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THE ROLE OF NEURONAL DNA METHYLTRANSFERASE 1 IN ENERGY
HOMEOSTASIS AND OBESITY.

by

EMILY C. BRUGGEMAN

Under the Direction of Bingzhong Xue, Ph.D.

ABSTRACT

Obesity is a grave disease that is increasing in global prevalence. Aberrant neuronal DNA methylation patterns have been implicated in the promotion of obesity development, but the role of neuronal DNA methyltransferases (Dnmts; enzymes that catalyze DNA methylation) in energy balance remains poorly understood. We investigated the role of neuronal Dnmt1 in normal energy regulation and obesity development using a novel Dnmt1 knockout mouse model, Dnmt1^{fl/fl} Synapsin1Cre (ND1KO), which specifically deletes Dnmt1 in neurons. ND1KO and fl/fl control littermates were fed either a standard chow diet or a high fat diet (HFD). We conducted a deep analysis to characterize both peripheral and central aspects of the ND1KO phenotype. We found that neuronal Dnmt1 deficiency reduced adiposity in chow-fed mice and

attenuated obesity in HFD-fed male mice. ND1KO male mice had reduced food intake and increased energy expenditure on the HFD. Furthermore, these mice had improved insulin sensitivity as measured by an insulin tolerance test. HFD-fed ND1KO mice had smaller fat pads and an upregulation of thermogenic genes in brown adipose tissue. These data suggest that neuronal Dnmt1 deletion increased diet-induced thermogenesis, which may explain the lean phenotype in HFD-fed ND1KO mice. Interestingly, we found that ND1KO male mice had elevated estrogen receptor- α gene expression in the hypothalamus, which previously has been shown to control body weight. Immunohistochemistry experiments revealed that estrogen receptor- α (ER α) protein expression was upregulated in the dorsomedial region of the VMH (VMHdm), a region which may mediate the central effect of leptin. Finally, we tested whether ND1KO mice had reduced methylation of the ER α gene promoter, which might explain the ER α upregulation. Neuronal Dnmt1 deficiency decreased methylation at two CpG sites on Exon A in chow-fed mice. Collectively, these data suggest that neuronal Dnmt1 regulates energy homeostasis through pathways controlling food intake and energy expenditure, and that ER α in the VMHdm may mediate these effects.

INDEX WORDS: Epigenetics, Estrogen receptor-alpha, Hypothalamus, Brown adipose tissue.

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EMILY C. BRUGGEMAN

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2016

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May 2016

DEDICATION

I would like to dedicate this dissertation to all people who suffer from overweight and obesity, and who feel stigmatized and misunderstood. It is my hope that our continuing knowledge and understanding of this disease will lead to greater compassion for such people.

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I first and foremost want to express my gratitude to Dr. Bingzhong Xue for being the advisor and mentor I needed—thank you for being patient, kind and absolutely wonderful. I have so much respect for you, and I admire your strength, brilliance and manner. To Aaron, Anne and Nancy, thank you for being a stellar dissertation committee! Each one of you have been fully engaged and thoughtful about my projects and have offered so much. I am grateful for your advice and guidance. Thank you to Dr. Hang Shi, for providing such an important perspective on my projects. I am grateful in so many ways to each member of my lab family: Dr. Xin Cui, Dr. Qiang Cao, Dr. Jia Jing, Dr. Rui Wu, Lin Zha, Xiaosong Yang, Yii-shyuan Chen, Anubama Rajan, Fenfen Li, Lizhi Fu, Shuping Kou, and newly adopted lab members Mary Schneider, Alex Thomas, Ngoc Ly Nguyen Everyone deserves such an excellent interpersonal lab environment to work in! Thank you to my undergraduate assistants, Ibitola Akinmurele and Arish Amersi. Massive thanks to Johnny Garretson for his support, advice and respect, and basically for everything. Thank you to Dr. Amy Ross, who was the first person I looked up to in graduate school, and has been invaluable to me in so many ways. I especially would like to express my gratefulness to Dr. Tim Bartness for giving me a step up, in so many ways, throughout the years. To my Dad, thank you for your unconditional love. To my Mom, thank you for raising me to be strong like you in order to get through these grueling 9 years. And to my sister, Lisa, and brother, David, for their love and support. Finally, I'm so grateful for Krista Hostetler, Aunt Jan and Seanna Oliver. And last but not least, thank you to my dear boyfriend, Victor, for doting on me, feeding me, bearing with me, and sharing me temporarily with my dissertation.

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LIST OF ABBREVIATIONS

ARC, arcuate nucleus of the hypothalamus

BAT, brown adipose tissue

BDNF, brain-derived neurotrophic factor

BMI, body mass index

ER α , estrogen receptor alpha

ESR1, estrogen receptor alpha gene

HFD, high fat diet

HYP, hypothalamus

LDL, low-density lipoprotein

LH, lateral hypothalamus

ND1KO, neuronal Dnmt1 knockout mouse

NE, norepinephrine

POMC, proopiomelanocortin

PVH, paraventricular hypothalamus

SNS, sympathetic nervous system

UCP1, uncoupling protein 1

V/DMH, ventromedial/dorsomedial hypothalamus

VMH, ventromedial hypothalamus

VMHdm, dorsomedial region of the VMH

VMHvl, ventrolateral region of the VMH

WAT, white adipose tissue

1 INTRODUCTION

1.1 The Obesity Disease

Obesity is a disease of energy imbalance characterized by the excessive storage of fat in the body. In 2014, 39% of the adult global population was estimated to be overweight, and 13% were estimated to be obese (defined as having a body mass index $\geq 30\text{kg/m}^2$) WHO (2015). Over the past 15 years, obesity prevalence in the U.S. has continued to increase in both adults and children (Ogden et al., 2014). Although the U.S. has one of the highest obesity rates of any country (Kelly et al., 2008), prevalence of this disease is climbing in South America, Europe, and even some African countries, as well (Stevens et al., 2012). One study even has purported that more individuals suffer from overweight and obesity than from malnutrition (Moore et al., 2010). Obesity predisposes people to developing a multitude of serious health problems, including dysfunctional lipid metabolism, cardiovascular diseases, insulin resistance, Type II diabetes, depression and even some cancers (Guh et al., 2009, Badimon et al., 2013, Martin-Rodriguez et al., 2015). Thus, obesity significantly affects a person's quality of life. Obesity and obesity-related diseases comprise an economic burden as well, with an estimated \$147 billion (~2 times the annual costs of healthy adults) in health care costs related to obesity in 2008 (Finkelstein et al., 2009).

1.2 Energy Regulation

Obesity results from an imbalance of energy intake and energy expenditure (Hill et al., 2012). Most organisms need to ingest food throughout the day in order to maintain the consistent blood glucose levels that are needed for cellular function. As these glucose levels fluctuate in between meals, or during fasting, however, multiple mechanisms accommodate for these drops in energy. Thus, the system as a whole works to maintain the appropriate level of blood sugar even during an energy-deficient state. Overall body weight tends to be relatively stable despite daily

fluctuations in exercise and caloric intake (Edholm et al., 1955), unless major, consistent changes are made (e.g., calorie restriction, exercise regimen) that surpass the point of compensation (Melzer et al., 2005).

A complex biochemistry regulates energy availability and storage. Following the ingestion of a meal, blood glucose levels rise, which triggers the release of insulin from the pancreas. Insulin is a hormone required by most cells in order to use glucose for energy. Blood glucose not immediately used is converted to lipids and stored in fat cells (white adipocytes), or is converted to glycogen in the liver for more accessible energy when needed. During fasting, blood glucose levels are increased by the liver in two ways: glycogenolysis (the breakdown of glycogen into glucose) and gluconeogenesis (the synthesis of glucose from substrates such as pyruvate or lactate) (Wahren and Ekberg, 2007). In addition, lipolysis in adipose tissue breaks down the stored triglyceride molecules into smaller, non-esterified (free) fatty acids that can be further metabolized into molecules to be used for cellular energy (Duncan et al., 2007, Fruhbeck et al., 2014).

In addition to circulating hormonal and metabolic cues, the nervous system also provides critical input to the periphery to maintain energy balance. During fed and fasted states, neural signals are generated to support either a catabolic or anabolic state (Kalsbeek et al., 2010, Seoane-Collazo et al., 2015). In an energy-excess state, food intake is inhibited while energy expenditure is increased (catabolic). Likewise, in an energy-deficient state, food intake is stimulated while energy expenditure is reduced and energy storage is promoted (anabolic). The autonomic nervous system innervates the pancreas, liver and adipose tissue to regulate metabolic processes such as insulin secretion, gluconeogenesis, lipolysis, lipogenesis and thermogenesis (Seoane-Collazo et al., 2015).

1.3 Epigenetics and DNA Methyltransferases.

Epigenetics is the study of factors that can repress or activate gene expression through chemical modifications to DNA or histones. DNA methylation, the most studied epigenetic modification, is the addition of methyl groups to the cytosine base of the DNA, producing 5'-methylcytosine (sometimes referred to as the “fifth DNA base”) (Hotchkiss, 1948, Wyatt, 1950). The discovery of 5'-methylcytosine dates back as far as 1925 (Johnson, 1925) and is associated with inactive chromatin and gene repression (Keshet et al., 1986). DNA is packaged in nucleosomes, comprised of a group of four histone dimers that act as spools around which the DNA is wrapped. Histones also can be methylated or acetylated on specific residues, which can repress or activate gene function (Dambacher et al., 2010). Epigenetic modifications occur as a result of environment or experience, and are stable and even heritable. In certain cases, however, DNA methylation can be reversed, although it tends to be more stable than dynamic (Haaf, 2006, Gavin et al., 2013, van der Wijst et al., 2015). Epigenetic modifications play an important role in orchestrating temporal and spatial regulation of gene expression, and this is especially crucial during development. DNA methylation is critical for organ development, because the repression of some genes allows for other genes to direct the course of cellular and tissue differentiation. DNA methylation is known to occur on cytosine residues that are followed by a guanine residue (CpG site), but it can also occur on cytosine residues not followed by a guanine (Sharma et al., 2015). Although CpG residues can appear as clusters, or CpG islands, about half of all CpG islands are unmethylated, and the majority of CpG sites outside of islands are methylated (Razin and Riggs, 1980). Importantly, gene promoter regions can be enriched with CpG sites, and when these are methylated, it can prevent the binding of transcription factors and proteins involved in transcription regulation (Doerfler et al., 1989).

DNA methyltransferases (Dnmts) are enzymes that catalyze the addition of methyl groups to DNA. There are three main Dnmts, and all of them are expressed throughout the brain, although to varying degrees in different regions (Simmons et al., 2013). Dnmt1 is considered the “maintenance” enzyme because it maintains methylation patterns in dividing cells. Dnmt1 has strong preference for hemi-methylated DNA, which occurs when the double-stranded DNA separates during the cell replication cycle (Guh et al., 2009). In the brain, Dnmt1 is mainly expressed in neurons, although some Dnmt1 expression has been demonstrated in microglia in an *in vitro* study (Chestnut et al., 2011). Dnmt3a and Dnmt3b are *de novo* methyltransferases, and they add new methyl groups to DNA where none existed before. Dnmt1 and Dnmt3b show greater brain expression during earlier development (through postnatal day 21), while Dnmt3a is expressed more highly in the adult brain (Chestnut et al., 2011, Simmons et al., 2013)

In the adult brain, Dnmt1 and Dnmt3a expression is higher than that of Dnmt3b, which is often difficult to detect. Although most of the brain consists of postmitotic cells, it is surprising that Dnmt1 is expressed moderately, even more so than Dnmt3b (Goto et al., 1994, Simmons et al., 2013). This might suggest additional roles for Dnmt1 other than maintaining methylation in dividing cells. In fact, several lines of evidence support this possibility. Neuronal deletion of both Dnmt1 and Dnmt3a (but not Dnmt3a alone) impairs synaptic plasticity and produces cognitive deficits in mice (Feng et al., 2010). Some have theorized that the combined actions of Dnmt3a and Dnmt1 synergistically produce complete *de novo* methylation on double-stranded DNA. This is likely, because Dnmt3a preferentially adds methylation to only one of the cytosine residues of the double-stranded DNA, which leaves a hemi-methylated DNA that is then rapidly methylated by Dnmt1 (Jeltsch and Jurkowska, 2014). Indeed, this is supported by a study demonstrating five-fold higher methylation activity levels on PCR fragments with Dnmt1 and Dnmt3a present, than with

either enzyme alone (Fatemi et al., 2002, Kim et al., 2002). In addition, *in vivo* evidence shows that double-knockout Dnmt3/Dnmt3b embryos still have some *de novo* methylation activity (Okano et al., 1999).

1.4 DNA Methylation and Obesity.

Aberrant DNA methylation and obesity have been repeatedly and reciprocally associated through both rodent and human studies. Converging evidence implicates aberrant neuronal methylation as a contributing factor in obesity development. For example, increasing methylation of the proopiomelanocortin (POMC; a key hypothalamic peptide in energy regulation) gene via deletion of methyl-CpG-binding protein 2 (MeCP2) in POMC neurons causes obesity (Wang et al., 2014). Hypothalamic deficiency of Dnmt3a causes metabolic dysfunction, hyperphagia, decreased energy expenditure and obesity (Kohno et al., 2014). Conversely, obesity and excess energy intake have been implicated as contributing factors in aberrant neuronal methylation. Overfeeding or a high fat diet (HFD) can cause hypermethylation of the POMC gene (Plagemann et al., 2009, Marco et al., 2013). An HFD increases histone deacetylases, which are associated with increased DNA methylation, in the hypothalamus (Funato et al., 2011) and reduces Dnmt3a expression in another region of the hypothalamus (Kohno et al., 2014). In corroboration of the POMC methylation studies in rodents, correlative evidence in humans (from blood cell analysis) shows an association between POMC hypermethylation and childhood obesity (Kuehnen et al., 2012). Finally, in obese males who have lost weight, POMC methylation is inversely correlated with the successful maintenance of this weight loss (Crujeiras et al., 2013).

Disconcertingly, the nutritional state of a pregnant female can lead to “metabolic programming” of the offspring. HFD-fed dams give birth to offspring that, even though they are

fed a normal chow diet, are overweight and have increased POMC methylation and reduced POMC expression (Marco et al., 2014). In addition, these offspring are more vulnerable to the deleterious effects of an HFD than offspring from normal chow-fed dams. The Dutch Hunger Winter was a famine that affected millions of people in 1944 (Tobi et al., 2014). It was later found that women who were pregnant during the famine (specifically during the first two trimesters) gave birth to infants that ironically had higher birth weights, despite the mothers' lack of food. In adulthood, these individuals had higher BMI, altered glucose response, and higher low density lipoprotein (LDL) and total cholesterols. Genome-wide methylation analysis conducted on blood from these people (compared with same-sex siblings not exposed prenatally to famine) pinpointed several differentially-methylated regions (on genes or gene promoters) involved in prenatal growth and fatty acid oxidation. Thus, one could hypothesize that the experience of famine, in utero, led to *de novo* methylation of genes that subsequently altered energy regulation.

The burgeoning field of epigenetics has revealed new insight regarding mechanisms through which diseases develop. Increasing evidence suggests that altered DNA methylation can promote weight storage, as well as predispose future generations to dysfunctional energy homeostasis. Obesity prevalence is rapidly increasing across the globe, and it is becoming clear that this disease is not easily reversed with diet and exercise regimens for many individuals who bear the burden of excess weight. The goal of this research is to understand how a major DNA methyltransferase, Dnmt1, in neurons regulates energy homeostasis and obesity development. Thus, we developed the following Specific Aims.

1.5 Specific Aims of this Dissertation

The overarching hypothesis for this proposal is that neuronal Dnmt1 regulates energy homeostasis and contributes to the development of obesity. Using a mouse model of neuronal

Dnmt1 deficiency, we studied in depth the metabolic phenotype of the neuronal Dnmt1 knockout mouse (ND1KO) under normal- and excess-energy conditions, as well as in an increased energy demand condition (cold exposure). We also studied the hypothalamic gene expression profile of the ND1KO mice. This dissertation is organized into two major Specific Aims that focus on answering the following questions:

SA1: How does neuronal Dnmt1 deficiency alter normal energy homeostasis and obesity development? We first predicted that neuronal Dnmt1 deficiency alters metabolism and energy regulation in normal chow-fed mice. We fed male and female ND1KO and fl/fl control mice a normal chow diet and measured body weight, body composition, glucose and insulin tolerance, fat pad weights and fat gene expression profile, as well as expression of hypothalamic peptides and hormones. For our second experiment, we predicted that neuronal Dnmt1 deficiency attenuates obesity and metabolic dysfunction produced by a high fat diet (HFD). We fed male and female ND1KO and fl/fl control mice an HFD and measured body weight, body composition, glucose and insulin tolerance, food intake and energy expenditure, fat pad weights and fat gene expression profile. For our third experiment, we predicted that neuronal Dnmt1 deficiency enhances brown fat thermogenic function during a state of increased energy demand, prolonged cold exposure. We subjected male ND1KO and fl/fl control mice to a cold environment (5°C) for 7 days and then measured body weight change, fat pad weights and fat gene expression profile.

SA2: How does neuronal Dnmt1 deficiency alter the central pathways that regulate energy homeostasis? The experiments in SA1 tested the role of neuronal Dnmt1 in energy regulation and obesity. The hypothalamus controls energy expenditure, food intake and glucose homeostasis in response to cues directly sensed through peripheral nerves and circulating

hormones and metabolites. The neuronal phenotype of the ND1KO mouse is unknown, given that it is a novel mouse model. Therefore, we characterized the hypothalamic phenotype of the ND1KO model. We first predicted that the ND1KO mouse displays alterations in the expression of peptide and hormone genes that have key roles in energy regulation. We quantified hypothalamic gene expression of the chow- and HFD-fed male ND1KO and fl/fl control mice from SA1. Estrogen receptor- α (ER α) in the central nervous system is well-known to play a role in body weight in both male and female rodents, specifically through actions in the ventromedial hypothalamus (VMH) (Xu et al., 2011, Sano et al., 2013). Thus, we secondarily predicted that ND1KO mice have upregulated ER α expression in the VMH. We quantified ER α gene and protein expression in the hypothalamus of chow- and HFD-fed ND1KO and fl/fl control mice. Finally, neuronal ER α expression is negatively correlated with methylation of its gene promoter (Westberry et al., 2010). Therefore, we predicted that ER α upregulation in the hypothalamus is associated with reduced ER α gene promoter methylation in ND1KO mice. We quantified ESR1 promoter methylation in the VMH/DMH of chow- and HFD-fed ND1KO and fl/fl control mice.

2 SPECIFIC AIM 1

2.1 Background

2.1.1 *The Adipose Organ*

Adipocytes function, in part, to store excess energy as lipids (triglycerides). When more energy is ingested than is currently required, it is stored in white adipocyte cells (called “white” adipocytes due to less mitochondria and vascularization compared with brown adipocytes) that contain a large unilocular lipid droplet (Cinti, 1999). White adipocytes are innervated by the sympathetic nervous system, as well as by sensory nerves, allowing communication to take place between the brain and the peripheral fat stores in both directions (Bartness and Song, 2007,

Bartness et al., 2010). In addition to storing energy, adipocytes have important endocrine functions, as well, such as adiponectin and leptin secretion (Adamczak and Wiecek, 2013).

In an energy deficient state, lipolysis is triggered through sympathetic neurons that activate adipocyte β -adrenergic receptors through the release of norepinephrine (NE) (White and Engel, 1958). NE-binding of β -adrenergic receptors triggers an intracellular signaling cascade ultimately leading to increased protein kinase A (PKA) activity, which phosphorylates the enzyme hormone-sensitive lipase (HSL) (Holm, 2003). Phosphorylated HSL has been relied upon as an indicator of stimulated lipolysis because HSL-deficient adipocytes show drastically reduced lipolysis in response to catecholamine stimulation *in vitro* (Wang et al., 2001). Phosphorylated HSL catalyzes the second step of adipocyte triglyceride breakdown, while two other lipases, adipose triglyceride lipase and monoacylglycerol lipase, are needed for complete triglyceride hydrolysis (Fredrikson et al., 1986, Duncan et al., 2007, Fruhbeck et al., 2014). Refeeding causes a reduction in lipolysis primarily through insulin signaling, which leads to the release of PKA activation (Duncan et al., 2007). In obese humans and genetically-obese rodents, basal lipolysis is increased while NE-stimulated lipolysis is decreased and HSL expression is reduced (Reynisdottir et al., 1995, Large et al., 1999). It is important to note that adipocytes are not simply storage vehicles for lipid droplets, but are constantly hydrolyzing and re-esterifying triglycerides at any given moment (hence, basal lipolysis). Thus, the adipose organ is more dynamic than one would assume.

