Spring 2021

Cold-Induced Metabolic Changes in Lean, NAG-1 Transgenic Mice

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COLD-INDUCED METABOLIC CHANGES IN LEAN, NAG-1 TRANSGENIC MICE

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A Thesis

Presented to

The Graduate Faculty

Central Washington University

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Biology

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by

Rachel Josette Davey

June 2021

CENTRAL WASHINGTON UNIVERSITY

Graduate Studies

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Dean of Graduate Studies

ABSTRACT

COLD-INDUCED METABOLIC CHANGES
IN LEAN, NAG-1 TRANSGENIC MICE

by

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Obesity is a multifaceted metabolic disorder with severe worldwide public health consequences. While obesity can often be treated using diet and exercise, there are complex genetic interactions driving obesity that don't always respond to such changes. One important research focus for addressing genetic factors is activating brown adipose tissue which increases thermogenesis, the process of burning stored fats to generate heat. Cold exposure is a known way to activate brown adipose tissue through β3-adrenergic signaling. This signaling pathway causes an upregulation of thermogenesis in brown adipocytes and beige adipocytes, which is white adipose tissue that "browns" and becomes metabolically active. In this study, transgenic mice that constitutively express the human non-steroidal anti-inflammatory gene 1 (NAG-1) were used as a model to further understand the mechanisms of increasing metabolism. At room temperature, NAG-1 mice have less adipose tissue, higher metabolic rates, and longer lifespans than their wildtype littermates. Wildtype and NAG-1 mice were subjected to a prolonged cold exposure at 10ºC for seven days. NAG-1 mice had lower body weights throughout the study compared to wildtype mice. NAG-1 mice had higher metabolic rates before and after cold exposure. Adipocyte size analysis indicated adult wildtype male mice have significantly larger white adipocytes after cold exposure than NAG-1 males. Quantitative real-time PCR indicated higher expression of *Glut4* and *Atgl*

in wildtype mice, indicating that NAG-1 does not upregulate expression of thermogenic genes in response to cold exposure. The data from these analyses suggest that NAG-1 does not act synergistically with the β-3 adrenergic stimulated pathway in cold response.

ACKNOWLEDGMENTS

I know that I will never be able to express my gratitude adequately with words, but it is certainly worth a try. First and foremost, I would like to acknowledge my committee chair and mentor, Dr. April Binder. Not only does she have a gift for explaining complex topics in a digestible way that boosts her students to a higher level of understanding and practice as research biologists, but she makes the entire process fun- something I have begun to truly appreciate the importance of. Her grace, patience, and unwavering support through one of the most difficult experiences of my life (graduate school and a pandemic?!) have changed who I am and who I strive to be in the best possible way. While the knowledge and techniques that she has instilled in me have given me confidence for my future as a scientist, the mentorship and perseverance I have been lucky enough to experience are absolutely invaluable for me as a human being. I will never be able to truly repay Dr. Binder and certainly would not have made it to this point without her.

I would also like to thank my wonderful committee members and advisors along the way- Dr. Irwin, Dr. Darda, Dr. Oppelt, and Dr. Carnell. Dr. Irwin was beyond generous with his time and equipment to allow me to perform the respirometry portion of this study. His insight into the analysis of the data collected was greatly appreciated and valued. Dr. Darda shared priceless advice that gave me hope to continue with histology after I thought I ruined all of my samples, and eventually led to the photos in this study! Dr. Oppelt brought incredible perspective and depth to the immunometabolism components of my research, but also

revitalized my love for science with her contagious passion for metabolism. Quite frankly, without Dr. Carnell I wouldn't be in this program. She helped me scramble to apply at the last minute and vouched for me as a student to Dr. Binder, who took me in when she certainly didn't have to. Dr. Carnell has opened my mind, broadened my horizons, and inspired me with her tireless efforts to ensure equity and access for all in science and education in general. I feel so lucky to have learned from all of these incredible people.

Massive thank you to Mukobe Lukwesa, Taylor Arndt, and Rebecca Follett for their help in the laboratory. They faced the difficulty of coordinating our efforts to further our data analysis in the midst of a pandemic and consistently stepped up to the plate. For the hundreds of samples processed, analyzed, sectioned and stained, they were with me. I really couldn't have asked for a better team. I would also like to thank the Binder Research Group for our supportive weekly meetings and synergy as a team to take care of the mice. Our morning laughs have sometimes been the only thing I've looked forward to in isolation.

Thank you also to the CWU Office of Graduate Studies and Research for their guidance and support, the Washington State Distinguished Fellowship in Biology for believing in and funding my project, and Provost Michelle DenBeste for awarding my research a grant that allowed me more means of analysis. To the unsung heroes of the Biology department-the staff (Mark, Jonathan, Kari, Stacey, Emil, Mari, Elena, Eric)- you are the soul that makes this department a family. You embraced me

wholeheartedly and I truly believe we would all fall apart without you. Thank you for everything.

Finally, I need to thank my family and friends who have been my biggest supporters. My mom, Marilynn, never doubted that I would get to this point for a second and answered every tear-ridden phone call, even when it was hard. My dad, Mike, was always available to give me a laugh and a reprieve from the world of academia. My brother Jace made custom playlists to help me study and endured endless FaceTimes to let me vent. My emotional support animals, Zola and Goose, snuggled me through the program. My best friends, Gage, Ivie and Jess, kept me functional as a human and took care of me when I couldn't. Thank you for being here for me, I love you all unconditionally.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

CHAPTER II

APPENDIX

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Metabolism is the sum of all chemical reactions in organisms that are essential for sustaining life. Perhaps the most familiar purpose of metabolism is to break down consumed food and drink into nutrients that can then be utilized as fuel by cells. Various organs have metabolic functions that work together to maintain homeostasis, ensuring a consistent blood glucose level that allows people to do everything from thinking to running a marathon. The regulation of metabolic homeostasis is intricate and highly vulnerable to disruptions from environmental and genetic factors. Such disruptions can lead to metabolic disorders, such as obesity, which is increasing at unprecedented rates globally (1).

Obesity and overweight are defined by the World Health Organization as abnormal or excessive fat accumulation that may impair health (2), however this definition deceives the complexities of the pathogenesis of obesity. According to the CDC, the prevalence of obesity among adults in the United States was 42.4% in 2017-2018 compared to a prevalence of 30.5% from 1999-2000 (3). It has been reported that approximately one-third of the world's population is either overweight or obese, with adult obesity exceeding 50% in many countries around the globe (4,5). Even more alarming is the pervasiveness of childhood obesity, which was reported in 2019 by the World Health Organization as affecting 41 million children under the age of five (6). An urgent concern of obesity is its causal relationship with several cancers, type II diabetes due to insulin resistance,

hypertension, cardiovascular disease, and respiratory effects (7). The increasing rates of obesity can be partially attributed to modernization and the rise of sedentary lifestyles in conjunction with highly calorie dense food intake, however there is strong evidence for a genetic component of the disease (8-12). While the understanding of causes of obesity continues to grow, the diverse interactions that are required for the regulation of fat tissue make it difficult to implicate the exact genetic components of the disease.

Pharmaceutical and other therapeutic interventions have been limited in their efficiency (13, 14). Further, calorie restriction and weight loss have been successful short-term, but many individuals regain the weight back which negates these as strategies for long-term success in fighting obesity (15). Current research focuses on the mechanisms by which energy is expended by the body, and aims to illuminate targets for effective treatments that could enhance these processes and burn the excess fat that characterizes obesity. To begin to address excessive fat, also known as adipose, it is paramount to first understand the types of adipose tissue and how they function normally. This builds the necessary context for understanding the pathophysiology of obesity and how this research addresses and contributes to obesity solutions.

Adipose Tissues

Adipose tissue is a dynamic organ that serves a variety of functions, including protection of organs, fat storage and maintaining energy balance. It is comprised of groups of adipocytes (fat cells), fibroblasts, macrophages, stromal

cells, and monocytes (16). Adipocytes are essential for nutrient homeostasis. In terms of survival, the body needs to ensure a consistent source of fuel despite varying availability of external resources. Adipocytes function not only to store energy, but also as regulators of energy metabolism. Adipose tissue is part of the brain-gut-adipose axis, an important integration of systems that allows for communication and response to metabolic signaling (17). Normally, the collaborators of the brain-gut-adipose axis help to coordinate the process of eating, from the feeling of hunger to the extraction of nutrients and excretion of waste from food consumed to satiate hunger. A disruption in the signals from adipose tissue have been linked to the cardiovascular and metabolic complications that are associated with obesity (18). Thus, adipose tissue is an adaptive, central organ that engages in constant metabolic reprogramming to respond to everchanging conditions.

There are two primary types of adipose tissue, white and brown, that are dispersed throughout the body (Figure 1). Both brown and white adipose have distinct morphological and secretory differences and almost antagonistic functions in the body. Where white adipose tissue primarily stores lipids, brown adipose tissue burns lipids in exchange for heat, a process familiarly known as thermogenesis.

Figure 1: Dispersal of white and brown fat in the human body. Reprinted from 'Developmental Origin of Fat' by S. Gesta, Y.H. Tseng, and C. R. Kahn, Cell 131.2 (2007). Permission to reproduce granted by Elsevier.

Recent research has indicated the presence of a third type of adipose tissue made of "beige", or brown-in-white, adipocytes which have been observed upon stimulation via exercise or cold exposure (19, 20-23). The origin of beige adipocytes is still highly debated in literature (24, 25). Regardless, both beige and brown adipocytes are considered metabolically active due to their ability to upregulate thermogenesis, making them attractive targets for obesity intervention. The process of generating heat by brown and beige adipocytes requires energy mobilization, which is provided by white adipocytes. This interconnection between adipose

tissues is essential not only for an understanding of effective therapies for metabolic dysfunction, but for sustaining health.

White Adipose Tissue

White adipose tissue (WAT) is found primarily in the abdomen (mesenteric, omental, perirenal), in the gonadal region and under the skin (subcutaneous) (26). The deposits of WAT are largely characterized as subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), which have been found to have differing development, formation, and functions (27, 28). Location and distribution of WAT is directly correlated to different functions and sex differences, discussed in more detail below. Morphologically, white adipocytes are spherical and unilocular, meaning that each cell contains one lipid droplet. They have a flattened nucleus and a thin layer of cytoplasm that encompasses the lipid droplet as well as few mitochondria and a low oxidative rate (23).

The main role of white adipose tissue (WAT) is to store energy in the form of triglycerides (TG) during energy consumption and to release fatty acids (FA) when energy expenditure exceeds energy intake. These processes are referred to as lipogenesis and lipolysis, respectively. Triglycerides prove to be an efficient form of energy storage as they are devoid of water and have over twice the energy density of carbohydrates and proteins (29).

The storage capacity of white adipocytes dynamically adjusts to caloric excess through the processes of hyperplasia, an increase in cell numbers, and hypertrophy, an increase in cell size (Figure 2) (30). Adipocytes continue to store

lipids until they reach a critical threshold, at which point they are hypertrophic. In this state, adipocytes release fatty acids and other signaling molecules to induce differentiation of preadipocytes, which increases the number of cells available for storage (31). Hyperplasia is the primary remodeling method used by WAT during growth stages. However in adult life, the capacity for differentiation of preadipocytes to become fully functional and mature diminishes (32). The balance of hyperplasia and hypertrophy is vital to metabolic health. Constant caloric excess results in rapid adipose tissue expansion and an increase in adipocyte size, which has been associated with induction of a chronic inflammatory response and adipocyte death observed in obesity (33). Adipocyte hypertrophy has been found to result in reduced angiogenesis, resulting in deficiencies in vasculature that ultimately lead to hypoxia-a trigger for inflammation in adipose tissue (34, 35).

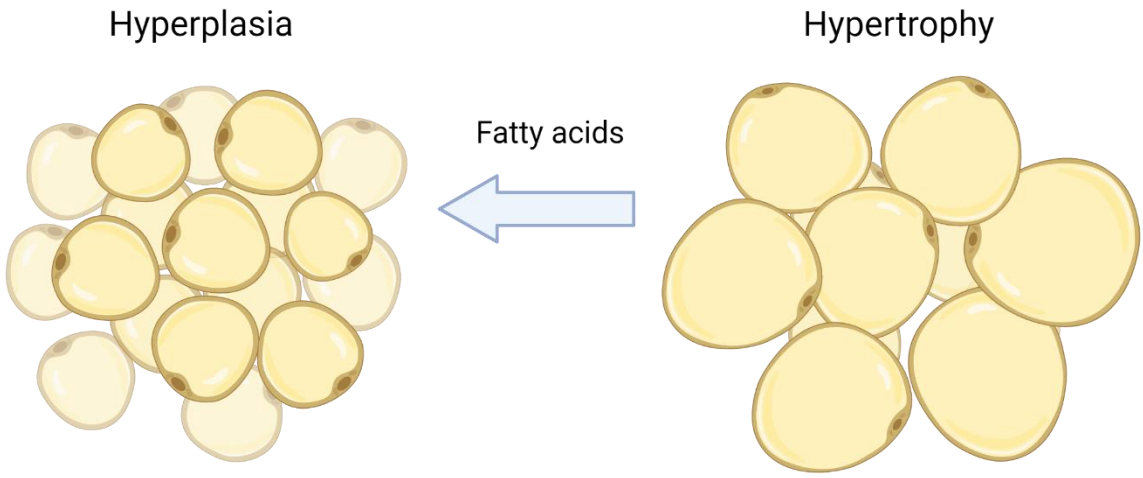

Figure 2: Hyperplasia and hypertrophy of WAT created with BioRender.com

White Adipose Tissue and The Immune System

Understanding the relationship between the immune system and WAT is highly important in the context of obesity. Obesity is characterized by chronic low-grade inflammation not only in adipose tissue, but also in skeletal muscle, the liver, intestine, brain, and pancreas islet (36-42). This was initially discovered in 1993, when increased levels of a proinflammatory cytokine called tumor necrosis factor alpha was observed WAT from obese humans and mice (43). Research has since suggested that the causal link between obesity and insulin resistance is due to the contributions of both innate and adaptive immune cells to the inflammation observed in obesity (36-42).

