the presence of BAT specific Cx3cr1+ macrophages that play a role in regulating axonal outgrowth [75] and the presence of a subset of Tregs (CD4+) that exhibited a differential gene expression in BAT versus visceral adipose tissue [93]. There is very little knowledge about the lymphatic system in BAT compared to WAT, and changes with obesity or cold likely differs between BAT and WAT as well, but this warrants further exploration. In total, cellular cross-talk in the adipose organ is clearly important for immunometabolic function and brain adipose communication, but our understanding is still at a very primitive stage.
IV. What makes a healthy nerve

Schwann cells are the glial cells of the peripheral nervous system; they are responsible for the formation of myelin sheaths around larger axons which allows for increased conduction velocity. Schwann cells also play an important role in peripheral nerve regeneration as they secrete neurotrophic factors (NTFs) to guide axonal regrowth. After nerve injury Schwann cells phagocytose damaged axons, guide nerve regrowth through secretion of NTFs and other growth factors, and remyelinate demyelinated nerves. In this sense, they behave similarly to macrophages in wound response. Furthermore, macrophage-Schwann cell crosstalk appears to be essential in the regenerative process of peripheral nerves. In rodent models of nerve injury (spinal and sciatic), [94] showed that Cx3cr1+ macrophages hone in to the injury site to clear debris and persist up to 3 weeks post injury, during which time Schwann cells increase in number and remyelinate injured axons. Ablation of macrophages prior to remyelination resulted in a lack of Schwann cell maturation (with concomitant increase in immature Schwann cells), a reduction in remyelination, and overall poorer electrical signal propagation [94]. The authors went on to demonstrate that a macrophage derived ligand, Gas6, promotes Schwann cell differentiation, thereby aiding in remyelination of regenerating nerves. Furthermore, macrophages persistently aggregate around denervated human nerves and express Gas6, suggesting that the mechanism may translate to human peripheral nerve regeneration. Macrophages also appear to induce vascular remodeling to provide a “bridge” by which Schwann cells can guide severed nerves to regrow [95]. Here the authors showed that macrophages (along with lower percentages of other cells, including neutrophils, fibroblasts and endothelial cells) aggregate between severed nerves in rats and secrete VEGF-A promoting development of polarized microvasculature within this
space that creates a bridge which Schwann cells can infiltrate and guide regenerating axons.

Schwann cells are present at nerve fibers in adipose tissue, and adipose SVF may serve as a potential Schwann cell reservoir [87]. Schwann cell-like cells (SCLCs) have been isolated from mouse i-scWAT. Adipose derived SCLCs expressed specific Schwann cell markers, secreted NTFs (nerve growth factor (NGF) and BDNF) in vitro, and were able to function in sciatic nerve regeneration in vivo, albeit not as effectively as sciatic nerve derived Schwann cells [87]. Overall, adipose derived SCLCs present an attractive source of peripheral nerve regenerating cells as it is much easier to obtain them than it is to isolate nerve fiber associated Schwann cells. SCLCs may also play a role in axonal plasticity within adipose tissue.

**Neurotrophic factors in maintaining nerve health**

As the name suggests neurotrophic factors are involved in nerve growth, but they are also necessary for nerve maintenance, survival and proliferation. NTFs are essential during development, but they also serve an important role in the mature animal. NGF belongs to the neurotrophin family of NTFs is perhaps the most well studied, as it was the first NTF to be described in the late 1950s by Rita Levi-Montalcini and colleague Stanley Cohen [96]. Since then, its role has been studied in the CNS, PNS, endocrine and immune systems, as reviewed by [97]. Other NTFs (i.e. Neurotrophin-3, BDNF, glial cell derived neurotrophin (GDNF), ciliary neurotrophic factor (CNTF)) appear to be less broadly active as NGF but this may be by virtue of being understudied compared to NGF, something
which is slowly being rectified. Nonetheless, global loss of any NT results in severe neuronal deficits and early postnatal death.

The neurotrophin family of NTFs, includes Neurotrophin-3 (NT-3), Neurotrophin-4/5 (NT-4/5), Neurotrophin-6, NT-6, Neurotrophin-7, NT-7, NGF, and BDNF, they signal through two classes of receptors, the tyrosine kinase family tropomyosin-related kinase (Trk) receptors, and pan-neurotrophin receptor p75NTR, which belongs to the TNF receptor superfamily [98]. Neurotrophins are selective to Trk receptor subtypes, NGF is specific to TrkA, BDNF and NT-4 bind to TrkB, and NT-3 preferentially binds to TrkC, but can bind to the other Trk receptors albeit with less efficiency, binding affinity to Trk receptors is regulated by p75NTR [98]. Trk receptors are expressed throughout the nervous system; neurotrophins are internalized by their respective Trk receptors at nerve terminals (via receptor mediate endocytosis) and can be transported long distances along the axon to the cell soma where nerve differentiation, survival and/or outgrowth is determined. This allows for long relay signaling that is essential for nerve maintenance in the PNS. Although NGF is inarguably important for the survival, differentiation, and maintenance of the PNS during development and in the adult, BDNF and NT-3 have been shown to play a key role in the myelination of peripheral nerves in developing and injured nerves [99, 100]. While the presence of BDNF was necessary for myelination and regeneration to occur, NT-3 appeared to modulate BDNF activity by inhibiting myelination [99, 100].

Although neurons and glial cells are predominant sources of NTF secretion, other cells types have been shown to secrete neurotrophins, especially after nerve injury, including fibroblasts and immune cells. Fibroblast have been shown to secrete NGF [101].
A multitude of hematopoietic cells have been shown to release NTFs. Mast cells synthesize, store and release NGF during nerve injury which is thought to play a role in sensory nociception [92]. Monocytes, as well as T and B cells can produce BDNF in human peripheral blood and in human inflammatory brain lesions [82]. Human CD4 + T cell clones (from peripheral blood) were shown to produce and release NGF in vitro [81]. In addition, eosinophils from human peripheral blood can produce NGF, NT3 and BDNF neurotrophic factors upon immunologic stimulus [90, 91].

Recently, some attention has been given to the role of NTFs in adipose tissue. It has been suggested that NGF is secreted from adipocytes and plays a role in sympathetic nerve plasticity during cold-induced browning of i-scWAT [102]. Adipose immune-cell derived BDNF has been demonstrated to be necessary for proper i-scWAT innervation, thermogenic function, and energy homeostasis (and is the focus of Chapter 4 of this thesis).

**Peripheral neuropathy & neural plasticity**

When nerves in the periphery become damaged, neuropathy can occur and lead to loss of nerve control in affected tissues resulting in organ/tissue damage. Peripheral neuropathy (PN) is a collective term for disorders that result in the progressive dying back of nerves, that usually begins with distal axons in the skin of hands and feet and moves inward. PN is accompanied by weakness, pain, numbness, and in severe cases can lead to limb amputation. Occurrence of PN is very common, varied causes can be hereditary, acquired, or idiopathic in origin, and include genetic disorders, traumatic injury,
chemotherapy treatment, environmental exposures, metabolic problems, and aging. Currently, diabetes is the greatest contributing factor to the condition, and when caused by it is referred to as diabetic PN (DPN). Despite its common occurrence in diabetic and pre-diabetic individuals the pathophysiology of DPN is not fully understood, treatment options are lacking and no cure exists, and the condition is often diagnosed late in disease progression. It was originally argued that impaired glucose control and glucolipotoxicity resulted in DPN [103], however this relationship is not very direct and the pathophysiology of peripheral neurodegeneration is much more complicated. Diabetes and obesity related microvascular dysfunction has been implicated in contributing to nerve damage; impaired blood flow to nerves can cause hypoxia and nerve death [104, 105]. Systemic inflammation, often occurring in diabetes and obesity, has also been shown to be a contributing factor in DPN development and progression in murine and human models [106]. The role of Schwann cells in DNP is gaining traction as a research focus [107]; and a few studies have examined the possibility of DNP treatment using NTFs with mixed results [108-110]. An NGF based treatment even reached phase III clinical trials, but was unfortunately unable to replicate efficacy from previous phase trials which were also plagued by off-target effects. The full story has been reviewed by [109] and reveals how much more we still need to understand about NTFs and their potential as a neuropathy treatment.

The PNS is remarkable in its ability to remodel, and axonal growth and remodeling can be seen through the nerve injury/regenerative process. Loss of function after nerve injury can be compensated by 1) the regrowth (and remyelination) of damaged axons, 2) increased branching of nearby undamaged axons, 3) rewiring of neural circuitry [111].
Although most investigation into axonal growth revolves around peripheral nerve repair and regeneration, there is some evidence of peripheral nerve remodeling that is not prompted by insult to the nerves. The term plasticity can be used to distinguish axonal growth in response to environmental stimuli that is not dependent on nerve injury. Another form of axonal growth is sprouting, which can be induced with or without nerve damage, and is often associated with hypersensitivity and increased pain sensation. Axonal growth of sensory and autonomic nerves, marked by an increase in nerve density as well as nerve size, occurs in patients with pancreatic cancer and pancreatitis, and is not caused by direct nerve injury [112]. The NTF neurturin, has been implicated in contributing to the axonal outgrowth in pancreatic cancer, through its production by pancreatic cancer cells [113]. However, axonal growth under these disease conditions is aberrant and often the cause of neuropathic pain [112, 114].

Evidence for adipose neuropathy and plasticity is discussed in more detail in Chapter 3.

Certain environmental factors have been shown to affect peripheral nerve plasticity. It is well appreciated that exercise produces a plethora of positive physiological changes, among which is BDNF mediated neurogenesis and synaptic plasticity in the hippocampus of rodents [115, 116]. Neurogenesis in the human brain is a bit trickier to study, and most research is correlative, however BDNF expression does increase in humans participating in moderate exercise [117] and is linked to improved cognition in adults [118]. Exercise also has a CNS mediated effect on peripheral nerves, by rewiring CNS neuronal networks to improve sympathetic and parasympathetic control of cardiovascular function [119]; a relationship that is well reviewed by [120]. Finally,
exercise has been shown to enhance axonal regeneration after nerve injury, and the process is dependent on neuron secreted BDNF [121]. However, this treadmill type exercise may only be effective in enhancing axonal regeneration in male mice and not have any effect on axon regeneration in females [122].

Cold-induced catecholamine release has been recently proposed as another modulator of peripheral nerve plasticity. Cao, et al [102], provided evidence that cold-exposure elicits an increase in synaptic nerve fiber density, and that NE release stimulated an increase in NGF within i-scWAT, however, adipocytes as the source of NGF was not convincing as they did not take into account the milieu of cells that reside within the adipose depots.

**Lipids and peripheral nerves**

Peripheral nerve bundles consist of 3 distinct connective tissue layers: the outermost epineurium which contains the nerve microvasculature and sheaths fascicle bundles; the perineurium, thin connective tissue than surrounds individual fascicles; and endoneurium, which encapsulates Schwann cells, fibroblasts, and individual axons. Adipocytes have been observed in the epineurium [123], and are a hypothesized fuel storage of fatty acids for the endoneurium. However, considering that adipocytes are endocrine cells they may be communicating in a paracrine fashion with other cells within the nerve bundle. The myelin sheath itself is very lipid laden (70-85% lipids), and although protein types of CNS and PNS myelin can vary, lipid composition is similar in both [124]. The predominant lipids in myelin are cholesterol, galactosylceramide (a glycosphingolipid),
and ethanolamine plasmalogen (a phospholipid) and comprise 65% of total lipid dry weight [125]. Myelin lipid composition can vary and has been shown to be affected by diet [126], but de novo fatty acid synthesis by Schwann cell also appears to be important in peripheral nerve myelination [127]. Schwann cells in mature peripheral nerves, also express genes for fatty acid metabolism and extracellular lipid transport [128].

Some lipids and/or their metabolites serve as endogenous sensitizers, inhibitors or activators of certain transient receptor potential (TRP) channels. TRP channels are Ca\(^{2+}\) permeable non-selective cation channels found on sensory neurons, and although they have been predominantly studied in relation to pain sensation, emerging research has shown that they play a crucial role in energy homeostasis [129, 130]. Agonism of certain sensory nerve TRP channels such as transient receptor potential vanilloid subtype 1 (TRPV1) has been shown to attenuate diet induced obesity through increased sympathetic tone via activation of the central nervous system thereby increasing browning of WAT (as reviewed by [130]). Multiple lipid species have been identified as endogenous agonists of TRPV1, including certain arachidonic acid metabolites (i.e. the eicosanoids: 5-HETE, 12-HETE) and phospholipid derivatives (i.e. lysophosphatidic acid), amongst others as reviewed by [131]. Transient receptor potential cation channel subfamily M member 8 (TRPM8) has been well studied as the primary ion channel on somatosensory nerves responsible for cold (and menthol) sensation. Its activation on both sensory nerves and BAT leads to thermogenesis and energy expenditure [132, 133]. TRPM8 can also be activated by membrane lipids such as phosphatidylinositol 4,5-bisphosphate (PIP2) as well as lysophospholipids as lysophosphatidylcholine, lysophosphatidylinositol and

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V. Sex differences to consider

Sex dimorphisms are known to exist with respect to energy homeostasis, obesity and its complications, although they have been historically understudied. Sexual dimorphism also present in the nervous system and extends to innervation of WAT. The physiological consequences of these sex differences are still being investigated.

Human females, of reproductive age, have a greater amount of energy expending BAT compared to males or postmenopausal females [134]. Women also have a higher percentage of body fat compared to males and yet are more resistant to metabolic diseases even on high fat/high sugar diets, a characteristic that is also observed in female rats and mice [135, 136]. Estrogens emerged as the most likely cause of this protective effect due to their reduction at postmenopausal age, as well as the sexually dimorphic expression of estrogen receptors in the hypothalamus (the CNS regulatory center of energy homeostasis) [137, 138]. However, studies implicating hormone protective effects are problematic since rodents do not experience the same type of reproductive senescence as humans do and ovariectomy produces a more violent effect than the gradual reduction in hormones experienced during reproductive senescence.

Immune system sex differences can contribute to the sexual dimorphism in diet induced obesity. As previously discussed, inflammation plays a key role in obesity related adipose tissue dysfunction. Sex differences in both innate and adaptive immune response
have been documented across a variety of species, and have been linked to hormonal as well as genetic mediators (including sex chromosome) [139]. Pro-inflammatory cytokine production by macrophages is higher in human and murine males, while macrophage activation, phagocytic capacity, and IL-10 production is higher in females [139, 140]. This would suggest females have a greater capacity to clear apoptotic tissue (such as with hypertrophic obese adipose) without entering a chronic state of low inflammation. Sex differences in the adaptive immune system include a T\(_H2\) cell bias in females, which has been linked to increased anti-inflammatory response, and obesity resistance. CD 4+ T cells, T cell proliferation, and B cell numbers are also higher in females [139], all these cell types are present in adipose tissue (Fig. 2), and have been demonstrated to be a source of NTF production upon immunological simulation; they may be playing a role in male versus female innervation of adipose tissue and is further discussed in Chapters 3 & 4.

Finally, sexual dimorphism exists in the human brain [141] and peripheral nervous system [142]. One of these PNS sexual dimorphisms may explain another adipose tissue sex dimorphism. gWAT of male mice does not readily undergo browning the way i-scWAT does, however, gWAT of female mice does. Coincidentally, NGF and BDNF are higher in female gWAT as is the amount of sympathetic innervation [143]. NGF was shown to increase in gWAT of females upon adrenergic stimulation, while presence of BDNF and sympathetic nerve activation appeared to be estrogen dependent [143]. These studies illustrate the physiologically consequential effects sex difference can have and underscore the necessity of including both sexes in research endeavors.
VI. Perspective

The historical and contemporary findings reviewed in this introduction, helped form and guide the hypotheses expounded and investigated in the subsequent chapters. Although it had been demonstrated that adipose tissue contained nerves, and that this innervation is critical for metabolic health, the nerve network and interaction of nerves with other adipose resident cells was still unknown. The question of whether adipose innervation was parenchymal or primarily perivascular was still a point of debate, and questions regarding the presence of myelinated nerves in adipose still remained. All assessment of adipose nerves hitherto, did not considered the distribution of nerves within the whole adipose depot, and where synapses were forming. Furthermore, the dynamics of adipose nerves had not been investigated at all; questions regarding adipose nerve remodeling - neuropathy, and neurite outgrowth, and what factors could control such actions remained open for exploration.

Since diet bears weight on adipose tissue remodeling, we designed experiments to determine if various dietary lipids and changes in their amount has any bearing on metabolic parameters including energy expenditure, which is greatly reliant on adipose innervation. Lipidomic analyses of adipose tissues, could provide insight into how various dietary fats are metabolized and sequestered in adipose tissue, allowing for further investigation regarding lipid nerve interactions in the adipose organ. These questions are explored in Chapter 2.

Loss of brain-adipose communication had been shown to …through surgical and chemical denervation studies. However, progressive impairment of brain-adipose
communication through pathophysiological means had not been explored. We know obesity leads to dysfunction of adipose tissue and overall metabolic distress, but could this dysfunction be related to an obesity related loss of adipose innervation? We know incidence of obesity and type 2 diabetes increases with age, but it remained unknown whether ordinary aging affects adipose innervation. Cold-induced noradrenergic stimulation had been shown to induce lipolysis and browning of adipose tissue, thereby increasing energy expenditure. Could this stimulation have any effect on the distribution of adipose nerves within a depot? Therefore, hypotheses regarding adipose innervation under basal, pathophysiological, or stimulated conditions are surveyed in Chapter 3.

Nerves require continuous signals from NFs in order to survive. NFs, can also regulated neurite remodeling and outgrowth. Various NFs exist and can come from various cellular sources. However, which NFs and what their cellular source is in adipose tissue had not been interrogated. Based on the support role of immune cells in adipose tissue, and the fact that microglia mediate neural and synaptic plasticly through the release of BDNF in the brain, we aimed to inspect if a similar mechanism may be in effect in adipose tissue. This hypothesis is probed in Chapter 4.
References


82. Kerschensteiner, M., et al., *Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a


Chapter 2:

A PEROXIDIZED OMEGA-3-ENRICHED POLYUNSATURATED DIET LEADS TO ADIPOSE AND METABOLIC DYSFUNCTION

Abstract

Consumption of diets that differ in fat type and amount, and sequestration of various fatty acids to tissues and organs, likely have effects on overall physiology and metabolic health. However, the contributions of dietary lipids to brain-adipose communication and adipose tissue function are poorly understood. We designed 6 custom diets that differed only in amount and type of dietary fat, with high or low levels of saturated fatty acids (SFA), omega-6 polyunsaturated fatty acids (n-6 PUFA), or omega-3 (n-3) PUFA. Mice fed the n-3 PUFA diet for 16 weeks displayed a striking reduction in weight gain, accompanied by smaller adipose depots and improved glucose sensitivity. Reduced body weight occurred despite lowered energy expenditure and no difference in food intake. Despite the apparent beneficial effects to whole body physiology, we have demonstrated for the first time that a peroxidized n-3 enriched diet led to lipotoxicity of white adipose tissue, as evidenced by increased fibrosis, lipofuscin, reduced anti-inflammatory markers, and loss of proper nerve supply. While healthful, n-3 fats are prone to peroxidation, and we observed peroxidated lipid metabolites in the adipose tissue of mice on these diets. Furthermore, using a lipidomics approach we have observed that brain, white adipose tissue, and brown adipose tissue accumulate lipid metabolites differently. The brain remained mostly shielded from changes in dietary fat type and
amount, but differences in adipose lipid metabolites across these six diets may have affected metabolic function and brain-adipose communication, as observed in this study.

**Introduction**

*Dietary Fatty Acids, Lipid Biochemistry and Metabolic Health*

Dietary fats, one of the 3 macromolecules in our food, are important for adding flavor to what we eat, for providing fuel for metabolism, and for enhancing the absorption of lipid-soluble vitamins such as A, D, E and K. Dietary fat-derived cellular lipids are also important as structural components for cellular membranes, which in turn affect cellular activities such as the function of transmembrane receptors. Additionally, fatty acids also play an important role as signaling molecules in the body, thereby affecting other cellular tasks. There are two main types of dietary fats: saturated, meaning all the carbons are bound with hydrogen atoms, and unsaturated, meaning some carbons lack hydrogen and are double-bonded to each other. Within unsaturated fats, there can be a single point of ‘unsaturation’ (or double-bond), termed monounsaturated, or multiple points, termed polyunsaturated. Omega-9 (n-9) fats are monounsaturated while Omega-3 (n-3) and Omega-6 (n-6) are polyunsaturated. While mammals are able to form the double-bonds necessary for the synthesis of n-9 fatty acids, they lack the necessary enzymes (i.e. Δ15-desaturase) that form the cis double-bonds which define n-3 and n-6 PUFAs [1]. This inability to synthesize these fatty-acids implies that they must be derived from diet and are therefore ‘essential’. The essential n-6 PUFA is linoleic acid (LA, 18:2n-6)), which can be converted to the longer chain arachadonic acid (AA, 20:4n-6). The parent fatty acid of n-3 PUFAs is alpha-linolenic acid (ALA), which can be converted to eicosapentaenoic
acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Although conversion of LA to AA, as well as conversion of ALA to EPA and DHA, occurs in vivo, this process is extremely limited. Both LA and ALA compete for the same elongase and desaturase enzymes (i.e. Δ5-desaturase, Δ6-desaturase, elongase, etc.) in the synthesis of longer PUFAs such as AA and EPA [1]. Conversion rates in humans of ALA to EPA or DHA are reported between 5-10% and less than 1%, respectively [2]. Therefore, consumption of EPA and DHA is also considered essential in our diet, but humans often do not take in sufficient amounts of these lipids. Biologically important fatty acids also include eicosanoids (such as prostaglandins, leukotrienes, and thromboxanes – derived from AA or EPA), DHA, fatty esters, and fatty amides, which include endocannabinoid lipid ligands. Most, if not all, of these fatty acids have been implicated in metabolic physiology.

Over time, the Western diet has reduced intake of n-3 PUFAs, and increased intake of n-6 PUFAs and saturated fats [3]. Correlational studies have indicated that diets higher in n-3 PUFAs (such as Inuit diets enriched for whale or seal meats, or the Mediterranean diet which is high in fish, olives, nuts, whole grains, and produce) result in lower risk for cardiovascular diseases [4]. Thus, the Western diet as it looks today with lower n-3 and higher n-6 PUFAs, may be predisposing us to cardiovascular complications and other metabolic diseases. n-3 PUFAs are important for cell membrane fluidity and function, hormone production, regulating inflammation, and binding nuclear receptors – all of which are implicated in cardiometabolic function. The brain is second only to adipose tissues in terms of its lipid content, and thus understanding how dietary fats affect both brain and adipose lipid metabolites and their cellular functions is an important research goal.
The complete repertoire of physiological and metabolic effects of differing fatty acid composition in the diet is also not well understood. For example, we do know that certain essential fatty acids (such as DHA and EPA) are important for brain development and neural plasticity (reviewed in [5]), but we do not yet know how these fatty acids affect other cellular and molecular mechanisms in discrete brain regions, or if peripheral nerve function is also affected. In addition, effects on cardiovascular risk or health may also be secondary to how these diets impact neural function, whole-body metabolism, energy balance, or adiposity – but these have not been delineated in the research literature. Finally, consumption of low n-3 PUFA diets that may or may not be comprised of peroxidized n-3s also confounds the nutritional literature.

Therefore, the goal of this study was to investigate how differing dietary fat type and amount resulted in changes to brain and adipose lipid composition, as well as brain-adipose communication (by investigating adipose innervation status). We hypothesized that diets enriched for saturated vs n-6 or n-3 PUFAs would result in different fatty acid profiles in adipose vs. brain, and that the diets would also differentially affect metabolic function and energy balance regulation. An unexpected, but nonetheless insightful, outcome of this work was the discovery that peroxidized n-3 PUFA diets have a detrimental effect on white adipose tissue (WAT) health, which may negatively impact the peripheral nerves that are resident in this tissue, and thereby result in impaired brain-adipose communication.
Materials and Methods

Mice, Metabolic Phenotyping, and in vivo Analyses

Dietary fat interventions and food intake

Adult (10 week old) C57BL/6J male mice were fed custom diets (designed with Research Diets (New Brunswick, NJ); as described in Table 1-2), for 16 weeks. All groups had N=6 mice, except for high n-3 PUFA which had 8 animals. Mice were housed 2-3 per cage, at room temperature. Body weight was measured weekly. Food intake was measured daily for 7 days, then weekly until 28 days. Adiposity was assessed at the end of the study, and hypothalamus, subcutaneous white adipose tissue (scWAT) and intrascapular brown adipose tissue (iBAT) depots were harvested and sent for lipidomics analysis with Berg, which included a measure of non-enzymatic (peroxidized) lipids.

