UNIVERSITY OF CALIFORNIA, SAN DIEGO

Maternal Western Diet Overrides Genetic Resistance to Obesity: Role of TRH-DE

A dissertation submitted in partial satisfaction of the
requirements for the degree
Doctor of Philosophy

in

Neurosciences

by

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2014

The dissertation of Jennifer Becker Frihauf is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

To Paul

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Chapter 2, in full, is currently in preparation for submission for publication as the manuscript Frihauf JB, Fekete EM, Nagy TR, Levin BE, Zorrilla EP. Maternal western diet promotes obesity even in male offspring of obesity-resistant rat dams: early endocrine markers. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Maternal Western Diet Overrides Genetic Resistance to Obesity: Role of TRH-DE

by

Jennifer Becker Frihauf

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Professor Eric Zorrilla, Chair
Professor Vivian Hook, Co-Chair

The dramatic rise in obesity rates in the United States over the last 30 years is likely a result of a combination of genetic and environmental factors. Genetic background can predispose an individual to obesity, while an environment rich in energy dense foods can promote overconsumption. Working in conjunction, genes and environment alter the regulatory systems responsible for maintaining energy balance. It has become increasingly clear that environmental factors can affect energy homeostasis beginning in utero. Exposure to a high-fat, high-carbohydrate diet in the perinatal period increases obesity risk. Maternal overnutrition and obesity or associated complications putatively mediate the obesogenic effects of perinatal energy-dense diet on developing offspring. It remains

unclear whether energy-dense diets promote offspring obesity even if the mother remains slim and does not overeat.

The work described in this dissertation uses an animal model of genetic obesity risk and resistance to determine the relative contributions of genes and environment and the importance of maternal diet per se in obesity susceptibility, to identify novel hypothalamic genes overexpressed in association with increased genetic or environmental obesity risk, and to determine the functional significance of those genes. Results showed that a high-fat, high-carbohydrate Western diet developmental environment promotes obesity not only in offspring from obesity-prone (DIO) mothers, but also in those from obesity-resistant (DR) dams, implicating a deleterious role for Western diet in and of itself. DR dams did not overeat or become obese while eating a Western diet during pregnancy and lactation, yet their offspring had increased body weight and adiposity as early as one day after birth, and decreased energy expenditure and increased fat gain in response to high-fat diet in adulthood. Microarray analysis of gene expression in the lateral hypothalamus identified TRHDE, which codes for thyrotropin-releasing hormone degrading enzyme (TRH-DE), as being overexpressed in both DIO and DR-Western offspring, indicating it may play a role in increased obesity susceptibility. In vivo behavioral studies showed that the thyrotropin-releasing hormone (TRH) system may be dysfunctional in DR-Western offspring, and normal TRH function is restored with administration of a TRH-DE inhibitor. This indicates that the overexpression of TRHDE in DR-Western offspring may be functionally related to increased obesity risk, providing a novel target for obesity treatment research.

Chapter 1

Introduction

Obesity is a large and growing problem in the United States. Obesity increases risk for cardiovascular disease, metabolic syndrome, type 2 diabetes and poor quality of life [1, 2, 3, 4, 5]. Among children and adolescents, obesity rates have almost tripled in the United States since 1980 [6]. Obese children are at risk for cardiovascular disease [7], type 2 diabetes [8], and social and psychological problems [9], as well as for severe obesity in adulthood [10]. A better understanding of early causes and markers of obesity risk is needed.

Obesity occurs when energy homeostasis is disrupted. The biological systems responsible for maintaining energy homeostasis are reviewed below.

1.1 Control of energy balance

The control of energy balance, that is, consuming the appropriate number of calories for the number of calories expended, is achieved through a network of hormones and neuropeptides acting along the gut-brain axis (Figure 1.1).

Peripheral signals

Hormones from the gut play a major role in the regulation of food intake by signaling short- and long-term energy needs to the brainstem and mediobasal hypothalamus [11, 12]. Appetite-regulating neuropeptide systems in the brainstem and hypothalamus are activated or inhibited according to peripheral signals, several of which are discussed

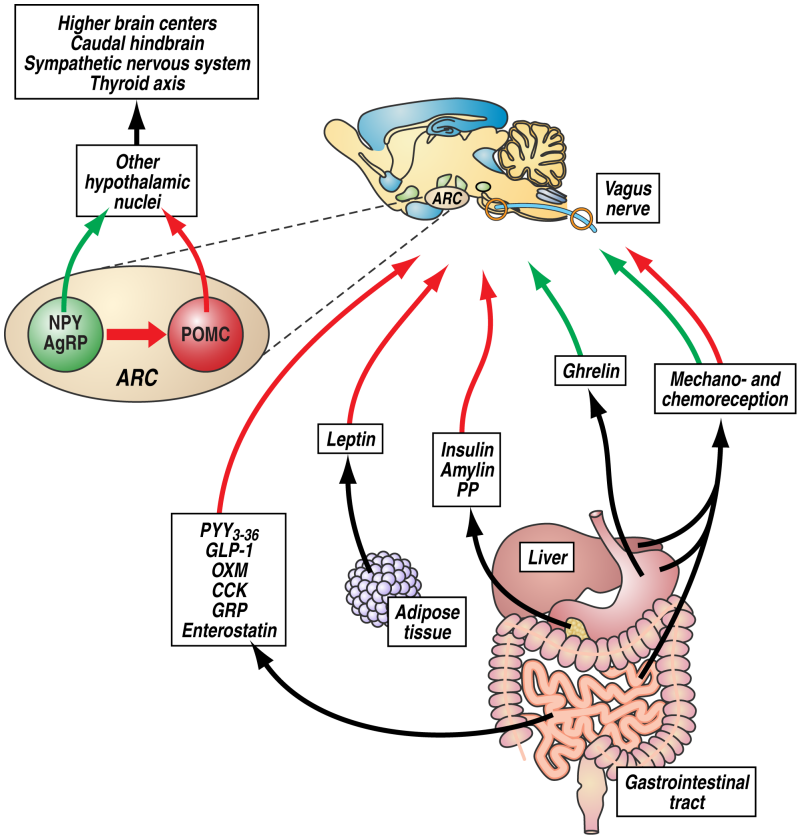

Figure 1.1: Role of peripheral signals in the regulation of food intake. (Adapted from Murphy and Bloom, Gut hormones and the regulation of energy homeostasis. *Nature* 444, 854–859, 14 December 2006.) Following a meal, PYY_{3-36}, glucagon-like peptide-1 (GLP-1), oxyntomodulin (OXM), cholecystokinin (CCK), gastrin-releasing peptide (GRP) and enterostatin are released from the stomach, while insulin, amylin, and pancreatic polypeptide (PP) are released from the pancreas, signaling to the brain to inhibit food intake. The anorectic signal leptin is released from adipose tissue in proportion to body fat. During a fast, ghrelin is released from the stomach to stimulate food intake. Signals are released from the liver and stomach to the mechano- and chemoreceptors in the vagus nerve to signal hunger or satiety. The arcuate nucleus of the hypothalamus (ARC) integrates the peripheral signals, and NPY/AgRP and POMC neurons in the ARC signal to other hypothalamic nuclei and brain regions. Green arrow indicates stimulatory effect and red arrow indicates inhibitory effect on appetite.

below. Peripheral signals from the pancreas, stomach, gastrointestinal tract, and adipose tissue allow for homeostatic control of appetite and energy expenditure.

LEPTIN

Leptin is an important protein hormone produced by adipose tissue. It binds to 6 receptor types, LepRa-LepRf, but LepRb is the only receptor that contains active intracellular signaling domains. Leptin levels in the blood are proportional to body fat content. An increase in body fat leads to an increase in leptin, which leads to a decrease in food intake and increase in energy expenditure. As with insulin receptors, LepRb receptors are abundant in the hypothalamus and inhibit neuropeptide systems that induce food intake, leading to decreased food intake when leptin is detected. Leptin deficiency causes obesity and hyperphagia, and leptin resistance is common in obese humans. Obesity can lead to high levels of leptin, to which the body becomes resistant over time, resulting in a failure of leptin to decrease food intake and increase energy expenditure. Leptin levels decrease during weight loss, leading to increased food intake.

INSULIN

Insulin is a 51-amino acid peptide hormone synthesized in the pancreas and released from pancreatic β-cells following a meal. It signals cells to take up glucose from the blood, storing it as glycogen. Insulin reduces food intake and is an adiposity signal, increasing with increases in body fat stores. Insulin resistance can occur with excessive weight gain. If the pancreas fails to compensate for insulin resistance by raising basal insulin levels as well as raising insulin levels postprandially, then hyperglycemia can occur. This relation contributes to the association of type 2 diabetes with obesity. Insulin receptors in the hypothalamus inhibit neuropeptide systems that increase food intake, leading to a decrease in food intake when insulin is detected.

GLUCAGON-LIKE PEPTIDE-1

Glucagon-like peptide-1 (GLP-1) is derived from the precursor protein preproglucagon and is secreted by L cells in the small intestine following food intake. The most common form of GLP-1 *in vivo* is GLP-$1_{7-36amide}$, which can act both peripherally and

centrally. Peripheral GLP-1 is an incretin, acting on GLP-1 receptors (Glp1r) in the beta cells of the pancreas to stimulate insulin secretion and decrease glucagon secretion. Central GLP-1 is localized in the NTS and area postrema and acts as a neuropeptide to control food intake and whole body glucose in hyperglycemic conditions. It decreases food intake and feelings of hunger, and also has effects on autonomic function. The GLP-1 competitive antagonist exendin-4_{9-39}, a truncated form of the GLP-1 agonist exendin-4, increases food intake when administered acutely and increases body weight when administered chronically.

AMYLIN

Amylin, also known as islet amyloid polypeptide, is a 37-amino acid peptide that is released from pancreatic β-cells along with insulin following a meal. It is thought to be involved in glucose homeostasis, but also reduces food intake when administered at high levels peripherally.

Brain signals

Neuropeptides in the brain also play a major role in the control of food intake and energy balance [13, 14]. The brainstem receives peripheral information about the current state of energy balance from peripheral endocrine feedback signals as well as direct neural innervations. Brainstem circuitry integrates these neural and hormonal indicators from the gut and elsewhere, communicates with other brain regions such as the hypothalamus, and regulates food intake according to energy needs. Brainstem motor systems involving the nucleus tractus solitarius (NTS) are involved in the control of feeding and respond to gut hormones via cranial nerves. The dorsal vagal complex is also of particular importance in the integration of neural signals [15].

Along with the brainstem, the hypothalamus is a crucial brain region for the regulation of food intake and energy homeostasis, integrating signals from the hindbrain, periphery, and limbic regions. In the dual-center model of appetite control, the ventromedial hypothalamic nucleus (VMH) was hypothesized to be the satiety center of the brain, whereas the lateral hypothalamic nucleus (LH) was represented as the hunger center. While these regions are still recognized to have important roles in energy home-

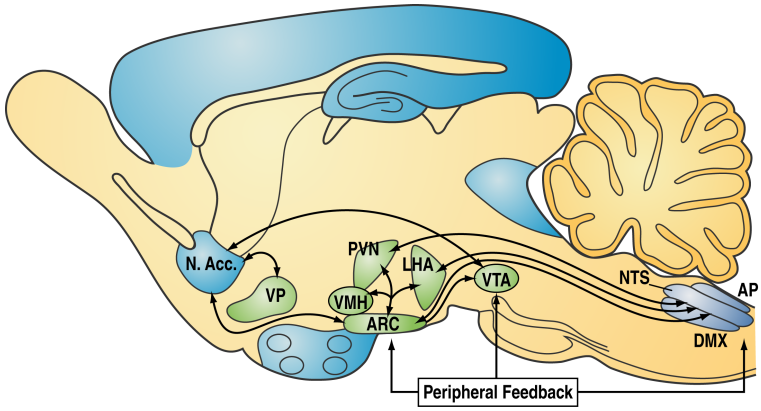

Figure 1.2: Neural circuitry of regulation of food intake and energy homeostasis. (Adapted from Morton, et al, Central nervous system control of food intake and body weight. *Nature* 443, 289–295, 21 September 2006.) Peripheral feedback converges on the arcuate nucleus of the hypothalamus (ARC), the ventral tegmental area (VTA), and the nucleus of the solitary tract (NTS). Reciprocal connections among hypothalamic nuclei, reward pathways, and the caudal brainstem allow for coordinated control of food intake and energy homeostasis. AP = area postrema; DMX = dorsal motor nucleus of the vagus nerve; LHA = lateral hypothalamus; VMH = ventromedial hypothalamus; PVN = paraventricular nucleus; VP = ventral pallidum; N. Acc = Nucleus accumbens.

ostasis, a more complex appetite-regulating network has been identified in hypothalamus that involves not only the VMH, and LH, but also major roles for the arcuate nucleus (Arc) and the paraventricular nucleus (PVN). The hypothalamic control of food intake is achieved through the transmission of orexigenic or anorexigenic neuropeptides from neurons localized in these and other hypothalamic nuclei (Figure 1.2).

NEUROPEPTIDE Y

Neuropeptide Y is found in high concentrations in the hypothalamus and is the major orexigenic peptide in the brain. It is synthesized in large part by neurons in the arcuate nucleus, which project to PVN, dorsomedial nucleus (DMN), and the median preoptic area. Neurons expressing NPY also express agouti-related protein (AgRP), another potent orexigenic neuropeptide. Injection of NPY into the ventricles or hypothalamus increases food intake, increases meal duration, reduces latency to eat, increases the motivation to obtain food, and delays satiety. Neurons in Arc, PVN, and hindbrain detect available energy via interactions with peripheral hormones and central peptides, and

activate the NPY system if not enough energy is available to fulfill the body's metabolic needs. Leptin and insulin form a negative feedback loop with NPY to regulate energy homeostasis [16].

AGOUTI-RELATED PROTEIN

Agouti-related protein (AgRP) is a neuropeptide released by NPY/AgRP neurons in the arcuate nucleus of the hypothalamus. It plays a significant role in energy balance, in part through its inverse agonist action at anorectic melanocortin receptors 3 and 4. AgRP binds to MC3-R and MC4-R, antagonizing anorectic melanocortins such as α-melanocyte-stimulating hormone (α-MSH) that bind to MC3-R and MC4-R. Central administration of AgRP increases food intake potently and with long-lasting effects. Obese mice have increased levels of hyperthalamic AgRP, and mice overexpressing AgRP are obese and hyperphagic. Like NPY, AgRP is downregulated by leptin and insulin, with increased leptin and insulin levels leading to decreased AgRP levels. Conversely, administration of the appetite-stimulating hormone ghrelin upregulates AgRP expression and induces c-fos expression in NPY/AgRP neurons.

OREXINS

Orexins, also known as hypocretins, are orexigenic neuropeptides involved in the regulation of the sleep-wake cycle and feeding. Two hypocretins, orexin A, a 28-amino acid peptide, and orexin B, a 33-amino acid peptide, are synthesized in LH. The orexins bind to two orexin receptor subtypes, OX1-R in VMH and Arc, and OX2-R in PVN and hindbrain. Injection of Orexin A and B into the ventricles or hypothalamus can increase food intake, but less potently than does NPY. Orexins also increase drinking, food seeking, and spontaneous activity. The orexin system is bidirectionally connected to the NPY system. NPY expression is increased with intraventricular injection of orexins, and NPY Y1 and Y5 receptor antagonists reduce the orexigenic effects of orexin injection. GABA is also involved in the modulation of orexin activation. GABA neurons co-express orexin, and orexin neurons are activated by a GABA agonist.

Orexin neurons are glucosensitive and respond to changes in blood glucose levels rapidly, making them an early hypothalamic factor for triggering food ingestion. Glu-

cosensitivity makes orexins highly sensitive to changes in food intake. A reduction in food intake leads to increased orexin concentrations in LH, increased orexin gene expression, and increased expression of orexin receptors. As with NPY, orexins are also sensitive to changes in leptin levels. Leptin inhibits orexin gene expression, so an increase in leptin due to satiety or increased adiposity suppresses orexin activity in the hypothalamus, leading to decreased food intake.

MELANIN-CONCENTRATING HORMONE

Melanin-concentrating hormone (MCH) is a 19-amino acid peptide that is synthesized in the LH and binds to two receptor types, MCH-R1 and MCH-R2. MCH neurons are directly connected to orexin neurons and contain OX1-R receptors, as well as cannabinoid CB1 receptors. Central injection of MCH increases food intake, and overexpression of MCH leads to obesity and insulin resistance. MCH is less potent than NPY when injected, but as potent as orexins. Chronic MCH administration increases food intake and leads to weight gain when animals are fed a high fat diet. Increased food intake is caused by action at the MCH-R1 receptor, which is widely distributed in the brain.

MCH neurons are not glucosensitive, but they are affected by food restriction. 24-hour fasting leads to increased expression of MCH. As with NPY and orexins, MCH is inhibited by leptin. Central injection of leptin leads to decreased MCH expression, decreasing orexigenic signaling. MCH also interacts with other hypothalamic neuropeptides. For example, injection of MCH increases NPY expression and stimulates the release of NPY, while it inhibits the release of α-MSH.

MELANOCORTINS

Melanocortins are anorexigenic peptides cleaved from the pro-opiomelanocortin (POMC) precursor polypeptide that can bind to MC1-MC4 receptors. The most abundant melanocortin found in the hypothalamus is α-MSH. POMC-expressing neurons in the arcuate nucleus comprise a distinct population of neurons than those that express NPY/AgRP, and also co-express cocaine and amphetamine-related transcript (CART). Similar to NPY/AgRP neurons, POMC neurons appear to be nodal points of peripheral

feedback to the CNS, but in this case subserving an anorectic role, and show major ascending projections to the PVN and LH.

Activation of the POMC system leads to anorexia. Synthetic MC3 and MC4 receptor agonists suppress food intake, whereas antagonists for those receptors increase intake. AgRP is a natural antagonist for melanocortin receptors and thereby increases food intake. POMC/CART neurons contain leptin and insulin receptors and are thus sensitive to changes in leptin and insulin levels. Unlike NPY, POMC expression is increased with increases in leptin levels that come as a result of overfeeding and weight gain. Injection of leptin into Arc also increases POMC expression. Leptin or insulin deficiency decreases POMC expression, leading to promotion of food intake.

POMC neurons receive inhibitory GABAergic collateral input from appetite-stimulatory NPY/AgRP neurons such that stimulation of NPY/AgRP neurons facilitates food intake both through direct effects of the orexigenic pathway and by inhibiting the anorexigenic pathway. A reciprocal inhibition of NPY/AgRP neurons by POMC neurons has not been identified, an absent regulatory mechanism that some have speculated contributes to the less strict control over positive energy balance.

Western diet and/or obesity can cause alterations in the peripheral and central signaling described above. Those alterations can then disrupt the balance of food intake and energy expenditure even further, increasing an individuals obesity susceptibility and making weight loss difficult. Details of the roles of the above signals in obesity risk are described in Chapters 2 and 3.

Hypothalamus-pituitary-thyroid axis

The hypothalamus-pituitary-thyroid (HPT) axis also plays an important role in maintaining energy homeostasis. Thyrotropin-releasing hormone (TRH) is a tripeptide hormone that is released from neurons in the paraventricular nucleus of the hypothalamus (PVN) to the pituitary, where it causes release of thyroid-stimulating hormone (TSH) [17, 18]. TSH then stimulates the release of thyroid hormones triiodothyronine (T3) and thyroxine (T4) from the thyroid gland into the bloodstream. T3 and T4 have effects on many biological processes, including control of basal metabolic rate and metabolism of protein, fat and carbohydrate. Up to 30% of basal energy expenditure is regulated by

thyroid hormones [19, 20, 21].

TRH secretion is peripherally controlled by negative feedback from T3 and T4, and centrally controlled by feeding-related neuropeptides and hormones [22]. TRH-releasing neurons are located in the paraventricular nucleus (PVN) of the hypothalamus and lateral hypothalamus [23] and project to other hypothalamic nuclei, including arcuate, dorsomedial and ventromedial, the central amygdala, the bed nucleus of the stria terminalis, and several other regions [140]. TRH neurons in the PVN receive input from two types of neurons involved in regulation of energy balance: POMC/CART neurons and NPY/AgRP neurons. Both types of neurons are leptin-sensitive, but activation of POMC/CART neurons increases energy expenditure, while activation of NPY/AgRP neurons decreases energy expenditure. Surprisingly, it was recently shown that TRH neurons are one of two major sources of excitatory input to AgRP neurons [25]. Stimulation of TRH neurons leads to excitation of AgRP neurons, which leads to increased feeding, even in sated mice. The ability of TRH to stimulate food intake via AgRP neuronal activation was previously unknown, and calls into question the mechanism by which central TRH administration acutely suppresses feeding . TRH also inhibits orexigenic melanin-concentration hormone (MCH) neurons via activation of local GABA neurons [26], which could contribute to the anorectic effects of central TRH administration. Neurons expressing the precursor peptide to TRH, pre-pro-TRH, are located in the lateral hypothalamus and are stimulated by leptin administration [27]. Decreased expression of pre-pro-TRH leads to increased food intake.

The TRH system, in conjunction with the peripheral and central signals reviewed above, controls energy expenditure. The role of TRH and its degrading enzyme, thyrotropin-releasing hormone degrading enzyme (TRH-DE), in obesity risk is described in Chapter 4.

1.2 Factors contributing to obesity risk

Energy homeostasis is a delicate balance and easily disrupted. In times of limited resources, too few calories and micronutrients may be consumed, tipping the balance in the negative direction and leading to malnourishment. In the modern Western world,

many people live in an environment of excess resources and consume too many calories, leading to obesity. However, whether a person becomes obese is often not just a simple matter of consuming more calories than he expends. The causes of obesity are complex and likely arise out of a combination of genetic and environmental factors [28, 29].

GENETIC FACTORS

Genetic background can predispose a person to obesity. Studies have demonstrated the importance of genetic background in obesity risk in both humans and animals. In humans, 40 to 70% of obesity-related traits are heritable [30, 31]. There are many genes that together determine an individuals obese phenotype, with each gene likely having a small impact on its own [32].

To model human obesity, Barry Levin and colleagues have developed a polygenic animal model of obesity risk and resistance by selectively breeding Sprague-Dawley rats based on their response to consumption of a 32% high-energy diet [33, 34]. The two resulting strains, diet-induced obese (DIO) and diet-resistant (DR) rats, have reliably responded to the diet by becoming obese (DIO) or remaining lean (DR) for over 40 generations. Obesity susceptibility in the DIO rats is associated with altered expression of a number of genes and changes in brain systems that control energy balance [35], a model which closely resembles human obesity.

ENVIRONMENTAL FACTORS

Environmental factors can make it more likely for a person to gain weight, and more difficult for a person to lose weight. In recent years, early life environment has emerged as particularly important in shaping an individuals propensity for obesity. The nutritional environment during in utero development, breastfeeding and early childhood influences the development of energy regulatory systems. Maternal diet during pregnancy and lactation has accordingly been well-studied as a key factor in shaping offspring energy homeostasis. Maternal undernutrition and associated intra-uterine growth restriction of the fetus can lead to offspring obesity later in life [36]. On the other hand, maternal high-fat diet consumption, excessive weight gain and gestational diabetes increase offspring susceptibility to obesity and insulin resistance in humans [37, 38, 39, 40] and animals

[41, 42, 43, 44]. Previous studies have emphasized overnutrition [45, 46, 47] or the resulting maternal obesity [48, 49] as proximate mediators. It remains unclear whether energy-dense diets promote offspring obesity even if the mother remains slim and does not overeat.

1.3 Specific Aims

Ultimately, the high rate of obesity heritability in humans cannot be explained by genetics alone, but likely arises from an interaction of genes and environment [50]. Genetic background and perinatal environment work together to increase obesity risk. The work described in this dissertation aims to determine the relative contributions of genes and environment to obesity susceptibility, and identify a novel target for obesity treatment. Studies sought to:

1. Determine the effects of maternal Western diet on genetically obesity prone and obesity resistant offspring

2. Identify a novel hypothalamic gene with increased expression associated with obesity susceptibility

3. Determine the functional significance of that gene as it relates to energy balance.

Chapter 1 includes material from Frihauf JB, Fekete EM, Zorrilla EP. Control of food intake. 2010. Encyclopedia of Behavioral Neuroscience, Academic Press, Amsterdam. The dissertation author was the primary investigator and author of this book chapter.

Chapter 2

Effects of Maternal Western Diet on Male Offspring Adiposity, Endocrine Measures and Energy Expenditure

Among children and adolescents, obesity rates have almost tripled in the United States since 1980 [6]. Obese children are at risk for cardiovascular disease [51], type 2 diabetes [8], and social and psychological problems [9], as well as for severe obesity in adulthood [10]. A better understanding of early causes and markers of obesity risk is needed.

In humans, 40-70% of obesity-related traits are heritable [30, 31]. Accordingly, Levin and colleagues developed a polygenic model of obesity risk vs. resistance by selectively breeding outbred Sprague-Dawley rats for weight gain response to a "Western" diet [33, 34]. The resulting lines are prone or resistant to diet-induced obesity (DIO and DR, respectively) and model regulatory systems implicated in human obesity [35].

Perinatal environment also influences the development of energy regulatory systems. For example, maternal high-fat diet consumption, weight gain and gestational diabetes increase offspring susceptibility to obesity in humans [37, 40] and animals [41, 43]. Previous studies have emphasized overnutrition [45, 46, 47] or the resulting maternal

obesity [48, 49] as proximate mediators. It remains unclear whether energy-dense diets promote offspring obesity even if the mother remains slim and does not overeat. In one study, rat dams received a very high-fat (60% kcal fat) diet, but were pair-fed to the caloric intake of chow-fed dams, thereby preventing maternal obesity [52]. Offspring did not show increased adiposity or body weight vs. those of chow-fed dams. However, forced caloric restriction may have interfered with the effects of high-fat diet consumption. Indeed, in non-human primates, developing offspring of diet-resistant, lean mothers chronically fed a high-fat (35% kcal) diet showed increased liver triglycerides, hepatic oxidative stress, and body weight at 130 days gestation [53]. Understanding the role played by diet is important because clinical recommendations and lay attention emphasize weight milestones [54] and caloric intake []InsOfMed05, InsOfMed92, more than diet composition, during pregnancy.

Accordingly, the current study examines whether early Western diet exposure in obesity-prone (DIO) and obesity-resistant (DR) rats increases offspring susceptibility to obesity. Offspring body weight, adiposity, food intake, whole-body energy metabolism, and key endocrine regulators of energy homeostasis (leptin, insulin, glucagon-like peptide-1 [GLP-1], and amylin) are examined. Because DR rats do not overeat or become obese when fed a Western diet, the study can evaluate the effects of Western diet per se without concurrent maternal obesity or food restriction. The present study tests the alternate hypotheses that: 1) Only offspring of obesity-prone dams will show perinatal Western diet-induced increases in obesity risk (implicating a necessary role for maternal obesity), vs. 2) Perinatal Western diet will increase obesity risk even in offspring of obesity-resistant dams (implicating a role for Western diet independent of maternal obesity/overnutrition). Unlike previous maternal diet studies of DIO and DR lines [57, 58, 59, 60], the current study examines offspring from very early in life through mid-adulthood (P1-200 days) and novelly finds that perinatal Western diet increases obesity risk even in offspring of obesity-resistant DR dams.

2.1 Methods

Subjects

DR and DIO breeders, offspring of rats from the original colonies of DIO and DR rats bred by Levin et al [33, 34], were born at The Scripps Research Institute. Rats resided in wire-topped plastic cages in a 12:12-hr light-dark cycle (600 lights on), humidity- (60%) and temperature- (220C) controlled vivarium with access to chow (LM-485 7012, 17% [kcal] fat, 58% carbohydrate, 25% protein; 3.1 kcal/g; 16% saturated, 26% monounsaturated and 58% polyunsaturated fats; 2.7% kcal from saturated fat, Harlan, Indianapolis, IN) and water ad libitum prior to experiments. Procedures adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and the *Principles of Laboratory Animal Care* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Diet and breeding

At 46-47 days of age, female DR and DIO rats were randomly divided into Western vs. chow diet conditions, yielding 4 groups: DR-Chow (n=28), DR-Western (n=16), DIO-Chow (n=28) and DIO-Western (n=16). Western diet dams received a moderate-fat diet *ad libitum* (Research Diets D12266B, 32% fat, 51% carbohydrate, 17% protein; 4.41 kcal/g; 26% saturated, 27% monounsaturated and 47% polyunsaturated fats, 8.5% kcal from saturated fat, New Brunswick, NJ). Chow dams received chow. The Western diet resembles the dietary intake of adult American women in fat (32-33%), saturated fat (10-11%), carbohydrate (50-53%) and protein (15-16%) proportions, http://www.cdc.gov/nchs/data/ad/ad334.pdf).

After 54 days of diet exposure, during which food and body weight were measured 3 times weekly, females were housed with males of the same genotype. Males were removed following pregnancy confirmation; dams continued to receive their experimental diet until weaning. The day of birth was termed postnatal day 0 (P0). Litters were culled to 8 pups ($\sim$1:1 sex ratio) at P1. All offspring were weaned onto standard chow on P23.

Because only DIO females that were comparatively resistant to the Western diet produced offspring (see Results, Section 2.2), the DIO-Western group had selective attri-

tion of obesity risk factors. Therefore, interpretation of the DIO-Western offspring data is complicated by this caveat, so we focus attention on the 3 other treatment groups in the Results and Discussion (Sections 2.2 and 2.3).

Maternal blood collection

Just before mating, fasted (16 hr overday) dams were tail bled for pre-pregnancy hormone analysis. After weaning, isoflurane-anesthesized dams were decapitated; trunk blood was collected for post-pregnancy hormone analysis. Blood was collected into polypropylene tubes containing chilled 0.5 M EDTA (10% v); plasma was isolated via centrifugation and stored at $-80°$C.