Adipocytes themselves can secrete cytokines (43-46), but recent studies have shown macrophages and other immune cells in WAT are responsible for the secretion of most of the inflammatory cytokines observed in obesity (37-42). Macrophages reside in lean adipose conditions normally but change their phenotype to become proinflammatory in obesity (47). This has led to the classification of adipose tissue macrophages into two subtypes: M1 macrophages with a proinflammatory identity and M2 macrophages that have been associated with anti-inflammatory properties (48). In healthy and lean adipose tissue, M2 macrophages work with T regulatory cells to instigate signaling cascades that result in the release of anti-inflammatory mediators to contribute to insulin sensitivity and inhibit dysregulation of the tissue (49). In obesity, however, there is a much higher prevalence of M1 macrophages that infiltrate adipose tissue. Hypertrophic

adipocytes, such as those observed in obesity, undergo necrotic-like adipocyte death (50). In the tissue surrounding necrotic adipocytes, macrophages are polarized to become M1 macrophages that form a "crown-like structure" that has become a hallmark of obesity (47). M1 macrophages then release inflammatory cytokines that propagate systemic inflammation that plays a major role in the development of obesity and insulin resistance (47, 52). As our understanding of the interactions of adipose tissue and the immune system are still evolving, it is undeniable that chronic low-grade inflammation is inextricable from obesity and the development of secondary metabolic disorders.

WAT and Its Endocrine Capabilities

Another key function of WAT is its ability to secrete a variety of hormones and cytokines, better known as adipokines. WAT effectively communicates with the rest of the body through these adipokines and via neuronal inputs, which is important for its role in the brain-gut-adipose axis (17, 53). Of particular interest is leptin, a protein that is secreted by adipocytes that increases energy expenditure while decreasing consumption and storage (44). There are receptors for leptin on the hypothalamus in the brain that have been found to control satiety and energy balance. Leptin receptors have also been found on other organs, including adipocytes themselves, supporting that leptin secretion acts locally as an autocrine factor in conjunction with its paracrine signaling (53). It has been shown that administration of leptin to hypothalamic regions decreases food intake and body weight in mice (54). Leptin has shown to have further applications through

stimulation of hematopoiesis and angiogenesis, which help to regulate immune response and influence the sympathetic nervous system (55). Adipocytes that respond to high leptin concentrations have shown to deplete up to 95% of their stored lipids (44). In an obese phenotype, leptin resistance is observed. Consequentially, the communication between WAT and the brain is disrupted, and ultimately promotes insulin resistance (44). Leptin has been successfully used as a therapeutic to treat leptin-deficient obese individuals but has had varying success rates in hyperleptinemic obese individuals (56). While leptin is far from the only important adipokine secreted by WAT, it is a protein of focus for its role in obesity.

Brown Adipose Tissue

Brown adipose tissue (BAT) is specialized for energy expenditure and is a key nonshivering thermogenic tissue. Historically, it was believed that brown adipose tissue was only present in humans during infancy to combat lethal hypothermia, however many studies have shown that active BAT remains in adulthood, albeit a decreased amount (19-23, 57). In adult humans, BAT has been found in the upper abdominal, cervical, supraclavicular, axillary, paravertebral, and mediastinal regions (58) (Figure 1). Brown adipocytes are smaller than white adipocytes, contain many small lipid droplets (multilocular), are very mitochondria-rich and are highly vascularized and innervated (29). Brown adipocytes also have high rates of glucose uptake and fatty acid oxidation (59).

BAT and Thermogenesis

BAT is considered metabolically active due to the ability of the mitochondria to utilize thermogenesis. This process has been characterized by the expression of uncoupling protein 1 (UCP-1), found in the inner mitochondrial membrane (Figure 3). As the name suggests, UCP-1 uncouples the hydrogen gradient used by the electron transport chain for ATP production, releasing energy in the form of heat. Regulation of UCP-1 in mitochondria occurs through a competitive interaction between purine nucleotides, primarily ATP, and long-chain fatty acids (LCFA)(60). At normal concentrations, flow of protons through UCP-1 is inhibited and oxidative phosphorylation continues to produce ATP. When levels of LCFAs are increased, purine nucleotide inhibition is overridden and the dispersal of the proton gradient is facilitated through a fatty acid/hydrogen ion symport mechanism (14). The increase in LCFAs in circulation occurs through lipolysis of WAT, which allows LCFA to be taken up and used by mitochondria as fuel and for activation of UCP-1 (14). Adaptive thermogenesis is heat production in response to external stimuli, such as cold temperatures or diet, and serves to protect from hypothermia and maintain energy balance after changes in diet (61). Increasing the number or activity of brown adipocytes has been a novel approach to increase whole-body energy expenditure to combat obesity (23).

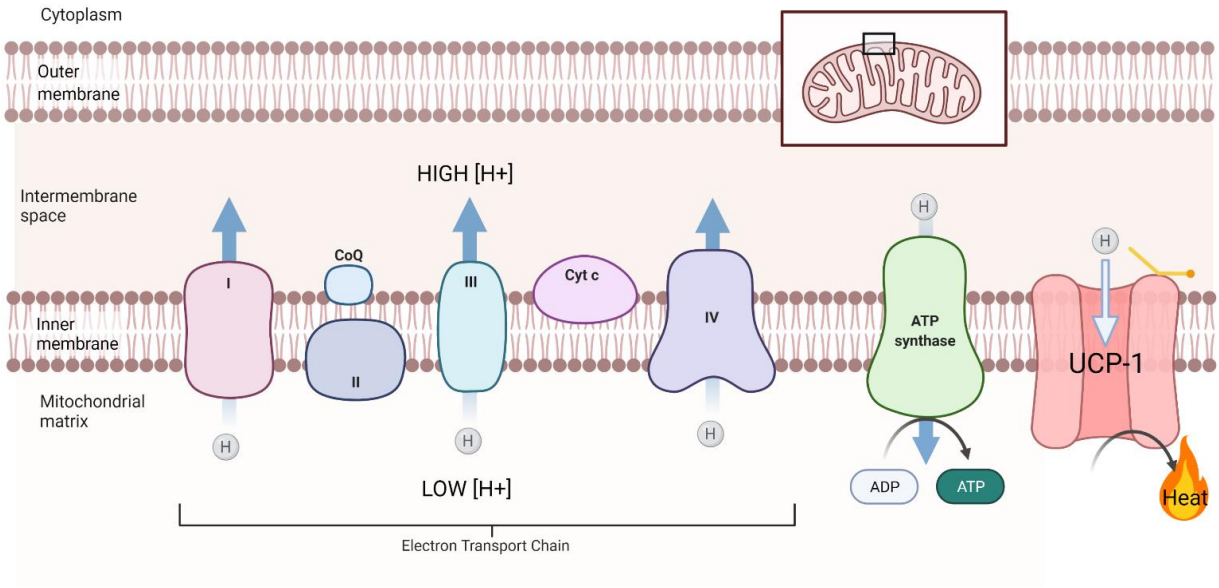

Figure 3: Expression of UCP-1 during thermogenesis uncouples the hydrogen gradient used in oxidative phosphorylation to produce heat. UCP-1 is stimulated by the binding of a long-chain fatty acid. Created on BioRender.com.

Beige Adipocytes

Beige (or brown-in-white (62), also known as brite) adipocytes are recently reported in literature as adipocytes that have a basal level of UCP-1 expression that is comparable to that of white adipocytes, but upregulate UCP-1 similarly to classical brown adipocytes upon cold exposure, administration of a β3 adrenergic receptor agonists or exercise (63). They have central nuclei, multilocular lipid droplets, and are mitochondria-rich (64). These adipocytes are characterized by their thermogenic capabilities, and their origin is debated. Mature adipocytes of all types are considered to be postmitotic, which limits the possibilities of beige adipocyte development to conversion of mature adipocytes or differentiation from resident precursor populations (24).

Interconversion of White Adipocytes to Beige Adipocytes

One theory for the origin of beige adipocytes is the direct interconversion of a mature white adipocyte to a brown adipocyte phenotype, deemed transdifferentiation in the literature (65-67). This idea is supported by reports that cell numbers remain constant during beiging, which would exclude proliferation of the current adipocyte population (68, 69). Another finding that supports this mechanism is the observation of beige adipocytes after cold exposure, which could strengthen the concept that white adipocytes actively convert to a brown phenotype when stimulated (65). However, upon warm adaptation, it has been found that beige adipocytes can change their morphology and gene expression profiles to that of white adipocytes (25). By using a UCP-1-CreER x ROSA26-tdRFP mouse model, RFP+ adipocytes that were labeled during cold exposure were purified using FACS sorting. It was found that a second cold exposure after warm adaptation beiged the same adipocytes as the first cold exposure (25). This is aligned with the idea that distinct deposits of WAT are capable of interconverting between phenotypes. Regardless, it has been shown that white and beige adipocytes have remarkable plasticity.

Precursor Cells Differentiate to Become Beige Adipocytes

Another theory is that beige adipocytes are recruited *de novo* from precursors that are already present in the tissue (70). Reports finding distinct precursor populations in WAT depots support this claim, as these precursors could differentiate into beige adipocytes upon stimulation (63, 71-73). Through RNA

sequencing and genome-wide expression analyses, molecular markers have been found and associated solely with beige adipocytes, which suggests differences in development (74). Similarly, it has been suggested that distinct populations of pre-beige adipocytes exist in the vasculature of adipose tissue comparable to those of pre-white adipocytes (28). A strong supporting study of this model observed beige precursor cells in explants of SAT that upon transplantation, enhanced glucose homeostasis (75).

Unification of Theories

A recent study has even suggested a unification of the two ideologies, arguing that beige adipocytes initially arise from progenitors, but then interconvert between "dormant beige" and "active beige" phenotypes (24). This study also addresses discrepancies in results in the field, emphasizing that the diverse methods applied by different researchers makes data and conclusions difficult to compare (24). Other noteworthy findings suggest that genetic variance strongly influences the amount of beige adipocyte populations in mice (76-78) and that the mode of beige cell recruitment may be dependent on differences between physiological and pharmacological activation (79). The research surrounding beige adipocytes is an everchanging landscape with the constant goal of finding a strategy to recruit more beige adipocytes *in vivo*.

UCP-1 Independent Thermogenesis

Classically, energy expenditure through thermogenesis was considered to be entirely dependent on the upregulation of UCP-1. While this is still the most

dominant form, it is clear that there are additional mechanisms through which thermogenesis occurs. Beige adipocytes were essential in elucidating these alternative forms of thermogenesis, as their low basal level of UCP-1 expression was initially believed to only marginally contribute to energy homeostasis (80). One of the established UCP-1 independent thermogenic processes occurs through futile Ca^{2+} cycling in the mitochondria-associated membrane of beige adipocytes (81). Another method is through creatine and creatine-mediated ATP hydrolysis. This initiates futile cycling of production and consumption of ATP in the mitochondria (82). The research of these alternative thermogenic mechanisms is relatively new. Their contributions to energy expenditure have yet to be fully explained, but seem to be highly important in understanding the process of thermogenesis in its entirety.

Sexual Dimorphism in Adipose Tissue

It has been shown that sexual dimorphism, or differences between males and females, contributes to adipose tissue remodeling including changes in metabolic activity, number and size of adipocytes (83-86). Generally, women have larger adipose deposits in the peripheral and subcutaneous regions (widely referred to as "pear" form) while men are more likely to have excess adipose tissue in the abdominal region and in visceral deposits (widely known as "apple" form) (87). While hereditary factors and race have been shown to contribute to differences in fat distribution, the main catalysts of sexual dimorphism in adipose tissue are hormones (88).

Variation in body compositions between males and females begins in infancy and continues through the process of development (89) driven by sex-specific hormonal differences (87). Aging propels adipose tissue distribution towards larger accumulations in visceral adipose tissue and non-adipose tissues, which is correlated with metabolic dysfunction (90). In women, this phenotype is observed at higher rates, likely due to a decrease in estrogen levels associated with post-menopausal hormonal changes that shift fat tissue towards an obese phenotype (91). A similar effect is observed in males due to declining serum levels of testosterone with aging that results in a decrease of SAT and an increase in VAT (92). Sex steroids have been shown to strongly influence lipid uptake, leptin production, lipolysis and development and differentiation of adipocytes (93). As these processes are integral in maintaining adipose tissue health, it is reasonable that a disruption in hormones can induce metabolic dysfunction.

Cold Exposure Mechanism and Precedence

Cold exposure is the most well-studied method of activating BAT and beige adipocytes (19-23). It is well documented that cold stimulus leads to the activation of the sympathetic nervous system, which releases norepinephrine that activates β-adrenergic receptors (Figure 4).

