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Anatomical Organization and Distinction of the Sympathetic Inputs to Interscapular Brown Adipose Tissue and Inguinal White Adipose Tissue in the Mouse

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ANATOMICAL ORGANIZATION AND DISTINCTION OF THE SYMPATHETIC INPUTS TO INTERSCAPULAR BROWN ADIPOSE TISSUE AND INGUINAL WHITE ADIPOSE TISSUE IN THE MOUSE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Biological Sciences

by Clara Jane Huesing
B.S., Franciscan University, 2015
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# Table of Contents

Acknowledgements ........................................................................................................... ii

List of Acronyms ................................................................................................................ iv

Abstract ............................................................................................................................... vii

Chapter 1. Introduction ........................................................................................................ 1
   1.1. Characterization of adipose tissue ........................................................................ 1
   1.2. The peripheral nervous system connection to adipose end-organs .................... 7
   1.3. The role of the sympathetic nervous system in adipose tissue ......................... 8
   1.4. Research focus ....................................................................................................... 11

Chapter 2. Sympathetic Innervation of Interscapular Brown Adipose Tissue in the Mouse .................................................................................................................... 13
   2.1. Introduction ............................................................................................................ 13
   2.2. Materials and methods ......................................................................................... 14
   2.3. Results ................................................................................................................... 19
   2.4. Discussion ............................................................................................................. 26

Chapter 3. Sympathetic Innervation of Inguinal White Adipose Tissue in the Mouse .... 31
   3.1. Introduction ............................................................................................................ 31
   3.2. Materials and methods ......................................................................................... 33
   3.3. Results ................................................................................................................... 38
   3.4. Discussion ............................................................................................................. 49

Chapter 4. Summary, Outlook, and Significance ................................................................. 60
   4.1. Research summary ............................................................................................... 60
   4.2. Outlook ................................................................................................................. 67
   4.3. Significance of research ....................................................................................... 71

Appendix A. Copyright Information for Chapter 2 .............................................................. 73

Appendix B. Supplemental Data for Chapter 2 ................................................................. 79

Appendix C. Supplemental Data for Chapter 3 ................................................................. 81

Reference List .................................................................................................................... 85

Vita ................................................................................................................................... 103
List of Acronyms

5-HT: 5-hydroxytryptamine

ACFN: anterior cutaneous femoral nerve

AgRP: Agouti-related peptide

ANS: autonomic nervous system

ATGL: adipose triglyceride lipase

BAT: brown adipose tissue

CA: central autonomic nuclei

cAMP: cyclic adenosine monophosphate

CG: celiac ganglion

CGRP: calcitonin gene-related peptide

CNS: central nervous system

DBE: dibenzyl ether

DCM: dichloromethane

DRG: dorsal root ganglion

EGFP: enhanced green fluorescent protein

EE: energy expenditure

FFA: free fatty acid
GFP: green fluorescent protein

HSL: hormone sensitive lipase

iBAT: interscapular brown adipose tissue

ICl: intercalated nucleus

ILI: ilioinguinal

IML: intermediolateral nucleus of the spinal cord

iWAT: inguinal white adipose tissue

LCFN: lateral cutaneous femoral nerve

MAG: monoacylglycerol

MeOH: methanol

NE: norepinephrine

NST: non-shivering thermogenesis

PAPy: parapyramidal area

PKA: protein kinase A

POMC: proopiomelanocortin

PNS: peripheral nervous system

PRV: pseudorabies virus

PRV\textsuperscript{GFP}: pseudorabies virus-encoding green fluorescent protein
rRPA: rostral raphe pallidus

SC: spinal cord

SChG: sympathetic chain ganglion

SNS: sympathetic nervous system

β3-AR: beta-3 adrenoreceptor

TAG: triacylglycerol

TH: tyrosine hydroylase

UCP1: uncoupling protein 1

WAT: white adipose tissue
Abstract

Adipose tissue is a dynamic endocrine organ that contains both the problem and the solution to discovering new therapies for individuals with obesity. Excessive adipose tissue disrupts energy balance and causes a host of comorbidities like insulin resistance and hypertension. On the other hand, adipose tissue is essential for life. During periods of energy demand, sympathetic nerve activity in brown and white adipose tissue stimulates thermogenesis and lipolysis; both of which decrease body weight and improve metabolic health. Therefore, comprehensive insight into the organization of sympathetic innervation and how it communicates with adipocytes is critical in order to utilize its capacity to reduce body weight and improve metabolic health. Recent literature enhances the confusion about the neuronal origin of postganglionic sympathetic innervation, and little is known about preganglionic inputs. Here we inject a retrograde transsynaptic tracer, pseudorabies virus (PRV), into the right interscapular brown adipose tissue (iBAT) pad and the inguinal WAT (iWAT) pad of mice to label pre- and postganglionic neurons that innervate iBAT and iWAT, respectively. To ensure a thorough investigation into the sympathetic structures, we established a whole-body tissue clearance method combined with a double immunohistological detection to identify sympathetic nerves and adipose tissue-related sympathetic circuits. Three-dimensional images generated with light sheet microscopy allowed for an unprecedented view of abdominal sympathetic structures. iBAT postganglionic innervation was found to be restricted to sympathetic chain ganglia (SChG) from the stellate ganglion (SG) to thoracic ganglion 5 (T5), while preganglionic innervation was found from T1-T5. iWAT postganglionic innervation was found at T12 to lumbar
ganglion 1 (L1), while preganglionic innervation was found from T7-T10. Importantly, PRV labeling was not identified in the prevertebral celiac ganglia. These novel observations demonstrate that the population of pre- and postganglionic neurons innervating iBAT and iWAT are distinct from one another.
Chapter 1. Introduction

1.1. Characterization of adipose tissue

1.1.1. Evolution of adipose tissue

The field of adipose tissue research has seen tremendous advancements within the last 50 years, likely due in large part to the increased prevalence of obesity and metabolic syndrome in humans. As the community at-large struggles to identify an efficacious treatment for obesity, it has become clear that adipose tissue is much more intricate than once hypothesized\textsuperscript{1-8}. In fact, forms of adipose tissue can be observed across the taxonomic ranks. For this reason, researchers study the phylogenetics of adipose tissue with the idea that the evolution of adipose tissue can help answer questions that prevail in the field today.

Indeed, studies have provided intriguing findings about the similarities in whole-body metabolic regulation between invertebrates and vertebrates. Numerous species of invertebrates possess specialized tissues that regulate metabolism, the most thoroughly investigated being the “fat body.” Reminiscent of vertebrate adipocyte function, the insect fat body stores lipids as triacylglycerol (TAG), and releases their breakdown products when demand is signaled from other tissues\textsuperscript{9,10}. Similar to adipose tissue, the fat body has a role in a variety of biological functions that include nutrition, reproduction, longevity, and production of antimicrobial peptides\textsuperscript{10}. Additionally, two significant signaling molecules in metabolism, Neuropeptide Y and insulin, have also been identified in invertebrates\textsuperscript{11-13}. Interestingly, the invertebrate sea squirt, \textit{Ciona}, has most of the signals and receptors implicated in appetite and energy storage in mammals\textsuperscript{14,15}. 
Although it is evident that core similarities between the fat body and adipose tissue exist, it is believed that adipocytes appeared during early vertebrate evolution, indicating that the rapid mobilization of the lipid droplet in adipocytes, otherwise known as lipolysis, is unique to vertebrates\(^9\). Additionally, sympathetic nervous system (SNS) innervation of adipose tissue is a potent regulator of lipolysis, a neural system that invertebrates either have a primitive form of, or do not possess at all\(^{15}\).

Differences within the vertebrate subphyla exist, too. Endothermy, endogenous heat production, is an evolved capability that allows organisms to self-regulate in order to achieve appropriate physiological body temperature. Only mammals and birds are universally endothermic, though some species of fish are capable of endothermy. Shivering through muscular exertion and uncoupled oxidative metabolism that occurs in brown adipose tissue are examples of endothermy. These two functions are achieved from the mobilization of fatty acids out of energy stores located in the adipocyte and thus, underscores the critical importance of adipose tissue in vertebrates.

Moreover, phasic obesity is a critical physiological behavior exhibited in mammals, bears for example. Similar to pathological obesity seen in humans, both arise from overeating. During periods of obesity, animals become hyperphagic and sometimes sedentary\(^{15}\), leading to decreased sympathetic tone in white adipose tissue (WAT) and increased SNS outflow to brown adipose tissue (BAT)\(^{16}\). However, this type of obesity is transient, controlled, and followed by prolonged periods of fasting\(^{15}\). During fasting, sympathetic tone in WAT increases because norepinephrine (NE) release increases in response to acute bouts of starvation\(^{17}\), causing mobilization of lipid stores into tissues like muscle\(^{18}\). Additionally, intense locomotor activity can be observed after the
hyperphagia, acting in concert with the fasting period, leading to weight loss\textsuperscript{15}. Given the bouts of increased locomotor activity and fasting after prolonged hyperphagia, the natural cycles of obesity in wild animals never leads to diabetes or cardiovascular disease\textsuperscript{15}, making it an ideal reference for information about structure, composition, neural, and endocrine activity in metabolically healthy adipose tissue. Taken together, it is evident that adipose tissue is critical to sustaining life, as its presence is conserved across the evolutionary spectrum.

1.1.2. Adipose tissue location, type, morphology

Adipose tissue is a complex endocrine organ that exists as subcutaneous (superficial) and visceral (deep) depots throughout the body\textsuperscript{8}. In adult rodents, subcutaneous adipose tissue is divided as either anterior or posterior. Anterior subcutaneous adipose tissue is situated at the level of the scapulae and divided into distinct depots: interscapular, subscapular, axillary, and cervical. Posterior subcutaneous adipose tissue is formed by three depots: dorso-lumbar, inguinal, and gluteal. The dorso-lumbar and inguinal depots are commonly treated as one depot and referred to as inguinal white adipose tissue (iWAT) and thus, will be referred to as such herein. The primary visceral depots are contained within the abdomen and are categorized as mediastinic, mesenteric, perigonadal, perirenal, and retroperitoneal.

Additionally, adipose tissue is further classified by color; brown, white, or beige (Fig.1.1). BAT consists of ellipsoid-shaped brown adipocytes (ranging from 15-50µm in diameter) which contain numerous, small lipid droplets (Fig.1.1.A)\textsuperscript{8}. Brown adipocytes
Figure 1.1. Cellular morphology reveals three adipose tissue types. After a 7-day cold challenge (10°C), adipose depots were collected and paraffin-embedded for sectioning (5µm thickness). In order to visualize morphology, tissues were stained with hematoxylin and eosin (H&E). iBAT contains almost entirely brown adipocytes, as evidenced by the multilocular cells, which results in an increase in the amount of plasma membrane (dense pink stain) (A). B) depicts a population of white adipocytes in iWAT, as evidenced by the large lipid droplet (absence of stain) and the nuclei pushed to the perimeter of the cell (blue stain). A population of beige adipocytes was located in the same iWAT fat pad as the white adipocytes in (B). Beige adipocytes can be identified by their heterogeneous appearance. There is a presence of brown adipocytes (dense pink stain) as well as white adipocytes (absence of stain) interspersed amongst each other (C). Confocal microscopy (5x objective) was used to capture images. Both adipose depots (iBAT, iWAT) were sourced from the same mouse.

are rich with mitochondria that contain uncoupling protein 1 (UCP1), the key molecule responsible for nonshivering thermogenesis (NST).
White adipose tissue is comprised of spherical adipocytes (ranging from 20-160µm in diameter depending on location) that are almost entirely occupied by a large lipid droplet, causing the nucleus and its few mitochondria to be located along the periphery of the cell (Fig. 1.1.B)\(^8\).

Within the last decade, evidence has demonstrated that when exposed to certain stimuli (e.g. SNS activation), WAT has the capability to transition to acquire BAT–like features, otherwise known as a beige or brite adipocyte\(^{19,20}\). More specifically, beige adipocytes are located amongst WAT and are purported to derive from precursors of white adipocytes\(^{21}\), but they have a different transcriptional program than white adipocytes\(^{22}\). Like BAT, beige adipocytes are thermogenic; they possess abundant mitochondria and have a multilocular lipid droplet morphology (Fig. 1.1.C)\(^{23}\). It should be noted that the cellular origin of beige adipocytes continues to be a topic of debate\(^{22,24-27}\).

The relative amount of each fat tissue type is influenced by a variety of factors like: body size and composition, metabolic rate, eating habits, nutrient selection, and physical activity. To add, the amount of each tissue type influences its overall functional capacity, and thus, one’s overall metabolic health.

1.1.3. Functions of adipose tissue

BAT’s principal function is heat production through NST, a form of heat production not associated with muscle activity, but nevertheless burns calories. The unique thermogenic capacity of BAT comes from the large number of mitochondria and high expression of UCP1 present in brown adipocytes. UCP1 is activated by free fatty acids (FFAs), increasing the conductance of the inner mitochondrial membrane\(^{28}\) and because anions on FFAs cannot dissociate from UCP1, the uncoupler acts as a H\(^+\)
carrier, causing mitochondria to generate heat instead of ATP\textsuperscript{29}. In order to regulate body temperature, human infants have prominent depots of brown fat that dissipate with age\textsuperscript{20}, whilst small rodents maintain their large depots throughout their life\textsuperscript{30}. Adult humans with increased amounts of BAT are leaner and the presence of brown fat counteracts obesity and metabolic syndrome in rodents\textsuperscript{26}, further underscoring the potency of BAT.

