The Role of Leptin in Body Weight Regulation

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ABSTRACT

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Leptin is an adipocyte-derived hormone which circulates in concentrations that are closely correlated with amounts of body fat. It provides a chronic signal to the central nervous system (CNS) regarding quantity of stored body fat and as such it is involved in the regulation of long term energy homeostasis. Leptin also declines abruptly when negative energy is imposed, providing a signal of incipient threats to the adequacy of fat stores.

Humans and mice maintain body weight (fat) at remarkably stable levels without conscious effort to adjust food intake or energy expenditure. Changes in body weight induced by either overfeeding or dietary restriction are rapidly reversed when free feeding is resumed, indicating that altered body weight is accompanied by physiological adjustments that oppose this change. The "set-point" that is being defended depends on individuals' genetic makeup and developmental environment during the perinatal period.

Several aspects of leptin physiology were investigated in the work presented in this dissertation including:

- the effects of transient hyperleptinemia at specific developmental periods on subsequent body weight set point in mice;
- regulation of body weight in the absence of leptin in mice;
- genetic contributors to circulating leptin concentrations in human and mice, and;
- the efficacy of an MC4R agonist – a downstream target of leptin – on maintenance of reduced body weight in mice.

Chapter 2 and 3. The effects of transient hyperleptinemia at specific developmental periods on subsequent body weight set point in mice

To assess whether leptin *per se* influences the body weight set point and whether there is a critical time window for such effects, we generated a transgenic mouse in which non-invasive induction of transient hyperleptinemia is dissociated from adiposity. This transgenic mouse uses a TET-ON system in which transgenic (CMV-driven) leptin expression is regulated by exposure to doxycycline (dox) in a dose-responsive manner that can be rapidly turned on and off. Circulating leptin concentrations can be elevated to those in a high fat-fed obese mouse within one day and either sustained indefinitely or restored to baseline concentrations within 24 hours. Acute overexpression of leptin in the adult transgenic mice reduces food intake and causes transient weight loss – confirming that the transgenic leptin is bioactive and capable of triggering anticipated physiological responses. This leptin transgenic mouse enables reversible increases in circulating leptin to virtually any level at any point in development.

Using this system we investigated the physiological consequences of developmentally timed transient hyperleptinemia on subsequent apparent set point for adiposity. Specifically, we evaluated the physiological effects of elevated leptin during adulthood, "adolescence" and the immediate postnatal period on the defense of body weight (adiposity) later in life and on the susceptibility to gain weight when offered a highly palatable diet *ad libitum*. We showed that inducing chronic hyperleptinemia in adult or "adolescent" mice does not increase the set point of defended body weight when excess leptin is removed; however, transient elevation of circulating leptin in the immediate postnatal period increases the hyperphagic response of the offspring to a highly palatable diet 7 weeks later, and renders animals more susceptible to obesity as adults. We demonstrated that leptin *per se* is capable of influencing the susceptibility of mice to gain weight

on high fat diet; however, these effects are restricted to a critical time window which we identified to be the immediate postnatal period.

Chapter 4. Regulation of body weight in the absence of leptin in mice

Leptin-deficient *Lep*$^{ob/ob}$ mice show metabolic compensation for lost weight and they appear to defend body fat by leptin-independent mechanisms. We attempted to identify mechanisms involved in leptin-independent regulation of body weight. *Lep*$^{ob/ob}$ mice were either fed *ad libitum* or calorie restricted to lose 20% of body weight. Calorie-restricted mice reduced energy expenditure and, when released to *ad libitum* feeding, regained fat and lean mass (to the levels of *ad libitum* controls) within 5 weeks. Calorie-restricted mice did so while their *ad libitum* caloric intake was equal to that of the control animals. These results confirm that, in congenitally leptin deficient animals, leptin is not required for compensatory reduction in energy expenditure accompanying weight loss, but suggest that the hyperphagia of the weight-reduced state is leptin-dependent.

Chapter 5. Genetic contributors to circulating leptin concentrations in human and mice

While circulating leptin concentrations correlate closely with body fat, at any given level of adiposity, there is substantial variation in circulating leptin. We collaborated with Dr. Ruth Loos – professor of Environmental Medicine & Public Health at Icahn School of Medicine at Mount Sinai – and her associates who carried out a genome-wide association study of circulating leptin concentrations adjusted for body mass and composition, and identified five loci associated with reduced circulating leptin concentrations [1]. The aim of the study was to identify and functionally assess potentially causal gene(s) within each implicated region. Our aim was to identify genes that modify leptin production/release in a manner that might account for reduced circulating leptin concentrations and hence predisposition to obesity. We developed an assay to

directly measure effects of the candidate genes in *ex vivo* adipose tissue explants on production and secretion of leptin. Using siRNAs, we knocked down expression of these genes in perigonadal adipose tissue explants from mice fed high fat diet and demonstrated that *Adig*, located in the *SLC32A1* locus, modulates leptin production and secretion [1]. These studies provide a prototype for the functional deconvolution of groups of genes identified by genome-wide association studies in which a specific cell type can be implicated.

Chapter 6. The efficacy of an MC4R agonist – a downstream target of leptin – on maintenance of reduced body weight in mice

Finally, we investigated the efficacy of an MC4R agonist in maintenance of reduced body weight in mice [2]. Weight loss is difficult to maintain due to physiological adaptations in energy expenditure and drive to eat that accompany this state. Exogenous leptin sufficient to restore circulating levels to those preceding weight (fat) loss reverses many of the relevant phenotypes. MC4R is a downstream target of leptin signaling and is central in energy homeostasis. In collaboration with scientists at AstraZeneca, we studied the effectiveness of a novel peptide MC4R agonist in maintenance of reduced body weight compared to its use in inducing weight loss. In the weight reduced state, 5x lower doses of the same molecule were comparably efficacious to a higher dose in the *ad libitum* state [2]. This protocol provides a model for evaluating the mechanisms and quantitative efficacy of weight-maintenance strategies and agents. These data support the concept that the pharmacology of the weight reduced state may be more tractable than that designed to induce weight loss.