Offspring blood and tissue collection

At P1, P9, P16 and P23, anaesthetized (CO_2 inhalation) male offspring were decapitated; brains were removed, snap frozen in ice-cold isopentane and stored at $-80°$C for DNA methylation analysis. Trunk plasma was stored for hormone analysis. To avoid litter effects [61], no more than one pup from a given litter was sacrificed at each age. To reduce confounding effects of litter size [62], litter size was equated across experimental groups, with typically 8, 7, and 6 pups through P9, P16 and P23, respectively. Carcasses were stored at $-20°$C for body composition analysis.

Fat pad and body composition analysis

Frozen carcasses were shipped to the University of Alabama at Birmingham, where inguinal, subcutaneous, mesenteric, retroperitoneal, gonadal, and brown adipose tissue fat pads were weighed and returned to the carcass for composition analysis. Total body water, fat mass, and fat-free dry mass were determined per Harris & Martin (1984). Adult, live offspring body composition was measured via nuclear magnetic resonance imaging (EchoMRI-1100, Software version 2008.01.18M, Echo Medical Systems, Houston, TX).

Table 2.1: Analyte sensitivities and intra-assay coefficients of variation

Analyte	**Sensitivity**	**Coefficient of variation**
Insulin	55.6 pM	3.8-10.6%
Leptin	6.2 pM	3.8-10.6%
Amylin	6.2 pM	3.8-10.6%
GLP-1	6.2 pM	3.8-10.6%
Adiponectin	40.7 pg/ml	$< 4\%$
Glucose	10μm - 640μm	Not available
Triglycerides		
NEFA		
Free T-3	0.5 pg/ml	3.1-4.9%
Free T-4	0.5 ng/dl	3.25-10.98%

Hormones and metabolic markers

Plasma insulin, leptin, amylin, GLP-1 and adiponectin were quantified by species-specific microbead immunoassays per the manufacturer's instructions (RENDO-85K, RADPK-81K-ADPN) (EMD Millipore Corporation, Billerica, MA) using a Luminex 100 IS (Luminex Corporation, Austin, TX). All samples from a given age were analyzed on the same plate; additionally, insulin, leptin, amylin and GLP-1 were quantified in multiplex format. Adiponectin was assayed separately. Free plasma levels of triiodothyronine (T3) and thyroxine (T4) were quantified by competitive ELISA (EIA-2385, EIA-2386, DRG International, Springfield, NJ). Non-esterified fatty acids (NEFA) and triglycerides were quantified by enzymatic colorometric detection assays (SFA-1, STG-1-NC, Zen-Bio, Inc, Chapel Hill, NC). Plasma glucose was quantified by glucose oxidation assay (SKU 1200031002, Eton Bioscience Inc., San Diego, CA). Assay sensitivities and coefficients of variation are reported in Table 2.1.

Food intake

Home cage intake of dams and adult offspring was determined using a scale of 0.1 g precision. Individual intake of group-housed animals was estimated as: total cage intake / # of rats in the cage. Degrees of freedom were adjusted in statistical analyses.

Indirect calorimetry

Open-circuit indirect calorimetry was performed (24 hr) on acclimated (3 days), singly-housed rats using a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Each clear chamber ($32 \times 20 \times 19$ cm) had a water sipper, chow tray connected to a balance, plastic-mesh floor, and 24 photobeams (2.5 cm apart, 9 and 14 cm above floor). Chamber exhaust was sampled every 10 min for 50 sec through O_2 and CO_2 sensors, from which oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were estimated. Sensors were pre-calibrated with known concentrations of O_2, CO_2, and N_2 (Praxair, Inc., Danbury, CT). Respiratory quotient (RQ) and heat formation were calculated as in [63]. Heat formation was covariate-normalized for lean mass [64].

DNA methylation

Whole hypothalamus was dissected from P1 brains. Coronal brain sections (800 μm) from P23 offspring were sliced using a cryostat, and bilateral lateral hypothalamus were dissected. DNA was extracted (DNA Masterpure DNA Purification Kit, Epicentre, Madison, WI) and total DNA methylation was quantified fluorometrically (Methylflash Methylated DNA Quantification Kit, Epigentek, Farmingdale, NY).

Statistics

Two-way analysis of variance (ANOVA) was used, with Genotype and Maternal Diet as between-subjects factors and cohort as a covariate; body weight was a covariate for food intake and indirect calorimetry measures. Pairwise comparisons were performed after Genotype $\times$ Maternal Diet interactions. Because home cage intake of adult DIO-Western offspring was not measured due to small sample size, separate 1-way ANOVAs tested effects of Maternal Diet (within DR offspring) or Genotype (within Chow offspring) on adult food intake; cohort was a covariate. Pregnancy outcome (births vs. no births) was analyzed using χ^2-analyses. Pearson correlations related hormones to body fat measures; significant differences between correlations were identified using the Fisher r-to-z transformation. DIO-Western offspring were excluded from correlation analysis because too few rats had both endocrine and fat pad data (n=4-5). Litter was the unit

of analysis [61]. To determine whether fat mass was disproportionately increased relative to the rest of body mass, ANCOVA analysis was used, covarying for lean mass or fat-free mass, as determined from qNMR or chemical analysis of body composition, respectively. The ANCOVA method avoids the problems associated with the ratio-based measure of % body fat [65], but % body weight scores also are provided in Table **??** to allow comparison with previous studies. To stabilize variance, carcass fat was log-transformed prior to ANCOVA analysis, and data are presented after back-transformation. Relative (lean or fat-free mass normalized) measures of fat mass are presented as the least square means + standard errors from ANCOVA analysis.

2.2 Results

Maternal pre-pregnancy food intake, body weight and endocrine measures

Table 2.2 shows that DIO dams ate more ($F(1, 44) = 54.907$) and weighed more ($F(1, 28) = 87.323$) than DR dams during the 54 days of diet exposure ($p < 0.001$). Western diet exposure increased pre-pregnancy body weight in DIO, but not DR, rats ($p < 0.03$, Diet $\times$ Genotype: $F(1, 28) = 5.853$). Pre-mating energy intake of Western diet was not greater than that of chow diet (effects involving Diet, $p > 0.20$).

Western diet increased pre-mating leptin levels in DIO ($p < 0.03$, $F(1, 22) = 5.595$), but not DR, dams (Diet $\times$ Genotype: $p = 0.05$, $F(1, 22) = 4.202$). A Genotype trend reflected greater pre-mating leptin levels in DIO vs. DR dams ($p < 0.07$, $F(1, 22) = 3.690$). There were no significant effects involving Genotype or Diet on pre-pregnancy amylin, GLP-1 or insulin levels (Table 2.2).

Table 2.2: Body weight, food intake and endocrine profile during pre-pregnancy and pregnancy for dams that gave birth. Data express LS Mean ±Standard error, covarying for cohort. * = Diet main effect, $p < 0.05$, # = Genotype main effect, $p < 0.05$, & = Genotype × Diet interaction, $p < 0.05$.

Measure	**DR Chow**	**DR Western**	**DIO Chow**	**DIO Western**
Pre-pregnancy				
Daily intake, kcal	49.2±1.3	47.3 ±1.3	60.1 ±1.5$^{\#}$	59.1 ±1.9$^{\#}$
Pre-mating wt, g	213 ±5	202 ±5	257 ±6$^{\#\&}$	276 ±9$^{*\#}$
Amylin, pM	23.9 ±5.2	22.3 ±4.4	38.4 ±5.2	19.5 ±9.8
GLP-1, pM	37.5 ±8.2	33.9 ±6.5	61.5 ±8.2	30.3 ±15.3
Insulin, pM	31.9 ±6.7	29.5 ±5.3	47.8 ±6.7	25.3 ±12.4
Leptin, pM	22.8 ±6.6	19.2 ±5.5	25.2 ±7.1	70.2 ±12.3$^{*\&}$
Pregnancy				
Daily intake, kcal	71.9 ±3.2	77.8 ±3.2	98.6 ±5.8$^{\#}$	92.1 ±0.4$^{\#}$
Pre-birth weight, g	314 ±6	307 ±8	390 ±6$^{\#}$	405 ±14$^{\#}$
Total wt gain, g	106 ±4	113 ±4	145 ±5$^{\#}$	136 ±8$^{\#}$
Days to birth	26.0 ±0.7	30.4 ±0.9$^{\&}$	26.7 ±0.8	26.4 ±1.8
Litter size, pups	9.6 ±0.5	10.4 ±0.6	11.6 ±0.6	10.2 ±0.8
M:F Sex ratio	0.42 ±0.03	0.53 ± 0.10	0.49 ± 0.50	0.60 ± 0.06
Total litter weight, g	60.8 ± 2.5	69.0 ± 4.0	76.3 ± 4.3$^{\#}$	70.1 ± 5.4$^{\#}$
Post-pregnancy				
Post-birth weight, g	230 ±5	241 ±7*	294 ±5$^{\#}$	333 ±14$^{\#}$
Weight loss at birth, g	87 ±3	76 ±5	99 ±4	89 ±13
Weaning weight, g	266 ±4	264 ±6	305 ±5$^{\#}$	296 ±11$^{\#}$
Wt loss at weaning, g	51 ±4	64 ±6*	90 ±5$^{\#}$	112 ±11$^{*\#}$
Amylin, pM	21.8 ±2.2	23.6 ±2.0	18.1 ±1.5	21.9 ±3.1
GLP-1, pM	13.1 ±1.1	13.1 ±0.2	12.6 ±0.1	13.0 ±0.3
Insulin, pM	58.5 ±8.4	63.2 ±12.7	70.0 ±9.2	89.6 ±19.0
Leptin, pM	35.3 ±7.2	61.2 ±12.3*	72.2 ±8.6$^{\#}$	120.7±17.0$^{*\#}$
Glucose, μm	4454 ±275	4597 ±442	4930 ±299	4615 ±572
Triglycerides, μm	0.18 ±0.03	0.28±0.05	0.29±0.04	0.59±0.09
NEFA, μm	15.4 ±1.6	26.9 ±2.1	16.0 ±1.9	29.4 ±3.4
Free T3, pg/ml	2.3 ±0.5	1.2 ±0.8	2.7 ±0.4	0.6 ±0.9
Free T4, ng/dl	1.5 ±0.1	1.6 ±0.2	1.5 ±0.1	1.4 ±0.2

Pregnancy outcomes

DIO-Western females produced a litter within 60 days of mate pairing less often than did DR-Western females (43.4 vs. 93.8%, $\chi^2(1) = 6.237$, $p = 0.013$), a difference not significant in chow-fed females (67.9 vs. 85.2%, $p = 0.44$). DIO-Western females that failed to produce litters ate significantly more (66 vs. 59 kcal/day, $p < 0.005$, $F(1, 11) = 13.253$) and descriptively weighed more pre-mating (296 g vs. 276 g) than successful DIO-Western dams. Thus, the lower birth rate of DIO-Western females may reflect obesity-related infertility [66]. Rather than preventing birth altogether as in the DIO line, Western diet prolonged the number of days until birth in DR dams. Reported days until birth is days from mating, not days from impregnation, as estrous cycles were not synchronized prior to mating. DIO dams birthed heavier litters than DR dams. The number of pups and sex ratio did not significantly differ by Genotype or Diet (Table 2.2).

Maternal food intake, body weight and endocrine measures during and after pregnancy

As shown in Table 2.2, Western vs. Chow diet dams did not differ in intake or weight gain during pregnancy. Western diet dams weighed more the day after parturition than Chow dams (Diet: $p < 0.01$, $F(1, 38) = 7.833$), however. With respect to genotype, DIO dams ate more (Genotype: $p < 0.001$, $F(2, 21) = 11.265$) and gained more weight during pregnancy than DR dams ($p < 0.001$, $F(1, 28) = 31.362$) and also weighed more than DR dams through weaning (Table 2.2, $p < 0.001$, $F(1.35) = 26.638$).

By weaning (P23), Western dams lost more weight than Chow dams ($p < 0.05$, $F(1, 19) = 5.813$), and DIO dams lost more weight than DR dams ($p < 0.01$, $F(1, 24) = 11.314$). As shown in Table 2.2, irrespective of genotype, at weaning Western dams had increased plasma levels of leptin ($p < 0.01$, $F(1, 33) = 7.70$), NEFA and triglycerides ($p < 0.001$, $F(1, 30) > 12.29$) and decreased levels of free T3 ($p < 0.05$, $F(1, 31) = 14.40$) vs. chow-fed dams. Irrespective of diet, DIO dams had higher leptin ($p < 0.001$, $F(1, 33) = 17.725$) and triglyceride ($p < 0.01$, $F(1, 30) = 13.650$), but not NEFA, levels than DR dams. There were no effects involving Genotype or Diet on dam levels of amylin, GLP-1, insulin, glucose or free T4 at weaning (Table 2.2).

Thus, Western diet impaired the reproduction and potentiated the elevated

weight gain and leptin and triglyceride levels of DIO dams. In contrast, as desired, Western diet did not increase body weight, weight gain or caloric intake of DR dams during pregnancy. Despite this, Western diet ultimately decreased free T3 levels and increased maternal post-birth (P1) weight, maternal weight loss across lactation, and circulating leptin, triglyceride, and NEFA levels of DR dams by weaning.

Offspring body weight and composition

As shown in Figures 2.1 and 2.2 and Tables 2.3 and 2.4, despite no excess weight gain or energy intake in DR dams, maternal Western diet increased the adiposity of DR offspring from P1 through weaning.

By P1, maternal Western diet increased the adiposity of DR offspring. There were Genotype × Diet interactions on absolute (g) and relative fat (fat-free mass normalized) in carcass composition analysis (ps < 0.05, Fs$(1, 22) > 5.152$) (Table 2.3), reflected in greater inguinal fat pad mass ($p < 0.01$, $F(1, 22) = 8.071$) (Table 2.4). Figure 2.1B shows that Western diet increased adiposity in DR offspring to the elevated levels of DIO pups, while showing no further effect in DIO offspring.

Genotype effects indicated that DIO pups were heavier ($p < 0.01$, $F(1, 23) = 8.330$) and fatter than DR pups on P1, especially in the chow-fed condition. DIO pups had greater absolute and relative carcass fat than DR pups (Fs$(1, 22) > 4.533$, ps < 0.05) (Table 2.3). Accordingly, absolute and relative subcutaneous and retroperitoneal white fat pad and brown adipose tissue (BAT) weights were greater in DIO than DR pups (Fs$(1, 21) > 5.706$, ps < 0.05) (Figures 2.1 and 2.2 and Table 2.4).

On P9, Western diet offspring were fatter than Chow offspring in both genotypes. Maternal Western diet increased the pups' absolute and relative carcass fat (ps < 0.05, Fs$(1, 35) > 5.046$) (Table 2.3), as well as the absolute and relative weights of subcutaneous, total visceral (mesenteric, retroperitoneal, and gonadal), total non-visceral, and total white fat pads (ps < 0.05, Fs$(1, 32) > 3.791$) (Figures 2.1 and 2.2 and Table 2.4). Genotype effects reflected that P9 DIO offspring were heavier ($p < 0.01$, $F(1, 36) = 7.740$) and had greater absolute fat ($p < 0.05$, $F(1, 35) = 6.732$) (Table 2.3) and absolute weights of inguinal, subcutaneous, total visceral fat, total white fat pads and BAT (ps < 0.05, Fs$(1, 35) > 5.567$) (Table 2.3). Genotype differences in relative fat

measures approached, but did not reach, significance (ps = 0.06-0.07)

By P16, Genotype effects had further diminished, whereas Western diet effects had increased. Maternal Western diet increased P16 offspring body weight ($p < 0.05$, $F(1, 31) = 4.250$) and absolute and relative carcass fat (ps < 0.001, Fs$(1, 30) > 14.471$) (Table 2.3). Accordingly, Western diet increased the absolute and relative weights of inguinal, subcutaneous, gonadal, retroperitoneal, total visceral, total non-visceral and total white fat pads (ps < 0.05, Fs$(1, 31) > 5.015$) (Figures 2.1 and 2.2 and Table 2.4). In contrast, DIO offspring were no longer heavier or fatter overall than DR animals (Table 2.3). Still, DIO pups had heavier absolute retroperitoneal, BAT and total visceral fat pads vs. DR pups (ps < 0.05, Fs$(1, 31) > 6.780$) (Figures 2.1 and 2.2 and Table 2.4).

At P23, Western diet offspring were heavier ($p < 0.001$, $F(1, 47) = 44.220$) than Chow offspring in both genotypes. Though Western diet offspring had greater fat-free dry carcass mass ($p < 0.001$, $F(1, 47) = 15.833$), they also were fatter, with increased total and relative carcass fat (ps < 0.001, Fs$(1, 46) > 171.594$) (Table 2.3) and disproportionately heavier inguinal, subcutaneous, BAT, gonadal, retroperitoneal, total visceral, total non-visceral and total white fat pads (ps < 0.001, Fs$(1, 46) > 22.924$) (Figure 2.1 and 2.2 and Table 2.4).

Offspring leptin, insulin, adiponectin, amylin and GLP-1 levels

Table 2.5 shows Genotype effects on energy regulatory hormone levels throughout the pre-weanling period, whereas Western diet effects did not appear until P23.

On P1, Genotype effects indicated that DIO pups had higher plasma amylin ($p < 0.01$, $F(1, 34) = 7.774$), GLP-1 ($p < 0.05$, $F(1, 33) = 7.185$), leptin ($F(1, 29) = 4.420$), insulin ($p < 0.005$, $F(1, 32) = 9.347$), and adiponectin ($p < 0.005$, $F(1, 31) = 10.243$) than did DR offspring.

On P9, DIO offspring continued to show higher plasma GLP-1 ($p < 0.005$, $F(1, 12) = 11.613$) and leptin ($p < 0.001$, $F(1, 13) = 18.714$) than DR offspring. Maternal Western diet increased plasma leptin only in DIO offspring to levels exceeding all other groups (Genotype $\times$ Diet: $p < 0.005$, $F(1, 13) = 12.274$).

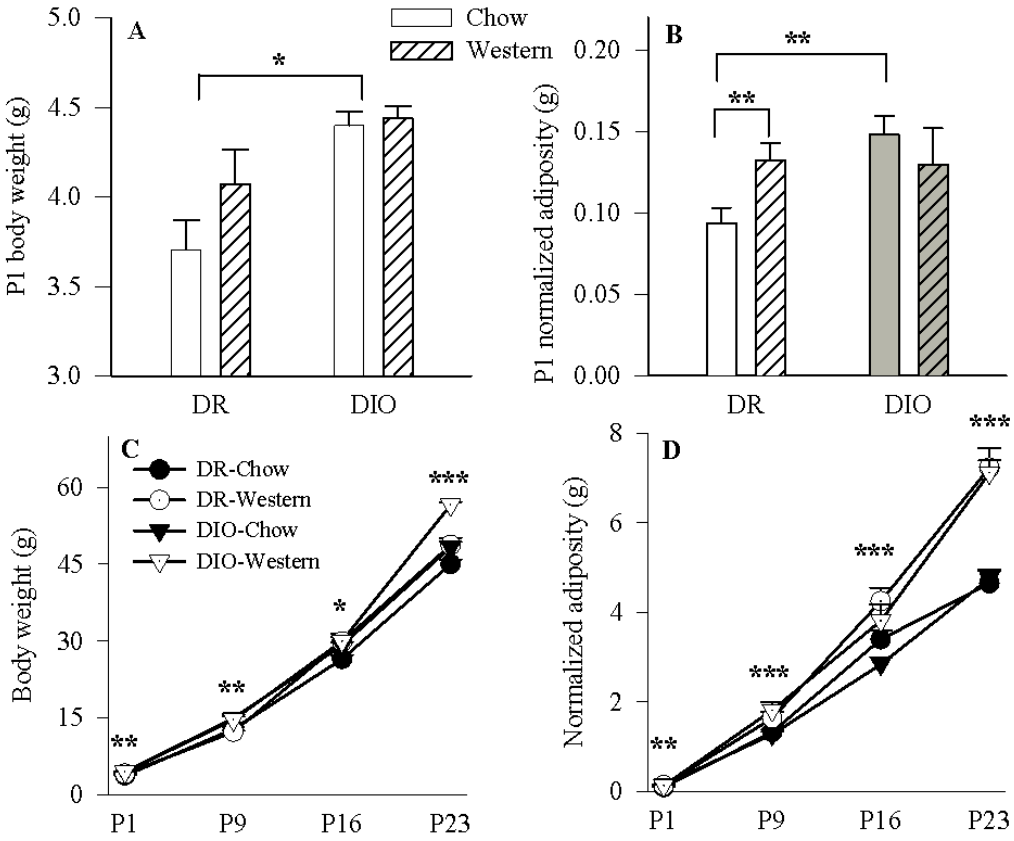

Figure 2.1: Effect of maternal Western diet and genotype on pre-weanling body weight and adiposity of offspring from genetically diet-induced obesity-prone (DIO) and resistant (DR) lines. (a) DIO pups weighed more than DR pups at P1; (b) Chemical analysis of body composition showed that DIO-Chow pups have greater relative adiposity (fat mass normalized for fat-free mass) than DR-Chow pups, and maternal Western diet selectively increased adiposity of DR pups to the higher levels of DIO pups at P1; (c) DIO pups weighed more than DR pups at P1 and P9; by P16, Western diet offspring were heavier in both genotypes thereafter, with DIO-Western offspring heaviest at P23; (d) maternal Western diet selectively increased relative adiposity only in DR pups at P1 but comparably increased relative adiposity of both genotypes thereafter through P23. Data are expressed as M + SEM. n = 4-10 per group. (a-d) * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

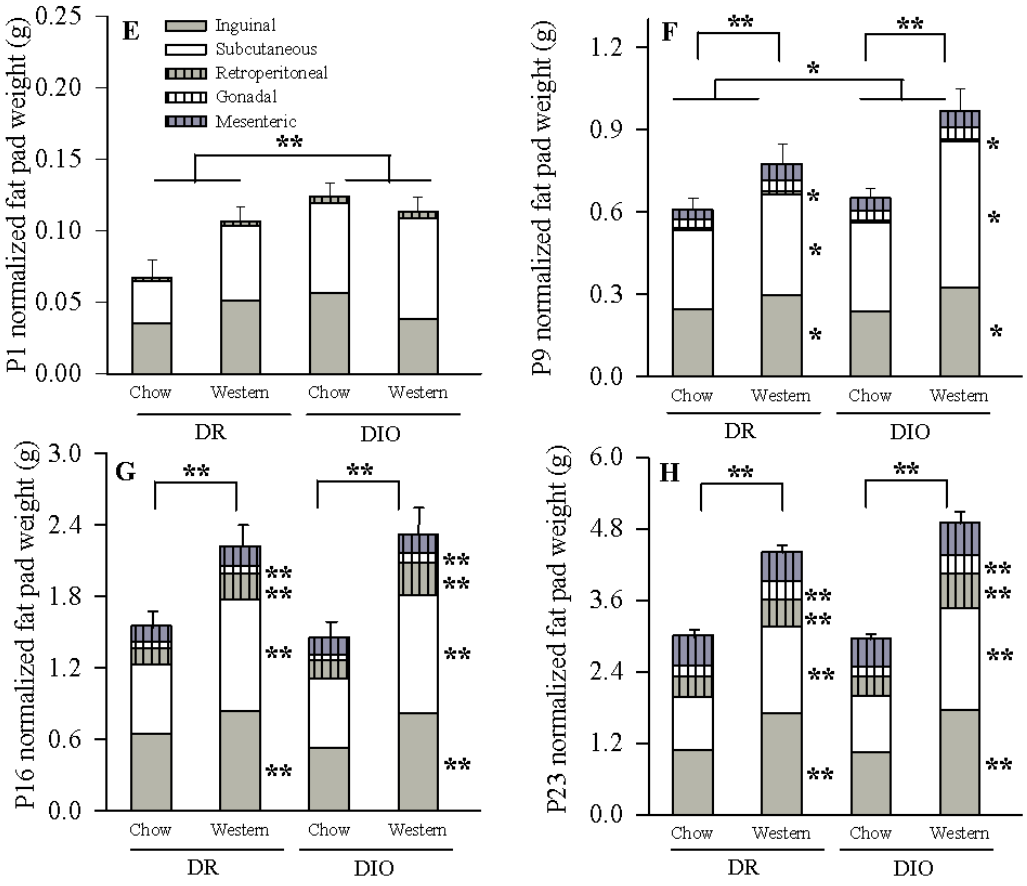

Figure 2.2: Effect of maternal Western diet and genotype on pre-weanling body weight and adiposity of offspring from genetically diet-induced obesity-prone (DIO) and resistant (DR) lines. (e) At P1, DIO pups had disproportionately heavier subcutaneous, retroperitoneal and total white fat pads than DR pups; (f) At P9, maternal Western diet offspring had disproportionately heavier inguinal, subcutaneous, retroperitoneal and total white fat pads than chow offspring, DIO genotype pups also had disproportionately heavier subcutaneous and total white fat pads than DR pups; At P16 (g) and P23 (h), maternal Western diet offspring had disproportionately heavier inguinal, subcutaneous, gonadal, retroperitoneal, total visceral, total non-visceral and total white fat pads than chow offspring. Data are expressed as M + SEM. n = 4-10 per group. (e) * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, (g-h) ** = $p < 0.001$.

Table 2.3: Chemical analysis of offspring carcass composition from postnatal day 1 (P1) to weaning (P23).

Measure	DR Chow	DR Western	DIO Chow	DIO Western
P1				
Carcass weight, g	3.7 ±0.2	4.1 ±0.2	4.4 ±0.1$^{\#}$	4.4 ±0.1$^{\#}$
Norm fat, mg	101 ±12	135 ±10$^{*\&}$	156 ±9$^{\#}$	142 ±20$^{\#}$
Fat, mg	84 ±12	132 ±11$^{*\&}$	171 ±11$^{\#}$	153 ±47$^{\#}$
FFDM, mg	555 ±25	554 ±25	620 ±15	591 ±18
Ash, mg	68 ±3	70 ±3	73 ±2	72 ±3
Water, g	3.1 ±0.1	3.4 ±0.2	3.6 ±0.1$^{\#}$	3.7 ±0.1$^{\#}$
P9				
Carcass weight, g	13 ±0.4	12 ±0.2	15 ±0.5$^{\#}$	15 ±0.6$^{\#}$
Norm fat, g	1.4 ±0.07	1.6 ±0.1*	1.3 ±0.06	1.8 ±0.1*
Fat, g	1.2 ±0.1	1.4 ±0.2*	1.5 ±0.1$^{\#}$	1.9 ±0.1$^{*\#}$
FFDM, g	2.2 ±0.1	2.1 ±0.1	2.5 ±0.1$^{\#}$	2.4 ±0.1$^{\#}$
Ash, mg	246 ±8	229 ±13	282 ±11$^{\#}$	275 ±11$^{\#}$
Water, g	9.4 ±0.3	8.8 ±0.2	11 ±0.3$^{\#}$	10 ±0.5$^{\#}$
P16				
Carcass weight, g	27 ±0.6	30 ±1.5*	29 ±0.8	30 ±0.9*
Norm fat, g	3.4 ±0.2	4.4 ±0.3*	2.9 ±0.2	3.9 ±0.3*
Fat, g	3.1 ±0.1	4.5 ±0.5*	3.2 ±0.3	4.1 ±0.4*
FFDM, g	5.2 ±0.1	5.6 ±0.2	5.7 ±0.2$^{\#}$	6.0 ±0.2$^{\#}$
Ash, g	0.6 ±0.02	0.6 ±0.03	0.6 ±0.02	0.7 ±0.01
Water, g	18 ±0.4	19 ±0.9	20 ±0.5	20 ±0.6
P23				
Carcass weight, g	39 ±0.8	44 ±1.3*	42 ±0.9	52 ±0.8$^{*\#\&}$
Norm fat, g	4.8 ±0.2	7.3 ±0.2*	4.8 ±0.1	7.6 ±0.3*
Fat, g	4.3 ±0.1	7.3 ±0.5*	4.8 ±0.2	8.8 ±0.2$^{*\#\&}$
FFDM, g	8.4 ±0.2	8.9 ±0.2*	9.1 ±0.2$^{\#}$	10 ±0.1$^{*\#}$
Ash, g	1.0 ±0.02	1.1 ±0.03	1.1 ±0.03	1.3 ±0.02
Water, g	27 ±0.5	28 ±0.8	28 ±0.5	32 ±0.6$^{*\#\&}$

Note: Data express LS Mean ±Standard error, covarying for cohort. FFDM = Fat-free dry mass, Norm = fat-free mass normalized. Carcasses are headless. * = Diet main effect, $p < 0.05$, # = Genotype main effect, $p < 0.05$, & = Genotype × Diet interaction, $p < 0.05$.

Table 2.4: Weight (mg) of offspring fat pads from postnatal day 1 (P1) to weaning (P23). Data express LS Mean ±Standard error, covarying for cohort, BAT = brown adipose tissue, Norm = fat-free mass normalized, * = Diet main effect, $p < 0.05$, # = Genotype main effect, $p < 0.05$, & = Genotype × Diet interaction, $p < 0.05$.