WAT is extensively distributed throughout the body and accumulates ectopically in situations of adipose dysfunction\textsuperscript{31}. Not only is WAT a depot for lipid storage, but it also has the capacity to undergo lipolysis and control energy homeostasis through secretion of leptin and a host of other molecules\textsuperscript{32-35}. Similar to WAT, beige adipocytes possess very low levels of UCP1. However, upon stimulation, UCP1 levels in beige fat can be induced to a level that is thermogenically functional. Shabalina \textit{et al} (2013) found that chronic cold exposure (4 weeks at 4°C) did not result in significant differences in mitochondrial respiratory chain protein levels between iBAT and beige fat extracted from iWAT. Given the high capacity of the respiratory chain found in BAT, a similar level found in beige fat provides evidence for UCP1 function. Under the same conditions, rates of oxidative phosphorylation in mitochondria did not differ between iBAT and beige fat, indicating that the UCP1 expression in beige fat has an inherent potential for NST\textsuperscript{21}. Therefore, it has been theorized that stimulating the development of beige adipocytes could alleviate the effects of excessive WAT and improve metabolic health\textsuperscript{36}. 
1.2. **The peripheral nervous system connection to adipose end-organs**

The major growth in the field of adipose biology has revealed numerous bioactive peptides secreted and expressed by adipose tissue\textsuperscript{37}. Most notably, the identification of leptin, a hormone almost exclusively secreted from adipocytes\textsuperscript{38}, has validated the importance of adipose tissue in proper metabolic function. In 1994, Dr. Jeffrey Friedman’s lab identified that the voracious appetite and subsequent obesity observed in \textit{ob/ob} mice was caused by a mutation in the gene encoding for leptin, causing an inability to produce the hormone\textsuperscript{39}. When \textit{ob/ob} mice are treated with exogenous leptin, significant weight loss and reversal of metabolic defects were observed\textsuperscript{40}. Unfortunately, leptin treatment is not an effective weight loss treatment in animals or humans that properly encode for leptin because leptin levels are in direct proportion to adipose tissue mass, and can actually create leptin resistance in obese individuals\textsuperscript{40,41}. Although \textit{ob/ob} mice are only one representative model of human obesity, they are critical for studies that aim to understand how leptin regulates food intake, metabolism, and obesity as the effects of leptin resistance (or surplus of leptin) can mimic those of leptin deficiency.

In metabolically healthy individuals, leptin functions as a metabolic signal of energy sufficiency\textsuperscript{41,42}. Hyperleptinemia is associated with metabolic disorders. Recently, Zhao \textit{et al.} (2019) demonstrated that reducing plasma leptin levels in the context of obesity recovered hypothalamic leptin sensitivity while reducing weight gain and enhancing insulin sensitivity\textsuperscript{43}. During periods of starvation, leptin concentrations decrease, thereby signaling the hypothalamus to increase food intake. In contrast, when energy stores are fulfilled, leptin concentrations increase and signal the hypothalamus to decrease food intake. In addition to the extensive literature characterizing leptin’s inhibitory effect on
food intake, leptin also mediates energy expenditure (EE). Early work described an increase in body temperature and physical activity when ob/ob mice were administered leptin\textsuperscript{40,44}. A later study attributed the leptin-induced effect on EE to the activation of sympathetic nerves innervating BAT, a thermogenic tissue\textsuperscript{44,45}. Furthermore, leptin induction of UCP1 is lost after BAT sympathectomy\textsuperscript{45}.

1.3. The role of the sympathetic nervous system in adipose tissue

In response to changes in nutritional or hormonal status, adipocytes have the unique capability to both accumulate and store large amounts of triglyceride (lipogenesis) within the lipid droplet, or hydrolyze the stored triglyceride (lipolysis) so it can be utilized as energy substrate in other tissues\textsuperscript{46}. Since adipose tissue represents the largest energy reserve in the body\textsuperscript{47}, the balance between lipogenesis and lipolysis largely determines total fat accumulation in the body. Thus, intense research interest lies in pinpointing the underlying mechanisms and players involved in lipogenesis and lipolysis in order to develop therapeutic treatments for obesity.

1.3.1. Sympathetic regulation of brown adipose tissue

Amongst the numerous factors that have the capacity to influence the brown adipocyte, NE is considered the most important and most studied. NE plays an important role in NST, BAT’s key function\textsuperscript{48}. In order to gain further insight as to the importance of sympathetic innervation in BAT, researchers performed a surgical denervation to the interscapular brown adipose tissue (iBAT), the largest brown fat depot in the body\textsuperscript{49,50}. Unilateral denervation of iBAT caused a significant decrease in mass and protein content of the iBAT, indicating that the hypertrophy and the increased thermogenic
capacity of brown fat induced by cold-adaptation depends entirely upon the presence of sympathetic innervation within the tissue\textsuperscript{49}. In another experiment, researchers injected SR-59230A, a beta-3 adrenergic receptor (\(\beta_3\)-AR) antagonist, which blocks NE from binding to the receptor and therefore, blocks sympathetically-mediated BAT thermogenesis. In a dose-dependent manner, as concentration of SR-59230A increased, the average iBAT and body temperature of the rats significantly decreased compared to the controls that were injected with water. These findings demonstrate that a profound amount of thermogenesis is provided by sympathetic stimulation\textsuperscript{51}.

1.3.2. Sympathetic regulation of white adipose tissue

Interestingly, the SNS has emerged as a key regulator in WAT function, namely its action in lipolysis (Figure 1.2). Upon sympathetic activation, release of the catecholamine, NE, from post-ganglionic sympathetic nerve fibers innervating adipose tissue binds to G-protein coupled alpha- and beta-adrenoceptors located on the surface of adipocytes. In mice, NE predominantly stimulates \(\beta_3\)-ARs\textsuperscript{52,53} activating adenylate cyclase, increasing cyclic adenosine monophosphate (cAMP), which stimulates protein kinase a (PKA) to phosphorylate hormone-sensitive lipase (HSL). Concurrently with HSL, adipose triglyceride lipase (ATGL) and monoacylglycerol (MAG) participate to hydrolyze the lipid droplet (triacylglycerol, TAG)\textsuperscript{54}. The resulting products are three fatty acids and one glycerol molecule, which exit the adipocyte and enter the bloodstream. The FFAs are taken up by other tissues, ultimately utilized for ATP generation while the glycerol is taken up by the liver where it inevitably gets routed to the glycolysis pathway\textsuperscript{55}. 
In fact, a substantial amount of literature has demonstrated the necessity of sympathetic innervation into WAT. In early work studying the SNS regulation of WAT, researchers postulated that the relationship between the SNS and the adrenal medulla, the source of catecholamine production and secretion, was crucial for the “fight or flight” response; raising blood glucose, mobilizing FFAs from AT, and increasing metabolic rate\(^6^6\). To the contrary, a mounting body of evidence has demonstrated that mobilization of FFAs requires direct sympathetic activation, opposing the idea that circulating catecholamines secreted from the adrenal medulla alone are sufficient\(^6^6\). To elaborate, adrenal demedullation in rats did not block NE turnover (a method used to measure sympathetic activation) during cold exposure\(^5^7\). Furthermore, sympathetic and ganglionic blockade experiments targeting the fat pad was demonstrated to inhibit the mobilization of lipid\(^5^9\), indicating the importance of direct sympathetic innervation. To add, Shi \textit{et al.} concluded that when iWAT was surgically denervated, white adipocytes displayed a significant increase in both hypertrophy and hyperplasia, concluding that intact sympathetic innervation can inhibit fat pad growth and proliferation in the iWAT\(^6^1\).

Although it is clear that the SNS plays a profound role in proper WAT functioning, it is important to realize that individuals with obesity have impaired WAT function. Wilfried Jänig describes that functional diseases like obesity, type 2 diabetes, and metabolic syndrome can be attributed to prolonged activation endured by the autonomic nervous system (ANS)\(^6^2\). Therefore, it is feasible to hypothesize that obesity diminishes or damages SNS innervation into the fat pad. Future strategies should attempt to restore metabolic function of WAT with the use of neuromodulation techniques that aim to increase the density of SNS innervation in adipose depots like iWAT.
1.4. Research focus

Our goal was to conduct a comprehensive study demonstrating the origin of sympathetic innervation to the iBAT and the iWAT in the mouse. To that end, we used the unique combination of reporter mice, viral retrograde tracing, large-volume tissue clearance, and light sheet microscopy to visualize the sympathetic innervation as well as the identity of the pre- and postganglionic inputs of the fat pads. Indeed, in our iBAT innervation study, we found that postganglionic neurons emanating from the fused stellate/T1 sympathetic chain ganglia innervate iBAT, but also extend to T5 sympathetic chain ganglia. Our study is the first to systematically analyze the extent of pre-ganglionic neurons in the intermediolateral nucleus of the spinal cord (IML) that innervate iBAT (level T2-T6).

We have also utilized the previously described methodologies to map the sympathetic innervation of iWAT. Further, we provide insight to the direct pre- and post-ganglionic sympathetic input of iWAT, while simultaneously presenting these findings with surrounding anatomical context in great detail. As a result of injecting PRV into the iWAT, we concluded that sympathetic nerves that originate from T12, T13, and L1 sympathetic chain ganglia are the primary contributors supplying the iWAT depot, while pre-ganglionic neurons in the IML at thoracic level T8-T10 appear to be the most common pre-ganglionic inputs communicating to iWAT. Notably, both data sets clarify that iBAT and iWAT receive pre-and postganglionic input from distinct anatomical levels that do not overlap, which is in contrast to the previously published data\textsuperscript{63}.

Lastly, it was important to account for viral contamination. To confirm that injecting PRV was required for viral expression, we designed a negative control that
entailed dripping an equal amount of virus directly onto the fat pad. No evidence of PRV labeling could be detected in the sympathetic chain ganglia, dorsal root ganglia, IML, or celiac plexus in the negative controls. Furthermore, with observation under a stereomicroscope, we did not detect expression of PRV anywhere else in the abdominal cavity. These results demonstrate that the virus did not “leak” and randomly express PRV if the virus were to make surface contact with nearby structures.

In sum, using transgenic reporter mice, retrograde tracing, whole-body tissue clearance, and lightsheet microscopy we present comprehensive insight to the SNS mapping of iBAT and iWAT in adult mice. These findings will be indispensable when strategizing future experiments targeting fat pads that involve the use of neuromodulation techniques like pharmacogenetics or optogenetics.
2.1. Introduction

Interest in BAT as a site for burning off excess calories in the fight against obesity flared up 35 years ago\cite{64,66} and returned recently after a long hiatus\cite{66,67}. While it has been clear that brown fat thermogenesis is important for the maintenance of body temperature for most homeothermic animals and human infants, a significant contribution in adult humans has only recently been suggested by the rediscovery of significant BAT depots\cite{64,67-72}. Besides responsiveness to photoperiod and ambient temperature\cite{66,73,74}, BAT activity in adult humans increases after a high-calorie meal\cite{75}. This latter finding revives Rothwell and Stock’s original idea of “Luxuskonsumption,” or the ability of BAT to burn off extra calories and prevent obesity\cite{76}.

In rodents, iBAT is the largest depot, with smaller depots in the mediastinum, along the cervical and thoracic aorta, and around the kidneys\cite{77}. In humans, BAT is less centralized than in rodents, with significant depots in supraclavicular, neck, and paraspinal regions\cite{67,78,79}. Based on numerous experiments with denervation of the interscapular pads in rodents, as well as pharmacological studies using β3-adrenergic agonists and blockers, the main driver of BAT thermogenesis seems to be its noradrenergic sympathetic innervation\cite{80-88}. Furthermore, a recent study demonstrating

increased thermogenesis by electrical field stimulation of the dorsal surface of iBAT in rats\textsuperscript{89} suggests that stimulation of noradrenaline release from sympathetic nerve terminals is required for this effect. The functional necessity of the SNS for intact BAT function is demonstrated by surgical and chemical denervation methods, which block or greatly reduce most cold-induced BAT adaptations like UCP1 induction, increased blood flow, higher mitochondrial density, enhanced glucose uptake, and activation of thyroid-activating enzymes\textsuperscript{90}.

Thus, there is an increasing need to visualize the sympathetic innervation of BAT efficiently to make this tissue accessible for peripheral nerve stimulation, verification of viral labeling extent, and specialized pharmacotherapy. Here, we identify different reporter mice that efficiently visualize peripheral sympathetic innervation and establish the use of immunohistochemical double labeling and tissue clearing for large thoracic specimens to identify the location and distribution of postganglionic sympathetic neurons innervating iBAT in the mouse using the widely-utilized pseudorabies virus (PRV).

2.2. Materials and methods

2.2.1. Animals

Transgenic tgdh-Cre mice (stock #: 032081-UCD,Tg(Dbh-Cre)KH212Gsat/Mmucd, Mutant Mouse Resource and Research Center, breeding pairs were obtained from Dr. Derbenev, Tulane University), TH-IRES-Cre mice (EM: 00254; B6.129×1-Thtm1(Cre)Te/Kieg; European Mouse Mutant Archive; breeding pairs were obtained from Dr. Louis deLecea, Stanford University) crossed with Rosa-Tomatofl/flmice (stock #: 007914; B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, Jackson Laboratories)
to generate TH:Tomato reporter mice. Both male and female offspring were group-
housed at a 12h:12h light/dark cycle with ad-lib access to food and water unless stated
otherwise. Animal genotypes were confirmed by PCR from tail biopsies DNA (tgDbh-
Cre mice: transgene forward 5′-AATGGCAGAGTGGGGTTGGG-3′; transgene reverse 5′-
CGGCAAAACGGACAGAAGCATT-3′; TH-IRESCre: Cre reverse 5′-GAT-ACC-TGG-CCT-
GGT-CTG-3′; wild-type/Cre forward 5′-CAC-CCT-GAC-CCA-AGC- ACT-3′; wild-type
reverse 5′-CTT-TCC-TTC-CTT-TAT-GAT-3′; Rosa-Tomato-flmice: wild-type
forward 5′-AAG GGA GCT GCA GTG GAG TA-3′; wild-type reverse 5′-CGG AAA ATC
TTGT GGG AAG TC-3′; mutant reverse 5′-GTC ATT AAA GCA GCATAT CC-3′; mutant
forward 5′-CTG TTC CTGTAC GGC ATG G-3′). The Pennington Biomedical Institutional
Animal Care and Use Committee approved all animal experiments.

2.2.2. PRV infection of the iBAT

In Figure 1A, the experimental steps are summarized schematically. The iBAT (n=9
mice; 7F,2M) received a unilateral injection with green fluorescent protein (GFP)
expressing PRV (PRV-GFP, viral titer, 1×10^9 pfu/mL, Lot #2007, 5×100 nL, kindly
provided by the National Center for Experimental Neuroanatomy with Neurotropic
Viruses, Pittsburgh, PA). Mice were anesthetized with isoflurane/oxygen and iBAT was
exposed by an intrascapular incision. The virus was then injected with a pulled glass
pipette (tip diameter ~5μm) attached to a 0.5-μL Hamilton syringe. Five separate
injections of 100 nL were distributed over the right iBAT depot, holding the pipette in
place for 10s to prevent backflow. Injection sites were then dried with gauze to prevent
leakage to surrounding tissue and the circulation. Mice were single-housed post viral
infection for 72 and 96h, and then perfused and processed as described below.
Controls included dripping the same total amount of PRV (500 nL) onto the surface of the iBAT depot (n=2) or surgical (n=2) denervation of iBAT.