In an energy-excess state, adipocytes first expand as more lipids enter into the cell, causing cellular hypertrophy. If excessive energy intake continues, the adipose tissue can undergo hyperplasia with the proliferation of more fat cells that differentiate from adipocyte precursor cells, to store these lipids (Bjorntorp et al., 1971). Eventually, with the expansion of individual cells as well as the tissue as a whole, the adipose organ becomes dysfunctional and inflamed (Vazquez-

Vela et al., 2008, Wensveen et al., 2015). Adipose tissue macrophages switch from an anti-inflammatory to a pro-inflammatory phenotype and increase the production of inflammatory molecules (e.g., tumor necrosis factor- α , interleukin-6, monocyte chemoattractant protein 1) (Lumeng et al., 2007). Adipose tissue capillary density is reduced in obese people (Pasarica et al., 2009), suggesting reduced oxygen supply to the adipocytes. Fibrosis can even develop in the dysfunctional adipose tissue (Khan et al., 2009). Thus, in an obese state, fat cells cannot function properly and excess fat storage becomes a disease.

2.1.2 Brown Adipose Tissue and Thermogenesis.

Brown adipose tissue functions to produce heat; thus, it expends rather than stores energy. Brown fat is called such due to the presence of numerous mitochondria and vasculature that lend it a darker appearance than white fat, and it is characterized by multilocular lipid droplets (Cinti, 1999, Cannon and Nedergaard, 2004). In rodents, the major brown fat depot is found in the intrascapular region, while other smaller depots are around some visceral organs. In humans, brown fat can be found in paracervical, paraspinal, supraclavicular, mediastinal and perirenal regions (Cypess et al., 2009). In very early development (infants), humans have more brown fat, but as adults they display reduced and less active BAT, especially when obese (Oberkofler et al., 1997, Vijgen et al., 2011). Brown fat expends energy through a process called non-shivering thermogenesis (Cannon and Nedergaard, 2004). Fatty acids that are oxidized through the mitochondria typically result in the production of ATP (cellular energy). In brown fat, however, uncoupling protein-1 (UCP1) creates a leak in the mitochondrial membrane, and this energy is released as heat instead of ATP. BAT is highly vascularized in order for the heat to be circulated throughout the body. Non-shivering thermogenesis is an important process to keeping the body warm during cold, and for increasing core temperature during a fever.

Brown fat is heavily innervated by the sympathetic nervous system (SNS). These neuronal projections release norepinephrine onto the brown fat, which stimulates β -3 adrenergic receptors. The resulting intracellular signaling cascade leads to fatty acid release through lipolysis, and to increased thermogenic capacity (e.g., mitochondriogenesis, UCP1 upregulation, increased BAT cells, upregulation of genes involved in BAT differentiation) (Cannon and Nedergaard, 2004). In addition to being the thermogenic substrate, fatty acids directly activate UCP1 (Nicholls and Locke, 1984). Acute SNS activation increases thermogenesis, while chronic SNS activation can lead to proliferation of preadipocytes and differentiation of BAT cells (Nechad et al., 1987, Bronnikov et al., 1992).

Cold exposure robustly stimulates thermogenesis. Cold is sensed through thermo-receptors on the skin, and these neuronal signals are transmitted to the preoptic area, which releases inhibition of the dorsomedial hypothalamus (DMH) (Morrison et al., 2012). DMH neurons send efferent projections to the rostral ventrolateral medulla (RVM), which activate sympathetic premotor neurons leading to BAT (Morrison, 1999). In addition, the raphe pallidus activates sympathetic preganglion neurons in the spinal column, which stimulate thermogenesis in brown fat. A number of elegant trans-neuronal tract tracing experiments have shown the neuronal pathways leading to BAT innervation (Bamshad et al., 1999, Bartness et al., 2005), and these pathways will be discussed in Specific Aim 2 Background section. In addition to cold, thermogenesis is increased acutely following a meal, as well as in diet-induced obese rodents (Cannon and Nedergaard, 2004).

Increased thermogenesis can be directly quantified by BAT temperature. However, changes in the BAT gene profile can indicate the possibility of increased thermogenic or mitochondrial capacity. Sympathetic nervous stimulation of BAT upregulates UCP1, but also

upregulates cytochrome c oxidase (COX1), acyl-coA-oxidase (ACOX), carnitine palmitoyltransferase 1B (CPT1B) (mitochondrial function genes), deiodinase iodothyronine Type II (DIO2), otopetrin 1 (OTOP1), and cell death-inducing DFFA-like effector A (CIDEA) (Nam and Cooper, 2015). Increased thermogenic activity also is accompanied by elevated lipolysis and lipogenesis (Yu et al., 2002, Mottillo et al., 2014), which is often reflected through the gene profile. Protein expression, however, is a more meaningful indicator of thermogenic capacity. The ratio of phosphorylated hormone-sensitive lipase (HSL) to total HSL protein is indicative of increased lipolysis in BAT, which can occur through elevated SNS drive to BAT (Laury et al., 1987).

Interestingly, in certain circumstances (e.g., prolonged cold exposure, pharmacological stimulation) some white adipocytes can even demonstrate function and appearance similar to brown fat, termed “browning” (Harms and Seale, 2013). These cells are called beige or brite (“brown-like-in-white”) cells because they are darker in appearance than regular white adipocytes, and they have thermogenic capabilities like brown adipocytes (Okamatsu-Ogura et al., 2013). The genetic program can indicate browning, through upregulation of genes such as peroxisome proliferator-activated receptor alpha (PPAR α), PPAR γ C1 α (PGC1 α), PPAR γ C1 β (PGC1 β), PR domain-containing 16 (PRDM16) and UCP1. Whether beige cells arise from a switch in the genetic program of white adipocytes, or whether they exist from the beginning as cells with a different functional potential is unclear (Sanchez-Gurmaches et al., 2016). The phenomenon of browning is a major target for obesity research because human BAT displays a high level of beige-specific molecular markers (Sharp et al., 2012). Drugs or therapies that may cause browning within white fat, or increase the thermogenic potential in BAT, would be an incredibly efficient way to reverse obesity.

The nervous system is vastly connected to the peripheral organs and controls many aspects of metabolism via innervation of WAT, BAT, as well as non-adipose organs. The following experiments tested mice that were neuronally-deficient for Dnmt1 in conditions of normal energy, excess energy and increased energy demand to determine how neuronal Dnmt1 deficiency can affect energy intake, storage, and expenditure.

2.2 Methods

2.2.1 *Knockout Mice.*

Neuronal Dnmt1-deficient mice were generated by mating Dnmt1^{fl/fl} mice (Jackson-Grusby 2001) with Synapsin1Cre^{+/-} mice (Hoesche C 1993). SynCre^{+/-} mice express Cre recombinase enzyme in the brain, spinal cord and dorsal root ganglion neurons beginning at E12.5 (Zhu 2001). Male and female pups were either Dnmt1^{fl/fl}SynCre^{+/-} (neuronal knockout [ND1KO]) or Dnmt1^{fl/fl}SynCre^{-/-} (no knockout, control [fl/fl]). We also studied a Dnmt^{+/+}SynCre^{+/-} (normal Dnmt1 expression) to confirm that the expression of Synapsin1Cre recombinase alone does not produce any phenotype. At 6 weeks of age (adulthood), male and female ND1KO and fl/fl littermates were fed *ad libitum* either a normal chow diet (Purina #5001; LabDiet; St. Louis, MO) or a high fat diet (HFD, 60% fat #12492. Research Diets; New Brunswick, NJ).

2.2.2 *Physiological Measurements.*

Body weight was measured weekly throughout the duration of the experiment. Glucose and insulin tolerance testing was performed after 16-18 weeks on the diets. For the glucose tolerance test (GTT), the mice were fasted for 16 hours overnight. Blood was obtained from tail nick to measure the glucose concentration using a OneTouch Ultra Blood Glucose Meter and test strips (LifeScan, Inc.; Milpitas, CA). After a baseline blood glucose measurement, mice were

intraperitoneally injected with a 20% dextrose solution (1g/kg) and subsequent measurements were taken at 15, 30, 60, 90 and 120 minutes following the glucose injection. For the insulin tolerance test (ITT), the mice were fasted for 4 hours then a baseline blood glucose measurement was taken. Mice were intraperitoneally injected with either 1.0U/kg (males) or 0.8U/kg (females) dose of 0.25U/mL humulin (Eli Lilly; Indianapolis, IN). Energy expenditure, oxygen consumption, CO₂ production, respiratory exchange ratio, and physical activity level data were collected using a TSE PhenoMaster metabolic chamber system (TSE Systems; Chesterfield, MO) after 22-24 weeks of HFD feeding (males) or 26-28 weeks of HFD-feeding (females). Body composition of relative lean and fat mass was obtained *in vivo* using time-domain nuclear magnetic resonance technology (TD-NMR) by a MiniSpec machine (Bruker; Spring, TX) after 24.5 weeks on the normal diet and 22 weeks on the HFD. Mice were euthanized using CO₂ inhalation and were decapitated. Blood was collected for serum and stored at -80⁰ C. Fat pads (brown, epididymal, subcutaneous) and liver were dissected and weighed, then were frozen in liquid nitrogen. A sample of each fat pad and of the liver was placed in formalin for later histological analysis.

2.2.3 Cold Exposure.

For the cold exposure experiment, we bred two separate cohorts of mice in order to have enough statistical power; male ND1KO and fl/fl control littermates ~8-9 weeks of age were used. Fifteen ND1KO and 8 fl/fl control mice were single-housed with nothing in the cage except corn cob bedding, and were subjected to a 5°C environment for 7 days. After 7 days, we removed the mice from the cold room individually and immediately sacrificed them. We harvested and weighed the brown, epididymal and subcutaneous fat tissues, and formalin-fixed a sample of each fat pad, while the remaining fat was frozen in liquid nitrogen.

2.2.4 Gene Expression.

Fat tissues were removed from -80°C storage and placed in liquid nitrogen. Each fat sample was removed from the liquid nitrogen and immediately homogenized in TRI Reagent (Molecular Research Center; Cincinnati, OH) using a handheld homogenizer. RNA was isolated using isopropanol precipitation, purified, and the concentration was measured using a Nanodrop. Gene expression was quantified by real-time qPCR using a Stratagene Mx3005P qPCR System (Agilent Technologies; Santa Clara, CA), ABI Universal PCR Master Mix (Applied Biosystems; Foster City, CA) and gene expression probes purchased from Applied BioSystems. Each assay included the reference gene cyclophilin as an internal control. All data was analyzed using the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001).

2.2.5 Protein Quantification.

Fat tissues were removed individually from liquid nitrogen and were immediately placed into RIPA buffer with protease inhibitor cocktails and homogenized using a handheld homogenizer. Protein concentration was quantified using a DC Protein Assay (BioRad; Hercules, CA) and protein samples were denatured at 90-100° for 10 minutes. Western blots were performed by loading 20-30µg of protein onto a 4-15% gradient polyacrylamide gel (Criterion TGX; Bio-Rad; Hercules, CA), which was then transferred to a PVDF membrane. We immunoblotted the membranes first by blocking with 5% nonfat milk, followed by antibodies against UCP1 (#23841; Abcam; Cambridge, MA), HSL (#4107S; Cell Signaling; Danvers, MA) or pHSL (serine 660) (#4126S; Cell Signaling) overnight at 4°C, followed by AlexaFluor goat anti-rabbit 680 secondary antibody (Thermofisher Scientific A21109) at 1:5,000 concentration for 3 hours. Bands were visualized and quantified using an Odyssey Fc Imaging System (Li-Cor;

Lincoln, NE) with background subtracted. All membranes also were immunoblotted with α -tubulin antibody at 1:500 (#2144S; Cell Signaling) as a normalization control.

2.2.6 Serum Assays.

Serum free fatty acids were quantified using an HR Series NEFA kit (Wako Diagnostics; Richmond, VA), and triglycerides were quantified using an L-Type Triglycerides M kit (Wako Diagnostics; Richmond, VA) kit. Serum leptin and insulin were quantified using ELISA kits (Cat. # 90030 and 90080, CrystalChem; Downers Grove, IL).

2.2.7 Histology.

After formalin fixation, fat tissues were dehydrated through a series of increasing isopropanols, and were embedded in paraffin blocks. BAT, SQ and EPI tissues were cut manually on a rotating microtome at 6 μ m width and mounted to SuperPlus slides. Tissue morphology was visualized by hematoxylin and eosin staining (Sigma-Aldrich; St. Louis, MO). UCP1 immunohistochemistry was performed using UCP1 antibody at 1:150 concentration (Abcam ab10983), and VectaStain and DAB Substrate kits (Vector Laboratories; Burlingame, CA). Fat histology images were captured using an Olympus DP73 photomicroscope and CellSens software (Olympus). Representative images were enhanced using ImageJ Software (NIH; Bethesda, MD). All images within an experiment were collected and processed using the same parameters.

2.2.8 Statistics.

Data were analyzed using Excel 2013 and were presented as means \pm the standard error of the mean. Statistical significance between the genotypes was tested using Student's *t*-tests or One-Way ANOVAs with IBM SPSS Statistics 20. Repeated-measures ANOVA was performed on data from metabolic chamber experiments, glucose/insulin tolerance, and weekly body weight. Follow-

up specific tests were performed on glucose/insulin tolerance test data using a Bonferroni correction if the main effect was significant.

2.3 Results.

2.3.1 Neuronal Dnmt1 deficiency reduces adiposity.

We generated neuronal-specific Dnmt1 knockout mice by breeding Dnmt1^{fl/fl} mice with Synapsin-Cre^{+/-} mice. We quantified Dnmt1 gene expression in several different hypothalamic nuclei of HFD-fed male ND1KO mice (V/DMH and PVH shown) to test the knockdown efficiency of our mouse model. Dnmt1 gene expression was significantly reduced in these regions (Fig. 1). To confirm that there were no compensatory increases in the other Dnmts as a result of Dnmt1 knockdown, we also quantified Dnmt3a and Dnmt3b gene expression. We found no significant changes in Dnmt3a or Dnmt3b expression (Fig. 1). There remained some Dnmt1 expression in these regions in the ND1KO mice presumably due to the presence of Dnmt1 expression in

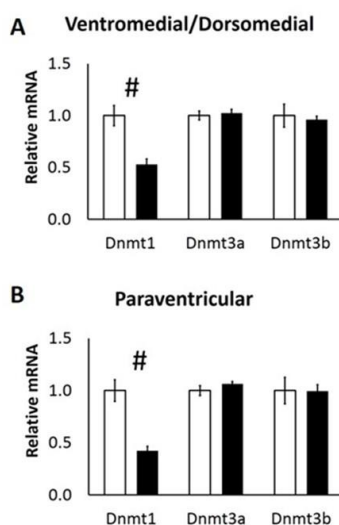


Figure 1. Dnmt mRNA expression in the hypothalamus.

Dnmt1, Dnmt3a and Dnmt3b gene expression in the ventromedial/dorsomedial (A) and paraventricular (B) hypothalamic regions of male ND1KO and fl/fl mice fed an HFD.

non-neuronal cells, such as glia. We first tested the role of neuronal Dnmt1 in energy regulation under normal metabolic demands.

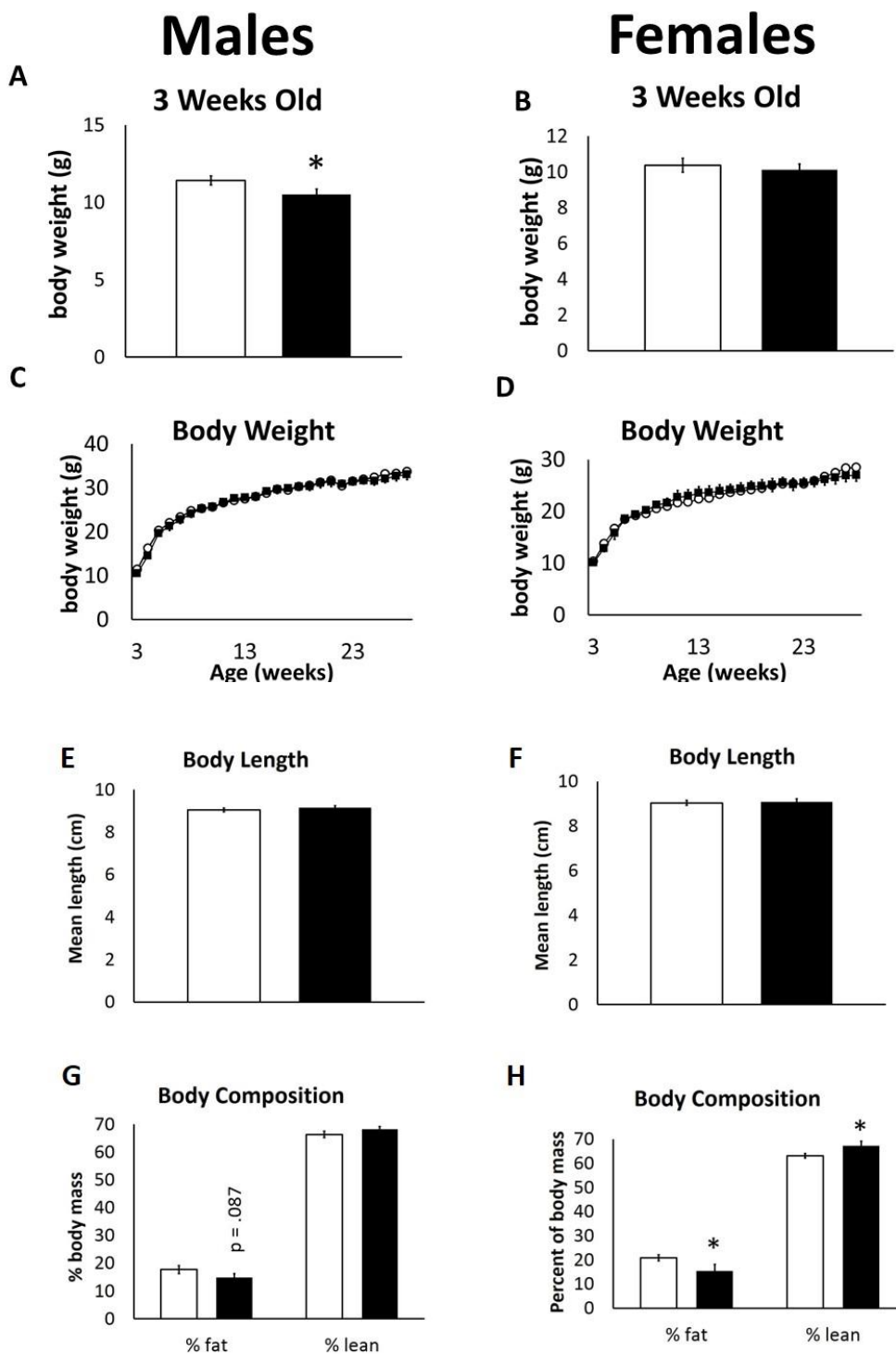


Figure 2. Body weight, length and composition of chow-fed mice.

Body weight at 3 weeks of age (A, B), weekly body weight (C, D), body length (E, F) and body composition (G, H) of male and female fl/fl and NDIKO mice fed a chow diet.

$Dnmt1^{fl/fl}SynCre$ male (ND1KO= 8, fl/fl= 8) and female (fl/fl= 10, ND1KO= 7) mice and fl/fl littermates were fed a standard chow diet (LabDiet 5001; St Louis, MO) from weaning onward. ND1KO male mice weighed less at weaning (3 weeks old; Fig. 2A), but this difference disappeared by the time they were 5 weeks old (Fig. 2C). Female ND1KO mice showed no difference in body weight (Fig. 2B-D). Neuronal $Dnmt1$ deficiency did not affect body length in either male or female mice (Fig. 2E-F). We confirmed that the body weight difference in male ND1KO mice was due to the lack of $Dnmt1$ specifically, in that the presence of Synapsin-Cre recombinase by itself did not affect body weight in male or female mice (Suppl. Fig. 1).