Measure	DR Chow	DR Western	DIO Chow	DIO Western
P1				
Norm white fat pads	67 ±13	106 ±10	124 ±10$^{\#}$	113 ±20$^{\#}$
Total white fat pads	44 ±12	101 ±15$^{*\&}$	134 ±10$^{\#}$	126 ±0.1$^{\#}$
Inguinal	23 ±7	49 ±7$^{\&*}$	61 ±4$^{\&\#}$	43 ±1
Subcutaneous	20 ±5	51 ±9*	69±7$^{\#}$	79 ±0.4$^{*\&}$
Retroperitoneal	1.2 ±0.7	2.5 ±0.6	4.6 ±0.3$^{\#}$	4.4 ±1.3$^{\#}$
Norm BAT	53 ±5	56 ±4	76 ±4$^{\#}$	65 ±9$^{\#}$
BAT	47±4	55 ±4	81 ±6$^{\#}$	70 ±10$^{\#}$
P9				
Norm white fat pads	609 ±43	774 ±83*	662 ±42	965 ±90*
Total white fat pads	559 ±40	658 ±123*	746 ±60$^{\#}$	999 ±46$^{*\#}$
Inguinal	225 ±12	254 ±43	274 ±18$^{\#}$	336 ±9$^{\#}$
Subcutaneous	265 ±29	311 ±76*	371 ±38$^{\#}$	551 ±62$^{*\#}$
Retroperitoneal	35 ±3	52 ±12*	55 ±5	61 ±10*
Gonadal	6 ±1	10 ±5	9 ±1	7 ±3
Mesenteric	27 ±4	31 ±7	37 ±4	44 ±3
Norm BAT	86 ±4	101 ±7	105 ±4	102 ±8
BAT	82 ±4	94 ±6	107 ±4$^{\#}$	104 ±3$^{\#}$
P16				
Norm white fat pads	134 ±12	161 ±7*	127 ±12	214 ±4*
Total white fat pads	1336 ±81	2277 ±275*	1589 ±161	2437 ±212*
Inguinal	555 ±31	859 ±78*	572 ±64	854 ±71*
Subcutaneous	501 ±44	959 ±182*	644 ±78	1046 ±92*
Retroperitoneal	118 ±13	222 ±23*	162 ±16$^{\#}$	288 ±40$^{*\#}$
Gonadal	47 ±5	70 ±10*	54 ±5	87 ±15*
Mesenteric	115 ±9	166 ±12*	157 ±12	164 ±3*
Norm BAT	143 ±9	169 ±10	180 ±8$^{\#}$	193 ±14$^{\#}$
BAT	137 ±5	171 ±13*	184 ±8$^{\#}$	196 ±24$^{*\#}$
P23				
Norm white fat pads	3011 ±108	4404 ±115*	2960 ±97	4900 ±198*
Total white fat pads	2660 ±120	4397 ±217*	2957 ±170	5860 ±228$^{*\#\&}$
Inguinal	961 ±433	1690 ±92*	1043 ±50	2100 ±84$^{*\#\&}$
Subcutaneous	781 ±44	1456 ±101*	947 ±76	2050 ±78$^{*\#\&}$
Retroperitoneal	300 ±18	461 ±22*	335 ±22	696 ±36$^{*\#\&}$
Gonadal	168 ±10	308 ±16*	165 ±13	368 ±33*
Mesenteric	451 ±29	481 ±20	469 ±23	647 ±56$^{*\#\&}$
Norm BAT	227 ±8	273 ±9*	278 ±7$^{\#}$	334 ±15$^{*\#}$
BAT	220 ±7	273 ±10*	278 ±8$^{\#}$	351 ±9$^{*\#}$

Table 2.5: Offspring plasma concentration (pM) of hormones from postnatal day 1 (P1) to weaning (P23).

Hormone	**DR Chow**	**DR Western**	**DIO Chow**	**DIO Western**
P1				
Adiponectin	18.1 ±1.5	17.7 ±1.3	24.1 ±2.0$^{\#}$	23.9 ±2.3$^{\#}$
Amylin	16.0 ±0.3	16.2 ±0.4	18.5 ±1.1$^{\#}$	17.0 ±0.6$^{\#}$
GLP-1	39.8 ±7.0	51.5 ±12.7	117.2 ±21.9$^{\#}$	68.5 ±15.6$^{\#}$
Insulin	49.3 ±15.4	100.9 ±29.8	231.0 ±44.3$^{\#}$	257.4 ±128.8$^{\#}$
Leptin	40.3 ±8.5	144.8 ±33.7	193.6 ±37.1$^{\#}$	204.4 ±99.9$^{\#}$
P9				
Adiponectin	38.9 ±1.3	36.7 ±6.1	38.4 ±1.3	46.0 ±0.3$^{*\&}$
Amylin	14.4 ±0.1	14.3 ±0.03	14.5 ±0.1	14.4 ±0.1
GLP-1	23.3 ±0.9	22.2 ±0.8	43.5 ±6.5$^{\#}$	42.0 ±3.8$^{\#}$
Insulin	70.5 ±11.1	50.4 ±15.8	109.6 ±11.9$^{\#}$	127.1 ±32.1$^{\#}$
Leptin	308.8 ±36.8	158.3 ±28.6	355.1 ±43.8$^{\#}$	599.1 ±38.9$^{*\#\&}$
P16				
Adiponectin	38.5 ±1.9$^{\#}$	40.5 ±1.9$^{\#}$	33.2 ±1.7	35.1 ±0.7
Amylin	13.6 ±0.4	14.5 ±0.8	13.4 ±0.3	13.1 ±0.2
GLP-1	19.1 ±0.7	20.8 ±1.1	18.4 ±0.7	18.0 ±0.7
Insulin	62.9 ±7.3	65.5 ±9.3	67.0 ±12.6	76.6 ±7.4
Leptin	195.2 ±23.1	249.8 ±30.8	290.6 ±39.7$^{\#}$	299.3 ±52.8$^{\#}$
P23				
Adiponectin	32.1 ±0.9	44.5 ±2.2*	32.8 ±1.6	50.6 ±2.3*
Amylin	22.1 ±2.9$^{\&}$	18.5 ±1.1	19.3 ±1.5	21.8 ±2.0$^{\&}$
GLP-1	20.4 ±0.9$^{\&}$	17.1 ±0.6	18.5 ±0.6	20.9 ±3.2$^{\&}$
Insulin	70.4 ±8.0	121.0 ±19.5*	99.0 ±8.2$^{\#}$	191.1 ±33.3$^{*\#}$
Leptin	155.1 ±8.0	262.7 ±28.1*	288.0 ±23.3	523.4 ±125.6*

Note: Data express LS Mean ±Standard error, covarying for cohort. * = Diet main effect, $p < 0.05$, # = Genotype main effect, $p < 0.05$, & = Genotype × Diet interaction, $p < 0.05$.

By P16, Genotype effects for GLP-1 and adiponectin had reversed in direction from younger ages. GLP-1 levels, which were higher in DIO than DR offspring on P1 and P9, now trended higher in DR offspring at P16 ($p = 0.06$, $F(1, 24) = 3.910$). Similarly, adiponectin levels, which had been greater in DIO than DR offspring on P1, were now higher in DR than DIO offspring on P16 ($p < 0.05$, $F(1, 16) = 6.966$). DIO pups continued to show higher plasma leptin than DR pups ($p < 0.05$, $F(1, 22) = 5.002$).

By P23, maternal Western diet effects appeared. Western diet offspring of both genotypes showed higher plasma insulin (Diet: $p < 0.001$, $F(1, 28) = 19.209$), adiponectin ($p < 0.001$, $F(1, 30) = 43.069$) and leptin ($p < 0.005$, $F(1, 24) = 11.986$) than Chow offspring. In addition, plasma leptin (Genotype: $p = 0.001$, $F(1, 24) = 14.266$) and insulin ($p < 0.05$, $F(1, 28) = 4.298$) were greater in DIO than DR pups. Despite equivalent body weight and adiposity at this age, DIO-Chow pups had 80% and 38% higher levels of leptin and insulin, respectively, than DR-Chow weanlings (Table 2.5). Genotype $\times$ Diet interactions reflected that Western diet increased amylin ($p < 0.05$, $F(1, 27) = 6.252$) and GLP-1 ($p < 0.05$, $F(1, 27) = 6.060$) levels in DIO offspring, while reducing levels in DR offspring.

Endocrine-adiposity correlations

As shown in Figure 2.3, maternal Western diet also disrupted otherwise seen relations of endocrine measures to adiposity. Specifically, GLP-1 levels correlated negatively with total carcass fat in DR-Chow offspring ($r[7] = -0.83$, $p < 0.05$), but positively with carcass fat in both DIO-Chow ($r[11] = 0.74$, $p < 0.01$) and DR-Western offspring ($r[7] = 0.662$). Also, whereas amylin levels correlated negatively with gonadal fat in DR-Chow offspring ($r[6] = -0.80$, $p < 0.05$) and positively in DIO-Chow offspring ($r[11] = 0.75$, $p < 0.01$), maternal Western diet abrogated any relation of amylin to gonadal fat in DR offspring ($r[7] = -0.09$) (Figure 2.3).

Furthermore, plasma leptin normally correlated positively with subcutaneous ($r[28] = 0.52$, $p < 0.005$) and total white fat pads ($r[28] = 0.57$, $p < 0.005$) across offspring. Such correlations were evident in DIO-Chow (rs = 0.53, 0.47) and DR-Chow (rs = 0.74, 0.60), but not DR-Western (rs = 0.05, 0.02), offspring (Figure 2.3). Similarly, insulin levels normally correlated positively with visceral fat pad mass across all offspring ($r[32] = 0.62$, $p < 0.005$), and in both DIO-Chow ($r = 0.44$) and DR-Chow groups ($r = 0.48$). Maternal Western diet, instead, produced an inverse relation of insulin with visceral fat pad mass in DR-Western offspring ($r = -0.53$) (not shown). Finally, across all weanlings, plasma adiponectin correlated positively with fat measures (rs = 0.51-0.79, ps < 0.01). A positive relationship was seen with relative carcass fat in DR-Chow ($r[12] = 0.76$) and DIO-Chow ($r[11] = 0.79$), but not DR-Western ($r[9] = -0.45$).

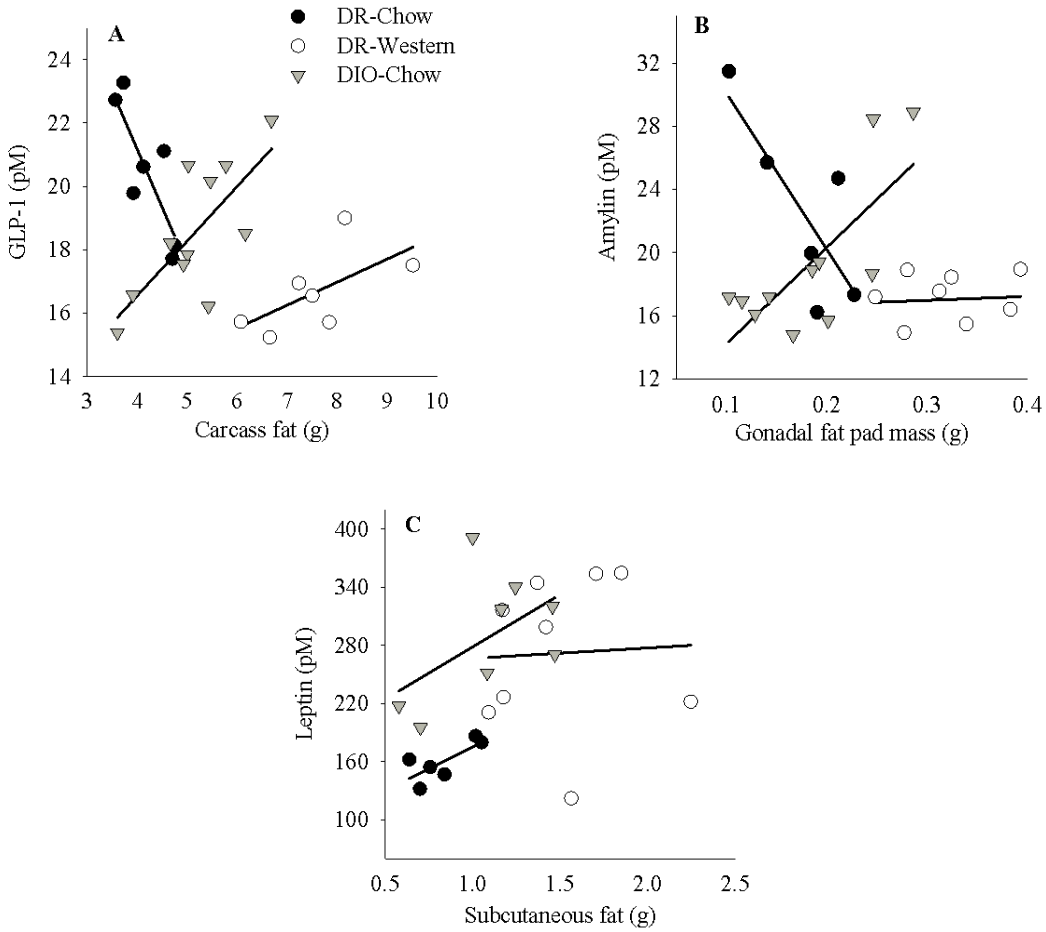

Figure 2.3: Scatterplot showing genotype differences in and the effects of maternal Western diet on the relations of GLP-1, amylin and leptin with body fat measures at weaning. GLP-1 correlated negatively with total carcass fat in DR-Chow offspring (r=-0.83, p¡0.05), but positively in DIO-Chow and DR-Western offspring (r=0.74 and 0.66, respectively). Amylin correlated positively with gonadal fat in DIO-Chow weanlings (r=0.75, p¡ 0.05) and negatively in DR-Chow (r= -0.80, p¡0.05) offspring. In contrast, no reliable correlation was seen in DR-Western offspring (r= 0.09). Leptin correlated positively with subcutaneous body fat in DIO-Chow (r=0.53) and DR-Chow offspring (r=0.74), but not in DR-Western offspring (r=0.05). Regression-lines illustrate the relation for each experimental group. n = 6-11 per group (see also text).

Maternal leptin and weight loss predict offspring body weight, adiposity and endocrine status

As Table 2.2 shows, Western dams lost more weight from the day before birth to weaning and showed greater post-weaning leptin levels than chow-fed dams. Dams with

Table 2.6: Offspring weight and body composition in adolescence and early adulthood.

Measure	DR Chow	DR Western	DIO Chow	DIO Western
Day 37				
Body weight, g	142 ±3.3	139 ±4.7	153 ±3.9	150 ±12.4
Day 60				
Body weight, g	317 ±4.5	321 ±5.9	369 ±5.0$^{\#}$	374 ±8.2$^{\#}$
Lean mass, g	244 ±5.3	255 ±5.3	283 ±5.3$^{\#}$	282 ±5.3$^{\#}$
Fat mass, g	30 ±1.9	29 ±1.9	40 ±1.9$^{\#}$	44 ±2.2$^{\#}$
Norm. fat, g	35 ±2.0	31 ±1.7	36 ±1.9	40 ±2.0$^{\#\&}$

Note: Data express LS Mean ±Standard error, covarying for cohort. Norm. = lean mass normalized. * = Diet main effect, $p < 0.05$, # = Genotype main effect, $p < 0.05$, & = Genotype × Diet interaction, $p < 0.05$.

greater leptin levels had heavier weanlings with more subcutaneous fat ($r[35] = 0.44$ and 0.46, $p < 0.01$). Dams with greater weight loss at weaning had heavier weanlings ($r[39] = 0.43$, ps < 0.01) with higher plasma leptin ($r[27] = 0.47$, $p < 0.05$).

Adult body weight and composition

All offspring were weaned onto chow. By day 37, there were no effects of Genotype or Diet on body weight. At 2 months of age, DIO offspring were heavier than DR offspring, but only DIO-Western offspring were disproportionately fattier than DR-Western offspring, with no difference in normalized fat between DIO-Chow and DR-Chow (Table 2.6). In contrast, at 150 days of age, maternal Western diet offspring of both genotypes were heavier (Diet: $p < 0.001$, $F(1, 51) = 16.900$) and disproportionately fatter ($p = 0.009$, $F(1, 51) = 7.315$) than maternal Chow diet offspring (Figure 2.4).

Food intake

Within the week after weaning (P25-30), DR-Western offspring showed greater 2-day chow intake (M±SEM: 23.4±0.9 kcal/day) than did DR-Chow offspring (19.7±0.9 kcal/day) ($p < 0.05$, $F(1, 6) = 8.696$). Figure 2.4 shows that mid-adult (P150-P160) DR-Western offspring also ate ∼10 kcal/day more chow in their home cages (or 14%) than did DR-Chow offspring ($p < 0.05$, $F(1, 18) = 5.379$). DIO-Chow rats ate ∼17

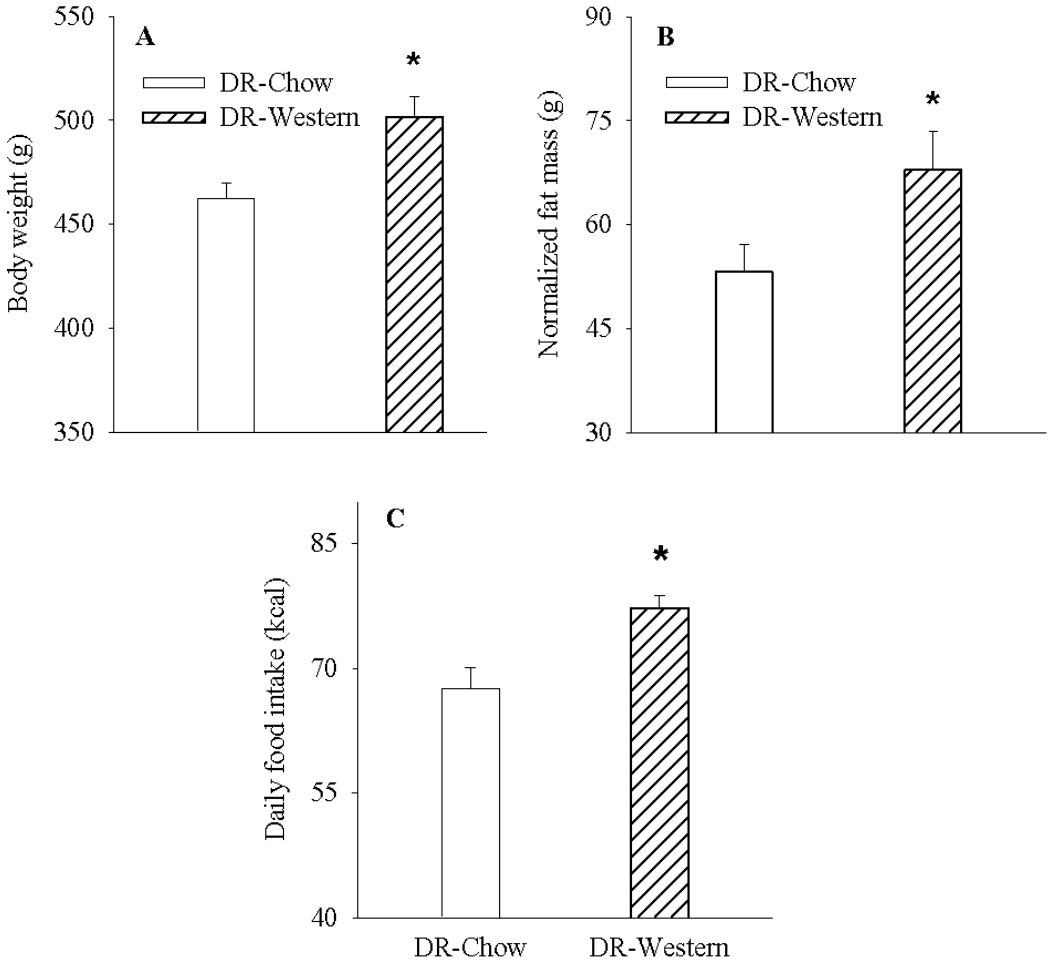

Figure 2.4: Maternal Western diet increased (a) body weight, (b) adiposity, or fat mass after covarying for lean mass in quantitative nuclear magnetic resonance body composition analysis, and (c) daily home cage chow intake in 150-day old DR offspring. All offspring were fed only standard chow beginning from weaning. Food intake is the average daily intake across 7 days of daily measurement. Data are expressed as $M+$ SEM. $n = 8$ per group. * = maternal Diet effect, $p < 0.05$

kcal/day more chow (25%) than did DR-Chow offspring ($p < 0.001$, $F(1, 22) = 17.449$) in their home cages and also showed greater daily intake while housed in the indirect calorimetry chambers ($p < 0.05$, $F(1, 16) = 6.316$). Cosinor analysis revealed that adult DIO offspring had a greater amplitude, MESOR ($ps < 0.05$, $F(1, 16) > 8.1$) and peak ($p < 0.01$, $F(1, 16) = 15.873$) of their circadian rhythm of feeding than DR offspring (Table 2.7).

Table 2.7: Cosinor analysis of offspring energy expenditure and food intake.

Measure	DR Chow	DR Western	DIO Chow	DIO Western
Day 25–30				
Energy expenditure				
Amplitude	0.13 ±0.02	0.30 ±0.03*	–	–
MESOR	2.0 ±0.1	1.8 ±0.1	–	–
Peak	2.1 ±0.1	2.0 ±0.1	–	–
Nadir	1.9 ±0.1	1.5 ±0.1	–	–
r^2	0.33 ±0.09	0.73 ±0.09*	–	–
Day 60–90				
Energy expenditure				
Amplitude	0.45 ±0.04	0.25 ±0.04*	0.34 ±0.04#	0.19 ±0.04*#
MESOR	2.0 ±0.06	1.9 ±0.05*	2.1 ±0.06	1.8 ±0.06*
Peak	2.4 ±0.06	2.1 ±0.05*	2.5 ±0.06	2.0 ±0.06*
Nadir	1.5 ±0.08	1.6 ±0.07	1.8 ±0.08	1.6 ±0.08
r^2	0.81 ±0.13	0.58 ±0.12	0.61 ±0.13	0.45 ±0.13
Food intake				
Amplitude	1.5 ±0.2	1.8 ±0.2	2.0 ±0.2#	2.4 ±0.2#
MESOR	2.6 ±0.2	2.4 ±0.2	3.0 ±0.2#	3.1 ±0.2#
Peak	4.1 ±0.3	4.2 ±0.2	5.0 ±0.3#	5.5 ±0.3#
Nadir	1.1 ±0.3	0.6 ±0.3	1.1 ±0.3	0.7 ±0.3
r^2	0.50 ±0.12	0.39 ±0.11	0.47 ±0.12	0.52 ±0.12

Note: Data express LS Mean ±Standard error, covarying for cohort. * = Diet main effect, $p < 0.05$, # = Genotype main effect, $p < 0.05$, & = Genotype × Diet interaction, $p < 0.05$.

Energy expenditure

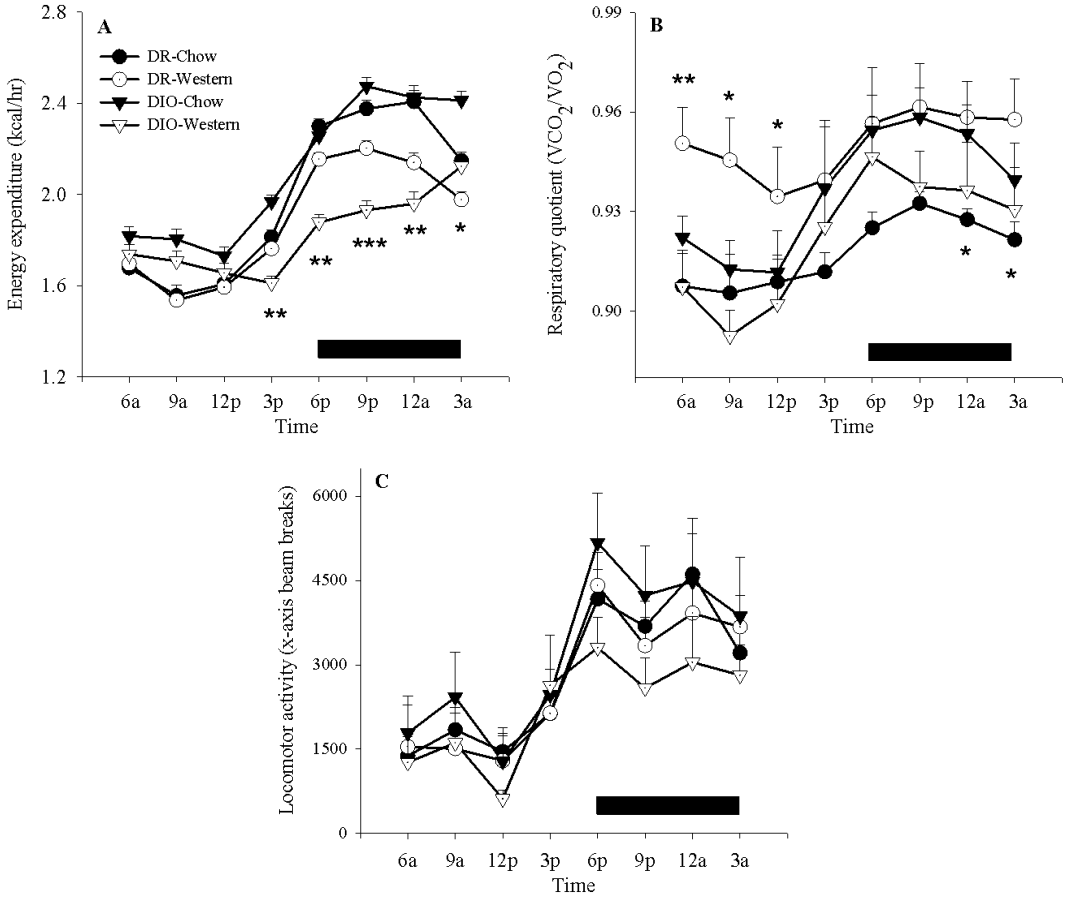

Figure 2.5: Effects of maternal Western diet and genotype on energy expenditure, respiratory quotient and locomotor activity in young adult (day 60-90) offspring. (a) Western diet offspring showed lower daily energy expenditure than did Chow diet offspring ($p = 0.003$), with significant differences evident during the dark ($p < 0.001$), and not light ($p > 0.15$), cycle; pairwise Diet p values ¡ 0.01 from T4 – T8. There was no Genotype effect on energy expenditure. (b) Western diet selectively increased respiratory quotient in DR offspring across the 24-hour period. (c) Neither maternal Western diet nor Genotype significantly altered locomotor activity of young adult offspring. $n =$ 6 per group. Symbols indicate * = maternal Diet effect $p < 0.05$, ** = $p < 0.01$, ** = $p < 0.001$; & = Diet × Genotype interaction $p < 0.05$, && = $p < 0.01$.

Maternal Western diet did not alter the total daily energy expenditure or respiratory quotient of weanlings (P25–P30). However, cosinor analysis revealed that the daily rhythm of DR-Western offspring energy expenditure did have a lower nadir ($p < 0.05$, $F(1, 5) = 6.689$) and greater amplitude ($p < 0.01$, $F(1, 5) = 21.620$) at this age (Ta-

ble 2.7). By 2-3 months of age, before maternal Western diet offspring weighed more or were fatter than chow-fed offspring, maternal Western diet offspring of both genotypes expended less energy during the dark cycle than controls (Figure 2.5A), leading to reduced daily energy expenditure. DIO-Western energy expenditure was even lower than that of DR-Western offspring. Respiratory quotient was higher for young adult DR-Western than DR-Chow offspring (Figure 2.5B), indicating relatively less lipid utilization as a fuel substrate. Locomotion did not differ significantly among groups (Figure 2.5C), indicating that maternal Western diet reduced offspring energy expenditure independent of locomotor activity. As shown in Table 2.7, cosinor analysis revealed that maternal Western diet decreased the MESOR ($p < 0.01$, $F(1, 17) = 12.854$), amplitude ($p < 0.001$, $F(1, 17) = 21.221$) and peak ($p < 0.001$, $F(1, 17) = 42.508$) of the circadian rhythm of energy expenditure. DIO offspring showed a lower amplitude than DR offspring ($p < 0.05$, $F(1, 17) = 5.619$).

DNA Methylation

Consistent with the presence of epigenetic changes in energy balance circuitry, at P1, but not P23, maternal Western diet and DIO genotype each independently reduced global DNA methylation in the lateral hypothalamus (ps < 0.05, Fs$(1, 17) > 6.17$).

2.3 Discussion

The present study shows that a maternal Western diet environment can promote offspring obesity in rats even when the mother shows little overt effect of the diet. Maternal Western diet increased offspring body weight and adiposity, including visceral fat, not only in obesity-prone DIO rats, but also in offspring of obesity-resistant DR dams. Thus, maternal Western diet partly overrode the offspring's genetic resistance to diet-induced obesity. Increased obesity in offspring has previously been reported following maternal exposure to high-fat and/or energy-dense diets [46, 48, 49]. Teratogenic effects typically have been attributed to maternal overnutrition or obesity (e.g., [58, 60]). Here, in contrast, we show in rats that exposure to a typical Western diet alone can produce metabolic changes in the offspring that promote obesity, despite the

mother showing normal energy intake, body weight and pregnancy weight gain. Thus, maternal Western diet covertly "programmed" increased risk of adiposity in offspring of genetically-resistant mothers.

Table 2.8: Body composition percentages (% of body weight). Data express LS Mean ±Standard error, covarying for cohort. FFDM = Fat-free dry mass, Carcasses are headless. * = Diet main effect, $p < 0.05$, # = Genotype main effect, $p < 0.05$, & = Genotype × Diet interaction, $p < 0.05$.