Overall, the major conclusions from these studies are that:

- transient hyperleptinemia during the postnatal period can influence the susceptibility of mice to diet-induced obesity in adulthood;

- factors other than leptin contribute to body weight regulation in leptin deficient mice;
- functional, biological assays can be used to identify causal genes in genome-wide association study identified loci, and;
- pharmacological agents to maintain reduced weight may be a tractable target for treatment of obesity.

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LIST OF ABBREVIATIONS

1TG	Single transgenic
2TG	Double transgenic
Adig	Adipogenin
AgRP	Agouti related protein
AL	*Ad libitum*
Anti-Anti	Antibiotic-Antimycotic
AP	Area postrema
ARH	Arcuate nucleus of hypothalamus
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BMI	Body mass index
BW	Body weight
CCK	Cholecystokinin
CGL	Congenital Generalized Lipodystrophy
CNS	Central nervous system
CR	Caloric restriction
CSF	Cerebrospinal fluid
DIO	Diet-induced obesity
DMH	Dorsomedial hypothalamus
DMV	Dorsal motor nucleus of the vagus
Dox	Doxycycline
DUO	Duodenum

EE	Energy expenditure
eQTL	Cis-expression quantitative trait locus
ER	Endoplasmic reticulum
ES	Embryonic stem
FFA	Free fatty acids
FFM	Fat-free mass
FI	Food intake
FM	Fat mass
GHSR	Growth hormone secretagogue receptor
GI	Gastrointestinal tract
GLP-1	Glucagon-like peptide-1
GWAS	Genome-wide association study
HFD	High fat diet
HYPO	Hypothalamus
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
ILE	Ileum
IP	Intraperitoneal
Jak	Janus kinase
JEJ	Jejunum
KID	Kidney
LEP	Leptin

Lep$^{ob/ob}$	Leptin deficient mouse
LepRb	Leptin receptor long form
Lepr$^{db/db}$	Leptin receptor deficient mouse
LHA	Lateral hypothalamic area
LIV	Liver
MAF	Minor allele frequency
MBH	Mediobasal hypothalamus
MC3R	Melanocortin receptor 3
MC4R	Melanocortin receptor 4
ME	Median eminence
MHC I	Myosin heavy chain isoform I
MTII	Melanotan II
MyD88	Myeloid differentiation primary response gene 88
Nac	Nucleus accumbens
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated β cells
NPY	Neuropeptide Y
NREE	Non-resting energy expenditure
NTS	Nucleus tractus solitarius
PC1	Prohormone convertase 1 protein
PC2	Prohormone convertase 2 protein
PFK	Phosphofructokinase
PG2Fα	Prostaglandin 2Fα
PGAT	Perigonadal adipose tissue

PGT	Prostaglandin transporter
PMv	Ventral premammillary nuclei
PNS	Parasympathetic nervous system
POMC	Pro-opiomelanocortin
Ptgrf	Prostaglandin 2Fα receptor
PTP1B	Protein-tyrosine phosphatase 1B
PVH	Paraventricular nucleus of the hypothalamus
R26	Rosa26 locus
REE	Resting energy expenditure
RER	Respiratory exchange ratio
rtTA	Reverse tet-transactivator
SCAT	Subcutaneous adipose tissue
SERCA2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
SF-1	Steroidogenic factor 1
SFA	Saturated fatty acids
siRNA	small interfering RNA
SN	Substantia nigra
SNP	Single-nucleotide polymorphism
SNS	Sympathetic nervous system
SOCS3	Suppressor of cytokine signaling 3
SPL	Spleen
STAT	Signal transducer and activator of transcription
STO	Stomach

TEE	Total energy expenditure
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TRE	Tetracycline-responsive elements
VMH	Ventromedial nucleus of hypothalamus
VTA	Ventral tegmental area
WHR	Waist-hip ratio
WR	Weight-reduced
WT	Wild-type
α-MSH	α-melanocyte stimulating hormone

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Chapter 1

"Introduction"

Obesity and Energy Homeostasis

Obesity is a major public concern in the United States and in other parts of the world [3]. More than sixty percent of US adults are overweight or obese [4], increasing the risk for a number of medical and psychiatric disorders including type II diabetes mellitus, hypertension, stroke, coronary heart disease, and certain types of cancer [5, 6]. Transient obesity is not without risk; midlife obesity is associated with increased risk of dementia and Alzheimer's disease in later life [7], even if obesity is later rectified [8]. Additionally, obesity puts a substantial economic burden on society [9]; it is estimated that over $190 billion is spent annually on health care costs of obesity and related diseases [10], hence the need for effective prevention and treatment.

Since 1988, the most dramatic increases in obesity rates have occurred in adolescents and children. In the United States, obesity increased among adolescents aged 12 to 19 from 11% in 1988-1994 to 21% in 2013-2014. Extreme obesity in the same age group had a more dramatic increase, from 3% in 1988-1994 to 9% in 2013-2014 [11].