2.2.3. Perfusion and fluorescence-guided dissection of relevant tissue blocks

Mice injected with PRV were perfused at 3–4 days post viral infection. Perfusion and immunohistochemistry were performed as previously described\(^9\). Briefly, mice were deeply anesthetized with isoflurane and transcardially perfused with ice-cold physiological saline followed by 10% buffered formalin.

Thoracic organs were removed and successful infection was verified with a fluorescent stereomicroscope (Nikon, SMZ25, Melville, NY) and only animals with visible sympathetic chain ganglia infection were further included in tissue dissection and analysis. The BAT was removed and the entire spinal cord was further cleared of excessive muscle mass. A laminectomy was performed to allow imaging of preganglionic IML neurons. The spinal cord was cut at the thoracic level T7/T8 to accommodate optimal imaging capacities. The tissue was post-fixed in formalin overnight and transferred to PBS-azide (2% sodium azide in PBS). All PRV-GFP–infected tissue was dehydrated in methanol (20%, 40%, 60%, 80%, and 100%, 1 h each) and stored in 100% methanol until further processing for staining and clearing as described below.

2.2.4. Immunohistochemistry and tissue clearing

Immunohistochemical staining was performed following the method from Renier et al. (https://idisco.info/idisco-protocol/updatehistory/) with modifications. Briefly, after methanol dehydration, tissues were treated with 5% H2 O2 in methanol (MeOH)
overnight at 4 °C (1 volume 30% H2O2 /5 volumes methanol, ice-cold), followed by washes in 100% methanol for 1h and rehydrated in a series of MeOH /PBS (80%, 60%, 40%, and 20%) for 1h each. Samples were further incubated in 1 x PBS/0.2% Triton X-100 twice (PTx.2 solution) for 1h each and permeabilized in 400 mL PTx.2, 11.5 g glycine, 100 mL DMSO at 37 °C with shaking for 2 days. See Appendix B for a complete list of antibodies used. After that, tissues were blocked in 42 mL PTx.2, 3 mL donkey serum (Jackson ImmunoResearch, West Grove, PA), and 5 mL DMSO over 2.5 days at 37 °C with shaking. Then, tissues were incubated with primary antibodies (chicken anti-GFP [1:400], Abcam, Cambridge, MA; rabbit anti-TH [1:400], Millipore, Burlington, MA) in PBS with 20% Tween 20 and 10 mg/mL heparin (PTwH)/5% DMSO/3% donkey serum at 37 °C with shaking for at least 7 days. Following primary staining, tissue samples were washed in PtWH 4–5 times in 60-min increments and then incubated in PtwH overnight. Samples were then subjected to secondary staining in PTwH/3% donkey serum for 1.5 weeks. Following primary staining, tissue samples were washed in PtwH 4–5 times 1h or until the following day.

Samples were dehydrated in MeOH/H2O series, incubated in 66% dichloromethane (DCM, SigmaAldrich, St. Louis, MO)/33% MeOH at room temperature, and then in 100% DCM for 15 min twice with agitating on a rocker to rinse out any remaining MeOH. Tissues were stored in dibenzyl ether (DBE, Sigma-Aldrich) until imaging.

2.2.5. Microscopy and image processing

Spine sample imaging utilizing both a Leica SP5confocal microscope (Leica Biosystems Inc., BuffaloGrove, IL) and a LaVision light sheet microscope (LaVision BioTec,
Bielefeld, Germany). Specimen imaging in the organic solvent required immersion of the microscope objective in it, which is a capability of the light sheet but not the confocal microscope. Therefore, we used a handcrafted chamber for the confocal microscope to allow safe specimen access without damaging the equipment. Overview 3D image stacks were generated for the spine with a dorsal view to reveal PRV labeling in the IML column. 3D image stacks with a ventral view revealed thoracic and lumbar sympathetic chain ganglia and the celiac ganglion (CG). Images of tyrosine hydroxylase (TH) and PRV labeling were collected for all samples. Higher magnification images were generated (10×) for PRV-positive sympathetic chain ganglia and the corresponding dorsal root ganglia (DRG), as indicated in figures, as well as for PRV-positive IML neurons. Note, even though confocal and lightsheet microscopy both have advantages and disadvantages, at this level of analysis we used both systems interchangeable and both systems were sufficient to obtain single-cell resolution of pseudorabies virus-labeled postganglionic neurons.

2.2.6. Quantitative analyses of postganglionic neurons

Among all investigated animals with successful PRV labeling, no evidence was found for pre- or post-ganglionic PRV labeling beyond thoracic level T8 and T7, respectively. Thus, we performed the systematic analysis only for the rostral portion of the spinal cord (the stellate ganglion to the sympathetic chain ganglion T8).

Overview images (5x magnification/1.0 zoom factor) showing the ventral (sympathetic chain ganglia) and dorsal (IML) view of the spinal cord were aligned to determine the thoracic levels of sympathetic chain ganglia and IML PRV labeling. We further investigated 10x magnification images to analyze PRV-labeled portions in more
Individual sympathetic chain ganglia were further quantified for the number of PRV-labeled neurons as a measure of infection efficiency and consistency for individual cases. 3D images included DRG for each sympathetic chain ganglia level and allowed evaluation for PRV labeling of sensory cell bodies. Within sympathetic chain ganglia, we estimated cell counts for PRV-positive neurons in z-stack images manually with the Adobe Photoshop count tool (AdobePhotoshopCS6, SanJose,CA), and an automated 3D analysis of cell counts utilizing the Imaris 9.2 spot counting feature (Bitplane AG, Concord,MA).

2.3. Results

2.3.1. Whole body in situ imaging and dissection of sympathetic nervous system components using TH:Tomato reporter mice

In order to guide the dissection of the thoracic specimen that would retain components of the SNS, we wanted to capitalize on the powerful ability of reporter gene expression. Most reporter lines are well characterized for their expression profile in the central nervous system (CNS), while reporter expression in peripheral nerves remains unexplored. We found that knock-in TH:Tomato mice and transgenic Dbh:Tomato mice both showed strong fluorescence in noradrenergic nerves. After formalin fixation and removing the viscera, the bilateral sympathetic chain ganglia could be identified using a fluorescent stereomicroscope with a long working distance (Fig. 2.1.A). Knock-in TH:Tomato mice (Fig. 2.1.B) showed stronger fluorescence compared to transgenic Dbh:Tomato mice (Fig. 2.1.C), but both mouse lines showed bright fluorescence, revealing sympathetic chain ganglia and interconnecting nerve strands even at lower magnification.
Figure 2.1. Overview of experimental design, dissection technique, and whole body tissue clearing. (A) Flow diagram of experimental design. (B and C) Ventral views of eviscerated, perfused TH:Tomato mouse (B), Dbh:Tomato mouse (C) using stereomicroscope. (D) Ventral view of eviscerated, perfused wild-type mouse 96 h post-PRV-GFP injection into the right iBAT pad using stereomicroscope. Note labeling of right, but not left sympathetic chain ganglia. (E) Confocal microscope image of the whole upper body of a mouse after iDISCO immunohistochemistry with TH and tissue clearing. Note labeling of the bilateral sympathetic chain, neck nerves, and brain areas. TH, tyrosine hydroxylase; DBH, dopamine beta-hydroxylase; PRV, pseudorabies virus; GFP, green fluorescent protein; SG, stellate ganglion; T1−T7, ganglia for thoracic levels 1−7.

In contrast, our initial attempts using transgenic TH:Tomato mice (B6.Cg-7630403G23RikTg(Th-cre)1Tmd/J, Stock# 008601) showed only sporadic fluorescent signal with only one or two Tomato-positive cells per thoracic sympathetic ganglia (data not shown). Thus, in our hands, knock-in TH:Tomato mice and transgenic Dbh:Tomato mice were most useful to visualize sympathetic chain ganglia, and associated nerves, and to clean ganglia of obscuring tissues (e.g., ganglia adipose tissue). Sympathetic
chain ganglia can be visualized without reporter gene expression, but the celiac/mesenteric ganglia complex is not visible without fluorescent labeling and abdominal chain ganglia are easily confused regarding to their thoracic and lumbar levels without fluorescent guidance specifically for the untrained experimenter.

2.3.2. Clearing of large tissue blocks reveals 3D anatomy of sympathetic chain and other components of the SNS

Earlier work in embryos or newborn mice has used whole body clearing techniques to visualize sympathetic chain ganglia; however, this technique has not been adapted to substantially larger adult mice with excessive muscle and adipose tissue mass. However, recent histological advances have promoted tissue clearance for a larger specimen like the brain and human embryos, suggesting that these techniques might also be suitable to visualize sympathetic chain ganglia in adult animals. We generated tissue blocks that contained specific components of the SNS of adult mice and subjected them to a modified iDISCO clearing protocol with immunohistochemistry for TH and other markers. This revealed for the first time in adult mice the undisturbed three-dimensional structure of the bilateral sympathetic chain and intercostal nerves, as well as adrenergic nerves and neurons in the neck and brain (Fig. 2.1.E and Supplementary Video S1, online only). Specifically, using classical or light sheet confocal microscopy, the individual anatomy of the stellate to T13 and lumbar sympathetic chain ganglia and interconnecting nerve strands were revealed in their three-dimensional space on a background of rib and vertebral column structure, which did not completely clear.
2.3.3. Pseudorabies virus mapping of iBAT-innervating neurons in sympathetic chain ganglia and spinal cord

PRV has been widely used to identify the ANS innervation of peripheral tissues and is the retrograde tracer of choice\textsuperscript{93}. We injected GFP-conjugated PRV unilaterally into the right iBAT pad. Our focus was on first- and second order neurons only (pre- and postganglionic neurons), so that the survival time after virus injections was kept short with 3–4 days. Labeling in the SG and upper thoracic sympathetic chain ganglia on the ipsilateral injection side could be seen in fixed and eviscerated whole animal preparations using a fluorescence stereomicroscope (Fig. 2.1.C), which guided dissection as above. After iDISCO clearance with the immunohistochemical enhancement of viral GFP and TH, many more details became visible with classical and light sheet confocal microscopy (Figs. 2.2 and 2.3).
Figure 2.3. Postganglionic sympathetic neurons innervating iBAT in the mouse. (A and B) Light sheet microscope images of two examples of retrogradely PRV-labeled postganglionic sympathetic neurons in the fused right stellate/T1 ganglion after unilateral PRV injections into the right iBAT pad. Inset shows retrogradely PRV-labeled (green) and unlabeled (red) neurons at higher magnification (asterisks depict examples of colocalized neurons). (C–H) Confocal microscope images of individual sympathetic chain ganglia at thoracic levels T2–T7 in the same mouse for which the stellate ganglion is shown in (B). Note strong labeling in T2–T4 and much weaker labeling in T5 and T6 ganglia. TH, tyrosine hydroxylase; PRV, pseudorabies virus; SG, stellate ganglion; T1–T7, ganglia for thoracic levels 1–7.

Specifically, there was strong labeling not only in the caudal portion of the SG, but also in the thoracic chain ganglia from T1 (typically fused to the SG) to T5, and in some cases scattered labeling up to T7. Labeling was limited to the ipsilateral side of the injection, even though we observed rare cases of bilateral labeling, likely due to accidental injection into the adjacent and merged left iBAT section. Labeling in the T5 to T7 ganglia became increasingly sparse. Higher magnification images clearly revealed individual PRV-infected neurons among noninfected TH-positive neurons (Fig. 2.3).
Figure 2.4. Controls for nonspecific labeling. (A and B) Light sheet microscope images of the fused right stellate/T1 ganglion showing the absence of PRV-labeled neurons (green) in mouse with PRV injections into surgically denervated right iBAT pad. (A') Same ganglion with TH fluorescence (red) to show the outline of the ganglion. The sympathetic innervation (TH labeling) is degraded in the denervated iBAT pad, in contrast to the intact iBAT pad (B). (C) Light sheet microscope image of a mouse with successful PRV labeling in sympathetic chain ganglia shows a complete absence of PRV labeling in the celiac ganglion (CG). (D–G) Confocal microscope images of a mouse with successful PRV labeling in sympathetic chain ganglia show sparse PRV labeling (green dots) in T3-DRG (D), T4-DRG (E), T5-DRG (F), and T6-DRG (G). TH, tyrosine hydroxylase; PRV, pseudorabies virus; SG, stellate ganglion; DRG, dorsal root ganglion; T1–T7, ganglia for thoracic levels 1–7.

In addition, PRV-labeled nerve fibers within intercostal nerves and ganglia connecting nerve strands could be discerned on the background of TH-positive nerves.

Importantly, injection of the same viral dose in mice with prior surgical iBAT denervation (Fig. 2.4.A, B), or dribbling it over the surface of iBAT (data not shown), did not result in any discernible labeling in any sympathetic chain ganglia.
TH labeling also revealed the location of the DRG as well as the paravertebral ganglia and we inspected these ganglia for PRV-labeled neurons.

We found no indication of PRV labeling in paravertebral abdominal ganglia, such as the celiac (Fig. 2.4.C) and splanchnic nerves, even though a few iBAT projecting DRG neurons were identified with PRV labeling (Fig. 2.4.D–G).

Quantitative cell count analysis of nine successful PRV injections revealed that about half of PRV-labeled neurons (approximately 789 cells) were located in the fused stellate/T1 ganglion and the other half was distributed over T2–T5 (approximately 723 cells, Fig. 2.5, Appendix B). However, the number of labeled neurons in the stellate/T1 ganglia varied widely between animals, indicating either a biological variation or incomplete labeling.