Measure	DR Chow	DR Western	DIO Chow	DIO Western
P1				
% Carcass fat	2.2 ±0.3	3.2 ±0.2$^{*\&}$	3.9 ±0.2$^{\#}$	3.5 ±1.1$^{\#}$
% White fat pads	1.3 ±0.2	2.4 ±0.3	3.0 ±0.2$^{\#}$	2.8 ±0.04$^{\#}$
% BAT	1.3 ±0.1	1.4 ±0.1	1.8 ±0.1$^{\#}$	1.6 ±0.2$^{\#}$
% FFDM	15 ±0.1$^{*\#}$	14 ±0.3$^{\#}$	14 ±0.2*	13 ±0.2
% Ash	1.8 ±0.03$^{\#}$	1.7 ±0.04$^{\#}$	1.7 ±0.03	1.6 ±0.04
% Water	83 ±0.2	83 ±0.3	82 ±0.2	83 ±0.9
P9				
% Carcass fat	9.6 ±0.3	12 ±1.5*	9.9 ±0.4	13 ±0.2*
% White fat pads	4.3 ±0.2	5.4 ±1.0*	5.2 ±0.3$^{\#}$	6.9 ±0.5$^{*\#}$
% BAT	0.64 ±0.03	0.77 ±0.06	0.75 ±0.02	0.72 ±0.05
% FFDM	17 ±0.4	17 ±0.6	17 ±0.4	17 ±0.1
% Ash	1.9 ±0.03	1.9 ±0.1	1.9 ±0.1	1.9 ±0.02
% Water	73 ±0.5*	72 ±0.9	73 ±0.6*	70 ±0.2
P16				
% Carcass fat	12 ±0.3	15 ±1.0*	11 ±0.7	14 ±1.1*
% White fat pads	5.0 ±0.3	7.5 ±0.7*	5.4 ±0.4	8.1 ±0.6*
% BAT	0.52 ±0.02	0.58 ±0.05	0.63 ±0.02$^{\#}$	0.65 ±0.07$^{\#}$
% FFDM	20 ±0.2	19 ±0.3	20 ±0.3	20 ±0.9
% Ash	2.2 ±0.04	2.2 ±0.1	2.2 ±0.03	2.2 ±0.1
% Water	69 ±0.3*	66 ±0.7	70 ±0.5	66 ±1.0*
P23				
% Carcass fat	11 ±0.2	16 ±0.4*	11 ±0.3	17 ±0.3*
% White fat pads	5.9 ±0.2	9.0 ±0.3*	6.1 ±0.2	10.3 ±0.3$^{*\#\&}$
% BAT	0.49 ±0.02	0.56 ±0.02*	0.58 ±0.02$^{\#}$	0.62 ±0.02$^{*\#}$
% FFDM	21 ±0.1*	20 ±0.3	22 ±0.1*	20 ±0.1
% Ash	2.6 ±0.02*	2.5 ±0.1	2.6 ±0.02*	2.5 ±0.02
% Water	68 ±0.1$^{*\#}$	64 ±0.3$^{\#}$	67 ±0.3*	63 ±0.3
Day 60				
% Fat	9.5 ±0.4	8.8 ±0.4	10.8 ±0.4$^{\#}$	11.6 ±0.5$^{\#}$
% Lean	77 ±0.3	78 ±0.3$^{*\&}$	77 ±0.3$^{*\&}$	75 ±0.4

While DR maternal Western diet dams did not show greater energy intake, pre-pregnancy body weight or leptin levels, or weight gain during pregnancy, they were distinguishable by several more covert characteristics. DR-Western dams had greater weight the day after birth, greater weight loss during the lactation period, higher levels of leptin, triglycerides and NEFA at weaning and lower free T3 at weaning. Elevated maternal circulating levels of leptin, triglycerides and NEFA in utero and/or during lactation may have developmentally affected DR-Western offspring by programming them for greater fat accumulation and/or leptin resistance [67]. For example, high materal leptin levels may have disrupted offspring thyroid function or development [68, 69], leading to reduced metabolic rate later in life. The increased maternal weight loss through lactation may have altered offspring development via greater transfer of energy or markers of lipid metabolism to the suckling pups.

Early and latent effects of maternal diet

The overt effects of maternal Western diet on offspring followed a discontinuous time course in otherwise obesity-resistant rats. That is, effects of maternal Western diet on body weight and adiposity went away during adolescence (P37) and early adulthood (P60). Yet, indirect calorimetry at 2-3 months of age, when Western offspring showed normal body weight and body composition, revealed a latent metabolic vulnerability [70, 71] of decreased daily energy expenditure and lipid utilization, and weanling Western offspring also ate more food. Subsequently, by mid-adulthood (P150), maternal Western diet offspring still ate more and again weighed more and were fatter than controls. Similarly, Howie, et al, found that offspring of dams fed a high-fat diet during pregnancy and lactation were normal-weight during adolescence but became overweight by adulthood [72]. The results resonate with findings in humans that overweight [73] and weight gain [74] in childhood predict adult obesity. They novelly suggest, however, that a battle with overweight may remain ahead even if children "thin out" during adolescence and young adulthood, times of rapid growth, because latent, early programmed metabolic vulnerabilities persist.

Maternal diet effects on endocrine profile and energy expenditure

Early Western diet, independent of maternal obesity or overeating, increased leptin, insulin and adiponectin levels in weanlings of both genotypes. The hyperinsulinemia and hyperleptinemia observed in Western offspring resembles findings from previous studies [60]; the present study showed that these differences are not seen very early in life (P1, P9), and, contrary to previous interpretations, do not depend upon maternal obesity or excess caloric intake because they were seen in DR offspring. In adulthood, adiponectin, an adipocytokine known for its insulin-sensitizing properties, correlates inversely with fat mass. In human neonates, however, cord and serum adiponectin levels correlate positively with body weight [75, 76], and adiposity [76], consistent with the positive relations seen here in weanlings. The results support the hypothesis that the endocrine triad of hyperleptinemia, hyperinsulinemia and hyperadiponectinemia early in life predicts increased vulnerability for later weight gain and obesity [77, 78]. Leptin levels normally peak between P7-P10 in rat neonates [79], and leptin antagonism during the neonatal period influences long-term body weight and composition [80]. Perhaps the atypical leptin profile of DR-Western offspring as compared to other groups during this critical developmental period contributed to their increased obesity risk later in life.

Maternal Western diet also disrupted the normal relations of several energy regulatory hormones (GLP-1, amylin, leptin, insulin, adiponectin) with adipose measures. In chow-fed weanlings of both genotypes, plasma leptin and insulin correlated positively with subcutaneous and visceral fat pad mass, respectively, consistent with findings in young children [81, 82, 83]. In contrast, these putative adiposity signals [84] were no longer positively correlated with fat pad measures in Western diet offspring, suggesting disruption of the hormones' regulatory relations. Similarly, maternal Western diet eliminated genotype differences that otherwise were seen in the correlations of amylin and GLP-1 with body fat. Specifically, levels of the incretin gut hormone GLP-1 correlated positively with total carcass fat in chow-fed DIO weanlings, but negatively in DR weanlings. The pancreatic hormone amylin, co-secreted with insulin, likewise correlated positively with gonadal fat in DIO weanlings, but negatively in DR weanlings. Maternal Western diet eliminated the inverse relation of amylin to gonadal fat in DR rats and produced a DIO-like, positive relation of GLP-1 to overall adiposity. The genotype dif-

ferences of chow-fed offspring in these correlations are consistent with the putative role of amylin as a negative feedback regulator of adiposity [85, 86] and of prandially-secreted GLP-1 as a central negative feedback control of food intake [87, 88]. In particular, the negative correlations seen in obesity-resistant, chow-fed DR rats may reflect efficacious negative feedback that is disrupted by the maternal Western diet environment. Conversely, the positive relation in the obesity-prone DIO line and, for GLP-1, DR-Western offspring, may indicate relative signaling resistance [89].

Maternal Western diet reduced offspring whole-body energy expenditure and relative utilization of lipid as a fuel substrate, but the physiological mechanisms for these effects remain to be determined. Changes in metabolism and lipid utilization of adipose tissue and skeletal muscle are plausible candidates, and future studies can address this by examining AMPK phosphorylation/activation, lipoprotein lipase activity, or mitochondrial bioenergetics in relevant tissue.

Comparison to previous studies

The present findings superficially appear to contrast from previous reports that maternal Western diet increases body weight and adiposity and alters endocrine profiles only in DIO and not DR offspring [57, 60]. A key procedural difference between studies is that dams in the present study were fed the Western diet for almost 8 weeks prior to mating, beginning from adolescence; those in previous studies received the diet for only 4 weeks from young adulthood. Other procedural differences here include the use of more dams to reduce litter effects [61] and litter sizes of 6–8, rather than 10, pups across lactation [90]. Finally, and perhaps most relevant, offspring in the previous studies were examined from 4-16 weeks of age. At similar ages in the present study (37, 60 and 90 days) maternal Western diet likewise did not alter offspring body weight or adiposity. Rather, diet effects were evident throughout the preweaning period (P1–P23), consistent with previous findings in P16 DR offspring [60], and, novelly, not again until mid-adulthood (150 days). Thus, overt effects of Western diet in adult DR offspring may have been missed in previous studies due to their time course across development.

Time course of vulnerability to maternal diet

To model societal conditions, Western diet was provided to adolescent dams before mating as well as during pregnancy and lactation. Therefore, it is not clear which time period of dietary exposure is critical for the present outcomes. Maternal diet influenced morphometric measures by P1 in DR offspring, suggesting a prenatal action. On the other hand, a postnatal action may contribute because diet-related differences in offspring body weight, adiposity, and regulatory hormones became more prominent postnatally. Differences grew from P1 to P16, during which offspring nutrition was derived almost exclusively from breast milk, and from P16 to P23, during which pups increasingly consume the home cage diet [91]. Breast milk of dams fed a 60% kcal fat diet is higher in fat, protein and overall nutritional content than the milk of chow dams, and offspring of high-fat dams consume more milk per kg body weight [92]. Indeed, a previous cross-fostering study in outbred rodents showed that a postnatal high-fat diet promoted offspring adiposity more than a prenatal high-fat diet [93]. Still, effects that first emerge late in the postnatal period might still reflect in utero or even pre-conception epigenetic programming. Cross-fostering and embryo transplant studies can resolve these uncertainties [94, 95] to allow identification of programming molecular mechanisms.

Genotype differences in dams and pups

Two sets of genotype differences in dams merit further discussion. First, DIO dams ate more, weighed more prior to pregnancy, gained more weight during pregnancy, lost more weight across lactation, and had higher plasma leptin than did DR dams. Each of these factors might contribute to the obesity-risk of their offspring above and beyond genomic modes of transmission. Second, Western diet selectively impaired the ability of DIO females to reproduce, especially in dams that ate more and had higher leptin levels, potentially representing a model of obesity infertility [71]. Paternal phenotype also likely contributed to the observed genotype differences. Paternal lineage and associated obesity risk were an intended part of the offspring genotype.

Genotype differences in the early adiposity and endocrine profiles of chow-fed DIO vs. DR preweanlings also merit mention, because they may mark genetic risk for adult obesity. On P1 and P9, DIO pups were heavier and fatter than DR pups, differences that

were associated with greater amylin, GLP-1, leptin, insulin and adiponectin levels. At P16, a transition was evident. DIO pups were no longer heavier or overall fatter than DR pups and showed lower GLP-1 and adiponectin levels than DR offspring. By weaning, chow-reared DIO and DR pups were indistinguishable from one another in body weight and adiposity, yet DIO offspring remained hyperleptinemic and hyperinsulinemic. As adolescents (P37), chow-fed DIO rats still showed similar body weights to DR rats, but, by young adulthood (2-3 months), DIO rats ate more, weighed more and were fatter than DR rats. The remitting-relapsing time course of overweight and fattiness in the obesity-prone DIO line, like that induced by maternal Western diet, reinforces the concept that overt morphometric and endocrine markers of obesity risk can be detected very early in life, but may normalize during growth to puberty or adolescence, before manifesting as increased obesity risk in adulthood. Both the inherited and Western-diet induced forms of increased obesity risk were associated with hyperinsulinemia and hyperleptinemia at weaning, supporting the hypothesis that early postnatal resistance to leptin or insulin may foreshadow adult obesity [77, 78].

Possible epigenetic mechanism

A possible mechanism for the effects of maternal diet on offspring outcome is epigenetic modifications to the genome. Early-life environment can program individuals for neurodevelopmental disorders and obesity [96], and alterations in DNA methylation can increase risk for metabolic disorders by altering the expression of energy regulatory genes [97]. Indeed, the primate epigenome is altered by maternal high fat diet [98], and epigenetic modifications to circadian rhythm-related genes can alter energy expenditure [99]. In the present study, global DNA methylation levels were reduced in the lateral? hypothalamus of P1 offspring by either maternal Western diet or DIO genotype, suggestive of major neurodevelopmental effects in this region. The lateral hypothalamus, a key node in the regulation of energy homeostasis, reciprocally connects to other hypothalamic nuclei that subserve the control of food intake and energy intake as well as the the caudal hindbrain. Accordingly, neurons in the the lateral hypothalamus abundantly express known regulators of energy balance, including leptin receptors, melanin-concentration hormone, and orexin [100, 101, 102]. Ongoing studies in our laboratory are examining

the effects of maternal Western diet on the expression and methylation of specific genes in the lateral hypothalamus that may modulate obesity risk.

Prevailing dietary guidelines for pregnancy and lactation recommend a diet of no more than 30-35% (%kcal) fat [55, 54], fat proportions that resemble a typical Western diet. Current obstetric practice and popular guidelines emphasize screening for overweight, BMI, pregnancy weight gain, glucose intolerance, or gestational diabetes [54, 103]. The present findings indicate that, in the rat, maternal dietary fat proportions as low as 32% lead to metabolic changes in the offspring that may promote obesity [70, 71], even while the body weight, weight gain, energy intake, and fasting insulin status of the mother appear normal. The results emphasize the importance of diet *per se*. Additional research is needed to identify ranges of maternal dietary fat in humans that are not associated with increased offspring overweight. Nutritional counseling should be standard care for all prospective and lactating mothers, not only those showing signs of overweight or metabolic disturbance.

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Chapter 2, in full, is currently in preparation for submission for publication as the manuscript Frihauf JB, Fekete EM, Nagy TR, Levin BE, Zorrilla EP. Maternal western diet promotes obesity even in male offspring of obesity-resistant rat dams: early endocrine markers. The dissertation author was the primary investigator and author of this paper.

Chapter 3

Effects of Maternal Western Diet on Female Offspring Adiposity, Endocrine Measures and Energy Expenditure

Obesity is a large and growing problem in the United States. Obesity increases risk for cardiovascular disease, metabolic syndrome, type 2 diabetes and poor quality of life [1, 2, 3, 4, 5]. The goal of this study is to provide a better understanding of early-life factors that may contribute to obesity risk.

Because obesity is highly heritable [30, 31], the current study uses the DIO-DR rat model of obesity risk and resistance developed by Barry Levin [33, 34] to assess the contributions of genetic factors to obesity susceptibility. The obesity-prone DIO line was selectively bred from Spague-Dawley rats that responded to a high-energy "Western" diet by gaining excess fat, while the obesity-resistant DR line did not gain fat after high-energy diet exposure. The two lines provide a polygenic animal model of human obesity [35].

While genetic factors likely play a large role in determining obesity susceptibility, environmental factors also contribute to obesity risk. The perinatal environment has been shown to be important in the development of energy balance systems [104, 105].

Maternal obesity during pregnancy and lactation as a result of overnutrition increases offspring obesity susceptibility [48, 49]. Most studies have emphasized maternal obesity and not maternal diet per se, so the effects of maternal high-energy diet consumption during pregnancy in the absence of maternal obesity remain unclear. Previous maternal diet studies in DIO and DR rats [57, 106] found few effects of maternal high-energy diet in diet-resistant DR offspring, but only male offspring were studied, and only from 4-16 weeks of age. The current study seeks to determine the relative contributions of genetic background and perinatal environment by studying the effects of maternal diet in young and adult female DIO and DR offspring.

Sex differences in offspring response to maternal overnutrition have been reported, highlighting the importance of studying female offspring. In rabbits, maternal high fat diet alters placental gene expression and fatty acid concentrations sex-specifically [107]. In mice, maternal obesity during gestation but not lactation was sufficient to induce overweight, insulin resistance and hyperleptinemia in male offspring but not female offspring. In a study on offspring metabolic profiles, male offspring of high-fat diet-induced obese mice had higher triglyceride, leptin and insulin levels compared to female offspring [108]. A multi-generational study showed that third generation female but not male offspring of high-fat-fed dams have large bodies and insulin insensitivity, suggesting sex-specific epigenetic programming by maternal diet [109]. Several other studies have shown sex specificity in epigenetic programming by prenatal maternal stress or diet [110, 111]. Therefore this study reports data from female offspring as a companion to the concurrently published study on male offspring.

Maternal overweight during gestation leads to hyperleptinemia, hyperinsulinemia, alterations in adiponectin signaling [112], and an exacerbated response to high-fat diet [113, 114, 115, 116] in adult offspring. Previous studies were done only in male offspring and examined the effects of maternal obesity. Studies have not been done to assess the long-term effects of maternal diet, with or without accompanying maternal obesity, in females. The current study seeks to determine the distinct effects of maternal diet and maternal obesity in female offspring.

3.1 Methods

Subjects

DR and DIO breeders, offspring of rats from the original colonies of DIO and DR rats bred by Levin et al [33, 34], were born at The Scripps Research Institute. Rats resided in wire-topped plastic cages in a 12:12-hr light-dark cycle (600 lights on), humidity- (60%) and temperature- (22°C) controlled vivarium with access to chow (LM-485 7012, 17% [kcal] fat, 58% carbohydrate, 25% protein; 3.1 kcal/g; 16% saturated, 26% monounsaturated and 58% polyunsaturated fats; 2.7% kcal from saturated fat, Harlan, Indianapolis, IN) and water ad libitum prior to experiments. Procedures adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and the *Principles of Laboratory Animal Care* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Diet and breeding

At 46–47 days of age, female DR and DIO rats were randomly divided into Western vs. chow diet conditions, yielding 4 groups: DR-Chow (n=28), DR-Western (n=16), DIO-Chow (n=28) and DIO-Western (n=16). Western diet dams received a moderate-fat diet ad libitum (Research Diets D12266B, 32% fat, 51% carbohydrate, 17% protein; 4.41 kcal/g; 26% saturated, 27% monounsaturated and 47% polyunsaturated fats, 8.5% kcal from saturated fat, New Brunswick, NJ). Chow dams received chow. The Western diet resembles the dietary intake of adult American women in fat (32-33%), saturated fat (10-11%), carbohydrate (50-53%) and protein (15-16%) proportions, http://www.cdc.gov/nchs/data/ad/ad334.pdf).

After 54 days of diet exposure, during which food and body weight were measured 3 times weekly, females were housed with males of the same genotype. Males were removed following pregnancy confirmation; dams continued to receive their experimental diet until weaning. The day of birth was termed postnatal day 0 (P0). Litters were culled to 8 pups (∼1:1 sex ratio) at P1. All offspring were weaned onto standard chow on P23.

Dam characteristics are reported in the companion paper describing the outcome of male offspring.

Because only DIO females that were comparatively resistant to the Western diet produced offspring (see Results), the DIO-Western group had selective attrition of obesity risk factors. Therefore, interpretation of the DIO-Western offspring data is complicated by this caveat, so we focus attention on the 3 other treatment groups in the Results and Discussion (Sections 3.2 and 3.3).

Body weight and composition

At P1, P4, P9, P16 and P23, all female pups from each litter were weighed (0.01 g precision scale), to determine the average female pup weight for each litter. Adult offspring body composition was measured via nuclear magnetic resonance imaging (EchoMRI-1100, Software version 2008.01.18M, Echo Medical Systems, Houston, TX). For measurements in adulthood, offspring were weighed individually within 3 days of the nominal age; the mean age at weighing did not differ between groups.

Chow intake

Home cage chow intake of adult female offspring was determined daily using a scale of 0.1 g precision for 1 week. Individual intake of group-housed animals was estimated as: total cage intake / # of rats in the cage. Degrees of freedom were adjusted in statistical analyses.

High fat diet challenge

To determine the effects of a high-fat diet challenge in adulthood (P210 days), individually-housed female DIO-Chow, DR-Chow and DR-Western offspring received ad libitum purified high-fat diet (HF, Research Diets D12451, 20% protein, 35% carbohydrate, 45% fat, 4.73 kcal/g) or low-fat diet (LF, Research Diets D12450B, 20% protein, 70% carbohydrate, 10% fat, 3.85 kcal/g), instead of chow, for 4 weeks. Food and water intake were measured 3 times per week, body weight was measured weekly, and body composition was measured via EchoMRI before diet access began and after 4 weeks of diet exposure.

Glucose gavage and endocrine assays

To determine the effects of maternal diet on offspring response to glucose in adulthood, fasted (16 hr overday) rats were gavaged with a glucose solution (1.2 g glucose/kg). To control for estrous cycle-related variability, estrous cycles were synchronized as previously described [117]. Briefly, rats were given two doses (2 μg each) of the gonadotropin releasing hormone (GnRH) agonist [d-Trp^6, Pro^9-NEt]-GnRH to simulate the GnRH surge at proestrus. Glucose gavage was done during diestrus. Tail blood glucose levels were determined via glucometer (Accu-Chek Active, Roche Diagnostics) pre-gavage (fasted) and at 15, 30, 60 and 120 min post-gavage. Additional tail blood was collected into polypropylene tubes containing 0.5 M EDTA (10% v), protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and DPP-IV inhibitor (Millipore, Billerica, MA) pre-gavage and at 15, 30 and 60 min post-gavage. Plasma was isolated via centrifugation and stored at -80°C for quantitation of insulin, leptin, amylin, GLP-1 and adiponectin levels by species-specific microbead immunoassays per the manufacturer's instructions (RENDO-85K, RADPK-81K-ADPN) (EMD Millipore Corporation, Billerica, MA) using a Luminex 100 IS (Luminex Corporation, Austin, TX). Insulin, leptin, amylin and GLP-1 were quantified in multiplex format. Adiponectin was assayed separately. The sensitivity of the multiplex assay is 55.6 pM for insulin and 6.2 pM for the other analytes with intra-assay coefficient of variations ranging 3.8-10.6%. The adiponectin assay has a sensitivity of 40.7 pg/ml and an intra-assay coefficient of variation of ¡4%.

Indirect calorimetry

Open-circuit indirect calorimetry was performed (24 hr) on acclimated (3 days), singly-housed rats using a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Each clear chamber (32 × 20 × 19 cm) had a water sipper, chow tray connected to a balance, plastic-mesh floor, and 24 photobeams (2.5 cm apart, 9 and 14 cm above floor). Chamber exhaust was sampled every 10 min for 50 sec through O_2 and CO_2 sensors, from which oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were estimated. Sensors were pre-calibrated with known concentrations of O_2, CO_2, and N_2 (Praxair, Inc., Danbury, CT). Respiratory quotient (RQ) and heat formation were calculated as in [63]. Heat formation was covariate-

normalized for lean mass [64].

Statistical analysis

Litter was the unit of analysis [61]. Two-way analysis of variance (ANOVA) was used to identify group differences in pre-weaning body weight, post-weaning body weight and composition and indirect calorimetry measures, with Genotype and Maternal Diet as between-subjects factors and cohort as a covariate. Pairwise comparisons were performed after Genotype × Maternal Diet interactions. DIO-Western offspring were excluded from several analyses when samples sizes were unacceptably low (n=2) due to low pregnancy success rate and possible effects of selective attrition (see companion paper). Separate 1-way ANOVAs tested effects of Maternal Diet (within DR offspring) or Genotype (within Chow offspring) on home cage chow intake, body composition, blood glucose levels, hormone levels, high-fat diet intake and weight gain; cohort was a covariate. To determine whether fat mass was disproportionately increased relative to the rest of body mass, ANCOVA analysis was used, covarying for lean mass or fat-free mass, as determined from qNMR or chemical analysis of body composition, respectively. The ANCOVA method avoids the problems associated with the ratio-based measure of % body fat [65], but % body weight scores also are provided in Supplemental Data to allow comparison with previous studies. To stabilize variance, carcass fat was log-transformed prior to ANCOVA analysis, and data are presented after back-transformation. Relative (lean or fat-free mass normalized) measures of fat mass are presented as the least square means + standard errors from ANCOVA analysis.

Homeostatic model assessment (HOMA2) was used to estimate beta cell function (%B) and insulin sensitivity (%S) [118, 119] with the University of Oxford HOMA2 calculator: http://www.dtu.ox.ac.uk/homacalculator/. 1-way ANOVAs were performed on log[HOMA2] values.

3.2 Results

Pre-weaning body weight

As shown in Table 3.1, significant maternal Diet effects reflected that Western diet female offspring weighed more than chow female offspring at P23 ($p < 0.005, F(1, 40) = 10.087$) and gained more weight between P16 and P23 ($p < 0.005, F(1, 40) = 11.938$). There were no significant effects of maternal Diet on offspring body weight at P1 or P9.

As shown in Table 3.1, main effects of Genotype indicated that DIO female offspring were heavier than DR female offspring at P16 ($p < 0.05, F(1, 40) = 5.981$) and P23 ($p < 0.005, F(1, 40) = 10.731$) and gained more weight between P16 and P23 ($p < 0.005, F(1, 40) = 7.935$). There were no significant effects of Genotype at P1 or P9.

Post-weaning body weight and composition—Chow maintenance

Despite all offspring receiving the same chow diet from weaning, there was a trend for a Diet × Genotype interaction on weight gain from adolescence through young adulthood (D35–D150) ($p = 0.059, F(1, 16) = 4.121$) (Table 3.1). Specifically, DR-Western offspring tended to gain more weight than DR-Chow offspring, whereas DIO-Western offspring tended to gain less weight than DIO-Chow offspring. As a result, DR-Western female offspring weighed significantly more than DR-Chow offspring by D150 ($p < 0.01, F(1, 11) = 14.212$). Maternal Western diet increased the total fat mass ($p < 0.01, F(1, 10) = 10.804$) and total lean mass ($p < 0.05, F(1, 10) = 9.712$), but not relative fat, of adult DR female offspring (D150; Figure 3.1).

Genotype effects increased with age, with DIO offspring heavier than DR offspring at D35, D70 and D150 (ps $< 0.0001, F$s$(1, 17) > 25.8$). Weight gain from D35 to D150 was much greater in DIO than DR female offspring ($p < 0.0001, F(1, 16) = 45.019$) (Table 3.1). Adult DIO-Chow female offspring had disproportionately greater total fat ($p < 0.0001, F(1, 17) = 172.607$) and relative fat ($p < 0.0001, F(1, 17) = 35.381$) than DR-Chow offspring. Lean mass was also greater in DIO-Chow offspring ($p < 0.0001, F(1, 17) = 40.413$) (Figure 3.1).

Table 3.1: Offspring body weight and composition from postnatal day 1 (P1) to adulthood.

Measure	**DR Chow**	**DR Western**	**DIO Chow**	**DIO Western**
Pre-weanling body wt, g				
P1	6.3 ±0.2	6.4 ±0.2	6.5 ±0.2	6.8 ±0.3
P9	17.6 ±0.4	18.6 ±0.7	19.3 ±0.5	19.7 ±1.1
P16	33.7 ±0.6	36.1 ±0.9	36.8 ±0.7$^{\#}$	38.2 ±1.6$^{\#}$
P23	54.9 ±0.9	58.9 ±1.3*	59.1 ±1.0$^{\#}$	64.3 ±2.2$^{*\#}$
Adult body weight, g				
P35	114 ±2	117 ±3	132 ±3$^{\#}$	134 ±5$^{\#}$
P70	182 ±6	200 ±7	243 ±8$^{\#}$	234 ±13$^{\#}$
P150	241 ±6	272 ±6$^{*\&}$	351 ±8$^{\#}$	326 ±27$^{\#\&}$
Pre-weanling wt gain, g				
P1 to P9	11.3 ±0.5	13.1 ±0.8	12.8 ±0.5	12.8 ±1.1
P9 to P16	16.4 ±0.4	16.9 ±0.7	17.5 ±0.4	18.4 ±1.0
P16 to P23	21.1 ±0.5	22.8 ±0.7*	22.3 ±0.5$^{\#}$	26.2 ±1.2$^{*\#}$
Adult weight gain, g				
P35 to P70	68 ±3	74 ±4	111 ±4$^{\#}$	100 ±6$^{\#}$
P70 to P150	50 ±5	65 ±7	105 ±7$^{\#}$	92 ±10$^{\#}$
P35 to P150	119 ±8	139 ±10	217 ±10$^{\#}$	192 ±16$^{\#}$
Adult body composition				
Total fat, g	20 ±1	27 ±2*	73 ±3$^{\#}$	
Total lean, g	181±4	201 ±5*	232 ±5$^{\#}$	
Normalized fat	22 ±1	25 ±2	64 ±4$^{\#}$	

Note: Data express LS Mean ±Standard error, covarying for cohort. * = Diet main effect, $p < 0.05$, # = Genotype main effect, $p < 0.05$, & = Genotype × Diet interaction, $p < 0.05$.

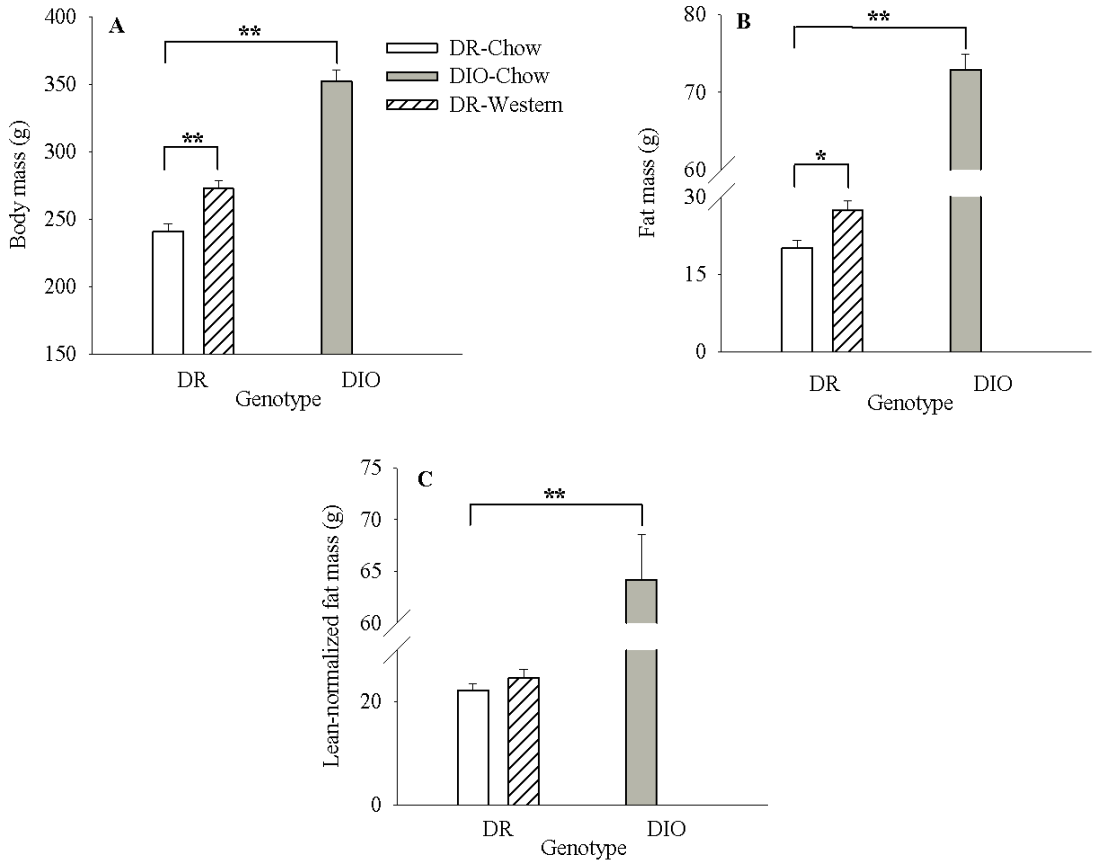

Figure 3.1: Effect of maternal Western diet or diet-induced obesty-prone (DIO) genotype on adult female offspring body weight and composition. (a) Diet-resistant offspring of Western diet dams (DR-Western) and DIO-Chow offspring weighed more than DR offspring of Chow diet dams (DR-Chow); (b) DR-Western and DIO-Chow offspring offspring had greater absolute fat mass than DR-Chow offspring; (c) DIO offspring had greater fat mass normalized for lean mass than DR-Chow offspring.