CLAMS

Metabolic cage analyses were conducted in a Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH), for measurement of oxygen consumption (VO₂) and carbon dioxide production (VCO₂), from which both respiratory exchange ratio (RER), and energy expenditure (heat) were calculated: RER=VCO₂/VO₂; *Energy expenditure (heat) = CV * VO₂ cal/hr, where CV is the “caloric value” as given by CV = (3.815 + 1.232) * RER.* Animal were single housed in a bedding free cage, at room temperature on a 12hr light/dark cycle. Mice were acclimated for 24-48hrs, after which VO₂, VCO₂, RER, and Heat were measured every 15min for 3 days (72hrs). Waveform analysis of CLAMS data was performed by matching every 15min measurement across all three 24hr-cycles. Two-way repeated measures analysis of
variance (RM, ANOVA) was performed for average VO$_2$, VCO$_2$, RER, and Heat per group. An uncorrected Fisher’s Least Significance Difference test was performed for each time point between dietary groups as a post-hoc test. Interaction P values are reported, which represent differences in 24hr data between groups, as well as multiple comparison results for differences which were only day/night phase specific.

*Lipidomics analysis of peroxidized lipid content in adipose*

Mass-spectrometry lipidomic analysis of scWAT was performed by Berg (Boston, MA). After dietary intervention, tissues were harvested, frozen on liquid nitrogen and shipped to Berg, where structural and signaling lipids within tissues were identified using the LC-MS/MS platforms, as previously described [6-8]. Only structural lipidomics across all 3 tissues was used as a dataset for comparison across tissue and diet.

*Thiobarbituric Acid Reactive Substances (TBARS) Assay*

To independently determine peroxidation of our n-3 PUFA diets, a malondialdehyde (MDA, a common by-product of lipid peroxidation) production assay was performed using a Cayman Chemical (Ann Arbor, MI) TBARS Assay Kit, per manufacturer’s instructions.

*Western Blot*

Protein lysates were prepared by homogenizing frozen tissue in RIPA buffer using a Bullet Blender (Next Advance, Averill Park, NY). Primary antibodies, including anti-TH (Millipore Cat. # AB152; Merck Millipore, Burlington, MA) and anti-β-actin (Abcam Cat. # ab8227), were both used at 1:1000 dilution. Secondary antibody was anti-rabbit HRP (Cell Signaling Ct # 7074), used at a 1:3000 dilution. Blots were visualized with enhanced
chemiluminescence (ECL; Pierce) on a Syngene G:BOX. TH protein expression was normalized to β-actin and quantified in Image J.

**Gene Expression (qPCR)**

RNA was isolated from whole tissue depots using Trizol reagent, and total RNA extracted using a Zymo (Irvine, CA) kit. RNA yield was determined on a Nanodrop; cDNA was synthesized using a High Capacity Synthesis Kit (Applied Biosystems, Foster City, CA). Real-time quantitative (q)PCR was performed with SYBR Green (Bio-Rad, Hercules, CA) on a CFX96 instrument (Bio-Rad, Hercules, CA). Primer sequences are listed in Supplemental Table S1.

**Statistical Analysis**

Statistical calculations were carried out in Excel (Microsoft, Redmond, WA) or GraphPad Prism (La Jolla, CA) programs, utilizing ANOVA or Student’s T-test as indications of significance. For effects of diet and tissue type, non-metric multidimensional scaling (NMDS) ordination to summarize structure of lipid species was performed. Bray-curtis dissimilarity indices, to reflect differences in abundances of lipid species, were calculated on loge(+1)-transformed data. Differences in lipid species structure between diet, tissue type, and their interaction was tested using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations using the adonis function in the vegan package [9] in R [10]. Other statistical analyses are described in the relevant Methods section above and Supplemental Materials. Error bars are SEMs. For all figures, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Further detailed Methods can be found in APPENDIX A: Chapter 2 Supplemental Materials

Results

Improved glucose tolerance and reduced weight gain in mice fed an n-3 PUFA diet, despite lower energy expenditure.

In order to test the effects on energy balance when differing dietary fat type and amount, we designed 6 custom diets that contained either high or low levels of saturated (SFA), n-6 or n-3 PUFAs, without differing sucrose or protein content (Table 1). Male C57BL/6 mice at 10 weeks old were fed the 6 diets for 16 weeks. By the end of the feeding period, mice on the high SFA and n-6 PUFA diets had gained significantly more weight compared to their low-fat counterparts, but mice on the high n-3 PUFA diet weighed less than animals on the 5 other diets (Fig. 1A). This difference in weight gain can also be seen across the entire time-course of the study, in terms of percent change in body weight each week and average body weight each week (Suppl. Fig. S1A-B).

The differences in body weight at the conclusion of the study were mirrored by the weights of subcutaneous WAT (scWAT) as well as perigonadal WAT (pgWAT) depots, both of which were higher in the high-SFA and high n-6 PUFA mice versus their low-fat diet counterparts, but did not differ between the high n-3 PUFA and low n-3 PUFA mice (Fig. 1B-C). Consistent with reduced weight gain, the high n-3 PUFA mice had a significantly improved glucose tolerance test (GTT) at week 4 of the diet, versus mice on the high-SFA or high n-6 PUFA diets (Fig. 1D), but at this time-point the body weights had not yet significantly diverged (Suppl. Fig. S1A). Despite a lower body weight at the
end of the 16wk feeding period, the high n-3 PUFA mice did not eat fewer calories over
the first 4 weeks of the diets (cumulative food intake; Fig. 1E).

When comparing metabolic cage measurements between the high n-3 PUFA and
high n-6 PUFA mice at week 7 of the diets, it was apparent that the high n-3 PUFA mice
had lower energy expenditure, as indicated by a lower VO$_2$ (Fig. 2A), VCO$_2$ (Fig. 2B), and
heat production (Fig. 2C). These differences were not apparent in low n-3 PUFA vs low
n-6 PUFA mice (at week 8 of the diets, Suppl. Fig. S2 A-D), indicating that this was a
function of dietary fat amount, as well as dietary fat type. Interestingly, the respiratory
exchange ratio (RER) measurement, an indication of fuel utilization, demonstrated a
significant interaction difference across the 24-hour period for the high and low n-6 vs n-
3 PUFA diets (Fig. 2D and Suppl. Fig. S2D), with a lower RER observed overall during
the dark phase for the low n-3 PUFA animals only (Supply. Fig. S2D). These findings
indicated greater utilization of lipids as fuel by the n-3 PUFA mice, since RER was closer
to 0.7 than to 1.0, which supports the finding that n-3 PUFA fed mice had smaller WAT
depots and likely had higher rates of lipolysis.

**Adipose tissue effects of the PUFA-enriched diets**

Hematoxylin staining and histological analysis of scWAT depots indicated that the
high-SFA and high n-6 PUFA tissues were indistinguishable in terms of cell size, crown-
like structures, and browning pattern (Fig. 3A). However, the high n-3 PUFA mice
displayed more browning (or, development of inducible and multilocular beige/brite
adipocytes), smaller cell size, and marked inclusions of lipofuscin stain (arrows, Fig. 3A),
a plastic-like aggregate in the liposomes that is a sign of tissue malfunction. Lipofuscin
stain was observed more strongly in some areas of the n-3 PUFA tissue than others, as demonstrated by the multiple representative images (Fig. 3A). Lipofuscin is known to accumulate with tissue damage and with age, and represents a type of lysosome storage disease [11-13] that is often considered a sign of aging, inflammation, and tissue degeneration. In both scWAT and perirenal white adipose tissues (prWAT) from mice fed the n-3 PUFA diet, large areas of lipofuscin accumulation can be seen through bright field microscopy as iridescent 'oil slicks' between adipocytes (Fig. 3A and 5B). Lipofuscin is also known to be autofluorescent [11], and fluorescent microscopy revealed highly intense autofluorescent signals only in WAT of the n-3 PUFA animals, and not those on an SFA diet (Fig. 3C).

In addition, trichrome stain revealed that the high n-3 PUFA mice had significantly more collagen (bluish-purple; Fig. 3B), indicating more tissue fibrosis, an accepted indicator of dysregulated adipose tissue. Trichrome stain also revealed more crown-like structures around the adipocytes of n-3 PUFA mice, indicating greater immune cell infiltration in the stromovascular fraction for this diet. Crown-like structures are a common occurrence in adipose under high fat feeding, and are formed from macrophage efferocytosis of dying cells, which increases in obesity due to increased inflammation and hypoxia. It is not surprising to see crown-like structures surrounding large adipocytes (ie: with the hypertrophy observed in mice fed an obesogenic diet), however, we were surprised at the higher extent of crown-like structures that accompanied the small adipocytes with the n-3 PUFA diet (Fig. 3B).
Peroxidation of an n-3 enriched diet

Despite the fact that animals fed a high n-3 PUFA diet appeared to exhibit the reported beneficial metabolic effects, such as decreased body and adipose weight, as well as better glucose tolerance compared to animals fed a high SFA diet (Fig. 1), after closer inspection of the tissues, we observed that adipose tissue damage and dysfunction had occurred (Fig. 3) and that the n-3 PUFA diet had become peroxidized. Specifically, two independent approaches confirmed that the n-3 PUFA diets themselves, as well as the tissues from mice fed this diet, exhibited signs of peroxidized lipid species. A mass-spectroscopy lipidomics approach revealed a greater concentration of lipid metabolite species that had been produced through non-enzymatic oxidation in scWAT of mice fed the high- and low n-3 PUFA diets (Fig. 4A).

In support of this, a TBARS assay measuring malondialdehyde (MDA), a common byproduct of peroxidation, demonstrated peroxidation of the n-3 PUFA diet itself, as well as the menhaden fish oil that was used as the n-3 source in the diet (Fig. 4B). A threshold of 50uM MDA was set based on previously published data indicating this amount of MDA accumulation is sufficient to cause intercellular oxidative stress [14, 15]. Interestingly, a high level of peroxidation was similarly observed in common fish oil supplements and food items that are high in n-3 PUFAs (tinned sardines and salmon), but this was not observed in the n-6 PUFA or SFA diets (Fig. 4B), nor other canned fish such as tuna in water or in oil, or kippered herring.
Metabolic dysregulation after a peroxidized n-3 PUFA diet

To observe the effects of consuming a peroxidized n-3 PUFA diet, we measured gene expression in the perigonadal WAT (pgWAT), a depot more likely to exhibit insulin resistance and inflammation, and found significantly reduced anti-inflammatory cytokines il4, il10, and il13 in animals fed our n-3 PUFA diet compared to the other two high-fat diets (Fig. 5A). These similarly fit with the prevailing narrative that n-3 PUFA diets are metabolically healthy. By contrast, we observed a decrease in the Schwann cell marker sox10 with the high n-3 PUFA diet, indicating a negative impact on myelination and nerve health (Fig. 5A). This was further substantiated by a decrease in tissue levels of brain derived neurotrophic factor (bdnf), as well as decreased expression of synaptic markers (synapsin I & II, synaptophysin, psd95; Fig. 5A). Together these indicate harm to the peripheral nerves innervating adipose tissue.

Since sympathetic nerve stimulation is necessary for activation of uncoupling protein 1 (ucp1) and thermogenesis in brown or beige adipocytes, we analyzed expression of genes related to ‘browning’ and thermogenic function in WAT. Not surprisingly, ucp1 expression was reduced in WAT from animals fed high n-3 PUFA diet; as was the enzyme elongation of very long chain fatty acid (elovl3), an important component in early lipid recruitment during ‘browning’ [16] (Fig. 5A). Surprisingly, we saw an increase in pgc1a, indicating that the ‘browning’ processes may be triggered by the n-3 PUFA diet, but in the absence of proper sympathetic stimulation, thermogenesis could not be supported.
The browning pattern (defined by presence of multilocular cells, distinct from lipofuscin and crown-like structures) in perirenal WAT (prWAT; Fig. 5B) was distinct from scWAT (Fig. 3A). These data are summarized in Table 3.

While we did not run lipidomics analysis on prWAT, there remains the possibility that diet-derived lipids accumulate differently in the two WAT depots and are responsible for the differences in browning patterns that were observed.

Consistent with our hypothesis that a peroxidized diet damages nerve health, we observed a decrease in protein expression of the sympathetic activity marker tyrosine hydroxylase (TH) in the interscapular WAT (iWAT) depot of high n-3 PUFA animals, when compared to those on a high SFA diet (Fig. 5C). This may reflect impaired communication between brain and adipose, or a compensatory reduction in sympathetic outflow given the dysregulated lipolysis observed in the WAT of mice fed the n-3 PUFA diet. In addition, despite displaying an increase in circulating levels of insulin-sensitizing adiponectin in the low n-3 PUFA diet, there was no increase maintained with the high n-3 PUFA diet (Fig. 5D), which was mirrored by a trend for a decrease in circulating levels of the anti-inflammatory cytokine IL10 in the high n-3 PUFA fed mice (Fig. 5E). Gene expression of il10 in WAT was significantly decreased in the n-3 PUFA mice (Fig. 5A).

**Tissue distribution of dietary fats and TRP-activating lipids**

Mass-spectroscopy structural lipidomics data were compared by non-metric multidimensional scaling (NMDS), across all 3 high-fat diets for all 3 tissues tested (WAT, BAT, and hypothalamus). These data indicated that hypothalamus was least likely to display differences in lipid diversity due to diet, whereas WAT had the most lipid diversity
due to diet (Fig. 6A). The concentration of anti-inflammatory lipid metabolite species was increased the most in scWAT by the n-6 PUFA diet, followed by the n-3 PUFA diet, but interestingly the pro-inflammatory lipids were also increased the most by an n-6 PUFA diet (Fig. 6B). A subset of the anti-inflammatory lipids, including TRP-channel inhibitors such as maresin 1 and the resolvins RvD1 and RvD2, were increased the most by the n-3 PUFA diet (Fig. 6C). In addition, TRPM8-activating lipids, including lysophosphatidylcholine (LPC), lysophosphatidylinositol (LPI) and lysophosphatidylserine (LPS) were increased in n-6 and n-3 PUFA diets. However, since TRP calcium channels are largely found on sensory nerves, it is likely that some of the response we observed in scWAT could be due to increased TRPM8 activation of WAT-resident sensory nerves by the high PUFA diets. Despite the potential for increased sensory nerve feedback of lipid stores to brain, the n-6 PUFA mice were not protected from weight gain and increased adipocyte cell size (Fig. 1 and Fig. 3), but they were protected from the changes in gene expression that were observed in scWAT of n-3 PUFA mice and did not display a reduction in peripheral nerve markers (Fig. 5A). This is likely because the n-6 PUFA diet was not peroxidized.

**Discussion**

**Diet peroxidation and metabolic health**

Very little is currently known about how varying dietary fat type and amount affects tissue distribution of lipids and lipid metabolites, and how tissue lipid composition affects brain-adipose communication and metabolic control. We have demonstrated that a high n-3 PUFA diet, typically considered the most metabolically healthy fat, does lead to less
weight gain and improved glucose tolerance, and that this occurs without consuming fewer calories and despite a decrease in energy expenditure. Given that there was a demonstrable negative impact on scWAT tissue integrity due to the peroxidized n-3 PUFA diet, we believe that altered WAT lipolysis underlies the weight reduction in these mice and not a shift in energy balance to alter appetite or overall metabolic rate. This fits with the smaller depot size and increased expression of lipolytic genes (atgl, hsl, Fig. 5A) in WAT, and the fact that increased lipid utilization and reduction in fat pad size are hallmarks of certain illnesses [17,18]. In addition, the peroxidation of the n-3 PUFA diet led to signs of peripheral nerve damage in the WAT and lipofuscin stain. Thus, the beneficial effects of this diet may have been outweighed by the negative consequences of the peroxidation.

Most surprising was the observation that despite numerous attempts to mitigate it (see details Suppl. Methods), the n-3 PUFA diets readily undergo peroxidation, a detrimental non-enzymatic degradation pathway that has been described only sparingly in the literature [15, 19] but is well-known to labs studying n-3 fats. Despite careful procedures undertaken by the menhaden fish oil processor, as well as the mouse diet manufacturer to reduce or eliminate this peroxidation process, we measured peroxidized lipid byproducts in both the fresh fish oil (from a new, sealed vial) and the fresh n-3 PUFA diet (from a new, sealed bag). By determining that the fish oil source was minimally peroxidized, we were able to conclude that the majority of peroxidation must have occurred during the diet manufacturing process, since peroxidation creates a chain reaction leading to more peroxidation, and diet manufacturing does put the food under mechanical pressure and the presence of oxygen, despite maintaining room
temperatures and avoiding heat. We also observed peroxidized lipid metabolites in scWAT of mice fed the n-3 PUFA diet. This tenacious process of peroxidation takes an otherwise healthy polyunsaturated fatty acid and turns it into a lipotoxic product, which we have demonstrated to correlate with WAT lipofuscin stain, inflammation, and signs of tissue denervation (including a reduction in neurotrophic and synaptic markers in WAT).

To date, several studies have reported that a hyperglycemic state can lead to glucotoxicity of nerves [20] and now our data support the notion of lipotoxicity to nerves as another likely cause of neuropathy.

Given the observation that only n-3 PUFA diets undergo peroxidation (ie: not SFA or n-6 PUFA), and the finding that fish oil in many processed forms exhibits high levels of peroxidized lipids (as we have measured in human fish food products), our new findings regarding lipotoxicity and neuropathy in WAT hold potentially striking importance for human nutrition and dietary health. This could especially be true for pets, who on a regular basis eat fish-based foods that may have become peroxidized. In addition, since so many people take fish oil supplements for the vitamin-D and healthy fat components, the high level of peroxidation in the supplements we measured indicate that the health benefits may be outweighed by the risks. Furthermore, although the World Health Organization has set levels of acceptable total oxidation in fish oil supplements, and the European Pharmacopoeia Standards specify both peroxidation and total oxidation standards, there are currently no set standards for peroxidation or total oxidation levels in fish oil supplements for the U.S., resulting in no market control of products available for public consumption. In one study in New Zealand, it was found that fish oil supplements not only had less of the beneficial EPA and DHA than reported (less than 67% on
average), they also were peroxidized at a rate of 83%, with some at toxic levels [21]. Another study from Norway determined that most fish oil supplements tested were above the recommended limit of 2meq/kg of peroxide values (29 out of 33 samples tested [19].

**Dietary fat type & brain-adipose communication**

Adipose tissue is able to sequester toxins, store fat-soluble vitamins, and maintain triglyceride stores in lipid droplets that structurally resemble lipoproteins, as they are studded with biologically important proteins (reviewed in [16]). The intake of excess diet results in neutral lipid storage as cholesterol esters, triglycerides, and fatty acids in adipose lipid droplets. However, with obesity comes mitochondrial dysfunction, a decrease in fatty acid oxidation, and defects in degradation of triglycerides by lipolysis. Thus, the tissue lipid stores swell and lipid droplets grow in size, as do the adipocyte cells themselves, thus leading to eventual metabolic dysregulation.

While adipose tissue is the most lipid-laden tissue in the body, the brain contains the second-most amount of lipid. The dry weight of the brain has around 50-80% lipid, with essential fatty acids comprising about 15-30% of that total. Dietary fat type and amount is known to be very important for brain health, including proper brain development, and also impacts neurodegenerative conditions such as with aging [22]. Thus, unsurprisingly, parallel to the decrease of n-3 PUFAs in western diets, there has also been an increase in neurological disorders [23]. Diets high in n-3 or supplemented with n-3 appear to result in improved cognition (reviewed in [24]), but it is not known how they affect adipose tissue integrity and function.
Neuronal membranes are made from a choline-phosphocoline-phosphatidylcholine mixture requiring synthesis from the long-chain PUFAs DHA, EPA, and AA (reviewed in [24]). Membranes making up synapses, dendrites, mitochondria and vesicles are also rich in n-3 and n-6 PUFAs. In the absence of these essential fats in our diet, other fatty acids fill in the structural role in neurons, thus changing cellular behavior [23]. Non-membrane lipids in the brain include steroid hormones and eicosanoids, which act as biomessengers. Long-chain PUFAs are required for neural plasticity and neural protection, whereas lipids like ceramides may be lipotoxic [25]. On a high-fat diet there is increased hypothalamic fatty acid uptake but very low beta-oxidation, resulting in excess fatty acid storage and potential lipotoxicity [25], whereas adipose tissue can take up and store lipids in a more efficient manner, as well as oxidize them as needed for fuel. Adipose tissue lipids also likely have an effect on the structure and function of the peripheral nerves that innervate them, but this has not been explored.

**Signaling lipids and sensory nerve TRP channels**

Sensory nerves, including those innervating adipose depots and mediating adipose to brain communication, contain specialized calcium channels of the transient receptor potential family (TRPs). Sensory nerves contain the following TRP family members: TRPV1-4, TRPA1, TRPM8, TRPC1/C3/C6 (reviewed in [26]). These TRP channels are important for metabolic processes like thermogenesis (TRPM8) and adipose tissue function (TRPV1-3; reviewed in [27]). Some of these TRPs respond to lipid ligands, and may also play a role in synaptic/neural plasticity (reviewed in [28]). We have found that n-3 PUFA diets, perhaps exacerbated by peroxidation, produce signaling lipids in the resolvin family (RVD1 and D2, and Maresin 1), which are known to inhibit sensory nerve
TRP channels. These lipids would also normally exhibit a beneficial anti-inflammatory role, but the peroxidation of the n-3 diet may be masking that beneficial effect. By contrast, n-6 PUFA diets display the opposite and are enriched for HODE-family metabolites that are known to activate sensory nerve TRP channels [26]. Maresin 1, a macrophage-derived mediator of inflammation resolution [29] can act to inhibit TRPV1 [30]. Maresins are produced by macrophages in response to the oxygenation of DHA with 14-HDHA serving as an intermediate step and precursor to maresin1 biogenesis [29, 31] and 14-HDHA is significantly increased in n-3 PUFA groups.

In addition, we observed that PUFA diets lead to increased LPC, LPI and LPS in WAT. These lipids serve as TRPM8 activators [32]. TRPM8 is a cold-activated channel [33] expressed in WAT, and when activated can induce browning of WAT, thereby increasing energy expenditure.