We measured *in vivo* body composition using time-domain nuclear magnetic resonance technology (TD-NMR). Male ND1KO mice had a tendency for reduced fat percentage (Fig 2G), whereas female ND1KO mice had significantly less fat percentage and a higher percentage of lean mass (Fig. 2H). The distribution and variability of body weight of males and females at 28 weeks of age is illustrated in Fig. 3A-B. In male ND1KO mice, retroperitoneal and brown adipose tissue pads weighed significantly less (Fig. 4A), and epididymal and subcutaneous

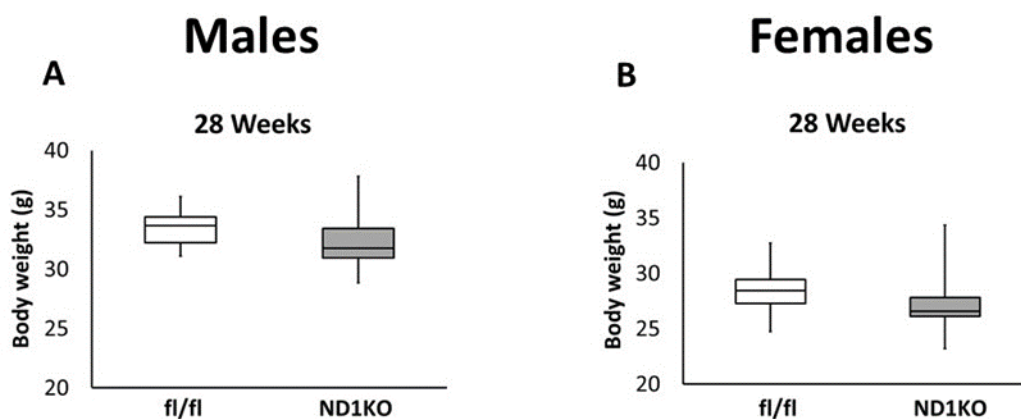


Figure 3. Body weight distribution of chow-fed fl/fl and ND1KO mice.
Body weight distribution at 28 weeks of age of male (A) and female (B) chow-fed fl/fl and ND1KO mice.

(inguinal) adipose tissue tended to weigh less. When normalized to body weight, all fat pads weighed less in male ND1KO mice (Fig. 4C). In female ND1KO mice, perigonadal, subcutaneous, and brown adipose tissue pads weighed significantly less (Fig. 4B). When normalized to body weight, all fat pads weighed less in female ND1KO mice (Fig. 4D) Neuronal Dnmt1 deletion did not affect liver mass in male or female mice (Fig 4C, D).

Next, we tested whether ND1KO mice had altered glucose or insulin dynamics. Glucose and insulin tolerance did not differ between the male genotypes (Fig. 5A, C, E, G), although female D1KO mice had slightly higher glucose blood concentration during the glucose tolerance test (Fig. 5B, 5D) but had no difference in insulin tolerance (Fig. 5F, 5H).

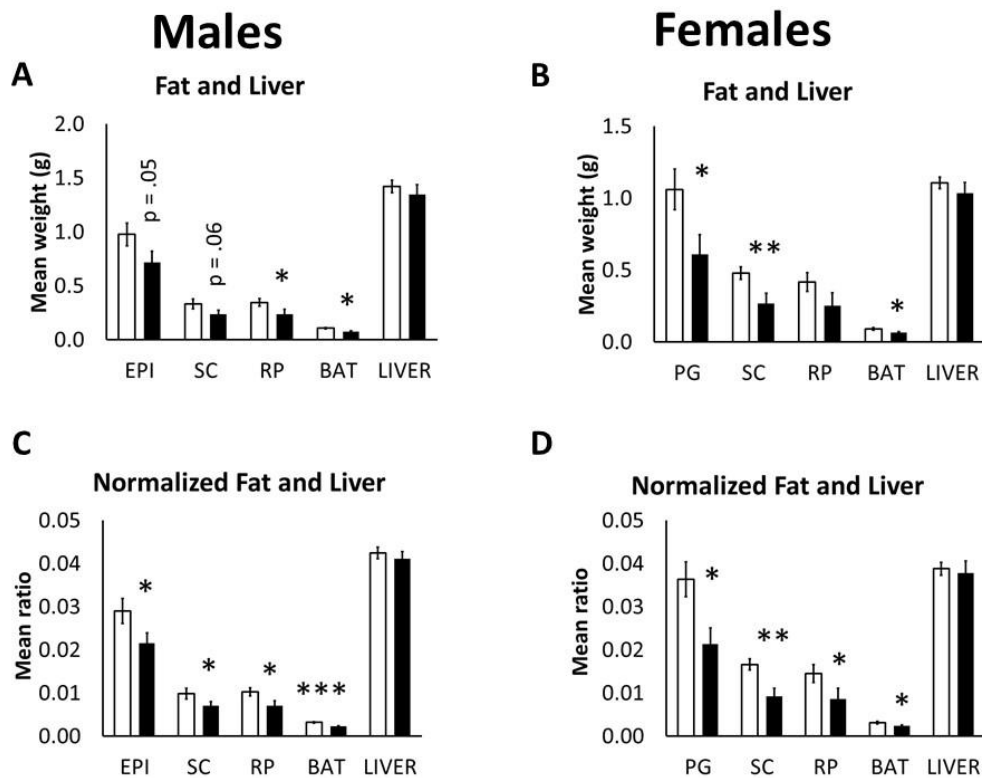


Figure 4. Fat pad and liver weights of chow-fed fl/fl and ND1KO mice. Epididymal (or perigonadal), subcutaneous, retroperitoneal and brown adipose tissue weights in male (A) and female (B) ND1KO and fl/fl control mice. Fat tissues weights normalized to body weight (C, D). * $p < .05$, ** $p < .01$, *** $p < .001$.

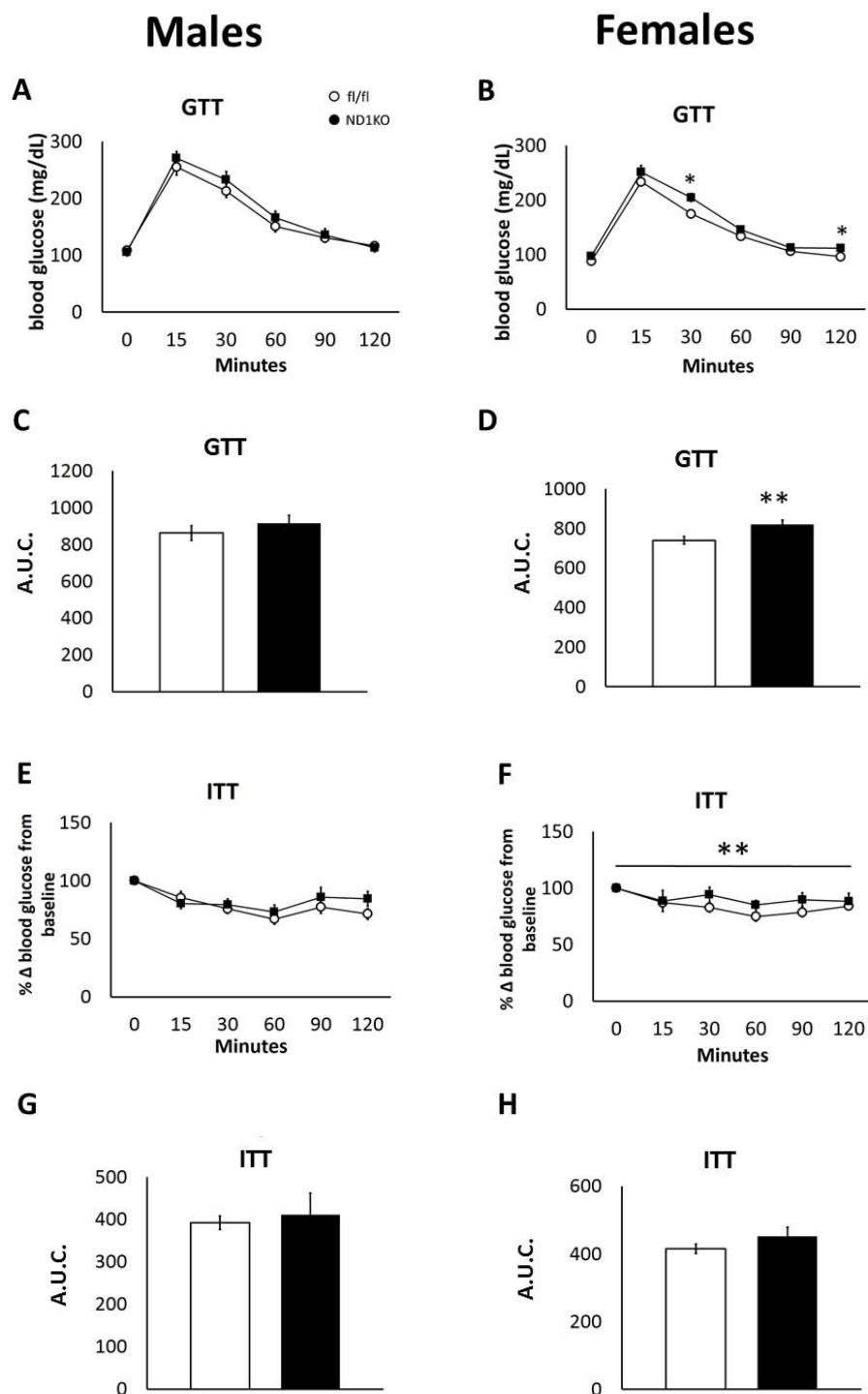


Figure 5. Glucose and insulin tolerance tests (GTT/ITT) of chow-fed fl/fl and ND1KO mice.

Blood glucose concentrations over two hours following an i.p. glucose infusion, and mean area under the curve of blood glucose concentrations during the test, in male (A, C) and female (B, D) chow-fed fl/fl and ND1KO mice. Blood glucose concentrations over two hours following an i.p. insulin infusion, and mean area under the curve of the blood glucose concentrations during the test, in male (E, G) and female (F, H) chow-fed fl/fl and ND1KO mice. * $p < .05$, ** $p < .01$.

2.3.2 Neuronal *Dnmt1* deficiency attenuates HFD-induced obesity.

To test the role of neuronal *Dnmt1* in obesity development, we fed male and female ND1KO and fl/fl mice a 60% high fat diet (HFD; Research Diets D12492; New Brunswick, NJ) starting at 6 weeks of age. ND1KO male mice weighed significantly less after two weeks on the HFD (Fig. 6A), and continued to weigh less throughout the duration of the experiment (the maximal weight difference occurred at 11 weeks on the HFD).

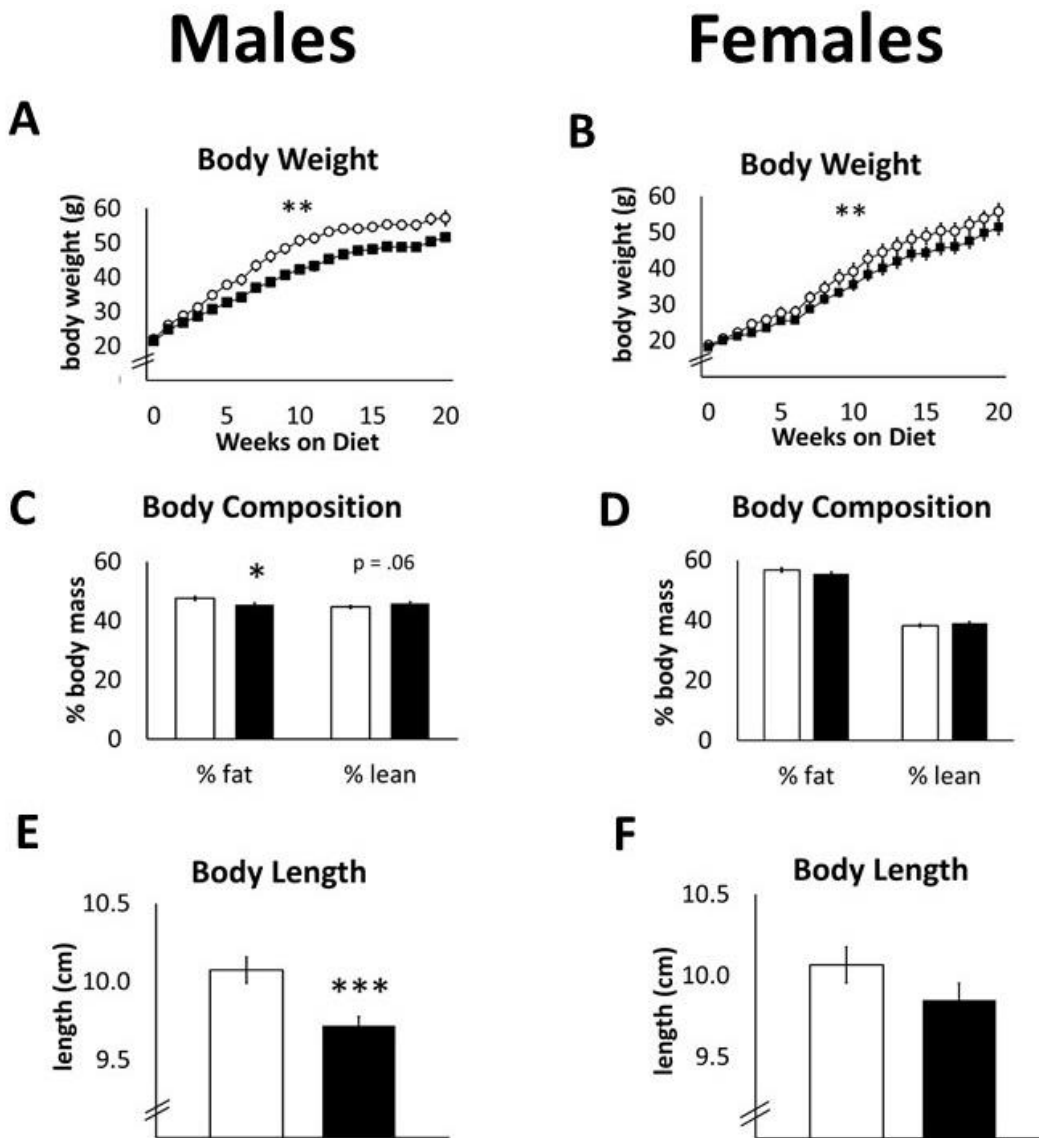


Figure 6. Body weight, length and composition of HFD-fed fl/fl and ND1KO mice.

Weekly body weight (A, B), body composition (C, D) and body length (E, F) of male and female HFD-fed fl/fl and ND1KO mice. * $p < .05$, ** $p < .01$, *** $p < .001$.

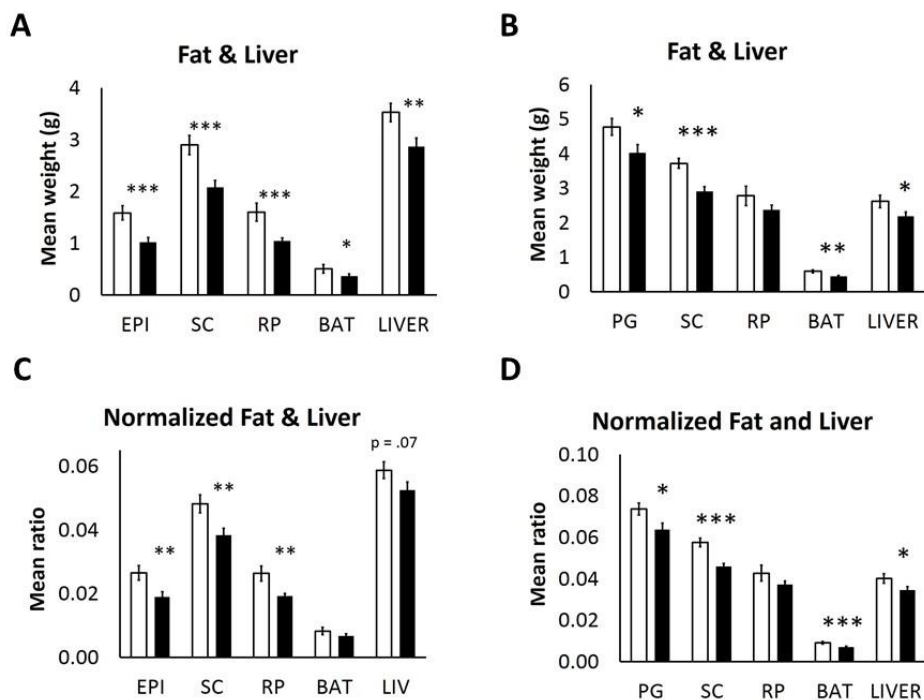


Figure 7. Fat pad and liver weights of HFD-fed fl/fl and ND1KO mice.

Epididymal/perigonadal, subcutaneous, retroperitoneal white adipose tissues, brown adipose tissue and liver weights (G, H); and fat and liver weights normalized to body weight of male and female HFD-fed fl/fl and ND1KO mice. * $p < .05$, ** $p < .01$, *** $p < .001$.

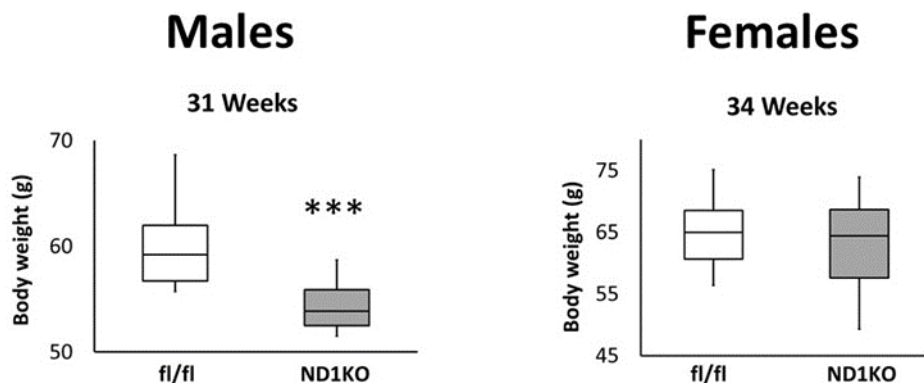


Figure 8. Body weight distribution of HFD-fed fl/fl and ND1KO mice.

Body weight distribution at 31-34 weeks of age of male and female HFD-fed fl/fl and ND1KO mice. *** $p < .001$.

Unlike the chow-fed ND1KO mice, HFD-fed ND1KO mice had reduced body length (Fig. 6E).

Male mice also had significantly reduced percentage of body fat, with a tendency for an increase

in lean mass (Fig. 6C). HFD-fed female ND1KO mice had significant differences in body weight

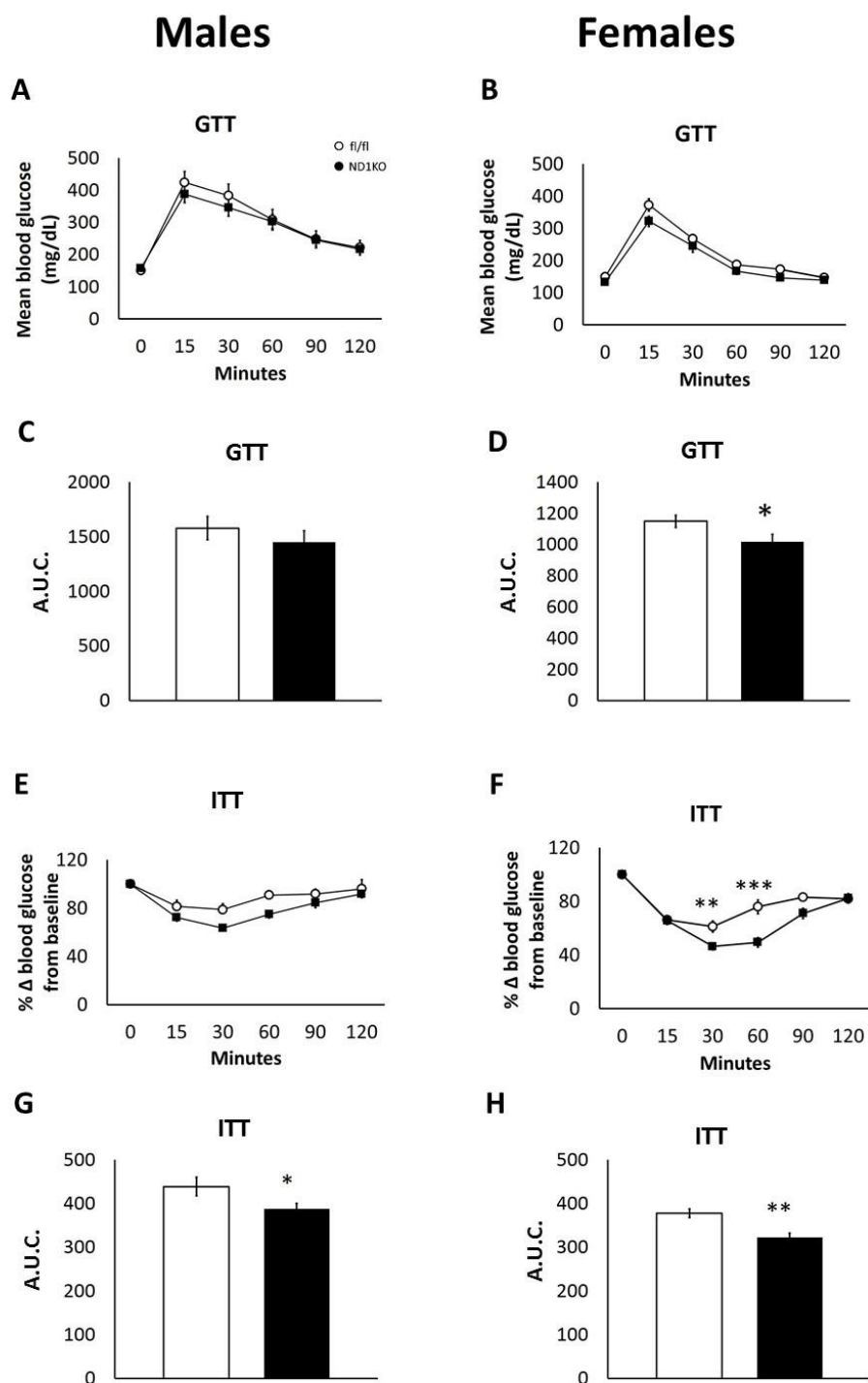


Figure 9. Glucose and insulin tolerance tests (GTT/ITT) for HFD-fed fl/fl and ND1KO mice.