Adult food intake—Chow maintenance

Maternal Western diet did not influence the chow intake of adult DR female offspring, whereas substantial Genotype effects reflected that DIO-Chow offspring showed much greater daily home cage chow intake than did DR-Chow offspring ($p < 0.0001, F(1, 10) = 105.161$; M ± SEM: DR-Western: 55.4 ± 1.5, DR-Chow: 51.6 ±1.4, DIO-Chow: 72.3 ±1.6 kcal). DIO-Chow females still tended to eat more than DR-chow females even after covarying for body weight ($p = 0.056$).

Blood glucose and hormone levels—Chow maintenance

As shown in Figure 3.2 and Table 3.2, maternal Western diet did not influence fasting blood glucose levels (BGL) or HOMA2 estimates of beta cell function (%B) or insulin sensitivity (%S) in adult DR female offspring. However, Western diet increased the area under the curve for the glucose fall, from 60 min to 120 min post-gavage vs. DR-Chow offspring ($p < 0.05, F(1, 15) = 4.758$).

Main effects of maternal Diet indicated that plasma leptin levels were higher in DR-Western than DR-Chow offspring ($p < 0.05, F(1, 8) = 8.839$) across the entire course of the glucose gavage; a similar maternal Diet trend was seen for insulin across the time course ($p = 0.089, F(1, 8) = 3.639$) and at fasting baseline ($p = 0.064 F(1, 9) = 4.438$) (Figure 3.2).

Genotype main effects reflected that DIO offspring had higher insulin ($p < 0.01, F(1, 8) = 13.65$) and leptin ($p < 0.01, F(1, 8) = 13.833$) levels during the glucose gavage than did DR offspring. Insulin area under the curve was greater in DIO offspring for the rise (0-30 min) and fall (30-60 min) (ps < 0.01, Fs$(1, 8) > 12.1$). DIO offspring also had greater beta cell function (%B) and lower insulin sensitivity (%S) than DR offspring as estimated by the HOMA2 model (ps $< 0.05, F(1, 8) > 9.9$) (Table 3.2). As expected, there were within-subject effects of Time on insulin ($p < 0.001$, $F(3, 24) = 19.212$) and leptin ($p < 0.05$, $F(3, 24) = 3.218$) levels post-glucose gavage (Figure 3.2).

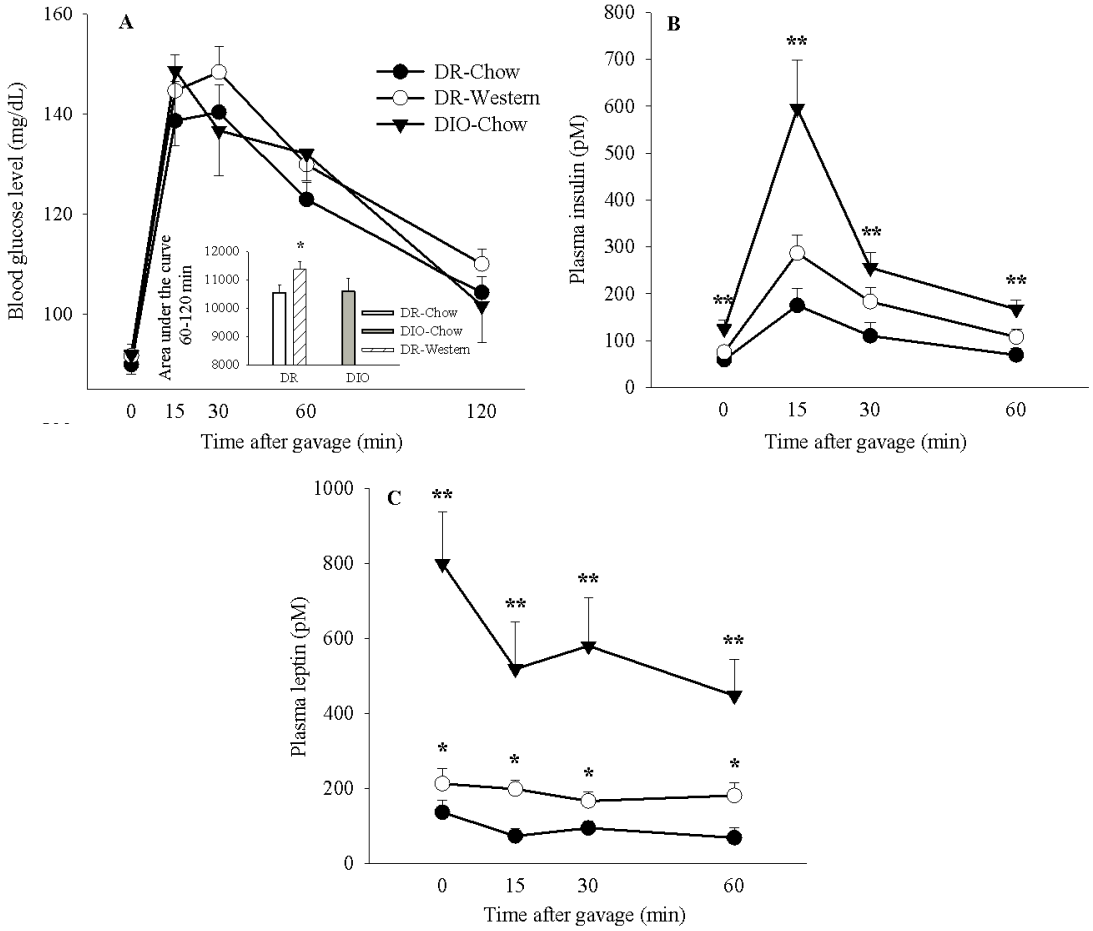

Figure 3.2: Effects of maternal Western diet or DIO genotype on plasma glucose, leptin and insulin levels prior to and following glucose gavage. (a) DR-Western offspring had increased blood glucose area under the curve (AUC) for the fall period (60–120 post-gavage) compared to DR-Chow offspring; (b) DIO-Chow offspring had higher plasma insulin levels than DR-Chow offspring fasting and for 60 minutes after the glucose gavage; (c) DR-Western and DIO-Chow offspring had higher plasma leptin levels than DR-Chow offspring fasting and for 60 minutes after the glucose gavage.

Table 3.2: Offspring glucose gavage endocrine measures. Data express LS Mean ±Standard error, covarying for cohort. * = Diet main effect, $p < 0.05$, # = Genotype main effect.

Measure, Time after gavage	**DR Chow**	**DR Western**	**DIO Chow**
PRE-HF DIET			
Glucose, ug/dL			
Fasting	92 ±2	92 ±3	92 ±4
15 min	139 ±8	145 ±7	149 ±15
30 min	140 ±6	148 ±5	137 ±9
60 min	123 ±3	130 ±3	132 ±5
120 min	104 ±3	110 ±3	102 ±7
Rise AUC	3880 ±119	3969 ±129	3968 ±201
Fall AUC	10,542 ±256	11,368 ±276*	10,579 ±473
Insulin, pM			
Fasting	59 ±13	75 ±14	125 ±19#
15 min	175 ±36	286 ±39	595 ±103#
30 min	110 ±28	183 ±31	255 ±33#
60 min	69 ±15	107 ±16	166 ±19#
HOMA2			
LOG[%B], fasting	2.0 ±0.5	2.0 ±0.05	2.2 ±0.05#
LOG[%S], fasting	1.9 ±0.08	2.0 ±0.07	1.7 ±0.08#
Leptin, pM			
Fasting	136 ±33	212 ±40*	799 ±139#
15 min	73 ±19	198 ±23*	519 ±124#
30 min	94 ±20	166 ±24*	580 ±129#
60 min	68 ±28	181 ±34*	447 ±97#
Amylin, pM			
Fasting	20 ±3	23 ±3	29 ±7
15 min	20 ±3	24 ±3	31 ±6
30 min	24 ±2	20 ±2	23 ±3
60 min	24 ±2	21 ±2	31 ±8
GLP-1, pM			
Fasting	20 ±2	20 ±2	19 ±2
15 min	21 ±3	19 ±3	20 ±3
30 min	20 ±2	19 ±2	20 ±3
60 min	21 ±2	20 ±2	20 ±3

Note: Table continued on next page.

Table 3.2: Offspring glucose gavage endocrine measures (*continued*).

Measure, Time after gavage	**DR Chow**	**DR Western**	**DIO Chow**
POST-HF DIET			
Glucose, ug/dL			
Fasting	86 ±6	103 ±4	96 ±4
15 min	134 ±10	133 ±7	134 ±15
30 min	134 ±14	141 ±10	138 ±17
60 min	121 ±17	130 ±12	130 ±11
120 min	109 ±10	117 ±7	114 ±8
Rise AUC	3775 ±257	3975 ±199	3770 ±217
Fall AUC	11,160 ±458	12,383 ±397	11,360 ±864
Insulin, pM			
Fasting	40 ±16	66 ±10	190 ±77
15 min	87 ±46	116 ±33	157 ±19
30 min	59 ±40	160 ±31	268 ±32
60 min	64 ±21	94 ±16	156 ±58
HOMA2			
LOG[%B], fasting	1.8 ±0.06	1.9 ±0.05	2.3 ±0.1
LOG[%S], fasting	2.2 ±0.09	1.9 ±0.08	1.5 ±0.1
Leptin, pM			
Fasting	184 ±83	333 ±64	2086 ±366$^{\#}$
15 min	156 ±94	336 ±73	1888 ±657$^{\#}$
30 min	92 ±103	369 ±81	1917 ±37$^{\#}$
60 min	167 ±92	292 ±72	1165 ±364$^{\#}$
Amylin, pM			
Fasting	5.7 ±1.1	5.5 ±0.9	8.0 ±1.7
15 min	5.8 ±1.5	5.5 ±1.2	6.5 ±1.8
30 min	6.6 ±1.2	5.4 ±1.0	7.1 ±1.8
60 min	6.5 ±1.5	5.4 ±1.2	6.5 ±1.8
GLP-1, pM			
Fasting	6.0 ±0.5	5.5 ±0.4	6.9 ±1.0
15 min	5.6 ±0.4	5.5 ±0.3	6.9 ±0.6
30 min	5.7 ±0.7	5.7 ±0.5	7.0 ±0.8
60 min	5.5 ±0.8	5.7 ±0.6	6.5 ±0.6

Indirect calorimetry—Chow maintenance

As shown in Figure 3.3, maternal Western Diet decreased daily energy expenditure in adult female offspring ($p < 0.05, F(1, 19) = 5.036$). There was also a strong trend for maternal Western diet to increase the respiratory quotient (RQ) of adult female offspring ($p = 0.055, F(1, 20) = 4.14$), consistent with reduced utilization of lipid as a fuel substrate. These effects cannot be explained by differences in locomotor activity, as there was no effect of maternal Diet on activity levels.

Although there was an effect of Genotype on activity levels ($p = 0.01$, $F(1, 20) = 8.0$), whereby female DIO offspring moved less than DR offspring, this did not manifest in a Genotype difference in total daily energy expenditure (Figure 3.3).

Body weight, intake, body composition and response to glucose: High-fat diet challenge

Maternal Western diet and DIO genotype each independently exaggerated responses to the 4 week high-fat diet challenge. Thus, main effects of Genotype and Maternal Diet were seen on energy intake, body weight, body composition and the endocrine response to glucose gavage, as detailed below.

INTAKE

As shown in Figure 3.4, during the 4 weeks of high-fat diet access, DIO-Chow and DR-Western offspring both ate more high-fat diet than did DR-Chow offspring across all weeks ($p < 0.01$, $F(1, 11) = 16.873$ and $p < 0.05$, $F(1, 16) = 4.535$, respectively). There was a significant effect of Week in all groups consuming high-fat diet, with animals eating most in the first week, and progressively less in each following week (ps $< 0.001, F$s$(1, 48) > 16.1$). There were no group differences in low-fat control diet intake.

BODY WEIGHT

DIO-Chow and DR-Western offspring weighed more each week and gained more weight than DR-Chow rats when fed purified high-fat (DIO-Chow: ps < 0.0001, Fs$(1, 14) > 104.14$; DR-Western: ps < 0.01, Fs$(1, 16) > 12.0$). They also gained signif-

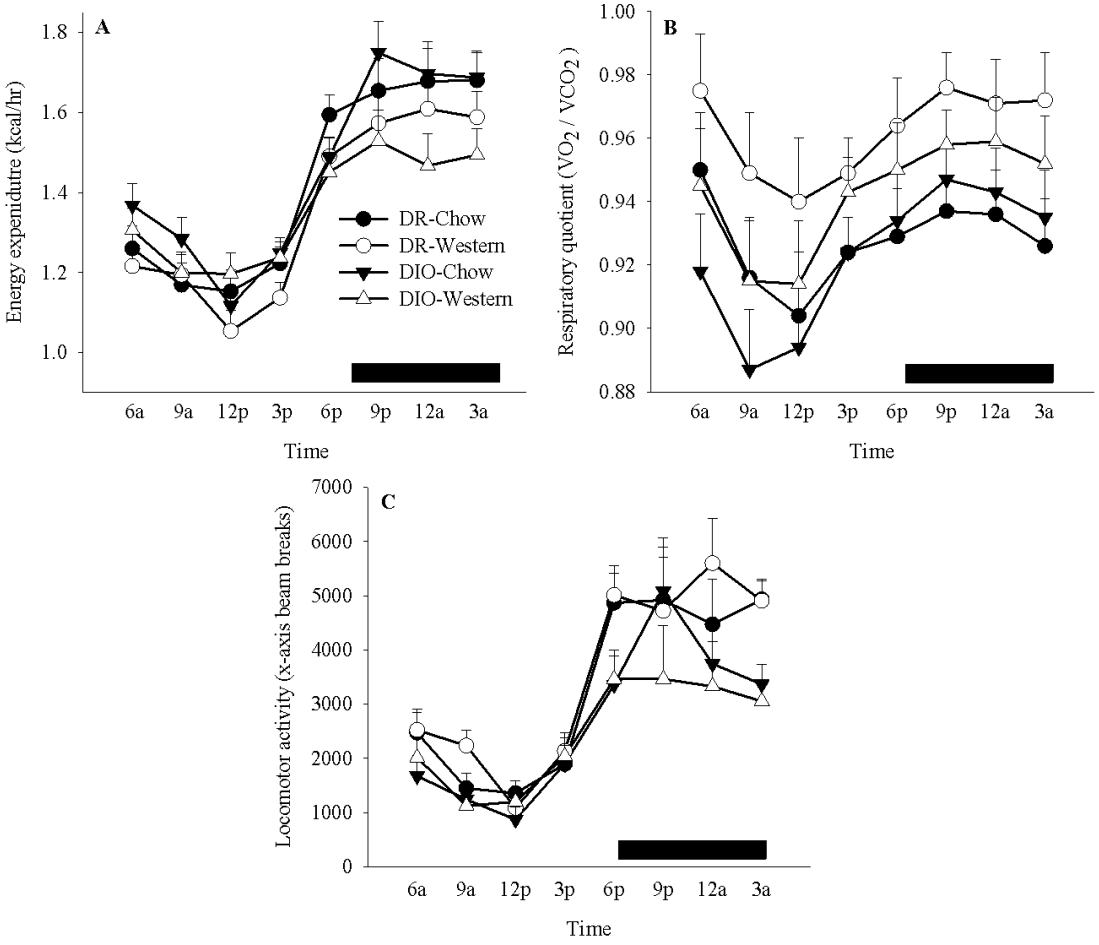

Figure 3.3: Effects of maternal Western diet and DIO genotype on energy expenditure and locomotor activity. (a) DR-Western and DIO-Western offspring had lower energy expenditure over a 24-hour period than DR-Chow and DIO-Chow offspring; (b) DR-Western and DIO-Western offspring tended ($p = 0.055$) to have lower respiratory quotient than DR-Chow and DIO-Chow offspring over the 24-hour test period; (c) DIO offspring had lower locomotor activity than DR offspring during the dark cycle.

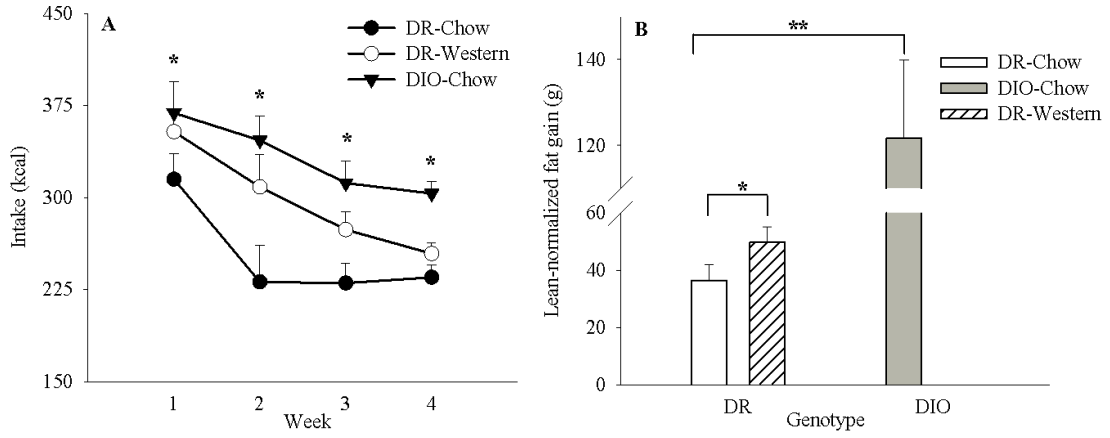

Figure 3.4: Effects of maternal Western diet or DIO genotype on food intake and fat mass gain during 4-week high-fat (45%) diet challenge. (a) DR-Western and DIO-Chow offspring ate more high-fat diet than DR-Chow offspring for each of the 4 weeks; (b) DR-Western and DIO-Chow offspring gained more lean-normalized fat mass over 4 weeks of high-fat diet consumption.

icantly more weight, though to a lesser degree, when fed purified low-fat control (DIO-Chow: ps $< 0.05, F\text{s}(1, 11) > 6.4$; DR-Western: ps $< 0.05, F\text{s}(1, 15) > 4.6$) (Figure 3.4).

BODY COMPOSITION

DR-Western offspring had greater absolute fat mass ($p < 0.05, F(1, 15) = 7.246$) and gained disproportionately more absolute fat ($p < 0.05, F(1, 15) = 6.018$) and relative fat ($p < 0.05, F(1, 14) = 4.373$) than DR-Chow offspring after 4 weeks of high-fat diet exposure (Figure 3.4).

Similarly, DIO-Chow offspring had greater absolute and relative fat mass (ps $< 0.001, F\text{s}(1, 14) > 24.5$) and gained more absolute and relative fat (ps $< 0.01, F\text{s}(1, 13) > 9.5$) than DR-Chow offspring after 4 weeks of high-fat diet exposure (Figure 3.4).

GLUCOSE GAVAGE

Following high-fat diet exposure, main effects of maternal Diet indicated that DR-Western offspring had greater leptin levels than DR-Chow offspring ($p < 0.05$, $F(1, 11) = 7.128$). DR-Western also offspring had greater beta cell function (%B) and lower insulin sensitivity (%S) than DR-Chow offspring as estimated by the HOMA2 model (ps < 0.05, $F(1, 9) > 8.3$) (Table 3.2).

There were strong trends for maternal Diet effects on fasting blood glucose ($p = 0.052$, $F(1,5) = 4.464$) (Table 3.2) and insulin area under the curve (AUC) in both the rise (0-30 min, $p = 0.057$, $F(1,11) = 4.519$) and fall (30-60 min, $p = 0.05, F(1,11) = 4.853$) phases (Table 3.2), with DR-Western AUC greater than DR-Chow AUC.

DIO offspring had higher leptin levels than DR offspring at each time point measured ($p < 0.05$, $F(1,3) = 13.384$), and like DR-Western offspring, had greater beta cell function (%B) and lower insulin sensitivity (%S) than DR-Chow offspring as estimated by the HOMA2 model (ps < 0.01, $F(1,7) > 25.2$). Insulin AUC rise and fall phases were greater for DIO-Chow than DR-Chow offspring (ps < 0.01, $Fs(1,8) > 21.2$) (Table 3.2).

There was no effect of maternal Diet or DIO Genotype on amylin or GLP-1 levels.

3.3 Discussion

The current study shows that maternal Western diet can increase adiposity and alter endocrine profiles in female offspring, even when the offspring are genetically resistant to diet-induced obesity. Maternal Western diet led to heavier litters, more rapid weight gain during adolescence, heavier juvenile offspring, heavier adult offspring, increased insulin and leptin levels, and greater fat gain after high-fat diet exposure. This was true not only in DIO female offspring, but also in DR female offspring. Together with the study presented in the companion paper, the present study shows that maternal Western diet can partially override genetic resistance to obesity in both male and female offspring.

Time course of maternal diet effects

Maternal diet effects on offspring body weight and adiposity were first seen early in life and lasted well into adulthood. Adult DR-Western offspring are heavier than DR-Chow offspring, though not disproportionately fatter. Early life exposure to Western diet makes DR offspring larger, even though they are genetically resistant to the effects of a high-energy diet. Maternal obesity is not necessary for long-lasting effects of maternal Western diet, as reported in the companion paper.

Maternal diet effects on leptin and insulin

Though not as dramatic as the Genotype effects, there are maternal Diet effects on leptin levels, reflecting greater total fat in DR-Western vs. DR-Chow offspring and possibly indicating leptin resistance. Following high-fat diet exposure in adulthood, DR-Western had higher fasting glucose levels than DR-Chow. Previous studies have shown that offspring of dams fed a high-fat diet during gestation are obese, hyperleptinemic and glucose intolerant [120, 121, 122]. This may be due to differences in milk composition, as maternal high-fat diet increases protein, cholesterol and triglyceride content of milk [121], and post-natal high-fat diet impairs offspring glucose tolerance and increases offspring leptin and adiposity more than pre-natal high-fat diet [93]. The present study novelly shows that maternal diet can have these effects in the absence of maternal obesity.

There was also a strong trend for maternal Diet effect on insulin ($p = 0.064$) prior to high-fat diet exposure in adult offspring. This may indicate risk for insulin resistance. As reported in the companion paper, dam pre- and post-pregnancy insulin levels are normal, so offspring insulin levels are affected without maternal levels being high.

Maternal diet effects on response to high-fat diet

DR-Western offspring had an increased risk for diet-induced obesity, as shown in the significantly greater weight and fat gain following high-fat diet exposure in adulthood. This was one of the strongest effects of maternal Western diet. It may indicate that individuals exposed to a high-fat, high-carbohydrate diet early in life should be careful to avoid such diets later in life, even if they are genetically resistant to weight gain.

Previous studies have shown an exacerbated response to adult high-fat diet exposure following maternal high-fat diet exposure in male offspring [114]. However, the high-fat diet-fed dams in those studies were obese during pregnancy, while the DR-Western dams in the current study remained lean, as reported in the companion paper. The present study shows that maternal obesity is not necessary for maternal diet to increase diet-induced obesity risk in female offspring.

Differences in males and females

Previous studies have shown that male offspring are more susceptible to the obesogenic effects of maternal obesity and/or high-fat diet [108, 123] than female offspring. This sexual dimorphism was not found in the current studies. Male and female DR-Western offspring showed effects of maternal Diet from early life through adulthood. Males and females both had increased body weight, increased food intake, increased absolute fat and decreased energy expenditure. Adult male DR-Western offspring did, however, show greater differences than female offspring in relative fat.

Genotype differences

As expected, DIO offspring were heavier and fatter than DR offspring. This is somewhat different from past studies because DIO rats were heavier from birth, even without exposure (maternal or otherwise) to a high-energy diet. Previous studies have shown, however, that chow-maintained DIO rats become hyperphagic and overweight as adults [57, 33, 34], so this result is not entirely unexpected. DIO rats are hyperleptinemic and hyperinsulinemic, as has been previously reported [124, 125, 58], possibly reflecting a pre-diabetic state of leptin and insulin resistance. HOMA2 analysis revealed lower insulin sensitivity, further suggesting pre-diabetes in DIO rats. Surprisingly, DIO rats were not glucose intolerant, even after the 4 week exposure to high-fat diet.

DIO-Western offspring

DIO-Western female offspring were large as pre-weanlings and at D35, but then started to decrease in body weight compared to DIO-Chow offspring. This may be a result of selective attrition in the DIO-Western dams. As reported in the companion paper, DIO-Western dams had a significantly lower birth rate than all other groups. Those that did give birth ate less and weighed less than those that did not. The successful dams and their offspring may have been less susceptible to obesity risk than the unsuccessful dams. DIO-Western offspring data should be interpreted with this caveat in mind.

Importance of diet

The present study shows that a Western diet in the absence of maternal obesity can lead to increased obesity susceptibility in female offspring, emphasizing the intrinsic importance of diet. It has previously been shown that a healthy maternal diet can benefit offspring, even when the dam is obese. Female offspring of obese, diabetic dams are resistant to diet-induced obesity if the dam is fed a low-fat control diet during pregnancy and lactation [126]. Together with the current study, this study showed that diet is important independent of maternal obesity and should be emphasized as an important health measure during pregnancy.

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Chapter 4

The Role of TRH-DE in Maternal Diet Effects on Offspring Outcome

In recent years, maternal nutritional status during pregnancy and lactation has been emphasized as a key environmental factor that can affect offspring outcome [127, 128, 129, 130, 131]. In particular, maternal overnutrition with associated obesity has been shown to increase offspring obesity risk [132, 133, 134, 40]. We have studied the role of maternal Western diet in offspring obesity susceptibility, and in Chapters 2 and 3 reported the importance of maternal diet *per se*, in the absence of maternal obesity. In the current chapter, we investigate a novel mechanism for the effect of maternal Western diet on increased offspring adiposity.

While maternal diet is one important environmental factor, genetic factors are also key in determining obesity risk. As in previous studies, here we used the DR-DIO polygenic model of obesity risk in conjunction with maternal Western diet to determine relative contributions of genetic and environmental factors to obesity susceptibility. DIO-Chow, DIO-Western, DR-Chow and DR-Western Genotype-Maternal Diet groups were initially studied. However, because of poor breeding success and selective attrition, DIO-Western offspring were excluded from the studies reported in this chapter. Initial microarray gene expression analysis was done on tissue from DIO-Chow, DR-Western and DR-Chow offspring in order to identify genes associated with both genetic obesity risk

(ie upregulated in DIO-Chow offspring) and environmental obesity risk (ie upregulated in DR-Western offspring). Because the most interesting results reported in Chapters 2 and 3 showed maternal Western diet had strong effects even in diet-resistant DR offspring, behavioral studies testing the functional significance of the genes identified with microarray analysis were done only on DR-Western and DR-Chow offspring. In doing this we shifted the focus of our study to the effects of maternal Western diet, rather than the effects of genetic background, on offspring outcome.

Possible mechanisms for the effects of maternal diet on offspring obesity risk are not well-understood. The current study aims to further understanding of changes in the brain that may disrupt energy balance as a result of maternal Western diet. The hypothalamus is a key brain region for the control of energy expenditure and food intake. The lateral hypothalamus (LH), in particular, is important for integration of feeding signals and maintenance of energy balance [135]. Neurons in the LH sense available glucose and insulin, respond to gut hormones such as ghrelin, and receive input from leptin-producing neurons in the arcuate nucleus. It contains distinct populations of orexin/hypocretin neurons and melanin-concentrating hormone neurons [136], both of which regulate food intake and thermogenesis.

The goal of the current study was to identify novel hypothalamic genes that are overexpressed in association with increased obesity susceptibility related to genetic or maternal diet factors via microarray analysis. The gene we identified, *trhde*, codes for thyrotropin-releasing hormone degrading enzyme (TRH-DE), a membrane-bound ectoenzyme located in the brain and pituitary that highly specifically degrades thyrotropin-releasing hormone (TRH) [137, 138]. TRH plays a well-studied role in the control of energy balance via peripheral and central mechanisms. For peripheral action, it is released from neurons in the paraventricular nucleus of the hypothalamus (PVN) to the pituitary, where it causes release of thyroid-stimulating hormone (TSH) [17, 18]. TSH then stimulates the release of thyroid hormones triiodothyronine (T3) and thyroxine (T4) from the thyroid gland into the bloodstream. T3 and T4 have effects on many biological processes, including control of basal metabolic rate and metabolism of protein, fat and carbohydrate. For central action, PVN TRH neurons project to other hypothalamic nuclei including the lateral hypothalamus, the amygdala, nucleus accumbens and the

hippocampus, where it acts on neurons controlling food intake, energy expenditure and nutrient sensing [139, 140].

The TRH system has long been known to play an important role energy balance. Hypothyroidism in relation to obesity has been well-studied [141, 142]. However, the link between maternal Western diet and disruptions to the TRH system has not been well-studied, and the mechanism is unknown. Here, we novelly report that maternal Western diet alters the behavioral response to central TRH administration, an effect that may be mediated by overexpression of *trhde*.