**Conclusions**

Taken together, our data indicate that differing dietary fat type and amount results in a distinct pattern of lipid distribution between brain, white, and brown adipose tissues, as well as differences in metabolic function. This may be due to altered brain-adipose communication, as peripheral nerves in adipose depots appear to be affected by the dietary fat components. Finally, the unexpected finding that n-3 PUFA diets were peroxidized, leading to accumulation of peroxidized lipid species in adipose, fibrosis, and lipofuscin, likely explains the blunted metabolic benefit (such as lower energy expenditure) that would normally be conferred by this diet.
Figure 1

A. % Change in Body Weight

B. scWAT/Body Weight

C. pgWAT/Body Weight

D. GTT

E. Cumulative Food Intake over first 28 days of diet
Figure 1: Effects of differing dietary fat type and amount on body weight, adiposity, glucose tolerance and food intake. (A) Percent change of body weight across 16wks of dietary fat feeding. No difference in percent change of body weight was observed within low fat groups (denoted by A); percent change in body weight was comparable for mice on high SFA and high n-6 PUFA diets (denoted by B); percent change in body weight for mice on high n-3 PUFA diet was significantly lower than any other diet (denoted by C, with p=<0.0001 when compared to either high SFA or high n-6 PUFA). Data was analyzed by one-way ANOVA with Tukey’s post hoc test. (B-C) Adipose depot weight as a percentage of body weight: subcutaneous (scWAT) and perigonadal (pgWAT) adiposity was comparable across all low fat diets and high n-3 PUFA (denoted by A), while similar between mice on high SFA and high n-6 PUFA diets (denoted by B, p<0.001 when analyzed by one-way ANOVA with Tukey’s post hoc test). (D) Glucose tolerance test after 4 weeks of dietary intervention, high SFA and high n-6 PUFA displayed significantly slower glucose clearance at 30 and 70min. Data was analyzed by two-way ANOVA with uncorrected Fisher’s LSD post hoc. (E) Cumulative food intake was calculated as average kcal consumed by each mouse over a 28-day period. There was no difference in food intake within low or high fat diets. Data was analyzed by one-way ANOVA with Tukey’s post hoc. For all data, N=6-8 male mice were analyzed.
Figure 2

A. **VO2**

B. **VCO2**

C. **HEAT**

D. **RER**

- High n-3 PUFA
- High n-6 PUFA
**Figure 2: Metabolic Cage Measurements** Waveform analysis of metabolic cage measurements (CLAMS) taken at 15min increments for 3 days. Time of day is indicated on the x-axis, and animals were maintained on a 12 hr light/dark cycle (black bars indicate dark cycle). (A) Volume of oxygen (VO$_2$) consumption was higher for n-6 PUFA mice at multiple time points throughout the 24 hr cycle. (B) Volume of carbon dioxide (VCO$_2$) produced was greater for animals on a high n-6 PUFA diet compared to those on high n-3 PUFA, predominately during the light phase. (C) Energy expenditure as measured by heat was higher at various time points, but especially during the day, for the high n-6 PUFA animals. (D) A strong interaction for the whole 24hr cycle was observed for respiratory exchange as a ratio (RER) between the two groups, indicating greater use of carbohydrates for fuel by the high n-3 PUFA animals compared to high n-6 PUFA. Data analyzed by two-way repeated measures ANOVA with Fisher’s LSD test; N=4 per group. Error bars are SEMs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 3

A. Hematoxylin Staining of scWAT

B. Trichrome Staining (scWAT)

C. Lipofuscin Autofluorescence (scWAT)
Figure 3: Adipose tissue effects of differing dietary fat amounts

(A) Hematoxylin staining of subcutaneous adipose tissue showed no difference in cell size or browning between high SFA and high n-6 PUFA groups. Despite greater variation within the high n-3 PUFA, cell size is smaller overall and lipofuscin (arrows) is noticeable. Inset is a digital zoom of 10x magnification showing representative lipofuscin. (B) Trichrome staining of scWAT revealed fibrosis and higher concentration of crown-like structures in tissue of high n-3 PUFA fed animals compared to those on a high SFA diet. Inset is a 20x magnification of a representative crown-like structure. (C). Green autofluorescence in high n-3 PUFA diet animals indicative of increased lipofuscin content relative to high SFA diet. For all experiments, N=6-8 mice per group.
Figure 4. Peroxidation of the n-3 diet. (A) Lipidomics analysis revealed an increase in non-enzymatic oxidized (peroxidized) lipids in scWAT of mice fed n-3 PUFA enriched diets only (groups labeled B are significantly different than groups labeled A). These lipids included: 11-HEPE, 9-HEPE, 11-HETE, 9-HETE, 8-HETE, 11-HDHA, 8-iso PGF2a, and 5-iPF2a-VI. Lipidomics data of non-enzymatic oxidized lipids were analyzed by ANOVA, with Tukey’s post hoc test, N=6-8 per group. (B) Peroxidation was confirmed by a TBARs assay, which compared the mouse diets themselves to both fish oil supplements and canned fish products. 50µM MDA was used as a threshold above which oxidative stress is exhibited in certain tissues. All data were run in technical replicates of n=3.
Figure 5

A. 
Gene Expression of pgWAT

B. 
Hematoxylin Staining of prWAT

C. 
iWAT Tyrosine Hydroxylase

D. 
Adiponectin

E. 
IL10
Figure 5: Adipose dysregulation in other depots due to peroxidized n-3 diet

(A) Gene expression analysis of pgWAT. Anti-inflammatory cytokines (il4, il10, il13), inflammatory markers (il6, tnfα), macrophage marker (f4/80), lipolysis markers (atgl, hsl), nerve related genes (sox10, bdnf, synapsin I, synapsin II, synaptophysin, psd95), and browning markers (ucp1, elovi3, pgc1α) were measured by qPCR and analyzed by two-tailed Student’s t-test, using Welch’s correction when variance was unequal, N=6-7 per group. Error bars are SEMs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (B) Hematoxylin staining of perirenal adipose tissue; signs of lipofuscin (arrows). (C) A decrease in sympathetic activation (TH protein levels) in the scWAT of n-3 PUFA mice, as measured by western blot. TH levels were normalized to β-actin housekeeper, and band densities were quantified in Image J and analyzed by two-tailed Student’s t-test. Error bars are SEMs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (D) ELISA measurements of circulating HMW adiponectin revealed higher levels in the lower n-3 PUFA mice only. (E) ELISA measurements of circulating anti-inflammatory IL10, with a trend for reduced IL10 in the high n-3 PUFA mice.
Figure 6

A. NMDS by Diet by Tissue

B. Total Inflammatory Lipid Species in scWAT

Anti-inflammatory

Concentration (nmole/mg protein)

Pro-inflammatory

Concentration (nmole/mg protein)

C. Anti-inflammatory TRP-inhibiting signaling lipids in scWAT

D. TRPM8 Activating Lipids
Figure 6: Tissue Distribution of Lipids

(A) A total of 2106 structural lipid species measured by lipidomics from hypothalamus, BAT and WAT across all 3 high-fat diets were compared using NMDS in R, revealing non-overlapping differences in lipid species composition among the three tissues. In addition, hypothalamus had little difference in lipid composition regardless of diet, whereas WAT had the most diversity in lipid composition depending on diet. (B) Total concentration of anti- and pro-inflammatory lipid species as measured in scWAT. (C) Levels of anti-inflammatory TRP inhibiting lipids in scWAT. (D) Levels of TRPM8 activating lipids in scWAT. All data generated by mass spectroscopy at Berg.
### Table 1: Composition of Custom Diets

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Table 2: Typical Fatty Acid Profile of Diets Based on Data from the Manufacturers of the Fat Sources

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<td><strong>1.1</strong></td>
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Table 3: Browning patterns across WAT depots by diet, as indicated by multilocular histology

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<th>Diet</th>
<th>scWAT Browning Pattern</th>
<th>prWAT Browning Pattern</th>
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<tr>
<td>Low SFA</td>
<td>3/6 mice exhibit browning</td>
<td>3/6 mice exhibit browning; large patches</td>
</tr>
<tr>
<td>High SFA</td>
<td>No browning observed</td>
<td>Some smaller patches of browning in a couple of animals</td>
</tr>
<tr>
<td>Low n-6 PUFA</td>
<td>All mice exhibit some extent of browning, but only small patches</td>
<td>1/6 mice exhibited browning; large patches</td>
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<tr>
<td>High n-6 PUFA</td>
<td>Little to no browning observed</td>
<td>5/6 mice exhibited browning</td>
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<tr>
<td>Low n-3 PUFA</td>
<td>No browning observed</td>
<td>All tissues exhibited some browning; 2/6 had large patches</td>
</tr>
<tr>
<td>High n-3 PUFA</td>
<td>No browning observed</td>
<td>No browning observed</td>
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References


Chapter 3:

NEUROPATHY AND NEURAL PLASTICITY IN THE SUBCUTANEOUS WHITE ADIPOSE DEPOT

Abstract

The difficulty in obtaining as well as maintaining weight loss, together with the impairment of metabolic control in conditions like diabetes and cardiovascular disease, may represent pathological situations of inadequate neural communication between the brain and peripheral organs and tissues. Innervation of adipose tissues by peripheral nerves provides a means of communication between the master metabolic regulator in the brain (chiefly the hypothalamus), and energy-expending and energy-storing cells in the body (primarily adipocytes). Although chemical and surgical denervation studies have clearly demonstrated how crucial adipose tissue neural innervation is for maintaining proper metabolic health, we have uncovered that adipose tissue becomes neuropathic in various conditions of metabolic dysregulation. Here, utilizing both human and mouse adipose tissues, we present evidence of adipose tissue neuropathy, or loss of innervation, under pathophysiological conditions such as obesity, diabetes, and aging, all of which are concomitant with insult to the adipose organ and metabolic dysfunction. Neuropathy is indicated by loss of nerve fiber protein expression, reduction in synaptic markers, and lower neurotrophic factor expression in adipose tissue. Aging-related adipose neuropathy particularly results in loss of innervation around the tissue vasculature. These findings underscore that peripheral neuropathy is not restricted to classic tissues like the skin of distal extremities, and that loss of innervation to adipose may trigger or exacerbate
metabolic diseases. In addition, we have demonstrated stimulation of adipose tissue neural plasticity with exercise, cold exposure or local AAV-mediated neurotrophic factor treatment, which may ameliorate adipose neuropathy and be a potential therapeutic option to re-innervate adipose and restore metabolic health.

**Introduction**

Since body weight regulation involves a precise balance between energy intake and energy expenditure, and requires coordination and control stemming from the central nervous system (CNS), the brain needs to adequately communicate with peripheral organs and tissues via peripheral nerves in order to maintain metabolic health. The CNS is able to control energy expenditure by signaling motivation to exercise or seek certain foods, driving sympathetic nervous system (SNS) activation of energy expending processes in the peripheral tissues of the body, and through receiving feedback from tissue sensory nerves, like those from adipose. Sensory nerves in white adipose tissue (WAT) are thought to communicate the status of energy stores to the brain, in order to help regulate energy intake versus expenditure. In WAT, SNS activation serves to increase lipolysis, *de novo* adipogenesis, and a process termed ‘browning’, whereby uncoupling protein 1 (UCP1)-positive brown adipocytes appear in WAT depots and contribute to energy expenditure via thermogenesis. In brown adipose tissue (BAT), SNS activation also drives non-shivering thermogenesis via activation of mitochondrial UCP1, which is a brown adipocyte-specific gene and required for this energy expending process in both classical and inducible/recruitable brown adipocytes. During thermogenesis, BAT must utilize fatty acid fuels, which are both stored in the multilocular lipid droplets of
brown adipocytes and are also obtained via circulating lipids that are released from WAT through lipolysis\textsuperscript{5,6}.

Surgical and chemical denervation studies have demonstrated the importance of adipose tissue nerves, and denervation leads to a loss of proper metabolic control\textsuperscript{3,7,8}. Denervation of WAT also leads to an increase in tissue mass and adipocyte cell number\textsuperscript{9-11}. Therefore, the regulation of lipid stores in both BAT and WAT and the activation of energy expenditure through CNS-SNS communication are essential for proper body weight maintenance and metabolic health. It is not yet understood what other aspects of adipose tissue function are under neural control, and whether or not the nerves that innervate adipose tissue can modify their connections under physiological or pathophysiological conditions. Therefore, these questions regarding adipose nerve remodeling warrant further investigation.

The nerves that innervate white adipose tissues include numerous peripheral nerve subtypes, such as sensory, parasympathetic and sympathetic nerves\textsuperscript{12-14}. Although the number of studies assessing adipose innervation are increasing, further demonstrating the importance of brain-adipose communication\textsuperscript{15-17}, it is still unclear which neurotransmitters and neuropeptides, aside from the well-studied norepinephrine, are synaptically released in adipose tissue and onto which receptor-expressing cell types. A better understanding of how the peripheral nerves in adipose tissue are regulated is important for the field, including differences in nerve plasticity between innervation of BAT and WAT, as well as sex differences in innervation density.

In other tissues, and in many organisms, peripheral nerves are appreciated as plastic (ie: able to undergo remodeling of neurites and synapses in response to stimuli),
or neuropathic (dying-back in pathophysiological conditions). For example, with distal peripheral neuropathy nerves in the skin of distal extremities can die back through an unclear process, resulting in pain, loss of sensation, and severe discomfort. The process begins in the skin and moves inward. Neuropathy can be caused by aging, certain drugs (such as antibiotics and chemotherapy agents), or diabetes. Diabetic neuropathy is especially prominent, affecting over 50% of diabetic individuals, and often leads to limb amputation. Aging is associated with a loss of metabolic regulation and an increased propensity for diabetes, and is independently associated with peripheral neuropathy. The debilitating aspects of peripheral neuropathy are largely due to the inability to prevent or treat these conditions, and the inability to halt and reverse the neurodegeneration. Standard clinical approaches include pain management or glucose regulation (with diabetic neuropathy), but no therapies are currently approved to mitigate nerve death or to stimulate peripheral nerve re-growth or re-myelination. It is important to understand whether or not neuropathy can extend below the skin into underlying adipose tissue, which may exacerbate metabolic disease.

Given the close association between situations of metabolic dysregulation (aging, obesity, diabetes) and peripheral neuropathy, and the clear importance of adipose tissue innervation for metabolic homeostasis, we sought to determine if adipose tissue nerves also undergo neuropathy with these conditions. To do this, we assessed human adipose tissue samples across a range of ages and body mass indices (BMIs), and also utilized mouse models of aging and obesity/diabetes. We hypothesized that age and obesity/diabetes would be positively correlated with a loss of proper innervation of adipose tissues. We also sought to determine which interventions could stimulate re-
innervation of adipose tissue in situations of adipose neuropathy, through a beneficial and physiological process of nerve plasticity.

**Methods**

*Mice, Metabolic Phenotyping, and in vivo Analyses*

**Young/Aged Sedentary/Exercised mice**

Age-matched C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME; stock number 000664 Black 6) were aged to 16 months. Body weight and adiposity were compared to age-matched C57BL/6J male mice at 10-12 weeks old.

**Young Sedentary/Exercised mice**

Young (12-15 week old) C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME; stock number 000664 Black 6) were age and body weight matched before randomized assignation to either sedentary or exercised groups.

**Voluntary running-wheel exercise**

Young and aged animals were single-housed in running wheel cages that allowed ad libitum access to running, for a period of 7 days. Control (sedentary) animals were either single-caged with locked running wheels, or caged in pairs without a running wheel.

**BTBR ob/ob**

Male BTBR +/- (WT) and ob/ob mutant (MUT) mice (Jackson Laboratory, Bar Harbor, ME; BTBR.Cg-Lep^{ob}/WiscJ, stock number 004824) were fed a standard chow diet and
aged to a minimum of 12 weeks of age, when they exhibit a robust phenotype including obesity, diabetes, and hyperglycemia. These animals (aged 12-28 weeks) were then euthanized after Von Frey Analysis of tactile allodynia (see details in Supplement).

For tissue harvesting all animals were CO₂ euthanized followed by cervical dislocation.

**Human adipose tissue analyses**

Human adipose samples (subcutaneous and omental) were obtained from the Boston Nutrition Obesity Research Center (BNORC) adipose tissue core. Biopsies of scWAT and omental adipose were taken from patients during elective surgery. All patients were either non-diabetic or pre-diabetic, with the exception of four diabetic individuals, 2 of whom were in the BMI cohort and 2 in the aged cohort (indicated by asterisks in data plots; see Suppl. Tables S1-3 for relevant patient data). Frozen and fixed tissue (from only a subset of patients) samples were obtained for analyses, including histology and western blotting.

**Whole depot imaging and analysis**

Mouse inguinal subcutaneous adipose depots were removed intact and immunostained as described in Supplemental Materials. Entire depots were imaged for Fig. 4 with a 10x objective on a Leica TCS SP8 or DMI6000 confocal microscope (Leica Microsystems, Wetzlar, Germany), by tiling z-stacks of the entire depth of tissue. Images ranged between 542-3464 tiles per depot, the average lied at approximately 1000 tiles per depot. Tiles were individually Z-projected and background subtracted (using the rolling ball method). Processed tiles were then thresholded into binary images and skeletonized. To analyze arborization of adipose nerves, skeletons were assessed for the following parameters: innervation density, total number of branches, total skeleton length and
tortuosity. Branches less than 4μm in length were excluded from the analysis. All analyses were performed in FIJI image analysis software. Arborization parameters were normalized to tissue weight. Additional analyses of whole depots are described in Supplement.

Statistical Analysis

For all animal experiments, mice were randomized to treatment groups to ensure no difference in starting body weight. All plots represent mean +/-SEM. Statistical calculations were carried out in Excel or GraphPad Prism software (La Jolla, CA, USA), utilizing ANOVA, Linear Regression, or Student’s T-test as indications of significance (specified in Figure legends). Gene and protein expression data were normalized to a housekeeper and analyzed by either ANOVA or by Student’s t-test, two-tailed, using Welch’s correction when variance was unequal. Error bars are SEMs. For all figures, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Ethical Statement

All procedures and handling of animals were performed in accordance with the University of Maine’s Institutional Animal Care and Use Committee (IACUC), to comply with the guidelines of the PHS Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals. This study was approved by the University of Maine’s IACUC, under protocol A2017-09-04. Human tissue samples were obtained from the Boston Nutrition and Obesity Research Center (BNORC) and were de-identified prior to being provided to our laboratory. Tissues were collected by BNORC under their IRB-approved protocol.
Data Availability

The datasets generated and/or analyzed during the current study are available from the laboratory’s DataVerse site or by emailing the corresponding author.

Additional Methods can be found in APPENDIX B: Chapter 3 Supplemental Material.

Results

Obese and diabetic mice display subcutaneous white adipose tissue (scWAT) neuropathy

In order to investigate whether obesity/diabetes leads to adipose tissue neuropathy, we used BTBR mice with the ob/ob leptin-deficient mutation (MUT). These animals develop severe obesity, type 2 diabetes, hypercholesterolemia, and insulin resistance, with significantly increased body weight and blood glucose by 8 weeks of age. Although disease progression is slower in females when compared to males, both sexes eventually develop diabetes and peripheral neuropathy, with nerve conductance deficits and intraepidermal nerve fiber loss evident by 9 and 13 weeks, respectively. While this diabetic neuropathy has been well described in skin and paw, the effects on adipose innervation have not been assessed.
By 12 weeks of age, male BTBR MUT mice exhibited the expected increase in body weight when compared to BTBR +/- wild type (WT) mice (Fig. 1a), which was concurrent with increased adiposity (Fig. 1b). In a pilot cohort that also included heterozygous (+/- HET) BTBR mice, only MUT animals displayed a trend for increased body weight and inguinal scWAT weight, for both males and females (Suppl. Fig. S1a). Thus, HET mice were not included in subsequent analyses. Consistent with previous reporting, neuropathy was detectable in BTBR MUT mice around 12 weeks of age via a Von Frey mechanical nociceptive assay (male mice, Fig. 1c; female mice, Suppl. Fig. 1b). This test determines tactile sensitivity in the skin of the hind paw, as an indirect measure of small fiber peripheral neuropathy. These data revealed that male mice displayed a stronger phenotype of peripheral neuropathy of extremities (Fig. 1c compared to Suppl. Fig. 1b), consistent with previous reports.28

The pan-neuronal marker protein gene product 9.5 (PGP9.5, also known as ubiquitin C-terminal hydrolase L1 (UCHL-1)), was also greatly reduced in inguinal scWAT of MUT mice (Fig. 1d; Suppl. Fig. S1c), indicating a loss of total nerve supply. BTBR MUT mice also displayed a marked reduction in sympathetic activation in inguinal scWAT as evidenced by a substantial decrease in tyrosine hydroxylase (TH) protein levels (Fig. 1e, Suppl. Fig. S1d). TH is the rate-limiting enzyme in catecholamine biosynthesis, including norepinephrine, the main sympathetic nerve neurotransmitter in WAT, and an indicator of SNS activation. After 12 weeks of age, the loss in total inguinal scWAT innervation remained consistent in MUT mice (Fig. 1f), but TH continued to decrease in MUT mice aged to 24 weeks (Fig. 1g). As loss of synaptic connections is another important factor in peripheral neuropathy, we measured protein expression of the synaptic protein
postsynaptic density 95 (PSD95), and observed a reduction in PSD95 in 12-week-old MUT mice when compared to WT (Fig. 1h). PSD95 protein levels were also further decreased in 24-week-old MUT animals (Fig. 1h).

Because the energy expending interscapular brown adipose tissue (iBAT) is known to be highly innervated, we examined iBAT innervation in the BTBR mice. Protein expression of PGP9.5 revealed a significant decrease in total innervation of iBAT in BTBR MUT animals when compared to littermate controls (Fig. 1i). These finding were concurrent with a decrease in protein expression of TH in the iBAT of BTBR MUT mice indicating decreased sympathetic activation (Fig. 1j). iBAT gross morphology also revealed a marked increase in lipid accumulation, also called “whitening”, in both male and female mutant BTBR mice, but most prominently in male mice that exhibited the stronger neuropathy phenotype (Suppl. Fig. S1f). For BTBR MUT mice, iBAT gene expression showed a significant decrease in Ucp1, whose activation by the SNS is necessary for thermogenesis to occur, along with reductions in the brown adipocyte markers Cidea and Dio2, despite no decrease in synaptic markers (Synapsin I, Synapsin II, Synaptophysin, Psd95), or other indicators of nerve health such as the Schwann cell marker Sox10 and neurotrophic factor brain derived neurotrophic factor (Bdnf) (Suppl. Fig. S1g). The gene expression pattern in inguinal scWAT of BTBR MUT was opposite of what we observed in iBAT and included a coordinated trend for reduced expression of neural markers in MUT animals (Suppl. Fig. S1h). In peri-renal (pr) WAT, another WAT depot that readily undergoes browning, the gene expression pattern was similar to that of iBAT (Suppl. Fig. S1i), which is perhaps not surprising considering the higher similarity of prWAT to BAT compared to scWAT. Together, these data indicate depot-specific
differences related to innervation, and suggest that different mechanisms may be involved in maintaining nerve health in different adipose depots.

To determine whether peripheral neuropathy extended beyond adipose in BTBR MUT mice, we examined the neuromuscular junctions (NMJ). Immunofluorescent staining of NMJs revealed that BTBR MUT mice had fewer fully occupied NMJs in both the medial gastrocnemius (MG) and soleus (SOL) muscles when compared to WT littermates, accompanied by an increase in partially occupied junctions (Suppl. Fig. S2a-b). As there was no difference in unoccupied NMJs, neurodegeneration at the NMJ may be a slower neurodegenerative process with obesity and diabetes than in skin and underlying adipose.

**White adipose tissue from obese humans exhibits neuropathy**

Based on our findings in the BTBR mouse model we sought to determine if neuropathy exists in adipose tissue of obese humans. We investigated degree of innervation in human scWAT (and omental WAT) obtained from individuals who underwent elective surgery. Protein expression of the pan-neuronal marker PGP9.5 revealed a decreasing trend with increasing BMI in human scWAT adipose tissue (Fig. 1k), despite no difference in adipocyte diameter (Suppl. Fig. S3a, left panel), indicating that adipose neuropathy also exists in human tissues. Since we were unable to obtain an entire WAT depot from humans, in order to determine if innervation status was confounded by cell size, adipocyte diameter was quantified for the human samples and revealed no differences that may confound these analyses. In addition, for all innervation analyses, western blots are always controlled by equal protein loading for each sample.
and normalization to a housekeeping protein. For mouse, entire inguinal scWAT depots were homogenized to analyze the total level of innervation, however, this was not possible for human samples as we could only obtain a small biopsy. Therefore, normalized protein expression was plotted against average cell size for each corresponding sample, which showed no correlation (Suppl. Fig. S3b). Interestingly, post-translational modification of PGP9.5 appears to be restricted to scWAT of humans (Fig. 1k-l) since we did not observe multiple band sizes in any of our mouse models.

We also assessed degree of innervation in human omental adipose tissue, and found no significant difference in innervation with increasing BMI (Suppl. Fig. S3c), suggesting that adipose neuropathy may be restricted to scWAT depots. This fits with our data from mouse as well. However, due to the limited number of omental adipose samples available, along with the narrow range in BMI, further assessments may be warranted to confirm this observation. Nevertheless, these data show that human adipose tissue undergoes neuropathy with obesity similar to the mouse model.

**Aging leads to white adipose tissue neuropathy in humans and mice**

Since adiposity increases with age concordant to increased risk of developing type 2 diabetes, we sought to determine if there is a link between adipose neuropathy and aging. When investigating the relationship between adipose innervation and age in humans, linear regression analysis revealed a significant decrease in protein levels of the pan-neuronal marker PGP9.5 with increasing age in inguinal scWAT (Fig. 1l) despite no difference in adipocyte size with no correlation between average cell size and protein
expression (Suppl. Fig. S3a-b, right panels). Again this finding did not extend to omental WAT (Suppl. Fig. S3d).

To further explore this relationship, we employed an aged mouse model. Male C57BL/6J mice were aged to 16 months, and the state of their adipose innervation was compared to young mice at 10-12 weeks old. Protein expression of PGP9.5 in inguinal scWAT was decreased in 16 month old aged mice when compared to young mice at 10-12 weeks of age (Fig. 2a), together with a trend for decreased TH levels (Fig. 2b). Consistent with the metabolic consequences of aging, total body mass of aged mice was significantly greater than that of young animals, however, there was no significant difference in quadriceps muscle weight or adipose depot weight, although inguinal scWAT and perigonadal (pg)WAT depot weight did display a trend to be higher at this age (Suppl. Fig. S4a).

Since a state of chronic inflammation increases tissue dysfunction and is known to damage nerves, we measured levels of anti-inflammatory cytokines which are involved in inflammatory resolution in young and aged sedentary mice. A non-significant reduction in the anti-inflammatory cytokines Interleukin 13 (Il13), Interleukin 4 (Il4), but not Interleukin 10 (Il10), was observed in the 16 month old mice when compared to younger mice in the sedentary state (Fig. 2c), suggesting an altered inflammatory homeostasis in aged adipose tissue. Gene expression for presynaptic proteins (Synapsin I and II), the neurotrophic factor Bdnf, which supports growth and survival of nerves, as well as the Schwann cell marker Sox10, showed a coordinated trend for reduction in aged mice as well (Fig. 2c).
Considering that nerves and vasculature are often linked, we measured gene expression of vascular markers (Cd31, Vegfa) in young and aged sedentary mice and saw no difference with respect to age (Fig. 2c). We took advantage of vascular autofluorescence to visualize adipose blood vessels in combination with immunofluorescent staining of nerves with a new whole-depot 3D microscopy technique developed in our lab (see Supplemental Methods Section). This imaging revealed that aged mice exhibit an overall decrease in innervation with a striking loss of innervation around vasculature within their inguinal scWAT (Fig. 2d). In young mice, autofluorescent blood vessels (red/orange) are clearly shrouded by fine PGP9.5-expressing nerves (green, Fig. 2d, left panels), but by 16 months of age, the adipose vasculature shows a striking loss of this neuronal sheathing (Fig. 2d, right panels). Taken together, these data reveal that loss of proper adipose innervation may precipitate aging-related metabolic dysfunction.