To map the preganglionic neurons in the spinal cord that innervate iBAT, light sheet microscopy was performed on cleared tissue blocks from seven mice containing the spinal cord and the sympathetic chain (Fig. 2.6.A). PRV labeling in the IML column of the spinal cord was found between rostrocaudal levels T2–T8 (Fig. 2.6.B). None of these seven mice showed preganglionic labeled neurons at the level of the stellate/T1 ganglia or beyond T9, all seven mice showed labeling at levels T2–T6, and labeling from T7 to T8 was found in a subset of animals (n=6,5 respectively). Thus, the representation of preganglionic neurons innervating iBAT is slightly shifted to more caudal levels as compared to the corresponding postganglionic neurons (Fig. 2.6.C)
Figure 2.5. Quantitative distribution of sympathetic postganglionic neurons innervating iBAT in the mouse. Mean ± SEM of number of retrogradely labeled neurons in mice (n = 9) unilaterally injected with PRV into the left iBAT pad. Note that about half the retrogradely labeled neurons are located in the fused stellate/T1 ganglion and the other half is distributed over T1–T5 ganglia.

2.4. Discussion

Brown fat thermogenesis is an evolutionarily conserved mechanism to maintain body temperature in mammals and has the potential to convert surplus energy into heat for better energy balance control in an environment of plenty\textsuperscript{76,94}. In addition, BAT may be the source of factors and hormones that play important roles in metabolic health\textsuperscript{91,95-97}. Because sympathetic drive is required for optimal BAT function\textsuperscript{98,99}, identification of the sympathetic pre- and postganglionic neurons selectively innervating BAT is important. Here, we combine classical pseudorabies retrograde tracing with transgenic mouse models, whole tissue clearing, and confocal light sheet microscopy to show the specific distribution of sympathetic postganglionic neurons innervating the iBAT in the mouse.
We identified suitable reporter mice to guide initial dissection of large tissue blocks containing the critical components, which greatly increases confidence in anatomical dissection and sectioning techniques. We further adapt iDISCO methods to immunohistochemically stain and perform tissue clearance of the entire spinal cord and sympathetic chain in the adult mouse. This approach is able to visualize the entire SNS and its major components undisturbed in situ, and greatly facilitates the unbiased and transparent evaluation of pre- and postganglionic contribution to iBAT innervation.

Contrary to findings in the rat\textsuperscript{100,101} and Siberian hamster\textsuperscript{102}, we found iBAT-innervating sympathetic postganglionic neurons not just in the SG but also in the T2–T5 sympathetic chain ganglia. Interestingly, within the SG, the rostral pole is suspiciously void of neurons innervating iBAT. The rostral pole is known to harbor postganglionic neurons that innervate the heart\textsuperscript{103,104}. In contrast, the more caudal portions of the SG together with the T1, T2, T3, T4, and T5 chain ganglia are densely packed with iBAT-innervating neurons. Future studies will further investigate how the sympathetic nerves leaving these individual ganglia relate to the organization of end-organ iBAT innervation.

Importantly, we did not find any iBAT-innervating postganglionic neurons in the lower sympathetic chain ganglia as reported for the Siberian hamster\textsuperscript{63}. This is consistent with the overall development of sympathetic innervation that clearly dissociates the origin of sympathetic innervation in anterior (thorax and forelimbs), posterior (hind limb), and abdominal (situ) sites\textsuperscript{105}, and classic views of autonomic innervation\textsuperscript{100}. A few PRV-labeled neurons were found in DRG, and it is not clear whether these are true sympathetic efferent neurons or inadvertently anterogradely labeled dorsal root afferents.
Figure 2.6. Location of sympathetic preganglionic neurons innervating iBAT and schematic diagram of sympathetic outflow pattern to iBAT in the mouse. (A and A’) Light sheet microscope image through spinal cord showing the location of preganglionic neurons (green) on the right side of the intermediolateral column (A), and merged image of PRV and TH (A’) in a mouse with unilateral injection of PRV into the left iBAT pad. (B) Semiquantitative assessment of location of postganglionic neurons in the spinal cord relative to the rostrocaudal level. Note the caudal-ward shift in representation with no preganglionic neurons at the level of the fused stellate/T1, T2, and T9 sympathetic chain ganglion. (C) Schematic diagram depicting the organization of sympathetic outflow to iBAT in the mouse. IML, intermediolateral column of the spinal cord.

Given the very small number of these neurons, they are not likely to be of major physiological importance. Our analysis of preganglionic neurons in the spinal cord is in general agreement with studies in the rat\textsuperscript{100,106} and Siberian hamster\textsuperscript{63}, which all report the SG as a major contribution to the sympathetic innervation of the iBAT. However, our data highlight the regional distribution of PRV labeling within the SG and the lack of PRV labeling in the dorsal pole. Furthermore, we clarify that thoracic ganglia from the
stellate until T5 significantly contribute to iBAT innervation, with scattered PRV labeling in T6/T7 and no contribution was observed below T8 chain ganglia, contrary to a report by Nguyen et al. We also clarified that the prevertebral CG did not innervate iBAT, which has been recently suggested as the main innervation site of adipose tissue. Our study is also the first to systematically investigate the anatomical extent of preganglionic iBAT innervating neurons in the IML (levels T2–T6).

Our study demonstrates the usefulness and points out some caveats of genetically altered mouse models for the analysis of ANS functional anatomy that should be very helpful for other studies. The TH-IRES-Cre knock-in and the transgenic DbhCre mice were both useful reporter driver in the SNS, while the transgenic TH-Cre mouse resulted only in sporadic cell bodies with reporter expression throughout the thoracic chain ganglia, which requires consideration when choosing Cre-driver lines for studies in peripheral sympathetic nerves. However, with appropriate Cre-driver lines, the red fluorescent signal in the reporter mice was strong enough to guide dissection of any component of the SNS in fresh or formalin perfused preparations under a fluorescent stereomicroscope. Other methods that have been used in the past are IP injections of Fluorogold, which label all neurons of the peripheral nervous system (sympathetic, parasympathetic, and sensory) in rats and mice, but genetic approaches hold promise for a better distinction of sympathetic, parasympathetic, sensory, and enteric nervous system components and do not require dissection under harmful UV light.

Our study also highlights that immunohistochemical staining and tissue clearance protocols are successful in adult animals with excellent resolution for the peripheral
nervous system and greatly enhance the rigor and transparency of analyzed anatomical levels. With the ascent of neural modulation devices and therapies\textsuperscript{110-113}, it will be important to provide detailed, complete, and specific functional maps of peripheral nervous systems. To this end, analysis of large tissue blocks allowing complete spatial representation of all nerves and ganglia vis-à-vis specific target organs will be indispensable. The methodology described here should enable high-resolution 3D imaging of sympathetic and parasympathetic innervation of other important target organs, such as WAT, gut, pancreas, liver, kidneys, spleen, the urogenital tract, and the respiratory system.

One potential limitation of using mice and other small mammals as models to gain basic information on the functional anatomy of the ANS is the possibility of significant differences from humans. Specifically, comparisons of SNS innervation of BAT between rodents and humans are complicated by the fact that in humans it appears to be located and distributed quite differently than in rodents. Instead of the prominent iBAT pad in rodents, the most important accumulation of BAT in humans is supraclavicular, with additional depots in the neck and along the spinal cord vertebrae\textsuperscript{66,67}. Even though the different distribution will likely not affect the functional importance of BAT per se, future studies in mice to modulate sympathetic iBAT function are important approaches to understanding metabolic dynamics in humans.

In conclusion, using transgenic TH-IRES-Cre mice, whole tissue clearing, and confocal/light sheet microscopy, we provide a complete map of SNS innervation of the mouse iBAT depot that shows the location of postganglionic sympathetic neurons not confined to the SG, but also the first several sympathetic chain ganglia.
Chapter 3. Sympathetic Innervation of Inguinal White Adipose Tissue in the Mouse

3.1. Introduction

Historically, WAT was largely considered a storage depot for excess energy\textsuperscript{114}. However, given the increasing number of obese individuals plagued with metabolic syndrome, and the associated risk for life-threatening comorbidities like type 2 diabetes, cancer, and cardiovascular disease\textsuperscript{115}, intense research over the last decades has highlighted a more detailed picture of the sophisticated structures within adipose tissue. Mature adipocytes co-exist with connective tissue, stromovascular cells, immune cells as well as nerve fibers and terminals; all of these are involved in endocrine crosstalk within the tissue and across other organs\textsuperscript{41}.

Just as excess adipose tissue poses clear health risks, the lack of adipose tissue, lipodystrophy, also leads to severe metabolic dysfunction, like liver steatosis and insulin resistance\textsuperscript{116}. One of the main functions of adipocytes is to switch between energy storage in times of excess through lipogenesis and fat storage and serving as an energy source via lipolysis to provide energy during periods of low food intake (e.g. sleep, starvation). Since the sympathetic nervous system plays a critical role for the induction of lipolysis in adipocytes\textsuperscript{90,117,118}, it is a good candidate for obesity interventions.

Previous studies have used diverse tracing strategies in various animal models to identify the origin of sympathetic neurons that innervate the adipose tissue. In Siberian hamsters, the retrograde tracer, fluorogold was injected into iWAT and identified labeled cell bodies in the abdominal sympathetic chain ganglion T13\textsuperscript{119}. 

31
Later studies used the transsynaptic retrograde tracer, pseudorabies virus, in rats and showed labeling in the abdominal sympathetic chain ganglia T13 and L1\textsuperscript{100}. Conversely, a retrograde tracing study with cholera toxin b conducted in mice suggested that iWAT receives sympathetic inputs from the CG\textsuperscript{107}.

Given these inconsistent findings and the increased interest to target the peripheral nervous system with novel molecular genetic tools\textsuperscript{100,107,120-122}, the mouse will likely be the model of choice to target sympathetic innervation of iWAT. However, the mouse has not been a preferred model for dissection of the peripheral nervous system due to its small size. Published literature regarding the organization of the SNS in rodents is typically available from rats\textsuperscript{123}. Yet, in order to apply novel molecular-genetic and viral tools in the peripheral nervous system, the mouse is the model of choice due to the abundant availability of cre-driver and floxed mouse lines.

In this study, we aimed to develop a method that allows for a high level of scientific rigor and transparency when studying the origin of sympathetic innervation of iWAT in male and female mice. In a recent study, we adapted the tissue clearance and immunolabeling methodology, iDISCO\textsuperscript{92}, to clear the entire spinal cord (SC) and associated peripheral ganglia in the thorax and abdomen\textsuperscript{122}. This technique allowed for high-resolution imaging of the SNS within the abdomen, all the while leaving the sympathetic chain ganglia (SChG) largely intact and undisturbed. In this study, we combined whole body iDISCO with the retrograde transsynaptic tracer PRV to identify pre- and postganglionic sympathetic inputs to iWAT. We further include anatomical dissection techniques in reporter mice to provide a detailed anatomical map for the sympathetic innervation of iWAT.
Our PRV study clarifies the origin of postganglionic iWAT innervation from SChG T12-L1 and preganglionic inputs from the SC levels T7-T10 in the mouse. We show that the lateral cutaneous rami continue from iWAT to the skin, while fine nerves containing sympathetic axons branch off to the dorsolumbar portion of iWAT. Another major innervation is provided by the anterior and lateral femoral cutaneous nerves to the inguinal portion of iWAT. Our study may serve as a comprehensive map for future experiments that employ virally-driven neuromodulation techniques in adipose tissue for rigorous and transparent verification of virus infection.

3.2. Materials and methods

3.2.1. Animals

TH-IRE-Cre mice (EM: 00254; B6.129X1-Th\textsuperscript{tm1(Cre)Te}/Kieg; European Mouse Mutant Archive; breeding pairs were obtained from Dr. Luis de Lecea, Stanford University) were crossed with Rosa-EGFP\textsuperscript{fl/fl} reporter mice (B6;129S4-Gt(ROSA)26Sor<tm9(EGFP/Rpl10a)Amc>/J, Stock# 024750, Jackson Laboratory, Bar Harbor, ME) or Rosa-Tomato\textsuperscript{fl/fl} mice (stock #: 007914; B6.Cg-Gt(ROSA)26Sor\textsuperscript{tm14(CAG-tdTomato)Hze}/J, Jackson Laboratories, Bar Harbor, ME) to generate TH:EGFP or TH:Tomato reporter mice. Animal genotypes were confirmed by PCR from tail biopsies DNA (TH-IRE-Cre: Cre reverse 5'-GAT-ACC-TGG-CCT-GGT-CTG-3'; wild-type/Cre forward 5'-CAC-CCT-GAC-CCA-AGC-ACT-3'; wild-type reverse 5'-CTT-TCC-TTC-CTT-TAT-TGA-GAT-3'; Rosa-EGFP\textsuperscript{fl/fl} mice: wild-type forward 5'-AAG GGA GGA GCT GCA GTG GAG TA-3'; wild-type reverse 5'-CCG AAA ATC TGT GGG AAG TC-3'; mutant forward 5'-ATT GCA TCG CAT TGT CTG AG-3'; mutant reverse 5'-CCG AAA ATC TGT GGG AAG TC-3'; Rosa-Tomato\textsuperscript{fl/fl} mice: wild-type forward 5'-AAG GGA GCT GCA GTG GAG...
TA-3’; wild-type reverse 5’-CCG AAA ATC TGT GGG AAG TC-3’; mutant forward 5’-CTG TTC CTG TAC GGC ATG G-3’; mutant reverse 5’-GGC ATT AAA GCA GCG TAT CC-3’;). Both male and female mice were used in all experiments and all animals were group-housed at a 12h:12h light/dark cycle with ad lib access to food and water unless stated otherwise. The Institutional Animal Care and Use Committee approved all animal experiments.

3.2.2. Fluorescence-guided dissection of tissue

We initially performed anatomical dissections in order to clarify the overall organization of the lower thoracic and upper abdominal sympathetic nervous system as well as iWAT innervation. We used TH:GFP (n=1) and TH:tomato reporter (n=6, 3 males, 3 females; ranging from 6 to 20 weeks of age) lines for dissections under a fluorescent stereomicroscope (Nikon, SMZ25, Melville, NY). Mice were euthanized with an overdose of CO2 and blood was removed by cardiac perfusion with saline. Thoracic and abdominal organs were removed and images of the pre- and paravertebral structures were taken for documentation. In some cases, we carefully removed the adipose tissue that surrounds all sympathetic ganglia (“ganglia fat”) to enhance visibility and imaging of abdominal ganglia. We further identified and documented major innervating nerves and their origin by dissection under the fluorescent stereomicroscope. In some cases we used TH:tomato mice to enhance visibility of fine nerves.