Blood glucose concentrations over two hours following an i.p. glucose infusion, and mean area under the curve blood glucose concentrations during the test, in male (A, C) and female (B, D) HFD-fed fl/fl and ND1KO mice. Blood glucose concentrations over two hours following an i.p. insulin infusion, and mean area under the curve of the blood glucose concentrations during the test, in male (E, G) and female (F, H) HFD-fed fl/fl and ND1KO mice. * $p < .05$, ** $p < .01$, *** $p < .001$.

throughout the duration of the HFD as indicated by Repeated-Measures ANOVA (Fig. 6B);

however, final body weight at the end of the study did not significantly differ between the

genotypes. Female ND1KO mice showed no significant differences in body composition or body length compared to fl/fl female mice (Figs. 6D, F).

HFD-fed ND1KO male mice had significantly smaller epididymal, subcutaneous and retroperitoneal fat pads, even when normalized to body weight (Fig. 7A, C). Brown adipose tissue was significantly smaller, but not when normalized to body weight. Female ND1KO mice had significantly smaller perigonadal, subcutaneous and brown adipose tissue fat pads and these differences remained significant even after body weight normalization (Fig. 7B, D). Both male and female ND1KO mice had smaller liver weights (Fig. 7A-B), although after adjusting for body weight, male ND1KO mice only had a tendency for reduced liver weight (Fig. 7A). The distribution and variability of body weight of HFD-fed males and females at 31-34 weeks of age is illustrated in Fig. 8A-B.

We tested whether neuronal Dnmt1 deletion altered glucose and insulin dynamics in the HFD-fed mice, since diet-induced obesity causes glucose and insulin intolerance. In the HFD-fed male mice, there was no change in the ability to clear a glucose infusion from the blood between the two genotypes (Fig. 9A, C). HFD-fed female ND1KO mice showed a very mild improvement in overall glucose tolerance as seen in the area under the curve (Fig. 9B, D). In response to an insulin injection, however, male ND1KO mice had a lower area-under-the-curve (AUC) of blood glucose (Fig. 9E, G), and female ND1KO mice had a similar effect (Fig. 9F, H). Female ND1KO mice, however, showed a significant main effect of genotype throughout the duration of the insulin tolerance test, and follow-up tests at various time points revealed significant reductions in blood glucose at the 30- and 60-minute time points (Fig. 9F).

The following results pertain only to male ND1KO and fl/fl mice, since the largest phenotype was observed in only male ND1KO mice fed an HFD. We quantified serum

Table 1. Serum hormone and lipid measurements.

Group	Insulin ng/mL	Leptin ng/mL	Triglycerides mg/dL	NEFA mmol/L
Chow				
fl/fl	1.86 ± .4	9.69 ± 1.3	123.9 ± 10.4	0.65 ± .06
ND1KO	1.22 ± .2	7.42 ± 1.6	109.4 ± 14.4	0.58 ± .08
HFD				
fl/fl	24.6 ± 7.9	71.1 ± 4.4	96.8 ± 16.0	0.91 ± .07
ND1KO	31 ± 5.4	57.6 ± 2.4 **	112.3 ± 7.7	0.86 ± .04

Serum insulin, leptin, triglycerides and non-esterified fatty acids in fed-state of chow- and HFD-fed male fl/fl and ND1KO mice. ** p< .01.

concentrations of insulin, leptin, non-esterified (free) fatty acids, and triglycerides of the fl/fl and ND1KO male mice (Table 1). Neuronal Dnmt1 deficiency did not alter serum leptin or insulin concentrations in chow-fed mice, although there was a trend for ND1KO mice to have lower concentrations of both hormones. HFD-fed ND1KO mice had significantly lower serum leptin concentrations compared to HFD-fed fl/fl controls, as might be expected from reduced adiposity. Leptin is secreted in direct proportion to the amount of fat stores in the body (Jequier, 2002). Serum insulin was no different in HFD-fed ND1KO or fl/fl control mice, however. Neuronal Dnmt1 deficiency did not affect serum triglycerides or non-esterified fatty acids in either chow-fed or HFD-fed mice.

Consistent with their reduced obesity, HFD-fed ND1KO male mice consumed less food over a 7-day period (Fig. 10A). We placed the male mice in metabolic cages and found that ND1KO mice had higher energy expenditure during both light and dark periods than the fl/fl mice (Fig. 10B). In addition, ND1KO mice had higher physical activity level (Fig. 10C), and a decreased respiratory exchange ratio (RER) (Fig. 10D) during one light-dark cycle of the 7-day

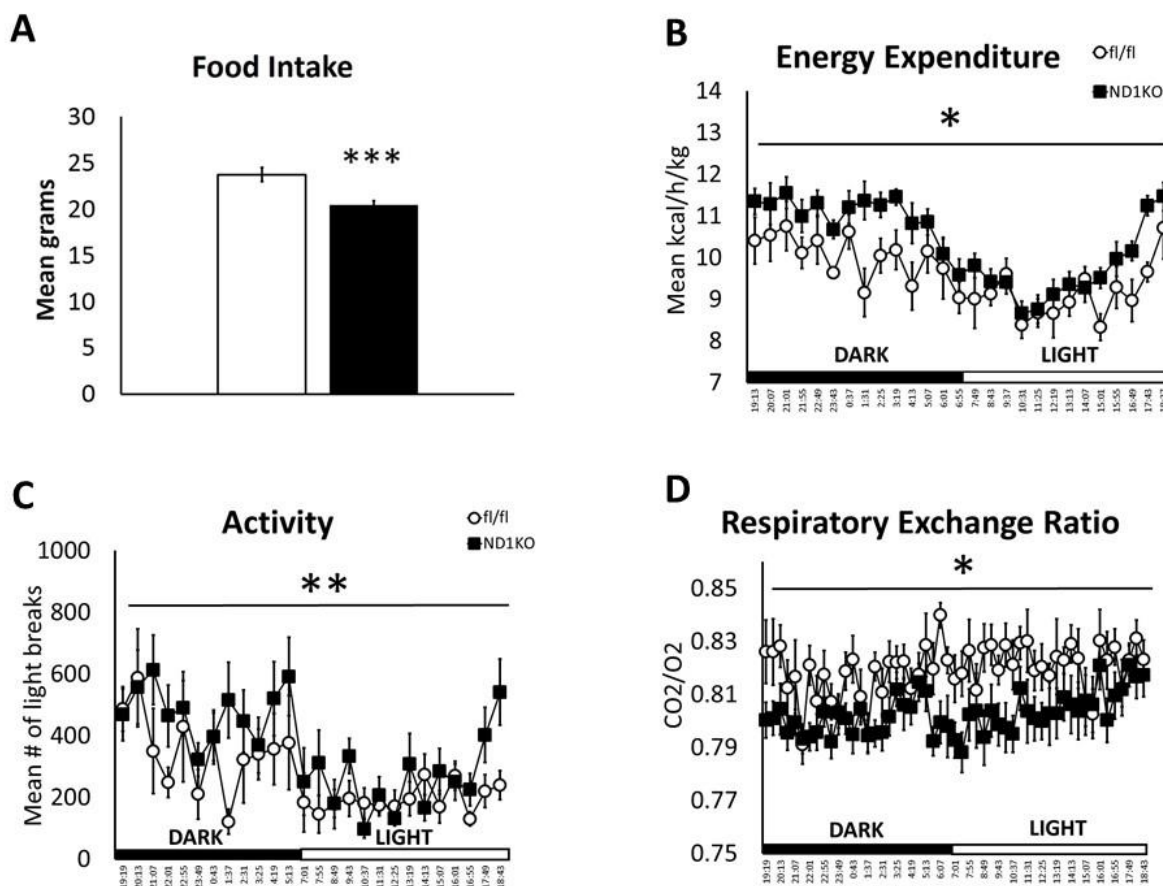


Figure 10. Food intake and energy expenditure of HFD-fed fl/fl and ND1KO mice.

Cumulative food intake over 8 days (A); energy expenditure (mean kilocalories/hour/kilogram) (B), physical activity (C) and respiratory exchange ratio (D) over a 24-hour period of male HFD-fed fl/fl and ND1KO mice.

* $p < .05$, ** $p < .01$, *** $p < .001$.

metabolic cage experiment, which indicates a preferential usage of fat acids as an energy source in ND1KO mice.

2.3.3 HFD-fed ND1KO male mice have upregulated BAT thermogenic genes.

Because neuronal Dnmt1 deficiency produced the most robust body weight differences in male mice fed an HFD, we characterized the ND1KO phenotype in further detail in HFD-fed males. On an HFD, ND1KO mice showed upregulated UCP1, OTOPI, CIDEA, PPAR γ and PRDM16 gene expression in BAT (Fig. 12). Collectively, these gene alterations suggest there could be increased mitochondrial oxidative capacity in BAT. Phosphorylation of HSL, a key

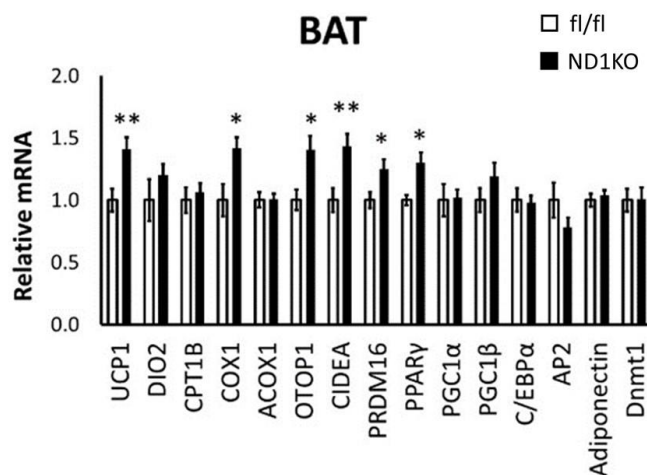


Figure 12. BAT gene expression in HFD-fed male fl/fl and ND1KO mice.

Expression of genes involved in thermogenesis and mitochondrial function in brown fat of male HFD-fed fl/fl and ND1KO mice. * $p < .05$, ** $p < .01$.

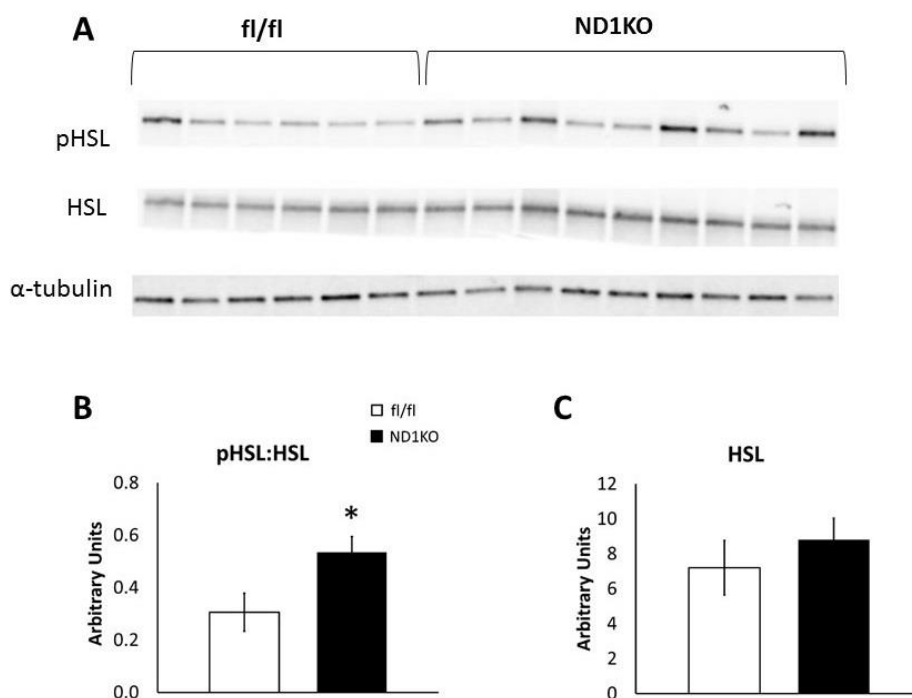


Figure 11. BAT protein expression of HSL and pHSL.

Western blot images of total HSL, phosphorylated (serine 660) HSL and α -tubulin (as a loading control) (A), and densitometry quantification of total HSL (B) and pHSL:HSL in male HFD-fed fl/fl and ND1KO mice. * $p < .05$.

enzyme in lipolysis, was significantly increased in BAT of HFD-fed ND1KO mice compared to fl/fl mice (Fig. 11), which indicates elevated activity of HSL and suggests increased lipolysis.

Consistent with changes in gene and protein expression, visual observation of hematoxylin and

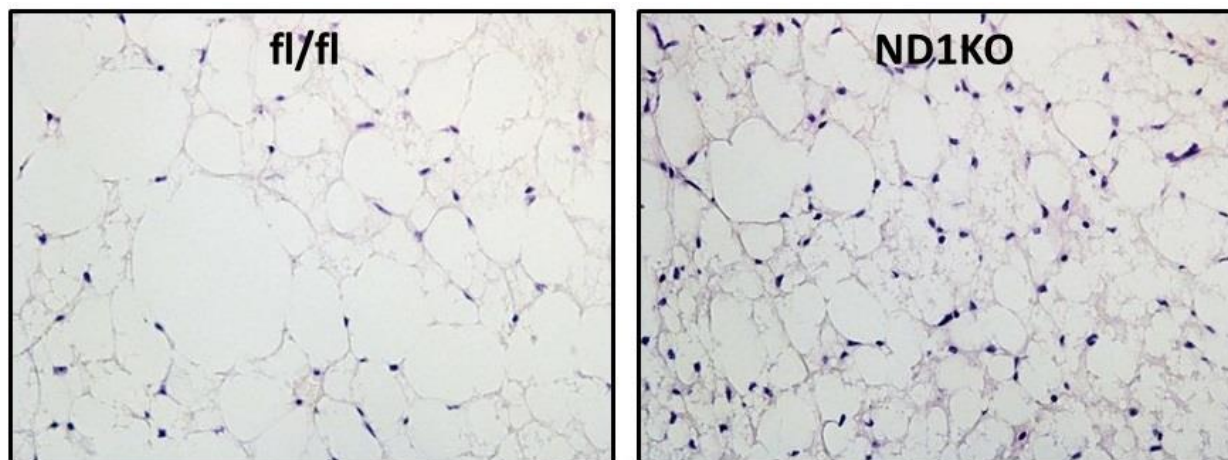


Figure 13. BAT morphology in HFD-fed male fl/fl and ND1KO mice.

Representative images of hematoxylin and eosin staining at 40X magnification of brown adipose tissue from HFD-fed male fl/fl and ND1KO mice.

eosin (H & E) staining of BAT from all mice in the experiment revealed a tendency for HFD-fed ND1KO mice to have smaller BAT cells as compared to fl/fl control mice, indicating less lipid accumulation in BAT (Fig. 13). There were no major differences in the gene profile of subcutaneous or epididymal WAT of HFD-fed male fl/fl and ND1KO mice (data not shown).

2.3.4 Effects of Cold Exposure on ND1KO mice.

Because ND1KO mice showed an upregulation of BAT genes when challenged with an HFD, suggesting BAT might have elevated mitochondrial capacity, we further tested the BAT phenotype in ND1KO mice by placing a separate cohort of ND1KO and fl/fl mice in a cold room for 7 days. Prior to the cold exposure, ND1KO mice had a tendency for reduced body weight (Fig. 14B). After the 7-day cold exposure, ND1KO mice had significantly reduced body weight compared to the fl/fl mice also subject to cold exposure (Fig 14B). There was no change in epididymal, subcutaneous or BAT fat pad weights between the genotypes when subjected to cold (Fig. 14C-F). Body weight and fat pad weight of age-matched fl/fl and ND1KO mice housed in a room temperature environment also are shown (Fig. 14A, C, E). In BAT, cold-exposed ND1KO mice had trended towards having more UCP1 and DIO2 expression, although these were not

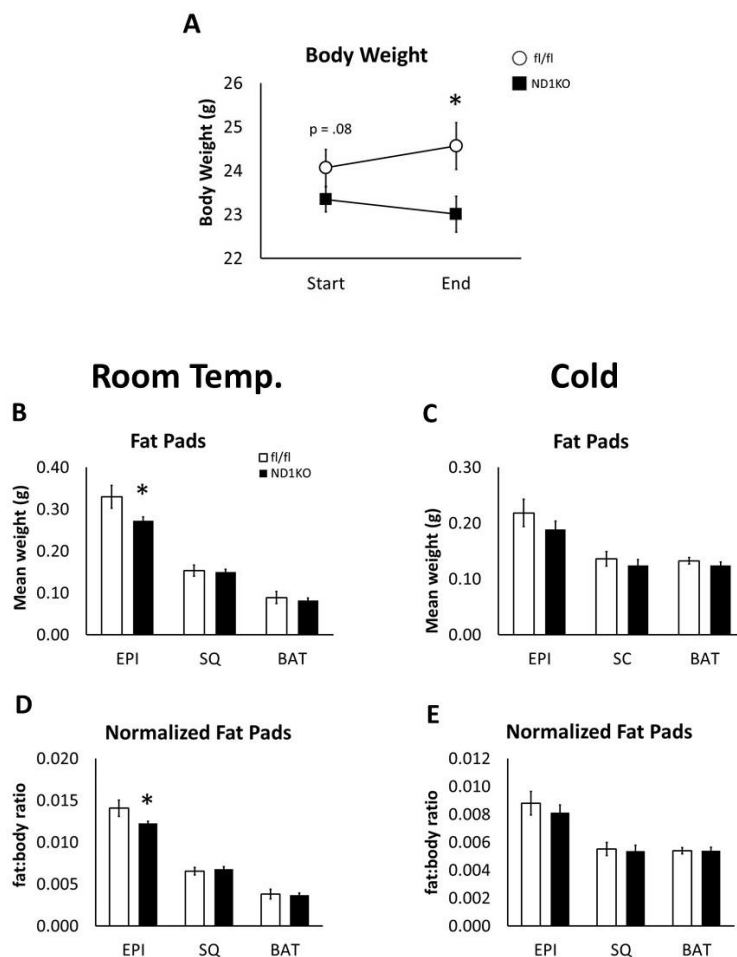


Figure 14. RT and cold-exposed body and fat pad weights of fl/fl and ND1KO mice.

Body weight of 8 week old room temperature-housed male fl/fl and ND1KO mice (A). Body weight of fl/fl and ND1KO mice before and after 7 day cold exposure (B); epididymal, subcutaneous and brown adipose tissue weights (C, D) and normalized fat pad weights of room temperature (C, E) and cold exposed (D, F) fl/fl and ND1KO mice. * $p < .05$.

significantly different (Fig. 15A, C). Western blots showed no significant change in UCP1 protein expression in BAT of cold-exposed or room-temperature ND1KO mice (data not shown).

In the subcutaneous tissue, cold-exposed ND1KO mice showed a strong tendency for increased UCP1 gene expression, which was just under the threshold of statistical significance, as well as a subthreshold tendency for increase in DIO2 (Fig. 15B, D). There was a considerable amount of variability in cold-induced expression of DIO2 and UCP1 in epididymal fat, however, and these did not differ between the ND1KO and fl/fl control mice (data not shown).