Childhood obesity is associated with both short term [12] and long term health effects [13]. In humans, adiposity decreases from the first year of life till its nadir at the age of 6–8 years, and then begins to increase again [14]. Several potential mechanisms have been suggested to explain how childhood obesity may predispose or cause adult disease. Total body fat and body fat distribution contribute to morbidity risk [15, 16]. Obesity lessens life expectancy markedly, especially among younger adults [17]. A 20-year-old white male with a body mass index (BMI)

greater than 45 is estimated to have 13 years of life lost due to obesity [17], shortening his life expectancy from 78 to 65, representing a 22% reduction in the remaining years of life [17]. Obesity is a result of chronic imbalances of food intake (FI) and energy expenditure (EE) leading to storage of excess energy as adipose tissue. The idea of total body fat regulation was first proposed by Kennedy in 1953 in his "lipostatic theory" where he suggested the existence of a signal from adipose tissue reporting fat mass to the hypothalamus [18]. Later, Coleman's parabiosis experiments in mice segregating for *ob* and *db* mutations with wild-type (WT) mice detected an apparent satiety factor in WT mice that was capable of reducing the body weight of *ob* mice but not of *db* mice, suggesting that the *ob* mutation encoded a ligand for which the *db* mutation was a receptor [19]. Two decades later, those genes were isolated by positional cloning and identified as leptin and leptin receptor, respectively [20-22], consistent with the lipostatic theory. Leptin is an adipokine which – in weight stable animals – circulates in direct proportion to fat stores, and informs the central nervous system (CNS) about the status of fat stores [20]. Changes in circulating leptin concentrations affect pathways that mediate energy homeostasis (intake, expenditure and partitioning of ingested calories) as well as thermoregulation and fertility [20, 23]. Circulating leptin exerts its effects on energy homeostasis through binding to its receptors that are expressed in many tissues, but predominantly in the brain [21].

Leptin

Leptin – a 167 amino acid hormone encoded by the *LEP* gene located on human chromosome 7 – is primarily produced by adipose tissue [20]. Low levels of leptin expression are detected in brown adipose tissue [24], stomach [25], placenta [26], mammary tissue [27] and ovaries [28]. In weight-stable mice and humans, circulating leptin concentrations are highly correlated with fat

mass. Plasma leptin follows a circadian rhythm (increases during dark cycle) [29] and responds to nutritional state. Fasting acutely decreases circulating leptin [30], while feeding increases leptin synthesis which is mediated, at least in part, by elevated circulating insulin during feeding [31]. Plasma leptin also depends on gender [32], fat distribution [33, 34], and the size of adipocytes [34, 35]. Several factors have been identified as regulators of leptin expression and secretion including inflammatory cytokines, glucocorticoids and insulin [36]. However, the molecular mechanism(s) of this regulation is not well understood.

While leptin concentrations at usual body weight are proportionate to the amount of stored fat, at any given level of adiposity there is some degree of discordance. In collaboration with Ruth Loos (professor of Environmental Medicine & Public Health at Icahn School of Medicine at Mount Sinai), we used this variability in circulating leptin to identify genetic loci that are associated with greater or lesser leptin production per unit of fat mass using a genome-wide association study (GWAS) approach [1]. We then developed a functional assay to analyze candidate causal genes that contribute to differences in circulating leptin. The details of these experiments are found in Chapter 5.

Leptin signaling

Early evidence confirming the importance of the hypothalamus in the regulation of body weight came from ablation of specific hypothalamic regions. The most pronounced phenotypes were seen when hypothalamic ventromedial nucleus (VMH) and lateral hypothalamic area (LHA) were lesioned, with the former leading to extreme obesity in rats and the latter resulting in anorexia and weight loss [37, 38]. Leptin is one of the major signals responsible for communication between fat stores and the CNS. In mice, there are six isoforms of leptin receptor

(LepRa-LepRf) all products of the *Lepr* gene, resulting from either alternative splicing of transcripts or posttranslational modifications and they all contain the extracellular domain [39, 40]. So-called "short forms" of LepR (LepRa, LepRc, LepRd, and LepRf) contain extracellular, transmembrane, and truncated intracellular domains. All isoforms except LepRe include the intracellular 'Box 1' motif and the Janus kinase (Jak) 2 tyrosine kinase binding domain [41]; however, only the LepRb isoform contains the 'Box 2'motif, required for efficient Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathway activation [42]. Soluble leptin receptor, LepRe, lacks both transmembrane and intracellular domains and acts as a major leptin-binding protein in the circulation [43, 44]. Leptin's action on energy intake and expenditure is primarily via LepRb signaling expressed in the central nervous system [45]; however other cell types outside of the CNS express LepRb including T-cells [46], the pancreatic β-cells [47], and epithelial cells lining the colon crypts [48].

LepRb is a type I cytokine receptor belonging to the IL-6 receptor family [49]. Leptin binds to the extracellular domain of LepRb, activating Jak2 which stimulates the phosphorylation of Tyr985, Tyr1077, and Tyr1138 residues in the intracellular portion of LepRb [50]. The most critical signaling pathway for leptin's action in energy homeostasis is the phosphorylation of Tyr1138 which recruits the STAT3 transcription factor [51]; phosphorylation of STAT3 promotes its translocation into the nucleus where it mediates expression of target genes, including *Socs3* (a feedback inhibitor of LepRb signaling) [42, 52]. Phosphorylation of Tyr985 recruits PTPN1, which initiates the ERK signaling cascade; Tyr 1077 phosphorylation induces STAT5 phosphorylation affecting transcription of target genes in the nucleus. LepRb signaling induction has feedback inhibition loops; these include suppressor of cytokine signaling 3 (SOC3)

which dephosphorylates Tyr985 [53] and protein-tyrosine phosphatase 1B (PTP1B) which dephosphorylates Jak2 and STAT3 [54]; both of these pathways act to inhibit LepRb action. LepRb is widely expressed in the mediobasal hypothalamus [55, 56]. Martin Myers' group confirmed the expression of the LepRb isoform in the brain using a leptin receptor reporter mouse [57]. They, and others, found many brain regions that express LepRb but the highest expression was found in the hypothalamic nuclei, including the arcuate (ARH), dorsomedial hypothalamus (DMH), VMH, LHA, and ventral premammillary nuclei (PMv) [57-59]. Other areas with high LepRb expression are the ventral tegmental area (VTA) involved in the reward system [60] and the brainstem (specifically nucleus of the solitary tract) which plays a role in modulating satiety [61].