3.2.3. PRV infection of iWAT

Mice (ranging from 6-15 wks old) were anesthetized with isoflurane/oxygen. A lower back incision was extended laterally on the right hindleg to reveal an adequate amount of iWAT sufficient for injection. Ten individual injections (each 100 nL, n=2 or 200 nL,
n=11) were distributed across the right iWAT depot with green fluorescent protein expressing PRV (PRV-GFP, viral titer, $1 \times 10^6$ viral molecules/mL, Lot #2007, kindly provided by the National Center for Experimental Neuroanatomy with Neurotropic Viruses; Pittsburgh, PA). Virus was injected with a beveled Hamilton® syringe (Model 7002 KH SYR, Reno, NV). Upon each injection, the syringe was held in place for 30-40 seconds to prevent backflow. Injection sites were dried with a cotton swab to mitigate leakage to surrounding tissue and circulation. Mice were single-housed post-viral infection for 96 hours. Note, our goal was to use minimal infection times, and we tested 10x100nl injection with different incubation times of 72 hrs (n=9) and 96 hrs (n=5), with no infection at 72 hrs and only 2 successfully infected animals at 96 hrs. Doubling the viral injection volume at 96 hrs incubation time increased the rate of successful infections (as observed under the dissecting microscope, 11/13). Control animals involved dripping the same total volume of PRV-GFP (1 µL, n=1 or 2 µL, n=4, 96 hrs incubation) onto the surface of the iWAT depot.

Perfusion and immunohistochemistry were performed as previously described\(^{122}\). Mice were deeply anesthetized with an overdose of isoflurane, followed by a transcardiac syringe perfusion with ice-cold physiological saline, then 10% neutral buffered formalin (Fisher, Passaic, New Jersey). Thoracic and abdominal organs were removed, which allowed for verification of successful infection when viewed with a fluorescent stereomicroscope (Nikon, SMZ25, Melville, NY). Only mice with visible sympathetic chain ganglia infection were included in further tissue dissection and analysis of sympathetic chain ganglia (n=13, 6 females, 7 males), intermediolateral nucleus of the spinal cord (IML) (n=11, 4 females, 7 males), dorsal root ganglia (DRG)
(n=13, 6 females, 7 males) and CG (n=8, 5 females, 3 males). The iWAT was removed for individual processing and excessive muscle mass was dissected from the spinal cord. A laminectomy was performed in order to image sympathetic preganglionic neurons. The spine and spinal cord were cut in half (roughly at the level of ribs 7 to 9) to accommodate the imaging capacity of both the light sheet and confocal microscope. Then, the tissue was post-fixed overnight in formalin, and stored in PBS-azide (0.02% Na-azide in PBS) 4°C until iDISCO processing.

3.2.3. Immunohistochemistry and tissue clearing

Immunohistochemical staining was performed following the iDISCO method from Renier et al (https://idisco.info/idisco-protocol/update-history/) with modifications. Tissues were dehydrated in a series of Methanol (MeOH)/H₂O mixtures (20%, 40%, 60%, 80%, 100%), then incubated overnight in a 66% dichloromethane (DCM) and 33% MeOH solution on a rocker (Southwest Science, model SBT30, Trenton, NJ). Tissues were treated with 5% H₂O₂ in MeOH overnight at 4°C. Following overnight incubation, samples were rehydrated in a series of 1hr MeOH/H₂O washes, then washed in PTx.2 solution (0.2% TritonX-100 in PBS). Samples were incubated in permeabilization solution (400mL PTx.2, 11.5g glycine, 100mL DMSO), followed by incubation in blocking solution (42 mL PTx.2, 5 mL DMSO, 3 mL NDS) at 37°C for two days in a shaking incubator (Corning LSE Product #6790, Corning, NY). Then, tissues were incubated with primary antibodies (chicken anti-GFP [1:400], Abcam, Cambridge, MA; rabbit anti-TH [1:400], Millipore, Burlington, MA; CD31/PECAM-1 [1:300], Novus Biologicals, Littleton, CO) in primary incubation solution (92 mL PTwH, 5 mL DMSO, 3 mL NDS) on a shaking incubator at 37°C for at least seven days. Following primary
staining, tissues underwent five, 60 minute washes in PTwH (1mL of 10mg/mL heparin stock solution, 2mL Tween-20, QS to 1L with PBS) then subjected to secondary staining (AlexaFluor 647 donkey anti-chicken [1:300], AlexaFluor 555 donkey anti-rabbit [1:300], Jackson ImmunoResearch, West Grove, PA, AlexaFluor 488 donkey anti-goat [1:300], ThermoFisher Scientific, Waltham, MA) in secondary incubation solution (PTwH with 3% NDS) for at least seven days at 37°C on a shaking incubator. Once labeling was confirmed, samples underwent the same PTwH wash step, then an additional round of MeOH dehydration (both washes described above). Tissues were incubated in 100% MeOH overnight and then submerged in 66% DCM/33% MeOH solution for three hours. Once completed, the samples underwent two, 100% DCM washes, and were then placed in dibenzyl ether (DBE), an organic solvent for at least three hours at room temperature for clearing (https://www.protocols.io/private/B7569F5C0050B3198C8BBC721EC210BC).

3.2.4. Microscopy and image processing

A Leica SP5, SP8 X (Leica Biosystems Inc., Buffalo Grove, IL), or Ultramicroscope II (LaVision BioTec, Bielefeld, Germany) were used to image all relevant structures. To allow for confocal imaging of DBE-cleared tissues, a chamber was manufactured in-house. 3-D overview images were generated of the spine and spinal cord in order to reveal PRV labeling in preganglionic sympathetic neurons (in the IML and medially) and DRG. The spine was repositioned with the ventral side up to allow for optimal imaging of thoracic and lumbar sympathetic chain ganglia, as well was the celiac ganglion. Lightsheet imaging was performed with a zoom factor of 0.63x or 1.6x (specified in figure captions). Images of TH and PRV labeling were collected for all samples, while
CD31 staining was not performed for all samples. Images from the far-red fluorescent channel were pseudocolored as green. Additionally, higher magnification (10x/0.40 NA) confocal images captured of PRV-positive sympathetic chain ganglia, their corresponding DRG, and PRV-positive preganglionic cell bodies.

3.3. Results

3.3.1. TH-IREs-cre/tomato mice reveal the organization of pre- and paravertebral ganglia in the thoracic and abdominal cavities

We aimed to visualize and document the overall organization of the thoracic and abdominal sympathetic nervous system in adult mice and utilized cre-driver mice for the sympathetic marker tyrosine hydroxylase (TH), to express the reporter genes EGFP-L10a (TH:EGFP mice) or tdTomato (TH:tomato mice). The ribosomal protein L10a targets green fluorescent protein (EGFP) expression to the cell body, so that TH:EGFP mice show strong labeling of cell body containing paravertebral ganglia (sympathetic chain ganglia) and prevertebral ganglia (celiac, suprarenal, aorticorenal and superior mesenteric ganglia); but no labeling in nerve fibers (Fig. 3.1.A,A’). In contrast, the cytoplasmatic tdTomato expression labels cell bodies and nerve fibers, revealing the connectivity of sympathetic chain and celiac complex in TH:tomato mice (Fig. 3.1.B,B’).

In the mouse, the splanchnic nerve consistently branched off at the level of the sympathetic chain ganglion T12 and the elongated interganglionic strands between T12 and T13 (piercing through the diaphragm) served as a prominent landmark to identify upper abdominal chain ganglia T13, and lumbar ganglia L1-L3 (Fig. 3.1.B). Abdominal chain ganglia required careful dissection to allow visualization for imaging (Fig. 3.1.B) as overlaying adipose tissue often obscured or blocked the fluorescent signal (Fig. 3.1.A)
ganglia caudal to T8 not visible). To fully visualize the abdominal chain ganglia, the
celiac-superior mesenteric complex was moved rostral in Fig. 3.1.B, while the
undisturbed view of the celiac complex is shown in Fig. 3.1.B’ with further details of the
aorticorenal ganglia and splanchnic nerves.

3.3.2. Large volume tissue clearance reveals 3-D anatomy of SNS

Tissue clearing is a well-established technique used to make an otherwise deep,
opaque sample optically transparent to improve anatomical analysis. In 2014, Renier et
al. modified the existing 3DISCO clearing protocol to allow the use of
immunofluorescence labeling in large solvent-cleared tissues. We modified the
iDISCO method to perform whole body histological staining for tyrosine hydroxylase
peptide (TH-peptide) and tissue clearing in adult mice, allowing for remarkable
visualization of sympathetic chain ganglia and their physical connections, as well as fine
innervation structures of the celiac-superior mesenteric plexus (Fig. 3.1.C). We also
note that this provides unparalleled anatomical detail and context for transparent and
rigorous reporting. Furthermore, TH-peptide immunostaining is a more reliable marker
of the sympathetic nervous system compared to direct TH-cre driven reporters. During
early development, tyrosine hydroxylase is transiently expressed in cholinergic
parasympathetic neurons, meaning that parasympathetic neurons may also be
labeled in TH-reporter mice with TH-cre induced reporter expression throughout
development. Fig. 3.1.C clearly demonstrates TH-peptide staining in iDISCO-cleared
tissue verifying the abdominal sympathetic organization.
Figure 3.1. Ventral views of sympathetic chain and prevertebral ganglia as seen under the fluorescence dissecting microscope (A-C) and in the light sheet microscope after iDISCO processing (D). A, A’. TH-EGFP reporter mouse (male, 8 weeks old, 0.5x objective) with ventral view of the sympathetic chain ganglia (T4 to T8) and abdominal prevertebral sympathetic ganglia (celiac superior mesenteric complex) at low magnification. Note, the lower sympathetic chain ganglia caudal to T8 are obscured by overlying fat tissue. The celiac superior mesenteric complex depicts the fused celiac and superior mesenteric ganglia as well as the left suprarenal, the left aorticorenal ganglia (A’) B. B’. TH-tomato mouse (male, 12 weeks old, 0.5x objective) with ventral views of lower thoracic sympathetic chain ganglia (T10 to T13) and upper lumbar sympathetic chain ganglia L1 to L3. The lumbar sympathetic chain ganglia can be seen after moving the overlying prevertebral ganglia to the side. (fig. cont’d)
In B’ the celiac superior-mesenteric complex is seen undisturbed, showing several details, e.g. splanchnic nerves and aorticorenal ganglion. Note several non-specifically labeled structures such as ribs and muscle fibers. C. Light sheet microscope extended focus view of cleared tissue block (iDISCO, 1.6x zoom factor) with tyrosine hydroxylase (TH-peptide) immunohistochemistry from a 12 weeks old, male mouse, showing the lower thoracic and upper lumbar chain ganglia, together with the overlaying celiac-superior mesenteric complex and connecting nerves. Note the detailed view of lower sympathetic chain ganglia, greater splanchnic nerves entering suprarenal and celiac-superior mesenteric ganglionic complex. Also note the left aorticorenal ganglion and intermesenteric plexus. A long interganglionic strand links sympathetic chain ganglia T12 and T13 through the diaphragm.

### 3.3.3. iWAT is innervated by postganglionic neurons located in sympathetic chain ganglia T12, T13, L1

We aimed to clarify the origin of sympathetic innervation of iWAT using iWAT-derived labeling with fluorescent PRV (PRV<sub>GFP</sub>) and iDISCO TH-peptide and GFP double staining. These methods provide a comprehensive analysis of overall sympathetic structures and their according labeling with PRV. We chose a minimal PRV infection time that was long enough to allow consistent labeling of sympathetic chain ganglia (4days, Fig. 3.2.A), but was too short to label supraspinal sympathetic premotor neurons (e.g. raphe pallidus). Under these conditions, labeling was restricted to post- and preganglionic neurons and spinal interneurons.

Light sheet and confocal microscope imaging allowed for three-dimensional visualization and analysis of the entire thorax and abdomen. Among the 13 analyzed animals (6 females, 7 males) with successful PRV labeling in sympathetic chain ganglia, we observed consistent labeling in ipsilateral sympathetic chain ganglia T12-L1 (Fig.3.2.B-D), even though some variability was observed for individual cases showing labeling in T7-T9 (1/13), T11 (3/13) and L2 (3/13) (Figure 3.2.D; Appendix C). No tracer
labeling was detected in contralateral ganglia. Additionally, we could not find any obvious differences between male and female mice at any level of iWAT innervation.

The celiac complex was analyzed in 8 animals, and 6/8 animals (75%) showed no signs of PRV infection, even though one female and one male showed minimal to moderate PRV-labeled cell bodies in the celiac ganglion (data not shown). We concluded that the celiac-superior mesenteric complex does not contribute to iWAT innervation. In the rare cases with PRV labeling in the celiac-superior mesenteric complex the injections may have unintentional punctured an indiscriminate area innervated by the celiac-superior mesenteric complex or PRV may have diffused from subcutaneous depots through the (in rodents typically open) vaginal process of the inguinal canal\textsuperscript{125}. 

Next, we looked for evidence of PRV labeling in DRG sensory neurons. In a similar study of interscapular brown adipose tissue derived PRV labeling, we identified a low, but consistent PRV labeling in the DRG\textsuperscript{122}. In contrast to interscapular brown adipose tissue (iBAT)-related labeling, DRG ganglia showed only occasional PRV labeling with typically <5 neurons per ganglion and most often observed in DRG T11 (46\% of analyzed animals) and T12 (30\% of analyzed animals, Appendix C). We noted that iWAT derived PRV was not co-localized with TH-positive DRG neurons which lack authentic catecholaminergic functions\textsuperscript{126}. Our finding is consistent with another report indicating that TH-positive DRG neurons, which often contained also calcitonin-gene related peptide (CGRP), may rather innervate the colorectum and urinary bladder although the majority of CGRP-positive axons in these organs apparently lacked TH immunoreactivity\textsuperscript{127}. 
Figure 3.2. Sympathetic postganglionic innervation of iWAT. A. Mice were injected with the retrograde, transsynaptic tracer PRV<sup>GFP</sup> into the right iWAT pad. Perfusion and tissue harvest was performed 3-4 days post-viral injection. Whole body iDISCO for TH peptide (B and C) and PRV (B' and C') imaged under a confocal microscope (10x objective) of a 12 weeks old female mouse. In the merged image (B'' and C'') retrogradely labeled postganglionic sympathetic neurons can be seen in the right chain ganglia, T13 and L1. D. Summary of ganglionic labeling (positive cases/total number of mice) showing most consistent labeling in T12 to L1.