Hematoxylin and eosin staining of BAT, subcutaneous and epididymal tissues was conducted on

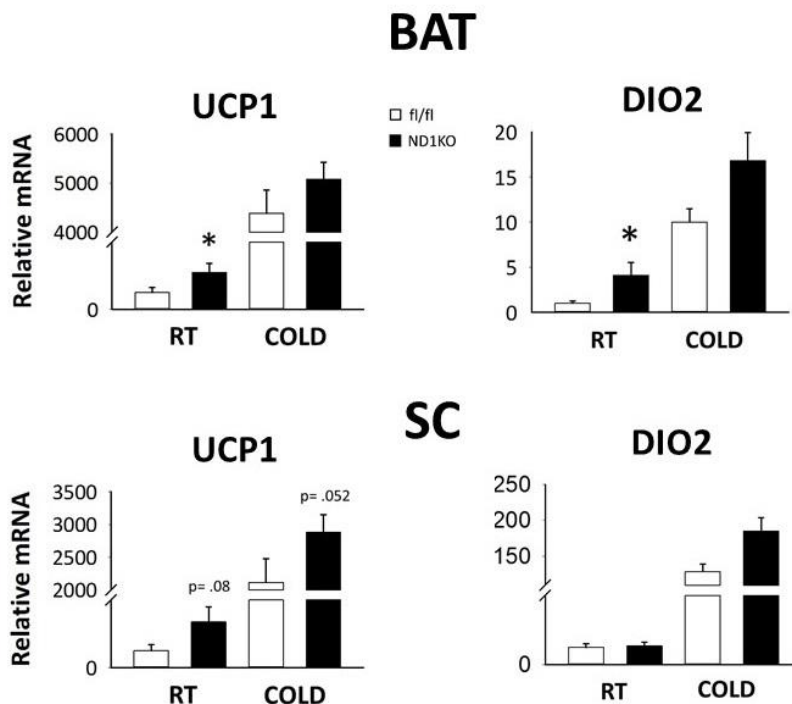


Figure 15. RT and cold-exposed BAT and SC gene expression of fl/fl and ND1KO mice.

UCP1 and DIO2 mRNA levels in BAT (A, B) and subcutaneous (C, D) tissue of room-temperature and cold-exposed fl/fl and ND1KO male mice (relative to room temperature fl/fl mice).

a random subset of four fl/fl and four ND1KO mice from both room-temperature and cold-exposure experiments. We did not observe any obvious differences in cell morphology, however, between the genotypes in either room-temperature or cold-exposed mice from these subsets (images not shown).

2.4 Summary of Specific Aim 1

Neuronal Dnmt1 deficiency reduced adiposity in both male and female mice when fed standard chow. When challenged with an HFD, male ND1KO mice showed attenuated obesity and reduced adiposity, with evidence of increased mitochondrial oxidative capacity in BAT. These changes were driven by reductions in food intake as well as elevated energy expenditure. When challenged with prolonged cold exposure, male ND1KO mice weighed less, but there was no difference in BAT, epididymal or subcutaneous fat pad weights. Thus, it appears that

neuronal Dnmt1 deficiency may affect BAT and reduce adiposity in an excess-energy condition (HFD), but not in a condition of increased energy demands (cold). Furthermore, the elevations in energy expenditure may be driven in part via enhanced mitochondrial oxidative capacity in BAT. Table 2 summarizes the overall peripheral phenotype produced by neuronal Dnmt1 deficiency in male mice in different energy states.

Table 2. Summary of ND1KO phenotype in various conditions.

ND1KO mice in various conditions:	Body Mass	Fat Pad Mass			Fat Gene/Protein Profile			Fat Cell Size		
		BAT	SQ	EPI	BAT	SQ	EPI	BAT	SQ	EPI
Chow (8 wks)	Trend ↓	--	--	↓	↑ UCP1	trend ↑ UCP1	--	ND	ND	ND
Chow (28 wks)	--	↓	↓	↓	--	Trend ↑ DIO2, PPAR γ	↑ UCP1, CIDEA	ND	ND	ND
HFD	↓	↓	↓	↓	↑ UCP1, COX1, OTOPI, CIDEA, PRDM16, PPAR γ , ↑ pHSL	--	Trend ↑ COX1	appear smaller	ND	ND
Cold	↓	--	--	--	--	trend ↑ UCP1, DIO2	--	ND	ND	ND

Summary of ND1KO body weight, fat pad weight, fat gene expression, and fat cell size in chow-fed (8 and 28 weeks of age), HFD-fed and cold-exposed conditions. ND= not determined.

3 SPECIFIC AIM 2

3.1 Background

3.1.1 The Central Nervous System.

The nervous system includes the central (brain and spinal cord) and peripheral (nerves extending from the spine) systems. In the peripheral nervous system, the somatic division controls nerves involved in muscle control, while the autonomic division controls sympathetic and parasympathetic nerves (Young et al., 2008). As energy demands fluctuate, both peripheral and

central neurons sense this information (e.g., glucose and hormone levels) and relay it on to brain nuclei that either stimulate or inhibit food intake and energy expenditure. The hypothalamus (HYP), a cluster of nuclei in the forebrain, regulates homeostatic processes including blood pressure, respiration, reproductive functions, thirst and hunger (Fulton, 1940). The arcuate nucleus (ARC), paraventricular hypothalamus (PVH), ventromedial and dorsomedial hypothalamus (VMH and DMH) and the lateral hypothalamus (LH) each receive and integrate a multitude of information regarding the metabolic state of the body (Karnani and Burdakov, 2011, Williams and Elmquist, 2012). Vagal and sensory input from the periphery is directly received by hindbrain neurons, and it is then relayed to the hypothalamic nuclei (Blevins and Baskin, 2010). Hypothalamic neurons also are in direct contact with circulating hormones and glucose in the blood and cerebrospinal fluid, which provide additional, often redundant metabolic information.

The association between the HYP and obesity was noted over 100 years ago from the coincidence of obesity in humans and animals with hypothalamic tumors (Bray and York, 1979). Since then, various hypotheses have evolved regarding how the hypothalamus regulates body weight. The “dual center hypothesis” developed from collective data (in particular, lesion and electrical recording studies) that implicated the lateral HYP as a hunger center and the ventromedial HYP as a satiety center. (Kennedy, 1950, Anand and Brobeck, 1951, Stellar, 1954) These effects were thought to be driven by changes in food intake, until further studies showed that this hypothesis was too simple to explain the role of the HYP in body weight (Han et al., 1965). The MONA LISA hypothesis (Most Obesities of unkNown Origin Are Low In Sympathetic Activity) then came into view, as autonomic dysfunction was implicated in hypothalamic obesity (Bray, 1991). For example, HYP lesions lead to reduced lipolysis (Inoue and Bray, 1977) (which occurs via sympathetic drive) and vagotomy reverses obesity caused by HYP lesions (Bray and

Nishizawa, 1978, Sims and Lorden, 1986). Our understanding of the role of the hypothalamus in energy regulation expanded with the discovery of the anatomy and projections of the paraventricular nucleus in the 1980s, followed by the characterization of the arcuate nucleus. Overall, each HYP nuclei have important, while not always distinctly separate, roles in regulating energy balance. The presence of redundant and overlapping pathways that lead to the same endpoint (such as stimulating food intake) ensures that even if the integrity of one pathway is compromised, an alternative pathway can compensate to send the same signal to the downstream neurons and organs.

3.1.1.1 Arcuate Nucleus.

The arcuate nucleus (ARC) contains agouti-related peptide/neuropeptide Y (AgRP/NPY) and proopiomelanocortin/cocaine-and-amphetamine-regulated-transcript (POMC/CART) neurons, which have opposite functions in terms of regulating food intake and energy storage. These neurons reside at the base of the third ventricle, in close proximity to circulating metabolites and hormones. Both AgRP and POMC neurons are highly glucose and leptin-responsive, in addition to receiving a variety of input from other brain areas (Belgardt et al., 2009). POMC neurons produce proopiomelanocortin peptide, which produces several cleavage products that known as melanocortins (including adrenocorticotrophic hormone and α -melanocyte-stimulating hormone [α -MSH]). Alpha-MSH is a key peptide that activates the melanocortin 3 and 4 receptors (MC4R) in the PVH. Activation of the melanocortin system leads to reduced food intake and increased energy expenditure (Rowland et al., 2010). Consistent with its anabolic role in energy balance, AgRP neurons antagonize MC4R in the PVH via a cleaved product of the AgRP protein (De Jonghe et al., 2011). The ARC neurons send key projections to

other hypothalamic nuclei (PVH, DMH and LH) to ultimately exert their effects on expending or saving energy.

3.1.1.2 The Paraventricular Nucleus.

The paraventricular nucleus resides at the top of the third ventricle, more rostral than the ARC or the VMH. The PVH is divided into two major groups of neurons: the magnocellular neurons (which project to the pituitary) and the parvocellular neurons (that receive input from the hindbrain) (Swanson and Sawchenko, 1980). The PVH is known for being the center of the melanocortin system, which regulates energy balance. A key discovery was the Agouti mutant mouse ($A^{y/a}$), which ectopically overexpresses the agouti gene (a melanocortin receptor antagonist) and develops hyperphagia and obesity (Sutton & Myers; Wolff 1986). Other studies reported that specific deletion of MC4R via adeno-associated virus in the PVH causes hyperphagia, body weight gain and adiposity, which may be mediated through single-minded-1 (Sim1)/glutamatergic cells (Shah et al., 2014). The role of the PVH in BAT thermogenesis has been controversial. Neural projections that innervate BAT have been retrogradely-traced to MC4R-expressing neurons in the PVH of hamsters (Song et al., 2008). Although activating MC4R neurons with melanotan II increases sympathetic outflow to BAT, as well as increases BAT temperature (Brito et al., 2007, Song et al., 2008), specific gain/loss-of-function studies involving MC4R in the PVH do not have any effect on energy expenditure (Balthasar et al., 2005, Garfield et al., 2015). PVH injections of glutamate increase BAT temperature in a dose-dependent manner (Amir, 1990), while in contrast PVH injections of N-methyl-D-aspartate (NMDA) inhibit cold-induced BAT sympathetic nerve activity (Madden and Morrison, 2009). PVH lesions reduce body temperature during a febrile response (Horn et al., 1994, Lu et al., 2001), yet have no effect on cold-induced thermogenesis (Horn et al., 1994). Collectively, these studies implicate varying roles

for the PVH in BAT thermogenesis, depending on the context, and depending on which sub-populations of PVH neurons are activated.

3.1.1.3 Ventromedial Hypothalamus.

The ventromedial hypothalamus (VMH), originally named the “satiety center” of the brain (until the functions of the PVH were discovered) is a complex region of the hypothalamus that regulates energy homeostasis as well as sexual and aggressive behavior (Kennedy, 1950, King, 2006, Lin et al., 2011, Lee et al., 2014). Collectively, studies conducted over the past few decades have provided evidence that this region is important in maintaining lean-ness (preventing obesity). Early experiments involving lesions and knife cuts of the VMH and its projections demonstrate that VMH dysfunction can cause obesity in both male and female rodents (Brobeck, 1946, Mufson et al., 1980). Further, the inclusion of rigorous controls (such as paired tube-feeding and hypophysectomy) (Han and Frohman, 1970, Cox and Powley, 1981) show that loss of VMH function causes metabolic disruption and fat gain (even when there is no weight change) that is not due to changes in food intake (Bernardis and Frohman, 1971).

The VMH is anatomically and functionally heterogeneous. Neuronal expression of steroidogenic factor-1 (SF1; important to the neuronal migration and development of the VMH) (Cheung et al., 2013) expression clearly distinguishes the VMH from surrounding regions. Within the VMH however, distinct neuronal sub-populations are mapped in different areas: leptin receptors are found in the dorsomedial VMH; insulin receptors in the central VMH, near the ARC; brain derived neurotrophic factor (BDNF) in the central and ventrolateral VMH; and estrogen receptor- α primarily in the ventrolateral VMH (Choi et al., 2013).

The role of the VMH in regulating energy homeostasis has been moderately researched, but much remains to be elucidated in terms of function and circuitry, given the complex nature of

the region. The VMH projects strongly to the DMH, and may mediate the effects of the preoptic area on the DMH with regard to stimulation of BAT thermogenesis (see SA1 Background on BAT and cold). Electrical stimulation of the VMH increases sympathetic nervous system drive to BAT, as well as BAT temperature (Perkins et al., 1981, Saito et al., 1987), while inactivation of the VMH via colchicine inhibits cold-induced BAT thermogenesis (Preston et al., 1989). Yet results from such experiments should be interpreted cautiously, as the significance of these findings is limited to the precision of the electrical and pharmacological VMH manipulations (i.e., these manipulations may inadvertently affect neighboring brain regions). The exact neuronal pathways of the VMH are not fully known, but it warrants mentioning that injection of a trans-neuronal tracer, pseudo-rabies virus into BAT shows very little infection in the VMH, even after 6 days (Bamshad et al., 1999). Thus, from these studies it would appear that the role of VMH in the stimulation of BAT thermogenesis is likely more of an indirect, mediatory one characterized by multi-synaptic pathways.

More precise manipulations have provided a clearer picture of the role of the VMH in energy regulation. SF1 neurons in particular, have been highlighted as having a key role in energy regulation. SF1 is expressed early in embryonic neurogenesis, and is essential to the formation of the ventromedial hypothalamus (Cheung et al., 2013). SF1-positive neurons are spread throughout the VMH, although a distinct sub-set of neurons in the ventrolateral part of the VMH do not express SF1 (Cheung et al., 2013). NestinCre-mediated deletion of SF1 (during gestation) causes late-onset obesity in both sexes, and increases diet-induced obesity via effects on energy expenditure but not food intake (Kim et al., 2011). Postnatal SF1 deletion also produces diet-induced obesity with a lack of compensatory increases in energy expenditure and no alteration in food intake (Kim et al., 2011). Further studies have established a role for leptin receptors in regulating body weight

through SF1 neurons. As evidenced by electrophysiological experiments, leptin depolarizes, and increases the firing rate, of SF1 neurons (Dhillon et al., 2006). Moreover, mice lacking leptin receptors in SF1 neurons display moderately increased body weight on a chow diet (Dhillon et al., 2006).

3.1.1.4 Dorsomedial Hypothalamus

Above the VMH resides the dorsomedial hypothalamus (DMH), directly posterior to the caudal PVH. The DMH receives projections from both the VMH and LH, and sends major projections to the PVH (Luiten and Room, 1980, ter Horst and Luiten, 1986). DMH neurons project to the rostral ventrolateral medulla (RVM) in the hindbrain, which activate sympathetic premotor neurons leading to BAT (Morrison, 1999). Early DMH lesion studies in rodents demonstrated reductions in food and water intake and reduced body weight, but normal body composition (Bernardis and Bellinger, 1998). Like other HYP nuclei, the DMH also has a role in mediating SNS drive to BAT, and both leptin signaling and neuropeptide Y (NPY) have been highlighted as mediatory pathways. Activating DMH leptin-receptor expressing neurons using pharmacogenetics techniques increases BAT and body temperature, energy expenditure and even physical activity (Rezai-Zadeh et al., 2014). Genetic knockdown of neuropeptide Y neurons in the DMH increases BAT temperature and stimulates WAT browning, which is associated with elevated NE secretion onto WAT (Bi, 2007). As outlined previously in the Specific Aim 1 Background, the DMH receives inhibitory projections from the preoptic area and during cold exposure, this inhibition is removed (Morrison et al., 2012). Outside of energy regulation, the DMH also plays a large role in regulating autonomic stimulation of the heart, particularly during stress (Fontes et al., 2014).

3.1.1.5 Lateral Hypothalamus

The lateral hypothalamus dominates all other HYP nuclei in size, extending in a rostral-caudal fashion from the preoptic area to the ventral tegmental area in the midbrain (Paxinos et al., 2001). Early lesion experiments of the LH resulted in a profound reduction in food and water intake, to the point of death, in rodents (Anand and Brobeck, 1951, Morrison et al., 1958) and gained the LH the label of “hunger center.” Since then, a broad connectivity with other parts of the brain has been defined, implicating roles for the LH in sleep/wake states, motivation, eating/drinking, via projections to and from forebrain, midbrain, and hindbrain structures (Brown et al., 2015). Major sub-populations of neurons in the LH include orexin neurons (feeding, arousal) (Kotz, 2006), melanin-concentrating hormone (MCH) neurons (stimulation of feeding), and MC4R neurons (glucose homeostasis and sympathetic activity) (Cui et al., 2012, Morgan et al., 2015). The LH has reciprocal connections with the ARC, and maintains direct and indirect connections to and from the DMH, VMH and PVH (Stuber and Wise, 2016). In summary, with regards to energy balance, the LH is involved in initiating food and drink consummatory behaviors and coordinates with the rest of the HYP to precipitate food intake and energy expenditure signals (van Dijk et al., 2011, Stuber and Wise, 2016).

It remains difficult to define a clear, unique function for any of the HYP regions discussed above, due to significant overlap in function. Although the disruption of energy balance is often incredibly pronounced when one of these nuclei is ablated, it is clear that the ARC, VMH, DMH, PVH and LH wear multiple hats in terms of their effects on energy homeostasis, which is important to a system that needs to be resilient in order for survival. The distinct nuclei, peptides/hormones, and connections of the hypothalamus orchestrate an incredibly complex set of functions that maintain stability despite the fluctuating energy demands of the organism.

3.1.2 Estrogen Receptor- α

3.1.2.1 ER α and Energy Regulation

Estrogen receptor α (ER α), encoded by the ESR1 gene, is a steroid nuclear receptor and transcription factor (Green et al., 1986). ER α is expressed throughout the periphery and brain in both males and females (Dechering et al., 2000, Mitra et al., 2003, Gillies and McArthur, 2010). Upon estrogen binding in the cytoplasm, ER α translocates to the cell nucleus where it functions as a transcription factor by binding to estrogen response elements (EREs) within the DNA (Kumar and Chambon, 1988) and by interacting with other transcription co-activators, such as steroid receptor coactivator-1 (Zhu et al., 2013, Roforth et al., 2014, Yi et al., 2015).

ER α has an important role in energy regulation. In mice of both sexes, global deletion of ER α produces obesity (Heine et al., 2000, Ohlsson et al., 2000), as does neuronal deletion of ER α (Xu et al., 2011). Importantly, specific VMH deletion of ER α increases body weight in both male and female mice (Musatov et al., 2007, Xu et al., 2011, Sano et al., 2013). VMH ER α expression in SF1 neurons affects energy expenditure in females (Xu et al., 2011). Additionally, VMH ER α expression inhibits AMPK activity, leading to increased SNS drive to BAT and thermogenesis (Martinez de Morentin et al., 2014). Although females have more ER α immunoreactivity than males in the ARC, there does not seem to be any sex difference in ER α expression in the adult VMH (Brock et al., 2015, Yu et al., 2015). ER α expression is highest in the ventrolateral portion of the VMH (VMHvl) (Mitra et al., 2003), where it positively regulates sexual behavior in females, (Musatov et al., 2006), and aggression and sexual behavior in males (Sano et al., 2013, Lee et al., 2014). Although males do not have nearly as much estrogen as females, estrogen can be synthesized from the conversion of testosterone via the enzyme aromatase, and this conversion can occur within the brain (Longcope et al., 1969).

3.1.2.2 ESR1 Promoter Methylation.

The mouse ESR1 gene shares a high degree of homology to the human ESR1 gene (White et al., 1987), including the promoter region (Kos et al., 2000). Five different splice variants of the ESR1 transcript, wherein the 5' untranslated region varies, have been defined in mice (Kos et al., 2000). The transcript variant spliced at exon C is expressed most abundantly in the brain (Kos et al., 2000, Prewitt and Wilson, 2007) (see Fig. 25). In the brain, decreases in ESR1 expression has been associated with increases in methylation on promoter exons A and C in both male and female mice throughout early development (Westberry and Wilson, 2012). Dysregulated ESR1 expression via methylation has been linked to several diseases, breast cancer and age-related atherosclerotic plaques (Post et al., 1999, Martinez-Galan et al., 2014). Interestingly, Dnmt1 has been shown to regulate ESR1 methylation and subsequent ER α expression in cultured human aortic cells (Wang et al., 2012).

Estrogen receptor- α expression in the VMH has a role in regulating weight gain in both male and female mice, and further, its expression has been negatively correlated with methylation of the ESR1 gene promoter in the cortex of the brain (Westberry et al., 2010). The following experiments were designed to test whether neuronal Dnmt1 deficiency affects ER α in the VMH, thus providing a possible lead for future mechanistic studies.

3.2 Methods

3.2.1 Gene Expression.

The brains harvested in Specific Aim 1 from normal- and HFD-fed mice were analyzed for gene expression using real-time PCR according to the methods used in SA1. Four hypothalamic regions were dissected (arcuate, medial, paraventricular and lateral) with the aid of a dissecting microscope according to the methods of Minokoshi et al., 2004. First, the brain was placed in a

block with 1mm grooves and a straight blade was placed in the exact midline of the brain creating a sagittal cut. Two additional blades were placed on each side at 1mm intervals from the midline blade. The arcuate, medial and paraventricular regions were dissected out from the right and left blades closest to the midline, and the lateral hypothalamus was dissected out from the farthest lateral right and left blades. The hypothalamic samples were frozen in liquid nitrogen and stored at -80° C for later gene analysis.