Leptin signaling in the arcuate nucleus of the hypothalamus

Leptin signaling via the Jak2/STAT3 pathway in pro-opiomelanocortin (POMC)- and Agouti related protein (AgRP)/ Neuropeptide Y (NPY)-expressing neurons, first order neurons located in the ARH, influences the neuroendocrine systems related to energy homeostasis [62]. Activation of the Jak2/STAT3 pathway by leptin signaling inhibits orexigenic pathways (decreases expression of *Npy* and *Agrp*) [63] and activates the anorectic pathways (increases *Pomc* and *Cart*) [64]. POMC is posttranslationally cleaved by proconvertases (PC1 and PC2) and other peptidases to multiple smaller peptides including β-endorphin and α-melanocyte stimulating hormone (α-MSH) [65]. α-MSH inhibits energy intake and stimulates energy expenditure via melanocortin 4 receptors (MC4R) and, to a lesser degree, melanocortin 3 receptors (MC3R) located on second order neurons (neurons that receive inputs directly from first order neurons) located in specific nuclei, including the PVH [66]. In contrast, AgRP is a

functional antagonist (inverse agonist) of the MC3R and MC4R receptors [67] opposing the effects of POMC. AgRP levels increase with fasting and decline following food intake or leptin administration [68, 69]. NPY stimulates appetite independently of MC4R; it is an agonist of NPY receptors with expression also controlled by nutritional status [62, 68]. The importance of POMC and AgRP neurons in the control of energy balance is supported by mutations in the *POMC* gene that lead to severe human obesity [70] and rodent knockouts are obese and insensitive to leptin [71]. Ablation of NPY/AgRP neurons in adult brains induces severe anorexia leading to death [72, 73]; however, ablating these neurons during development does not cause anorexia and subsequent death suggesting that compensatory signals can be established early.

In humans, mutations in the *MC4R* gene account for about 3% of severe, early-onset obesity and represent the most common cause of monogenic human obesity [74]. Similarly, *Mc4r* null mice are obese both due to increased energy intake and, to a lesser degree, by decreased energy expenditure. Conversely, intracerebroventricular (ICV) administration of MC3/4R agonist, MTII, to diet induced obese (DIO) mice inhibits food intake, induces weight loss and elevates energy expenditure [75] further supporting the role of melanocortin pathway in energy regulation.

MC4 receptor is expressed broadly across the rodent CNS [76, 77]; however, the paraventricular nucleus of the hypothalamus (PVH) contains MC4R-expressing neurons that regulate appetite [78, 79] whereas cholinergic MC4R neurons in the dorsal motor nucleus of the vagus (DMV, within the brainstem) and spinal cord mediate energy expenditure and glucose homeostasis [80].

Leptin signaling in other brain regions

In addition to the classic leptin signaling pathways in the ARH involved in weight-homeostatic neurocircuits, other brain regions (within and outside of the hypothalamus) express LepRb and

mediate leptin effects on energy homeostasis. LepRb expressing neurons in the DMH are important in regulation of energy expenditure via sympathetic activation of brown adipose tissue (BAT) thermogenesis [81]. Neurons expressing steroidogenic factor 1 (SF-1) are restricted to the VMH. A subset of these SF-1 neurons co-express LepRb; deletion of LepRb specifically in these SF-1 neurons results in both increased body weight and susceptibility to DIO via effects on body food intake and energy expenditure. This indicates a leptin VMH connection in regulating energy balance [82].

Brainstem regions – including the nucleus tractus solitarius (NTS) and the area postrema (AP) – are involved in feeding behavior [83, 84]. The brainstem is a critical area for the integration of gastrointestinal signals including vagal afferents and gut peptides such as glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK) [84]. LepRb is expressed in the NTS and AP; administration of leptin directly to the NTS suppresses food intake. Additionally, leptin acts synergistically with GLP-1 and CCK, released during feeding, to induce satiety after a meal [85, 86].

Leptin plays a role in hedonic circuits through its action on the mesolimbic dopamine system – one of the most critical brain reward pathways – to influence food reward and the motivation to eat. The anatomic core of this system is located in a subset of dopaminergic neurons in the VTA/substantia nigra (SN) which project to the striatum [nucleus accumbens (NAc), caudate/putamen], amygdala, and prefrontal cortex [87]. Leptin receptors (LepRb) are expressed in the VTA and the SN [88]; leptin inhibits LepRb neurons within the VTA and decreases food intake by decreasing food reward [60, 89]. A subset of GABAergic/inhibitory neurons within the LHA also express LepRb and innervate the VTA [90]. Direct LHA leptin injection confirms

leptin action on LHA LepRb neurons by decreasing food intake and body weight over a period of 24 hours [90].