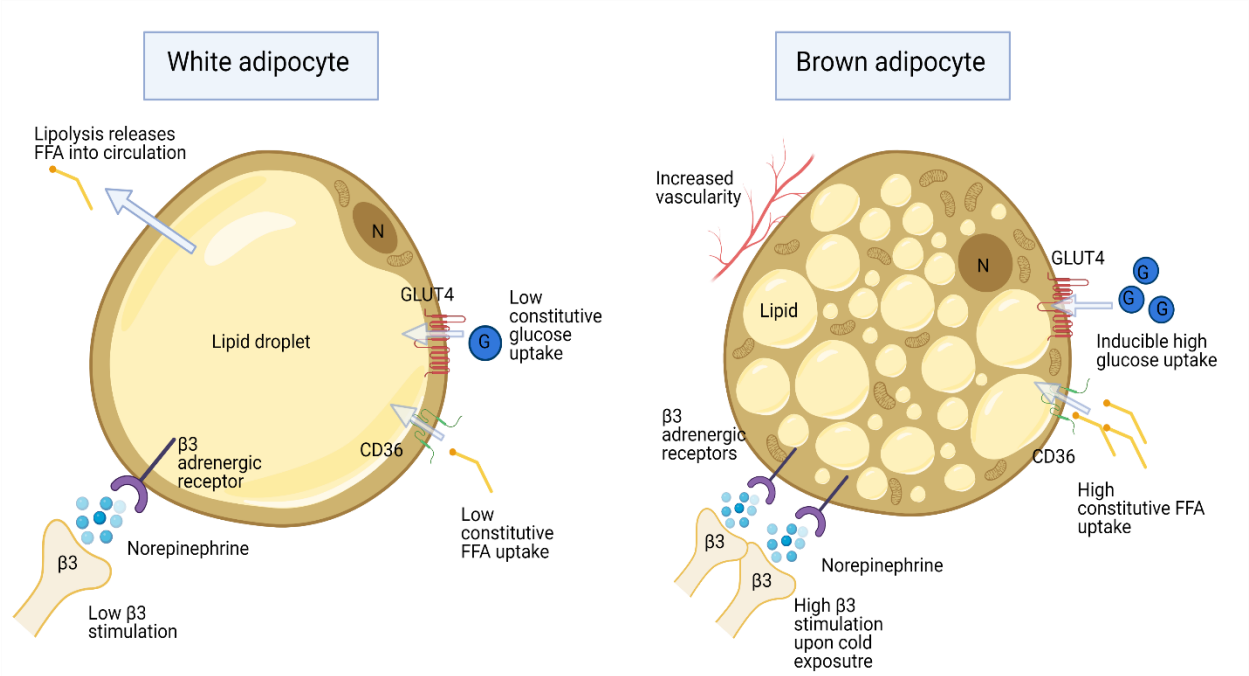

Figure 4: β-3-adrenergic signaling in white and brown adipocytes. Lipolysis in white adipocytes liberates free fatty acids into the bloodstream, where they can be taken up through the CD36 receptor of brown adipocytes. Brown adipocytes, upon high β3-adrenergic signaling, increase their glucose uptake through receptor GLUT4. The free fatty acids taken up by receptor CD36 are used by the mitochondria to fuel thermogenesis by being a substrate for the citric acid cycle as well as an activator of UCP-1. Vascularity is also increased in metabolically active brown adipocytes. Figure made using BioRender.com and adapted from (94).

Upon binding of norepinephrine β3 adrenergic receptors, the intracellular cyclic adenosine monophosphate (cAMP)- protein kinase A pathway is activated. This culminates in the phosphorylation of adipose triglyceride lipase and hormone-sensitive lipase which causes lipolysis of intracellular lipid droplets leading to increased FFA concentrations (95). Free fatty acids activate UCP-1, and also serve as substrates for thermogenesis. The same signaling pathway causes lipolysis in WAT, which mobilizes FFA for use by BAT and thermogenesis (95).

In conjunction with lipid metabolism, glucose utilization by BAT is significantly increased by sympathetic nervous system activation (95). It has been

suggested that glucose utilization is upregulated to compensate for the decrease in ATP production in mitochondria due to UCP-1 function (95). This idea is supported by studies that have shown inhibition of glycolysis significantly decreases thermogenesis (96, 97). Accordingly, glucose uptake in BAT is dependent on UCP-1 function (95). Insulin, which encourages the uptake of glucose into cells, is an important molecule in this process. Insulin resistance is a comorbidity of obesity, and leads to Type II diabetes (44). Thus, understanding the relationship of glucose to the process of thermogenesis is necessary to recognize how thermogenesis could be impaired in obese and insulin resistant phenotypes.

Another major occurrence during sympathetic nervous system signaling activation of BAT is the upregulation of thermogenic genes. These genes upregulate UCP-1 expression, mitochondrial biogenesis as well as lipolysis, hyperplasia and hypertrophy of adipose tissue (61). Recent research has also focused on how reactive oxygen species released by the mitochondria during cold-induced thermogenesis sensitize UCP-1 for further activation (98).

Studies utilizing both acute and prolonged cold exposure reported increased glucose uptake and improved insulin sensitivity in mouse and rat models (59, 99, 100). Cold exposure has been used as a tool for lineage-tracing beige adipocytes (25) and analyzing gene expression levels of BAT-associated genes (59, 99, 100). Despite the vast literature studying cold exposure in mice, rats, and even in humans, there have been no studies to date performed with the NAG-1 lean transgenic mouse model.

Lean Transgenic NAG-1 Mouse Model

Nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1) is known by many names, including growth differentiation factor 15 and macrophage inhibitory cytokine-1 and belongs to the transforming growth factor beta superfamily of proteins (105). It is known that transforming growth factor beta superfamily members modulate adipocyte differentiation and function and can induce or inhibit adipogenesis (106). NAG-1 expression has been reported in most tissues at low concentrations and is found in high levels in the serum as it is a secreted protein (107). Expression of NAG-1 is induced by anti-inflammatory drugs and chemicals known to have anti-tumorigenic activity, but the mechanism of its biological activity is poorly understood (107).

A transgenic mouse model was developed in 2006 that constitutively expresses human NAG-1 under the control of a chicken β-actin promoter (108). Transgenic NAG-1 mice have lower body weights, less white adipose tissue, are more metabolically active compared to their wildtype littermates (109). Further, they are resistant to genetic and diet-induced obesity and have improved insulin sensitivity and production, regardless of sex or diet (110). NAG-1 mice also have increased expression of key genes involved in the regulation of thermogenesis by adipose tissue, resulting in increased oxidative metabolism (110). Glucose tolerance tests in NAG-1 mice, after a 12-hour fasting period, have shown a lower initial spike in blood glucose levels with quicker returns to resting blood glucose levels. When these mice were then injected with insulin, they showed larger drops in blood

glucose levels. These data suggest that the expression of NAG-1 improves glucose tolerance (111). NAG-1 mice have also been found to have a reduced inflammatory response (107), which could contribute to their longevity compared to wildtype mice (112).

Further characterization of this transgenic model has shown that NAG-1 mice have higher oxygen utilization, VCO_2 and heat expenditure, consistent with having higher metabolic activity (110). NAG-1 mice have also been found to have lower levels of leptin, which contributes to their increased metabolism (110). Being that the NAG-1 transgenic mouse clearly proves to be a model for higher metabolic activity at room temperature, and that the mechanism through which NAG-1 acts is unclear, stimulating further thermogenesis in these mice could provide more information about the action of NAG-1. There are no previous studies of cold exposure with NAG-1 mice, which offers the novel opportunity to further understand this lean mouse model and its response to cold. If the metabolic activity of wildtype mice increases upon cold exposure to be similar to that of NAG-1 mice, that would inform us that NAG-1 overexpression does not increase metabolism in conjunction with β3 adrenergic stimulation. However, if the NAG-1 mice show a further elevation in activity over their wildtype littermates, this could indicate that NAG-1 acts on a pathway that works synergistically with β3 adrenergic signaling.

Indirect Calorimetry as a Method for Measuring Metabolic Rate

Indirect calorimetry has long dominated modern metabolic research concerning resting metabolic rates in mice (113). Respirometry is the most common

type of indirect calorimetry and estimates metabolic rate through the measurement of respiratory gas exchange between the animal and its environment (113). Cellular respiration utilizes O_2, carbohydrates, lipids and proteins as fuel sources for ATP production and produces the by-product of CO_2. By measuring the volumes of O_2 consumed and CO_2 produced, metabolic rate can be calculated (113). The respirometry system works as depicted in Figure 5. Essentially, air is pushed through a temperature-controlled system at a flow rate determined by the researcher. The air passes through scrubbers containing chemicals such as soda lime and ascarite to remove CO_2 and drierite to remove H_2O, as this can be detected by the CO_2 analyzer and result in loss of accuracy. The flow continues through a tube containing the mouse, which is also in a temperature-controlled apparatus. It passes through a final scrubber of magnesium perchlorate to remove H_2O before passing through O_2 and CO_2 analyzers. The data collected by the analyzers can then be used to determine metabolic rate.

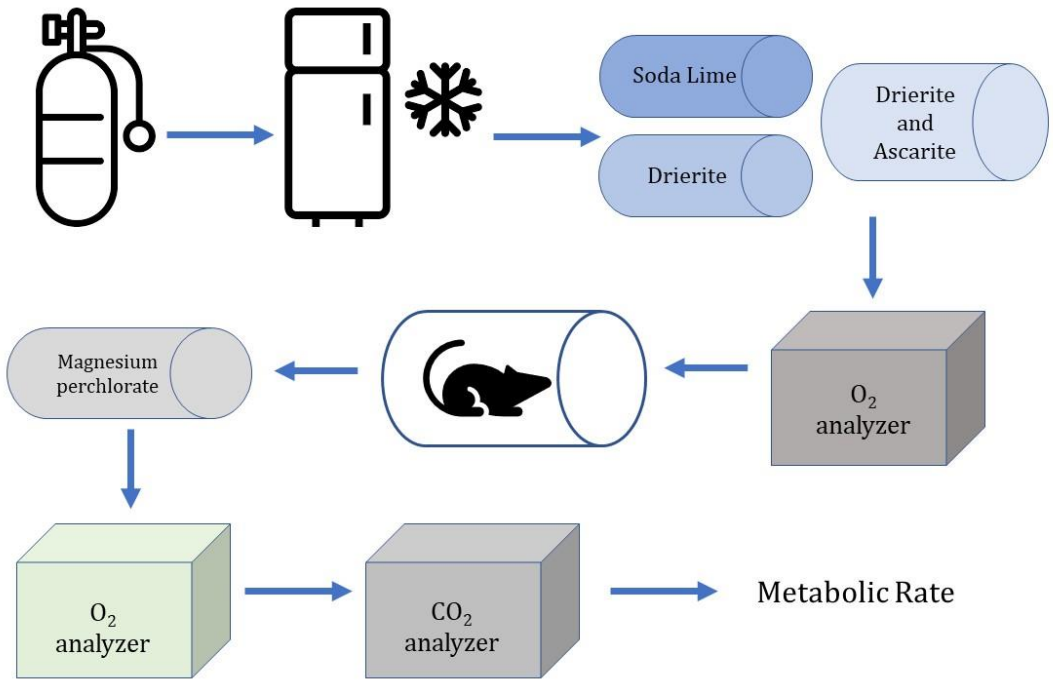

Figure 5: Respirometry apparatus for measuring metabolic rate.

Respirometry is a valuable method for comparing metabolic rates of the transgenic NAG-1 mice and their wildtype littermates. Of great importance is the ability to manipulate the temperature at which the mice are held, which allows for metabolic rates to be measured at room temperature before stimulus and at the end of a prolonged cold exposure. A direct comparison can then be made in each individual mouse to determine the influence of cold stimulus on the mice and their cellular respiration processes.

Conclusion

Obesity is a major world health concern with a consistent increase in prevalence. Therapies beyond diet and exercise are limited and have low efficacy. Obese individuals are at significantly higher risk for a host of metabolic disorders.

Presently, studies are finding that obesity has an associative relationship with increased COVID-19 hospitalizations and deaths (114). The discovery of BAT in adult humans stimulated research into activating thermogenesis as a therapeutic approach to combat obesity. Clearly, there are many layers of complexity to adipose tissue and its interactions with the immune system, the nervous system and even within itself. By equipping methods such as cold exposure to induce higher metabolic activity through thermogenesis, our collective understanding of the intricacies of the cellular processes that coordinate an increase in energy expenditure is becoming more robust. These studies are vital for understanding the pathways involved and how targeted pharmaceuticals or interventions can be effective for combatting the growing obesity epidemic.

The NAG-1 transgenic mouse makes for a strong model to understand metabolic dysfunction as they have protection from disorders such as obesity and insulin resistance. While the mechanism of protection is not clearly understood, NAG-1 mice may offer valuable insight through changes observed when comparing to wildtype littermates. Novel cold stimulus of NAG-1 mice has great potential to identify and further contribute to pathway analysis and mechanism clarity.

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CHAPTER II

COLD-INDUCED METABOLIC CHANGES

IN LEAN, TRANSGENIC MICE

JOURNAL-READY ARTICLE

FOR SUBMISSION TO:

OBESITY

EFFECTS OF PROLONGED COLD EXPOSURE ON NAG-1 TRANSGENIC MICE

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Abstract

In this study, transgenic mice that constitutively express the human non-steroidal anti-inflammatory gene 1 (NAG-1) were used as a model to further understand the mechanisms of increasing metabolism. Obesity is a multifaceted metabolic disorder with severe worldwide public health consequences. While obesity can often be treated using diet and exercise, there are complex genetic interactions driving obesity that don't always respond to such changes. One important research focus for addressing genetic factors is activating brown adipose tissue which increases thermogenesis, the process of burning stored fats to generate heat. Cold exposure is a known way to activate brown adipose tissue through β3 adrenergic signaling. This signaling pathway causes an upregulation of thermogenesis in brown adipocytes and beige adipocytes, which is white adipose tissue that "browns" and becomes metabolically active. At room temperature, NAG-1 mice have less adipose tissue, higher metabolic rates, and longer lifespans than their wildtype littermates. Wildtype and NAG-1 mice were subjected to a prolonged cold exposure at 10ºC for seven days. NAG-1 mice had lower body weights throughout the study compared to wildtype mice. NAG-1 mice had higher metabolic rates before and after cold exposure. Adipocyte size analysis indicated adult male wildtype mice have significantly larger white adipocytes after cold exposure than NAG-1 males. Quantitative real-time PCR indicated higher expression of *Glut4* and *Atgl* in wildtype mice, indicating that NAG-1 does not upregulate expression of thermogenic genes in response to cold exposure. The data from these analyses suggest that NAG-1 does not act synergistically with the β3 adrenergic pathway in cold response.