**Exercise attenuates age-related adipose neuropathy**

Exercise has been shown to increase circulating levels of the nerve survival factor BDNF. We therefore exposed young and aged mice to voluntary wheel running for 7 days and assessed the status of their adipose innervation. Two-way ANOVA analysis of gene expression revealed an interaction of exercise and age only for Synaptophysin, with exercise (p<0.0001) having a stronger effect than age (p=0.0213) (Fig. 2c). Exercise had an overall effect on the expression of Bdnf (p=0.0008), Synapsin II (p=0.0008), and Psd95 (p=0.0003) in axillary scWAT, and resulted in increased expression in both young and aged animals, suggesting an improvement in innervation (Fig. 2c). This occurred despite no changes in total body mass or adiposity before and after the short exercise intervention.
No difference in anti-inflammatory cytokines IL4, and IL13 was seen after exercise in either young or aged animals (Fig. 2c). Exercise also increased gene expression of vasculature markers Cd31 and Vegfa, but only in young animals (Fig. 2c), indicating that physical activity can increase both vascular and nerve supply to adipose, but perhaps is blunted with advancing age.

Protein levels of both PGP9.5 and TH in inguinal scWAT of young exercised mice were significantly increased when compared to young sedentary mice (Fig. 3a-b), indicating that neurite outgrowth has likely taken place, as well as sympathetic nerve activation. Exercise also resulted in a trend for increased protein expression of PGP9.5 and TH in aged animals (Fig. 3c-d), although this did not reach significance, further supporting a blunted effect with age. Direct comparison of the effect of exercise on inguinal scWAT innervation of young versus aged animals revealed no significant difference in response based on age, as both young and aged mice showed relatively similar levels of PGP9.5 and TH expression (Suppl. Fig. S4b-c). The effects of exercise on adipose tissue did not extend to BAT. Protein expression of PGP9.5, TH, and PSD95 in BAT did not differ between young sedentary and exercised groups (Suppl. Fig. S4d-f), nor did gene expression of nerve and synaptic markers (Suppl. Fig. S4g).

Exercise did not appear to have a specific effect on the vasculature innervation, but the levels of PGP9.5 around vasculature had high intra-individual variability (Fig. 3e). To further probe the effects of exercise on vascular innervation, a separate cohort with only young animals was subjected to 7 days of voluntary wheel running as described above. As observed previously (Suppl. Fig. S4a), there was no change observed in body weight, or adiposity between young sedentary and exercised animals (Suppl. Fig. S5a).
Consistent with other reports\textsuperscript{37,38} there was also no difference in skeletal muscle mass for either sedentary or exercised animals after 1 week of voluntary wheel running (Suppl. Fig. S5a). There was a trend for increased protein expression of the synaptic marker PSD95 in axillary scWAT (Suppl. Fig. S5b), consistent with the previously observed (Fig. 2c) increased gene expression of \textit{Psd95} in this depot in response to exercise. Although gene expression of innervation markers in inguinal scWAT was not as pronounced as in the axillary depot (Fig. 2c), there was an increased trend for Schwann cell marker \textit{Sox10}, neurotrophic factor \textit{Bdnf}, and post-synaptic marker \textit{Psd95} (Suppl. Fig. S5c). Innervation of blood vessels was assessed by immunostaining of PGP9.5 was combined with isolectin staining of vasculature in inguinal scWAT. Innervation of blood vessels that measured 50 µm or more in diameter was evaluated (Suppl. Fig. S5d-e). We found no difference in either percentage of blood vessels innervated (Suppl. Fig. S5a, left panel) or number of nerves per blood vessel (Suppl. Fig. S5a, right panel), between sedentary and exercised groups.

In order to determine changes to sensory nerves innervating WAT, we assessed gene expression in the T13-L1 dorsal root ganglion (DRG), which is known to innervate inguinal scWAT\textsuperscript{39,40} (Fig. 3f). The following neuropeptides were measured with or without exercise intervention: calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), pituitary adenylate cyclase-activating polypeptide (PACAP), pro-opiomelanocortin (POMC), proenkephalin (PENK), substance p (Tac1), and vasoactive intestinal peptide (Vip). We found that mRNA expression of \textit{Npy} was decreased in exercised mice (Fig. 3f). The effect of exercise on \textit{Cgrp} expression was highly variable, but linear regression showed that this neuropeptide increased with relation to amount of running the animal.
performed (Fig. 3g), which fits with reports of increased Cgrp expression in neurons of the T13/L1 ganglia upon inguinal scWAT cold induced ‘browning’.  

**Adipose tissue undergoes remodeling with cold exposure**

In addition to exercise as a means to promote adipose tissue neural plasticity, cold exposure has been demonstrated to increase TH+ nerve fibers in murine adipose tissue.  

Similarly, we also found that cold stimulation also increased adipose tissue innervation, as protein levels of PGP9.5 went up in scWAT after only 3 days of cold exposure compared to tissues from mice housed at room temperature or thermoneutrality (Fig. 4a). To examine the adipose nerve network under this innervation promoting condition, adult mice maintained at room temperature (RT) were compared to littermates that were either cold exposed for 10 days or cold exposed for 10 days and rewarmed for 1 week. Inguinal scWAT depots were immunostained with the pan-neuronal marker β-3-tubulin. At room temperature, a diffuse pattern of innervation can be discerned (Fig. 4b, left panel), with larger nerve bundles converging around the subilia lymph node (white arrow), similar to the staining pattern we observed with the other pan-neuronal marker, PGP9.5. The posterior end (right of subiliac lymph node in image) of the tissue did not appear to have as much innervation as the anterior portion (left of subiliac lymph node in image) or as the area surrounding the subiliac lymph node.

After cold exposure, a distinct change in the neural arborization pattern was observed (Fig. 4b, middle panel), particularly an increase in intensity around the lymph node. Since these 2D representations of 3D data do not convey accurate changes in neurite density, tissue z-stacks were analyzed for mean innervation density (calculated
as total axon length normalized to tissue weight) and this revealed a trend for increased innervation density in the 10 day cold exposed group compared to RT and rewarmed groups (Fig. 4b, right panel). However, taking an average of the entire tissue (as we did in Fig. 4b quantifications) blunts regional anatomical differences in innervation density, which we can observe when qualitatively assessing the tissues. This can also be seen when looking at the 2D representations in Fig. 4b, where the anterior and posterior portions display differences in innervation status after cold.

Of note, average innervation density per inguinal scWAT depot totaled up to 25 meters per tissue depot, underscoring the great density of nerve fibers contained in WAT depots. Furthermore, the inguinal scWAT depot can be considered to have 3 distinct anatomical regions, as illustrated in Fig. 4c. Region 1 is the anterior side of the depot and contains major branches of the thoracoepigastric vein (TEV) (Fig. 4c, blue lines/arrows); the subiliac drainage lymph node, often used as an orientation landmark in inguinal scWAT, is located in Region 2, as is the superficial caudal epigastric artery (SCEA, red lines/arrow); Region 3 is the posterior end of the tissue with smaller vasculature including branches of the common iliac vein. From the anterior to the posterior side of the tissue and travelling through the subiliac lymph node is a large branching network of nerves which we have called the subiliac transverse nerves (SiTN) (Fig. 4c, green lines/arrows). These nerves were present in the inguinal scWAT regardless of age, exercise intervention, or cold exposure, and travelled in parallel to the main vasculature in the depot. It is important to note that these subiliac transverse nerves are not the only nerves found within the inguinal adipose depot. Similar to previous observations in BAT\textsuperscript{42-44} and WAT\textsuperscript{13} thin nerve fibers extend throughout the inguinal adipose tissue. These nerves have
been observed running along vasculature, extending throughout the parenchymal space
and wrapping around individual adipocytes, and sympathetic nerve synapsing on
adipocytes has been recently proposed as the means of leptin driven lipolysis in scWAT\textsuperscript{15}

In separate experiments, we mimicked cold exposure by delivering the beta-3
adrenergic agonist CL 316,243 (‘CL’) to wild type mice, and measured secretion of the
neurotrophic factor BDNF from scWAT tissue explants. This approach revealed a
significant increase in BDNF secretion after 10-14 days of CL treatments (Fig. 4d),
supporting the notion that cold stimulation increases BDNF secretion from adipose, while
neuropathic states reduce BDNF expression in adipose. Increased hypothalamic BDNF
expression was previously shown to affect energy expenditure by increasing sympathetic
drive to scWAT, inducing browning of the depot and decreasing depot mass\textsuperscript{45}. To test
whether BDNF was capable of promoting these effects directly in the periphery by
increasing scWAT innervation, we used adeno-associated virus (AAV) to deliver BDNF
directly to the inguinal scWAT of BTBR MUT male mice at 16 weeks of age, when adipose
neuropathy is prominent. The AS/Rec2 dual cassette vector used to deliver the BDNF
transgene, transduces adipose tissue efficiently while restricting off-target transduction in
liver\textsuperscript{46}. Animals received a bolus injection of 1x10\textsuperscript{10} vg of AAV-BDNF in one inguinal
scWAT depot and a vehicle injection in the other inguinal scWAT. After two weeks, protein
expression of PGP9.5 was significantly increased in the fat pads that received virus
compared to the vehicle treated depots (Fig. 4e). No difference was seen in protein
expression of TH or PSD95 following AAV-BDNF treatment (Suppl. Fig. 6a-b) for this
dose and duration. Taken together, these data suggest a role for BDNF in promoting total
innervation on inguinal scWAT.
**Extent of adipose innervation is depot, but not sex, specific**

To determine whether a difference in adipose innervation between depots and sexes exists in healthy animals, 16 week old male and female control mice were cold exposed for 3 days. No difference between sexes was observed for sympathetic activation (TH) or synapses (PSD95) in inguinal scWAT and iBAT (Suppl. Fig. S7a-b), however, both TH and PSD95 protein expression were much greater in iBAT than inguinal scWAT. Similarly, protein expression of PGP9.5 and TH in axillary and inguinal scWAT depots was comparable between sexes (Suppl. Fig. S7c-d). However, while PGP9.5 expression remained the same between the depots (Suppl. Fig. S7c), axillary scWAT exhibited significantly more protein expression of TH than inguinal scWAT (Suppl. Fig. S7d), which correlates to the ‘browning’ potential of this particular WAT depot in comparison to inguinal WAT.  

Since we had seen decreases in BDNF in both obesity and age-related inguinal scWAT neuropathy (Fig. 2c, Suppl. Fig. S1h), an increase in BDNF in response to noradrenergic stimulation (Fig. 4d), and observed the ability of BDNF to increase total innervation as evidenced by AAV-BDNF delivery to inguinal scWAT (Fig. 4e), we decided to investigate whether there were sex differences in BDNF expression in inguinal scWAT. After a 3-day cold exposure, adult (16 weeks old) female mice (C57BL/6J) showed a much greater level of BDNF expression in inguinal scWAT than male mice, as measured by multiplex ELISA (Suppl. Fig. S7e). This fits with the apparent protection from neuropathy in female BTBR ob/ob mice (Suppl. Fig. S1b and 28), despite females having no difference in total innervation versus males (Suppl. Fig. S7), providing the possibility
that females have more adipose-secreted BDNF that maintains nerve integrity in neuropathic conditions.

Discussion

Our data have demonstrated that under certain pathophysiological conditions, including aging, obesity and diabetes, WAT from humans and mice does not maintain proper innervation and undergoes a process of nerve death that we call ‘adipose neuropathy.’ While the exact neuropathy phenotypes differed slightly between these metabolic conditions, the underlying theme was decreased adipose tissue health. In all cases, the neuropathy was accompanied by a loss of synaptic markers and a reduction in the local expression of the neurotrophic and nerve survival factor BDNF. These new findings have implications for the treatment of metabolic diseases, in that re-innervating adipose tissue may be required to properly regain metabolic control. Therapeutic interventions that act to support neurite outgrowth and synapse formation on adipocytes and stromovascular cells may assist with the efficacy of glucose-lowering drugs or diet and exercise interventions. In this study, we have found a promising novel treatment by locally delivering scWAT with AAV-BDNF, which appeared to succeed in re-innervating the tissues of diabetic mice. How this increase in innervation improves metabolic parameters in obesity is the focus of ongoing research.

Interestingly, while diet and exercise are often prescribed in concert, despite exercise not being a very effective means to reduce appetite, the myriad health benefits of exercise may help to bolster additional weight loss strategies. This may be in part by
mediating increased peripheral nerve plasticity, including in adipose depots, as we have demonstrated here. Furthermore, we have also demonstrated that cold exposure similarly boosts peripheral nerve plasticity in adipose, and may also be a strategy to enhance diet and exercise-based weight loss interventions, and help prevent obesity related adipose tissue neuropathy.

We have demonstrated that both mouse and human scWAT undergo neuropathy with aging and diabetes/obesity, and these data provide evidence that peripheral neuropathy is not restricted to classical tissues such as epidermis and distal limbs. Adipose tissue nerves are known to be essential for energy-expending processes such as lipolysis and thermogenesis, and loss of a proper nerve supply can have serious detrimental effects on metabolic control, which may exacerbate or initiate an insulin resistant state. Additionally, as recently demonstrated, leptin stimulated lipolysis is mediated at least in part by sympathetic innervation of scWAT, further underscoring how adipose neuropathy can contribute to metabolic dysfunction through obesity-induced leptin resistance.

In all pathophysiological states we studied, lack of adipose innervation was associated with one or more of the following: an increase in adipose depot size, loss of proper adipose tissue function and metabolic control, or altered cytokine inflammatory markers. These changes in WAT may be due to loss of synapses on adipocytes themselves, or on stromovascular cells resident in WAT, including the macrophages and other immune cells that are important for proper tissue function. Indeed, macrophages are known to express adrenergic receptors, and may also become denervated (neuropathic) in the models described here. Neuropathy appeared to be most severe in
the obese and diabetic BTBR ob/ob mice, which displayed additional lack of synaptic integrity in the neuromuscular junction and BAT, as well as in skin and underlying adipose.

In the aged mouse model, most striking was the loss of innervation around the vasculature of WAT, without any apparent deficits to the vasculature morphology or to vascular markers such as CD31 and VEGFa (at least at the mRNA level). However, these markers cannot attest to the functionality of adipose vasculature, which was not assessed here. Aging is known to cause alterations in the structure and function of vasculature and leads to decreased basal limb blood flow, which may result from loss of nerve supply to the blood vessels, but to our knowledge this has not yet been investigated. Exercise was able to at least partially restore the innervation around the adipose vasculature in the aged mice. Thus, exercise may be important for prevention of aging-related diabetes by maintaining adipose innervation and metabolic health, similarly to how exercise induces neural plasticity in the brain.

Beyond vasoregulation, it is still unclear exactly how nerves and blood vessels interact and whether their plasticity is functionally linked, and this warrants further investigation. Vasculature within the adipose tissue promotes metabolic health by preventing adipose tissue hypoxia and subsequent inflammation, and provides endocrine communication between adipose tissue and the rest of the body. Nerves also rely on vasculature for sustained health, since they become damaged in a hypoxic state, while vasculature relies on innervation for constriction and vasodilation of blood vessels. Since nerves, such as those in the sympathetic nervous system, regulate vascular control (i.e. vasoconstriction) and may also be involved in angiogenesis, adipose neuropathy may also have adverse effects on endocrine system communication with adipose tissue and
may lead to adipose hypoxia due to lack of proper vascular supply\textsuperscript{51}. The association here could also go in the other direction, with circulating factors affecting nerve supply (ie: glucose, lipids, hormones) – but these hypotheses require closer investigation.

Synaptic markers and the neurotrophic factor BDNF were reduced with adipose neuropathy, fitting with the loss of pan-neuronal protein expression in neuropathic WAT depots. Neurotrophic factors, such as BDNF, are important mediators of neural plasticity, essential for nerve survival and growth, neurite outgrowth and branching, and synaptogenesis. Loss of neurotrophic signaling can prompt neurite retraction, axonal degeneration, and nerve death. It is well known that exercise often leads to an increase of BDNF in the brain\textsuperscript{52,53} and in the peripheral circulation\textsuperscript{36} and that this improves health outcomes in patients with neurodegenerative diseases such as Parkinson’s\textsuperscript{54}. It has been shown that BDNF is expressed in adipose tissue, but it’s role there is vastly understudied\textsuperscript{55}. Nerve growth factor (NGF) has also been reported as a neurotrophic factor expressed in adipose tissue\textsuperscript{56} but until recently its role in adipose tissue had been unclear. Cao et al. recently argued that cold induced synaptic plasticity in scWAT is dependent on adipose derived NGF\textsuperscript{17}. Other neurotrophic factors are expressed in adipose depots but their functional significance is not currently known. Here we present a case for a potential role of BDNF in maintaining WAT peripheral nerve innervation. This idea is strengthened by the efficacy of AAV-BDNF treatments to scWAT and the striking increase in adipose BDNF in response to exercise, a known neurogenic intervention in the brain as well\textsuperscript{50}.

We and well as others\textsuperscript{17,31,41} have shown that noradrenergic stimulation via cold exposure increased nerve density in scWAT. Previously reported increases in sympathetic nerve fiber density in response to cold stimulation have been correlated to
areas of increased browning within the adipose tissue. Catecholamines elicit differential effects when signaling through α- or β-adrenergic receptors (AR) in adipose. ARs belong to the family of G-protein coupled receptors (GPCRs); the five ARs active in white and brown adipose are α-1 AR (ADRA1), α-2 AR (ADRA2), and β1-, β-2, β-3, AR (ADRB1/2/3). ADRB3 is the predominate βAR present in rodent adipose, through which sympathetic nerve released neurotransmitter, NE acts. βAR mediated signaling activates lipolysis through stimulating activity of adenylyl cyclase, thus increasing intracellular cyclic adenosine monophosphate (cAMP), which in turn stimulates cAMP dependent protein kinase activity that activates hormone sensitive lipase (HSL) by phosphorylation. By contrast, ADRA2 mediated signaling promotes adenylyl cyclase inhibition leading to antilipolytic effects.

Although catecholamines have a higher affinity for αARs than βARs, physiological effects of catecholamine signaling through ARs is largely determined by receptor abundance which varies between adipose depots. Catecholamine residence in peripheral tissues has been observed under certain disease states, although the mechanisms behind its occurrence have not been fully understood. In obese adipose tissue lipolytic catecholamine resistance has been associated with a decrease in βARs in adipose of human females, and has been attributed to decreased expression of βARs on fat cells, as well as increased sensitivity to ADRA2 signaling. There may be a similarity between lipolytic catecholamine resistance and insulin or leptin resistance, where receptors are no longer properly transducing the ligand signals.

A recently discovered subset of macrophages present in scWAT interacts with sympathetic nerves and degrades NE. These sympathetic nerve associated
macrophages or "SAMs" increase in abundance in obesity\textsuperscript{64} and their increased activity of NE degradation can be seen as a type of AR ligand sequestration, possibly contributing to lipolytic catecholamine resistance in obesity. NE degrading macrophages have also been reported to increase in aging,\textsuperscript{65} and aging is associated with decreased lipolysis in adipose tissue. These reports, combined with our findings presented here, all likely contribute to reduced lipolysis under the obese state. It is unknown whether loss of innervation leads to decreased βARs in increased NE degrading macrophages in adipose tissue, or visa verse, and this requires further investigation. However, lipolysis can also be affected by non-adrenergic signaling as recently reviewed\textsuperscript{66} and should be considered. Purinergic (via adenosine and ATP) signaling in adipose produces similarly diverse effects as NE signaling and is dependent on specific receptor activation. Adenosine appears to be a negative modulator of lipolysis in WAT, yet has pro-lipolytic activity in BAT.\textsuperscript{66} Other neurotransmitters and neuropeptides are expressed in adipose tissues, such as, NPY, Substance P, CGRP and others\textsuperscript{51}. It is currently unknown what role, if any, these play in lipolysis.

Finally, we have revealed that iBAT and scWAT differ in their patterns of innervation and response to neuropathic stimuli, and that male and female mice differ in scWAT innervation with a blunted response to neuropathy and higher levels of BDNF. Using a new technique for whole-depot imaging of adipose innervation, we have also revealed a consistent pattern of nerves in inguinal WAT (traversing the subiliac lymph node), which changes regionally in response to cold stimulation.

Taken together, we have demonstrated for the first time that adipose tissue nerves are able to undergo neuropathy in pathophysiological situations but can also undergo
neural plasticity in response to exercise or cold exposure. We have also identified BDNF as a locally produced peripheral nerve survival factor in WAT, which can therapeutically improve adipose neuropathy of diabetes. Changes in adipose innervation status were correlated with altered metabolic control and underscore the importance of adipose nerves for proper metabolic health.
Figure 1

a. **Body Weight**

![Graph showing body weight comparison between WT and ob/ob MUT.

b. **scWAT/Body Weight**

![Graph showing scWAT/body weight comparison between WT and ob/ob MUT.

c. **Von Frey Analysis**

![Graph showing von frey analysis for WT and ob/ob MUT.

d. **Inguinal scWAT PGP9.5**

![Graph showing PGP9.5 expression in inguinal scWAT.

e. **Inguinal scWAT TH**

![Graph showing TH expression in inguinal scWAT.

f. **Inguinal scWAT PGP9.5**

![Graph showing PGP9.5 expression in inguinal scWAT.

g. **Inguinal scWAT TH**

![Graph showing TH expression in inguinal scWAT.

h. **Inguinal scWAT PSD95**

![Graph showing PSD95 expression in inguinal scWAT.

i. **iBAT PGP9.5**

![Graph showing PGP9.5 expression in iBAT.

j. **iBAT TH**

![Graph showing TH expression in iBAT.

k. **Human scWAT PGP9.5**

![Graph showing PGP9.5 expression in human scWAT.

l. **Human scWAT PGP9.5**

![Graph showing PGP9.5 expression in human scWAT.

---

**Figure 1**

**a. Body Weight**

- Graph showing body weight comparison between BTBR WT and BTBR ob/ob MUT.

**b. scWAT/Body Weight**

- Graph showing scWAT/body weight comparison between BTBR WT and BTBR ob/ob MUT.

**c. Von Frey Analysis**

- Graph showing von frey analysis for WT and BTBR ob/ob MUT.

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- Graph showing TH expression in iBAT.

**k. Human scWAT PGP9.5**

- Graph showing PGP9.5 expression in human scWAT.