Congenital Leptin-deficient mice and humans

Congenital leptin (*Lep*$^{ob/ob}$) or leptin receptor (*Lepr*$^{db/db}$) deficiency renders mice morbidly obese due to both increased hyperphagia and decreased EE. Gross differences in body weight are detectable as early as 2 weeks of age. *Lep*$^{ob/ob}$ and *Lepr*$^{db/db}$ become hyperglycemic and hyperinsulinemic, they have defects in thermoregulation – apparent by decreased body temperature and a reduced thermogenic response to cold [91], and upon fasting or caloric restriction they become torpid [92]. Deficiency in leptin signaling causes hypogonadotropic hypogonadism resulting in infertility of both males and females [93]. Additionally, *Lep*$^{ob/ob}$ have impaired immunity [94]. Administration of exogenous leptin largely rescues the obesity and related phenotypes in *Lep*$^{ob/ob}$ mice. The wide range of phenotypes associated with congenital leptin deficiency highlights the physiological importance of leptin in regulating not only energy homeostasis but also multiple other systems.

The first two humans with homozygous frame shift mutations of LEP were identified in 1997 by O'Rahilly and colleagues; they were leptin-deficient, severely hyperphagic, and morbidly obese [95]. Mice lacking leptin reduce energy expenditure [91] but leptin deficient humans have no detectable changes in resting or free-living energy expenditure [96]. While inactivating leptin mutations are exceedingly rare, recombinant human leptin therapy is available and results in remarkable benefits for these patients including normalization of body weight and many of the associated comorbid phenotypes [97].

I investigated whether *Lep*$^{ob/ob}$ mice are capable of regulating body weight in the absence of leptin signaling (Chapter 4).

Leptin and metabolic adaptations (weight-reduced state)

Humans and rodents regulate and defend set-points (threshold levels below which metabolic-behavioral defense of body weight is invoked) of body fat without conscious effort to adjust food intake or energy expenditure [98]. In rodents and humans, changes in body weight imposed by either overfeeding or dietary restriction are rapidly reversed when free feeding is resumed [99-101]. Following a ten percent reduction in body weight, energy expenditure is approximately 20% lower than what would be estimated from changes in body mass and composition [99]. The magnitude of this response is comparable in obese and lean subjects indicating that the same metabolic adaptations are present regardless of the baseline (usual) body weight and adiposity. In addition to decreased EE, weight-reduced humans display other physiological adaptations that promote body weight regain including increased hunger, decreased circulating leptin and bioactive thyroid hormones, decreased sympathetic nervous system (SNS) tone, and increased parasympathetic nervous system (PNS) tone [99, 102, 103]. The difference in EE is accounted for primarily by decreased nonresting energy expenditure (NREE). Reduction in NREE is a result of increased skeletal muscle contractile efficiency during low intensity physical activity rather than increased physical activity [104]. Muscle biopsies from weight-reduced individuals have decreased activity of a glycolytic enzyme, phosphofructokinase (PFK) [105], increased expression of more efficient myosin heavy chain isoform (MHC I), and Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2, which contributes to muscle

efficiency independently of MHC I) [106]. These biochemical changes in muscle during maintenance of reduced weight are consistent with the decline in NREE reported *in vivo*.

fMRI studies show that humans in a weight-reduced state presented with food items display increased neural activity in brain areas associated with reward valuation and processing of visual food cues and reduced activity in areas involved in restraint compared to themselves at their usual weight [107].

These studies suggest that the weight-reduced state can be perceived as one of relative leptin insufficiency due to reduced body fat. Exogenous leptin is ineffective at weight loss induction in humans [108, 109], however, Leibel, Rosenbaum, and colleagues demonstrated that majority of phenotypes associated with the weight-reduced state – including EE reduction, increased hunger, decreased circulating thyroid hormones, decreased SNS tone, and brain fMRI patterns – are reversed with replacement doses of exogenous leptin sufficient to restore circulating concentrations of leptin to those present prior to weight loss [107, 110].

The phenotype of the weight-reduced state in mice is similar to that described in humans. Mice maintaining a reduced body weight show decreased EE, increased hunger, and increased food seeking behavior leading to greater *ad libitum* food intake. Weight-reduced mice have decreased excitatory synapses onto ARH POMC neurons [111], similar to changes seen in leptin deficient, $Lep^{ob/ob}$ mice [106, 112].

Leptin as a signal for sufficiency of body fat stores

Administration of supraphysiological doses (~10 fold above physiological concentrations) of leptin to lean or obese humans has limited capacity to induce weight loss [108] but supplementation of exogenous leptin in individuals in a weight-reduced state (a state of relative

leptin insufficiency) is effective in partially reversing the physiological adaptations [110] suggesting that the primary role of leptin is to defend fat stores, not to protect against fat gain. Leibel and associates proposed that the central nervous system responds in a threshold-like manner in response to alterations in circulating leptin concentrations [98]. Each individual has a different leptin threshold; genetic differences as well as developmental processes can affect both gene expression and neuroanatomical structure of circuits involved in setting the leptin threshold [98]. The extent to which the functionality of these molecules, cells and circuits can be influenced by "environmental" factors and the mechanisms by which these effects are conveyed remain unclear. Understanding the physiological and molecular mechanisms underlying these effects is important to the prevention and treatment of obesity.

Physiological and CNS changes due to elevation of body weight

In contrast to the physiological responses triggered during weight loss, gradually increasing body weight does not seem to invoke strong counter-regulatory mechanisms. DIO mice maintained at elevated adiposity for prolonged periods of time appear to defend a higher body weight (fat) when weight is reduced by hypocaloric feeding [111]. The threshold for minimum adiposity can be elevated; however the mechanisms driving this upward re-setting are unknown. Body weight (and fat) elevation is associated with many physiological changes such as increased free fatty acids, glucose, insulin, and proinflammatory cytokines; insulin resistance and decreased adiponectin and ghrelin. Additionally, increased adiposity results in fat-appropriate elevations in circulating leptin as well as decreased hypothalamic leptin signaling which has been suggested as a pathophysiological mechanism in the development of obesity [113].