We further ensured that “off-target” labeling was not due to an overall viral leakage, and dripped the same volume and titer of PRV onto iWAT tissue. In all five negative control animals, virus drip-on was insufficient to infect sympathetic chain ganglia, celiac-superior mesenteric complex, and DRG with PRV (Appendix C).
3.3.4. Preganglionic sympathetic neurons innervating iWAT are located in the IML of the spinal cord spanning from T7 to T10

In order to allow imaging of preganglionic neurons in the spinal cord, all animals received a laminectomy prior to staining procedures. We imaged specimens from their dorsal side with either light sheet or confocal microscopy (Fig. 3.3.A). PRV labeling was predominant in the ipsilateral IML, but we also noted consistent PRV labeling in the intercalated (ICl) and central autonomic (CA) nuclei and some contralateral cells (Fig. 3.3.A). It should be noted that PRV tracing from peripheral organs to the spinal cord also labels interneurons in addition to sympathetic preganglionic neurons, due to further propagation of the transsynaptic PRV infection\textsuperscript{128-130}. In line with that, and because tracer labeling was absent in contralateral SChG, we interpreted PRV labeled contralateral neurons as interneurons.

The spinal root entry defines the middle of the according spinal segment. In three mice, we verified that thoracic SChG from T1 to T12 correlate well with the level of the DRG (Fig. 3.3.B) and the according dorsal root entry into the spinal cord, which is shifted slightly rostral (Fig. 3.3.C). Thus, for data analysis of PRV labeling in iWAT related preganglionic neurons we used sympathetic chain ganglia as a proxy to determine their segmental level. Preganglionic labeling was restricted to the thoracic spinal cord, with consistent labeling at T7-T10 in the majority of analyzed animals (82-91%), even though some cases included more rostral (T5) or caudal (T11) labeling (36%) (Fig. 3.4.A, Appendix C). Thus, iWAT-related preganglionic neurons (T7-T10) show a significant segmental rostral shift with respect to postganglionic PRV labeled neurons (T13-L1) as highlighted schematically in Fig. 3.4.B.
Figure 3.3. Preganglionic sympathetic neurons related to iWAT in the spinal cord in iDISCO preparation. A: Most preganglionic neurons labeled transsynaptically with PRV from iWAT are located in the IML (white arrows), while a significant number is detected in the intercalated (filled arrow heads) and central autonomic nuclei (open arrowheads); confocal microscope, 10x objective. B: In a 3D stack with overlaying TH immunofluorescence, the levels of sympathetic chain ganglia (red) serve as proxies for spinal cord segments. The location of DRG and according SChG is indicated, but only faintly visible. Light sheet microscopy, 0.63x zoom factor. B’: In a slice image the individual dorsal root ganglia and according dorsal roots (white arrows) are visualized as they enter the SC, which defines the middle of the according spinal cord segment. Light sheet microscopy, 0.63x zoom factor. Note, that lower sympathetic chain ganglia may serve as a proxy for spinal segments due to the increasing elongation of spinal roots before they enter the spinal cord.
3.3.5. Dissection and iDISCO clearing reveal two major access routes of sympathetic nerves to iWAT

Next, we aimed to define the peripheral neural pathway, which can guide sympathetic postganglionic axons to the iWAT endorgan. Initially, we used bright field guided dissection and imaging to identify the sympathetic nerve inputs to dorsolumbar iWAT. We located the exit points of lateral cutaneous rami from intercostal nerves T11 to T13 through the fascia and traced them to iWAT and skin (Fig. 3.5.A) as highlighted schematically in Fig. 3.5.B. The dorsolateral portion of iWAT is further innervated by the ilioinguinal (ILI) and the lateral cutaneous femoral nerves (LCFN, Fig. 3.6.A,B), which are both derived from the lumbar plexus.

The inguinal portion of the iWAT is entirely innervated by nerves that are derived from the lumbar plexus, which provides overall innervation to the ventral thigh. Fig. 3.6.C shows the inguinal portion of the iWAT in more detail. The major nerves visible in this view are the femoral nerve mainly derived from ventral rami of spinal nerves (L1-3), from which the anterior cutaneous femoral nerve (ACFN) branches off and enters the inguinal division of the iWAT depot (Figure 3.6.C). Further details of the innervation are visible upon removal of the overlying WAT, showing further branching of the LCFN and anastomoses with a branch of the ACFN (Fig. 3.6.D).

The complexity of the intercostal nerves and lumbar plexus nerves carrying sympathetic postganglionic fibers is not fully represented by stereomicroscopic images. Thus, we further dissected the entire iWAT depot and performed iDISCO staining for TH peptide and CD31 for better visualization of incoming major nerve fibers and vasculature (Fig. 3.7.A, A').
Figure 3.4. Pre-and postganglionic innervation of iWAT. A: Diagrammatic representation of spinal cord segments (SCS) with PRV-positive preganglionic neurons (n positive / n total number of mice) in IML, IC and CA. Preganglionic iWAT innervation is observed from T5 to T12, but is most consistent across animals from T7 to T10. B: Diagrammatic synopsis of pre- (green) and postganglionic (red) iWAT innervation. Note, the diagram only includes consistent pre- and postganglionic labeling sites. Also note the significant segmental caudal shift of postganglionic PRV labeling with respect to preganglionic innervation.

For orientation, we adopted the anatomical labeling and distinction of two iWAT portions that are roughly separated by a prominent lymph node in the WAT depot\textsuperscript{131}: the dorsolumbar and inguinal depot. We consistently observed one major, prominent nerve entering the inguinal portion of iWAT that is consistent with the anterior cutaneous femoral nerve (Fig. 3.6.C). In the dorsolumbar portion, we identified several incoming nerves (Fig. 3.7.A depicts 3 incoming nerves with asterisk).
Figure 3.5. Sympathetic chain ganglia T11-T13 provide sympathetic innervation to dorsolumbar WAT via lateral cutaneous nerves. A: Distribution of lateral cutaneous rami of intercostal nerves T11 to T13 to dorsolateral iWAT (dl) as seen under the stereomicroscope (0.5x objective). Yellow dots indicate exit of dorsal cutaneous rami T11 to T13 through the external oblique abdominal muscle (exobl) and fascia as landmarks for spinal nerve identification. B: Scheme of lower thoracic spinal nerve branching emphasizing lateral cutaneous branches to iWAT.

We found no consistency in the anatomical orientation of these nerves within dissected iWAT tissue, but they are consistent with the lateral cutaneous rami T11-T13 documented earlier (Fig. 3.5.A).

Furthermore, we assembled a schematic of the intercostal and lumbar plexus nerves as observed by stereomicroscope dissection in the mouse (Fig. 3.7.B). We found that all lateral cutaneous rami of intercostal nerves T11-13 and cutaneous rami of the lumbar plexus provide innervation to the adipose tissue, skin, and vasculature. Thus, these innervation systems seem highly intertwined and should receive consideration specifically in denervation studies where innervation of vasculature, skin and adipocytes will be likely similarly affected.
3.3.6. Varicose innervation density is sparse among white adipocytes, but dense among brown adipocytes within iWAT

Apart from the prominent main innervation branches depicted in Fig. 3.7.A, a grainier TH-positive structure is visible. This structure seemed to represent areas with increased density of axonal varicosities, even though the resolution of light sheet or confocal images in cleared tissue samples was insufficient to fully resolve this. In a paraffin, longitudinal section through the iWAT the location of beige islands, that show increased accumulation of brown adipocytes (also known as beige or brite adipocytes), is visible (Fig. 3.8.A, purple arrows). Immunohistochemical staining of adjacent sections for TH-peptide and uncoupling protein 1 (UCP1) demonstrates the stark difference in axonal varicosity density of UCP1 void areas (Fig. 3.8.B, B’) versus UCP1-rich areas (Fig. 3.8.C,C’). Further details of an iDISCO cleared iWAT sample further depicts an area that shows neuronal branching with axonal varicosities into a beige island in close proximity to the lymph node (Fig. 3.8.D). For clarity, the image was reproduced as a schematic in (Fig. 3.8.E).

3.4. Discussion

Our study aimed to allow a comprehensive, rigorous and transparent evaluation of pre- and postganglionic sympathetic inputs to iWAT in male and female mice. Our goal was also that our work would be useful for future studies that use virus-driven neuromodulation in select adipose tissue depots.

Our PRV based study demonstrates sympathetic chain ganglia T12-L1 as major postganglionic input to iWAT, and is similar to data in other rodent models.\textsuperscript{100,119}
Figure 3.6. Peripheral nerves to right iWAT in a 3 week old female TH:tomato mouse in brightfield (A) and fluorescence (B-D) stereomicroscope, ventral view. A. Brightfield overview (0.5x objective) of the right dorsolateral (dl) and inguinal (ing) portions of iWAT. The thoracoepigastric vein (thev) is visible at the cranial tip of the dl and the superficial ilium circumflex vein (sicv) is visible at the dl/ing iWAT junction. The femoral nerve (FN) appears upon its passage under the inguinal ligament (dotted) through the muscular lacuna. Medially, the femoral artery and vein (avf) are passing through the vascular lacuna. B. The upper rectangle in A imaged at higher magnification under the fluorescent microscope (0.5x objective). Intercostal (subcostal) T13, iliohypogastric (IHG), the fine ilioinguinal (ILI, open arrowheads) and lateral cutaneous femoral (LCFN) nerves and the iliolumbar artery and vein (avil) travel to the dorsolateral portion. Dashed contour indicates lymph node (ly) at dl/ing junction; exobl denotes external oblique abdominal muscle and fascia. (fig. cont’d)
C. Lower rectangle of A imaged at higher magnification under the fluorescent microscope (0.5x objective). The LCFN is seen approaching the dl/ing junction of iWAT. The main branches of the femoral nerve (FNm – motor branch to thigh extensor muscles, SAPH – saphenous nerve, ACFN – anterior cutaneous femoral nerve) are visible. The ACFN enters the inguinal portion and eventually exits to skin (cut, asterisk). D. The rectangle in C imaged at higher magnification. Upon removing the overlying WAT, the LCFN divides. One branch (double asterisk) anastomoses with a fine branch of ACFN (open arrowhead) deep within the inguinal portion. Bright fluorescent artifacts at dl/ing junction typically occur after longer exposure on WAT surface.

Recent reports have suggested that iWAT in mice may be purely innervated by the celiac complex\textsuperscript{107,132}, even though the celiac ganglia are thought to provide sympathetic innervation for abdominal structures like liver, kidney and gastrointestinal organs\textsuperscript{133}. In our study, we rarely found labeling within celiac ganglia or the celiac-superior mesenteric complex. We note that tracer injections to the iWAT are particularly prone to tracer diffusion from subcutaneous depots through the vaginal process (in rodents typically open) of the inguinal canal into the peritoneal cavity\textsuperscript{125}. Specifically, large injection volumes and long incubation times may enhance the risk of diffusion through the inguinal canal, eventually labeling neurons that innervate intraperitoneal organs. Indeed, Jiang \textit{et al} used a total tracer volume of 5 µL choleratoxin B with a 7-day incubation time compared a total PRV injection volume of 1-2 µL and 4 days incubation time in the present study. This further highlights the importance of our study to define reproducible labeling patterns for endorgan innervation, so that future approaches for viral targeting of the peripheral nervous system continues the same rigor as established for virus spread in the CNS\textsuperscript{134-137}.

The findings presented here confirm postganglionic innervation from T13 and L1, which was found by others in rats and hamsters\textsuperscript{100,119}.
Figure 3.7. iDISCO tissue clearing shows main innervation branches to iWAT. A. Light sheet images of an iWAT depot, stained and cleared with iDISCO for TH-peptide (red = sympathetic innervation) and CD31 (green= vasculature and lymph node (ly)). The dorsolateral innervation shows several incoming branches (asterisk) that cannot be conclusively assigned to individual nerves, but should represent lateral cutaneous rami from T11-T13. In the inguinal portion one prominent incoming branch can be consistently identified that we conclude is the ACFN, which is also the most prominent nerve visible under the stereomicroscope. B. Schematic drawing of the lumbar sympathetic plexus that provides innervation to the thigh, including adipose tissue, vasculature and skin.

Another study in the hamster also found sympathetic inputs to iWAT from T12-L3, and additionally input from T1-T3 chain ganglia, with extensive overlap of sympathetic input to iBAT and iWAT. In contrast, the present study showed a more restricted input to iWAT (SChG T12-L1). None of our animals showed PRV labeling in rostral thoracic SChG (stellate/T1-T5), which we recently demonstrated as the main sympathetic input to iBAT. Importantly, our data suggests an anatomical separation of postganglionic input to iBAT vs iWAT. We speculate that the discrepancies of our study with Nguyen et
are likely due to the extended PRV incubation time (6 days vs. 4 days in our study), as their aim was also to study overlapping patterns in CNS structures that require longer incubation time. Thus, while the ability of PRV for transsynaptic labeling is an advantage to identify circuits, it is important to recognize the possibility for transsynaptic labeling within pre- and postganglionic neurons with increasing incubation time, and has been demonstrated for spinal cord interneurons\textsuperscript{128-130}.

We further show that the preganglionic input to iWAT (T7-T10) was also anatomically distinct from preganglionic input to iBAT (T2-T6)\textsuperscript{122}. We were unable to find other published work that anatomically defined the rostro-caudal extent of preganglionic input to iWAT or iBAT in the spinal cord. Importantly, our data suggest that both pre- and postganglionic inputs to iBAT and iWAT are anatomically separated.