**l. Human scWAT PGP9.5**

- Graph showing PGP9.5 expression in human scWAT.
Figure 1. Obesity and diabetes led to white adipose tissue neuropathy. The BTBR ob/ob (MUT) model of obesity and diabetes was compared to BTBR +/+ wild-type (WT) for body weight measurements (a), and adiposity (b). Body and tissue weight data were analyzed by two-tailed Student’s T-Test. Von Frey tactile alldynia analysis was performed on MUT and WT animals to determine onset of peripheral neuropathy (c). Von Frey data was analyzed by ANOVA with Sidak’s post hoc test. For (a-c), all males; WT N=8, 12-20 weeks old; MUT N=6, 12-24 weeks old. Protein levels of PGP9.5 (d), as well as TH (e) in inguinal scWAT of the MUT and WT mice were measured by western blotting. For (e), lane 5 was excluded from analyses due to uneven resolution of housekeeper. For (d-e), all males; WT N=5, 12-20 weeks old and MUT N=4, 12-20 weeks old. Protein expression of PGP9.5 (f), TH (g) and PSD95 (h) in inguinal scWAT of 12-25 weeks old WT, 12 week old MUT and 24-28 week old MUT was measured by western blotting. All males, N=3 per group. Protein expression of PGP9.5 (i) and TH (j) in iBAT was measured in MUT mice and compared to WT littermate controls; for (i-j) all 12 week old males, N=3 WT/MUT. For all western blots, data were normalized to housekeeper proteins β-tubulin or cyclophilin B, band intensities were quantified in Image J, and analyzed using a two-tailed Student’s T-Test. Error bars are SEMs. In human scWAT, protein levels of pan-neuronal marker PGP9.5 were measured by western blotting; linear regression analysis was performed for assessment of normalized protein levels compared to body mass index (BMI) (k) or age (25-38 years old) (l). For (k-l) N=9 BMI cohort, N=9 Age cohort. For (k-l) * indicates individuals with diagnosed diabetes. The majority of human samples were females, see Suppl. Table S1-S3 for clinical details. Western blot data were normalized to β-actin, band intensity were quantified in Image J, and analyzed by two-tailed Student’s T-Test. Error bars are SEMs, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 2

a. **Inguinal scWAT**

**PGP9.5**

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p = 0.0031

**β-Actin**

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b. **Inguinal scWAT**

**TH**

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p = 0.2914

**Cyclophilin B**

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c. **Gene Expression of Axillary scWAT**

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# = Aged Sed v Aged Run
¥ = Young Sed v Young Run
$ = Young Run v Aged Run


d. **Immunofluorescent Staining of Inguinal scWAT**

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Figure 2. Aging is associated with adipose tissue neuropathy. In young (10-12 weeks old) and aged (16 months old) sedentary male C57BL/6J mice, western blotting was used to measure protein expression of PGP9.5 in inguinal scWAT for assessment of total innervation (a), while TH was used to measure sympathetic activation (b). Protein expression was normalized to either β-actin or cyclophilin b, band intensity was quantified in Image J and analyzed by two-tailed Student’s T-Test; N=4 for young and aged groups. Gene expression of anti-inflammatory markers *Il4 and *Il13, *Il10, neurotrophic factor *Bdnf, synaptic markers (*Synapsin I and II, *Synaptophysin, *Psd95), as well as vascular markers (*Cd31, *Vegfa) was measured by qPCR in axillary scWAT of young and aged mice (c). For young and aged sedentary animals N=4, for young and aged exercised (run) mice, N=5 per group. Gene expression data expressed as fold change normalized to the housekeeping gene *Cyclophilin, was analyzed by two-way ANOVA, with Uncorrected Fisher’s LSD post hoc test, and significance denoted with * for young sedentary versus aged sedentary; # for aged sedentary versus aged run; ¥ for young sedentary versus young run; and $ for young run versus aged run. Whole depot immunofluorescent imaging of inguinal scWAT from sedentary animals for total innervation (PGP9.5 in green) and vasculature (red/orange; autofluorescence) was performed (d). Images captured at 10x and are representative of N=4 mice analyzed per group. Error bars are SEMs, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 3

a. Inguinal scWAT PGP9.5
Young Exercised

p=0.037

PGP9.5
β-Tubulin

Sedentary Run

b. Inguinal scWAT TH
Young Exercised

p=0.0451

TH/Cyclophilin B

Sedentary Run

c. Inguinal scWAT PGP9.5
Aged Exercised

p=0.2041

PGP9.5
β-Actin

Sedentary Run

d. Inguinal scWAT TH
Aged Exercised

p=0.1712

TH/β-Actin

Sedentary Run

e. Immunofluorescent Staining of
Inguinal scWAT Following Exercise

f. Gene Expression of T13-L1 DRGs
Young Exercised

Sed Run

Cgrp Npy Pacap Pomp Penk Tac1 Vip

g. Cgrp Fold Change Correlated to
Running Distance

R² = 0.9094

p=0.0119

km Fold Change
Figure 3. Exercise increases adipose innervation in young mice and attenuated loss of age-related adipose innervation. Young (10-12 weeks old) and aged (16 months old) male C57BL/6J mice were placed in running-wheel cages for 7 days with continuous access to a running wheel (run). To assess exercise effects on adipose innervation in young mice, protein expression in inguinal scWAT was measured by western blotting with PGP9.5 as a marker of total innervation (a), and TH as an indicator of sympathetic activation (b). Protein expression was normalized to either β-tubulin or cyclophilin b, band intensity was quantified in Image J and analyzed by two-tailed Student’s T-Test; N=4 for young sedentary and N=5-6 for exercised groups. Protein expression of PGP9.5 (c) and TH (d) in inguinal scWAT of aged sedentary and exercised mice was also determined by western blotting. Protein expression was normalized to β-Actin, band intensity was quantified in Image J and analyzed by two-tailed Student’s T-Test; N=4 for aged sedentary animals, N=5 for aged exercised (run) animals. Whole depot nerve and vasculature imaging of inguinal scWAT from exercise (run) animals was performed by combining immunostaining for PGP9.5 (green) with autofluorescence of vasculature (red/orange); images were captured at 10x (e). Images are representative of N=5 mice analyzed per group. Young (12-15 weeks old) male C57BL/6J mice were placed in running-wheel cages for 7 days with continuous access to a running wheel (run), control (sed) animals were placed in cages with a locked running wheel. Gene expression of neuropeptides in the T13-L1 DRGs was measured by qPCR, expressed as fold change normalized to the housekeeping gene Cyclophilin, and analyzed by Student’s T-test (f). Linear regression analysis was performed for Cgrp to assess the effect of running on changes in gene expression (g). Error bars are SEMs, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 4

a. Inguinal scWAT PGP9.5 Cold Exposed

![Graph showing PGP9.5 levels at 30°C, 23°C, and 4°C.]

b. Inguinal scWAT β-3-Tubulin Cold Exposed

Room Temp 10 days 5°C

![Images showing β-3-Tubulin expression at room temp and 5°C for 10 days.]

Mean Innervation Density

![Bar graph showing mean innervation density at different conditions.]

c. Regional Anatomy of the Inguinal scWAT

![Diagram illustrating regional anatomy with labels for TEV, SCEA, LN, and SITN.]

d. BDNF Secretion from Inguinal scWAT after CL316,243 (ADRβ3)

![Graph showing BDNF secretion levels with fold change for Veh and CL treatment.]

e. Inguinal scWAT PGP9.5

![Graph showing PGP9.5 expression levels for Veh and AAV-BDNF treatment.]

1. TEV
2. SCEA
3. LN
4. SITN
Figure 4. Cold exposure induces adipose nerve remodeling.

Adult (8 week old) wild-type C57BL/6J male mice were either cold exposed (at 5°C), maintained at room temperature, or at thermoneutrality (30°C) for 3 days. Changes in innervation were assessed by measuring protein expression of a pan-neuronal marker (PGP9.5) in inguinal scWAT via western blotting (a). Protein expression was normalized to β-actin, band intensity was quantified in Image J and analyzed by two-tailed Student’s T-Test; N=4 per group. In a separate experiment, adult (18-22 week old) male control male mice on a mixed genetic background were maintained either at room temperature (RT), at 5°C for 10 days, or at 5°C for 10 days and then returned to RT for 1 week (rewarmed). Entire depots were immunostained with β3-Tubulin and imaged on a Leica TCS SP8 or DMI6000 confocal microscope by tiling z-stacks through the entire depth of tissue (b). For quantification of arborization (b, right panel), tiles were individually Z-projected, background subtracted, thresholded into binary images, and skeletonized. Branches less than 4μm in length were excluded from the analysis. White arrows point to subiliac lymph node, red arrows indicate branches of the thoracoepigastric vein (TEV); anterior side (A) is left of subiliac lymph node, posterior side (P) is right of subiliac lymph node. Images are representative of N=3 per group, error bars are SEMs. Schematic of inguinal scWAT (c) divided into three anatomically distinct areas: 1. area anterior to subiliac lymph node (LN), 2. LN area, 3. area posterior to LN. Major vasculature is shown with the TEV67 illustrated as blue line and the superficial caudal epigastric artery (SCEA)68 and other vasculature illustrated as red lines. Branches of the subiliac transverse nerves (SiTN) are illustrated as green lines. Adult (12-13 weeks old) male C57BL/6J mice received either daily i.p. injections of ADRβ3 agonist CL316,243 (at 1.0 mg/kg BW), or vehicle (Veh) for 10-14 days; ex vivo secretions (collected at 1hr and 2hr) from inguinal scWAT explants were measured for BDNF by ELISA, analyzed by two-tailed Student’s T-Test, and presented as fold change in picograms (pg)/mL (d). Data is representative of multiple cohorts, N=5 (Veh), N=7 (CL316,243). Error bars are SEMs. 16 week old male BTBR MUT mice (N=3) received single injection of AAV-BDNF (1x10^{10} vg) into their left inguinal scWAT and an equal volume of vehicle into their right inguinal scWAT; protein expression of PGP9.5 in inguinal scWAT was measured by western blotting after 2 weeks (e). Protein expression was normalized to β-tubulin, band intensity was quantified in Image J and analyzed by two-tailed Student’s T-Test; error bars are SEMs.


Chapter 4:

MYELOID-SECRETED BDNF MAINTAINS INNERVATION OF INGUINAL ADIPOSE IN MALE MICE

Graphical Abstract

Myeloid lineage cells regulate nerve remodeling via secretion of BDNF

Abstract

Innervation of adipose tissue is essential for the proper function of this critical metabolic organ. Numerous surgical and chemical denervation studies have demonstrated how maintenance of brain-adipose communication through both sympathetic efferent and sensory afferent nerves helps regulate adipocyte size, cell number, lipolysis, and
‘browning’ of white adipose tissue. Neurotrophic factors are growth factors that promote neuron survival, regeneration and outgrowth of neurites on adult nerves. Peripheral blood immune cells have been shown to be a source of neurotrophic factors in humans and mice. Although a number of these cells also reside in the adipose stromal vascular fraction (SVF) it has remained unclear what role, if any, they play in adipose innervation. We have demonstrated that adipose resident myeloid lineage immune cells secrete brain derived neurotrophic factor (BDNF) and that deletion of this neurotrophic factor from myeloid cells led to a ‘genetic denervation’ of inguinal subcutaneous adipose tissue, thereby causing decreased energy expenditure and increased adipose mass. We and others have previously shown that noradrenergic stimulation via cold exposure increases adipose innervation in the inguinal depot. Here we have identified a subset of myeloid cells that are Cx3cr1+ monocytes/macrophages expressing adrenergic receptors. The quantity of these mobile immune cells increased in adipose tissue upon cold stimulation, fitting with their function to hone to sites of tissue injury, and these cells also expressed BDNF. We propose that these cold induced neuroimmune cells (CINCs) are key players in maintaining adipose innervation as well as promoting adipose nerve remodeling under adrenergic stimuli such as cold exposure.

Introduction

In order for the central nervous system (CNS) to regulate functions of distal organs, peripheral innervation of tissues needs to be maintained. It has been demonstrated numerous times that loss of innervation to the adipose organ leads to dysfunction of the
tissue and disrupts energy homeostasis. Denervation of brown adipose tissue (BAT) greatly impairs the energy expending process of adaptive thermogenesis [1-4], while denervation of white adipose tissue (WAT) results in fat mass accumulation via hyperplasia and impaired lipolysis [5-7]. Furthermore, we have previously shown that nerves in subcutaneous adipose tissue (scWAT) undergo remodeling in response to pathophysiological and environment stimuli [8]. While obesity and aging can lead to dying back (neuropathy) of adipose nerves, exercise and cold exposure served to enhance nerve growth and branching within the scWAT depot [8].

Nerve remodeling is facilitated by neurotrophic factors (NFs) both in the CNS [9] and in peripheral nerves [10]. NFs are nerve growth factors that support nerve health, survival, and plasticity. Brain derived neurotrophic factor (BDNF), is one member of the neurotrophin family of NFs which in mammals also includes nerve growth factor (NGF), Neurotrophin-3 (NT-3), and Neurotrophin-4/5 (NT-4/5). Neurotrophins signal predominantly through Trk receptors on nerves, through which they are endocytosed and, in peripheral nerves, transported in a retrograde manner to the nerve cell body. BDNF has been well studied for its role in hippocampal synaptic plasticity in the adult brain [11-13], as well as learning and exercise related neurogenesis [14]. BDNF is also an important modulator of energy balance through its actions in the hypothalamus [14]. In rats, deletion of Bdnf in the ventromedial and dorsomedial regions of the hypothalamus resulted in an obesity phenotype due to hyperphagic behavior [15]. Obesity is associated with lower serum levels of BDNF in humans, and animal studies have shown that central and peripheral administration of BDNF reduced food intake and hyperglycemia, and increased energy expenditure, via CNS mediated mechanisms [16-18]. As review by Xu and Xie,
2016, (genetic mutations in human BDNF and its receptor TrkB result in morbid early-onset obesity [19]; furthermore genome wide associated studies (GWAS) have identified single nucleotide polymorphisms (SNPs) in or near BDNF to be associated with increased BMI [19]. Despite these strong correlations between BDNF and its receptor TrkB and obesity it is predominately studied in CNS. However, a few studies have shown that NFs, including BDNF are present in adipose tissue [20-22] but the cellular source of BDNF in adipose has not been determined. An adipose specific (Adipoq-Cre) knock-out of BDNF and TrkB, only reduced TrkB expression in adipose, suggesting that mature adipocytes are not the cellular source of BDNF [23]. NFs are secreted by glial cells in the brain [24] and Schwann cells in peripheral nerves [25]. However, other cell types, predominantly immune cells, are also known sources of NFs [26-30].

As part of the immune response to injury immune cells are critical players in wound healing, regeneration, and remodeling of various tissues. They are an important component of the adipose organ where they modulate the inflammatory response, clear tissue of apoptotic cells, and mediate adipose tissue remodeling during obesity through an influx of monocytes (undifferentiated macrophages), neutrophils, T cells, B cells and mast cells [31-36]. Macrophages and their monocyte precursors are myeloid lineage immune cells and comprise the highest fraction of immune cells present in adipose tissue [37]. They are highly heterogeneous cells that are polarized by environmental stimuli to evoke differential responses within a tissue. In a simplistic paradigm, classically activated macrophages (M1) act in a pro-inflammatory manner, while alternatively activated macrophages (M2) produce an anti-inflammatory response and initiate tissue remodeling after injury. Both M1 and M2 cells retain phagocytic behavior. During obesity, M1 adipose
tissue macrophages (ATMs) greatly increase in number thus contributing to a chronic state of tissue inflammation [38]; and inflamed, insulin-resistant adipose tissue histology is characterized by macrophage crown-like structures surrounding hypertrophic, hypoxic and dying adipocytes. On the other hand, it has been suggested that cold-induced browning of adipose promotes an M2 phenotype in ATMs [38] possibly promoting tissue remodeling and may be a source of NFs in adipose tissue.

Although myeloid cells, including monocytes and macrophages, from peripheral blood have been shown to store and release NFs [39] it is still unclear what role these immune cells play in peripheral nerve innervation, and it remains unknown how and if adipose-resident immune cells are stimulated to release NFs that act locally. Microglia, or the CNS resident myeloid cells that are most similar to macrophages, are an accepted source of BDNF in the brain [40] and increase secretion of BDNF in response to neuroinflammation [41]. Microglial derived BDNF in the CNS promotes hippocampal synaptic plasticity [40] and neurogenesis [42]. We hypothesized that myeloid lineage cells may play a similar role in adipose tissue, and generated a myeloid specific BDNF knock-out mouse model, by crossing LysMcre− and BDNFfl/fl mice. Here we report a role for myeloid derived BDNF in the specific maintenance of adipose innervation, and identify a subpopulation of macrophages (Cx3cr1+) that infiltrate adipose tissue in response to cold stimulation and express BDNF.
Materials and Methods

Mice, Metabolic Phenotyping, and in vivo Analyses

Animals

The following mouse strains were obtained from The Jackson Laboratory: C57BL/6J (Stock # 000664); LysM<sup>cre−</sup> (B6.129P2-Lyz2/J, Stock # 004781); BDNF<sup>fl/fl</sup> (Bdnf<sup>tm3Jae</sup>/J, Stock # 004339); R26R-EYFP (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J, Stock # 006148. Animals with myeloid specific deletion of BDNF (LysM<sup>cre−</sup>:BDNF<sup>−/−</sup>) were generated in our facility by crossing mice heterozygous for the myeloid -specific cre transgene (LysM<sup>cre−</sup>) with mice homozygous for floxed BDNF. Reporter mice were generated by crossing LysM<sup>cre−</sup>x BDNF<sup>fl/fl</sup> x R26R-EYFP. Animals were housed 3-5 to a cage providing for socialization, in a monitored temperature and humidity-controlled environment with 12/12hr light/dark cycle. Cages were replaced weekly, ad libitum access to food and water was maintained. For all studies animals were sacrificed using CO<sub>2</sub> followed by cervical dislocation.

Dietary fat interventions and food intake

Adult male LysM<sup>cre−</sup>:BDNF<sup>−/−</sup> mice were a 45% HFD diet from Research Diets (New Brunswick, NJ) for up to 11 weeks. Mice were housed 2-3 per cage, at room temperature. Body weight was measured weekly. Food intake was measured daily for 7 days, then weekly until the end of the experiment. Adiposity was assessed at the end of the study.
Metabolic cage analyses were conducted in a Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH), for measurement of oxygen consumption (VO$_2$) and carbon dioxide production (VCO$_2$), from which both respiratory exchange ratio (RER), and energy expenditure (heat) were calculated: 

$$RER = \frac{VCO_2}{VO_2}; \text{ Energy expenditure (heat) } = CV \times VO_2 \text{ cal/hr}, \text{ where } CV \text{ is the “caloric value” as given by } CV = (3.815 + 1.232) \times RER.$$

Animals were single housed in a bedding free cage, at room temperature on a 12hr light/dark cycle. Mice were acclimatized for 24-48hrs, after which VO$_2$, VCO$_2$, RER, and Heat were measured every 15min for 3 days (72hrs). Waveform analysis of CLAMS data was performed by matching every 15min measurement across all three 24hr-cycles. Two-way repeated measures analysis of variance (RM, ANOVA) was performed for average VO$_2$, VCO$_2$, RER, and Heat per group. An uncorrected Fisher’s Least Significance Difference test was performed for each time point between dietary groups as a post-hoc test. Interaction P values are reported, which represent differences in 24hr data between groups, as well as multiple comparison results for differences which were only day/night phase specific.

**Cold Exposure Experiments**

All cold exposure was carried out in a diurnal incubator (Caron, Marietta, OH, USA) at 5°C, and a 12hr light/dark cycle. Animals were housed two to a cage and continuously cold exposed for 3 - 14 days.
**CL Injections**

Adult (12-13 week old) male C57BL/6 mice received daily i.p. injections of ADRβ3 agonist CL316,243 (Tocris Bioscience, Bristol, U.K.; Cat # 1499), at 1.0 mg/kg BW or an equivalent amount of sterile saline, for 10-14 days.

**Glucose Tolerance Test (GTT)**

Animals were fasted overnight for 16hr, after which they received an intraperitoneal (i.p.) bolus injection of 1g/kg glucose. Blood glucose was measured using tail vein blood with a hand-held glucometer (OneTouch UltraMini, LifeScan, Milpitas, CA, Johnson&Johnson, New Brunswick, NJ), at time 0 and at intervals of 15 minutes, 30 minutes, and 60 minutes after injection.

**Western Blot**

Protein lysates were prepared by homogenizing frozen whole adipose depots in RIPA buffer using a Bullet Blender (Next Advance, Averill Park, NY), followed by Bradford Assay, and preparation of equal-concentration lysates in Laemmli buffer. 60ug of protein was loaded per lane of a 10% polyacrylamide gel, and following gel running, proteins were transferred to PVDF membranes for antibody incubation. Primary antibodies used included: anti-PGP9.5 (Abcam, Cambridge, U.K. Cat. #10404 and #108986) used at a 1:1000 and 1:500 dilutions respectively; anti-UCP1 (Abcam, Cambridge, U.K. Cat. #10983) used at 1:1000 dilution, anti-TH (Millipore Cat. # AB152; Merck Millipore, Burlington, MA), anti-β-tubulin (Cell Signaling Technology, Danvers, MA, USA; Cat. # 2146BC), were all used at 1:1000 dilution. Secondary antibody was anti-rabbit HRP (Cell
Signaling Ct # 7074), used at a 1:3000 dilution. Blots were visualized with enhanced chemiluminescence (ECL; Pierce) on a Syngene G:BOX. Protein expression of PGP9.5, TH, and UCP1 was normalized to either -β-tubulin or β-actin and quantified in Image J.

**Collection of adipose secretions and BDNF ELISA**

BAT depots were dissected, weighed and minced in a petri dish containing DMEM (high-glucose, serum-free). Minced tissue was transferred to a 15mL conical tube, with 5mL DMEM (loosely capped to keep tissue oxygenated) and placed in a shaking water bath at 37°C. Secretions were collected at time 0, 1hr, 2hrs, and 3hrs (1mL collected from conical tube at each time point and replaced with 1mL fresh DMEM). Secretions were stored at -80°C until processing. For ELISA, protein secretions were concentrated using Amicon ® Ultra Centrifugal Filters, Ultracel ® -100K (Millipore, Burlington, MA USA; Cat. # UFC510096), per manufacturer’s instructions. Mouse BDNF PicoKine™ ELISA Kit (Boster Biological Technology, Pleasanton, CA, USA; Cat# EK0309) was used per manufacturer’s instruction to determine amount of BDNF present in adipose active secretions.

**Thyroid hormone ELISA**

Mouse sera were used to measure circulating levels of thyroxine (T4) and triiodothyronine (T3). Circulating concentrations were determined by Enzyme-Linked Immunosorbant
Assays (ELISA) at Maine Medical Center Research Institute’s Core Facilities (Scarborough, ME).

**SVF isolation**

Bilateral whole inguinal adipose depots were quickly dissected and weighed. Tissue was minced in 37°C pre-warmed DMEM (high glucose, serum free) containing 2mg/mL Roche Collagenase A (Millipore-Sigma, St. Louis, MO; Cat# 10103586001) at a volume of 10mL/depot. Minced tissue with collagenase containing DMEM was placed in a 50mL conical tube and transferred to a shaking water bath (350/min rotation) at 37°C. Every 10min cells were dispersed by vortex and pipette mixing. Full dissociation was usually achieved within 2hrs, when adipocytes were clearly visible and all tissue was dissociated. Dissociated media was poured through 100um cell strainers, rinsed with DMEM and centrifuged at 500g for 10min to separate adipocytes from SVF. After centrifugation adipocytes were collected (found floating on top); remainder of DMEM was removed sparing the SVF pellet. SVF pellet was incubated with 500uL of RBC lysis buffer on ice for 2min, after which 2mL od DMEM with serum was added to stop lysis. Cells were centrifuged at 500g for 5min at 4°C, and either collected for RNA or resuspended in FACS/MACS buffer for cell sorting.

**Magnetic-activated Cell Sorting (MACS)**

SVF from bilateral whole inguinal adipose depots was isolated as described above and resuspended in degassed buffer (1XPBS pH7.2, 0.5% BSA and 2mM EDTA). Single-cell suspensions were sorted on the MidiMACS Quadro magnetic-activated cell separator system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufactures
instructions. Briefly, cells were stained with primary PE-conjugated antibody, CD11b-PE (Cat #130-098-087); a 1:10 antibody dilution per $10^7$ cells was used. Cells were incubated for 10min at 4-8°C, washed, and centrifuged at 300g for 10min. Washed cell pellet was resuspended in a 1:10 dilution of anti-PE microbeads included in Anti-PE MultiSort Kit (Cat. #130-090-757). Following 15min incubation at 4-8°C, cells were washed and centrifuged as described above. Cells were resuspending in 500µL of buffer and passed through LS columns of the MACS separator. LS columns were prepped according to manufacturer’s suggestion. Cells were washed 3 times, collected cells (CD11b-) were collected. LS columns were removed from the MACS separator and flushed with 5mL of buffer to release the magnetically labeled cell fraction (CD11b+). MicroBeads were removed using MicroSort release reagent included in Anti-PE MultiSort Kit (Cat. #130-090-757). MicroBead free CD11b+ cell fraction was labeled for the secondary marker, Anti-F4/80-APC (Cat. #130-102-942), following the same procedure as described for the primary marker, except that Anti-APC MicroBeads (Cat. #130-090-855) were used.

Fluorescence-activated Cell Sorting (FACS)

SVF from bilateral whole inguinal adipose depots was isolated as described above. Cells were resuspend in FACS buffer (HBSS, 5mM EDTA, 2 %FBS). Single cell suspensions were incubated in an “antibody cocktail” against 23 cell surface markers for 20min at 4°C. Cells were centrifuged at 500g for 10min, washed, resuspended and sorted with flow cytometry on a five-laser 30-parameter FACSymphony A5 cytometer (BD Biocsiences, San Jose, USA). For sorted cells, SVF was gated on CD45 and CD11b; CD45+CD11b- represented the non-myeloid population; CD45+CD11b+ myeloid fraction was gated on Ly6c, followed by CCR2 and Cx3cr1.
Gene Expression (qPCR)

RNA was isolated from whole tissue depots using Trizol reagent, and total RNA extracted using a Zymo (Irvine, CA) kit. RNA yield was determined on a Nanodrop; cDNA was synthesized using a High Capacity Synthesis Kit (Applied Biosystems, Foster City, CA). Real-time quantitative (q)PCR was performed with SYBR Green (Bio-Rad, Hercules, CA) on a CFX96 instrument (Bio-Rad, Hercules, CA).