Introduction

Obesity is a multi-faceted, complex disease that was designated as a global epidemic by the World Health Organization in 1997 (1). Despite the pressing nature of obesity as a serious public health concern, prevalence has continued to increase globally at unprecedented rates (2). Approximately one-third of the world's population is either obese or overweight, with adult obesity exceeding 50% in many countries (3, 4). An urgent concern of obesity is its causal relationship with several types of cancers, Type II diabetes due to insulin resistance, hypertension, cardiovascular disease, and respiratory effects (5). Further adding to the global burden of obesity and its comorbidities are the psychological and social consequences that affect all ages and socio-economic groups indiscriminately (6).

Obesity is defined as abnormal or excessive fat accumulation that may impair health (1). While modernization and the rise of sedentary lifestyles in conjunction with calorie dense food intake are contributing causes, there is also strong evidence for a genetic component of the disease (7-11). Current pharmaceutical and therapeutic interventions have had limited efficiency (12, 13). Caloric restriction and weight loss have been successful short-term solutions, but many individuals regain weight which negates these strategies for long-term success in fighting obesity (14). Current research focuses on the mechanisms through which energy is expended by the body and aims to illuminate targets for effective treatments that could enhance these processes to burn the excess fat that characterizes obesity.

Brown adipose tissue (BAT) is metabolically active due to the ability of the mitochondria to undergo thermogenesis, the process of burning stored lipids for heat. Historically, it was believed that brown adipose tissue was only present in humans during infancy to combat lethal hypothermia, however many studies have shown that active BAT remains in adulthood, albeit a decreased amount (15-21). Thus, increasing the number or activity of brown adipocytes has been a novel approach to increase whole-body energy expenditure to combat obesity (22). Brite (or brown-in-white (23), also known as beige) adipocytes are adipocytes that have a basal level of UCP-1 expression and phenotype that is comparable to that of white adipocytes. However, beige adipocytes upregulate UCP-1 similarly to classical brown adipocytes upon cold exposure, administration of a β3 adrenergic receptor agonists or exercise (24). Studies utilizing both acute and prolonged cold exposure reported increased glucose uptake and improved insulin sensitivity in mouse and rat models (25-27). Cold exposure has been used as a tool for lineage-tracing brite adipocytes (28) and analyzing gene expression levels of BAT-associated genes (25-32). Despite the vast literature studying cold exposure in mice, there have been no studies to date performed with the NAG-1 lean transgenic mouse model and prolonged cold exposure.

Nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1), also called growth differentiation factor 15 and macrophage inhibitory cytokine-1, belongs to the transforming growth factor beta superfamily of proteins (33). It is known that transforming growth factor beta superfamily members modulate adipocyte differentiation and function and can induce or inhibit adipogenesis (34). Expression

of NAG-1 is induced by anti-inflammatory drugs and chemicals known to have anti-tumorigenic activity, but the mechanism of its biological activity is poorly understood (35). At room temperature, NAG-1 transgenic mice have lower body weights and less white adipose tissue, are more metabolically active than wildtype age-matched littermates, and are resistant to genetic and diet-induced obesity (36, 37). NAG-1 mice also have increased expression of key genes involved in the regulation of thermogenesis by adipose tissue, resulting in increased oxidative metabolism (37).

Being that the NAG-1 transgenic mouse is a model for higher metabolic activity at room temperature, and that the mechanism through which NAG-1 acts is unclear, using cold to stimulate thermogenesis could provide more information about the action of NAG-1. There are no previous studies of prolonged cold exposure with NAG-1 mice, which offers the novel opportunity to further understand this lean mouse model and its response to cold. If the metabolic activity of wildtype mice responds to cold by matching or being similar to that of NAG-1 mice, this would inform us that NAG-1 overexpression does not respond to β3 adrenergic stimulation to further increase metabolism. However, if the NAG-1 mice show a further elevation in activity over their wildtype littermates, this could indicate that NAG-1 acts on a pathway that works synergistically with β3 adrenergic signaling.

A previous study performed in our lab investigated the effects of an acute, four-hour cold exposure on NAG-1 prepubertal and adult mice in both females and

males (Qvigstad A, 2018, unpublished). The metabolic rates of the study groups were measured after cold exposure. Prepubertal males, both wildtype (WT) and NAG-1, displayed significantly higher metabolic rates than adult males after cold exposure. NAG-1 adult mice also trended towards having higher metabolic rates than WT littermates at both 25°C and 10°C regardless of sex, however these differences were not observed in the prepubertal groups. One major conclusion from these data are that NAG-1 mice develop their increased metabolic rate at room temperature that contributes to their phenotypic differences from WT littermates as they age. Also, the drastic increases in the metabolic rates of the prepubertal males over adult males in response to cold exposure was considered evidence for a higher prevalence of brown fat in younger animals. The observation of differences between genotype, age, and sex informed the design of the current study and warranted further investigation.

Transgenic NAG-1 and WT mice (females, males, prepubertal and adult for both genotypes) were held at 10°C for seven days. I hypothesized that after the prolonged cold exposure, NAG-1 mice would have further increased metabolic rates, higher glucose tolerance, smaller adipocytes and increased expression of thermogenic genes, suggesting interactive effects of NAG-1 overexpression and cold response. I found that NAG-1 mice had lower body weights, however, estrus cycles and glucose tolerance were similar to those of WT mice after cold exposure. White adipose tissue in wildtype mice had higher expression levels of both *Glut4* and *Atgl* and no significant differences in expression were observed in other thermogenic genes. NAG-1 mice did have higher metabolic rates after cold exposure. Taken

together, this study indicates that NAG-1 mice do not have a more exaggerated increase in metabolism due to cold exposure which suggests that NAG-1 does not act synergistically with the β3 signaling pathway upon cold exposure.

Materials and Methods

Animals

Male transgenic mice expressing the human NAG-1 gene on a C57BL/6 background were obtained from Dr. Tom Eling from the National Institute of Environmental Health Sciences and bred with female C57BL/6J mice at Central Washington University. Mice were kept on a 12-hour light/dark cycle. Mice were fed Mazuri Rat and Mouse diet and water *ad libitum.* At weaning, mice were genotyped as previously described (38). Mice were assigned to study groups with $n = 6$ for each group (adult female NAG-1, adult male NAG-1, adult female WT, adult male WT, prepubertal female NAG-1, prepubertal male NAG-1, prepubertal female WT and prepubertal male WT). Prepubertal mice in the study began their prolonged cold exposure at 21-22 days of age and adults at 13 weeks. To begin the cold exposure, mice were moved to a cold room set to 10°C, where they remained for seven days. Mice were checked twice daily throughout cold exposure and room temperature was recorded daily. Body weights were measured every other day of the study and analyzed via two-way ANOVA and repeated *t*tests. End of study body weight comparisons were assessed with Student's *t*tests. All animal care and use was approved by CWU Institutional Animal Care and Use Committee (IACUC protocol # 2020-083).

Vaginal Smears

Estrus cycles of adult females were assessed vaginal smears. Vaginal smears were performed on adult female NAG-1 and WT mice each morning of the seven-day cold exposure. Normal saline solution (0.86%) was rinsed over the vaginal opening of each mouse individually using an eyedropper and mounted onto a slide. The solution was then fixed and allowed to dry before further processing. The fixed slides were stained with hematoxylin and eosin (Sigma-Aldrich Corp., St. Louis, MO), dehydrated, and sealed with a glass coverslip. The stained slides were viewed under a compound microscope for scoring of the cell types present (39). Blind scoring was performed by two trained technicians and categorized as being predominantly proestrus, estrus, metestrus or diestrus. Proestrus slides were defined by the presence of large, nucleated cells, estrus as having anucleated cells, metestrus as the infiltration of leukocytes in nucleated and anucleated cells, and diestrus as having far fewer and mixed cells in comparison to the other stages. Data were percent of study spent in each stage and was analyzed by a one-way ANOVA.

Indirect Calorimetry

Respirometry was performed with all the mice at room temperature (22°C) before cold exposure and again in the cold (10°C) on the seventh day of cold exposure. Body weights of the mice were taken directly before each mouse went into the respirometry chamber. To measure the metabolic rates, air was passed through soda lime, drierite, and a drierite/ascarite combination scrubber at a flow

rate of 178.5 mL·min^{-1}. It then continued through a CO_2 analyzer (LI-COR, Lincoln, NE) before passing through the chamber containing the mouse in a temperature-controlled apparatus. The air flow continues out of the chamber, through a magnesium perchlorate scrubber before passing on to the final CO_2 analyzers (Sable Systems International, Las Vegas, NV). The data collected from the analyzers was plotted in real-time using ExpeData (Sable Systems International, Las Vegas, NV) and further processed using the ExpeData software to calculate the O_2 and CO_2 exchange for each mouse. VCO_2 outputs (mL/h) were corrected for body weight (in kg) and two-way ANOVAs were performed, followed by Student's *t*tests.

Glucose Tolerance Test

Mice were fasted overnight for 16 hours. An initial fasting blood glucose level was taken using tail blood with a glucometer (Nipro Diagnostics, Ft. Lauderdale, FL.) following manufacturer's instructions. Mice were then injected intraperitoneally with 2000 mg/kg of dextrose in sterile saline solution. Blood glucose levels were monitored every twenty minutes for the first hour, then every hour for the next two hours via blood from the tail using a glucometer (Nipro Diagnostics, Ft. Lauderdale, FL.). A two-way ANOVA was performed to determine significance, followed by repeated *t*tests. Fasting blood glucose levels were analyzed by Student's *t*test.

Tissue Collection

Following glucose tolerance tests, mice were euthanized using CO_2 inhalation followed by cervical dislocation as a secondary method. Final body

weights were immediately obtained before tissue collection. Inguinal white adipose and interscapular brown adipose were collected from each mouse. Adipose samples collected for histological analysis were stored in 10% formalin at 4°C until further processing. The remaining adipose samples were stored at -80°C until needed. A tail sample was also collected for genotype confirmation.

Gene Expression

White and brown adipose tissue samples were collected from the mice and stored at -80ºC until ready for use. Samples were thawed with 300 µL of TRIzol® reagent (Invitrogen, Carlsbad, CA), homogenized, then stored at -80ºC for 20 hours. Samples were thawed and homogenized again, then 700 µL of TRIzol® was added to the samples and RNA was isolated following the manufacturer's TRIzol® reagent protocol. RNA was quantified on a spectrophotometer and checked for purity via the 260/280 ratio, with a value of 1.7-2.0 indicating RNA that could proceed to cDNA synthesis. RNA that was not within this range of values was subjected to a repurification process utilizing 3M sodium acetate (40).

RNA (1 µg) was reverse transcribed into cDNA using the Verso cDNA synthesis kit and following manufacturer's protocol (ThermoFisher Scientific, Waltham, MA) and stored at 4ºC until needed for quantitative real-time PCR.

The cDNA samples were diluted with 80 µL of DEPC-H_2O and qRT-PCR was performed using primer sequences (Supplemental Table 1) received from Integrated DNA Technologies (Coralville, IA) and PowerUp™ SYBR®Green Master Mix (Applied Biosystems, Waltham, MA.) according to manufacturer's protocols.

Each sample was ran in duplicate with 5 μL of template cDNA. Plates were run using QuantStudio3 machine with a Comparative-Ct-SYBR program (ThermoFisher Scientific, Waltham, MA). C_t values were normalized to Rpl7, which was used as an internal control. Data were analyzed with two-way ANOVAs and Student's *t*tests.

Histology and Adipocyte Size

Tissues were fixed in 10% formalin immediately after collection and stored at 4°C until further use. Tissues were dehydrated through a series of ethanol washes, cleared with Citrisolv (Sigma-Aldrich Corp., St. Louis, MO), and infiltrated with paraffin for two days prior to embedding. Tissues were sectioned to 8 μM slices, with every tenth section mounted to a slide (n = 3 for each sample). The samples were stained following standard H&E staining protocols (41) and cover slipped.

Images of stained sections were taken using an EVOS XL microscope. Two sections were selected and utilized for analysis for each mouse. Area of adipocytes in the white adipose samples were measured using the Watershed Algorithm package on ImageJ. The areas of the two sections were averaged for each animal. Data were analyzed with one-way ANOVA and Student's *t* tests.