Several mechanisms have been implicated in obesity-attenuated responsiveness to leptin including reduced leptin transport across the blood-brain barrier (BBB) [114, 115] and processes that can reduce LepRb cellular signaling including increased hypothalamic free fatty acids (FFA) [116, 117], endoplasmic reticulum (ER) stress [118, 119], inflammation [119, 120], feedback inhibition [121], and, more likely, the interplay among these processes.

The ratio of cerebrospinal fluid (CSF) to serum leptin concentration in lean humans is about 4 fold greater than that of obese individuals [122]. DIO rodents respond to centrally administered leptin (ICV injections), but not to peripherally administered leptin (subcutaneous pumps or intraperitoneal injections) [123, 124]. Recent studies have indicated that leptin enters hypothalamic neurons via tanycytes – specialized hypothalamic glial cells – located in the median eminence (ME) that extend from the surface of the 3rd ventricle to the fenestrated capillaries [115]. The ME is a circumventricular organ located at the base of the hypothalamus, which serves as an interface between the portal blood and the hypothalamus and allows circulating factors to enter the CSF and mediobasal hypothalamic (MBH) neurons [125]. Balland *et al.* demonstrated that blood-borne leptin first enters tanycytes through LepR (tanycytes express all isoforms of LepR) but its entrance is not dependent on LepRb signaling cascade [115]. Leptin is then released from tanycytes to the CSF by a process requiring activation of ERK pathway and can then reach MBH neurons [115]. In mice lacking the long form of LepR, *Lep*$^{db/db}$, and in HFD-fed mice, leptin accumulates in tanycytes in the ME but fails to appear in the neurons of the MBH [115]. Additionally, activating ERK signaling cascade in tanycytes rescues leptin's release into the CSF and results in activation of the MBH neurons [115]. These findings suggest that leptin signaling in the hypothalamus may be diminished due to disruption of leptin transport to the CNS due to HFD feeding.

Leptin signaling is negatively regulated by SOCS3 [121] which belongs to a family of cytokine-inducible inhibitors of signaling and binds directly to the Tyr985 amino acid of LepRb, inhibiting leptin-induced STAT3 signaling; it has therefore been implicated in cellular leptin resistance. In DIO rodents, *Socs3* expression is increased specifically in the hypothalamus when the animals are initially exposed to HFD [126]. Mice with POMC-specific *Socs3* overexpression are fatter than WT mice when on a chow diet [127]; neuronal deletion of SOCS3 results in decreased weight gain on HFD [128].

Providing a link between calorically dense foods (such as HFD) and disruption of energy balance, De Souza and colleagues showed that HFD feeding promotes hypothalamic inflammation in rodents [120]. Earlier reports established that HFD feeding leads to chronic low-grade inflammation in peripheral tissues including adipose tissue [129, 130] and liver [131, 132] which then contributes to insulin resistance and glucose intolerance [133]. Preceding any large changes in body weight or reduced leptin signaling, hypothalamic inflammation develops within the first few days of HFD feeding [134]. HFD feeding induces hypothalamic inflammation by activating the proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), Interleukin 1 beta (IL-1β), and Interleukin 6 (IL-6), and inflammatory signaling pathways including IκB kinase/ nuclear factor kappa-light-chain-enhancer of activated β cells (IKKβ/NF-κB) and Toll-like receptor 4/ myeloid differentiation primary response gene 88 (TLR4/MyD88) in the hypothalamus [119, 120]. Saturated fatty acids (SFA) can directly induce inflammatory response via the TLR4/MyD88 pathway; disruption of this pathway protects mice against DIO and cellular leptin resistance [116, 117]. TLR4/MyD88 further signals via the IKKβ/NF-κB which induces a proinflammatory program in the hypothalamus. Specifically activating IKKβ in the brain leads to increased food intake and weight gain due to attenuated leptin signaling [119].

Endoplasmic reticulum (ER) stress was implicated as a downstream target of SFA-induced TLR4 signaling, ultimately leading to hypothalamic inflammatory activation [117]. Ozcan *et al.* demonstrated that increased circulating cytokines and free fatty acids as a result of exposure to HFD contributes to the induction of ER stress in the hypothalamus of DIO mice and subsequently decreases leptin signaling [118]. Inflammatory and ER stress pathways are involved directly with leptin signaling; activation of NF-κB and elevated ER stress upregulates the previously mentioned inhibitor of LepRb, *Socs3* [119]. The interplay of these various cellular pathways are complex and it remains unclear which factors are the primary drivers as opposed to secondary responders in triggering attenuation of cellular leptin signaling in the hypothalamus. Further support for the pathogenic role of HFD feeding in promoting hypothalamic inflammation is provided by studies demonstrating accumulation of activated glial cells in the hypothalamus of DIO rodents [134]. Glial cells include astrocytes, microglia, tanycytes, polydendrocytes, and oligodendrocytes and are the most abundant cell type in the CNS [135]. They are involved in various processes in the CNS critical during development, health and disease [136]. Microglia are tissue-resident macrophages in the CNS which play a role in synaptic pruning, cellular debris phagocytosis, immune surveillance and inflammation. Astrocytes contribute to nutrient support, neurotransmitter reuptake, neurovascular coupling and gliotransmitter (chemical transmitter released from glial cells) production [136]. Glial activation – also known as gliosis – refers to morphological (amoeboid form) and gene expression changes in microlia and astrocytes. In an activated state, microglia express major histocompatability complex proteins and inducible nitric oxide [137], whereas astrocytes induce expression of specific structural proteins such as glial fibrillary acidic protein (GFAP) and vimentin [138]. Within the first week of HFD consumption, hypothalamic inflammation is induced [120] and, over the same time period, mice accumulate