4.1 Methods

Subjects

DR and DIO breeders, offspring of rats from the original colonies of DIO and DR rats bred by Levin et al [33, 34], were born at The Scripps Research Institute. Rats resided in wire-topped plastic cages in a 12:12-hr light-dark cycle (600 lights on), humidity- (60%) and temperature- (220°C) controlled vivarium with access to chow (LM-485 7012, 17% [kcal] fat, 58% carbohydrate, 25% protein; 3.1 kcal/g; 16% saturated, 26% monounsaturated and 58% polyunsaturated fats; 2.7% kcal from saturated fat, Harlan, Indianapolis, IN) and water ad libitum prior to experiments. Procedures adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and the *Principles of Laboratory Animal Care* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Diet and breeding

At 46-47 days of age, female DR and DIO rats were randomly divided into Western vs. chow diet conditions, yielding 4 groups: DR-Chow (n=28), DR-Western (n=16), DIO-Chow (n=28) and DIO-Western (n=16). Western diet dams received a moderate-fat diet ad libitum (Research Diets D12266B, 32% fat, 51% carbohydrate, 17% protein; 4.41 kcal/g; 26% saturated, 27% monounsaturated and 47% polyunsaturated fats, 8.5% kcal from saturated fat, New Brunswick, NJ). Chow dams received chow. The Western diet resembles the dietary intake of adult American women in fat

(32-33%), saturated fat (10-11%), carbohydrate (50-53%) and protein (15-16%) proportions, (http://www.cdc.gov/nchs/data/ad/ad334.pdf).

After 54 days of diet exposure, during which food and body weight were measured 3 times weekly, females were housed with males of the same genotype. Males were removed following pregnancy confirmation; dams continued to receive their experimental diet until weaning. The day of birth was termed postnatal day 0 (P0). Litters were culled to 8 pups (∼1:1 sex ratio) at P1. All offspring were weaned onto standard chow on P23.

Because only DIO females that were comparatively resistant to the Western diet produced offspring (see Chapter 2), the DIO-Western group had selective attrition of obesity risk factors. Therefore, DIO-Western data was excluded from analysis. The microarray and qPCR studies only included DIO-Chow, DR-Chow and DR-Western groups. All other studies only included DR-Chow and DR-Western groups.

Offspring blood and tissue collection

At P23, anaesthetized (CO_2 inhalation) male offspring were decapitated. For gene expression analysis, brains were removed, sliced in half sagitally along the midline, and stored in RNAlater at -20 °C. For western blot analysis, brains were snap frozen in ice-cold isopentane and stored at -80°C. Trunk plasma was stored at -80°C for hormone analysis. In adulthood, male offspring were isoflurane-anesthetized and decapitated. Brains were rapidly removed and sliced with a wire matrix. LH tissue was dissected from 2 slices with a 12 gauge metal tube.

Lateral hypothalamus tissue collection

To collect tissue from the lateral hypothalamus (LH) for gene expression analysis of P23 offspring, one hemisphere from each P23 brain collected and stored in RNA later was sliced using a Vibratome (500 μm slice, followed by a 400 μm spacer, followed by a 600 μm slice). Punches were taken from the lateral hypothalamus of the 500 and 600 μm slices using a 14 gauge metal tube and stored at -80°C. For Western blot analysis, whole frozen brains were sliced using a Cryostat, with slice thicknesses and punches the same as above.

RNA extraction, retro-transcription and microarray analysis

RNA was extracted from P23 LH tissue using the QIAGEN RNEasy Lipid Mini Kit (QIAGEN, Hilden, Germany). RNA quality and concentration were measured by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The 3 highest quality and most concentrated RNA samples from each of the 3 groups (DR-Chow, DR-Western and DIO-Chow) were used for microarray analysis. RNA was retro-transcribed to cDNA. Each of the 9 samples was run on a separate Affymetrix Rat Gene 1.0 microarray chip (RaGene-1_0-st-v1 chip, Affymetrix, Inc., Santa Clara, CA, USA).

Quantitative polymerase chain reaction

Primers were designed using Oligo Primer Analysis Software (Molecular Biology Insights, Inc., Cascade, CO, USA) and optimized the 6 genes with the highest fold change when comparing DR-Chow to DIO-Chow and DR-Chow to DR-Western with XRAY microarray data analysis for microarray target confirmation, and for TRH receptors R1 and R2 (Table 4.1). Quantitative real-time polymerase chain reaction (qPCR) was performed on all LH cDNA samples (n = 7-8) for each of the genes using SYBR Green SYBR Select Master Mix (Life Technologies, Inc., Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cyclophilin A was the internal standard.

Western blot

P23 LH tissue was homogenized via sonication in lysis buffer and protein was quantitated via protein assay (Bio-Rad DC, Hercules, CA, USA). Western blots were performed for TRH-DE and β-tubulin proteins. Bands were analyzed semi-quantitatively using ImageJ (National Institutes of Health). TRH-DE band density was adjusted for same-subject β-tubulin band density.

Intra-cerebroventricular surgeries

Stainless steel cannulae were stereotaxically implanted into the lateral ventricle of isoflurane-anesthetized DR-Chow and DR-Western offspring aged 50 days (+5 incline skull; from Bregma: -0.6 A/P, +/- 2.0 M/L, -3.3 D/V). Cannula placement was tested by administration of 100 ng Angiotensin II in 5μl saline over 1 min. Placement was

considered correct if the animal drank more than 5 mL water in less than 20 min following administration.

Peptides

Angiotensin II was generously provided by Dr. Jean Rivier (The Salk Institute, La Jolla, CA, USA). TRH (Pyr-His-Pro-NH2) was purchased from Bachem (Torrance, CA, USA). The highly potent TRH-DE inhibitor Glp-Asn-Pro-7-amido-4-methylcoumarin (GAP-AMC) [143] was generously provided by Dr. Julie Kelly (University College, Dublin, Ireland).

TRH administration study

Vehicle, 4.167 μg, 12.5 μg or 37.5 μg TRH dissolved in 0.1% bacteriostatic saline was injected into the lateral ventricle (5 μl over 1 min with 1 min hold) in a Latin square design with a 10 μl Hamilton syringe (Reno, NV, USA) attached with PE 50 tubing to a 28 gauge injector that projected 3 mm beyond the guide cannula. Rats were injected at dark cycle onset and returned to indirect calorimetry chambers immediately following injections.

TRH-DE inhibitor administration studies

Vehicle, 12.5 μg TRH, 5 μg TRH-DE inhibitor, or both TRH and TRH-DE inhibitor were administered as above. Each rat received two injections per day (vehicle + vehicle, TRH + vehicle, TRH-DE inhibitor + vehicle, or TRH + TRH-DE inhibitor) with 1 minute between infusions. For indirect calorimetry study, DR-Chow and DR-Western offspring were injected at dark cycle onset and returned to indirect calorimetry chambers immediately following injections. For plasma free T3 and free T4 assays, DR rats with no maternal diet history (chow-reared) were returned to home cages after injections and tail blood was collected into microcentrifuge tubes with 1% volume 0.5 M EDTA at 30 and 60 minutes after injections. Blood was centrifuged and plasma was stored at -80°C.

Rat indirect calorimetry

Open-circuit indirect calorimetry was performed (24 hr) on acclimated (3 days), singly-housed DR-Chow and DR-Western offspring using a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Each clear chamber (32×20×19 cm) had a water sipper, chow tray connected to a balance, plastic-mesh floor, and 24 photobeams (2.5 cm apart, 9 and 14 cm above floor). Chamber exhaust was sampled every 10 min for 50 sec through O_2 and CO_2 sensors, from which oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were estimated. Sensors were pre-calibrated with known concentrations of O_2, CO_2, and N_2 (Praxair, Inc., Danbury, CT). Respiratory quotient (RQ) and heat formation were calculated as in [63]. Heat formation was covariate-normalized for lean mass [64]

Hormone analysis

Free plasma levels of triiodothyronine (T3) (0.5 pg/ml sensitivity, 3.1-4.9% CV) and thyroxine (T4) (0.5 ng/dl sensitivity, 3.25-10.89% CV) were quantified by competitive ELISA (DRG International, Springfield, NJ).

Trhde knock-out mice

Heterozygote Trhde mutant (+/-) $\text{Trhde}^{\text{tm1Lex/MMUCD}}$ mice on a B6/129s5 background were obtained from the UC Davis Mutant Mouse Resource Regional Center (MMRRC) and mated. The resulting adult male and female null mutant mice (Trhde (-/-), *trhde* KO), wild-type (WT), and heterozygous littermates were used in this study. PCR was used to determine the genotype of the mice [primer sequences: (wild-type forward) 5'-CTC TCG GAC CCG TGG GCT G-3', (wild-type reverse) 5'-GCA TCA AAT TGT AGT GCA AGC G-3', (null forward) 5'- GCA GCG CAT CGC CTT CTA TC-3', (null reverse) 5'- CCA CTG TAA CCT GAG AAG TTG-3'; PCR conditions: 35 cylces of 95°C (30 sec), 60°C (30 sec) and 72°C (75 sec)]. Mice were group-housed in Macrolon shoebox cages with ad libitum access to food and water in a humidity- and temperature (22 °C)-controlled vivarium on a 12-h reverse light cycle (lights on 6:00 am).

Mouse indirect calorimetry

Open-circuit indirect calorimetry was performed (24 hr) on acclimated (3 days), singly-housed mice using a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Each clear chamber (20×10×12.5 cm) had a water sipper, chow tray connected to a balance, plastic-mesh floor, and 16 photobeams (1.3 cm apart, 3.3 and 7.3 cm above floor). Chamber exhaust was sampled every 16 min for 60 sec through O_2 and CO_2 sensors, from which oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were estimated. Sensors were pre-calibrated with known concentrations of O_2, CO_2, and N_2 (Praxair, Inc., Danbury, CT). Respiratory quotient (RQ) and heat formation were calculated as in [63]. Heat formation was covariate-normalized for lean mass [64].

Body composition analysis

Mouse body composition was measured via nuclear magnetic resonance imaging (EchoMRI-1100, Software version 2008.01.18M, Echo Medical Systems, Houston, TX).

Chow intake

Home cage chow intake of adult male and female was determined daily using a scale of 0.1 g precision for 1 week. Individual intake of group-housed animals was estimated as: total cage intake / # of mice in the cage. Degrees of freedom were adjusted in statistical analyses.

High fat diet challenge

To determine the effects of a high-fat diet challenge, individually-housed male and female mice received ad libitum purified high-fat (HF, Research Diets D12451, 20% protein, 35% carbohydrate, 45% fat, 4.73 kcal/g) instead of chow for 4 weeks. Food and water intake was measured 3 times per week, body weight was measured weekly, and body composition was measured via EchoMRI before diet access began and after 4 weeks of diet exposure.

Statistics

Separate one-way analyses of variance (ANOVAs) tested effects of Maternal Diet (within DR offspring) or Genotype (within Chow offspring) on microarray gene expression and TRH-DE protein expression. Gene expression data were z-score normalized and analyzed by ANCOVA with Cyclophilin A expression levels as a covariate. Two-way ANOVAs were used to determine effects of Maternal Diet and Dose for TRH and TRH-DE inhibitor administration studies. Lean mass was a covariate for indirect calorimetry studies. All other data was analyzed using one-way ANOVA to determine effects of Maternal Diet (rat studies) or gene knock-out (mouse studies), with repeated-measures as indicated.

Statistical analysis of microarray data

Two methods were used to analyze microarray data. First, the chips were normalized with Robust Multichip Average (RMA) in the Expression Console package from Affymetrix. RMA was used to convert the intensity values to expression values [144, 145]. The transcripts were filtered from the lowest standard deviation so that the top two-thirds of the variants remained. ANOVA was performed within the Bioconductor project and the R program software (R is available as Free Software under the terms of the Free Software Foundation's GNU General Public License). The fold changes and standard errors were estimated by fitting a linear model for each gene and empirical Bayes smoothing was applied to the standard errors for all the samples at the same time. The linear modeling approach and the empirical Bayes statistics as implemented in the Limma package in the R software were employed for differential expression analysis. Statistics were obtained for transcripts with the multiple testing adjusted (Benjamini-Hochberg) p-values level of 0.05. Filtering was performed so that probe-sets with a fold change of < 1.4 were eliminated from the results.

The data analysis was also performed using XRAY (version 3.2) software, the Excel add-in from Biotique Systems Inc. (Reno, NV, USA). The input files were normalized with full quantile normalization [146]. Background is established from a pool of probes designed for that purpose. Background probes are stratified by CG content and each probe score was corrected for background by subtracting the median expression score of

background probes with similar GC content. For this analysis, only 'Core' probe-sets were analyzed include probe-sets that correspond to high quality genomic features like RefSeq (www.ncbi.nlm.nih.gov) or Ensembl (www.ensembl.org) transcripts.

Mixed Model, Nested Analysis of Variance (Montgomery, 2006) was used to identify genes with group specific gene expression or alternative splicing.

To correct for the multiple testing, the Benjamini and Hochberg False Discovery Rate (FDR) method was used as outlined by Benjamini and Hochberg [147] that controls the family wide error rate in a weak sense.

Pathway analysis of microarray results was completed using the Gene Set Enrichment Analysis tool from the Broad Institute.

4.2 Results

Pathway analysis

Pathway analysis was performed to identify sets of genes involved in the same biological processes that are all upregulated or downregulated in association with risk for obesity. Two comparisons were made: DR-Chow vs. DR-Western and DR-Chow vs. DIO-Chow. The pathways containing sets of genes that were up- or downregulated involved general cell energy and metabolism, such as pathways for ribosomal proteins, ATPases and cellular metabolism. These pathways were too general to identify genes for further analysis in functional studies. Because of this, gene analysis was prioritized based on XRAY fold change data.

Quantitative PCR

Because pathway analysis did not yield results that could be further explored for functional relevance to obesity risk, the 6 genes with the highest fold changes for DIO-Chow and DR-Western vs. DR-Chow expression in the LH were identified using XRAY analysis. Table 4.1 shows gene names and fold changes. Quantitative PCR was performed on all P23 LH cDNA samples (n = 7-8) to confirm differences in gene expression. Cyclophilin A-normalized gene expression levels are shown in Figures 4.1 and 4.2. The greatest expression level difference was found for thyrotropin-releasing hormone

degrading enzyme (*trhde*) (DR-Chow vs. DR-Western $p < 0.01$, $F(1, 11) = 10.194$; DR-Chow vs. DIO-Chow $p < 0.01$, $F(1, 9) = 11.539$).

TRH-DE western blot

There were no significant differences in TRH-DE protein expression in the lateral hypothalamus of P23 DR-Chow, DR-Western and DIO-Chow offspring as determined by western blot analysis.

TRH receptor qPCR

There were no significant differences in TRH receptor-1 or TRH receptor-2 expression in the lateral hypothalamus of P23 or adult (D120) DR-Chow and DR-Western offspring.

TRH administration indirect calorimetry

The first the sum of the total energy expenditure for 60 minutes of indirect calorimetry data was used for analysis, as previous studies have shown that TRH is most behaviorally effective for 20-60 minutes after central administration [151, 153, 154]. As shown in Figures 4.1 and 4.2, there was a significant Diet effect ($p < 0.05$, $F(1, 9) = 4.994$) and a strong trend for Dose effect ($p = 0.055$, $F(1, 9) = 2.747$) on total energy expenditure for 60 minutes following TRH administration. Pairwise comparisons revealed significant differences in total energy expenditure between vehicle and each of the 3 TRH doses for DR-Chow offspring (ps < 0.001), while there were significant differences only at the 4.17 μg and 37.5 μg doses for DR-Western offspring (ps < 0.05). There was a significant difference between DR-Chow and DR-Western offspring total energy expenditure at the 12.5 μg and 37.5 μg dose (ps < 0.05).

TRH administration increased total locomotor activity similarly in DR-Chow and DR-Western offspring (Figures 4.1 and 4.2), with a significant effect of Dose ($p < 0.001$) but no effect of Diet.

TRH-DE inhibitor administration indirect calorimetry

As shown in Figures 4.1 and 4.2, there were Diet and Dose effects on total energy expenditure for 60 minutes following icv administration of TRH, TRH-DE inhibitor or both TRH and TRH-DE inhibitor (ps < 0.01). While administration of TRH alone increased energy expenditure in DR-Chow offspring, it did not increase energy expenditure in DR-Western offspring. TRH-DE inhibitor alone moderately increased energy expenditure in both DR-Chow and DR-Western offspring, and the combination of TRH and TRH-DE inhibitor significantly increased energy expenditure in both groups (ps < 0.05).

Table 4.1: Genes targeted for further study based on microarray results. Fold change is from XRAY analysis.

Gene name	**Gene symbol**	**Diet fold change**	**Genotype fold change**	**Primers 5'-3'**
Guanine deaminase	*gda*	2.31	3.91	CCC AAC TCT AAT CTG TCG C TCT GTC CCA AGC CCT ATC
Transthyretin	*ttr*	1.91	1.51	GCT GGA CTG ATA TTT GCG TCT ACG GCC ACA TCG ACA G
Thyrotropin releasing hormone degrading enzyme	*trhde*	1.81	1.71	TGC CCT GAT AGA GAA CGA AGT GGC CTT GTA GAT TGG T
GABA-4 receptor α-subunit	*gabra4*	1.51	1.81	TGC CTG GCG ACT TGT T GAC GCA GTC TGT TGT CAT AAC
Regulator of G-protein signaling 4	*rgs4*	1.51	1.61	GGC CAA GAA GAT CTA CAA T AAA CAG GTT ATC GTG GG
Fat mass and obesity associated gene	*fto*	1.31	1.51	AGA CAC TTG GCT TCC TTA CCT GCA GCT CCT CGG GTA TG
Thyrotropin releasing hormone receptor 1	*trhr1*			CGT GGA AAA ATG ACT CAA ATC CAT AAA AGG GCA AAC
Thyrotropin releasing hormone receptor 2	*trhr2*			TGG GCA TCA CCT ACT TCC CCT GCG ATG ATC CGT TT

DR offspring free T3 and T4 levels

As shown in Table 4.2, P23 DR-Western offspring had lower free T3 plasma levels than DR-Chow offspring ($p < 0.05$, $F(1, 22) = 5.119$). There was no effect of maternal Diet on free T4 levels.

There were no significant effects of icv administration of TRH, TRH-DE inhibitor or both TRH and TRH-DE inhibitor on free T3 or free T4 plasma levels in chow-reared DR rats (Table 4.2).

TRH-DE knock-out mice body composition, chow intake and indirect calorimetry

55 day-old mutant mice lacking the *trhde* gene (KO mice) weighed less than their wild-type and heterozygous littermates ($p < 0.01$, $F(1, 13) = 11.113$). This was driven by a difference in lean mass ($p = 0.001$, $F(1, 13) = 17.801$), with no genotype effect on fat mass.

Body composition data collected from older *trhde* KO mice (Day 107) by Lexicon Genentech and available on the NIH MMRRC database (http://mmrrc.mousebiology.org /phenotype/Genentech/PRT357N1.html) showed that *trhde* KO mice had significantly lower body fat than their wild-type and heterozygous littermates ($p < 0.05$), indicating that differences in body composition may emerge as the mice age.

Trhde KO mice ate less chow in their home cages than their wild-type and heterozygous littermates (Figure 4) ($p < 0.05$, $F(1, 13) = 5.826$).

As shown in Figure 4.4, *trhde* KO mice had greater average energy expenditure over a 24-hour period ($p < 0.05$, $F(1, 11) = 7.029$), as well as greater total 24-hour energy expenditure ($p < 0.05$, $F(1, 11) = 6.988$), an effect that was more significant for the dark cycle ($p = 0.019$, $F(1, 11) = 7.48$) than for the light cycle ($p = 0.051$, $F(1, 11) = 4.818$). KO mice tended to have higher total locomotor activity during the dark cycle ($p = 0.053$, $F(1, 13) = 4.511$), but not over the 24-hour period. There was no effect of genotype on average respiratory quotient.

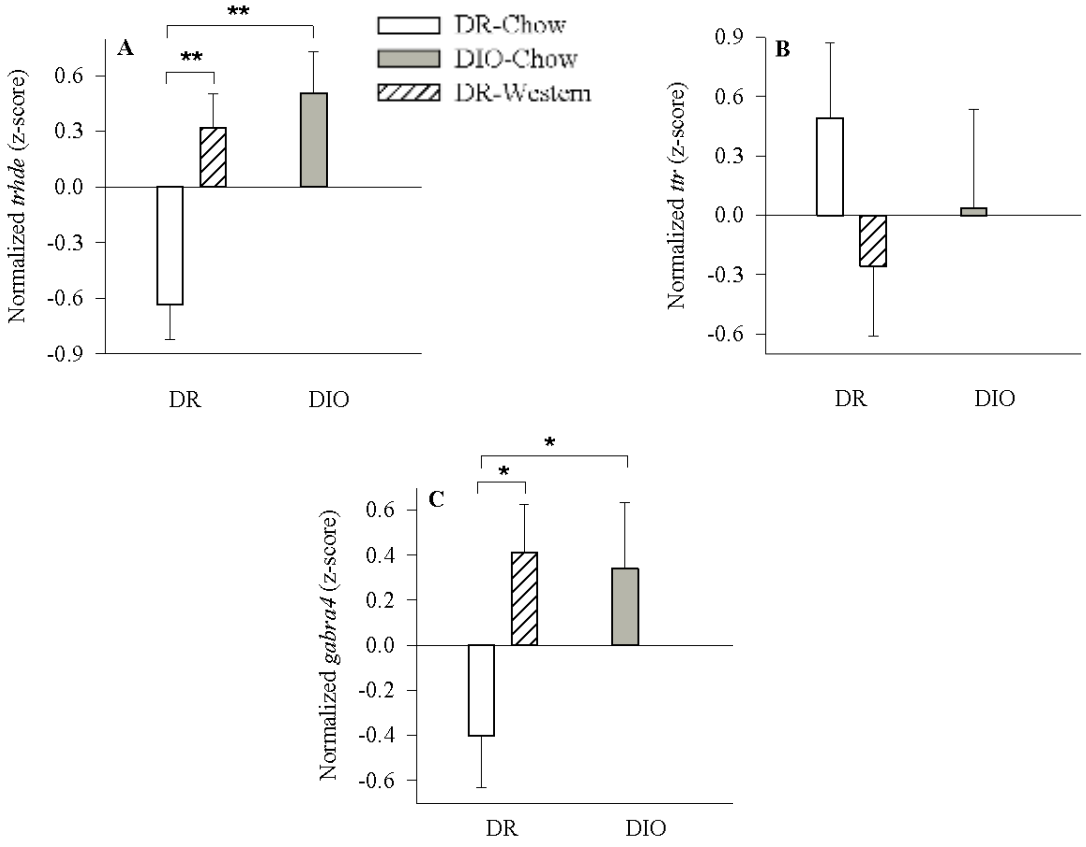

Figure 4.1: Gene expression levels in DR-Chow, DR-Western and DIO-Chow offspring. Genes were identified by microarray analysis ($n = 3$) as more highly expressed in DIO-Chow and DR-Western offspring than DR-Chow offspring. Data shown is from confirmatory qPCR (n =7-8), z-score normalized with same-subject Cyclophilin A expression as a covariate. (a) *trhde* is more highly expressed in DIO-Chow and DR-Western offspring than DR-Chow offspring; (b) *ttr* expression is not significantly different among the groups; (c) *gabra4* is more highly expressed in DIO-Chow and DR-Western offspring than DR-Chow offspring.

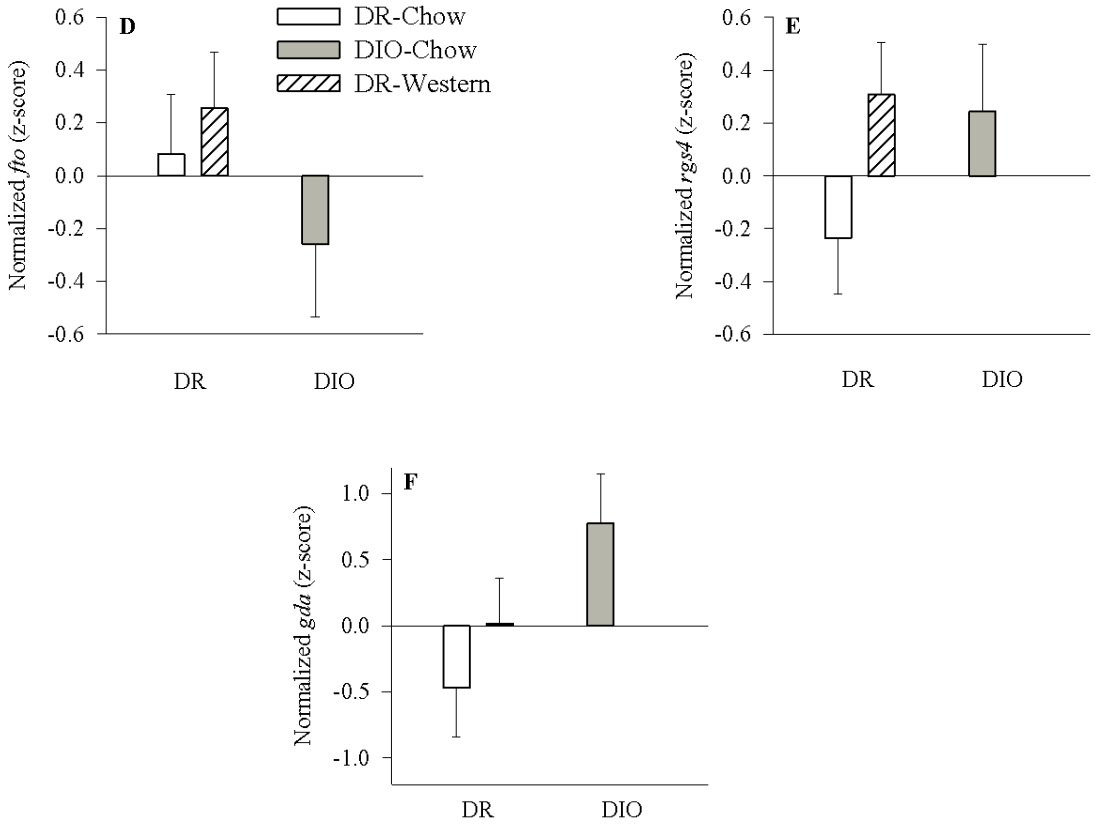

Figure 4.2: Gene expression levels in DR-Chow, DR-Western and DIO-Chow offspring. Genes were identified by microarray analysis ($n = 3$) as more highly expressed in DIO-Chow and DR-Western offspring than DR-Chow offspring. Data shown is from confirmatory qPCR (n =7-8), z-score normalized with same-subject Cyclophilin A expression as a covariate. (d) *fto* expression is not significantly different among the groups; (e) *rgs4* expression is not significantly different among the groups; (f) *gda* expression is not significantly different among the groups.

Table 4.2: Free T3 and free T4 levels.

	Free T3 (pg/dl)		Free T4 (pg/dl)	
P23 DR offspring				
DR-Chow	6.8 ±0.3		1.3 ±0.1	
DR-Western	5.9 ±0.3*		1.1 ±0.1	
Adult DR rats	**30 min**	**60 min**	**30 min**	**60 min**
Vehicle	2.0 ±0.6	2.3 ±0.6	2.4 ±0.4	2.0 ±0.4
TRH	2.9 ±0.6	2.7 ±0.6	2.8 ±0.4	2.4 ±0.4
Inhibitor	2.1 ±0.6	1.5 ±0.6	2.1 ±0.3	2.3 ±0.4
TRH + TRHDE inhibitor	2.1 ±0.6	3.5 ±0.6	2.3 ±0.4	2.3 ±0.3
***trhde* mutant mice**				
Knock-out	5.8 ±0.8		1.8 ±0.4	
Wild-type	4.7 ±1.2		1.9 ±0.6	
Heterozygous	4.7 ±0.9		1.2 ±0.5	

Note: Data shown are LS Mean ± standard error, covarying for cohort. * = Diet effect, $p < 0.05$.

TRH-DE knock-out mice high-fat diet challenge

Knock-out mice ate less high-fat diet than their wild-type and heterozygous littermates on an average daily kcal basis over the course of study ($p < 0.05$, $F(1, 13) = 7.975$) (Figure 4). Knock-out mice also weighed less ($p < 0.05$, $F(1, 7) = 6.03$) and gained less fat ($p < 0.01$, $F(1, 7) = 29.087$) than their wild-type littermates (Figure 4). There was no difference in lean mass gain.

TRH-DE knock-out mice free T3 and T4 levels

There were no significant differences among *trhde* knock-out, wild type and heterozygous mice in free T3 or free T4 plasma levels (Table 4.2).