Results

NAG-1 Adult Mice Weigh Significantly Less Than Their Wildtype Littermates

NAG-1 mice have been shown to weigh less than their wildtype littermates (35-37, 42). To determine whether this is observed during a prolonged cold

exposure, body weights were measured every other day during cold exposure. Interestingly in the prepubertal females, we observed the WT mice weighing significantly more than the NAG-1 mice at every measurement with the exception of the first day (Figure 1A), with significant interactions between time points ($p < 0.0001$) and genotype ($p = 0.0068$) determined by two-way ANOVA. No significance was observed with repeated t-tests of the prepubertal males over the course of the study ($p = 0.052$) or by genotype ($p = 0.0852$) according to two-way ANOVA analysis (Figure 1B). However, when comparing body weights at the end of the study, NAG-1 prepubertal males had a mean final weight of 10.74 ± 0.59 g compared to a mean of 13.23 ± 0.87 g of their WT littermates which yielded statistical significance when analyzed with a t-test ($p = 0.039$). The difference of the mean body weights between NAG-1 over WT prepubertal mice increased during the study. Conversely, NAG-1 transgenic adult mice weighed significantly less than their wildtype littermates, regardless of sex (Figure 1C, 1D). Although this has been reported numerous times with the NAG-1 transgenic mice at room temperature (35-37, 42), these data show this transgenic model maintains their lower body weights even in cold.

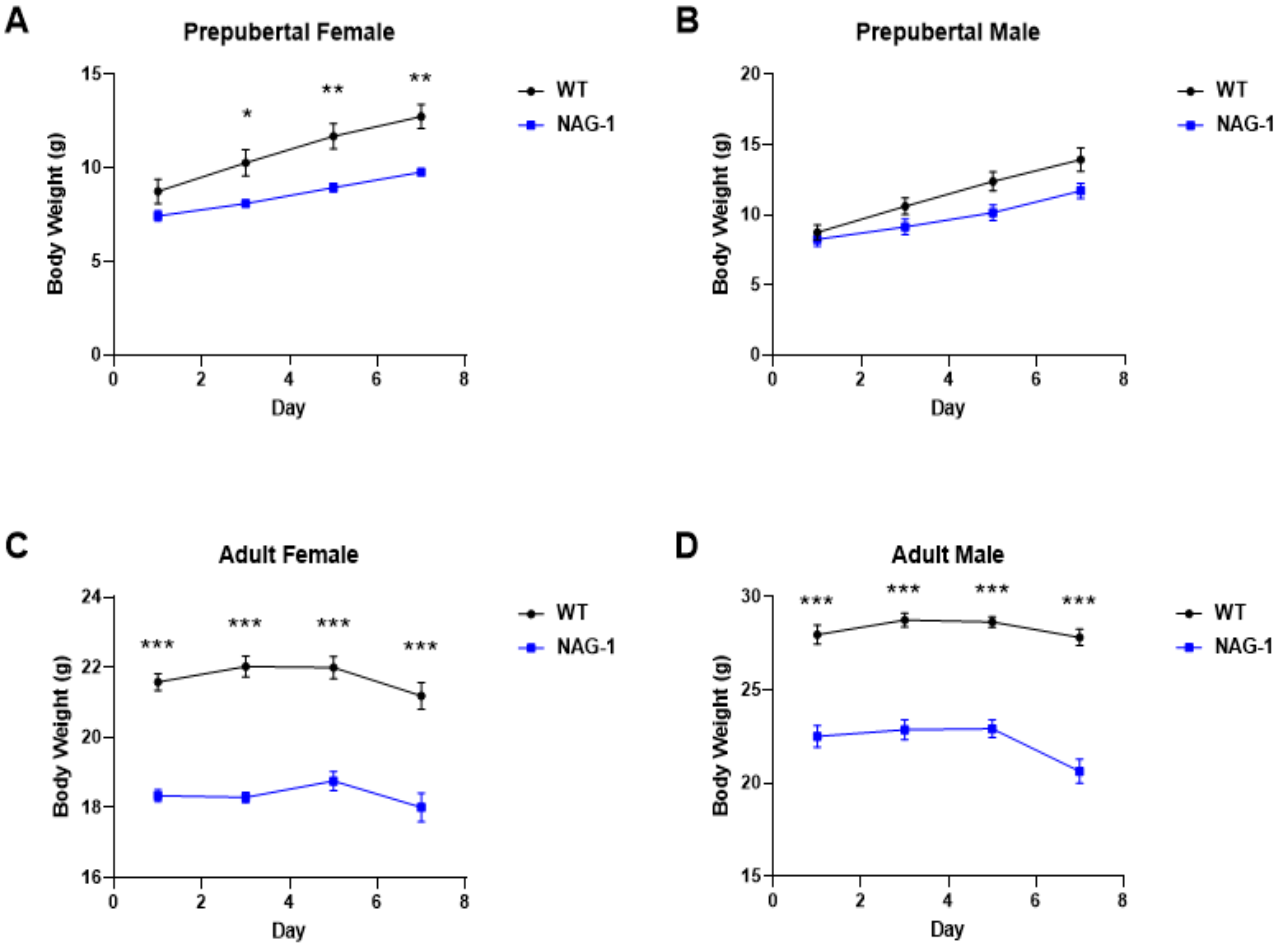

Figure 1: Body Weights During Cold Exposure. Mice were weighed every other day over the course of the prolonged cold exposure. **A**) Prepubertal female body weights in WT (n = 6) and NAG-1 mice (n = 6). **B**) Prepubertal male WT (n = 6) and NAG-1 mice (n = 6). **C**) Adult female WT mice (n = 6) and NAG-1 littermates (n = 6). **D**) Adult male WT mice (n = 6) and NAG-1 littermates (n = 5). Data shown is the average ± SEM and significance is denoted by results of repeated Student's t-tests. Significance determined as follows: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$

<u>Estrus Cycles Show No Significant Differences Between NAG-1 and WT Females</u>

Mice typically complete one full estrus cycle over four days, with a complete cycle ending in oocyte release (39). Monitoring estrus cycles serves as an indirect measure of fertility that cold exposure has been suggested to disrupt via ovarian

abnormalities as well as changes in morphology of the uterus (43). Differences in the cycles between NAG-1 and WT females could indicate that the disruption observed in previous cold exposure studies (43) is altered due to overexpression of NAG-1. Vaginal cytology in NAG-1 (n = 4) and WT mice (n = 6) (Figure 2C-F) showed no significant differences in the completion of estrus cycles when analyzed with a one-way ANOVA, which indicates that any effects of cold exposure on reproduction happen regardless of genotype (Figure 2A-B). It is important to note that two NAG-1 females were not cycling at all, with all seven days of the study spent in diestrus. These mice (n = 2) were excluded from the statistical analysis.

Differences in Glucose Tolerance After Cold Exposure Are Insignificant

One measure of metabolic activity is glucose tolerance. It has been shown that NAG-1 adult mice have higher glucose tolerance and glucose efficiency over time than WT mice (37, 42). To test glucose tolerance in mice that have been subjected to seven days of cold exposure, we performed an intraperitoneal glucose tolerance test on the morning of the eighth day of the study. No statistical significance was observed in glucose tolerance between the NAG-1 and WT mice (Figure 3A-D). Two-way ANOVAs of each group show no significant variation due to genotype, but do show significance in each group over time ($p<0.05$), which is expected as we are measuring response to a glucose injection. Upon further analysis, prepubertal NAG-1 males had a significantly lower average fasting blood glucose level of 104.0 ± 4.97 mg/dL compared to WT mice, which had an average of 144.3 ± 6.82 mg/dL ($p=0.0026$). This trend was observed in the adult male mice as well with NAG-1 males displaying an average fasting blood glucose level of 124.0 ±

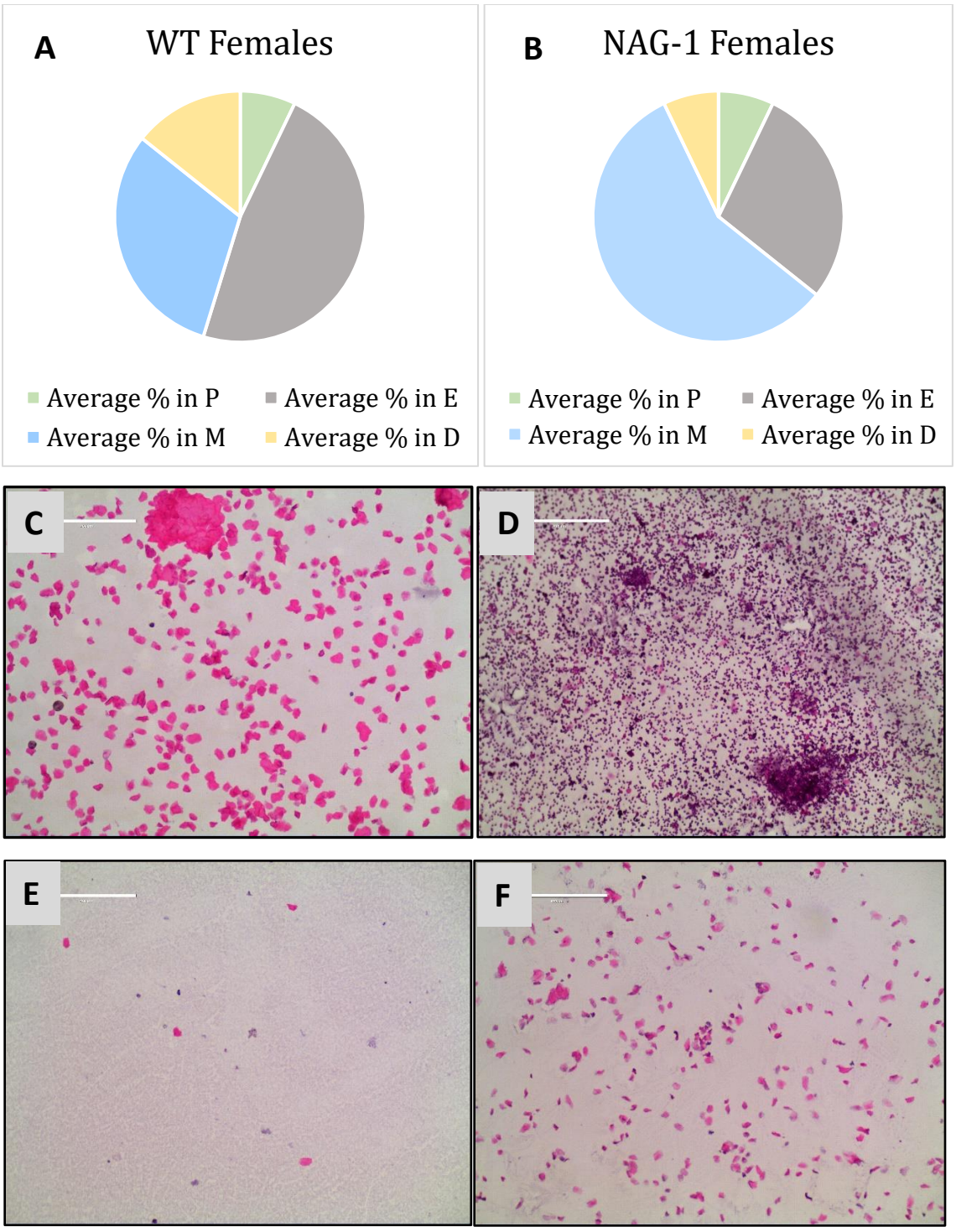

Figure 2: Estrus Cycles in Mice During Cold Exposure. Estrus cycle analysis serves as an indirect measure of fertility. **A**) WT females and **B**) NAG-1 females (P, Proestrus; E, Estrus; M, Metestrus; D, Diestrus). **C-F**) Examples of vaginal washes from **C**) estrus, **D**) metestrus, **E**) diestrus and **F**) proestrus. Data shown is percent of study spent in each stage of the estrus cycle analyzed with a one-way ANOVA. Images were taken at 100x and scale bars represent 400µm.

27.2 mg/dL, which was significantly lower than that of the WT mice at 221.5 ± 25.9 mg/dL (p=0.0267). Adult male wildtype mice also showed a slower return to fasting blood glucose levels than NAG-1 adult males (Figure 3D). Differences in fasting blood glucose were not observed in the adult nor the prepubertal female groups. Increase in glucose levels post-injection showed similar increases in blood glucose levels in all animals, although the WT adult males took longer to respond to this stimulus than the NAG-1 males did (Figure 3D).

NAG-1 Mice Have Higher Metabolic Rates After Cold Exposure

Indirect calorimetry, such as respirometry, has been a well-established method to measure resting metabolic rates in mice (44). By quantifying the respiratory gas exchange between the animal and its environment, the metabolic rate can be calculated (44). NAG-1 mice have been characterized to have significantly higher metabolic rates than WT mice at room temperature (37). Cold exposure, both acute and prolonged, has been shown to increase metabolic rates in mice (29). In this study, metabolic rates of the mice were measured at room temperature before cold exposure (Supplemental Figure 1). NAG-1 mice had notably higher metabolic rates than their WT littermates, with statistical significance in every group except for the prepubertal females (Supplemental Figure 1). These findings in the adult NAG-1 mice support previously reported results (37) observed in metabolic rate.