activated microglia and astrocytes in the MBH [134] circumstantially suggesting that gliosis mediates the inflammatory response. Indeed, expression of proinflammatory genes in microglia is elevated in the hypothalami of HFD-fed mice and ablation of microglia normalizes hypothalamic inflammation and neuronal stress [139, 140]. Astrocytes, when activated by CNS insults such as brain injury or ischemia, also produce proinflammatory cytokines (TNF-α, IL-1β, and IL-6) [141, 142]. Some *in vitro* studies suggest that SFA can activate proinflammatory signals in cultured astrocytes [143]. However, it is unclear whether this response occurs *in vivo* with astrocyte activation resulting from HFD feeding. Activation of microglia and astrocytes usually occurs together and there is certainly crosstalk between them; proinflammatory factors secreted from microglia can signal to astrocytes and astrocytes can communicate with microglia via factors that they release such as colony stimulating factor-1 (CSF-1) and ATP [136]. Surprisingly, C57Bl6 female mice do not exhibit central inflammatory and gliosis response when exposed to HFD [144]. While female mice gain weight on HFD, they do so to a lesser extent compared to male mice [145]. Recently, Dorfman *et al.* demonstrated that females on HFD do not show a decrease in CX3CL1, a chemokine secreted by neurons to maintain microglial quiescence, as occurs in male mice on HFD. When exposed to HFD, CX3CL1-deficient females show activation of microglia and weight gain comparable to that observed in male mice [146] indicating that decreased gliosis and weight gain on HFD in female mice may be mediated, at least in part, via the CX3CL1-CX3CR1 pathway.

In rodents, during postnatal weeks 2 and 3, glia cell number markedly increases [147] coinciding with the leptin surge [148]. Astrocytes express LepRb [149, 150]. Exogenous leptin administration between P8-P12 increases proliferation of astrocytes in the hypothalamus and astrocyte-specific deletion of LepRb results in decreased astrogenesis [151]. Conditional deletion

of LepRb in adult mouse astrocytes leads to morphological changes in astrocytes and increases synaptic inputs onto hypothalamic POMC and AgRP neurons [149]. Additionally, these mice display a decrease in leptin-induced suppression of food intake [149]. These results suggest a direct impact of leptin on astrocyte development and function in adult mice.

Lep$^{ob/ob}$ (leptin deficient) mice become severely obese on chow diets, and have reduced activated microglia compared to WT mice on chow or on HFD [152]. Exposing *Lep*$^{ob/ob}$ mice to HFD increases microglial markers to a lesser degree than HFD feeding in WT mice [152]. These findings suggest that leptin, HFD, or the interaction of both is required for HFD-induced gliosis. Astrocyte activation was not evaluated in this study. Martin Myers and colleagues showed that 10-days of continuous systemic leptin infusion in WT mice fed a chow diet was sufficient to induce microglial and astrocyte activation in the hypothalamus (personal communication). This finding implicates a direct role of leptin in gliosis, suggesting that elevated leptin *per se* (which is physiologically increased by obesity) may contribute to hypothalamic inflammation and subsequently to attenuated leptin signaling.

It remains unclear whether CNS changes associated with HFD feeding and/or obesity described above are secondary to increased adiposity or if they are contributing factors to the development and/or perpetuation of obesity. Determining causality is challenging due to the interrelated nature of obesity, physiologic, neuronal, and structural changes but the disentanglement of these factors is critical in understanding the pathophysiology of obesity in an environment characterized by calorically dense and highly palatable diet.

Part of my PhD research, focused on the investigation of hyperleptinemia *per se* as a contributing factor to the elevation of body weight threshold. This work is described in more detail in Chapter 3 of this thesis.

Developmental influence on body weight

Investigation of the pathways linking perinatal environments and future health outcomes can be helpful in prevention of obesity and obesity-related diseases. Adverse or suboptimal environment during fetal/early development leads to increased risk of disease in adult life. In 1990, based on historical cohort studies, David Barker noted that infants who are small at birth are at higher risk for increased blood pressure and other adverse cardiovascular endpoints in adulthood [153]. The Dutch Hunger Winter of 1944-45 provides a unique opportunity to investigate how gestational undernutrition impacts the risk of disease later in life. The data collected from the offspring of women exposed to famine at different stages of gestation demonstrates that the timing of undernutrition in the programming of adult disease is critical. Only those fetuses exposed to famine during early gestation had a higher prevalence of obesity and cardiovascular disease [154]. Undernutrition during late gestation resulted in smaller birth weight and that cohort continued to be smaller as adults with lower rates of obesity [154, 155].

In the current environment, nutritional excess, rather than scarcity is prevalent. Epidemiological studies indicate that maternal obesity (even accounting for shared genotype) predisposes offspring to obesity and metabolic disease [156-158]. The underlying cellular and molecular mechanism(s) for these effects of maternal programming of adult disease are unclear. Rodent studies have indicated that maternal HFD feeding and/or obesity during critical developmental periods leads to obesity and metabolic disease including insulin resistance and hepatic steatosis in the offspring later in life [159-163]. As discussed in the previous section, many physiological and molecular alterations occur as a result of obesity and HFD feeding and these changes may have profound effects on the offspring during gestation and in the postnatal period. The

developing hypothalamus may be especially influenced by the hormonal milieu; suboptimal concentrations of various hormones may affect neurogenesis (alter the number of neurons born during gestation that are involved in feeding behavior), axonal outgrowth of neurons participating in feeding circuits during early postnatal period, or epigenetic modifications to genes important in body weight regulation [164]. The pathways predicted to be primarily affected by such perinatal programming are those critical in energy homeostasis; such as those involving leptin- and insulin-responsive POMC and AgRP neurons [64, 72].