Histology

Adipose

Immunofluorescent staining of 10% buffered formalin fixed, paraffin-embedded, 7uM sections of adipose tissues was performed for detection of UCP1 (1:500, Abcam, Cambridge, UK. Cat. #10983). Alexa 488 (2.5μg.mL, Molecular Probes, Eugene, OR, USA, Cat. # A11070) was used as secondary antibody. Typogen Black staining was used to quench tissue autofluorescence (prior to antibody incubation) and also provided visualization of cell size, browning (multilocularity) and crown-like structures. Stained sections were mounted using Millipore mounting fluid (Burlington, MA USA; Cat. # 5013) and 1 1/5 coverslips, and imaged on a Nikon Eclipse E400 epiflourescent microscope equipped with Nikon DS-fi2 camera.

Neuromuscular junction immunofluorescence, imaging, and analysis

Soleus and medial gastrocnemius muscles were removed and fixed in a 2% PFA at 4°C for 2 hours. Tissues were rinsed with 1XPBS and incubated in blocking buffer (1XPBS/2.5%BSA/0.5-1%Triton) at 4°C for at least 24 hours and up to 7 days. Following
blocking muscles were teased, tendons and fat removed, and tissue was flattened by being placed between two tightly-bound glass slides for at least 30 minutes at 4°C. Tissues were transferred to fresh blocking buffer at 4°C for at least 12 hours. Immunostaining of innervation with primary antibodies was performed overnight at 4°C, followed by 1XPBS washes on a rotating platform at 4°C replacing PBS every 1hr for a total of 4-6hrs. Tissues were incubated with secondary fluorescent antibodies in similar fashion as primary antibodies. Primary antibodies included: neurofilament-M (2H3, 1:500) and synaptic vesicles (SV2, 1:250) from Developmental Studies Hybridoma Bank, (University of Iowa, USA). Secondary antibodies included: Alexa Fluor 488 at 1:500 (A21121) and alpha-bungarotoxin (BTX)-conjugated to Alexa Fluor 594 at 1:1000 (B13423) from Molecular Probes (Eugene, OR, USA). Tissues were mounted on microscope slides using Millipore mounting fluid (Burlington, MA USA; Cat. # 5013) and 1 1/5 coverslips then sealed and allowed to set overnight. Stained sections were imaged on a Nikon Eclipse E400 epifluorescent microscope equipped with Nikon DS-fi2 camera. Brightness, contrast, and sharpness were adjusted in Microsoft PowerPoint. Up to 100 NMJs were counted for each tissue, statistics were conducted in GraphPad PRISM software (La Jolla, CA, USA) using the multiple t-tests (one-per row) function.

Statistical Analysis

For all animal experiments, mice were randomized to treatment groups to ensure no difference in starting body weight. All plots represent mean +/-SEM. Statistical calculations were carried out in Excel or GraphPad Prism software (La Jolla, CA, USA), utilizing ANOVA, Linear Regression, or Student’s T-test as indications of significance (specified in Figure legends). Gene and protein expression data were normalized to a
housekeeper and analyzed by either ANOVA or by Student’s t-test, two-tailed, using Welch’s correction when variance was unequal. Error bars are SEMs. For all figures, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**Ethical Statement**

All procedures and handling of animals were performed in accordance with the University of Maine’s Institutional Animal Care and Use Committee (IACUC), to comply with the guidelines of the PHS Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals. This study was approved by the University of Maine’s IACUC, under protocol A2017-09-04.

**Results**

**BDNF is expressed in adipose SVF**

We previously demonstrated that BDNF levels increase in adipose tissue in response to noradrenergic stimulation [8]. Administration of the β-3 adrenergic receptor (ADRβ3) agonist CL 316,243 to the inguinal scWAT resulted in an increase of BDNF secretion in the tissue [8]. We also showed that adeno-associated virus (AAV)-mediated gene delivery of BDNF to inguinal scWAT of BTBR ob/ob mice (which we exhibited adipose neuropathy) served to increase innervation within the adipose tissue [8] and is a potential treatment. We now endeavored to determine which adipose compartment and cell type was the source of local BDNF secretion in scWAT. Adipose tissue is a heterogeneous organ and although adipocytes are the main cell type, other cell types are contained within the
stromal vascular fraction (SVF) of adipose tissue. Adipose SVF consists predominantly of hematopoietic lineage cells including ATMs [36, 37] but also contains preadipocytes, vascular endothelial cells, and pericytes. To determine the compartmental source of adipose BDNF, adult male C57BL/6 mice were cold exposed and SVF was isolated from mature adipocytes of inguinal scWAT. Gene expression analysis revealed that Bdnf was almost exclusively expressed in the SVF, while other common NFs showed no difference of expression between SVF and mature adipocyte fractions (Fig. 1A). These data, along with other previous findings [8], suggested neuroimmune interactions in adipose tissue.

To further probe the role of BNDF in adipose tissue, we created a knock-out (KO) mouse model using Cre-Lox technology. LysM<sup>cre/-</sup>:mice were bred to BDNF<sup>fl/fl</sup> mice to generate LysM<sup>cre/-</sup>:BDNF<sup>fl/fl</sup> (KO) animals, which lacked BDNF in myeloid lineage cells. Compared to littermate controls, KO animals exhibited a significant, although not complete, decrease of Bdnf in adipose SVF as measured by gene expression (Fig. 1B). Since myeloid lineage cells are expressed in the brain, and BDNF has been shown to play a role in energy balance via CNS control of satiety, we also investigated whether our KO model affected BDNF expression in the hypothalamus. Gene expression of Bdnf in the hypothalamus did not differ between KO animals and their littermate controls (Fig. 1C).

**LysM<sup>cre/-</sup>:BDNF<sup>fl/fl</sup> (KO) mice show lowered energy expenditure under basal conditions and impaired response to cold exposure**

Physiological assessment using metabolic cages performed on KO animals and their littermate controls in the basal state revealed that KO mice had significantly lower energy
expenditure (Fig. 1D) despite showing no difference in body weight or adiposity (Suppl. Fig. S1A). Since a decrease in energy expenditure could be indicative of impaired sympathetic drive, we stimulated sympathetic nerve activity via cold exposure. When adult (22-23 week old) male mice were cold challenged at 5°C for 4 days, KO and control animals maintained similar body weight (Fig. 2A, left panel). However, KO animals had significantly increased adiposity (Fig. 2A, right panel). As cold exposure stimulates catecholamine-induced lipolysis, mediated through sympathetic nerves, we next investigated innervation of the inguinal scWAT depot following a 7-day cold challenge. Protein expression of the pan-neuronal marker PGP9.5 was markedly reduced in inguinal scWAT of male KO mice compared to littermate controls (Fig. 2B, left panel). Protein expression of tyrosine hydroxylase (TH), a marker of sympathetic activation, was also drastically reduced in inguinal scWAT of KO animals compared to controls (Fig. 2B, right panel). Cold exposure induces expression of uncoupling protein 1 (UCP1) as it is required for adaptive thermogenesis, due to its ability to uncouple the mitochondrial respiratory chain resulting in a proton leak and heat production. UCP1 is therefore a unique marker of BAT and browning in scWAT. Gene expression of Ucp1 in inguinal scWAT of 7-day cold exposed mice was reduced in KO animals compared to littermate controls (Fig. 2C). Thyroid hormone potentiates SNS activation of thermogenesis in BAT; no changes in thyroid hormone were observed between KO and CON animals (Fig. 2D). Histological assessment of inguinal scWAT revealed what appeared like increased multilocularity suggestive of browning in KO animals (Fig. 2E, Typogen Black staining). However, there was a strikingly lack of UCP1 expression in these areas (Fig. 2E). Considering denervation of WAT increases hyperplasia the observed browning could more likely be
areas in increased preadipocytes. Taken together, these data suggested a genetic denervation of inguinal scWAT and diminished thermogenic response in inguinal scWAT in KO mice.

**Genetic denervation of scWAT in LysM<sup>cre−/−;BDNF<sup>fl/fl</sup> (KO) mice is depot specific**

We next sought to determine whether genetic denervation of scWAT in KO animals was restricted to adipose tissue or extended to other organs. BDNF is a known myokine, and muscle is an energy expending tissue. We assessed innervation of fast twitch (gastrocnemius) and slow twitch (soleus) muscle in CON and KO, by investigating occupancy of neuromuscular junctions (NMJs) at basal conditions in adult male mice. Immunostaining of the presynaptic nerve and vesicles (neurofilament and SV2, respectively), and postsynaptic acetylcholine receptors was performed to allow visualization of MNJ (Suppl. Fig. S1B, left panel). Following counts of occupied, partially occupied, and unoccupied NMJs it was determined that there was no evidence of neurodegeneration in the NMJ of KO animals (Suppl. Fig. S1B, right panel). In the same animals we also assessed axon numbers of spinal (L5 ventral root), motor, and sensory nerves through cross-section imaging (Suppl. Fig. S1C). A lower axon count could reflect neuronal death, but no difference was observed between CON and KO animals.

Although thyroid hormones were similar between CON and KO animals (Fig. 2D), suggesting no effect of our KO on BAT thermogenesis, myeloid cells are present in this tissue. We therefore wanted to evaluate whether a lack of BDNF in BAT myeloid cells would have an effect on tissue function. BAT of 7-day cold exposed 12-25 week old male CON and KO mice was evaluated. Protein expression of UCP1, TH, and PGP9.5 did not
differ between KO mice and littermate controls (Suppl. Fig. S2A), which suggested BAT function was neither impaired nor was the tissue denervated. Histological assessment of BAT revealed no difference in cellular morphology, or UCP1 expression (Suppl. Fig. S2B). These results suggested that BDNF may not play a modulatory role in BAT innervation. Indeed, when adult male C57BL/6 mice were cold exposed or treated with the pharmacological β-3 adrenergic receptor (ADRβ3) agonist, CL316,243, no difference in BDNF secretion for BAT was observed when compared to basal conditions (Suppl. Fig. S2C). Together, these data supported scWAT depot specificity of our genetic denervation model.

**HFD feeding exacerbates fat mass accumulation in LysMcre/-:BDNFfl/fl (KO) Mice**

Loss of sympathetic innervation to inguinal scWAT has been shown to increase depot mass [43], however in our genetic model of scWAT innervation, no difference in adiposity was observed under basal conditions (Suppl. Fig. S1A) despite the observed decrease in energy expenditure (Fig. 1D). We next metabolically challenged CON and KO through administration of a 45% high fat diet (HFD). Adult (25 week old) male CON and KO mice were placed on a HFD for 3-11 weeks, to assess adipose integrity and energy balance. At 3 weeks of HFD feeding animals were assessed in metabolic cages. HFD resulted in only a slight decrease in energy expenditure in KO mice compared to littermate controls (Fig. 3A, top panel). However, KO mice showed a higher RER than CON animals during the light cycle, indicative of preferential metabolism of carbohydrates over lipids for fuel (Fig. 3A, bottom panel). These data fits with studies demonstrating that adipose nerves are important for lipolysis [44] and that denervation would shift fuel preference to
carbohydrates. These physiological differences between CON and KO animals were observed despite no difference in food intake or change in body weight (Fig. 3B). After 6 weeks of HFD, KO mice displayed worsened glucose control compared to CON animals (Fig. 3C). By 11 week of HFD feeding KO animals displayed greater adiposity than littermate controls (Fig. 3D). Taken together, the challenge of HFD feeding exacerbated fat accumulation in inguinal scWAT of KO animals, possibly contributing to the observed impairment of glucose control.

**Cold-induced neuroimmune cells (CINCs) are recruited to scWAT and express BDNF**

After demonstrating the significance of myeloid derived BDNF to scWAT we sought to determine which myeloid cells were the source of BDNF. Considering their multifaceted role in adipose tissue and source of BDNF in the brain, we hypothesized that macrophages were the leading source of BDNF in scWAT. Since previous studies indicated that BDNF is increased in scWAT with noradrenergic stimulation [8] CD11b+ F4/80+ macrophages were isolated from SVF of inguinal scWAT of room temperature and 5-day cold exposed C57BL/6 adult (12 week old) male mice. Bdnf gene expression did not differ between room temperature and cold exposed CD11b+ F4/80+ macrophages (Fig. 4A). Although F4/80+ is considered a pan-macrophage marker, it was too broad to reveal subpopulation changes, and does not effectively mark monocytes (macrophage precursors), that could be infiltrating the tissue. Based on these findings we applied a different approach to determining which myeloid cells are the source of scWAT BDNF. Adult (12 week old) female control animals were maintained at room temperature or cold exposed for 10 days. Inguinal scWAT SVF was isolated and FACS sorted using a 23-
antibody cocktail against myeloid lineage cells. Surprising, cold exposure did not have an effect on either M1 or M2 ATMs (Fig 4B). Instead, the greatest increase in macrophage related cell populations was in Ly6c+ monocytes. Both Ly6C+ CCR2+ and Ly6C+ CCR2+ Cx3cr1+ increased in inguinal scWAT following cold exposure (Fig. 4C). Spanning-tree Progression Analysis of Density-normalized Events (SPADE) analysis of room temperature and cold exposed SVF was performed to assess changes throughout all myeloid lineage cells in response to cold (Fig. D). SPADE analysis revealed cold-induced enrichment in CCR2+ and Cx3cr1+ populations. SPADE analysis also revealed that Mrc1/CD64hi cells decrease in response to noradrenergic stimulation, the significance of which remains to be investigated (Fig. 4D).

To confirm that CCR2+ Cxc3cr1+ monocytes/macrophages were the source of BDNF, we sorted out Ly6c+CCR2+Cx3cr1+ and Ly6c+CCR2+Cx3cr1- from inguinal scWAT SVF of 14 day cold exposed adult (12-13 week old) male C57BL/6 mice. Although the previously observed cold induced elevation of Ly6c+CCR2+Cx3cr1- in inguinal scWAT was not observed in the male cohort, Ly6c+CCR2+Cx3cr1+ did increase in response to cold (Fig. 4E). We measured Bdnf gene expression in cold induced Ly6c+CCR2+Cx3cr1+ (Cx3cr1+) cells and found that they showed a mild trend for increased expression of Bdnf compared to cold exposed non-myeloid cells (Fig. 4F).

Adrβ3 gene expression in Cx3cr1+ cells confirmed the presence of norepinephrine (NE) receptor on these cells indicating the potential to be responsive to SNS stimulation (Suppl. Fig. S3).
Taken together, these data indicated that Cx3cr1+ cells are cold-induced neuroimmune cells (CINCs) that have the potential to be stimulated by sympathetic nerves, and express BDNF.

**Discussion**

Here we present evidence of the necessity of myeloid-derived BDNF in maintaining inguinal scWAT innervation. Loss of BDNF from LysM+ myeloid cells resulted in a severe decrease in total innervation of the inguinal adipose tissue as measured by the pan-neuronal marker PGP9.5, and a near complete denervation of sympathetic nerves (as measured by TH protein expression (Fig. 2B). We attribute this ‘genetic denervation’ as a cause for the observed decrease in energy expenditure exhibited under basal conditions (Fig. 1D), impaired thermogenic potential evidenced by lack of UCP1 induction and impaired lipolysis during cold stimulation (Fig 2A, C, E), and worsened response to HDF (Fig. 3). These phenotypes fit with the known roles of adipose nerves in regulating proper adipogenesis, lipolysis, thermogenesis and overall metabolic health in adipose tissues.

Loss of sympathetic innervation to adipose tissue inevitably results in a decrease in catecholamine induced lipolysis, as sympathetic nerve-released NE signals through ADRβ3 on adipocytes to initiate a signaling cascade that results in increased activity of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), key enzymes involved in intracellular breakdown of triacylglycerols [45]. Cold exposure suggested impaired lipolysis in scWAT as KO animals had greater scWAT adiposity than littermate
controls (Fig.2 A). Cold exposure leads to SNS stimulation of scWAT resulting in browning and lipolysis. Lipolysis of scWAT provides lipids to BAT via the circularity system which are used as fuel for adaptive thermogenesis. This usually result in deceased scWAT fat mass. HFD feeding also resulted in greater accumulation of scWAT mass in KO mice compared to controls (Fig. 3D). This, combined with HFD RER data indicating preferential metabolism of carbohydrates as fuel in KO animals (Fig. 3A) further suggests impaired lipolysis in scWAT.

Under basal conditions KO animals showed lower energy expenditure as measured by heat production (Fig 1D) than when challenged by HFD (Fig. 3A). However, obesity often results in excess lipid accumulation in BAT and ectopic lipid storage [46]. This may be providing BAT with an increased fuel source, resulting in the HFD induced partial recovery of the decreased energy expenditure observed under basal conditions. Amongst the physiological pressures of a HFD, there are alterations to the immune milieu within adipose tissue. Diet-induced obesity results in chromic low grade adipose inflammation due to infiltration of pro-inflammatory immune cells to the tissue, and ATM content increase from 10-15% (present in lean mice) to ~50% [31, 47]. Phenotypic switching of CD4+ T cells, recruitment T cell and B cells precedes macrophage infiltration, and macrophage polarity is affected to exhibit a more inflammatory phenotype. The interactions between these infiltrating cells and BDNF secreting myeloid cells is unknown; and are potential caveats to the observed phenotype of our myeloid BDNF knockout model.
We initially hypothesized that anti-inflammatory (M2) ATMs were the source of scWAT BDNF. However, we observed no changes in M1 or M2 populations in scWAT after cold exposure (Fig. 4B), instead we saw an increase in Ly6c+CCR2+Cx3cr1+ monocytes in both male and female mice (Fig. 4C-D). We have named these cells CINCs since they are recruited to scWAT by cold, and express BDNF. Surprisingly, it would appear that CINCs are a pro-inflammatory cell type as Ly6c+ is a marker of inflammatory monocytes. Although pro-inflammatory cell infiltration to adipose tissue is usually a harbinger of metabolic dysfunction, an acute inflammatory response is necessary in wound healing and tissue remodeling. By utilizing an unbiased 23 antibody labeling approach to assessing adipose SVF, we have discovered subsets of myeloid cells that respond to environmental stimuli providing further granularity to the myriad of immune cells types active in adipose.

Recently, a Cx3cr1+ population of ATMs had been described in association with sympathetic nerves in adipose tissue [48]. These sympathetic nerve associated macrophages (SAMs) were found to regulate catecholamine levels on adipose tissue by phagocytosing and degrading norepinephrine (NE) [48]. Whether SAMs are the same cells as our CINCs remains uncertain, as other distinguishing markers like Ly6C and CCR2 were not assessed. Similar to CINCs, SAMs do exhibit pro-inflammatory markers. SAMs hone to WAT in the obese state but we see CINCs hone to WAT with cold/noradrenergic-stimulation, indicating they may have opposing roles. Fitting with this, SAMs sequester NE while CINCs express BDNF. One could speculate then, that SAMs serve to decrease SNS input to the tissue while CINCs serve to increase innervation of WAT. On the other hand, Cx3cr1+ macrophages have been shown to play diverse and
even opposing roles in the intestines [49], a phenotypic plasticity which may also be present in adipose tissue. One thing that is clear is that the M1/M2 paradigm of ATM classification is an oversimplification of the functionally distinct populations of macrophages active in adipose tissue. However, further markers beyond Cx3cr1+ are necessary to fully understand and phenotype the rich variety of macrophages in adipose. Compounding the difficulty in clearly delineating macrophage populations is that macrophage activation is a dynamic process. Phenotypic switching appears to be sequential, responding to microenvironment stimuli [50] and may be dependent on spatiotemporal differences in tissue resident immune cell subtypes [51]. Single-cell RNAseq of adipose immune cells may provide more precise information necessary for further phenotyping CINCs.

We postulate that CINCs contribute to nerve remodeling under noradrenergic stimulation, leading to BDNF release. However, BDNF can also be secreted by nerves, and it remains unclear what the relationship, if any, is between CINC secreted BDNF and BDNF that may be released from adipose nerves. Cao, et al have recently suggested that sympathetic nerve plasticity is dependent on cold induced adipose derived NGF [52]. Although they provide evidence that NGF is involved to some degree in promoting cold induced sympathetic nerve density and browning of scWAT, there is insufficient evidence as to the cellular source of NGF. After determining that NGF gene expression increased in the first couple of days of cold exposure, Cao, et al used an NGF neutralizing antibody to prevent NGF activity and observed decreased sympathetic nerve density in response to cold. Several caveats to this study exist, cold-induced sympathetic nerve density was not assessed in the whole adipose depot, and we know that regional innervation patterns
exist from our previous works as well as others [53]. Furthermore, NGF neutralizing antibody was administered systemically, and there was no control for off-target effects, including any that may have affected hypothalamic control of sympathetic drive to scWAT. However, their approach in preventing TrkA receptor function in adipose nerves and showing decreased browning and sympathetic nerve density lends credence to the notion that NGF plays a role in adipose innervation. Considering the variety of NFs and the multitude of cell types in adipose tissue it would not be surprising if both NGF and BDNF play a role in adipose innervation through different mechanisms, and the presence of one NFs within as tissue does not have to preclude the physiological role of another.
Figure 1

A. Gene Expression of Neurotrophic Factors in Adipose Compartments

B. BDNF Gene Expression in Inguinal scWAT SVF of LysMCre:BDNF<−/< (KO) mice

C. BDNF Gene Expression in Hypothalamus

D. Basal Heat (CLAMS)
Figure 1. BDNF is expressed in the SVF of adipose; \( \text{LysM}^{\text{cre-}:BDNF^{fl/fl}} \) (KO) mice have lower energy expenditure. Adult male CB57/BL6 mice were cold exposed at 5°C for XX days and SVF was isolated from mature adipocytes of the inguinal scWAT depot. Differences in gene expression between adipose compartments of neurotrophic factors, \( Bdnf, \ ngf, \) and \( \text{Vegfa} \), is shown (A). Data analyzed by Student’s t-test, two-tailed, N=4. BDNF gene expression in adipose SVF of \( \text{LysM}^{\text{cre-}:BDNF^{fl/fl}} \) (CON) versus \( \text{LysM}^{\text{cre-}:BDNF^{fl/fl}} \) (KO) mice (B). Data analyzed by Student’s t-test, two-tailed, N=4 per group. Gene expression of \( Bdnf \) in hypothalamus of \( \text{LysM}^{\text{cre-}:BDNF^{fl/fl}} \) (CON) versus \( \text{LysM}^{\text{cre-}:BDNF^{fl/fl}} \) (KO) mice. Data analyzed by Student’s t-test, two-tailed, N=5 CON; N=6 KO. Adult (8-12 week old) male CON and KO mice were assessed in metabolic cages (CLAMS). Waveform analysis of metabolic cage measurements taken at 15min increments for 48 hrs (D). Time of day is indicated on the x-axis, animals were maintained on a 12 hr light/dark cycle (black bars indicate dark cycle). KO mice displayed lower energy expenditure represented as heat calculated from measures of \( \text{VO}_2 \) and \( \text{VCO}_2 \) over the whole 24hr (D). For all error bars are SEMs.
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scWAT/Body Weight after Cold Exposure

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B. Inguinal scWAT Innervation after Cold Exposure

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C. UCP1 Gene Expression in Inguinal scWAT after Cold Exposure

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D. Circulating Thyroid Hormones after Cold Exposure

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E. Immunofluorescent Staining of Inguinal scWAT Following Cold Exposure

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Figure 2. LysMcre-:BDNF^{fl/fl} (KO) have increased adiposity and impaired response to cold due to genetic denervation of scWAT. Adult (22-23 week old) male LysM^{-/-}:BDNF^{fl/fl} (CON) and LysMcre-:BDNF^{fl/fl} (KO) mice were cold exposed at 5°C for 4 days; body weight and adiposity were compared between CON and KO groups (A). Body and tissue weight data were analyzed by two-tailed Student’s T-Test, N=5 CON, N=8 KO. Protein expression of PGP9.5 and tyrosine hydroxylase (TH) in inguinal scWAT was measured by western blotting from adult (12-25 week old) 7-day cold (5°C) exposed WT/CON and KO male animals (B). β-tubulin was used as a loading control for normalization. Data were analyzed by two-tailed Student’s T-Test, N=3 WT/CON, N=4 KO, * denotes data that was removed from analysis due to lack of expression of loading control. Gene expression of UCP1 was measured in adult (12-25 week old) 7-day cold (5°C) exposed WT/CON and KO males (C). Data were analyzed by two-tailed Student’s T-Test, N=5 WT/CON, N=5 KO. Circulating thyroid hormones, thyroxine (T4) and triiodothyronine (T3), were measured by ELISA from serum of adult (22-23 week old) 4-day cold (5°C) exposed CON and KO male mice (D). Data were analyzed by One-way ANOVA, with Tukey’s multiple comparisons test, N=5 CON, N=8 KO. Immunofluorescent staining for UCP1 was performed on inguinal scWAT sections of adult (22-23 week old) male CON and KO mice following 4-day cold (5°C) exposure (E). Typogen black, used to quench lipid autofluorescence, provided staining cell morphology which was visualized under brightfield microscopy. Images were acquired with a 10X objective for a total magnification of 100X, and are representative of N=5 CON, N=8 KO. For all error bars are SEMs.
Figure 3

A. Heat 45% HFD (3 wks)

B. Food Intake 45% HFD (7 days)

C. GTT 45% HFD (6 wks)

D. scWAT/BW 45% HFD (11 wks)
Figure 3. LysMcre/-:BDNFfl/fl (KO) showed accelerated fat accumulation on an 45% HFD. Adult (25 week old) male LysMcre/-:BDNFfl/fl (CON) and LysMcre/-:BDNFfl/fl (KO) were challenged with a 45% HFD for 3 weeks before undergoing physiological assessment in metabolic cages (A). Energy expenditure as measured by heat was lower for KO versus CON only for a short period during the dark cycle (A, top panel). Respiratory exchange as a ratio (RER) between the two groups, indicated greater use of carbohydrates for fuel by KO animals during the light cycle. Data presented as waveform analysis of measurements taken at 15min increments for 3 days. Time of day is indicated on the x-axis, and animals were maintained on a 12 hr light/dark cycle (black bars indicate dark cycle). Data analyzed by two-way repeated measures ANOVA with Fisher’s LSD test; N=4 per group. Adult (25 week old) male CON and KO animals were placed on a 45% HFD, daily food intake (represented as cumulative food intake) (B, top panel) was measured for the first week of HFD feeding. Percent change in body weight (B, bottom panel) was measured for the first 7 days of HFD feeding. Data were analyzed by two-tailed Student’s T-Test, N=6 CON, N=4 KO. Glucose tolerance testing was performed at 6 weeks of HFD feeding (C). Data were analyzed by Two-way ANOVA, with Tukey’s multiple comparisons test, N=4 CON, N=4 KO. Adiposity was measured for CON and KO animals after 11 weeks of HFD feeding as a percentage of scWAT over body weight (D). Data were analyzed by two-tailed Student’s T-Test, N=4 CON, N=4 KO. For all error bars are SEMs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 4

A. 