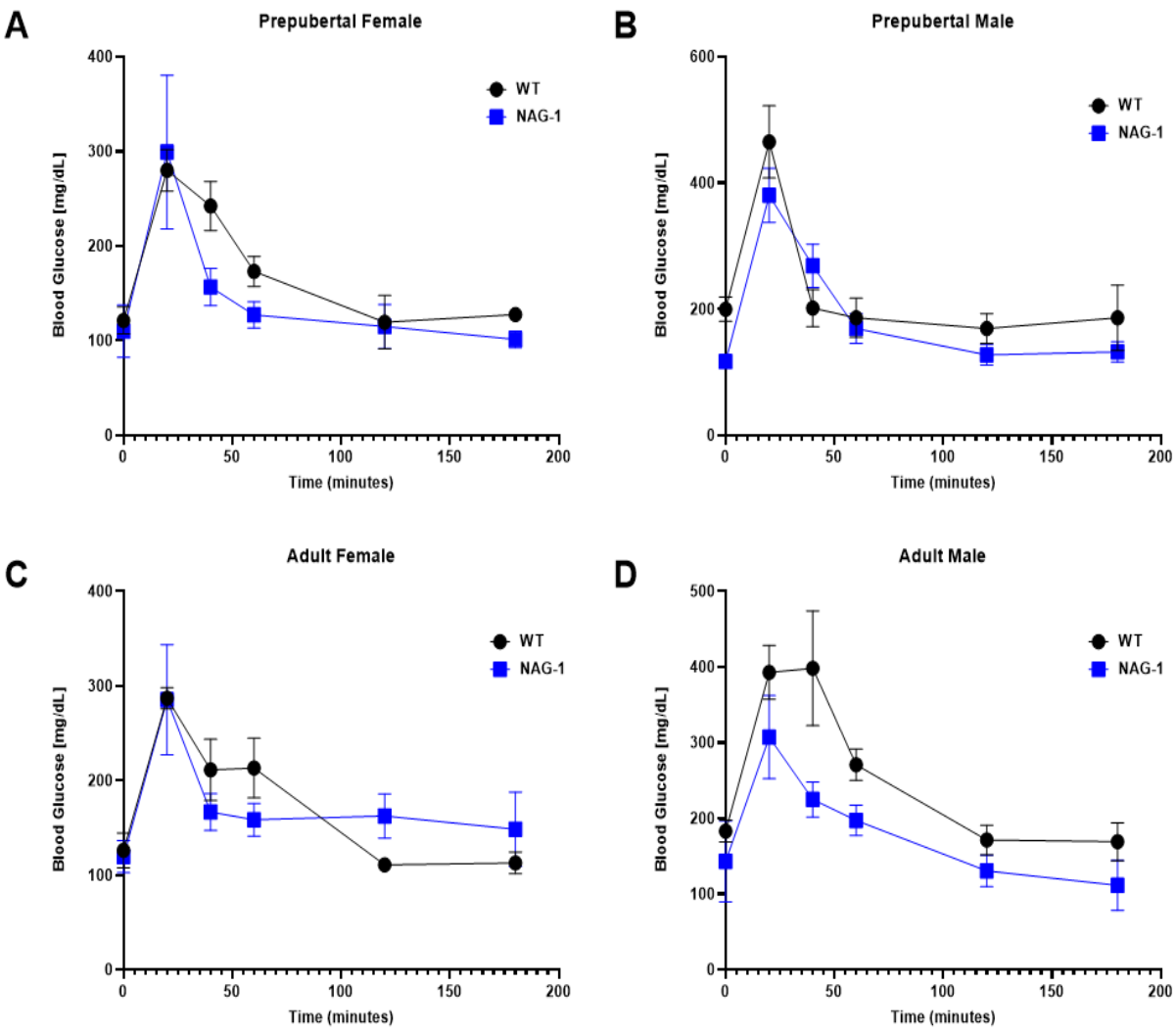

Figure 3: Intraperitoneal Glucose Tolerance Tests. Mice were fasted overnight, and fasting blood glucose was measured through blood collected from the tail. After fasting blood glucose was measured, animals received 2000 mg/kg of glucose via intraperitoneal injection on the morning of the eighth day of the study. Blood glucose levels were monitored every twenty minutes for the first hour, then every hour for the next two hours. **A**) Prepubertal female NAG-1 mice (n = 4) compared to WT (n = 4). **B**) Prepubertal male NAG-1 mice (n = 3) compared to WT (n = 3).**C**) Adult female NAG-1 mice (n = 3) and WT mice (n = 3). **D**) Adult male WT mice (n = 4) compared to NAG-1 mice (n = 3). Data shown in with average ± SEM and was analyzed by two-way ANOVA and repeated *t*tests.

To determine whether the increases in metabolic rate from NAG-1 and cold exposure act synergistically, respirometry was performed again in the cold on the seventh day of exposure (Figure 4). The metabolic rates of both NAG-1 and WT adult

females increased compared to their room temperature rates, but the difference between the rates was no longer significant after cold exposure. Sex and genotype were determined to be significant sources of variation in the prepubertal mice (p = 0.020 and p = 0.005, respectively) and in the adult mice (p < 0.0001 and p = 0.0019, respectively) via two-way ANOVA. Adult female NAG-1 mice had an average metabolic rate of 150.5 ± 4.624 $mL \cdot kg^{-1} \cdot h^{-1}$ compared to that of the adult WT females of 142.4 ± 3.203 mL·kg-1·h-1 (p = 0.2012, Figure 4A). Adult NAG-1 males had markedly higher average metabolic rates of 134.8 ± 6.028 $mL \cdot kg^{-1} \cdot h^{-1}$ than WT mice at 112.0 ± 1.238 $mL \cdot kg^{-1} \cdot h^{-1}$ (p = 0.0028, Figure 4B). Average metabolic rate of prepubertal female NAG-1 mice was 259.5 ± 3.08 $mL \cdot kg^{-1} \cdot h^{-1}$ which was decidedly higher than the average rate of WT prepubertal females at 214.5 ± 8.67 $mL \cdot kg^{-1} \cdot h^{-1}$ (p = 0.0006, Figure 4C). Prepubertal male mice maintained these differences too, with NAG-1 mice having an average metabolic rate of 239.3 ± 9.62 $mL \cdot kg^{-1} \cdot h^{-1}$ compared to an average WT metabolic rate of 199.8 ± 9.645 $mL \cdot kg^{-1} \cdot h^{-1}$ (p = 0.0245, Figure 4D). While the trends of NAG-1 having higher metabolic rates was observed in cold exposure, the difference between the NAG-1 and WT mice was not further increased which suggests that NAG-1 and cold exposure do not act synergistically to increase metabolic rates.

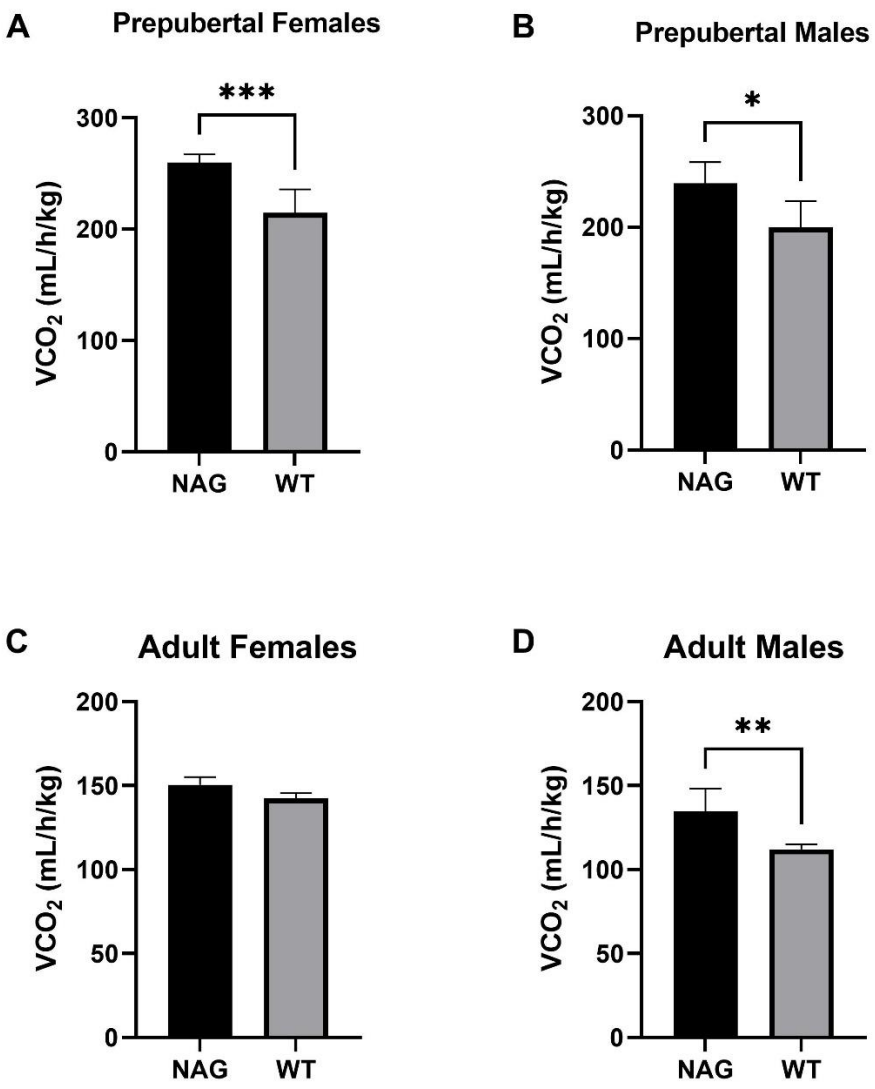

Figure 4 6: NAG-1 Mice Have Higher Metabolic Rates After Seven Days of 10°C. Indirect respirometry measures the exchange of respiratory gases between the animal and the environment, allowing for calculation of metabolic rate . **A**) NAG-1 prepubertal females (n = 6) compared to WT prepubertal females (n = 6). **B**) Prepubertal male NAG-1 mice (n = 4) and prepubertal male WT mice (n = 6). **C**) NAG-1 adult females (n = 6) and WT adult females (n = 5). **D**) NAG-1 adult males (n = 5), WT adult males (n = 6). Data shown is the VCO_2 that has been corrected for body weight, average ± SEM. Analysis via two-way ANOVA and Student's t-test. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Beiging of White Adipose Tissue Observed in NAG-1 and WT Mice

It has been reported that white adipose tissue "beiges" in response to cold exposure (23, 24, 28, 29), an observation that is morphologically characterized by

the presence of central nuclei, multilocular lipid droplets and are mitochondria-rich (24). Representative histological images of prepubertal WAT are shown in Figure 5 and adult WAT in Figure 6. The beige morphology was qualitatively observed in NAG-1 and WT mice alike, especially in the prepubertal mice (Figure 5). Perhaps one of the more distinct examples of beiging can be observed in the adult female WT mice (Figure 6D), with a discrete population of multilocular, brown-like adipocytes within unilocular adipocytes.

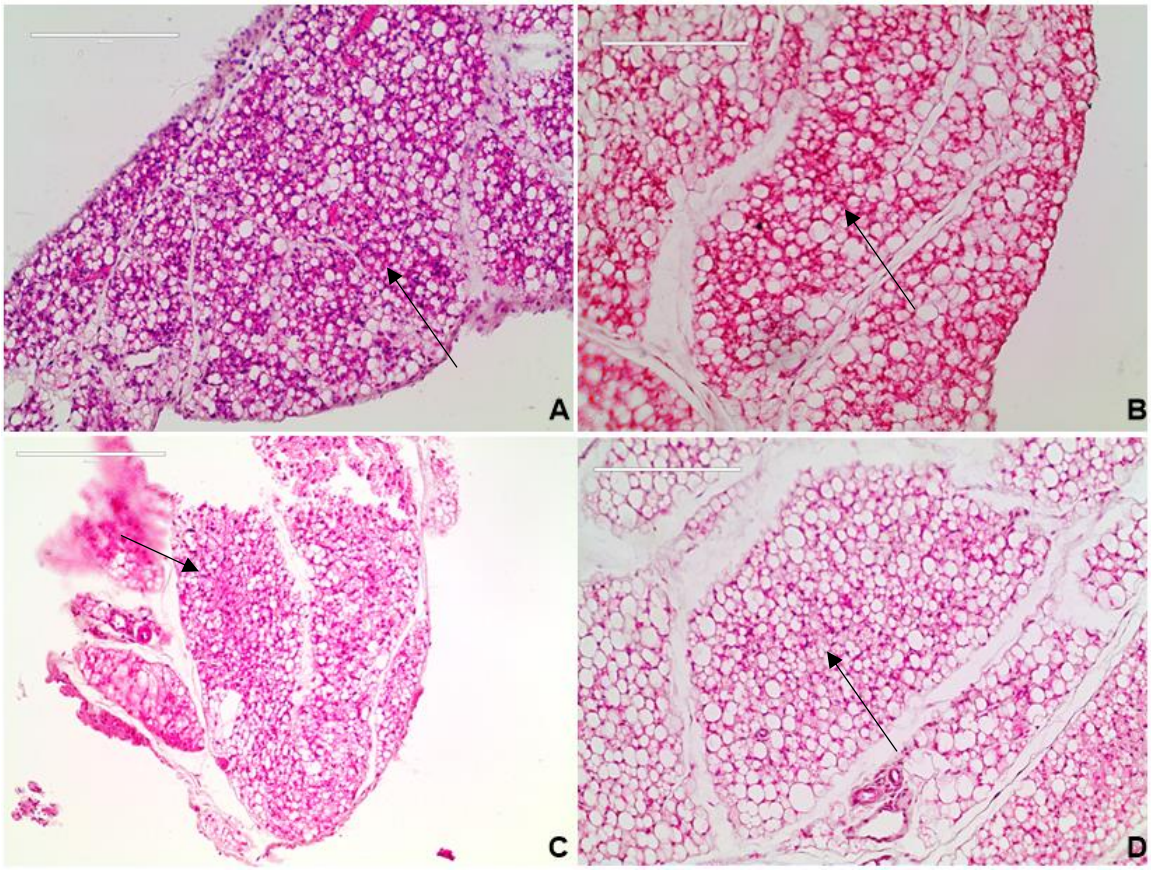

Figure 5: Prepubertal White Adipocyte Histology After Cold Exposure. Representative H&E stained images of white adipocytes from A) NAG-1 males, B) WT males, C) NAG-1 females and D) WT females. White adipocytes can be seen with deposits of beige adipocytes, marked with arrows. Images were taken at 200x magnification with scale bars representing 200μm.