3.2.2 Immunohistochemistry.

Fourteen ND1KO and fl/fl control littermates were fed a chow diet, while a separate cohort of sixteen ND1KO and fl/fl mice were fed an HFD. Body weight was recorded weekly. After 11-12 weeks on the respective diets, the mice were sacrificed and the brains were processed for immunofluorescence. Mice were transcardially perfused with a heparinized saline/DEPC H₂O solution followed by 4% paraformaldehyde. Brains were soaked in 4% paraformaldehyde in 4^o C for 2-3 hours, then were placed in 18% sucrose/DEPC water until they sunk. The brains were frozen and sectioned on a cryostat at 20 μ m and mounted onto slides. Immunofluorescence was conducted using primary antibodies to target estrogen receptor- α (#06-935; EMD Millipore; Merck KgaA, Darmstadt, Germany) at a 1:20,000 concentration, or neuron-specific nuclear protein (NeuN) at a 1:1,000 concentration (#MAB377; EMD Millipore) overnight at 4^oC. AlexaFluor secondary antibodies (#A21207, #A21202; ThermoFisher Scientific; Waltham, MA) were used at a 1:5,000 concentration for 3 hours at room temperature. Slides were mounted with Prolong Diamond Antifade Mountant with DAPI. Images were acquired the following day using an Olympus DP73 fluorescent photomicroscope and CellSens software (Olympus). ER α -positive cells in the VMHvl and VMHdm were counted using Photoshop CS3, and using the NeuN image of the same section overlaid as a guide for the

boundary of the VMH. The thalamus was used as an anatomical negative control. Cell counts per section were averaged for each brain, and the mean of these averages was calculated for each group. Student's t-test were used to compare differences between fl/fl and ND1KO groups. We set the significance level at $p < .025$ for the VMHdm cell counts, because this was an unplanned, additional comparison.

3.2.3 ESR1 Methylation.

ND1KO and fl/fl control littermates were fed either a chow diet (n= 16) or an HFD (n=20) for 12 weeks starting from weaning. Mice were sacrificed and the hypothalamus was dissected according to the methods described above. DNA was isolated from the V/DMH region using phenol/chloroform followed by isopropanol precipitation. Bisulfite conversion was performed using an Epiect Bisulfite Kit (Cat. #59104, Qiagen. Valencia, CA) and 2 μ g of DNA from each sample. The ESR1 promoter Exon A was amplified using primers from EpiGenDX (ADS911). Pyrosequencing and analysis for ESR1 mouse promoter Exon A was performed by EpiGenDX (Hopkinton, MA). One-Way ANOVAs were used to test for significance between the genotypes at each CpG site. For the ESR1 methylation positive control study, C57BL/6 mice were bred in house and brains were harvested at P5 and P18 (P0 as the day of birth) according to the methods of Westberry et al. (Westberry et al., 2010). Briefly, cortex was dissected with the aid of a dissecting microscope from approximately Bregma 1.20 to -0.38. Cortex samples were frozen in liquid nitrogen and stored at -80° C until processed. The same methods for DNA isolation, PCR and pyrosequencing were used for the control study as for the experimental study. Two-Way ANOVAs were used to test the effect of age and sex at each CpG site of Exon A (according to Westberry et al., 2010).

3.3 Results.

3.3.1 *ND1KO* male mice have altered hypothalamic gene expression.

We screened the expression of a variety of hypothalamic genes involved in energy regulation. In chow-fed *ND1KO* mice, there were no significant changes in gene expression in the arcuate (ARC), paraventricular (PVH), ventromedial/dorsomedial (V/DMH) or lateral (LH) hypothalamic nuclei (Fig. 16A-D). Similarly, in HFD-fed mice, there was no change in ARC gene expression (Fig. 17A). In the PVH of HFD-fed mice however, neuronal *Dnmt1* deficiency caused an upregulation of the vesicular GABA transporter gene (*vGAT*) (Fig. 17B). In the V/DMH, corticotropin-releasing hormone and the vesicular glutamate transporter 2

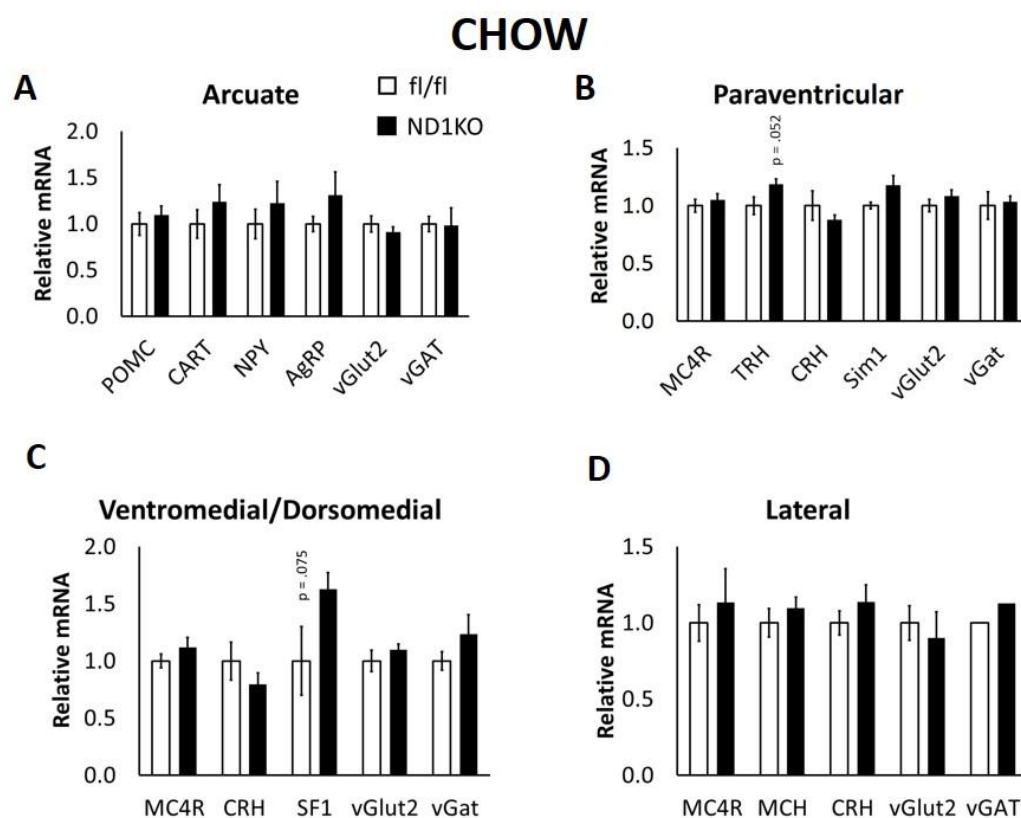


Figure 16. Hypothalamic gene expression in chow-fed *fl/fl* and *ND1KO* male mice.

Relative mRNA expression of genes involved in energy regulation in the arcuate (A), paraventricular (B), ventromedial/dorsomedial (C), and lateral (D) hypothalamic regions in chow-fed *fl/fl* and *ND1KO* mice.

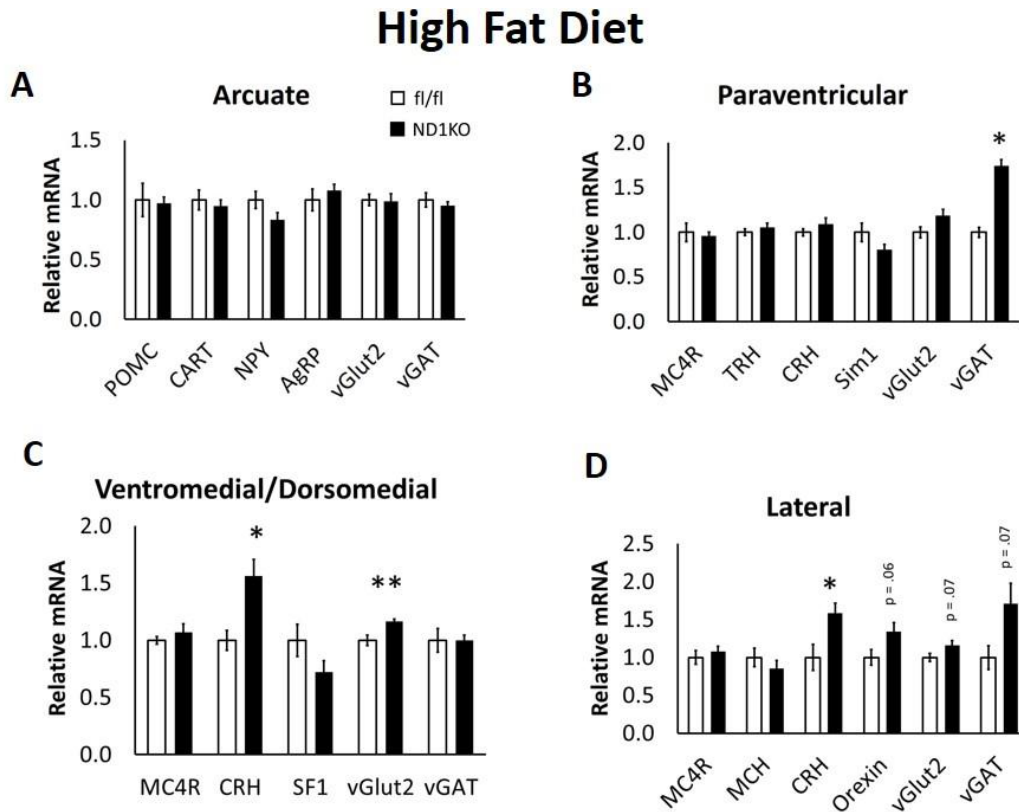


Figure 17. Hypothalamic gene expression in HFD-fed fl/fl and ND1KO male mice.

Relative mRNA expression of genes involved in energy regulation in the arcuate (A), paraventricular (B), ventromedial/dorsomedial (C), and lateral (D) hypothalamic regions in chow-fed fl/fl and ND1KO mice. * $p < .05$, ** $p < .01$.

vGlut2) genes were upregulated (Fig 17C). Finally, in the LH, HFD-fed ND1KO mice had increased CRH gene expression, and there was a tendency towards upregulation of orexin, vGlut2 and vGAT (Fig 17D).

Estrogen receptor- α (ESR1 gene) expression in the VMH is involved in body weight control in both male and female mice (Xu et al., 2011, Sano et al., 2013). Therefore, we quantified ESR1 expression in the hypothalamic nuclei to test whether this gene might be mediating the lean phenotype in ND1KO male mice. Chow-fed ND1KO mice had significantly increased ESR1 in the V/DMH and PVH (Fig. 18A). Similarly, HFD-fed ND1KO mice had increased ESR1 expression in the V/DMH, but had an upregulation of ESR1 in the LH instead of the PVH (Fig. 18B). Simultaneous assay results indicated that the HFD did not affect ESR1 gene

expression in the V/DMH in fl/fl mice, as indirectly compared to the chow-fed fl/fl mice (although these cohorts were not fed the diets at the same time; data not shown).

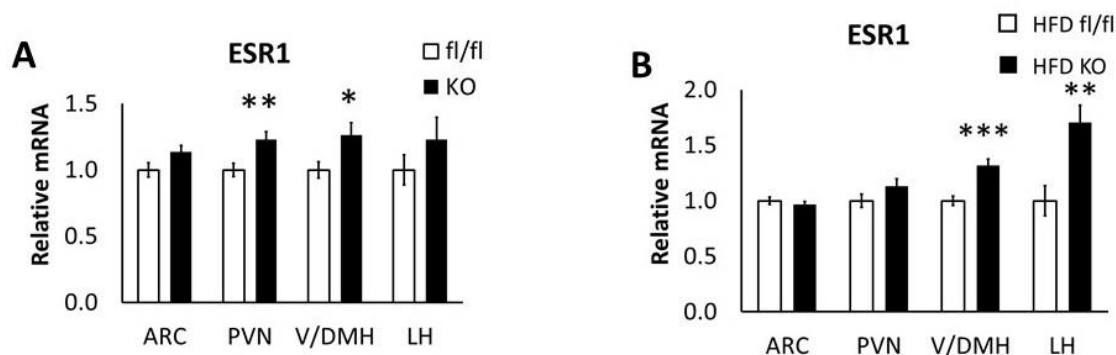


Figure 18. Hypothalamic ESR1 gene expression.

Relative ESR1 mRNA levels in the different hypothalamic regions of chow-fed (A) and HFD-fed (B) fl/fl and ND1KO male mice

3.3.2 ND1KO ER α Immunofluorescence.

We next sought to precisely locate where ER α was upregulated in the V/DMH of ND1KO mice. Previous studies have demonstrated that VMH ER α expression is greater in the ventrolateral portion of the VMH (VMHvl) than in the central or dorsal portions (Mitra et al., 2003). In addition, VMH ER α has been implicated in regulation of body weight (Sano et al., 2013), whereas we cannot find any published evidence of ER α expression in the DMH regulating body weight. Thus, we hypothesized that ER α expression would be upregulated specifically in the VMHvl. Two separate cohorts of male ND1KO mice and fl/fl control littermates were fed either a normal chow diet or an HFD beginning at 6 weeks of age for 12 weeks (the time point that coincides with the largest body weight reduction observed in SA1). After sacrifice, perfused brains were sectioned and processed for immunohistochemistry using an antibody targeting ER α .

High Fat Diet

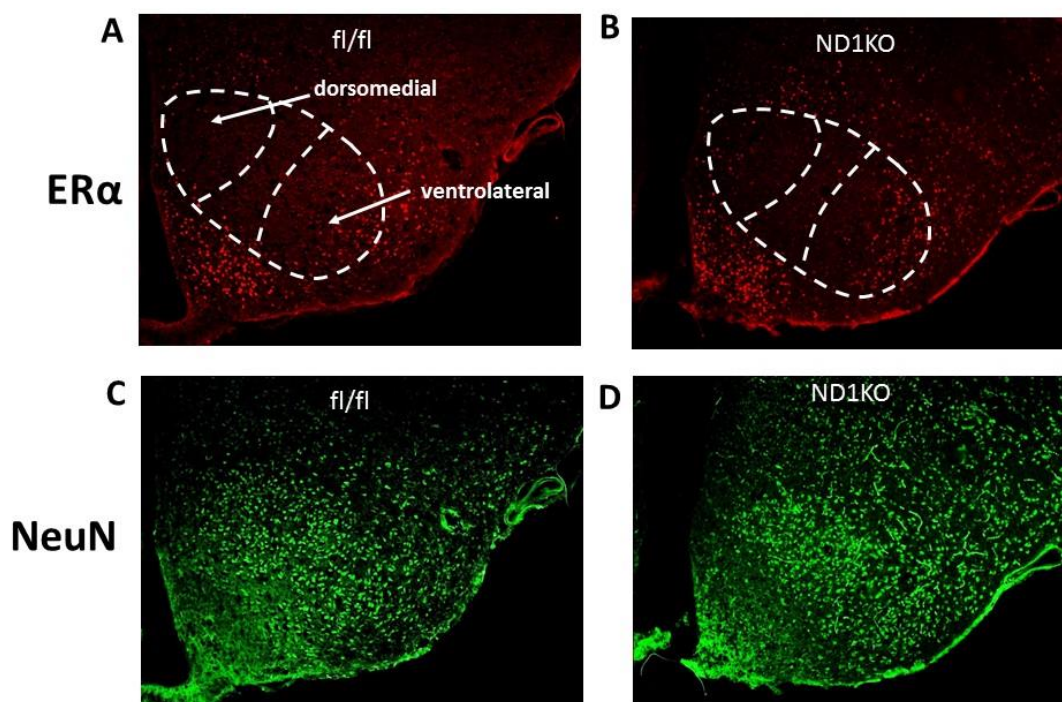


Figure 19. Estrogen receptor- α and NeuN immunohistochemistry (10X) in the VMH.

Estrogen receptor- α (red, top) and NeuN (neuronal nuclei marker; green, bottom) immunohistochemistry in the VMH at 10X magnification in HFD-fed fl/fl (A, C) and ND1KO (B, D) male mice. White-dashed lines show the boundary of the VMH, with ventrolateral and dorsomedial borders. Images were enhanced for clarity of presentation using PhotoShop CS3.

ER α -positive cells were manually counted in the VMHvl, VMHdm as well as in the ARC (to serve as a negative control, because ND1KO mice show no change in ARC ESR1 gene expression). We chose sections within Bregma -1.58 to -1.82 (Paxinos et al., 2001) because this area has the highest number of immunohistochemically-labeled ER α -positive cells (Sano et al., 2013). The pattern of staining was consistent with prior reports of ER α immunoreactivity in the VMH (Fig. 19A,B) (Brock et al., 2015, Yu et al., 2015). Contrary to our hypothesis, the number of ER α -positive cells in the VMHvl did not significantly differ between the genotypes in chow-fed mice (Fig. 20A). In fact, when fed an HFD, ND1KO mice had significantly *less* ER α expression in the VMHvl (Fig. 20B). We then tested whether ER α protein was increased in the dorsomedial VMH (VMHdm). Although ND1KO mice fed a normal diet showed no significant

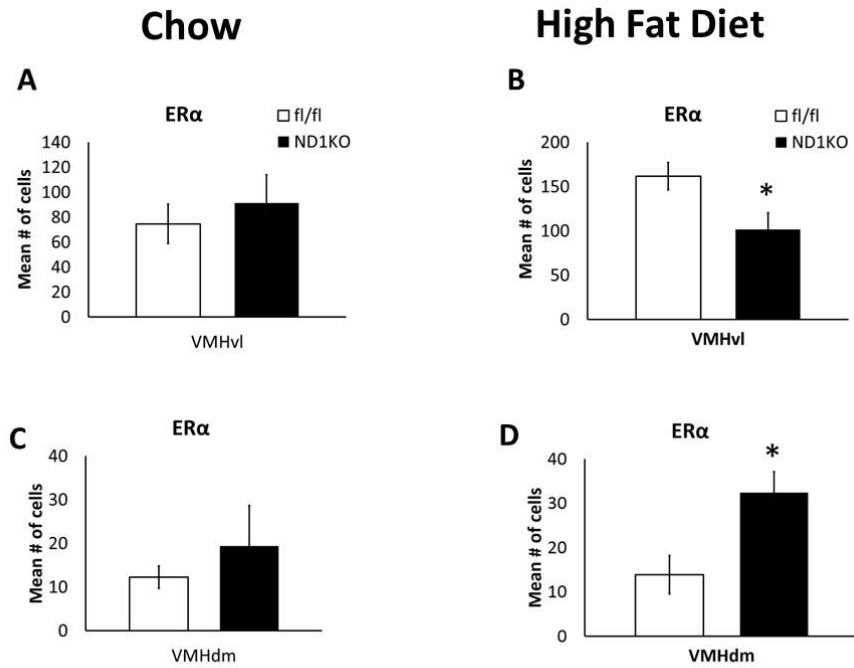


Figure 20. Estrogen receptor- α cell counts in the VMH.

Estrogen receptor- α cell counts in the ventrolateral and dorsomedial VMH of chow-fed (A, C) and HFD-fed (B, D) fl/fl and ND1KO male mice. * $p < .05$.

High Fat Diet

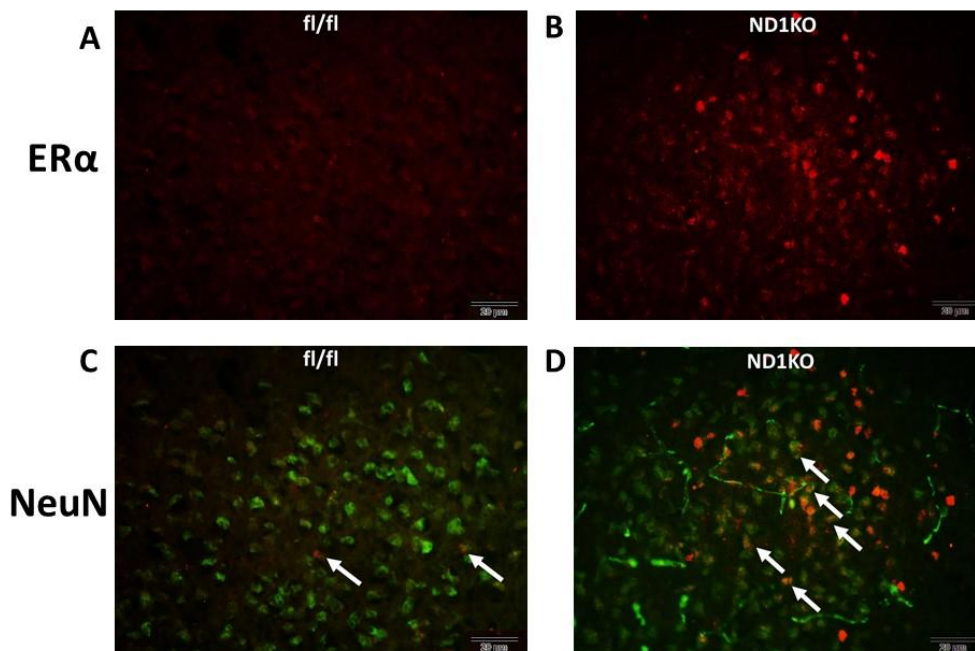


Figure 21. Estrogen receptor- α and NeuN colocalization (40X) in the VMHdm.