**Bdnf Gene Expression in F4/80+ Macrophages**

B. **Macrophage polarity**

C. **Monocyte changes**

D. **SPADE Analysis**

E. **Cold Induced CINCs**

F. **Bdnf Gene Expression in CCR2+Cx3Cr1+ Macrophages**
Figure 4. Cold induced neuroimmune cells (CINCs) hone in to inguinal scWAT and express Bdnf. Adult (12 week old) male C57BL/6 were either maintained at room temperature (RT) or cold exposed (5°C) for 5 days, ATMs from inguinal scWAT depots were isolated using magnetic-activated cell sorting (MACS) by positive selection of CD11b+ followed by F4/80+ cells. Bdnf gene expression in doubly labeled CD11b+ F4/80+ macrophages was compared between RT and cold exposed animals (A). Data were analyzed by two-tailed Student’s T-Test, N=4 per group. Adult (12 week old) female control animal were either maintained at room temperature (RT) or cold exposed (5°C) for 10 days, SVF from bilateral inguinal scWAT was isolated and FACS sorted using a 23 cell surface marker panel for myeloid lineage immune cells (B-D). Changes in M1/M2 polarity (B) and Ly6C+ macrophage precursors/monocytes (C) were measured between RT and cold exposed animals. Data were analyzed by two-tailed Student’s T-Test, N=3 per groups. Spanning-tree Progression Analysis of Density-normalized Events (SPADE) analysis was performed to identify myeloid lineage cell population changes in response to cold exposure; Ly6c+CCR2+ and Ly6c+CCR2+Cx3cr1+ were identified as adipose cold induced neuroimmune cells (CINCs) (E). Data representative of N=3 per group. Adult (12-13 week old) male C57BL/6 were either maintained at room temperature (RT) or cold exposed (5°C) for 14 days; SVF from bilateral inguinal scWAT was isolated and FACS sorted (E-F). Previously identified CINCs, Ly6c+CCR2+Cx3cr1- and Ly6c+CCR2+Cx3cr1+ cells were compared between RT and cold exposed animals (E). Bdnf gene expression was measured in Ly6c+CCR2+Cx3cr1+ cells (F). Data analyzed by two-tailed Student’s T-Test, N=4 per group. For all error bars are SEMs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Supplementary Figure S1

A. 

**Basal Body Weight**

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**Inguinal scWAT/Body Weight**

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**Neuromuscular Junction (NMJ) Assessment**

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**Spinal, Motor, Sensory Nerve Assessment**

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n.s. - not significant
Supplemental Figure S1. Further basal phenotyping of \textit{LysM^{cre/-}:BDNF^{fl/fl}} (KO) animals. Adult (29 week old) male \textit{LysM^{cre/-}:BDNF^{fl/fl}} (CON) and \textit{LysM^{cre/-}:BDNF^{fl/fl}} (KO) were assessed under basal condition for body weight and subcutaneous adiposity (A). Data analyzed by two-tailed Student’s T-Test, N=5 CON, N=6 KO. Immunostaining of the presynaptic nerve and vesicles (neurofilament and SV2, in green) and postsynaptic acetylcholine receptors (in red) was performed on fast twitch (medial gastrocnemius) and slow twitch (soleus) muscles to allow visualization and assessment of the neuromuscular junction (NMJ) (B). Data analyzed by two-tailed Student’s T-Test, and representative of male 23-25 week old CON and KO mice, N=3 per group. For the same group, axon numbers of spinal (L5 ventral root), motor, and sensory nerves were counted through cross-section electron microscopy imaging (C). For all error bars are SEMs.
Supplementary Figure S2

A. BAT 7-day cold (males)

UCP1  
TH  
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CON  |  KO

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B. BAT histology and UCP1 immunostaining 5-day cold (males)

Typogen Black  
UCP1 (green)  

CON  |  KO


C. BDNF Secretions from BAT

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n.s.
Supplemental Figure S2. Assessment of BAT in LysMcre:BDNFfl/fl (KO) animals. Adult (12-25 week old) male LysMcre:BDNFfl/fl (CON) and LysMcre:BDNFfl/fl (KO) animals were cold (5°C) exposed in diurnal chambers for 5-7 days (A-B). Protein expression of UCP1, tyrosine hydroxylase (TH) and PGP9.5 in BAT was compared between CON and KO mice (A). Data analyzed by two-tailed Student’s T-Test, N=6 CON, N=8 KO. Immunostaining for UCP1 (green) was performed on paraffin embedded cut sections of BAT from CON and KO animals (B). Typogen black staining shows cell morphology, images taken with 40X objective for total magnification of 400X. Data representative of N=6 CON, N=8 KO. Adult (12-13 weeks old) male C57BL/6J mice were maintained at room temperature (RT) or cold exposed (5°C) for 10 days, or received either daily i.p. injections of ADRβ3 agonist CL316,243 (at 1.0 mg/kg BW), or vehicle for 14 days. Ex vivo secretions (collected at 1hr and 2hr) from BAT explants were measured for BDNF by ELISA, and not analyzed due to low N of control animals, N=2 RT/VEH, N=4 Cold/CL.
Adrβ3 gene expression in CINCs. Adult (12-13 week old) male C57BL/6 were either maintained at room temperature (RT) or cold exposed (5°C) for 14 days; SVF from bilateral inguinal scWAT was isolated and FACS sorted to isolate CINCs. Adrβ3 gene expression was measured in Rt and cold exposed Ly6c+CCR2+Cx3cr1+ cells (F). Data analyzed by two-tailed Student’s T-Test, N=4 per group. For all error bars are SEMs.
References


OVERALL CONCLUSION

The work presented here intensifies our understanding of brain-adipose communication and brings to light many more questions. We have provided a deeper knowledge of adipose neuroanatomy; and by gaining a fuller appreciation of form we are allowed more insight into function, thus informing further mechanistic studies regarding adipose innervation and the role it plays in maintaining energy homeostasis. We have shown that adipose innervation is heterogenous within a depot, yet overall patterned, and that it can undergo remodeling under certain conditions. Although we see adipose nerves within parenchymal space, and interacting with vasculature and cells within the SFV, we have yet to establish how and where synaptic connections are formed in adipose tissue, and what affects their establishment. Formation of these synaptic contacts under pathophysiological conditions remains to be investigated. We have shown adipose neuropathy under conditions of obesity, diabetes, dietary toxicity, and aging. However, the etiology of each requires further probing. We have shown that the neurotrophic factor BDNF is diminished in neuropathic adipose tissue, and that AAV-mediated BDNF gene therapy may be beneficial in reestablishing adipose innervation under obesity/diabetes related adipose neuropathy. Although, we do not yet know what mechanisms drive this observed decrease in BDNF, it is clear that BDNF plays an important role in adipose innervation. We have presented evidence that myeloid cell derived BDNF is required for adult adipose innervation, and that animals that lacking BDNF in myeloid cells exhibit a genetic denervation of their subcutaneous adipose tissue. As with surgical or chemical adipose denervation, this genetic denervation is accompanied by impaired energy expenditure and metabolic function.
We have also shown in mouse models that adipose BDNF is increased by exercise and cold exposure, and that this increase is accompanied by an increase in adipose innervation. These findings further implicate BDNF as a regulator of adipose innervation. During our search for the source adipose BDNF, we discovered a subset of immune cells, CINCs, that hone into adipose tissue in response to cold exposure and express adrenergic receptors as well as BDNF. We believe these cells represent the neuroimmune junction in regulating adipose innervation. Further investigation into how CINCs are recruited to adipose and the evidence of their sexual dimorphism requires continued investigation. Insight into the recruit of CINCs may come from investigating the adipose lymph node. The subiliac lymph node is the main drainage lymph node in inguinal adipose tissue, and a nexus of immune cells. As interstitial fluid drains though the lymph node, fatty acids, hormones, neurotransmitters, and other solutes interact with immune cells in the lymph nodes. This provides another point of communication between the adipose organ and the rest of the body as lymphatic vessels return tissue fluid to blood vessels. Through our exploration of adipose neuroanatomy, we made the observation that the subiliac lymph node is highly innervated. This innervation made be critical in the lymph node’s ability to properly drain adipose interstitial fluid which may be harboring critical information regarding the needs of the adipose organ. The role of the lymphatic system in adipose innervation is currently understudied and should be considered in future studies of neuroimmune interactions.

A common thread in the pathophysiological conditions of adipose neuropathy observed is inflammation. Obesity, diabetes, and dietary toxicity from a peroxidized Omega-3 diet all resulted in chronic inflammation of the adipose organ, and aging is
associated with the pro-inflammatory senescence associated secretory profile (SASP). Further studies aimed at resolving chronic inflammation in the adipose tissue may prove insightful in providing a mechanism for the onset of adipose neuropathy. Interestingly, inflammation may also play a role in promoting adipose innervation. Under conditions of exercise and cold exposure mediated noradrenergic stimulation the body undergoes an acute state on inflammation. Furthermore, the CINCs which we propose secrete BDNF to mediate neurite outgrowth in adipose tissue, exhibit a pro-inflammatory profile. This combined with the potential involvement of the lymphatic system in adipose innervation opens new areas of inquiry in the study in neuroimmune interactions in adipose innervation.
APPENDIX A: Supplemental Material Chapter 2

Extended Materials and Methods

Mice, Metabolic Phenotyping, and in vivo Analyses

Dietary fat interventions and food intake

Six diets enriched for either low or high saturated fatty acids (SFA), low or high omega-6 polyunsaturated fatty acids (n-6 PUFA) and low or high omega-3 polyunsaturated fatty acids (n-3 PUFA), with equal sugar and protein content were designed (with Research Diets, New Brunswick, NJ; Table 1). Low and high SFA and n-6 PUFA diets were the same as our previously published diets [1]. Coconut oil, a common source of saturated fat in obesity and diabetes studies [2], was used as the source of saturated fat in our custom diets. n-3 PUFA diet was created with menhaden fish oil, supplied by Nordic Naturals (Watsonville, CA). The menhaden oil provided by Nordic Naturals was produced in adherence with the European Pharmacopoeia Standard and voluntary standards set by the Global Organization for EPA and DHA Omega-3 (GOED), the International Fish Oils Standards (IFOS) program, World Health Organization (WHO) fish oil monograph, and California’s Proposition 65 (PROP 65) to ensure purity and quality. Menhaden oil was maintained under a protective nitrogen environment (oxygen-free) until the custom diet manufacturing process began to reduce risk of peroxidation. The manufacturing process itself, carried out by Research Diets, used heatless mechanical pellet formation which relied solely on pressure. Upon manufacture, diets were maintained at -20°C in airtight containers, and thawed in small batches for daily feeding. All custom diets were fed to adult (10 week old) C57BL/6J male mice for 16 weeks, concurrent with body weight and food intake measurements. All diet groups had N=6 mice, except for high n-3 PUFA
which had 8 animals. For the duration of the study, mice were caged 2-3 per cage. Food intake was measured every day for 7 days, then weekly until 28 days, and values for each cage were divided by the number of mice per cage to obtain a daily intake per mouse. Special care was taken to ensure accurate food intake data by thoroughly searching bedding for crumbled food pellets to obtain accurate food weights. Adiposity was assessed at the end of the study, and hypothalamus, subcutaneous white adipose tissue (scWAT) and intrascapular brown adipose tissue (iBAT) depots were harvested and sent for lipidomic analysis with Berg, which included a measure of non-enzymatic (peroxidized) lipids.

**Power Analysis**
Numbers of mice per group were based on power calculations and previously published data using a subset of these diets [1], given the predicted difference between groups that is expected (in terms of natural variations in body weight due to diet). For these studies, we used the G*Power 3.1 software, with an effect size of 5, power of 0.85, an alpha level of 0.05 and 10 degrees of freedom. We calculated based on the endpoint of variation in body weight, based on data from our previous experiments and wanting to detect a difference of 5g body weight for 3 groups (ie: all high fat or all low fat, thus an effect size of 5. This gave us N=6 per group.

**CLAMS**
Metabolic cage analyses were conducted in a Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH), allowing for measurement of oxygen consumption (VO$_2$) and carbon dioxide production (VCO$_2$), from which both respiratory exchange ratio (RER), and energy expenditure (heat) were
calculated: \( RER = \frac{VCO_2}{VO_2} \); \( Energy \ expenditure \ (heat) = CV \times VO_2 \text{ cal/hr}, \) where \( CV \) is the “caloric value” as given by \( CV = (3.815 + 1.232) \times RER \). Each animal was placed in a bedding free CLAMS cage and acclimated for 24-48hrs, after which VO2, VCO2, RER, and Heat were measured every 15min for 3 days (72hrs). Throughout the CLAMS assessment animals were housed at room temperate on a 12hr light/dark cycle. Waveform analysis of CLAMS data was performed by matching each 15min measurement for each of the three 24hr time-points measured for each animal. A two-way repeated measures analysis of variance (RM, ANOVA) was performed showing the average VO2, VCO2, RER, and Heat per group, allowing for better analysis of variation. An uncorrected Fishers Least Significance Difference test was performed for each time point between dietary groups as a post-hoc test. Interaction P values are reported, which represent differences in 24hr data between groups, as well as multiple comparison results for differences which were only day/night phase specific.

**Glucose Tolerance Test (GTT)**

Animals were fasted overnight for 16hr, after which they received an intraperitoneal (i.p.) bolus injection of 1g/kg glucose. Blood glucose was measured using tail vein blood with a hand held glucometer (OneTouch UltraMini, LifeScan, Milpitas, CA, Johnson&Johnson, New Brunswick, NJ), at time 0 and at intervals of 15 minutes, 30 minutes, and 70 minutes after injection.

**Thiobarbituric Acid Reactive Substances (TBARS) Assay**

To independently determine peroxidation of our n-3 PUFA diets, a malondialdehyde (MDA, a common by-product of lipid peroxidation) production assay was performed using a Caymen Chemical (Ann Arbor, MI) TBARS Assay Kit, per manufacturer’s instructions.
Several common fish food products along with omega-3 fish oil supplements (both for human consumption) were assayed concurrently with the mouse diets.

**Histology**

Hematoxylin staining of paraffin-embedded, 10uM slices of adipose tissues was performed to determine cell size and to assess browning (multilocularity) and crown-like structures. With hematoxylin staining lipofuscin appears as yellow-brown granules. Trichrome staining was performed using a kit for connective tissue (Abcam, Cambridge, UK). Autofluorescence of lipofuscin in paraffin-embedded adipose tissues was detected using a Nikon Eclipse E400 epifluorescent microscope equipped with Nikon DS-fi2 camera.

**ELISA**

Mouse sera were used to measure circulating levels of Interleukin 10 (IL10) and adiponectin (High molecular weight (HMW)). Circulating concentrations were determined by Enzyme-Linked Immunosorbant Assays (ELISA) at Maine Medical Center Research Institute’s Core Facilities (Scarborough, ME).

**Western Blot**

Western blotting analysis was performed to measure protein expression of tyrosine hydroxylase (TH) in whole adipose tissue depots. Protein lysates were prepared by homogenizing frozen tissue in RIPA buffer using a Bullet Blender (Next Advance, Averill Park, NY). Primary antibodies utilized included anti-TH (Millipore Cat. # AB152; Merck Millipore, Burlington, MA) and anti-®-actin (Abcam Cat. # ab8227) as housekeeper, which were both used at 1:1000 dillutions. Secondary antibody was anti-rabbit HRP (Cell Signaling Ct # 7074), used at a 1:3000 dilution. Blots were visualized with
enhanced chemiluminescence (ECL; Pierce) on a Syngene G:BOX. TH protein expression was normalized to β-actin and quantified in Image J.

**Gene Expression (qPCR)**

RNA was isolated from whole tissue depots using Trizol reagent; a Bullet Blender was used to enhance cell lysis, and total RNA extracted using a Zymo (Irvine, CA) kit. RNA yield was determined on a Nanodrop; cDNA was synthesized using a High Capacity Synthesis Kit (Applied Biosystems, Foster City, CA). Real-time quantitative (q)PCR was performed with SYBR Green (Bio-Rad, Hercules, CA) and the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). Relative quantification analysis and fold change was performed with all values normalized to levels of a housekeeper gene (cyclophilin). Primer sequences are listed in Supplemental Table S1.

**Statistical Analysis**

For all animal experiments, mice were randomized to treatment groups in order to avoid starting differences in body weight. All plots represent mean +/-SEM. Statistical calculations were carried out in Excel (Micosoft, Redmond, WA) or GraphPad (La Jolla, CA) Prism programs, utilizing ANOVA or Student’s T-test as indications of significance. GTT was analyzed by two-way ANOVA with Uncorrected Fisher’s Least Significant Difference test used to make multiple comparisons between dietary groups. TBARs data were analyzed by ANOVA with Bonferroni’s post hoc test. Gene and protein expression data were normalized to a housekeeper and analyzed by either ANOVA or by Student’s t-test, two-tailed, using Welch’s correction when variance was unequal. To test the effects of diet and tissue type, we used non-metric multidimensional scaling (NMDS) ordination to summarize structure of lipid species. Bray-curtis dissimilarity indices, to reflect
differences in abundances of lipid species, were calculated on log_e(+1)-transformed data. Differences in lipid species structure between diet, tissue type, and their interaction was tested using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations using the adonis function in the vegan package [3] in R [4]. Other statistical analyses are described in the relevant Methods section above. Error bars are SEMs. For all figures, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**Ethical Statement**

All procedures and handling of animals were performed in accordance with the University of Maine’s Institutional Animal Care and Use Committee (IACUC), to comply with the guidelines of the PHS Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals.
Supplemental Figures

Supplemental Figure S1: Body weight gain over time

A. Percent Change in Body Weight

B. Average Body Weight
Supplemental Figure S1: Body weight gain over time. (A) Body weight represented as percent change over time. By week 2 the high n-3 PUFA group had significantly diverged from high SFA and high n-6 PUFA (p=0.0014 and p=0.0065 respectively). By week 16 the change in body weight from the start of diet treatment had further diverged for the high n-3 PUFA animals when compared to high SFA and high n-6 PUFA (p>0.0001 and p=0.0012, respectively). Percent change in body weight was comparable between high SFA and high n-6 PUFA, and within all the low fat diets. (B) Average body weight (in grams) measured weekly for length of dietary treatment. N=6-8 mice per group.
Supplemental Figure S2: Metabolic Cage Measurements (CLAMS)

A. VO2

B. VCO2

C. HEAT

D. RER

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Low n-6 PUFA
Low n-3 PUFA
Supplemental Figure S2: Metabolic cage measurements for low PUFA groups. (A-C) Oxygen consumption (VO₂), carbon dioxide production (VCO₂), and energy expenditure (heat) did not vary between low n-3 PUFA and low n-6 PUFA diet groups. (D) Interaction of time and dietary group was observed for respiratory exchange ratio (RER), indicating a preference for lipid fuel utilization of the low n-3 PUFA animals during the dark phase. Data presented as waveforms of a 24hr cycle with measurements taken over 3 days, analyzed by two-way ANOVA with uncorrected Fisher’s LSD; black bars represent dark phase, N=4 per group. Error bars are SEMs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Supplemental Table S1: Primers used for qPCR

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Reference List


APPENDIX B: Supplemental Material Chapter 3

Supplemental Methods

Von Frey Analysis

A Von Frey mechanical nociceptive assay was performed on BTBR mice ranging from 12-24 weeks of age, to determine tactile sensitivity of hind paw skin, according to protocol described by Feldman et al. 2009. Briefly, each mouse was subjected to five filaments (Semmes-Weinstein evaluators) at varying strengths (4.56, 4.31, 4.08, 3.61, 2.36), which corresponded to specific target forces (4, 2, 1, 0.4, 0.02 – grams of force (g), (Stoelting Co., Wood Dale, IL)). For the Von Frey evaluations, mice were placed on top of a grid platform into individual clear-walled compartments and allowed to acclimate with no stimulus for at least 20 minutes. After the acclimation period, trials began and filaments were applied in order of decreasing strength. Each filament was applied to the mid-plantar surface of the hind paw and slight pressure applied until the mouse showed a response or the filament bent with force. Mouse response was recorded as either positive (immediate paw removal or paw licking when filament is applied), neutral (delayed paw removal), or negative (no reaction), and each filament strength test was performed in 5 cycles.

DRG collection

Animals were euthanized and T13-L1 DRGs were extracted as previously described[1]. Briefly, muscle, fat and soft tissue is cut away from the spinal column; the T13 DRG pair is readily located caudal to the most caudal ribs which were used as orienting landmarks. The spinal column was removed maintaining this orientation, cut along the midline and
the spinal cord was removed in a rostral to caudal direction. Meninges, which cover the ganglia were carefully removed as well. Bilateral T13-L1 DRGs were collected and immediately frozen in liquid nitrogen, for further processing as described in RNA Extraction and Gene Expression section below.

CL Injections

Adult (12-13 week old) male C57BL/6 mice received daily i.p. injections of ADRβ3 agonist CL316,243 (Tocris Bioscience, Bristol, U.K.; Cat # 1499), at 1.0 mg/kg BW or an equivalent amount of sterile saline, for 10-14 days.

Collection of adipose secretions and BDNF ELISA

Inguinal scWAT depots were dissected, weighed and minced in a petri dish containing DMEM (high-glucose, serum-free). Minced tissue was transferred to a 15mL conical tube, with 5mL DMEM (loosely capped to keep tissue oxygenated) and placed in a shaking water bath at 37°C. Secretions were collected at time 0, 1hr, 2hrs, and 3hrs (1mL collected from conical tube at each time point and replaced with 1mL fresh DMEM). Secretions were stored at -80°C until processing. For ELISA, protein secretions were concentrated using Amicon® Ultra Centrifugal Filters, Ultracel®-100K (Millipore, Burlington, MA USA; Cat. # UFC510096), per manufacturer's instructions. Mouse BDNF PicoKine™ ELISA Kit (Boster Biological Technology, Pleasanton, CA, USA; Cat# EK0309) was used per manufacturer’s instruction to determine amount of BDNF present in adipose active secretions.
**BDNF Multiplex ELISA**

BDNF expression in protein lysates of inguinal scWAT from 3-day cold exposed adult (16 weeks old) male and female C57BL/6J was measured using MILLIPLEX MAP Mouse Myokine Magnetic Bead Panel (Millipore, Burlington, MA USA, Cat. # MMYOMAG-74K; MILLIPLEX Magnetic Microspheres Cat. # RBDNF-MAG). Protein lysates were prepared as described under *Western Blotting* (below). The Luminex xMAP MAGPIX® system (Austin, TX, USA) was used to detect BDNF in protein samples, data was analyzed with MILLIPLEX® Analyst 5.1 software (Millipore, Burlington, MA USA) accounting for then 2-fold dilution, then normalized to total protein (previously determined by Bradford Assay).

**AAV Vector Construction and Package**

HA-tagged human BDNF cDNA was subcloned into a novel AAV plasmid of dual cassettes that restricts off-target transduction in liver [2]. The rAAV plasmid contains a vector expression cassette consisting of the CMV enhancer and CBA promoter, WPRE, and bovine growth hormone (bGH) poly-A flanked by AAV2 inverted terminal repeats. Engineered hybrid serotype Rec2 vectors were packaged and purified as described previously [3].