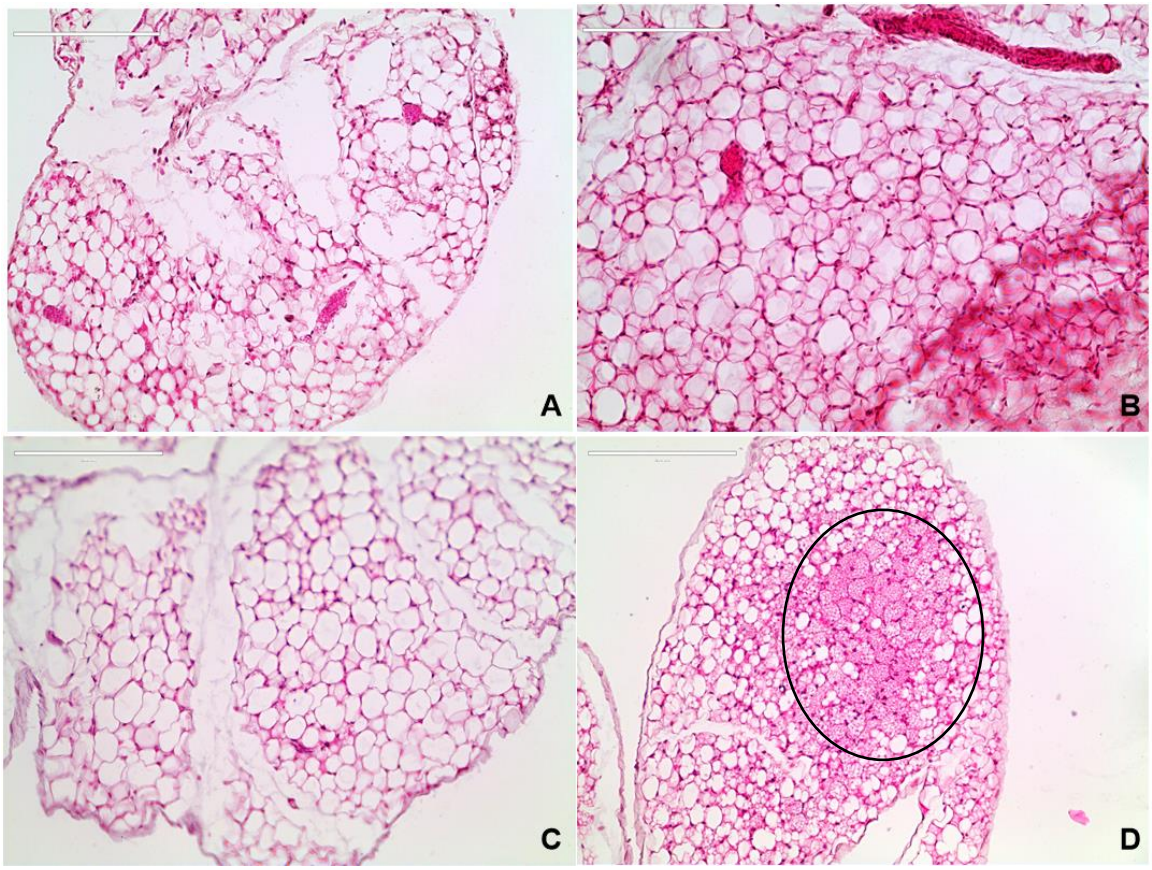

Figure 6: Adult White Adipocyte Histology After Cold Exposure. Representative H&E stained images of white adipocytes from A) NAG-1 males, B) WT males, C) NAG-1 females and D) WT females. Deposit of beige adipocytes circled in D. Images were taken at 200x magnification with scale bars representing 200μm.

Adult WT Males Have Significantly Larger Adipocytes After Cold Exposure

Adipocyte size has been correlated with metabolic activity, specifically with hypotrophic adipocytes linked to higher metabolic activity (45,46). In obese phenotypes, adipocytes are typically hypertrophic with excess lipid storage. Smaller lipid storages in WAT can indicate utilization of fat storage for metabolism, or can also be a sign of decreased differentiation and recruitment of preadipocytes (47). At room temperature, NAG-1 mice have smaller adipocytes with no differences in relative expression of adipogenesis markers compared to wildtype mice, suggesting

that differentiation of adipocytes in NAG-1 mice is likely unaffected by overexpression of NAG-1 (35). We measured the size of white adipocytes of both NAG-1 and WT mice after cold exposure (Figure 7). No significant differences were observed between the prepubertal males (NAG-1 mice 138.3 μm^2 ± 24.8, WT mice 206.2 μm^2 ± 10.2, $p = 0.06$) or the prepubertal females (NAG-1 mice 72.1 μm^2 ± 14.5, WT mice 231.0 μm^2 ± 78.4, $p = 0.22$) after cold exposure (Figure 7A). However, WT adult males had an average white adipocyte size of 1202.0 μm^2 ± 136.0 which was significantly more hypertrophic than the average white adipocyte size of NAG-1 mice of 449.7 μm^2 ± 110.5 ($p = 0.012$, Figure 7B). Interestingly, this difference was not observed in the adult females (NAG-1 mice 477.5 μm^2 ± 160.3, WT mice 619.4 μm^2 ± 111.5, $p = 0.51$).

Gene Expression Analysis Shows Significant Differences in Adult Males

Changes in the expression of genes associated with thermogenesis have been reported as a characteristic of white adipose tissue that is "beiging" or becoming metabolically active. One of these genes is Glucose transporter 4 (*Glut4*) which codes for a protein that transports glucose into the cell. An upregulation of the expression of *Glut4* can indicate higher rates of glucose transport that correlates with increased metabolic activity (48). Another gene of interest is adipose triglyceride lipase (*Atgl*), a lipolytic gene involved in the mobilization of fatty acids with higher expression rates linked to increases in thermogenesis (49).

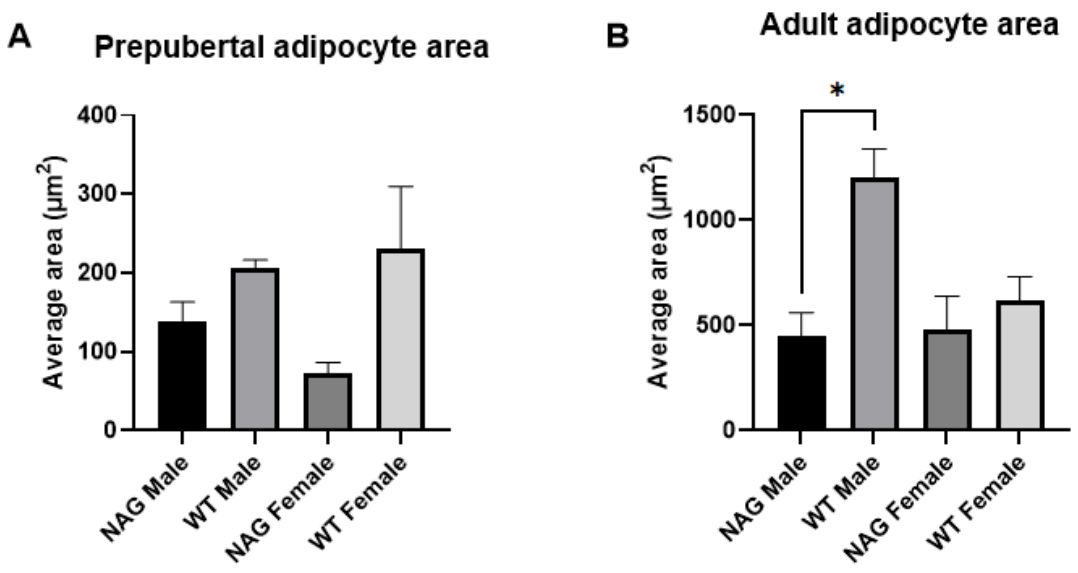

Figure 7: Average White Adipocyte Area After Cold Exposure of 10°C for Seven Days. Adipocyte size can serve as an indicator of metabolism, as smaller adipocytes indicate the utilization of fat storage for metabolism. Adipose area was calculated using ImageJ. A) Average white adipocyte area in prepubertal mice. B) Average area of white adipocytes in adult mice. Data shown is the average of adipocyte area from two histological sections for each animal ± SEM. Statistical significance determined by one-way ANOVA and Student's *t* test- *, $p < 0.05$.

Previous gene expression analysis of adult male NAG-1 mice performed at room temperature by Chrysovergis, et. al (2014) showed no significant differences in expression of *Glut4* but did find a significant increase in *Atgl* expression compared to WT mice (37). Cold exposure studies performed with C57BL/6 mice to investigate increased thermogenic activity of white adipose tissue have reported increased expression of *UCP-1*, *Pgc1α* and *PPARγ* in beiging white adipocytes (29).

To measure the effects of cold exposure on thermogenic gene expression in NAG-1 mice, RNA isolated from inguinal white adipose tissue was used to perform quantitative RT-PCR and expression levels of thermogenic genes were measured.

Adult male NAG-1 mice had significantly lower expression of both *Glut4* (Figure 3A, -0.6 fold, $p = 0.0029$) and *Atgl* (Figure 3B, -0.6 fold , $p = 0.0002$), and a significant difference between expression by sex was determined by two-way ANOVA ($p = 0.0259$ and $p = 0.048$, respectively). No significant differences were observed in expression of *Glut4* or *Atgl* in adult females (Figure 8A, 8B). Analysis of *UCP-1*, *Pgc1α* and *PPARγ* showed no significant differences in expression in either the adult female or male groups (Figure 8C, 8D, 8E).

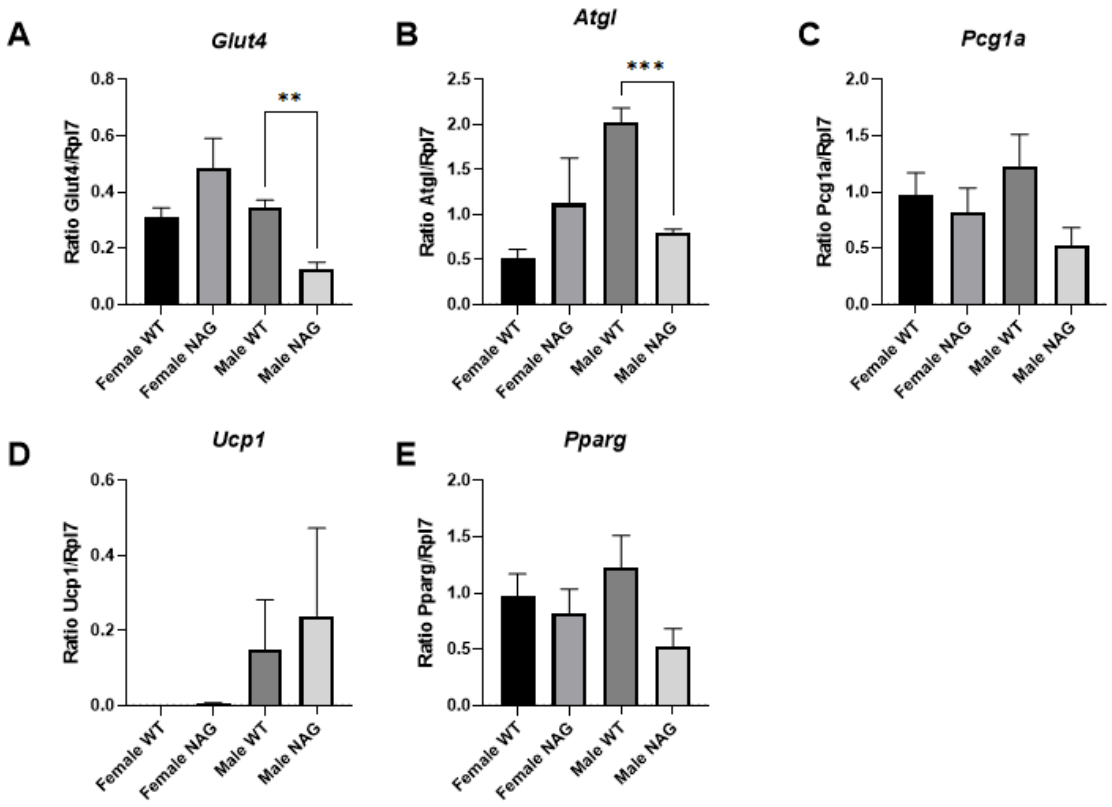

Figure 8: Gene Expression Changes in Adult WF. RNA was isolated from inguinal white adipose tissue and reverse transcribed. Expression was measured using quantitative real-time PCR. Data shown is ratio of Ct values of gene of interest compared to *Rpl7* (internal control) +/- SEM. Analyzed via two-way ANOVAs and Student's *t*test, **, $p < 0.01$, ***, $p < 0.001$.

Discussion

NAG-1 transgenic mice have been established as a metabolic model, with studies showing increased metabolic rates, lower body weights, higher expression of thermogenic genes and are resistant to diet and genetic-induced obesity when compared to age-matched WT littermates at room temperature (35-37, 50). Interestingly, this is the first study of NAG-1 mice undergoing prolonged cold exposure. The molecular pathways that respond to the stimulus of cold exposure to increase thermogenesis, and subsequently metabolism, are well documented (24-27). The aim of this study was to compare NAG-1 mice and WT mice after prolonged cold exposure to determine whether the mechanism through which NAG-1 increases metabolism acts synergistically with that of cold exposure. There are complex aspects that influence metabolism, including age and sex. To examine these mitigating factors, females and males as well as prepubertal and adults comprised the groups of this study.