Our data provide the anatomical basis to suggest that CNS inputs could differentially regulate the sympathetic tone to iBAT vs. iWAT. This is in line with a recent study indicating that hypothalamic \textit{proopiomelanocortin} (Pomc) expressing neurons and \textit{agouti related peptide} (Agrp) expressing neurons both regulate the sympathetic tone to iBAT, while sympathetic tone to iWAT was only regulated by Pomc neurons, but not Agrp neurons\textsuperscript{138}. This is also relevant, as several studies have now suggested that sympathetic activation of BAT might not be sufficient to induce BAT thermogenesis and might require additional sympathetic activation of WAT\textsuperscript{139-141}. Future studies will need to address if known preganglionic inputs from the CNS may selectively regulate iBAT and iWAT sympathetic circuits.
The clear anatomical separation of both pre- and postganglionic neurons innervating iWAT and iBAT, respectively, may facilitate future experimental studies on differential forebrain control of brown and white adipose tissues.

Sympathetic postganglionic axons travel via dorsal and ventral rami to their end organs, which combine sensory fibers from dorsal root ganglia and, via communicating rami, postganglionic fibers from SChG. Our anatomical dissections show that major incoming nerves to the dorsolumbar portion of iWAT are lateral cutaneous branches of intercostal nerves. Indeed, iWAT-related PRV labeling of SChG is consistent with the innervation from intercostal nerves T12 to L1, which represent ventral rami of spinal nerves in that area.

Likewise, we found that the inguinal portion of iWAT is innervated via nerves derived from the lumbar plexus, which is also formed by ventral rami of spinal nerves, with a branch of the femoral nerve (L1-3) and the lateral cutaneous nerve of the thigh (L2). iWAT-related postganglionic neurons in chain ganglia L1-L3 send their axon through the communicating rami into these nerves. However, also lower thoracic chain ganglia, e.g. T12 and T13 may connect to the lumbar plexus nerve, as a particular spinal nerve receives postganglionic axons from both, the next cranial and also the next caudal chain ganglia\textsuperscript{14}. As no PRV labeled postganglionic neurons were found in sympathetic ganglia caudal to L2, we speculate that this reflects PRV concentration into the dorsolumbar iWAT, which is consistent with our dorsal injection approach that might have unintentionally favored viral uptake into nerves to the dorsolumbar iWAT. Also, we cannot rule out that fat mass expansion with aging or obesity could further recruit innervation from more rostral or caudal sympathetic chain ganglia.
Interestingly, it has been noted by others that the dorsolumbar and inguinal portions show functional distinctions\textsuperscript{142}. The inguinal portion is more prone to beiging and shows denser sympathetic innervation than the dorsolumbar portion. Thus, it is interesting to speculate if and how the anterior femoral nerve contributes to the increased beiging. Our data are the first to provide detailed maps for sympathetic nerves of iWAT. We further translate these data to 3D iDISCO imaging data in iWAT, so that a more targeted investigation and distinction of dorsolumbar vs. inguinal innervation patterns is encouraged for future studies.

Importantly, varicose innervation density is strongly associated with adipose tissue structure. In iWAT, dense varicosities are mainly associated with arteries, while white adipocytes show sparse varicose innervation. In contrast, brown adipocytes clustered in beige islands, strongly correlate with increased varicose innervation pattern. Thus, dynamic interaction of neurotrophic repulsion and attraction, similar to autonomic innervation guidance during development\textsuperscript{105,143}, are likely responsible for the robust difference in innervation patterns of white and brown adipocytes, respectively. However, the dynamic changes of varicose endings at the interface of white and brown adipocytes remains unstudied. Some studies used the dense network of sympathetic nerves to measure iWAT innervation density\textsuperscript{107,131}, but this may not well represent the varicose innervation pattern at the interface of white and brown adipocytes, as it is the number of individual varicosities that dictate the amount of neurotransmitter release and thus, degree of sympathetic action\textsuperscript{144}.

Our data also clarify that nerves supplying the iWAT are continuous structures that transition through the adipose tissue further to the skin.
Figure 3.8. Varicose innervation density depends on white versus brown adipocyte innervation. A. Hematoxilin and Eosin staining (stains cytoplasmatic content purple) of a paraffin-embedded longitudinal section (5 μm) of iWAT shows the prominent lymph node (ly) at the junction of dorsolumbar (dl) and inguinal (ing) portions of iWAT (confocal microscopy, 5x objective). Areas with mainly white adipocytes appear light purple (rectangle to B), due to the reduced cytoplasmatic content of white adipocytes. Areas with darker purple stain depict areas with increased number of brown adipocytes (beige islands, purple arrows and rectangle to C), which contain more coplasm than white adipocytes. (fig. cont’d)
The upper square depicts an area with mostly white adipocytes. Staining of an adjacent section with TH-peptide (B) and the brown adipocyte marker, UCP1 (B’) shows that low TH-positive varicosities are found among UCP1-negative white adipocytes. The lower square depicts an area with mostly brown adipocytes. Staining of an adjacent section with TH-peptide (C) and UCP1 (C’) shows that high TH-positive varicosities are found among UCP1-positive brown adipocytes. D. Confocal image (5x objective) of iWAT iDISCO cleared and stained for TH-peptide and the vascular marker CD31. Apart from the major TH-positive nerves, a more grainy TH labeling of axonal varicosities is seen. Co-localization with CD31 shows dense varicosites associated with arterial vasculature. Beige islands with brown adipocytes can be identified by dense varicosities, while areas with mostly white adipocytes show low varicosity density. E. The schematic drawing of the confocal image in D clarifies the observed pattern of axonal varicosites associated with arteries, brown and white adipocytes.

Fine nerves branch off from these rami to reach deeper into the adipose tissue and provide a dense plexus of varicose axons especially to the smooth muscle media of small arteries and arterioles. Varicosities are sparse adjacent to white adipocytes, even though beige islands with brown adipocytes show a visibly denser varicosity pattern. It should be noted that surgical iWAT denervation, likely by cutting the lateral cutaneous rami and lumbar plexus incoming nerves \(^{145}\), will remove innervation to iWAT, but also to the skin and vasculature. To selectively cut iWAT-specific branches off the rami is likely not feasible, and therefore the confounding effects of skin and vasculature denervation should be considered in future surgical denervation studies. Alternatively, chemical denervation has been successfully used with comparable results to surgical denervation \(^{146}\), where the chemical toxin is directly injected into the adipose tissue depot. We predict that this method is more suitable to selectively denervate adipose tissue, while sparing innervation to further downstream structures like the skin that are jointly innervated by the same rami. We also suggest that future denervation studies (surgical or chemical) to include histological verification of the denervation extent with a focus on structures that share innervation via the same rami.
Furthermore, the precise anatomical identification of pre- and postganglionic innervation in the mouse should be a guide to verify correct viral labeling e.g. using virus-driven expression of chemo-or optogenetic constructs, that are based on retrograde tracing methods. Furthermore, animals should be inspected for off-target labeling in the celiac ganglion, as they are inconsistent with iWAT innervation. Also, we currently have little understanding if all iWAT innervating nerves serve the same purpose, and past electric recordings of adipose tissue innervation lacked anatomical definition which nerves were recorded and if they included skin and vascular innervation or if more specific iWAT related recording is feasible\textsuperscript{138}. Our work enables future studies to provide anatomical detail about the nerves targeted. Another consideration is also the density of axonal varicosities as they are variable within the iWAT depot with dense varicosities in areas with high brown adipocytes (beige islands) and sparse varicosities adjacent to white adipocytes. We speculate that the density of varicosities could affect viral uptake and retrograde tracing efficiency. Indeed, in an earlier study we found that PRV incubation times after injection into the iBAT required less incubation time\textsuperscript{122} than after injection into iWAT. Such dynamics will require more rigorous examination in the peripheral nervous system when comparing mouse models with increased or decreased innervation (e.g. due to obesity or chronic cold exposure).

In summary, our data provide a comprehensive study to precisely define pre- and postganglionic sympathetic inputs to inguinal and dorsolumbar adipose iWAT tissue in male and female mice that are distinct from pre-and postganglionic inputs to BAT.
These studies should serve as a guide for a rigorous and transparent method to verify proper viral infections for molecular genetic tracing of adipose tissue specific innervation, which is comparable to the rigorous approach commonly used in CNS studies.
Chapter 4. Summary, Outlook, and Significance

4.1. Research summary

4.1.1 Independent sympathetic inputs of iBAT and iWAT

In Chapters 2 and 3, we detailed the sympathetic innervation of iBAT and iWAT through the use of reporter mice, viral retrograde tracing, large-volume tissue clearance, and light sheet microscope. To our knowledge, an anatomical diagram that depicts the spatial orientation of SChG, the spinal cord and column, and the spinal nerves had not been generated for the mouse. In Fig. 4.1, a schematic accurately displays the distribution of these structures.

In addition, Fig. 4.1 comprises the viral tracing results from both iBAT and iWAT datasets. Visualizing the data in this manner emphasizes the separation of the pre- and postganglionic inputs between the two fat pads, as they arise from distinct anatomical levels. This is a significant shift from earlier views, which indicated that iBAT and iWAT may have joint innervation circuits. Our findings can be further substantiated upon examination of the physical location of each fat pad.

We report that the sympathetic postganglionic neurons that innervate iBAT are located at approximately the same vertebral level of their according preganglionic inputs (Figure 4.1). This is because the iBAT receives input from the SChG that emanate at the level of the upper thoracic vertebrae. Due to the articulations between the upper thoracic vertebrae and the ribs, these vertebrae are rigid in relation to other regions the of vertebral column, which have a greater degree of curvature.
Figure 4.1. The distinct pre- and postganglionic sympathetic inputs of iBAT and iWAT using PRV retrograde tracing. Unilateral PRV injection into the iBAT resulted in labeling of SChG SG-T5. As the SG is often described as being “fused” to SChG T1, labeling of preganglionic inputs in the spinal cord are found at the anatomical level T1-T5. When PRV is unilaterally injected into the iWAT, labeling is found in SChG T12-L1. During childhood development, the spinal cord no longer elongates as the vertebral column does. The spinal nerves move farther away from their original position relative to the spinal cord, which is why the preganglionic inputs of iWAT are found at anatomical level T12-L1.
During development, the degree of curvature in the spine decreases, which elongates the vertebral column\textsuperscript{148}. However, the spinal cord does not elongate equally, making the spinal cord shorter than the vertebral column\textsuperscript{149}. Therefore, pre- and postganglionic inputs of the iBAT appear at approximately the same vertebral level. In contrast, the postganglionic neurons that innervate iWAT are located caudal to their preganglionic neurons (Figure 4.1). This finding is in agreement with the understanding that postganglionic inputs innervate peripheral structures located at the same spinal segment\textsuperscript{150}, as seen with the SChG that innervate iWAT.

Furthermore, the spinal cord is organized into 31 segments that are defined by 31 pairs of nerves (cervical (8), thoracic (12), lumbar (5), sacral (5), and coccygeal (1)). Each spinal segment contains organs, skin, and muscles in which the according spinal nerve innervates\textsuperscript{148}. Overlap between neighboring segments exists\textsuperscript{151}, as the human sciatic nerve is formed by the combination of five spinal nerves (L4, L5, S1, S2, S3)\textsuperscript{152}, indicating five different spinal segments. However, the most caudal spinal nerve innervating iBAT is T5, and the most rostral spinal nerve innervating iWAT is T12. The distance between spinal segments T5 and T12 further supports our conclusion that iBAT and iWAT are innervated by differential sympathetic nerves that are unlikely to overlap.

One can speculate that previous work demonstrating an overlap in iBAT and iWAT sympathetic input could have arisen from virus incubation time. At most, we allowed for a 4d incubation period post-injection. Conversely, Nguyen et al. (2017) performed iBAT and iWAT injections in the same hamster and allowed for a 6d incubation time\textsuperscript{63}. Given that PRV is a retrograde transsynaptic tracer, the two additional
days could allow PRV that was injected into iWAT to also label the SChG innervating iBAT, causing a perceived overlap in sympathetic inputs.

Altogether, these findings give way to the possibility that a differential central control of the fat pads exist. In an experiment by Bell et al. (2017), deletion of leptin receptors (LEPR) on POMC and AgRP neurons partially blunted the typical, leptin-evoked increase in sympathetic nerve activity observed in BAT. However, loss of LEPR in AgRP neurons, but not POMC neurons, interfered with leptin-induced sympathetic nerve activity to iWAT and the liver\textsuperscript{138}. This data suggests that leptin engages different neuronal populations to control the sympathetic nerve activity of distinct tissues.

There is also data that a differential SNS activation of adipose tissues exist via melanocortin receptor stimulation\textsuperscript{153}. Melanocortins, which are peptide hormones adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormone (MSH) cleaved from POMC, control energy intake and expenditure. Upon central injection of melanotan II, a melanocortin 3/4-receptor agonist known to decrease adiposity, differential sympathetic drives to iBAT and iWAT were observed. Using norepinephrine turnover as a measurement of sympathetic drive, it was found that MTII increased sympathetic drive in iWAT, but not iBAT. Furthermore, central MTII did not increase sympathetic drive of epididymal WAT or retroperitoneal WAT. Taken together, these results not only demonstrate a difference between iWAT and iBAT, but also between WAT depots. To further substantiate these findings, a future study could use a melanocortin 4 receptor reporter (MC4R) mouse and our anatomical labeling maps of the IML to determine if their location corresponds to the location of the preganglionic cell bodies of iBAT and iWAT. If a significant discrepancy in the distribution of melanocortin
4 receptors is evident, this could be the first indication for why there was a difference in the sympathetic drives. This hypothesis could be tested by injecting PRV into the different fat pads of MC4R mice and analyzing the amount of co-localization between PRV and MC4R in the IML. Collectively, our viral tracing and anatomical data in conjunction to the findings described in the literature, strongly supports the notion that iBAT and iWAT possess distinct sympathetic inputs.

4.1.2. PRV-labeled preganglionic cells may provide new understanding of adipose depot connection to CNS

The combination of whole-body tissue clearance and high-resolution imaging resulted in the ability to image the entire thoracic and lumbar region of the spinal cord, distinguish spinal cord nuclei, and identify the vertebral level at which the PRV-labeled cells were located. As depicted in Fig. 4.2.A, the grey matter of the spinal cord is distinguished by nuclei and Rexed laminae. Spinal cord nuclei are determined by cell location, whereas Rexed laminae are ten layers that are grouped according to the cell’s structure and function. Also, PRV\textsuperscript{GFP} labeling was restricted to the right side of the spinal cord (Fig. 4.2.B), ipsilateral to the fat pad that received the injection, indicating that our tracer was specific and viral leakage did not occur.