Estrogen receptor- α (red, top) and NeuN (neuronal nuclei marker; green, bottom) colocalized immunohistochemistry in the VMHdm at 40X magnification in HFD-fed fl/fl (A, C) and ND1KO (B, D) male mice. White arrows mark examples of double-labeled ER α /NeuN cells. Images were enhanced for clarity of presentation using PhotoShop CS3.

change in ER α expression in the VMHdm, HFD-fed ND1KO mice had significantly *greater* ER α expression as compared to HFD-fed fl/fl control mice (Fig. 20D). Finally, we found no difference in ER α expression in the ARC of HFD-fed ND1KO and fl/fl control mice (data not shown), which is consistent with our ESR1 gene expression data in the ARC. Figure 21 illustrates ER α colocalized with neurons in the VMHdm of HFD-fed mice.

3.3.3 Chow and HFD-fed ND1KO ESR1 promoter methylation

Estrogen receptor- α expression in the cerebral cortex is regulated via methylation of the ESR1 gene promoter region (Westberry et al., 2010), specifically on Exons A and C (Fig. 22). Because ND1KO mice are neuronally-deficient in Dnmt1, an enzyme that methylates DNA, we tested whether ND1KO mice had reduced methylation of the ESR1 promoter region, which might explain the increase in ESR1 transcript (mRNA) seen in ND1KO mice. We microdissected V/DMH region, as we had done previously for our gene expression analysis, and processed this tissue for bisulfite conversion and subsequent pyrosequencing. The results indicated that chow-fed ND1KO mice had slightly, but significantly reduced methylation at CpG sites 6 and 7 of Exon A (Fig. 23). There was no difference in the overall methylation status of the promoter between the genotypes (data not shown). In addition, there were no significant differences in

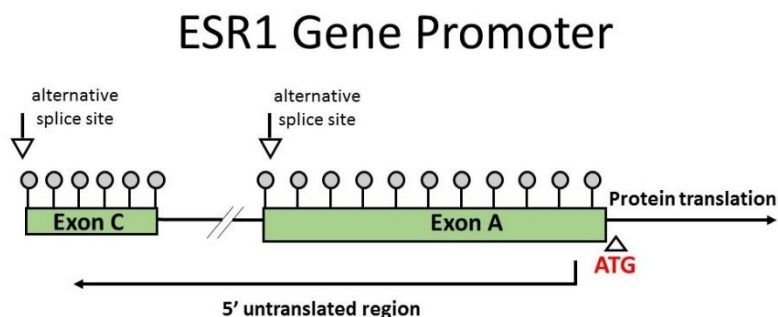


Figure 22. Map of ESR1 gene promoter CpG sites.

Illustration of CpG sites (grey circles) on exons A and C of the ESR1 gene 5' untranslated region, upstream of the protein translation start site.

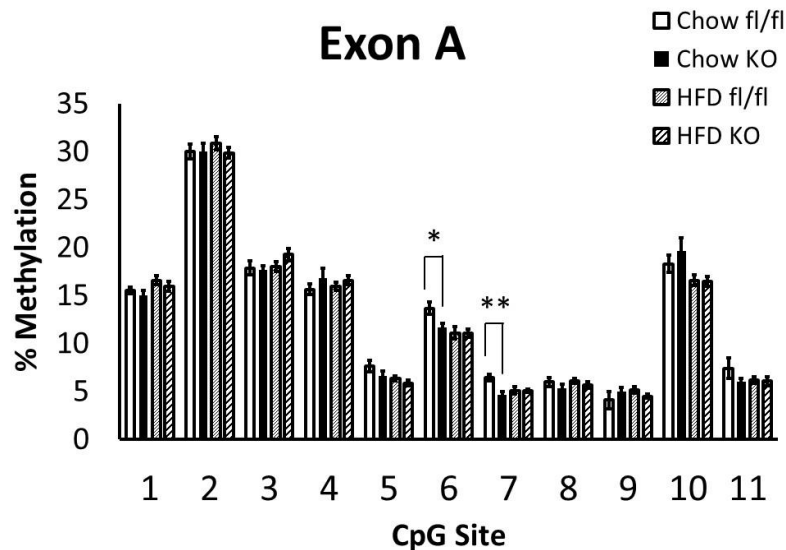


Figure 23. ESR1 Exon A promoter methylation.

ESR1 Exon A promoter methylation at CpG sites 1-11 in fl/fl and ND1KO male mice fed a chow or high-fat diet. * $p < .05$, ** $p < .01$.

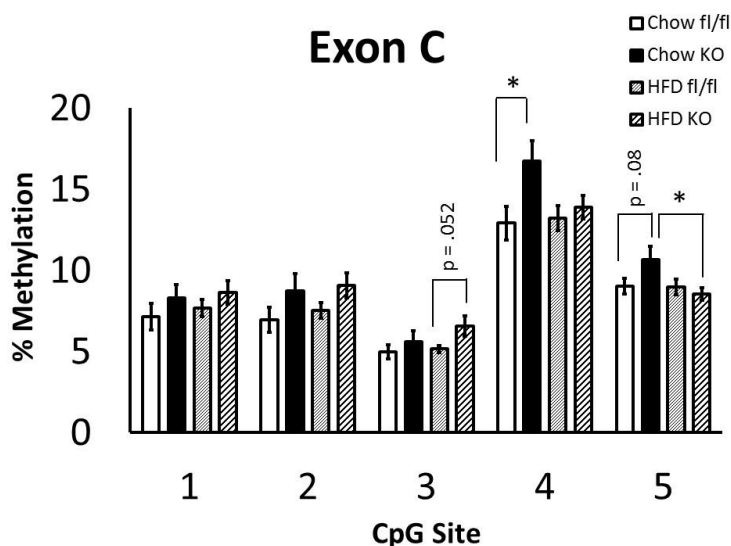


Figure 24. ESR1 Exon C promoter methylation.

ESR1 Exon C promoter methylation at CpG sites 1-5 in fl/fl and ND1KO male mice fed a chow or high-fat diet. * $p < .05$.

methylation between ND1KO and fl/fl mice fed an HFD. On Exon C, ND1KO mice showed a significant increase at CpG site 4, but this was not evident in HFD-fed ND1KO mice (Fig. 27).

Overall, on Exon C, we saw no consistent effect of either genotype or diet on the methylation pattern. As a positive control, we quantified the methylation of ESR1 Exon A in cerebral cortex of postnatal day 5 (P5) and postnatal day 18 (P18) C57BL/6 male and female

mice. It has been reported that the ESR1 promoter shows increases in methylation throughout early development (Westberry et al., 2010). Two-way ANOVAs testing the effect of sex and age across individual CpG sites revealed a main effect of age at CpG sites 1, 2, 6 and 8 of Exon A, with mice at age P18 having more methylation than those at P5 (Suppl. Fig. 3). Follow-up One-Way ANOVAs conducted separately for each sex showed that P18 females had more methylation at each of the sites that were significant in Two-Way ANOVA, while males showed a statistically significant increase at CpG site 2.

3.4 Summary of Specific Aim 2

Neuronal Dnmt1 deficiency produced remarkably little effect on the gene expression of major hypothalamic peptides involved in energy balance. Estrogen receptor- α gene (ESR1) expression, however, was upregulated in the V/DMH of both chow-fed and HFD-fed ND1KO male mice. Immunohistochemical analysis of ER α protein expression in the VMH revealed an upregulation of ER α in the VMHdm, but also a downregulation of ER α in the VMHvl of HFD-fed ND1KO mice. The ESR1 promoter showed small but significant methylation decreases at two CpG sites of Exon A in chow-fed ND1KO mice, but not in HFD-fed KO mice.

4 DISCUSSION

Aberrant neuronal gene methylation patterns are associated with obesity. For example, hypermethylation of POMC, an anorexigenic gene in the hypothalamus, correlates with obesity in both rodent and human studies (Plagemann et al., 2009, Crujeiras et al., 2013). Furthermore, increased expression of neuronal Dnmt enzymes have been linked to brain pathologies (Veldic et al., 2004), indicating a role for these enzymes in brain dysregulation. In the present study, we hypothesized that Dnmt1, a DNA methyltransferase ubiquitously expressed throughout the brain,

regulates energy homeostasis and obesity development. To our knowledge, the present study is the first to report that widespread neuronal deficiency of a DNA methyltransferase enzyme affects energy regulation, and in particular, attenuates diet-induced obesity development. In the present study, we found that neuronal Dnmt1 deficiency reduced adiposity in male and female mice fed a chow diet, and attenuated obesity in male mice fed an HFD. When fed a chow diet, ND1KO mice had smaller fat pad mass, and a tendency for reduced body fat percentage, but no difference in body weight. When fed an HFD, ND1KO mice had reduced body weight, smaller fat pad mass and improved insulin tolerance. These metabolic changes in HFD-fed mice were driven by reduced food consumption as well as increased energy expenditure.

4.1 Peripheral phenotype of ND1KO mice.

Under normal energy conditions, neuronal Dnmt1 deficiency reduced adiposity, which was evident in both BAT and WAT. The gene expression profile of BAT and WAT in ND1KO mice did not show changes that might explain the reduced adiposity, however. We conducted a preliminary visual analysis of H & E staining of BAT, epididymal and subcutaneous tissues from a subset of mice from each genotype, but we did not see any trends in reduced cell size or morphology. It is possible that ND1KO mice simply consumed less food on the chow diet, and for this reason, stored less fat. We did not quantify food intake in these mice because there was no difference in body weight throughout the course of the experiment. In our future studies, we will quantify food intake of fl/fl and ND1KO chow-fed mice.

We found the largest effect of neuronal Dnmt1 deficiency when the mice were in a state of positive energy balance—consuming an HFD. In these mice, BAT showed an upregulation of multiple genes involved in thermogenesis, indicating the stimulation of the thermogenic program. WAT showed no major changes in the gene program that would be indicative of browning,

however. In addition to reduced BAT mass, BAT cells tended to appear smaller in HFD-fed ND1KO mice. One might expect that the reduced BAT mass and cell size could be due to increased utilization of fatty acids as substrate for thermogenesis. Indeed, we found increased phosphorylation of hormone-sensitive lipase (HSL) in BAT of ND1KO mice fed an HFD. These findings are consistent with elevated sympathetic nervous system (SNS) drive to BAT, which may be a possible mechanism through which neuronal Dnmt1 deficiency alters BAT metabolism. A future experiment is in progress to test the hypothesis that ND1KO mice have elevated SNS drive to BAT by quantifying norepinephrine turnover in BAT tissue (Vaughan et al., 2014). Alternatively, we may sympathetically denervate brown and/or white fat (Vaughan et al., 2014) in fl/fl and ND1KO mice, to test whether removing the sympathetic drive to fat prevents the phenotype of ND1KO mice.

In prolonged cold exposure, there was surprisingly little indication of increased thermogenic programming in BAT or WAT. ND1KO mice weighed less after the cold treatment compared to cold-exposed fl/fl mice, but the fat pad mass did not differ between the genotypes. Although the effect was not robust, ND1KO mice had a tendency for increased UCP1 and DIO2 gene expression in subcutaneous WAT, suggesting that neuronal Dnmt1 deficiency could increase the potential for WAT browning, at least mildly. In the context of increased energy needs (cold environment), the purpose of SNS drive to WAT or BAT is to induce changes in the tissue that allow it to use energy to produce heat, and maintain the temperature stability of the animal. This is in contrast with a positive energy balance, in which increased heat is not necessary, but energy expenditure increases for other reasons (diet-induced thermogenesis, discussed below). Even though other parameters could have been analyzed for the cold exposure experiment (e.g., BAT

and core temperature), if neuronal Dnmt1 deficiency robustly altered metabolism in response to cold, it is likely that this would have been observed in the fat pads that we analyzed.

It is important to note that thermogenesis, while stimulated via sympathetic nerve projections from the spinal cord, may be stimulated upstream via different mechanisms depending on whether it is triggered through cold or other factors (Peterson et al., 2016). For example, cold is sensed through peripheral sensory nerves that project to the preoptic area, which then releases the DMH from inhibition, leading to increased sympathetic drive to BAT (Contreras et al., 2015). Other factors, such as ingesting a meal (postprandial thermogenesis) or consuming an HFD (diet-induced thermogenesis) also can increase sympathetic activation of BAT (Cannon and Nedergaard, 2004). Thus, ND1KO mice may have alterations in the neural pathways that regulate energy expenditure and BAT activity in the context of excess energy consumption, but not in the context of a cold environment. Cold exposure and HFD-feeding also can lead to differential gene expression in both BAT and WAT. For example, the gene chimerin (CMKLR1), which is positively correlated with obesity and metabolic dysfunction (Bozaoglu et al., 2007), is downregulated in cold exposure but upregulated from HFD-feeding in both BAT and WAT (Hansen et al., 2014). A final thought is that cold induces certain changes in BAT depending on how long the animal has been acclimated to the cold (Yu et al., 2002). It is possible that we may have seen differences between the genotypes if we analyzed the mice at a different time point of cold exposure.

ND1KO mice had reduced subcutaneous and perigonadal fat pad mass regardless of which diet they consumed. The reduced fat pad size observed in ND1KO mice could be from either a reduced number of fat cells, or reduced fat cell size. Although the fat pads weighed less in chow-fed ND1KO mice, in our preliminary studies we did not observe any obvious differences in the

cellular morphology of these fat tissues. If the adipocyte size did not differ between the genotypes, but the overall fat pads were smaller, than it is logical to assume that there was a reduced number of fat cells in the ND1KO mice. The most likely explanation is that neuronal Dnmt1 deficiency increases autonomic nervous system drive to fat tissues, which stimulates proliferation of energy-consuming BAT cells while inhibiting proliferation of energy storing WAT cells (Geloan et al., 1988, Jones et al., 1992, Foster and Bartness, 2006). Further experiments measuring the distribution of adipocyte size in the fl/fl and ND1KO mice are needed to elucidate whether and how neuronal Dnmt1 deletion affects adipocyte development.

In addition to increased metabolic activity in BAT or WAT, another contributing factor to the elevated energy expenditure of HFD-fed ND1KO mice is that they had a higher physical activity level. In fact, previous studies have highlighted certain genes in hypothalamic neurons as being important to controlling spontaneous physical activity, as well as energy intake/expenditure. TrkB (the BDNF receptor) (Ozek et al., 2015) or FoxO1 (Ren et al., 2013) expression in the hypothalamus, ROCK1 expression in AgRP neurons (Huang et al., 2013), and ER α expression in the VMHvl (Musatov et al., 2007, Correa et al., 2015) all have been reported to drive physical activity levels, in addition to energy homeostatic processes. Reduced physical activity is a well-documented effect of HFD-feeding (Tung et al., 2006). Neuronal Dnmt1 deficiency likely had a protective effect on the HFD-inhibition of physical activity, although we did not directly compare physical activity in the HFD-fed mice to that of chow-fed mice. Thus, increased glucose uptake and utilization in muscle from elevated physical activity also could contribute to the elevated energy expenditure in ND1KO mice, although this was not directly measured.

Changes in the adipose organs, whether brown or white, strongly affect glucose and insulin dynamics. On a chow diet, ND1KO mice showed no major change in serum insulin levels, in the

ability to clear a glucose injection from the blood, or to correct blood glucose levels after an insulin injection. ND1KO male mice had a tendency towards reduced insulin levels, although this was not significant. As expected with attenuated obesity, HFD-fed ND1KO mice had improved glucose and insulin dynamics. Male ND1KO mice showed a more efficient response to insulin in clearing glucose from the blood and maintaining glucose homeostasis. Although insulin resistance was not directly quantified, an HFD is a reliable model of insulin resistance and metabolic disorder (Fellmann et al., 2013, Williams et al., 2014); hence, we interpret this to mean the neuronal Dnmt1 deficiency attenuated the severity of, or protected the mice from, HFD-induced insulin resistance.

Increased energy expenditure was not entirely responsible for the attenuated obesity in HFD-fed ND1KO mice, as these mice also ingested less energy. An important follow-up study would be to pair-feed fl/fl and ND1KO mice during the first 3 months of the HFD, when the reduced obesity is starting to become evident in ND1KO mice. Pair-feeding would allow us to distinguish between the relative contributions of reduced energy intake vs. elevated energy expenditure in the attenuation of obesity. It is likely that alterations in food intake and energy expenditure contribute in similar degrees to the leaner phenotype, because neither had particularly huge effects. Further, we do not know whether changes in food intake and energy expenditure occurred consistently throughout the entire HFD experiment, or whether these effects were dynamic throughout the 25-week HFD.

4.2 Hypothalamic phenotype of ND1KO mice.

Food intake behavior is regulated by the central nervous system, and in particular, the melanocortin system. Given the previous studies linking POMC hypermethylation with obesity, we were surprised to see no changes in POMC, or any other major ARC genes. It would be interesting to quantify α -MSH protein in the ARC, since this post-transcriptional cleavage product

of the POMC gene is the signaling molecule that effects reductions in food intake via signaling the PVH. However, even if this were the case, one might expect to see compensatory changes in the PVH to accommodate for this increased signaling protein. In the V/DMH and LH regions, corticotropin-releasing hormone (CRH) was upregulated, which is an anorexigenic hormone. CRH certainly plays a large role in the inhibition of food intake, but this hormone is known to exert this effect by way of PVH projections, not the VMH. While the VMH certainly contains CRH receptors that mediate the inhibition of food intake (Nishiyama et al., 1999), the effect of upregulated CRH hormone in the V/DMH region is not clear. Thus, we did not observe any major changes in the hypothalamic gene profile screening that could at once explain the phenotype. An important consideration is that our analysis of the hypothalamic gene profile was conducted after a long duration of HFD-feeding. Thus, our “snapshot” of the hypothalamic gene profile reported here is one that includes any compensatory changes occurring over time on an HFD or with older age. It would be important to analyze the hypothalamic gene expression profile in both young adult ND1KO mice (8 weeks old) as well as ND1KO mice that have had a shorter exposure to the HFD.

An important mechanism to test in our future studies is whether neuronal Dnmt1 deficiency attenuates obesity by enhancing neuronal leptin sensitivity. Leptin has the well-known effect of reducing food intake and body weight (Campfield et al., 1995), and these effects have been strongly corroborated by studies involving the leptin-deficient *ob/ob* mouse (Hwa et al., 1997). An HFD causes both hyperleptinemia and central leptin resistance (Maffei et al., 1995, Munzberg et al., 2004). In the present study, HFD-fed ND1KO mice showed reduced serum leptin concentrations compared to those of HFD-fed *fl/fl* mice. This may be explained by the simple fact leptin is secreted in proportion to the amount of fat stored (Jequier, 2002) and ND1KO mice had less adiposity. Hyperleptinemia can lead to leptin resistance (although it is not required for it)

(White et al., 2009), and so it is important to determine whether the reduction in serum leptin also was associated with enhanced leptin signaling within the brain. The present finding that HFD-fed ND1KO mice had reductions in food intake and body weight is consistent with an enhanced leptin response (Campfield et al., 1995). Our future studies will test if leptin sensitivity is enhanced in ND1KO mice by quantifying food intake and body weight in response to leptin injections. If it is, then additional studies can build on this by investigating molecular components of the neuronal leptin signaling cascade (e.g., phosphorylated STAT3) in the hypothalamus (Bjorbaek and Kahn, 2004). Collective results from such studies can implicate neuronal leptin signaling as a possible mechanism in the ND1KO phenotype.

4.3 The ND1KO phenotype in females.

Under normal energy conditions, neuronal Dnmt1 deficiency reduced adiposity in females. Chow-fed ND1KO females had significantly reduced body fat composition and a slightly higher blood glucose during the glucose and insulin tolerance tests. In an excess energy condition (HFD), ND1KO females did not have significantly attenuated body weight at the end of the HFD, although they did not show differences in overall body fat composition and reduced fat pad weights. HFD-fed female ND1KO had significantly improved insulin tolerance throughout the insulin tolerance test, whereas there was no overall main effect of insulin tolerance in males (despite a lower A.U.C.).