It is known that at room temperature, NAG-1 adult mice weigh significantly less than age-matched WT littermates (35, 37, 50), however little is known about body weight differences between prepubertal NAG-1 and WT mice. This study demonstrates that adult NAG-1 mice maintain their distinctly lower body weights after seven days of cold exposure, regardless of sex. Prepubertal female NAG-1 mice did not initially weigh less than WT mice but as they aged, the weight differences became significant. Prepubertal male mice did not display significant differences in body weight until the final day of the study. The difference in response from the

prepubertal males and females suggests sexual dimorphism through development, which could be influenced by hormones or simply by the responses of the mice to cold stimulus. A plausible explanation for the lower body weights observed in NAG-1 mice would be lower consumption of food, however it was shown at room temperature that NAG-1 mice do not eat less than WT mice (37). In this study, food consumption was monitored and no significant differences were observed (data not shown).

Estrus cycle monitoring is a way to indirectly measure fertility of mice. Cold exposure has been suggested to disrupt estrus cycles, contribute to ovarian abnormalities as well as cause changes in morphology of the uterus (43). Estrus cycles in NAG-1 adult females compared to WT adult females showed little variation. However, there were two NAG-1 females who did not cycle throughout the study and remained in diestrus for the full seven days (data not shown). While this has the potential to indicate changes in disruption of estrus cycles in NAG-1 mice and WT mice, further studies would be needed to corroborate this finding. Also, on average mice complete the estrus cycle in four days. Longer cold exposure to allow for a bigger picture analysis and more time for completion of the estrus cycles would be ideal in informing us of the response of female adult mice to cold stimulus.

Another common measure of metabolic function is to determine an animal's glucose tolerance, as efficiency of glucose utilization can indicate metabolic health (42). Previous studies have found that NAG-1 mice have higher glucose tolerance, which was not corroborated by data in this study. Because the uptake of glucose is a

vital component to upregulation of thermogenesis, it is reasonable that this process is altered by cold exposure regardless of genotype. Another discrepancy between this study and previous reports is the age of the mice. Previous reports have assessed 16-week-old mice (37) and adults in this study were 13 weeks old. While no statistical differences were found in the overall response to glucose injection, both prepubertal and adult NAG-1 males had significantly lower fasting blood glucose levels, a finding that was not observed in female mice. This could represent sex differences in the utilization of glucose to adapt to cold exposure, which has been reported at room temperature (51).

Cellular respiration in humans and mice alike utilizes the intake of oxygen and burning of macromolecules to produce ATP and carbon dioxide. Indirect calorimetry, specifically respirometry, measures the volume of respiratory gases- O_2 and CO_2- exchanged in this process and allows for the calculation of metabolic rate. Previous studies performed with NAG-1 mice show they have higher metabolic rates at room temperature (37). NAG-1 mice after cold exposure also exhibited higher metabolic rates with significance in all groups except for the adult females. However, the differences between the metabolic rates of the NAG-1 and WT mice after a prolonged cold exposure were not further exaggerated over the differences observed at room temperature, indicating that NAG-1 overexpression does not further increase thermogenesis in response to cold exposure. There has been some concern voiced about the statistical methods that have become commonplace in metabolic rate analysis, specifically the correcting of metabolic rate by dividing by the animal's body weight in transgenic models (52). The points raised are valid,

especially the consideration that correcting for body weight when using animals with different genotypes assumes isometric body composition (52). However, due to literature precedence, particularly in metabolic rate measurements of NAG-1 mice, and the design of the study to compare NAG-1 mice directly to WT mice in each group, the statistical method of two-way ANOVAs and student t-tests was used in this study. Also, the NAG-1 mouse model composition has been well-documented at room temperature, which helps mitigate the concern of assumptions of body composition (35).

White adipose tissue stores lipids that can be mobilized as a fuel source for activation of UCP-1 in metabolically active tissue (45,46). For this reason, adipocyte size is correlated to metabolic activity, with smaller adipocytes indicating more metabolic activity (45,46). At room temperature, adult NAG-1 mice have been shown to have smaller white adipocytes without lower expression of adipogenesis genes, indicating that the difference in adipocyte size is likely not due to impairment in differentiation (35). We found no significant difference in adipocyte size for the prepubertal mice or in the adult females. Adult male wildtype mice, however, had significantly larger adipocytes than NAG-1 mice. Qualitatively, beiging of white adipose tissue to adapt morphological features of brown adipose tissue in response to cold stimulus was observed, particularly in the representative images from the prepubertal mice and the adult wildtype females. Because adipocyte size is a measure that is prone to individual variation, we hypothesize with a larger sample size that the distinction between NAG-1 and WT mice might be more pronounced. The significant difference observed in the adult males but not in the adult females

could be further evidence for sex differences in response to cold stimulus that result regardless of genotype. At room temperature, sex steroids have been shown to strongly influence lipid uptake, leptin production, lipolysis and development and differentiation of adipocytes (53). Because these processes are fundamental for adipocyte health, it is reasonable that these differences would be maintained after cold exposure and present as differences in adipocyte size.

Gene expression analysis is another crucial component to measuring the response of mice to cold exposure. We found that adult male WT mice showed significant increases in expression of *Glut4* and *Atgl* over NAG-1 mice. There were no significant differences in the adult female groups, or in the expression of uncoupling protein-1 (*UCP-1*), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*), and peroxisome proliferator activated receptor gamma (*PPARγ*). Previous reports of gene expression analysis performed on WAT isolated from NAG-1 mice at room temperature showed no noteworthy differences in expression of *Glut4* or *PPARγ,* but did find a significant increase in *Atgl* expression compared to WT mice (37). Because the metabolic rates of NAG-1 mice are already increased at room temperature (37), the gene expression data from this study might indicate that upregulation of thermogenic genes is not as integral for adaptation to cold exposure in NAG-1 mice as it is for wildtype mice. It could also suggest that NAG-1 mice adapt to the cold more quickly than their wildtype littermates, which could be assessed with gene expression analysis of NAG-1 males and WT males after an acute cold exposure.

The gene expression of the adult females relates to the respiration changes observed in the study. The expression of key thermogenic genes was not significantly different between NAG-1 and WT females, just as metabolic rates were not different after cold exposure, although they were at room temperature (Supplemental figure 1). The differences in gene expression suggests that males and females in the study respond differently to cold exposure. This has been shown to be the case in a cold exposure study with humans (54), which could be due to the influence of sex steroids on lipid uptake, lipolysis, development and differentiation of adipocytes (53).

In conclusion, this study aims to contribute to the growing understanding of how NAG-1 increases metabolism to identify targets for potential therapeutic treatments for obesity. It has been demonstrated at room temperature that induced NAG-1 overexpression in the NAG-1 transgenic mice upregulates expression of thermogenic genes, which protects against insulin resistance and obesity (37). Cold exposure stimulates the β3 adrenergic signaling pathway to upregulate thermogenesis and allow for adaptation to the cold environment. Herein, we show that NAG-1 mice maintain their lower body weights in cold exposure compared to WT mice, estrus cycles are comparable between NAG-1 and WT females in cold exposure and that higher glucose tolerance in NAG-1 mice is not observed after a prolonged cold exposure. Metabolic rates of NAG-1 mice are higher than WT mice after cold exposure, but not by a margin that indicates exaggerated response to cold. Gene expression analysis also suggests that NAG-1 mice do not have an increased response to cold exposure via upregulated expression of thermogenic genes. Taken

together, these data show that the mechanism through which NAG-1 increases metabolism does not act synergistically with the β3 adrenergic signaling pathway that is stimulated by cold exposure. There are many layers of complexity to adipose tissue and its interactions with the immune system, the nervous system and even within itself. Data from this study support the idea that sexual dimorphism and age contribute to adipose tissue remodeling, including changes in metabolic activity, and response to cold exposure. Further studies could utilize different time periods of cold exposure, investigate binding partners of NAG-1, and how NAG-1 influences immune cell activity observed in chronic inflammation associated with obesity. More research needs to be performed on the effects of NAG-1 overexpression to identify the mechanism of action, however additive interactions with the β3 adrenergic signaling pathway can be considered insignificant from the data presented in these studies.

Acknowledgments

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This study was supported by grants from the Washington State Distinguished Fellowship in Biology and from Central Washington University Provost, Michelle DenBeste's Faculty/Student Research Grant.

Disclosure Statement: The authors have nothing to disclose.

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https://doi.org/10.1016/j.jtherbio.2018.08.016

Appendix A

Table A1. Primer sequences for qRT-PCR

Gene	Forward Sequence	Reverse Sequence	Source
Rpl7	AGCTGGCCTTTGTCATCAGAA	GACGAAGGAGCTGCAGAACCT	Primer Harvard Bank
Glut4	GTGACTGGAACACTGGTCCTA	CCAGCCACGTTGCATTGTAG	[37]
Atgl	CAACGCCACTCACATCTACGG	GCACACCTCAATAATGTTGGCAC	[37]
PGC1α	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG	[30]
PPARγ	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT	[31]
UCP-1	ACGTCCCCTGCCATTTACTG	CACACACAGGCGCCTTAAAC	Primer Harvard Bank

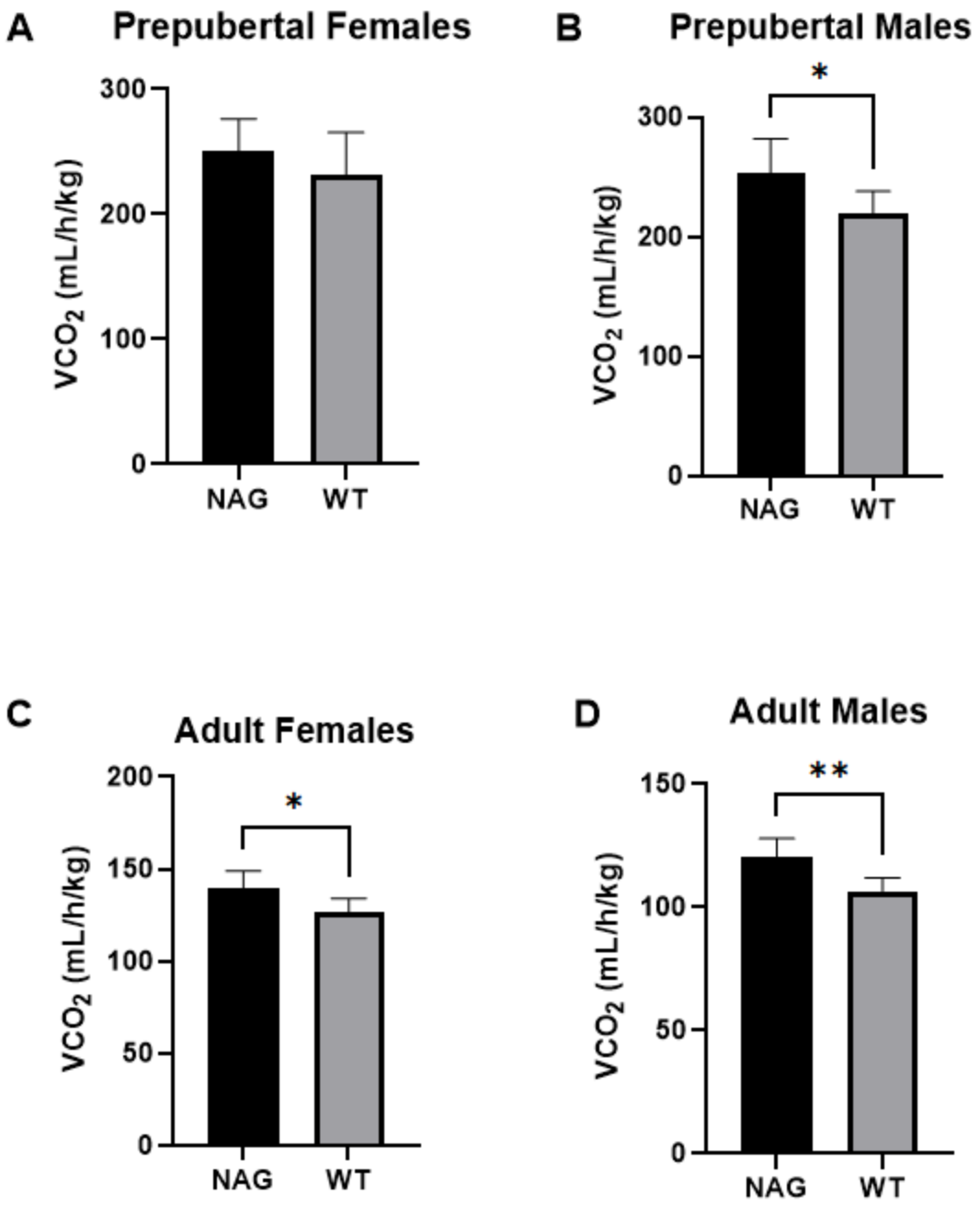

Figure A1. Metabolic Rates At Room Temperature. Indirect respirometry measures the exchange of respiratory gases between the animal and the environment, allowing for calculation of metabolic rate . A) NAG-1 prepubertal females (n = 6) compared to WT prepubertal females (n = 6) B)Prepubertal male NAG-1 mice (n = 4) and prepubertal male WT mice (n = 6) C)NAG-1 adult females (n = 6) and WT adult females (n = 5). D) NAG-1 adult males (n = 5), WT adult males (n = 6). Data shown is the VCO_2 that has been corrected for body weight +/- SEM. Analysis via two-way ANOVA and Student's t test-*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.