Our studies revealed that the positively labeled, preganglionic cells of the spinal cord were located in the IML, ICl, and CA; which can also be characterized as Lamina VII (IML, ICl) and X (CA). The IML, ICl and CA are the sites in which sympathetic preganglionic neurons are located\textsuperscript{154}, which provides as additional confirmation because these are the specific regions in the spinal cord that we observed PRV\textsuperscript{GFP} labeling (Fig. 4.2). Furthermore, it has been reported that the dendrites from the IML,
ICI, and CA form dendritic bundles that traverse the spinal cord\textsuperscript{154-156}, a detail that we could clearly observe in Fig. 4.2. Our findings in the spinal cord are meaningful in that they can provide additional understanding to the sympathetic circuitry innervating iBAT and iWAT.

In 2014, Madden and Morrison elegantly outlined a proposed thermoregulatory network of BAT\textsuperscript{157}. Ambient temperature is sensed by cold and warm cutaneous thermal sensory receptors that send information via afferent pathways to the preoptic area (POA). The POA functions to modulate the central thermoregulatory response by controlling sympathetic output to many organs, most notably BAT\textsuperscript{158}, and it has also been speculated that the POA is similarly involved in the browning of beige adipocytes found in WAT depots\textsuperscript{159}. In the instance of cold exposure, a stimulus for BAT activation, POA warm-sensing neurons inhibit BAT sympathoexcitatory neurons in the dorsomedial hypothalamus (DMH)\textsuperscript{157,158}. The disinhibition of the neurons in the DMH excite BAT sympathetic premotor neurons in the rostral raphe pallidus (rRPA) and parapyramidal area (PAPy). Serotonergic (5-hydroxytryptamine, 5-HT) premotor neurons descend from the rRPA and PAPy and terminate in close proximity to GABAergic interneurons in the CA of the spinal cord\textsuperscript{160}. Serotonin plays a vital role in the excitation of the BAT sympathetic preganglionic neurons in the spinal cord, as it has been demonstrated to potentiate the excitatory inputs to BAT sympathetic preganglionic neurons from the rRPA. It has been postulated that the serotonergic inputs descending from the rRPA inhibit 5-HT\textsubscript{1A} receptors located on GABAergic (inhibitory) neurons in the IML.
Figure 4.2. Peripheral PRV\textsuperscript{GFP} injections into iWAT fat pad labels the intermediolateral (IML), intercalated (ICl), and central autonomic (CA) nuclei of the spinal cord. A) In lamina VII, the preganglionic cell columns of the autonomic nervous system are located in the IML, ICl, and CA. The preganglionic sympathetic neurons in the IML extend their dendrites in a transverse manner, where they reach the ICl. The ICl acts to connect the IML to the CA. Unilateral injection of PRV into the fat pad resulted in PRV-labeled cell bodies in the IML, ICl, and CA (labeled in green, B). These findings indicate that PRV labeling in the spinal cord nuclei is ipsilateral to the injected fat pad and that PRV labeling is restricted to nuclei that contain sympathetic cell bodies.

Inhibition of GABAergic interneurons allows for increased sympathetic outflow, as the inhibitory effect of the GABAergic interneurons to BAT sympathetic preganglionic neurons is now absent\textsuperscript{157,161,162}. Since our methods enable one to simultaneously immunolabel and visualize the physical connections between spinal cord nuclei (Fig. 4.2.B), this poses as an intriguing opportunity to shed new light on the hypothesized circuit.

A future study could involve PRV injection into the iBAT of vesicular GABAergic transporter (VGAT) reporter mice.
Use of VGAT reporter mice will function as a marker of the precise location of the GABAergic cell population in the spinal cord, as it appears that the CA and IML are used interchangeably across different publications. Furthermore, previous studies lack photograph evidence documenting the spinal cord and its nuclei.\textsuperscript{157,160-162} Next, iDISCO processing would include an immunolabel for PRV\textsuperscript{GFP}, 5-HT, and the VGAT reporter mice.

First, we would seek to verify that PRV labeling was evident in SChG SG-T5. We would then determine if PRV\textsuperscript{GFP} cells (marker for BAT sympathetic preganglionic neurons) have a close association with VGAT-labeled fibers in the IML, which would provide evidence that GABAergic interneurons synapse onto BAT sympathetic preganglionic neurons. Next, verify if and where the cell bodies of the GABAergic interneurons of the spinal cord appear to make close contact with 5-HT labeled fibers. In the brain, we would analyze the cell bodies in the rRPA-PAPy for co-localization of PRV\textsuperscript{GFP} and 5-HT. If successful, this study would provide compelling visual evidence of the proposed pathway.

4.2. Outlook

4.2.1. Current status of adipose-specific SNS stimulation

Ideally, our goal is to utilize the results described in Chapters 2 and 3 to generate a tissue-specific SNS stimulation technique with the hypothesis that an iBAT- or iWAT-specific SNS stimulation via $\beta_3$-adrenoreceptors will stimulate thermogenesis or lipolysis in diet-induced obese mice. In turn, this would exclusively enhance adipose tissue function while avoiding the dangerous potential side effects of systemic $\beta_3$-adrenoreceptor agonist compounds\textsuperscript{163}. $\beta_3$-adrenoreceptor agonist drugs have fallen out
of favor because β3-adrenoreceptors have been indicated to participate in the pathogenesis of cardiac failure\textsuperscript{164-166}, so any compound that would exacerbate the development of cardiovascular disease is not ideal.

A major criticism in developing an intervention that exclusively stimulates the SNS in fat pads points to the evidence that the SNS is pathologically overactive in obesity.\textsuperscript{167} This suggests that increased stimulation via the SNS is not only ineffective for weight loss, but dangerous to human health. Metabolic syndrome (MetS) is a health condition characterized by the co-occurrence of obesity, hypertension, dyslipidemia, insulin resistance, and hyperglycemia. Interestingly, data from MetS patients is described as chronic sympathoexcitation, resulting in increased urinary noradrenaline levels, efferent muscle sympathetic nerve activity, and rates of plasma noradrenaline spillover. In fact, a handful of symptoms do provide evidence that could indicate sympathetic overactivity: insulin resistance, increase in HPA axis activity, leptin resistance.\textsuperscript{168} However, this does not address the status of sympathetic activity in adipose tissue.

The sympathetic tone in adipose tissue has been shown to be decreased in obese humans and rodents and have down-regulated β-adrenoreceptors in WAT.\textsuperscript{168} It is also believed that obese adipose tissues lose thermogenic capacity because of the expanding size of the depot, leading to impairment of the innervation and vascularization,\textsuperscript{169,170} and ultimately sympathetic dysfunction.\textsuperscript{171} As the functionality of sympathetic activity continues to be debated, it is more likely that both lines of thought exist contemporaneously, with some organs gaining an increase in sympathetic tone while others decrease.\textsuperscript{168}
Despite arguments to the contrary, SNS stimulation has been a long-standing rationale for obesity therapeutics. The challenge lies in identifying the correct combination of tools and molecules to target in order to achieve the desired effects in vivo, while also keeping human application in mind. Previously, Zeng et. al. utilized optogenetics and TH-cre mice to locally stimulate the sympathetic nerve endings in iWAT, concluding that leptin drives sympathetic activation, wherein sympathetic nerve fibers make direct contact with adipocytes to release NE. While this methodology did indeed result in lipolysis and fat mass reduction, implantation of the optical fiber was invasive as it was implanted down the entire length of the back and therefore, poses concerns about feasibility for long-term use. Also, this study was not conducted in diet-induced obese mice, so it remains to be seen if this method would prove successful in the mouse line that serves as a proxy for human obesity.

Additionally, there is data that credits intracellular glycolysis being essential to NST in iBAT when induced via acute optogenetic stimulation. The role of glucose in NST presents an interesting line of thought because iBAT is a substantial depot for glucose uptake. Individuals with type 2 diabetes have impaired glucose metabolism in BAT, which indicates a possible cellular mechanism for the hyperglycemia exhibited in those with type 2 diabetes. However, body weight loss was not considered and represents a significant drawback, as the experiments were conducted under anesthesia and hence, acute in nature (3hr). Monitoring body weight is paramount because it is estimated that 85% of people with type 2 diabetes are overweight or obese. To add, it has been routinely reported that the amount of BAT is inversely correlated with body mass in humans. Therefore, future work should implement
BAT stimulation in awake, freely-moving wild-type and db/db mice (type 2 diabetes mouse model) to gain a more accurate understanding of the relationship between intracellular glycolysis in BAT, NST, and the possible impact in type 2 diabetes.

Lastly, a recent publication provides proof-of-concept for wireless optogenetic implantation in iWAT. The wireless optogenetic device selectively stimulates calcium cycling in the iWAT of Adipo-ChR2 mice, initiating thermogenesis through the SERCA2b pump. This approach differs from our design as it targets adipocytes by exploiting the adiponectin gene expressed in every adipocyte versus targeting the SNS that innervates adipose tissue. Intriguingly, wireless optogenetics successfully increased whole-body EE in a thermoneutral environment and protected mice from obesity in the presence of HFD\(^{176}\). It must be noted that the success of this method is exclusive to the Adipo-Serca KO mouse line and not the wild-type littermate controls. Indeed, clinical trials using optogenetics-based therapies have been approved (clinicaltrials.gov NCT03326336)\(^{177}\), but it is not yet commonplace, and the addition of implanting a wireless device is another component to address. Taken together, a substantial undertaking lies in implementing this type of therapy in humans.

In our ongoing efforts to chemogenetically stimulate sympathetic nerves of iBAT or iWAT, we utilize the innervation maps described herein to routinely verify that the viral constructs that are injected into distinct adipose depots properly target their according sympathetic chain ganglia. Furthermore, the visual verification and documentation of the virally-labeled sympathetic chain ganglia is an excellent method that works to ensure that our experiments are carried out with scientific rigor, transparency, and reproducibility.
4.3. Significance of research

Extensive research has been conducted to identify the hypothalamic circuits involved in controlling the sympathetic nerve activity found in iBAT and iWAT. To that end, the sympathetic post-ganglionic inputs of iBAT and iWAT have also been documented\textsuperscript{100,101,119}. In Chapters 2 and 3, we utilized these findings to verify our own labeling results and then, ultimately improve, the method to properly identifying SChG and other relevant structures through the use of reporter mice, PRV retrograde tracing, iDISCO tissue clearance, and lightsheet/confocal microscopy. Furthermore, our conclusion that iBAT and iWAT are served by separate sympathetic inputs is supported by the mounting evidence that separate neuronal populations govern the activity of each adipose depot\textsuperscript{138,153}

Additionally, our results can contribute to improving the precision capabilities of technology like pharmaco- and optogenetics because our detailed innervation map enables one to conclude where the virus was uptaken in the fat pad. For example, our dissection studies revealed that iWAT has the potential to receive innervation from SChG T12-L4; however, the most frequently labeled SChG were T12, L1. In the future, we can test if concentrating injections to the inguinal portion of the iWAT results in a higher incidence of SChG L2, L3, and L4 labeling. On the other hand, it is also possible that differences in innervation density (varicosities) that exist in a fat pad (fig. 3.8) could be responsible for this effect.

In conclusion, we provide a complete map of SNS innervation to the iBAT and iWAT depot. These data document the identity and location of the pre- and postganglionic sympathetic neurons innervating iBAT and iWAT. We also hope that our
findings can be utilized as a comprehensive anatomical resource for those interested in SNS studies in the mouse.
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### Appendix B. Supplemental Data for Chapter 2

Detailed information on mouse models, pseudorabies virus and antibody used

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Appendix B. Supplemental Data for Chapter 2

Individual data for automated cell counts of PRV-GFP labeled neurons in sympathetic chain ganglia using IMARIS software

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Appendix C. Supplemental Data for Chapter 3

PRV labeling is occasionally found in dorsal root ganglia. Example of PRV labeling (green) in DRG at level T11 (A), T12 (B), T13 (C) and L1 (D). DRG neurons were easily identified by the existence of few, but consistent TH-positive neurons (red). PRV-positive DRG neurons were consistently not co-localized with TH. Light sheet images, 0.63x zoom factor.
Appendix C. Supplemental Data for Chapter 3

PRV injections into iWAT exclusively labels the sympathetic chain ganglia and IML. A-C. Negative control “drip-on” tests were conducted in 5 mice. PRV administered onto the iWAT via this method provided no evidence of PRV labeling in the sympathetic chain ganglia (A, A’, light sheet microscope, 0.63x zoom factor), celiac ganglion (B, B’, dissecting microscope, 0.5x objective), or iWAT (C,C’, light sheet microscope, 0.63x zoom factor).
Appendix C. Supplemental Data for Chapter 3

Individual results for all animals of PRV-positive neurons found in sympathetic chain ganglia (A), dorsal root ganglia (B), Celiac ganglia (C), Intermediolateral bundle (D).
Appendix C. Supplemental Data for Chapter 3

List of used resources.

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<th>Reagent type (species) or resource</th>
<th>Designation</th>
<th>Source or reference</th>
<th>Identifiers</th>
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Reference List


3 Shapiro, B. & Wertheimer, E. The metabolic activity of adipose tissue; a review. Metabolism **5**, 79-86 (1956).


7 Cinti, S. in *Adipose Tissue and Adipokines in Health and Disease* (ed G. Fantuzzi, Mazzone, T.) 3-19 (Humana Press, 2007).


Vita

Clara Huesing spent her childhood in the city of Aurora, Illinois. She returned to her birthplace of Louisiana to attend college. She received her Bachelor of Science degree from Franciscan Missionaries of Our Lady University in Baton Rouge, Louisiana. While pursuing her undergraduate degree, she worked in Dr. Heike Münzberg-Grüning’s laboratory at Pennington Biomedical Research Center. The combination of neurobiology, cutting-edge technology, and obesity research piqued her interest, so she made the decision to apply for the PhD program at Louisiana State University. It was in Dr. Heike Münzberg-Grüning’s laboratory where Clara remained and completed her doctoral studies. She anticipates graduating in August 2020.

When Clara is not researching, she enjoys doting on her English Bulldog, Louie, gardening, and making memories with her friends and family.