In the interpretation of these findings, it is important to consider that there are a number of differences in lipid metabolism between the sexes. Women have more body fat than men (Gallagher et al., 2000), a difference that is easily attributable to the increased energy requirements that come with pregnancy and gestation. Fat storage also is distributed differently between the sexes, with women storing more fat in subcutaneous regions while men store more fat in the

visceral/abdominal region (Palmer and Clegg, 2015). Although the rate of lipid oxidation is similar between the sexes, females have greater lipolysis rates (Schmidt et al., 2014), are more sensitive than males to norepinephrine-induced lipolysis (Schmidt et al., 2014), have greater triglyceride secretion and clearance, and higher fasting free fatty acid levels than males (Ter Horst et al., 2015).

The metabolic differences in general that exist between males and females could be due to the sexual differentiation of the brain circuits (or peripheral organs) controlling energy homeostasis, but are likely due to the greater presence of estrogen, as well. Estrogen is a major player in the sex differences seen in metabolism and energy regulation. For example, estrogen may enhance neuronal leptin sensitivity in females, which affects glucose homeostasis, food intake and energy expenditure (Clegg et al., 2006). In addition, aromatase-knockout or ER α -knockout mice of both sexes are more vulnerable to streptozotocin-induced diabetes, and estrogen reverses this effect in aromatase-knockout mice (Le May et al., 2006). Studies of women before and after menopause (when there is a drop in estrogen production) also have provided insight into the role of estrogen in fat regulation; specifically, fat distribution in post-menopausal females resembles that in males, with a shift from subcutaneous to visceral storage (Palmer and Clegg, 2015). Thus, estrogen makes a strong case for itself in terms of being a likely hormone that may regulate a majority of metabolic sex differences.

We can speculate that perhaps neuronal Dnmt1 deficiency enhanced an already-existing tendency for greater lipolysis in females, and this may have resulted in reduced adiposity in chow-fed females. Further, the lack of neuronal Dnmt1 may have altered autonomic nervous system regulation of pancreatic insulin secretion, thereby resulting in a slightly impaired ability to clear glucose from the blood in females. In the HFD-fed females, we might hypothesize that neuronal Dnmt1 deficiency could enhance the protective effects of estrogen (e.g., anti-inflammatory,

anorectic) in females fed an HFD; yet, female ND1KO mice showed no significant difference in body weight at the end of the HFD. A possibility is that perhaps estrogen already had the maximum protective effect in the HFD-fed females (anti-inflammatory, anorectic effects), and that this could not be further enhanced by neuronal Dnmt1 deficiency. The contribution of estrogen to the neuronal Dnmt1 phenotype seen in HFD-fed female mice could be tested by comparing the phenotype of ovariectomized versus intact ND1KO female mice. This is a critical study to conduct in both the chow and HFD conditions, as it could elucidate whether estrogen is necessary for the effects of neuronal Dnmt1 deficiency on adiposity in chow-fed females, as well as the lack of effect on overall body weight in HFD-fed females.

4.4 Remodeling of estrogen receptor- α in VMH of ND1KO mice.

In the present study, we found that neuronal Dnmt1 deficiency upregulated the estrogen receptor- α gene, ESR1 in the V/DMH region of both normal-fed and HFD-fed mice. Converging evidence implicates VMH ER α expression in body weight regulation of both male and female rodents (Xu et al., 2011, Sano et al., 2013). We hypothesized that neuronal deficiency upregulates ER α in the ventrolateral VMH (VMHvl) because the majority of VMH ER α is expressed in the ventrolateral portion (Mitra et al., 2003). Contrary to our hypothesis, however, we discovered that ER α protein was not upregulated in the VMHvl of HFD-fed ND1KO mice, but rather in the dorsomedial VMH (VMHdm). The VMHdm has a known role in the response to leptin. Although leptin receptors are expressed throughout the VMH (Caron et al., 2010), leptin stimulated pSTAT3, a downstream leptin signaling protein, is more robustly induced in the VMHdm (Elmqvist et al., 1998). Additionally, cFos (a marker of neuronal activation) is upregulated in VMHdm neurons within 2 hours following an i.v. leptin injection (Elmqvist et al., 1997). Specific deletion of ER α in SF1 neurons of the VMH produces obesity in female mice, but not in male mice (Xu et al.,

2011). Nonetheless, ER α knockdown using adeno-associated virus (AAV) delivery of a short hairpin RNA into the VMH does produce obesity in male mice (Sano et al., 2013). This knockdown model is not precise enough to determine a more specific location within the VMH through which ER α has this effect in males. Additional studies can build on this by immunohistochemically double-labelling ER α along with other proteins of interest (e.g., leptin receptor, BDNF) to characterize the VMH neurons in which ER α exerts its effect in males. To specifically test whether neuronal Dnmt1 deficiency attenuates body, at least in part, through ER α in the VMHdm, we could knock-down ER α in the VMHdm of males using AAV methods, for example. In addition, while not specific to just the VMHdm, knock-down of ER α in leptin receptor-expressing neurons would be a critical experiment to conduct.

Along with ER α upregulation in the VMHdm, ER α was downregulated in the VMHvl (and this effect was greater than the effect in the VMHdm) in HFD-fed ND1KO males. ER α expression in the VMHvl positively regulates sexual behavior and aggression in males (Sano et al., 2013, Lee et al., 2014). Thus, these results indicate that neuronal Dnmt1 deficiency may have altered these behaviors, although they were not measured in the present study. In chow-fed mice, we did not observe any significant effects of neuronal Dnmt1 deficiency on ER α expression in the VMHvl nor the VMHdm. Although there appeared to be a trend for the ND1KO mice to have more ER α in the VMHdm, there was too much variability. Notably, there was a robust (2-fold) increase in ER α protein expression in the VMHvl in HFD-fed fl/fl control mice as compared to chow-fed fl/fl control mice. A review of the literature yielded only one report in which a 4-month 42% HFD *reduced* ER α protein and mRNA in the overall hypothalamus of male mice (Morselli et al., 2014). The possibility exists that an HFD decreases ER α in the hypothalamus as a whole, but it still upregulates ER α expression in the smaller region of the VMHdm. Although we did not conduct a

count of ER α -positive cells, nor quantify ESR1 expression in the gross hypothalamus, our qPCR analyses of separate hypothalamic nuclei did not demonstrate decreases in gene expression in any of the nuclei. Similarly, a time-course study of mice fed a purified control diet or an HFD also showed only some increases, but no decreases, in ER α expression at the gene level (Supplemental Figure 2; unpublished data from Xue lab).

We noted that although ND1KO mice had increased ESR1 gene expression in the V/DMH, the separate cohort used for the immunohistochemical study showed both a reduction and an elevation in ER α protein in the VMHvl and VMHdm, respectively. There are several explanations for the observation that ER α expression was not correlated at the gene and protein levels in ND1KO mice. First, the ESR1 expression was quantified in a large region that included the DMH in addition to the VMH. Thus far, we have quantified the ER α protein in several regions of the VMH, but not the DMH. Second, the cohort of mice analyzed for ESR1 expression were fed an HFD for six months, while the cohort of mice analyzed for ER α protein were fed an HFD for only 3 months. As mentioned before, compensatory mechanisms might kick in after a prolonged duration of HFD-feeding, due to dynamic and static phases of the response to an HFD (Williams et al., 2014). And third, mRNA transcription is regulated by different mechanisms than protein translation; therefore, upregulation seen at the gene level may not always be reflected at the protein level. Regardless of these differences, it is more important to focus on the increase in ER α protein in discrete regions of the VMH, since the VMH contains heterogeneous sub-populations of neurons that have roles in discrete functions (sexual behavior, energy expenditure, etc.). Further, the measurement of gene expression primarily serves as an indicator that remodeling within a tissue or organ might be taking place, while ultimately the expression of proteins that may be inducing this remodeling is more important.

Neuronal expression of ESR1 is regulated via methylation of its promoter region in the cerebral cortex of developing mice (Westberry et al., 2010). We tested whether ESR1 upregulation in the V/DMH was associated with hypomethylation of this same promoter region. In chow-fed ND1KO mice, we found significantly decreased methylation at several CpG sites on Exon A. Even a single CpG site, if it is in a region critical to the binding of a transcription factor or other co-activator, may control expression of that gene. For example, in piglets, the tissue-specific expression of ESR1 is controlled by a single CpG site that is located at a transcription factor binding site (Furst et al., 2012). After an extensive review of the literature, we were unable to find any published reports on transcription factor binding sites within Exon A of ESR1; thus it is unknown if the decreased methylation at these two CpG sites regulates increased ESR1 expression in the normal-fed ND1KO mice. In Exon C, we saw no decreases in methylation due to either diet or genotype. It is not necessarily the case, to our knowledge, that methylation of the exon closest to the transcript splice site would have the greatest effect on gene regulation. Some have theorized that perhaps even intragenic (non-promoter) methylation might regulate gene expression in certain contexts (Ehrlich and Lacey, 2013). Additionally, although the primary ESR1 transcript in the brain is spliced at Exon C, Exon A is still included in this transcript, and methylation of either region might lead to differential ESR1 gene expression.

The HFD-fed ND1KO mice showed no decreases in methylation at any of the sites in Exon A or C as compared to HFD-fed fl/fl mice, which was surprising, given that they also showed upregulated ESR1 mRNA. A general assumption is that reduced methylation of a gene promoter correlates with an increased likelihood of that gene being expressed. Increased gene expression, however, may not necessarily correlate with reduced methylation. Although methylation can block key transcription sites that are needed for transcription, the lack of methylation is not the only

condition necessary for transcription to occur. An HFD may affect other aspects of the transcriptional machinery at the ESR1 promoter, and thus upregulate ESR1 expression without reducing methylation. Further, we note that the HFD did not increase ESR1 promoter methylation as compared to chow-fed mice. Thus, ESR1 transcription may not be regulated by these promoter regions specifically in the V/DMH region of the brain, as it is in the cerebral cortex (Westberry et al., 2010). As a final point, since we have observed differential regulation of ER α expression in different regions within the VMH, it is possible that robust differences in ESR1 promoter methylation exist between the genotypes, but only within the discrete VMHvl or the VMHdm regions. Consequently, if the VMHdm had increased methylation, and the VMHvl (or DMH) had reduced methylation, these differences would be cancelled out in the methylation analysis of the entire V/DMH hypothalamic region. Therefore, it will be important to conduct more spatially-precise analyses of ESR1 methylation in order to determine if ER α upregulation in the VMHdm is associated with reduced methylation of the ESR1 promoter in the VMHdm.

4.5 Comparison of ND1KO with other phenotypes.

The phenotype in ND1KO mice fed an HFD is relatively moderate compared to other models of metabolic disorders or obesity attenuation. For example, the leptin-deficient ob/ob mouse displays massive, early-onset obesity that is accompanied by the progressive development of insulin resistance and adipocyte hypertrophy and hyperplasia, which occurs even on a chow diet (Bray and York, 1979). The effects of leptin-deficiency in the ob/ob mouse on energy expenditure are hugely robust compared to the effects of neuronal Dnmt1 deficiency (Hwa et al., 1997). This is not surprising, given that ND1KO mice have pan-neuronal deletion of a ubiquitously-expressed enzyme that likely regulates multiple aspects of energy homeostasis in different brain regions. Thus, it is reasonable that widespread deletion of neuronal Dnmt1 would produce less robust

effects due to compensation of different regions. Site-specific PVH deletion of Dnmt3a produces obesity on a chow diet (Kohno et al., 2014), and this effect is much more robust than that of the ND1KO mouse. Thus, site-specific deletion of Dnmt1 may yield different, and more robust phenotypes than that of the ND1KO mouse. The ND1KO mouse is more similar to the MC4R knockout mouse, when comparing the effect size on energy expenditure (Ste Marie et al., 2000). Both of these models produce phenotypic changes in fat pads, body weight and body length via altering both food intake and energy expenditure, albeit opposite directional effects (Ste Marie et al., 2000, Butler and Cone, 2003).

4.6 Summary

In summary, the present study characterized both peripheral and central alterations in energy and metabolism that occur in a state of neuronal Dnmt1 deficiency, and investigated ER α as a possible central mechanism. To our knowledge, this is the first study to demonstrate that widespread neuronal deficiency of a DNA methyltransferase enzyme alters energy homeostasis. Neuronal Dnmt1 deficiency in mice resulted in a reduction of adiposity in both normal- and excess-energy conditions, thus suggesting that neuronal Dnmt1 may be involved in the development of obesity. BAT from ND1KO mice showed evidence of increased lipolysis and mitochondrial oxidative capacity, suggesting that neuronal Dnmt1 deficiency increased sympathetic drive to BAT. Thus, we have laid an excellent groundwork upon which we can conduct specific mechanistic studies to elucidate how neuronal Dnmt1 deficiency reduces diet-induced obesity. Similar to the HFD-fed ND1KO mice, chow-fed ND1KO mice have reduced adiposity and elevated ESR1 in the V/DMH. These consistent findings point to a possible common mechanism of reduced adiposity that may involve ER α . In HFD-fed mice, neuronal Dnmt1 deficiency induced a remodeling of the VMH in terms of ER α protein. The finding that ND1KO mice had elevated

ER α protein in the VMHdm, a leptin-sensitive region that may regulate diet-induced obesity, provides a strong rationale for focusing on this region in future mechanistic studies. We now are poised to test specific mechanisms by which neuronal Dnmt1 regulates adiposity. Future studies that branch off from the present work will provide key insight into how epigenetic alterations in the nervous system can lead to energy dysregulation and obesity.

Epigenetic modifications can cause dysregulation of systems within an organism. Importantly, however, epigenetic modifications also are dynamic, changing with environment and experience and thereby changing gene regulation. Thus, understanding the role of methylation dynamics in energy regulation and obesity has incredible consequence to developing ways to prevent and reverse obesity. The present study has uncovered a role for neuronal DNA methyltransferase 1 in regulating adiposity, and has elucidated many new research questions through which we can further our understanding of how epigenetics operate to influence the development of obesity.

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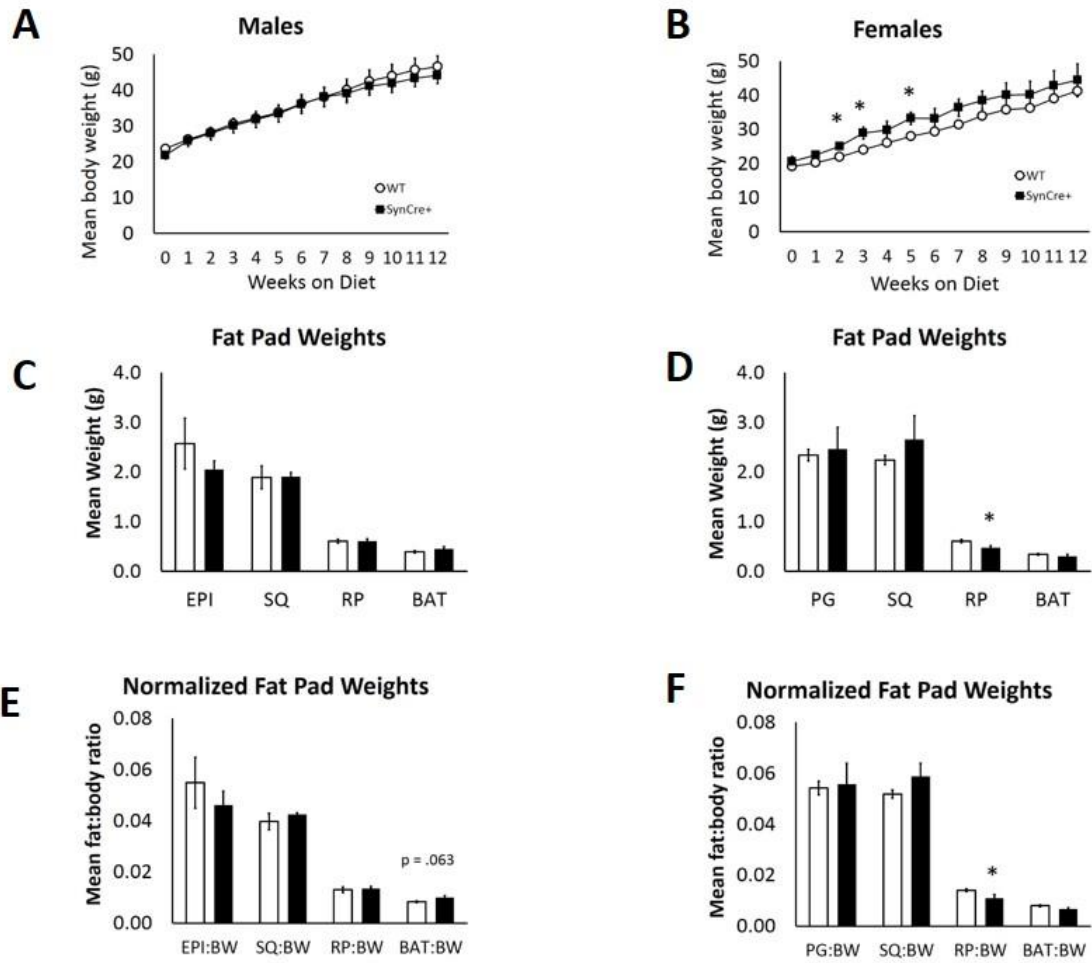
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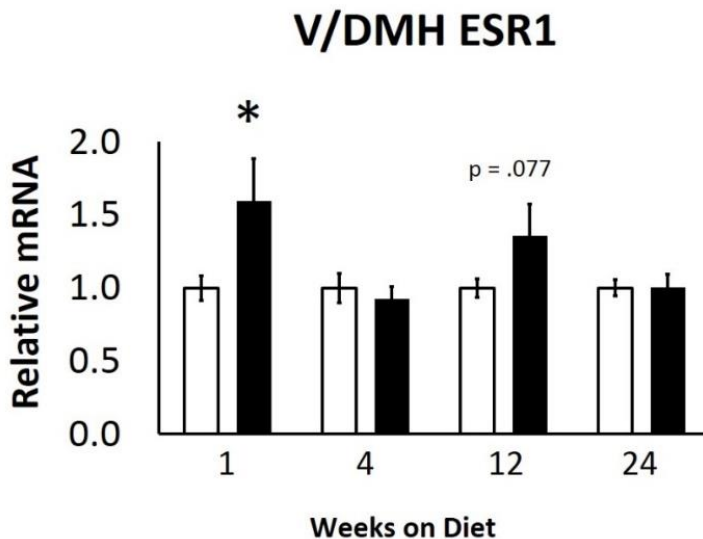
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APPENDICES

Appendix A Supplementary Figures

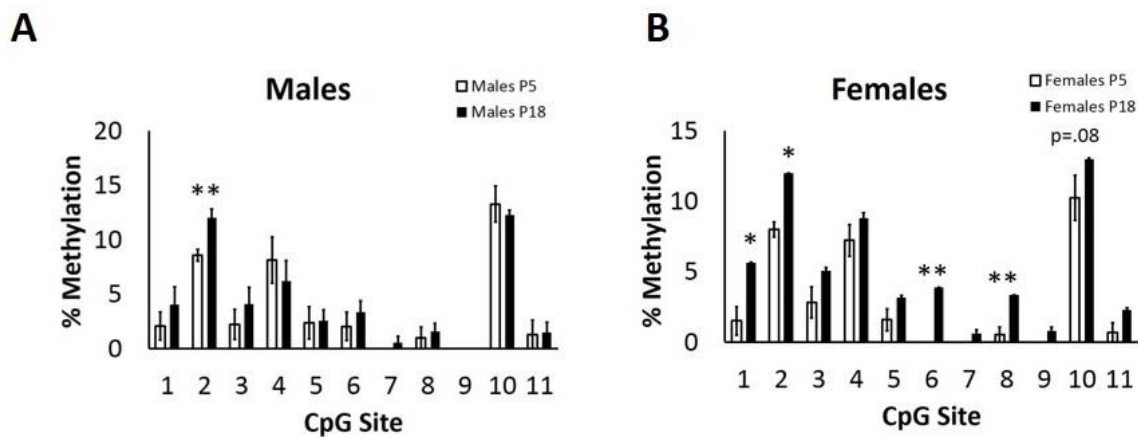
**Supplemental Figure 1. SynCre^{+/-} metabolic phenotype.**

Weekly body weight (A, B) fat pads (C, D) and normalized fat pads (E, F) of male and female SynCre^{+/-} (Dnmt1^{+/-}) mice.



Supplemental Figure 2. ESR1 mRNA expression at different time points during control or HFD feeding.

ESR1 mRNA expression in the ventromedial/dorsomedial hypothalamus of male mice fed either a purified control diet (10% fat) or an HFD (60% fat) for 1, 4, 12 or 24 weeks.



Supplemental Figure 3. ESR1 methylation in wildtype cortex.

Methylation of ESR1 Exon A from cerebral cortex of P5 and P18 males (A) and female (B) C57BL/6 (wildtype) mice.