4.3 Discussion

Analysis of gene expression in DR-Chow and DR-Western offspring identified *trhde* as a novel target for obesity susceptibility studies, and implicated the TRH sys-

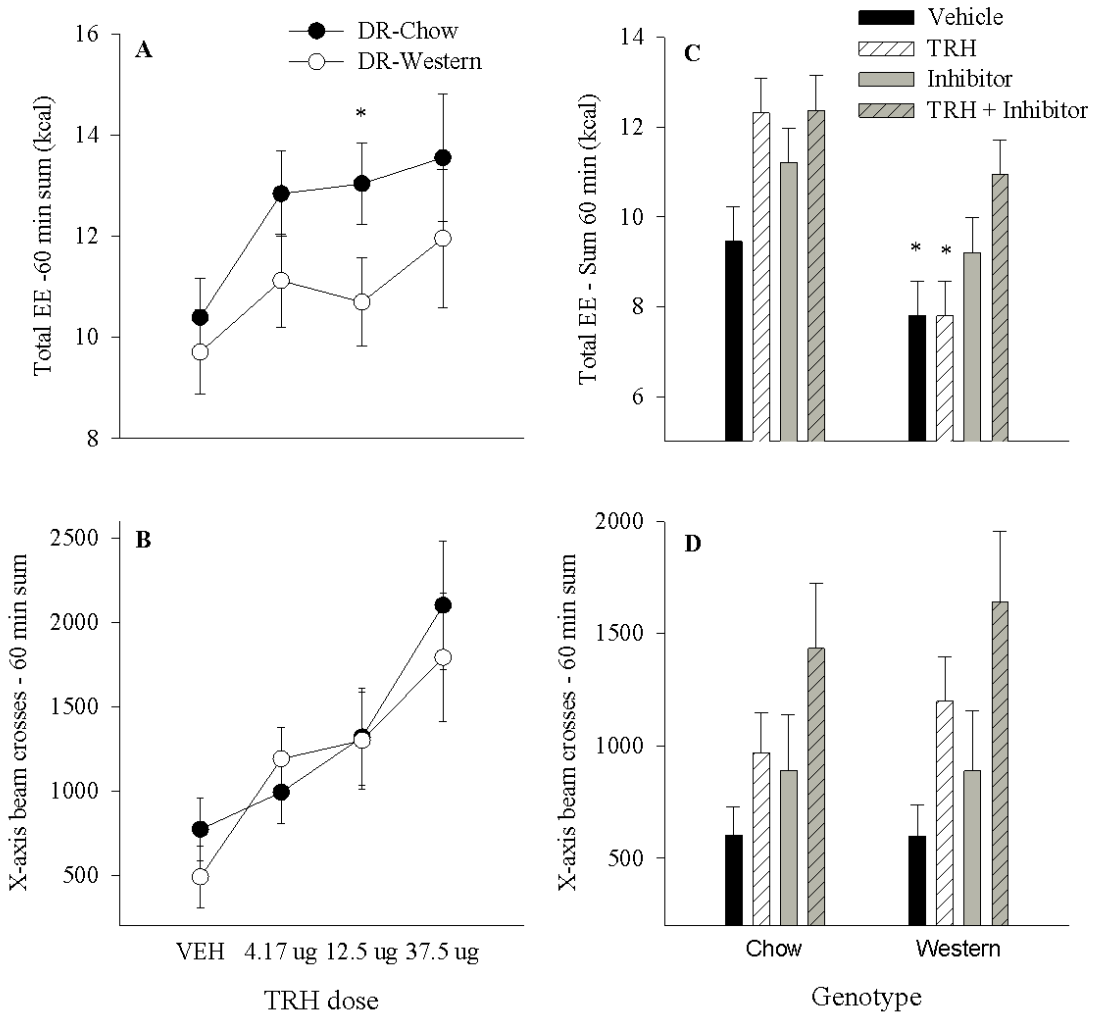

Figure 4.3: DR-Chow and DR-Western energy expenditure (EE) and locomotor response to TRH and TRH-DE inhibitor administration. (a) DR-Chow offspring increased total EE more than DR-Western offspring for 60 minutes following administration of the 12.5 μg dose of TRH; (b) DR-Chow and DR-Western offspring similarly increased total locomotor activity for 60 minutes following TRH administration; (c) DR-Western offspring did not increase total 60 min EE following TRH administration, but did increase total 60 min EE following combined TRH and TRH-DE administration; (d) DR-Chow and DR-Western offspring had similar patterns of locomotor response to TRH and/or TRH-DE administration.

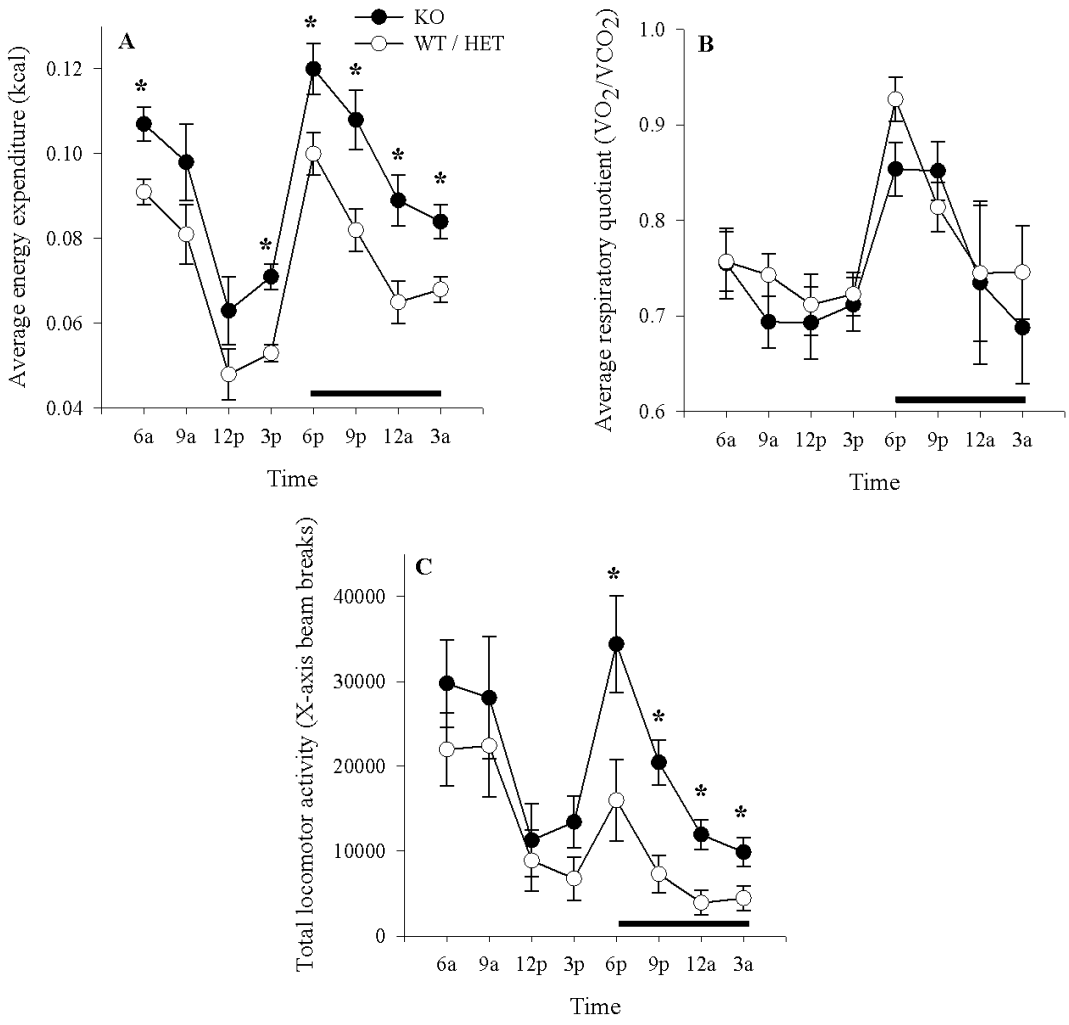

Figure 4.4: Energy expenditure, respiratory quotient and locomotor activity in *trhde* null mutant mice (KO) and their wild-type and heterozygous (WT/HET) littermates. (a) *trhde* KO mice had higher average energy expenditure over a 24-hour period than their WT/HET littermates; (b) There was no effect of *trhde* genotype on average respiratory quotient; (c) KO mice had greater locomotor activity during the dark cycle than their WT/HET littermates.

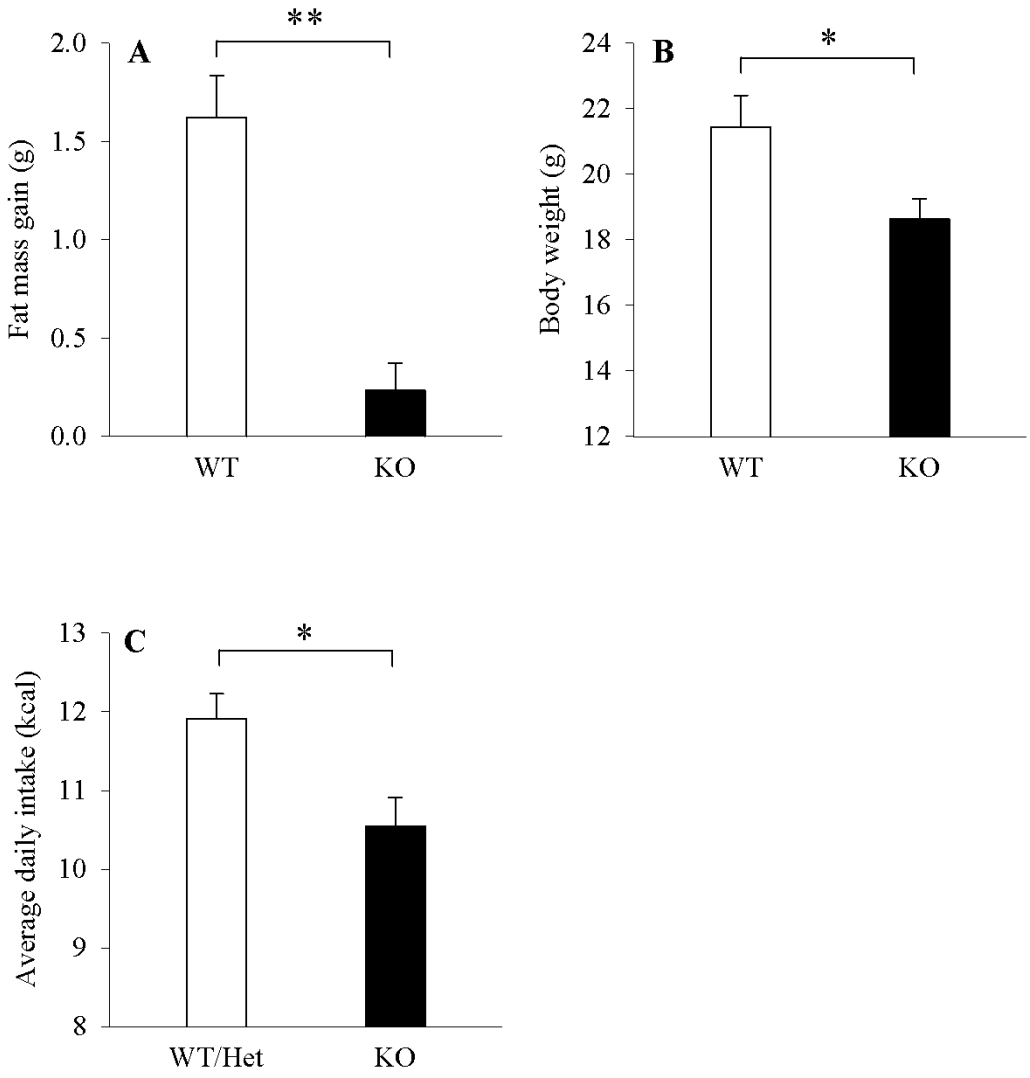

Figure 4.5: Response to high-fat diet challenge in *trhde* null mutant mice (KO) and their wild-type and heterozygous littermates (WT/HET) (a) *trhde* KO mice gained less fat than WT littermates; (b) *trhde* KO mice weighed less than WT littermates; (c) *trhde* KO mice ate less high-fat diet than WT/HET littermates daily.

tem as having a key role in maternal Western diet-induced obesity. Indirect calorimetry studies clearly showed that DR-Western offspring respond to TRH administration differently from DR-Chow offspring, indicating dysregulation of the TRH system by maternal Western diet. The difference in DR-Chow and DR-Western response to TRH administration was eliminated with concurrent administration of a TRH-DE inhibitor. TRH is known to play an important role in the control of energy balance and have a variety of behavioral effects. TRH administration decreases short-term food intake following acute administration [148, 149, 150, 151], increases food intake with long-term administration [152], and increases energy expenditure [151], body temperature [148], locomotor activity [153], anxiety-like behaviors [155] and wet dog shakes [156]. The current study showed that maternal Western diet alone, with no associated maternal obesity, can alter TRH effects on energy expenditure, likely through a central mechanism mediated by overexpression of *trhde*.

Central mechanisms of TRH action

TRH exerts effects on energy expenditure, locomotor activity and food intake not only via activation of the hypothalamus-pituitary-thyroid axis, but also via central mechanisms [139]. TRH-releasing neurons are located in the paraventricular nucleus (PVN) of the hypothalamus and lateral hypothalamus [23], and project to other hypothalamic nuclei, including arcuate, dorsomedial and ventromedial, the central amygdala, the bed nucleus of the stria terminalis, and several other regions [140]. TRH neurons receive input from two types of neurons involved in regulation of energy balance: pro-opiomelanocortin (POMC)/cocaine- and amphetamine-related transcript (CART) neurons and neuropeptide
Y (NPY)/agouti-related protein (AgRP) neurons. Both types of neurons are leptin-sensitive, but activation of POMC/CART neurons increases energy expenditure, while activation of NPY/AgRP neurons decreases energy expenditure. TRH neurons are leptin-sensitive. Surprisingly, it was recently shown that TRH neurons are one of two major sources of excitatory input to AgRP neurons [25]. Stimulation of TRH neurons leads to excitation of AgRP neurons, which leads to increased feeding, even in sated mice. The ability of TRH to stimulate food intake via AgRP neuronal activation was previously

unknown, and calls into question the mechanism by which central TRH administration acutely suppresses feeding . TRH also inhibits orexigenic melanin-concentration hormone (MCH) neurons via activation of local GABA neurons [26], which could contribute to the anorectic effects of central TRH administration. Neurons expressing the precursor peptide to TRH, pre-pro-TRH, are located in the lateral hypothalamus and are stimulated by leptin administration [27]. Decreased expression of pre-pro-TRH leads to increased food intake.

There are also central effects of TRH on energy balance outside of the hypothalamus. TRH and leptin work together to regulate adipose tissue metabolism. Serum TSH and leptin levels are correlated in obese subjects [157]. TRH application to the dorsal medulla in the hindbrain slightly increases brown adipose tissue (BAT) thermogenesis and core body temperature. TRH and leptin co-application increase BAT and core temperature 3 times more than TRH alone, indicating that TRH may interact with leptin in the brain to increase energy expenditure [158]. TRH potentiation of locomotor activity is mediated by dopamine release from pre-synaptic terminals. Pre-treatment with a dopamine antagonist blocks the effects of TRH on locomotor activity, and TRH administration increases dopamine activity in the nucleus accumbens, indicating TRH and dopamine in the accumbens work together to increase locomotor activity [159].

The neural circuits to which TRH neurons are connected suggest an intimate involvement with the central regulation of energy balance.

TRH-DE regulation of TRH

TRH-DE was identified and characterized as a highly-specific degrading ectoenzyme for TRH by Bauer and colleagues [137, 138]. TRH-DE is located on the surface of neurons throughout the brain, with highest expression levels in cortex, hypothalamus and medial habenula, as well as in the pituitary [160]. TRH-DE activity is increased under conditions that also activate TRH neurons in the hypothalamus, amygdala, accumbens, cortex, and hippocampus [161], suggesting that TRH-DE and TRH can be co-activated. Feedback from thyroid hormones can decrease TRH-DE levels in the pituitary [162], but not in the brain [163], which suggests that brain TRH-DE is influenced by central but not peripheral factors. TRH-DE reduces the amount of active TRH at

the synaptic cleft.

In accordance with previous studies showing that the behavioral effects of TRH administration are not related to the hypothalamus-pituitary-thyroid axis [154, 164], there was no effect of TRH or TRH-DE inhibitor administration on free T3 or free T4 levels, nor was there a difference in free T3 or free T4 levels between *trhde* wildtype and knock-out mice. This suggests that differences between DR-Chow and DR-Western offspring and *trhde* knock-out and wildtype mice are centrally mediated. Increased expression of *trhde* in DR-Western offspring may lead to increased degradation of TRH by TRH-DE, resulting in less available TRH in the extra-cellular space. Less TRH would lead to less activation of neural circuits known to be involved in heat formation that are activated by TRH.

When TRH-DE is inhibited, the amount of active TRH in the synapse is increased [165]. In the current study, it is hypothesized that overexpression of lateral hypothalamic *trhde* in DR-Western offspring resulted in higher levels of TRH-DE in the brain, which caused increased degradation of TRH, which led to a blunted response to TRH administration. Concurrent administration of a TRH-DE inhibitor blocked the excess TRH-DE, preventing it from degrading the TRH and restoring a normal behavioral response to TRH administration. TRH regulation of energy expenditure is dysfunctional in DR-Western offspring, but regains normal function with the administration of a TRH-DE inhibitor. This indicates that high levels of TRH-DE in DR-Western animals may be related to their long-term obesity risk (increased adiposity and decreased energy expenditure).

Loss of function study

In order to determine if *trhde* has a functional role in long-term risk for obesity, mice lacking *trhde* were studied. As predicted by our hypothesis, *trhde* null mutant mice were lighter, leaner and had greater energy expenditure than their wild-type littermates. This suggests that *trhde* is important in energy balance, and overexpression of *trhde* may lead to obesity.

Future studies are planned to determine the effects of *trhde* loss on the DR maternal Western diet model of obesity risk. TRH-DE inhibitor studies provided evidence

that excess TRH-DE is related to the failure of DR-Western offspring to respond to TRH administration. However, it is unclear if excess TRH-DE is the cause of long-term obesity susceptibility in DR-Western offspring. Future studies will knock down *trhde* expression in the lateral hypothalamus via bilateral microinfusions of a lentiviral vector transducing RNAi for *trhde* in P23 DR-Chow and DR-Western offspring.

Localization to lateral hypothalamus

While microarray and qPCR analysis identified differences in TRH-DE mRNA expression in the lateral hypothalamus, the importance of that region is unclear. qPCR was attempted on the PVN, ventro-medial hypothalamus, arcuate nucleus of the hypothalamus and accumbens, but because of the small amount of tissue yielded from unilateral dissection of P23 brains, the mRNA was not of sufficient quality or quantity to proceed with analysis for those regions. Further studies should be done to assess maternal Diet-induced gene expression changes in DR rats in other brain regions. Additionally, because TRH and TRH-DE inhibitor were administered to the lateral ventricle, the whole brain was affected by the administration. Site-specific administration or gene knock-down studies targeting the lateral hypothalamus will determine if the lateral hypothalamus is functionally essential in TRH-DE-mediated obesity risk in DR-Western offspring.

Epigenetic mechanism for *trhde* overexpression

One possible mechanism for the effect of maternal Western diet on offspring *trhde* expression is epigenetic modifications to the genome. Environmental factors, including maternal diet, have been shown to induce changes in histone acetylation and/or DNA methylation, which can alter gene expression levels [166, 167, 96]. Increased histone acetylation tends to increase gene expression, while increased methylation at the 5-carbon of the cytosine ring (5-mC) decreases gene expression [168]. As reported in Chapter 3, in order to determine if DNA methylation may be the cause of altered gene expression in DR-Western offspring, global 5-mC DNA methylation was measured in whole hypothalamus tissue from P1 offspring and lateral hypothalamus tissue from P23 DR-Chow and DR-Western offspring. At P1, DR-Chow offspring showed increased global

DNA methylation ($p = 0.024$). At P23, there was no significant difference in global DNA methylation.

A future gene-specific methylation study will determine if maternal Western diet alters methylation of the *trhde* gene. CpG islands in the promoter region and first coding exon of *trhde* will be targeted, as methylation of those regions has been shown to have the greatest impact on gene expression [169, 170].

While disruptions in the thyroid axis in association with obesity have been well-studied, underlying causes of thyroid hormone dysregulation are unknown. Specifically, the relationship between maternal diet and offspring thyroid function has not been well-studied. One study in non-human primates observed that maternal high-fat diet consumption during pregnancy and lactation decreased offspring T4 levels and altered expression of thyroid-related genes, possibly through epigenetic modifications to the genome [171]. The current study is the first to report maternal Western diet-associated overexpression of *trhde* and differences in behavioral response to central TRH administration. That the differences in behavioral response to TRH disappear with concurrent administration of a TRH-DE inhibitor indicates that the differences may be functionally related to elevated *trhde* levels. Increased *trhde* expression as a result of maternal Western diet is a novel mechanism for disruptions in the central thyroid system that lead to long-term obesity susceptibility.

Chapter 5

Conclusions

The goal of the work described in this dissertation was to identify a novel obesity treatment target by clarifying the roles of genetic and environmental factors in obesity risk. Specifically, studies aimed to: 1) determine the effects of maternal Western diet on genetically obesity prone and obesity resistant offspring; 2) identify a novel hypothalamic gene with increased expression associated with obesity susceptibility; 3) determine the functional significance of that gene as it relates to energy balance.

The studies described in Chapter 2 determined the effects of maternal Western diet and obesity-susceptible genotype on male offspring. As early as P1, maternal Western diet increased adiposity even in male offspring of obesity-resistant dams. The offspring were genetically programmed to resist obesity, yet became obese as a result of maternal Western diet. Offspring thinned out during adolescence, but the effects of maternal Western diet were again seen in adulthood, with adult offspring of obesity-resistant dams showing increased adiposity, increased food intake, decreased energy expenditure and decreased lipid utilization. This indicates that a high-fat, high-carbohydrate perinatal nutritional environment can override genetic resistance to obesity, with effects starting in infancy and lasting into adulthood. Results implicate perinatal environment and maternal diet per se as having greater impact on offspring outcome than previously appreciated.

The studies described in Chapter 3 determined the effects of maternal Western diet and obesity-susceptible genotype on female offspring. Female offspring of obesity-resistant dams had increased leptin and insulin levels, increased fat gain in response to

high-fat diet exposure and decreased energy expenditure. As in male offspring, energy balance systems were disrupted by maternal Western diet in female offspring, showing once again that environmental factors can overpower genetic factors.

Genetic background also had large effects on offspring obesity risk. Genetically obesity-susceptible male and female offspring were heavier and fatter than obesity-resistant male and female offspring from a young age, even if they were never exposed to a high-energy diet. This shows that genes also play an important role in determining obesity risk, as has been previously reported.

The studies described in Chapter 4 sought to identify a previously-unstudied gene that is overexpressed in association with genetic or environmental obesity risk. Microarray analysis was performed on cDNA generated from lateral hypothalamic tissue of obesity-susceptible DIO and DR-Western offspring. Genes that were more highly expressed in both DIO and DR-Western offspring than in DR-Chow offspring were targeted for analysis. The gene coding for TRH-DE had the greatest expression difference and so was chosen for further study. It was hypothesized that increased trhde expression was related to increased obesity risk. Because the effects of maternal Western diet in obesity-resistant DR offspring were the most interesting and novel results from Chapters 2 and 3, functional studies of TRH-DE were done only in DR offspring. Central administration of TRH and TRH-DE inhibitor showed that TRH regulation of energy expenditure is dysfunctional in DR-Western offspring, but regains normal function with the administration of a TRH-DE inhibitor. This indicates that overexpression of trhde in the brain is likely to be functionally related to obesity risk. Loss of function studies in trhde knock-out mice revealed that a total loss of trhde expression led to increased energy expenditure and decreased adiposity, providing further evidence that the hypothesis that trhde overexpression increases obesity risk by decreasing energy expenditure.

The work of this dissertation novelly demonstrated that maternal diet in and of itself, in the absence of maternal obesity, has the power to increase offspring obesity risk, even if offspring are genetically resistant to obesity. This has great implications for pregnancy healthcare in humans. Healthcare providers should emphasize the importance of diet to pregnant and lactating women and provide diet consultation services. Pregnancy guidelines should include and emphasize diet recommendations, rather than

focusing on weight gain, as is the current practice. Additionally, the work described in this dissertation identified a new central mechanism for TRH-related disruption of energy homeostasis. It has provided a new target for potential pharmaceutical treatment of obesity.

Bibliography

[1] **Kassi E, Pervanidou P, Kaltsas G, Chrousos G** 2011 Metabolic syndrome: definitions and controversies. BMC medicine 9:48

[2] **van Hout GC, van Oudheusden I, van Heck GL** 2004 Psychological profile of the morbidly obese. Obesity surgery 14:579-588

[3] **Hu FB, Manson JE, Stampfer MJ, Colditz G, Liu S, Solomon CG, Willett WC** 2001 Diet, lifestyle, and the risk of type 2 diabetes mellitus in women. The New England journal of medicine 345:790–797

[4] **Eeg-Olofsson K, Cederholm J, Nilsson PM, Zethelius B, Nunez L, Gudbjornsdottir S, Eliasson B** 2009 Risk of cardiovascular disease and mortality in overweight and obese patients with type 2 diabetes: an observational study in 13,087 patients. Diabetologia 52:65–73

[5] **Taylor VH, Forhan M, Vigod SN, McIntyre RS, Morrison KM** 2013 The impact of obesity on quality of life. Best practice & research Clinical endocrinology & metabolism 27:139–146

[6] **Ogden CL, Carroll MD, Curtin LR, Lamb MM, Flegal KM** 2010 Prevalence of high body mass index in US children and adolescents, 2007-2008. JAMA 303:242–249

[7] **Freedman DS, Mei Z, Srinivasan SR, Berenson GS, Dietz WH** 2007 Cardiovascular risk factors and excess adiposity among overweight children and adolescents: the Bogalusa Heart Study. J Pediatr 150:12–17 e12

[8] **Whitlock EP, Williams SB, Gold R, Smith PR, Shipman SA** 2005 Screening and interventions for childhood overweight: a summary of evidence for the US Preventive Services Task Force. Pediatrics 116:e125–144

[9] **Puder JJ, Munsch S** 2010 Psychological correlates of childhood obesity. Int J Obes (Lond) 34 Suppl 2:S37–43

[10] **Biro FM, Wien M** 2010 Childhood obesity and adult morbidities. Am J Clin Nutr 91:1499S–1505S

[11] **Chen HC, Roth JD, Schroeder BE, Weyer C** 2008 Role of islet-, gut-, and adipocyte-derived hormones in the central control of food intake and body weight: implications for an integrated neurohormonal approach to obesity pharmacotherapy. Current diabetes reviews 4:79–91

[12] **Hameed S, Dhillo WS, Bloom SR** 2009 Gut hormones and appetite control. Oral diseases 15:18–26

[13] **Ahima RS, Antwi DA** 2008 Brain regulation of appetite and satiety. Endocrinology and metabolism clinics of North America 37:811–823

[14] **Woods SC, D'Alessio DA** 2008 Central control of body weight and appetite. The Journal of clinical endocrinology and metabolism 93:S37–50

[15] **Berthoud HR** 2008 The vagus nerve, food intake and obesity. Regulatory peptides 149:15–25

[16] **Benoit SC, Tracy AL, Davis JF, Choi D, Clegg DJ** 2008 Novel functions of orexigenic hypothalamic peptides: from genes to behavior. Nutrition 24:843–847

[17] **Harris AR, Christianson D, Smith MS, Fang SL, Braverman LE, Vagenakis AG** 1978 The physiological role of thyrotropin-releasing hormone in the regulation of thyroid-stimulating hormone and prolactin secretion in the rat. The Journal of clinical investigation 61:441–448

[18] **Hall R, Amos J, Garry R, Buxton RL** 1970 Thyroid-stimulating hormone response to synthetic thyrotrophin releasing hormone in man. British medical journal 2:274–277

[19] **Silva JE** 1995 Thyroid hormone control of thermogenesis and energy balance. Thyroid : official journal of the American Thyroid Association 5:481–492

[20] **Danforth E, Jr., Burger A** 1984 The role of thyroid hormones in the control of energy expenditure. Clinics in endocrinology and metabolism 13:581–595

[21] **Silva JE** 2006 Thermogenic mechanisms and their hormonal regulation. Physiological reviews 86:435–464

[22] **Nillni EA** 2010 Regulation of the hypothalamic thyrotropin releasing hormone (TRH) neuron by neuronal and peripheral inputs. Frontiers in neuroendocrinology 31:134–156

[23] **Merchenthaler I** 1991 Co-localization of enkephalin and TRH in perifornical neurons of the rat hypothalamus that project to the lateral septum. Brain research 544:177-180

[24] **Wittmann G, Fuzesi T, Singru PS, Liposits Z, Lechan RM, Fekete C** 2009 Efferent projections of thyrotropin-releasing hormone-synthesizing neurons residing in the anterior parvocellular subdivision of the hypothalamic paraventricular nucleus. The Journal of comparative neurology 515:313-330

[25] **Krashes MJ, Shah BP, Madara JC, Olson DP, Strochlic DE, Garfield AS, Vong L, Pei H, Watabe-Uchida M, Uchida N, Liberles SD, Lowell BB** 2014 An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. Nature

[26] **Zhang X, van den Pol AN 2012** Thyrotropin-releasing hormone (TRH) inhibits melanin-concentrating hormone neurons: implications for TRH-mediated anorexic and arousal actions. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:3032-3043

[27] **Carbone DL, Zuloaga DG, Lacagnina AF, Handa RJ** 2012 Preprothyrotropin releasing hormone expressing neurons in the juxtaparaventricular region of the lateral hypothalamus are activated by leptin and altered by prenatal glucocorticoid exposure. Brain research 1477:19-26

[28] **Hill JO, Peters JC** 1998 Environmental contributions to the obesity epidemic. Science 280:1371–1374

[29] **Comuzzie AG, Allison DB** 1998 The search for human obesity genes. Science 280:1374–1377

[30] **Comuzzie AG, Blangero J, Mahaney MC, Mitchell BD, Stern MP, MacCluer JW** 1994 Genetic and environmental correlations among skinfold measures. Int J Obes Relat Metab Disord 18:413–418

[31] **Comuzzie AG, Blangero J, Mahaney MC, Mitchell BD, Hixson JE, Samollow PB, Stern MP, MacCluer JW** 1995 Major gene with sex-specific effects influences fat mass in Mexican Americans. Genet Epidemiol 12:475–488

[32] **Comuzzie AG, Higgins PB, Voruganti S, Cole S** 2010 Cutting the fat: the genetic dissection of body weight. Progress in molecular biology and translational science 94:197–212

[33] **Levin BE, Dunn-Meynell AA, Balkan B, Keesey RE** 1997 Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats. Am J Physiol 273:R725–730

[34] **Ricci MR, Levin BE** 2003 Ontogeny of diet-induced obesity in selectively bred Sprague-Dawley rats. Am J Physiol Regul Integr Comp Physiol 285:R610–618

[35] **Madsen AN, Hansen G, Paulsen SJ, Lykkegaard K, Tang-Christensen M, Hansen HS, Levin BE, Larsen PJ, Knudsen LB, Fosgerau K, Vrang N** 2010 Long-term characterization of the diet-induced obese and diet-resistant rat model: a polygenetic rat model mimicking the human obesity syndrome. J Endocrinol 206:287–296