**AAV-BDNF delivery**

16 week old male BTBR MUT mice were injected once in the left inguinal scWAT with $1 \times 10^{10}$ vg of AAV-BDNF, while the right inguinal scWAT received an equal volume injection of vehicle (AAV buffer). Virus was constructed by Dr. Lei Cao as previously described [2]. Animals, were carefully observed and scored for malaise for 48 hours after virus injection, and then observed daily, and showed no adverse reaction to the treatment.
After 2 weeks animals were sacrificed, inguinal scWAT depots were harvested and processed for western blot analysis.

**Mouse adipose tissue collection and analyses; immunostaining**

Mice were euthanized, whole subcutaneous white adipose tissue (scWAT) depots were carefully removed to remain intact depots, and fixed in 2% PFA at 4°C for 4hr-12hrs depending on thickness of tissue. The tissues were then rinsed for 10 minutes with 1X PBS w/ 10u/mL heparin, twice at 4°C. Tissues were incubated in blocking buffer (1XPBS/2.5% BSA/0.5-1% Triton) at 4°C at least overnight but no more than 7 days, depending on tissue size, with blocking buffer replaced every 24hr period. After blocking period, tissues were flattened by being placed between two large glass slides bound tightly together, for at least 30min but no more than 1.5hrs at 4°C. Tissues were next incubated with 0.03% Typogen Black for 20 minutes at room temperature on a rotating platform to minimize autofluorescence. Following Typogen Black incubation, tissues were washed with 1X PBS w/ 10u/mL heparin on rotating platform at 4°C replacing PBS every 1hr for a total of 4-6hrs, or until all unbound stain was removed. Immunostaining of innervation with primary antibodies was performed overnight at 4°C, and the following day tissues were washed with 1XPBS on a rotating platform at 4°C, replacing PBS every 1hr for a total of 4-6hrs followed by incubation with secondary fluorescent antibodies.

Primary antibodies included: PGP9.5 (1:1000, Abcam, Cambridge, U.K. Cat. #10404 and #108986); post synaptic density protein 95 (1:1000-1:5000, Abcam, Cambridge, U.K. Cat. #18258); TH (1:250, Millipore, Burlington, MA USA; Cat. # AB152); NAv1.8 (1:500, StressMarq, Victoria, BC, Canada; Cat #SMC-342D); neurofilament-M (2H3, 1:500),
beta-3 tubulin, (6G7, 1:250) and synaptic vesicles (SV2, 1:250) from Developmental Studies Hybridoma Bank, (University of Iowa, USA). Secondary antibodies included Alexa Fluor 488 at 1:500 and Alexa Fluor 594 at 1:1000 from Molecular Probes. For vascular autofluorescence visualization tissues were not washed in 1X PBS w/ 10u/mL heparin, prior to further immunostaining. For vascular staining, tissues were incubated with isolectin IB4 stain conjugated to Alexa 594 (ThermoFisher Scientific, Waltham, MA, USA; Cat # I21413) at 1μg/mL concentration overnight at room temperature. Tissues were washed in 1X PBS and mounted on slides. Images were acquired using Nikon Eclipse E400 epiflorescent microscope, Nikon A.1 confocal microscope (Nikon, Minato, Tokyo, Japan) or Leica TCS SP8 (Leica Microsystems, Wetzler, Germany) digital lightsheet/confocal microscope.

Neuromuscular junction immunofluorescence, imaging, and analysis

Following protocols provided by Greg Cox and Robert Burgess at Jackson Laboratory, mice were euthanized, both soleus and medial gastrocnemius tissues were carefully removed and fixed in a 2% PFA at 4°C for 2 hours. The tissues were then gently rinsed with 1XPBS and incubated in blocking buffer (1XPBS/2.5%BSA/0.5-1%Triton) at 4°C for at least 24 hours but no more than 7 days. After blocking period muscles were teased, tendons and fat were removed and muscle tissue was flattened by being placed between two tightly-bound glass slides for at least 30 minutes at 4°C. Tissues were next transferred to fresh blocking buffer at 4°C for at least 12 hours, but no more than 7 days. Immunostaining of innervation with primary antibodies was performed overnight at 4°C, the following day tissues were washed with 1XPBS on a rotating platform at 4°C replacing PBS every 1hr for a total of 4-6hrs. Tissues were then incubated with secondary
fluorescent antibodies overnight and washed again in 1XPBS on a rotating platform at 4°C replacing PBS every 1hr for a total of 4-6hrs. Primary antibodies included: neurofilament-M (2H3, 1:500) and synaptic vesicles (SV2, 1:250) from Developmental Studies Hybridoma Bank, (University of Iowa, USA). Secondary antibodies included: Alexa Fluor 488 at 1:500 (A21121) and alpha-bungarotoxin (BTX)-conjugated to Alexa Fluor 594 at 1:1000 (B13423) from Molecular Probes (Eugene, OR, USA). Tissues were next mounted on microscope slides using Millipore mounting fluid (Burlington, MA USA; Cat. # 5013) and 1 1/5 coverslips then sealed and allowed to set overnight. All images were acquired with Nikon Eclipse E400 epiflorescent microscope using an integrated ‘Real Time Manual EDF’ acquisition tool in Nikon Elements Software (Nikon, Minato, Tokyo, Japan). Insert images were cropped in FIJI. Brightness, contrast, and sharpness were adjusted in Microsoft PowerPoint. Up to 100 NMJs were counted for each tissue and statistics were conducted in GraphPad PRISM software (La Jolla, CA, USA) using the multiple t-tests (one-per row) function.

RNA Extraction and Gene Expression

Total RNA was isolated from tissues using a Trizol reagent (Zymo, Irvine, CA, USA; Cat. # R2050-1-200), Bullet Blender for lysis, and Zymo Miniprep kit (Zymo, Irvine, CA, USA; Cat. # R2052). RNA yield was determined on a Nanodrop and cDNA synthesized using High Capacity Synthesis Kit (Applied Biosystems, Foster City, CA, USA; Cat# 4368813). Real-time quantitative (q)PCR was performed with SYBR Green (Bio-Rad) and CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Relative quantification analysis and fold change was performed with all values normalized to levels of a housekeeper gene (cyclophilin). Primer sequences are listed in Supplemental Table S4.
**Western Blotting**

Protein expression was measured by western blotting analysis of tissue lysates. Whole adipose depots were homogenized in RIPA buffer with protease inhibitors in a Bullet Blender, followed by Bradford Assay, and preparation of equal-concentration lysates in Laemmlli buffer. 60ug was loaded per lane of a 10% polyacrylamide gel, and following gel running proteins were transferred to PVDF membranes for antibody incubations. Tyrosine hydroxylase (TH) antibody (Millipore Cat. # AB152) at 1:1000 dilution was used with both mouse and human protein lysates. Anti-PGP9.5 antibody (Abcam Cat. #ab10404 and #ab108986) was used at a 1:1000 and 1:500 dilutions respectively, were used with both mouse and human protein lysates. Anti-PSD95 antibody (Abcam Cat. #ab18258) at 1:750 dilution was used with both mouse and human protein lysates. TH, PGP9.5, and PSD95 protein expression was normalized to one of the following housekeeping proteins, all at 1:1000 dilutions: β-tubulin (Cell Signaling Technology, Danvers, MA, USA; Cat. # 2146BC), β-actin (Abcam, Cambridge, U.K.; Cat. # ab8227), cyclophilin B (Abcam, Cambridge, U.K.; Cat. #ab16045). Secondary antibody anti-rabbit HRP-linked (Cell Signaling Technology, Danvers, MA, USA; Cat. # 7074) at 1:3000 dilution was used for conjugation with all primaries. Blots were visualized on a Syngene G:BOX (Frederick, MD, USA). Ponceau S staining was performed after immunoblotting was complete using Ponceau S Solution (Sigma-Aldrich, St. Louis, MO, USA; Cat. # P7170-1L).
Cold Exposure Experiments

All cold exposure was carried out in a diurnal incubator (Caron, Marietta, OH, USA) at 5°C. Animals were housed two to a cage and continuously cold exposed for 3 - 14 days.

For whole adipose innervation imaging, 18-22 week old control male mice on a mixed genetic background were housed either at room temperature, cold exposed for 10 days, or cold exposed for 10 days and returned to room temperature for 1 week (‘rewarmed’).
Supplemental Figure S1 – Adiposity & neuropathy of BTBR ob/ob mutant (MUT) mice.

a. Body Weight (Males)  Body Weight (Females)  scWAT/Body Weight (Males)  scWAT/Body Weight (Females)

b. Von Frey (Females)

c. Inguinal scWAT PGP9.5

d. Inguinal scWAT TH

e. Ponceau Stain for Total Protein in BTBR ob/ob Inguinal scWAT

f. Interscapular Brown Adipose Tissue

Male

Female

HET  MUT  WT

HET  MUT  WT

HET  MUT  WT

HET  MUT  WT

g. BTBR ob/ob Gene Expression iBAT

h. BTBR ob/ob Gene Expression Inguinal scWAT

i. BTBR ob/ob Gene Expression prWAT
**Supplemental Figure S1: Adiposity & Neuropathy of BTBR ob/ob mutant (MUT) mice.** Male and female BTBR MUT mice were assessed for total body weight and adiposity (inguinal scWAT/body weight), and compared to WT or HET animals in a pilot cohort (a). Female BTBR WT, HET, and MUT were assessed for tactile allodynia via the Von Frey assay, an indirect measure of peripheral neuropathy (b). Protein expression of PGP9.5 (c) and TH (d) in inguinal scWAT of HET, MUT, and WT BTBR mice was measured via western blotting. For (a-d), males: MUT N=2; WT N=3; HET N=3; females: MUT: N=1; WT: N=3; HET: N=3; all mice were 12-24 weeks old. Data represents a pilot cohort to compare HET to MUT mice, and males to females, thus statistical analyses were not performed due to small sample sizes. Sample Ponceau S staining to demonstrate equal protein loading (e). Whole BAT depots were compared between female and male BTBR HET, MUT, and WT mice (f). Images are representative, males: MUT N=2; WT N=3; HET N=3; females: MUT N=1; WT N=3; HET N=3; all mice were 12-24 weeks old. Gene expression analysis of BAT, inguinal scWAT, and prWAT (g-i). Gene expression data were analyzed by two-tailed Student’s t-test, using Welch’s correction when variance was unequal, N=4-5 per group. Error bars are SEMs.
Supplemental Figure S2 – Adipose neuropathy of BTBR ob/ob mutant (MUT) mice.

a. Immunofluorescent Staining of NMJs

b. NMJs of MG and SOL

- WT MG
- MUT MG
- WT SOL
- MUT SOL

0 50 100
% Total NMJs

Occupied Partially Occupied Unoccupied
Supplemental Figure S2: Adipose neuropathy of BTBR ob/ob mutant (MUT) mice.

Neuromuscular Junction (NMJ) Analysis (a-b). Immunofluorescent staining of male BTBR WT and MUT neuromuscular junctions of the medial gastrocnemius (MG) and soleus (SOL) muscles was performed using neurofilament M (2H3) and synaptic vesicles (SV2) (in green) to visualize the pre-synaptic area, and α-bungarotoxin (in red) to visualize the post-synaptic area. Representative images at 40x magnification of BTBR ob/ob wild-type (WT) medial gastrocnemius (MG) (top left panel), and soleus (bottom left panel). Mutant (MUT) MG (top right panel) and SOL (bottom right panel) (a). Inserts are of occupied (left panels) and partially occupied (partially occupied) NMJs in representative images (a). Percent of total NMJs for WT and MUT animals in both MG and SOL muscles was calculated as an indicator of neuropathic state (b). Analysis shows multiple t-tests (per row) of replicate cohorts; N=7 for WT MG, N=6 for MUT MG, N=7 for WT SOL, and N=4 for MUT SOL. Error bars are SEMs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Supplemental Figure S3 – Human scWAT cell size and omental adipose innervation

a. Average Cell Diameter of Human scWAT Correlated to BMI and Age

R²=0.007700  
p=0.6447

R²=0.03984  
p=0.3498

b. Cell Diameter of scWAT Correlated to PGP9.5 When Assessed by BMI (Fig. 1k)

R²=0.0188  
p=0.7246

R²=0.0020  
p=0.9159

c. Human Omental PGP9.5

R²=0.06483  
p=0.5816

d. Human Omental PGP9.5

R²=0.03939  
p=0.6697
Supplemental Figure S3. Human scWAT cell size and omental adipose innervation. Average cell diameter in scWAT of human samples when assessed by BMI or age (a), and normalized protein expression plotted against average cell size for either BMI or age cohorts (b), corresponds to western blot data in Fig. 1g-h. Cell diameter measured from images of histological cross-sections of adipose tissue from each patient, averaged (n=3), and analyzed by linear regression. Human omental adipose samples were analyzed for protein expression of PGP9.5 via western blotting (c-d). Linear regression was performed with respect to BMI (c) or age (d). Protein expression was normalized to β-Actin and band intensities were quantified in Image J. Due to limitations of available samples from the BNORC adipose tissue core at Boston Medical Center, the BMI distribution of omental adipose was clustered around 40. Error bars are SEMs.
Supplemental Figure S4 – Body weight, inguinal scWAT, and BAT innervation of young/aged and sedentary/exercised (Run) mice

a. Body Weight, Inguinal scWAT/Body Weight, pgWAT/Body Weight, Quad Weight

b. Inguinal scWAT

PGP9.5

p=0.5802

PGP9.5

β-Actin

Young Run

Aged Run

c. Inguinal scWAT

TH/Cyclophilin B

p=0.6327

TH/Cyclophilin B

Tyrosine Hydroxylase

Cyclophilin B

Young Run

Aged Run

d. iBAT PGP9.5

PGP9.5/β-Tubulin

p=0.4054

PGP9.5

β-Tubulin

Young Sed

Young Run

e. iBAT TH

TH/Cyclophilin B

p=0.7408

TH/Cyclophilin B

Cyclophilin B

Young Sed

Young Run

f. iBAT PSD95

PSD95/β-Tubulin

p=0.3767

PSD95

β-Tubulin

Young Sed

Young Run

g. Gene Expression iBAT

Fold Change

iBAT

Scn10

Bdnf

Synaptotagmin

Synaptophysin

Phyn1

pde5a

Usp17

Vegfr1

Cd1

Young Sed

Young Run

207
Supplemental Figure S4: Body weight and inguinal scWAT innervation of young/aged and sedentary/exercised (run) C57BL/6J mice. Body weight, adiposity (inguinal scWAT/body weight & pgWAT/body weight), and quadricep muscle weight was measured for young (12 weeks old) and aged (16 month old) mice under sedentary (sed) and exercised (run) conditions (a). Young and aged sedentary (sed) groups, N=4; young and aged exercised (run) groups N=5. Body and tissue weight analyzed by one-way ANOVA, with Tukey post hoc, groups labeled with the same letter (A or B) are statistically similar, for body weight: $p=0.0473$ for young sed v. aged sed; $p=0.0240$ for young run v aged run. Protein expression PGP9.5 (b) and TH (c) in inguinal scWAT of young run versus aged run mice was determined by western blotting. Protein expression was normalized to β-Actin or Cyclophilin B; band density was quantified in Image J and analyzed by two-tailed Student's t-test. Protein expression of PGP9.5 (d), TH (e), and PSD95 (f) in BAT of young (12 week old) sedentary (young sed) versus young exercised (young run) mice was determined by western blotting. Protein expression was normalized to β-tubulin or cyclophilin B; band density was quantified in Image J and analyzed using a two-tailed Student's t-test. Gene expression analysis of BAT from young (12-15 week old) sedentary (young sed) versus young exercised (young run) male mice (g), gene expression was analyzed by two-tailed Student's t-test, N=4 for sedentary and N=6 for run. Error bars are SEMs.
Supplemental Figure S5 – Innervation of vasculature in young sedentary/exercised (Run) mice

a. Body Weight

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p = 0.3743

b. Axillary scWAT

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p = 0.1133

Gene Expression Inguinal scWAT

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Specific Innervation of Blood Vessels (≥50um)

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Specific Innervation of Blood Vessels (≥50um)

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p = 0.7341

# of Nerves per Blood Vessel

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Body Weight

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p = 0.1175

Axillary scWAT

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p = 0.3743
Supplemental Figure S5: Innervation of vasculature in young sedentary/exercised (run) mice. Body weight, adiposity (inguinal scWAT/body weight & pgWAT/body weight), and quadricep muscle weight was measured for young (12-15 weeks old) mice under sedentary (sed) and exercised (run) conditions (a). Body and tissue weight analyzed by one-way ANOVA with Tukey post hoc. N=4 for sedentary and N=5 for run groups. Protein expression for PSD95 in axillary scWAT was determined by western blotting. Protein expression was normalized to β-Tubulin; band density was quantified in Image J and analyzed by two-tailed Student’s t-test. Gene expression analysis of inguinal scWAT from young (12-15 week old) sedentary (young sed) versus young exercised (young run) male mice (g), gene expression was analyzed by two-tailed Student’s t-test, N=4 for sedentary and N=5 for run. Error bars are SEMs. Whole inguinal scWAT depots were collected from young C57BL6/J mice under sedentary and exercised (run) conditions. Tissue was stained with PGP9.5 (green) and Isolectin lb-4 (red) to analyze nerve and blood vessel interactions in WAT with exercise. Tissues were scanned at 10x for blood vessels 50um or greater in diameter. Up to twenty blood vessels were evaluated for innervation in each tissue (d-e). Percentage of innervated vessels with a diameter of ≥ 50um were evaluated per tissue (d, left panel). Analyzed with two-tailed Student’s T-Test. Error bars are SEMs. Innervation was further characterized by the number of resident nerves per blood vessel (d, right panel). Data was analyzed using a two-way ANOVA and Tukey’s multiple comparison test. Error bars are SEMs. Fluorescent imaging of blood vessel and nerve interactions at 4x, 10x, and 40x using an Nikon E400 epifluorescent microscope (e).
Supplemental Figure S6 – AAV mediated BDNF delivery to inguinal scWAT of BTBR MUT mice

a. Inguinal scWAT TH

b. Inguinal scWAT PSD95

$\text{TH}/\text{Cyclophilin B}$

$\text{PSD95}/\beta$-Tubulin

$p=0.6234$

$p=0.7007$
Supplemental Figure S6 – AAV mediated BDNF delivery to inguinal scWAT of BTBR MUT mice. Adult (16 week old) male BTBR MUT mice (N=3) received single injection of AAV-BDNF (1x10^10 vg) into their left inguinal scWAT and an equal volume of vehicle into their right inguinal scWAT. Two weeks post-injection, protein expression of TH (a) and PSD95 (b) in inguinal scWAT was measured by western blotting. Protein expression was normalized to cyclophilin or β-tubulin, band intensity was quantified in Image J and analyzed by two-tailed Student's T-Test. Error bars are SEMs.
Supplemental Figure S7 – Adipose innervation: Sex and depot comparison

a. Cold Exposed
Inguinal scWAT v. iBAT TH

p=0.0011
p=0.0019
n.s.

b. Cold Exposed
Inguinal scWAT v. iBAT PSD95

p=0.0039
p=0.0004
n.s.

C.

Cold Exposed Axillary v. Inguinal scWAT PGP9.5

p=0.2676

Sexes Combined
PGP9.5
Cyclophilin B

F M F M

Axillary Inguinal

D.

Cold Exposed
Axillary v. Inguinal scWAT TH

p=0.0009
p=0.0141
n.s.

Sexes Combined
TH

F M F M

Axillary Inguinal

E.

Cold Exposed
BDNF in Inguinal scWAT

p=0.0004

Male Female
Supplemental Figure S7: Adipose innervation: Sex and depot comparison

Adult (16 week old) male and female control mice on a C57BL/6J background were cold exposed (5°C) for 3 days. Protein expression of TH (a) and PDS95 (b) were measured in inguinal scWAT and BAT via western blotting. Protein expression of PGP9.5 (c) and pan-neuronal marker TH (d) was measured in axillary and inguinal scWAT for both sexes via western blotting. Protein expression was normalized to β-Tubulin or Cyclophilin B, band density was quantified in Image J and analyzed using a two-tailed Student’s t-test. Error bars are SEMs. BDNF expression in inguinal scWAT was measured for both sexes via multiplex ELISA assay (e).
Supplemental Table S1 - Human Cohort Data by Age

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1 Metabolic data from patients whose adipose tissues were assessed for innervation, data sorted by ascending age BMI = body mass index; BG Rand = random blood glucose; HDL = high density lipoprotein; LDL = low density lipoprotein; HgBA1c = hemoglobin A1c.
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<td>1g, S2c (BMI)</td>
<td>sqWAT</td>
<td>F</td>
<td>Panniculectomy</td>
<td>Hispanic</td>
<td>Non-diabetic</td>
</tr>
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<td>1g, S2c (BMI)</td>
<td>sqWAT</td>
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<td>African American</td>
<td>Non-diabetic</td>
</tr>
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<td>1h, S2c (age)</td>
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<td>1g, S2c (BMI)</td>
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<td>F</td>
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<tr>
<td>1g, S2a-b, c (BMI)</td>
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<td>F</td>
<td>Gastric Bypass</td>
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<td>Non-diabetic</td>
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<td>F</td>
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<td>F</td>
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<td>Non-diabetic</td>
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</table>

---

2 Patient tissue type analyzed, sex, elective surgical procedure, race or ethnicity, and diabetic state are presented along with which figure samples were used in. sqWAT = subcutaneous white adipose tissue; BMI = body mass index; F = female; M = male.
Supplemental Table S3 – Human Cohort Data Total Statistics

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<thead>
<tr>
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<th>Percent of Total</th>
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<tr>
<td><strong>Gender</strong></td>
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<td>Male</td>
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<tr>
<td>Female</td>
<td>95</td>
</tr>
<tr>
<td><strong>Ethnicity/Race</strong></td>
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<td>Hispanic</td>
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<tr>
<td>African American</td>
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<td>White</td>
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<tr>
<td>Unknown</td>
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<tr>
<td><strong>Age</strong></td>
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</tr>
<tr>
<td>20-29</td>
<td>15</td>
</tr>
<tr>
<td>30-39</td>
<td>45</td>
</tr>
<tr>
<td>40-49</td>
<td>25</td>
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<tr>
<td>50-59</td>
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<td>60+</td>
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<tr>
<td><strong>Menopausal</strong></td>
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<tr>
<td>Unknown</td>
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</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td></td>
</tr>
<tr>
<td>Pre-diabetic</td>
<td>10</td>
</tr>
<tr>
<td>Diabetic</td>
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<tr>
<td>Nondiabetic</td>
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<tr>
<td><strong>BMI</strong></td>
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<tr>
<td>Normal: &lt;25.0</td>
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<tr>
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<tr>
<td>Class I: 30.0-34.9</td>
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<tr>
<td>Class II: 35.0-39.9</td>
<td>20</td>
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<tr>
<td>Class III: &gt;40.0</td>
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<tr>
<td><strong>Obese</strong></td>
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<tr>
<td>Panniculectomy</td>
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<tr>
<td>Gastric Bypass</td>
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<tr>
<td>Gastrectomy Sleeve</td>
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</table>

Summarized statistics are shown for all patients in the study. Numbers represented as percent of total.
### Supplemental Table S4 - qPCR Primers

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<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<td>bdnf</td>
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<td>cyclophilin</td>
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<td>AAGACCACATGCTTGCCAT</td>
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<td>AGAAGCCGACGGGAGGAGAGAT</td>
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<tr>
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<td>AGC GGA AGT GAC CCA TGA CGT</td>
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<td>ACAGAGAGGGGCAGGCGAGT</td>
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<td>vip</td>
<td>AGTGTGCTGTCTCTCAGTGC</td>
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</tbody>
</table>
References


BIOGRAPHY OF THE AUTHOR

Magda was born in a deep and dark, green forest called NYC. It smelled like humanity. She misses that in balsam scented Maine. It’s complex. From there, Magda wandered around the forest seeking the ethereal things that didn’t smell as they seemed. Those things piqued her curiosity and spoke to her soul. This was Art.

Art asked the question of how things exist. Delirium made her think she could answer it, if she talked to Science. So, she studied Science. It was cryptic. And difficult. It argued with her for a long time until she realized that she could argue back. Science showed her doorways that she could access if she had the courage to ask the right questions.

When she chose a doorway, it wasn’t like it was before. She didn’t go in or out. She could choose another doorway if she wanted to. She could go back and forth until she found the right path. Science told her there was not just one direction. If you make a choice, it doesn’t have to be the final one. Your possibilities are endless as long as you have the courage to admit what you know and what you don’t.

Magda is a candidate for the Doctor of Philosophy degree in Biomedical Science from the University of Maine in May 2019.