[36] **Thorn SR, Rozance PJ, Brown LD, Hay WW, Jr.** 2011 The intrauterine growth restriction phenotype: fetal adaptations and potential implications for later life insulin resistance and diabetes. Seminars in reproductive medicine 29:225–236

[37] **Maffeis C, Micciolo R, Must A, Zaffanello M, Pinelli L** 1994 Parental and perinatal factors associated with childhood obesity in north-east Italy. Int J Obes Relat Metab Disord 18:301–305

[38] **Schaefer-Graf UM, Pawliczak J, Passow D, Hartmann R, Rossi R, Buhrer C, Harder T, Plagemann A, Vetter K, Kordonouri O** 2005 Birth weight and parental BMI predict overweight in children from mothers with gestational diabetes. Diabetes Care 28:1745–1750

[39] **Lawlor DA, Lichtenstein P, Fraser A, Langstrom N** 2011 Does maternal weight gain in pregnancy have long-term effects on offspring adiposity? A sibling study in a prospective cohort of 146,894 men from 136,050 families. Am J Clin Nutr 94:142–148

[40] **Boney CM, Verma A, Tucker R, Vohr BR** 2005 Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics 115:e290–296

[41] **Guo F, Jen KL** 1995 High-fat feeding during pregnancy and lactation affects offspring metabolism in rats. Physiol Behav 57:681–686

[42] **Massiera F, Barbry P, Guesnet P, Joly A, Luquet S, Moreilhon-Brest C, Mohsen-Kanson T, Amri EZ, Ailhaud G** 2010 A Western-like fat diet is sufficient to induce a gradual enhancement in fat mass over generations. J Lipid Res 51:2352–2361

[43] **Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH, Piersma AH, Ozanne SE, Twinn DF, Remacle C, Rowlerson A, Poston L, Taylor PD** 2008 Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. Hypertension 51:383–392

[44] **Catalano PM, Ehrenberg HM** 2006 The short- and long-term implications of maternal obesity on the mother and her offspring. BJOG 113:1126–1133

[45] **Rooney K, Ozanne SE** 2011 Maternal over-nutrition and offspring obesity predisposition: targets for preventative interventions. Int J Obes (Lond) 35:883–890

[46] **Krasnow SM, Nguyen ML, Marks DL** 2011 Increased maternal fat consumption during pregnancy alters body composition in neonatal mice. American journal of physiology Endocrinology and metabolism 301:E1243–1253

[47] **Dyer JS, Rosenfeld CR** 2011 Metabolic imprinting by prenatal, perinatal, and postnatal overnutrition: a review. Seminars in reproductive medicine 29:266–276

[48] **Li M, Sloboda DM, Vickers MH** 2011 Maternal obesity and developmental programming of metabolic disorders in offspring: evidence from animal models. Experimental diabetes research 2011:592408

[49] **Norman JE, Reynolds RM** 2011 The consequences of obesity and excess weight gain in pregnancy. The Proceedings of the Nutrition Society 70:450–456

[50] **Russo P, Lauria F, Siani A** 2010 Heritability of body weight: moving beyond genetics. Nutrition, metabolism, and cardiovascular diseases : NMCD 20:691–697

[51] **Freedman DS, Mei Z, Srinivasan SR, Berenson GS, Dietz WH** 2007 Cardiovascular risk factors and excess adiposity among overweight children and adolescents: the Bogalusa Heart Study. J Pediatr 150:12–17 e12

[52] **White CL, Purpera MN, Morrison CD** 2009 Maternal obesity is necessary for programming effect of high-fat diet on offspring. American journal of physiology Regulatory, integrative and comparative physiology 296:R1464–1472

[53] **McCurdy CE, Bishop JM, Williams SM, Grayson BE, Smith MS, Friedman JE, Grove KL** 2009 Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. The Journal of clinical investigation 119:323–335

[54] **Rasmussen KM, Yaktine AL, Institute of Medicine (U.S.). Committee to Reexamine IOM Pregnancy Weight Guidelines.** 2009 Weight gain during pregnancy : reexamining the guidelines. Washington, DC: National Academies Press

[55] **Institute of Medicine (U.S.). Panel on Macronutrients., Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes.** 2005 Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids. Washington, D.C.: National Academies Press

[56] **Institute of Medicine (U.S.). Committee on Nutritional Status During Pregnancy and Lactation. Subcommittee for a Clinical Application Guide.** 1992 Nutrition during pregnancy and lactation : an implementation guide. Washington, D.C.: National Academy Press

[57] **Levin BE, Govek E** 1998 Gestational obesity accentuates obesity in obesity-prone progeny. Am J Physiol 275:R1374–1379

[58] **Levin BE, Dunn-Meynell AA** 2002 Maternal obesity alters adiposity and monoamine function in genetically predisposed offspring. American journal of physiology Regulatory, integrative and comparative physiology 283:R1087–1093

[59] **Le Foll C, Irani BG, Magnan C, Dunn-Meynell A, Levin BE** 2009 Effects of maternal genotype and diet on offspring glucose and fatty acid-sensing ventromedial hypothalamic nucleus neurons. American journal of physiology Regulatory, integrative and comparative physiology 297:R1351–1357

[60] **Gorski JN, Dunn-Meynell AA, Levin BE** 2007 Maternal obesity increases hypothalamic leptin receptor expression and sensitivity in juvenile obesity-prone rats. American journal of physiology Regulatory, integrative and comparative physiology 292:R1782–1791

[61] **Zorrilla EP** 1997 Multiparous species present problems (and possibilities) to developmentalists. Developmental psychobiology 30:141–150

[62] **Knittle JL, Hirsch J** 1968 Effect of early nutrition on the development of rat epididymal fat pads: cellularity and metabolism. The Journal of clinical investigation 47:2091–2098

[63] **Zorrilla EP, Sanchez-Alavez M, Sugama S, Brennan M, Fernandez R, Bartfai T, Conti B** 2007 Interleukin-18 controls energy homeostasis by suppressing appetite and feed efficiency. Proceedings of the National Academy of Sciences of the United States of America 104:11097–11102

[64] **Wahlig JL, Bales ES, Jackman MR, Johnson GC, McManaman JL, Maclean PS** 2012 Impact of high-fat diet and obesity on energy balance and fuel utilization during the metabolic challenge of lactation. Obesity (Silver Spring) 20:65–75

[65] **Karp NA, Segonds-Pichon A, Gerdin AK, Ramirez-Solis R, White JK** 2012 The fallacy of ratio correction to address confounding factors. Laboratory animals 46:245–252

[66] **Pasquali R, Patton L, Gambineri A** 2007 Obesity and infertility. Current opinion in endocrinology, diabetes, and obesity 14:482–487

[67] **Lins MC, de Moura EG, Lisboa PC, Bonomo IT, Passos MC** 2005 Effects of maternal leptin treatment during lactation on the body weight and leptin resistance of adult offspring. Regulatory peptides 127:197–202

[68] **Passos MC, Lins MC, Lisboa PC, Toste FP, Bonomo IT, de Moura EG** 2007 Maternal leptin treatment during lactation programs the thyroid function of adult rats. Life sciences 80:1754–1758

[69] **Passos MC, Lisboa PC, Pereira-Toste F, Oliveira E, Moura EG** 2012 Developmental plasticity in thyroid function primed by maternal hyperleptinemia in early lactation: a time-course study in rats. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 44:520–526

[70] **Ravussin E, Lillioja S, Knowler WC, Christin L, Freymond D, Abbott WG, Boyce V, Howard BV, Bogardus C** 1988 Reduced rate of energy expenditure as a risk factor for body-weight gain. The New England journal of medicine 318:467–472

[71] **Zurlo F, Lillioja S, Esposito-Del Puente A, Nyomba BL, Raz I, Saad MF, Swinburn BA, Knowler WC, Bogardus C, Ravussin E** 1990 Low ratio of fat to carbohydrate oxidation as predictor of weight gain: study of 24-h RQ. Am J Physiol 259:E650–657

[72] **Howie GJ, Sloboda DM, Kamal T, Vickers MH** 2009 Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. The Journal of physiology 587:905–915

[73] **Sun SS, Liang R, Huang TT, Daniels SR, Arslanian S, Liu K, Grave GD, Siervogel RM** 2008 Childhood obesity predicts adult metabolic syndrome: the Fels Longitudinal Study. J Pediatr 152:191–200

[74] **Baird J, Fisher D, Lucas P, Kleijnen J, Roberts H, Law C** 2005 Being big or growing fast: systematic review of size and growth in infancy and later obesity. BMJ 331:929

[75] **Kotani Y, Yokota I, Kitamura S, Matsuda J, Naito E, Kuroda Y** 2004 Plasma adiponectin levels in newborns are higher than those in adults and positively correlated with birth weight. Clinical endocrinology 61:418–423

[76] **Tsai PJ, Yu CH, Hsu SP, Lee YH, Chiou CH, Hsu YW, Ho SC, Chu CH** 2004 Cord plasma concentrations of adiponectin and leptin in healthy term neonates: positive correlation with birthweight and neonatal adiposity. Clinical endocrinology 61:88–93

[77] **Kettaneh A, Heude B, Romon M, Oppert JM, Borys JM, Balkau B, Ducimetiere P, Charles MA** 2007 High plasma leptin predicts an increase in subcutaneous adiposity in children and adults. European journal of clinical nutrition 61:719–726

[78] **Savoye M, Dziura J, Castle J, DiPietro L, Tamborlane WV, Caprio S** 2002 Importance of plasma leptin in predicting future weight gain in obese children: a two-and-a-half-year longitudinal study. Int J Obes Relat Metab Disord 26:942–946

[79] **Ahima RS, Prabakaran D, Flier JS** 1998 Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. The Journal of clinical investigation 101:1020–1027

[80] **Beltrand J, Sloboda DM, Connor KL, Truong M, Vickers MH** 2012 The Effect of Neonatal Leptin Antagonism in Male Rat Offspring Is Dependent upon the Interaction between Prior Maternal Nutritional Status and Post-Weaning Diet. Journal of nutrition and metabolism 2012:296935

[81] **Freedman DS, Srinivasan SR, Burke GL, Shear CL, Smoak CG, Harsha DW, Webber LS, Berenson GS** 1987 Relation of body fat distribution to hyperinsulinemia in children and adolescents: the Bogalusa Heart Study. Am J Clin Nutr 46:403–410

[82] **Lonnerdal B, Havel PJ** 2000 Serum leptin concentrations in infants: effects of diet, sex, and adiposity. Am J Clin Nutr 72:484–489

[83] **Arslanian S, Suprasongsin C, Kalhan SC, Drash AL, Brna R, Janosky JE** 1998 Plasma leptin in children: relationship to puberty, gender, body composition, insulin sensitivity, and energy expenditure. Metabolism: clinical and experimental 47:309–312

[84] **Benoit SC, Clegg DJ, Seeley RJ, Woods SC** 2004 Insulin and leptin as adiposity signals. Recent progress in hormone research 59:267–285

[85] **Wielinga PY, Lowenstein C, Muff S, Munz M, Woods SC, Lutz TA** 2010 Central amylin acts as an adiposity signal to control body weight and energy expenditure. Physiol Behav 101:45–52

[86] **Lutz TA** 2010 The role of amylin in the control of energy homeostasis. American journal of physiology Regulatory, integrative and comparative physiology 298:R1475–1484

[87] **Barrera JG, Jones KR, Herman JP, D'Alessio DA, Woods SC, Seeley RJ** 2011 Hyperphagia and increased fat accumulation in two models of chronic CNS glucagon-like peptide-1 loss of function. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:3904–3913

[88] **Naslund E, Barkeling B, King N, Gutniak M, Blundell JE, Holst JJ, Rossner S, Hellstrom PM** 1999 Energy intake and appetite are suppressed by glucagon-like peptide-1 (GLP-1) in obese men. Int J Obes Relat Metab Disord 23:304–311

[89] **Boyle CN, Rossier MM, Lutz TA** 2011 Influence of high-fat feeding, diet-induced obesity, and hyperamylinemia on the sensitivity to acute amylin. Physiol Behav 104:20–28

[90] **Chen H, Simar D, Lambert K, Mercier J, Morris MJ** 2008 Maternal and postnatal overnutrition differentially impact appetite regulators and fuel metabolism. Endocrinology 149:5348–5356

[91] **Henning SJ** 1981 Postnatal development: coordination of feeding, digestion, and metabolism. Am J Physiol 241:G199–214

[92] **Purcell RH, Sun B, Pass LL, Power ML, Moran TH, Tamashiro KL** 2011 Maternal stress and high-fat diet effect on maternal behavior, milk composition, and pup ingestive behavior. Physiol Behav 104:474–479

[93] **Sun B, Purcell RH, Terrillion CE, Yan J, Moran TH, Tamashiro KL** 2012 Maternal high-fat diet during gestation or suckling differentially affects offspring leptin sensitivity and obesity. Diabetes 61:2833–2841

[94] **Fahrenkrog S, Harder T, Stolaczyk E, Melchior K, Franke K, Dudenhausen JW, Plagemann A** 2004 Cross-fostering to diabetic rat dams affects early development of mediobasal hypothalamic nuclei regulating food intake, body weight, and metabolism. The Journal of nutrition 134:648–654

[95] **Gorski JN, Dunn-Meynell AA, Hartman TG, Levin BE** 2006 Postnatal environment overrides genetic and prenatal factors influencing offspring obesity and insulin resistance. American journal of physiology Regulatory, integrative and comparative physiology 291:R768–778

[96] **Bale TL, Baram TZ, Brown AS, Goldstein JM, Insel TR, McCarthy MM, Nemeroff CB, Reyes TM, Simerly RB, Susser ES, Nestler EJ** 2010 Early life programming and neurodevelopmental disorders. Biological psychiatry 68:314–319

[97] **Barres R, Zierath JR** 2011 DNA methylation in metabolic disorders. Am J Clin Nutr 93:897S–900

[98] **Aagaard-Tillery KM, Grove K, Bishop J, Ke X, Fu Q, McKnight R, Lane RH** 2008 Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. Journal of molecular endocrinology 41:91–102

[99] **Suter M, Bocock P, Showalter L, Hu M, Shope C, McKnight R, Grove K, Lane R, Aagaard-Tillery K** 2011 Epigenomics: maternal high-fat diet exposure in utero disrupts peripheral circadian gene expression in nonhuman primates. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 25:714–726

[100] **Barson JR, Morganstern I, Leibowitz SF** 2013 Complementary roles of orexin and melanin-concentrating hormone in feeding behavior. International journal of endocrinology 2013:983964

[101] **Laque A, Zhang Y, Gettys S, Nguyen TA, Bui K, Morrison CD, Munzberg H** 2013 Leptin receptor neurons in the mouse hypothalamus are colocalized with the neuropeptide galanin and mediate anorexigenic leptin action. American journal of physiology Endocrinology and metabolism 304:E999–1011

[102] **Louis GW, Leinninger GM, Rhodes CJ, Myers MG, Jr.** 2010 Direct innervation and modulation of orexin neurons by lateral hypothalamic LepRb neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:11278–11287

[103] **Herring SJ, Rose MZ, Skouteris H, Oken E** 2012 Optimizing weight gain in pregnancy to prevent obesity in women and children. Diabetes, obesity & metabolism 14:195–203

[104] **Sullivan EL, Smith MS, Grove KL** 2011 Perinatal exposure to high-fat diet programs energy balance, metabolism and behavior in adulthood. Neuroendocrinology 93:1–8

[105] **Tamashiro KL, Moran TH** 2010 Perinatal environment and its influences on metabolic programming of offspring. Physiol Behav 100:560–566

[106] **Gorski JN, Dunn-Meynell AA, Levin BE** 2007 Maternal obesity increases hypothalamic leptin receptor expression and sensitivity in juvenile obesity-prone rats. American journal of physiology Regulatory, integrative and comparative physiology 292:R1782–1791

[107] **Tarrade A, Rousseau-Ralliard D, Aubriere MC, Peynot N, Dahirel M, Bertrand-Michel J, Aguirre-Lavin T, Morel O, Beaujean N, Duranthon V, Chavatte-Palmer P** 2013 Sexual dimorphism of the feto-placental phenotype in response to a high fat and control maternal diets in a rabbit model. PloS one 8:e83458

[108] **Ornellas F, Mello VS, Mandarim-de-Lacerda CA, Aguila MB** 2013 Sexual dimorphism in fat distribution and metabolic profile in mice offspring from diet-induced obese mothers. Life sciences 93:454–463

[109] **Dunn GA, Bale TL** 2011 Maternal high-fat diet effects on third-generation female body size via the paternal lineage. Endocrinology 152:2228–2236

[110] **Dunn GA, Morgan CP, Bale TL** 2011 Sex-specificity in transgenerational epigenetic programming. Hormones and behavior 59:290–295

[111] **Zambrano E, Martinez-Samayoa PM, Bautista CJ, Deas M, Guillen L, Rodriguez-Gonzalez GL, Guzman C, Larrea F, Nathanielsz PW** 2005 Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation. The Journal of physiology 566:225–236

[112] **Shankar K, Kang P, Harrell A, Zhong Y, Marecki JC, Ronis MJ, Badger TM** 2010 Maternal overweight programs insulin and adiponectin signaling in the offspring. Endocrinology 151:2577–2589

[113] **Shankar K, Harrell A, Liu X, Gilchrist JM, Ronis MJ, Badger TM** 2008 Maternal obesity at conception programs obesity in the offspring. American journal of physiology Regulatory, integrative and comparative physiology 294:R528–538

[114] **Kruse M, Seki Y, Vuguin PM, Du XQ, Fiallo A, Glenn AS, Singer S, Breuhahn K, Katz EB, Charron MJ** 2013 High-fat intake during pregnancy and lactation exacerbates high-fat diet-induced complications in male offspring in mice. Endocrinology 154:3565–3576

[115] **Tamashiro KL, Terrillion CE, Hyun J, Koenig JI, Moran TH** 2009 Prenatal stress or high-fat diet increases susceptibility to diet-induced obesity in rat offspring. Diabetes 58:1116–1125

[116] **Page KC, Malik RE, Ripple JA, Anday EK** 2009 Maternal and postweaning diet interaction alters hypothalamic gene expression and modulates response to a high-fat diet in male offspring. American journal of physiology Regulatory, integrative and comparative physiology 297:R1049–1057

[117] **Parylak SL, Cottone P, Sabino V, Rice KC, Zorrilla EP** 2012 Effects of CB1 and CRF1 receptor antagonists on binge-like eating in rats with limited access to a sweet fat diet: lack of withdrawal-like responses. Physiol Behav 107:231–242

[118] **Wallace TM, Levy JC, Matthews DR** 2004 Use and abuse of HOMA modeling. Diabetes Care 27:1487–1495

[119] **Quon MJ** 2001 Limitations of the fasting glucose to insulin ratio as an index of insulin sensitivity. The Journal of clinical endocrinology and metabolism 86:4615–4617

[120] **Khalyfa A, Carreras A, Hakim F, Cunningham JM, Wang Y, Gozal D** 2013 Effects of late gestational high-fat diet on body weight, metabolic regulation and adipokine expression in offspring. Int J Obes (Lond) 37:1481–1489

[121] **Franco JG, Fernandes TP, Rocha CP, Calvino C, Pazos-Moura CC, Lisboa PC, Moura EG**, Trevenzoli IH 2012 Maternal high-fat diet induces obesity and adrenal and thyroid dysfunction in male rat offspring at weaning. The Journal of physiology 590:5503–5518

[122] **Masuyama H, Hiramatsu Y** 2012 Effects of a high-fat diet exposure in utero on the metabolic syndrome-like phenomenon in mouse offspring through epigenetic changes in adipocytokine gene expression. Endocrinology 153:2823–2830

[123] **Dahlhoff M, Pfister S, Blutke A, Rozman J, Klingenspor M, Deutsch MJ, Rathkolb B, Fink B, Gimpfl M, de Angelis MH, Roscher AA, Wolf E, Ensenauer R** 2013 Peri-conceptional obesogenic exposure induces sex-specific programming of disease susceptibilities in adult mouse offspring. Biochimica et biophysica acta

[124] **Chang S, Graham B, Yakubu F, Lin D, Peters JC, Hill JO** 1990 Metabolic differences between obesity-prone and obesity-resistant rats. Am J Physiol 259:R1103–1110

[125] **Levin BE, Triscari J, Sullivan AC** 1983 Relationship between sympathetic activity and diet-induced obesity in two rat strains. Am J Physiol 245:R364–371

[126] **Gallou-Kabani C, Vige A, Gross MS, Boileau C, Rabes JP, Fruchart-Najib J, Jais JP, Junien C** 2007 Resistance to high-fat diet in the female progeny of obese mice fed a control diet during the periconceptual, gestation, and lactation periods. American journal of physiology Endocrinology and metabolism 292:E1095–1100

[127] **Wood-Bradley RJ, Henry SL, Vrselja A, Newman V, Armitage JA** 2013 Maternal dietary intake during pregnancy has longstanding consequences for the health of her offspring. Canadian journal of physiology and pharmacology 91:412–420

[128] **Remacle C, Bieswal F, Bol V, Reusens B** 2011 Developmental programming of adult obesity and cardiovascular disease in rodents by maternal nutrition imbalance. Am J Clin Nutr 94:1846S–1852S

[129] **Drummond EM, Gibney ER** 2013 Epigenetic regulation in obesity. Current opinion in clinical nutrition and metabolic care 16:392–397

[130] **Black RE, Victora CG, Walker SP, Bhutta ZA, Christian P, de Onis M, Ezzati M, Grantham-McGregor S, Katz J, Martorell R, Uauy R** 2013 Maternal and child undernutrition and overweight in low-income and middle-income countries. Lancet 382:427–451

[131] **Lukaszewski MA, Eberle D, Vieau D, Breton C** 2013 Nutritional manipulations in the perinatal period program adipose tissue in offspring. American journal of physiology Endocrinology and metabolism 305:E1195–1207

[132] **O'Reilly JR, Reynolds RM** 2013 The risk of maternal obesity to the long-term health of the offspring. Clinical endocrinology 78:9–16

[133] **Galliano D, Bellver J** 2013 Female obesity: short- and long-term consequences on the offspring. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology 29:626–631

[134] **Drake AJ, Reynolds RM** 2010 Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. Reproduction 140:387–398

[135] **Berthoud HR, Munzberg H** 2011 The lateral hypothalamus as integrator of metabolic and environmental needs: from electrical self-stimulation to optogenetics. Physiol Behav 104:29–39

[136] **Swanson LW, Sanchez-Watts G, Watts AG** 2005 Comparison of melanin-concentrating hormone and hypocretin/orexin mRNA expression patterns in a new parceling scheme of the lateral hypothalamic zone. Neuroscience letters 387:80–84

[137] **Schauder B, Schomburg L, Kohrle J, Bauer K** 1994 Cloning of a cDNA encoding an ectoenzyme that degrades thyrotropin-releasing hormone. Proceedings of the National Academy of Sciences of the United States of America 91:9534–9538

[138] **Bauer K** 1994 Purification and characterization of the thyrotropin-releasing-hormone-degrading ectoenzyme. European journal of biochemistry / FEBS 224:387–396

[139] **Lechan RM, Fekete C** 2006 The TRH neuron: a hypothalamic integrator of energy metabolism. Progress in brain research 153:209–235

[140] **Wittmann G, Fuzesi T, Singru PS, Liposits Z, Lechan RM, Fekete C** 2009 Efferent projections of thyrotropin-releasing hormone-synthesizing neurons residing in the anterior parvocellular subdivision of the hypothalamic paraventricular nucleus. The Journal of comparative neurology 515:313–330

[141] **Pearce EN** 2012 Thyroid hormone and obesity. Current opinion in endocrinology, diabetes, and obesity 19:408–413

[142] **Gertig AM, Niechcial E, Skowronska B** 2012 Thyroid axis alterations in childhood obesity. Pediatric endocrinology, diabetes, and metabolism 18:116–119

[143] **Kelly JA, Slator GR, Tipton KF, Williams CH, Bauer K** 2000 Kinetic investigation of the specificity of porcine brain thyrotropin-releasing hormone-degrading ectoenzyme for thyrotropin-releasing hormone-like peptides. The Journal of biological chemistry 275:16746–16751

[144] **Bolstad BM, Irizarry RA, Astrand M, Speed TP** 2003 A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185–193

[145] **Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP** 2003 Summaries of Affymetrix GeneChip probe level data. Nucleic acids research 31:e15

[146] **Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP** 2003 Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249–264

[147] **Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I** 2001 Controlling the false discovery rate in behavior genetics research. Behavioural brain research 125:279–284

[148] **Choi YH, Hartzell D, Azain MJ, Baile CA** 2002 TRH decreases food intake and increases water intake and body temperature in rats. Physiol Behav 77:1-4

[149] **Suzuki T, Kohno H, Sakurada T, Tadano T, Kisara K** 1982 Intracranial injection of thyrotropin releasing hormone (TRH) suppresses starvation-induced feeding and drinking in rats. Pharmacology, biochemistry, and behavior 17:249-253

[150] **Vijayan E, McCann SM** 1977 Suppression of feeding and drinking activity in rats following intraventricular injection of thyrotropin releasing hormone (TRH). Endocrinology 100:1727-1730

[151] **Schuhler S, Warner A, Finney N, Bennett GW, Ebling FJ, Brameld JM** 2007 Thyrotrophin-releasing hormone decreases feeding and increases body temperature, activity and oxygen consumption in Siberian hamsters. Journal of neuroendocrinology 19:239–249

[152] **Iglesias R, Llobera M, Montoya E** 1986 Long-term effects of TRH administration on food intake and body weight in the rat. Pharmacology, biochemistry, and behavior 24:1817-1819

[153] **Przegalinski E, Papla I, Czepiel K, Wydra K, Nowak E, Lason W, Filip M** 2004 Effect of thyrotropin-releasing hormone on the locomotor and sensitizing effects of cocaine in rats. Neuropeptides 38:48–54

[154] **Scalabrino GA, Hogan N, O'Boyle KM, Slator GR, Gregg DJ, Fitchett CM, Draper SM, Bennett GW, Hinkle PM, Bauer K, Williams CH, Tipton KF, Kelly JA** 2007 Discovery of a dual action first-in-class peptide that mimics and enhances CNS-mediated actions of thyrotropin-releasing hormone. Neuropharmacology 52:1472–1481

[155] **Thompson BL, Rosen JB** 2000 Effects of TRH on acoustic startle, conditioned fear and active avoidance in rats. Neuropeptides 34:38–44

[156] **Wei E, Sigel S, Loh H, Way EL** 1975 Thyrotrophin-releasing hormone and shaking behaviour in rat. Nature 253:739–740

[157] **Pinkney JH, Goodrick SJ, Katz J, Johnson AB, Lightman SL, Coppack SW, Mohamed-Ali V** 1998 Leptin and the pituitary-thyroid axis: a comparative study in lean, obese, hypothyroid and hyperthyroid subjects. Clinical endocrinology 49:583–588

[158] **Hermann GE, Barnes MJ, Rogers RC** 2006 Leptin and thyrotropin-releasing hormone: cooperative action in the hindbrain to activate brown adipose thermogenesis. Brain research 1117:118–124

[159] **Horita A, Carino MA, Lai H** 1986 Pharmacology of thyrotropin-releasing hormone. Annual review of pharmacology and toxicology 26:311–332

[160] **Heuer H, Ehrchen J, Bauer K, Schafer MK** 1998 Region-specific expression of thyrotrophin-releasing hormone-degrading ectoenzyme in the rat central nervous system and pituitary gland. The European journal of neuroscience 10:1465–1478

[161] **de Gortari P, Fernandez-Guardiola A, Martinez A, Cisneros M, Joseph-Bravo P** 1995 Changes in TRH and its degrading enzyme pyroglutamyl peptidase II, during the development of amygdaloid kindling. Brain research 679:144–150

[162] **Bauer K** 1988 Degradation and biological inactivation of thyrotropin releasing hormone (TRH): regulation of the membrane-bound TRH-degrading enzyme from rat anterior pituitary by estrogens and thyroid hormones. Biochimie 70:69–74

[163] **Ponce G, Charli JL, Pasten JA, Aceves C, Joseph-Bravo P** 1988 Tissue-specific regulation of pyroglutamate aminopeptidase II activity by thyroid hormones. Neuroendocrinology 48:211–213

[164] **Ervin GN, Schmitz SA, Nemeroff CB, Prange AJ, Jr.** 1981 Thyrotropin-releasing hormone and amphetamine produce different patterns of behavioral excitation in rats. European journal of pharmacology 72:35–43

[165] **Charli JL, Mendez M, Vargas MA, Cisneros M, Assai M, Joseph-Bravo P, Wilk S** 1989 Pyroglutamyl peptidase II inhibition specifically increases recovery of TRH released from rat brain slices. Neuropeptides 14:191–196

[166] **Sable P, Randhir K, Kale A, Chavan-Gautam P, Joshi S** 2013 Maternal micronutrients and brain global methylation patterns in the offspring. Nutritional neuroscience

[167] **Grissom N, Bowman N, Reyes TM** 2014 Epigenetic programming of reward function in offspring: a role for maternal diet. Mammalian genome : official journal of the International Mammalian Genome Society 25:41–48

[168] **Wolffe AP, Matzke MA** 1999 Epigenetics: regulation through repression. Science 286:481–486

[169] **Curradi M, Izzo A, Badaracco G, Landsberger N** 2002 Molecular mechanisms of gene silencing mediated by DNA methylation. Molecular and cellular biology 22:3157–3173

[170] **Gardiner-Garden M, Frommer M** 1987 CpG islands in vertebrate genomes. Journal of molecular biology 196:261–282

[171] **Suter MA, Sangi-Haghpeykar H, Showalter L, Shope C, Hu M, Brown K, Williams S, Harris RA, Grove KL, Lane RH, Aagaard KM** 2012 Maternal high-fat diet modulates the fetal thyroid axis and thyroid gene expression in a nonhuman primate model. Mol Endocrinol 26:2071–2080