The first critical period of hypothalamic development is *in-utero* when hypothalamic neurogenesis occurs and neuronal cell numbers are determined. In mice, most of hypothalamic neurogenesis for neurons involved in energy balance happens mid-gestation, between embryonic days 12 and 14 [165]. These neurons do not send their axonal projections to their target sites until the postnatal period, marking the second critical period during development of the hypothalamus [166, 167]. Simerly and colleagues demonstrated that at birth, the ARH projections are immature and these projections develop mostly during the second postnatal week of life [167]. The mature innervation from the ARH to the DMH and PVH is achieved by P6 and P12, respectively, whereas projections from ARH to LHA begin at P12 [167].

Leptin is an essential factor in the process of projection formation in the feeding circuits. $Lep^{ob/ob}$ mice have a severe reduction in the density of axons from ARH neurons to PVH, DMH, and LHA and this decrease persists through adulthood [166]. The phenotype of diminished ARH projections in $Lep^{ob/ob}$ mice can be rescued with exogenous leptin, however, the time window for this effect is restricted to the postnatal period (P4-P12). Specifically, Bouyer and Simerly defined that early exposure of $Lep^{ob/ob}$ mice to exogenous leptin rescues AgRP inputs to preautonomic but not the neuroendocrine neurons within the PVH indicating that in addition to its role in

formation of hypothalamic projections, leptin is also important in targeting of axons originating from the ARH to specific cell-types within the hypothalamic nuclei [168]. Administration of leptin to adult $Lep^{ob/ob}$ mice does not restore hypothalamic projection densities. The offspring of dams exposed to HFD during gestation and lactation [169] or during lactation only [170] gain more weight than controls. The density of AgRP immunoreactive fibers in the PVH are decreased in the offspring of HFD dams compared to chow fed dams [169]. Vogt *et al.* reported reduced fiber densities of AgRP and α-MSH projections from the ARH to three downstream hypothalamic areas: PVH, DMH and LHA in the offspring of dams fed HFD during lactation [170]. These data suggest that ambient leptin is critical in the development of hypothalamic feeding circuits.

Metabolic signals other than leptin (such as insulin and ghrelin) may also act as neurotrophic factors during early development. A single injection of insulin directly into the hypothalamus of P8 rats results in decreased neuronal density in the VMH of P15 pups and increased body weight gain as adults [171]. Carmody *et al.* demonstrated that HFD feeding, in addition to maternal insulin resistance (dams were heterozygous for a null allele of the insulin receptor), increases the number of POMC-expressing cells in the ARH of P9 offspring [172]. This suggests that insulin can influence the specification of POMC neurons in response to maternal HFD feeding [172]. Vogt *et al.* showed that elevated insulin resulting from maternal HFD exposure disrupts POMC neuron innervation to the preautonomic compartment of the PVH. Blocking insulin signaling specifically in POMC neurons, under conditions of maternal HFD feeding, rescues the projections in this circuit and corrects glucose intolerance [170], implicating the role of insulin in the development of hypothalamic circuits involved in glucose homeostasis.

Ghrelin's role has also been studied during mouse postnatal development. Ghrelin – an appetite stimulating circulating factor – is primarily produced in the stomach and signals through the growth hormone secretagogue receptor (GHSR) [173, 174]. GHSR is expressed most densely in the hypothalamus; administration of ghrelin activates neurons in brain areas known to control feeding (such as ARH, VMH, and PVH) [175]. Steculorum *et al*. reported that in WT mice circulating ghrelin levels during postnatal period start rising from P6 and reach adult concentrations by P14 [176]. They evaluated the physiological and neurobiological roles of ghrelin during postnatal development by either blocking ghrelin signaling from P4 to P22 or elevating circulating ghrelin concentrations from P4 to P12. Blocking ghrelin action resulted in enhanced overall fiber density in the PVH, including AgRP and αMSH immunoreactive fibers; administration of ghrelin had the opposite effects – innervation in the PVH was reduced. Surprisingly, both interventions resulted in increased body weight, fat, and blood glucose. While the overall density of AgRP and αMSH fibers in the PVH are affected in an opposite way by ghrelin and anti-ghrelin treatment, the ratio of orexigenic AgRP to anorexigenic αMSH is increased in both conditions which could explain the increased body weight in both manipulations [177]. These data emphasize that appropriate timing and magnitude of ghrelin's action is important in proper formation of hypothalamic neurocircuits and in control of body weight in adulthood [176].

The studies described above indicate that suboptimal concentrations of metabolic hormones including leptin, insulin, and ghrelin during the immediate postnatal period – a critical window for development of feeding circuits– are capable of permanently altering the neuroanatomical organization of the hypothalamic circuits essential in the control of body weight and glucose homeostasis. Whether hyperleptinemia *per se* can influence the formation of these circuits has

not been evaluated but is of obvious relevance. Investigating the role of these hormones during other developmental periods such as gestation and adolescence requires further investigation. To this end, during my doctoral training, I have made a transgenic mouse with the capability to precisely and temporally restrict hyperleptinemia completely dissociated from diet or obesity and its metabolic consequences and used it to address some of the questions raised in this section. These studies are described in the 2nd and 3rd chapter.

Pharmacology

Obesity is a chronic disease with serious health consequences. Modest weight loss of only 5-10% of body weight in obese patients has substantial health benefits including improvements in blood glucose, prevention of type 2 diabetes, reduction of blood pressure and triglycerides, and increasing HDL cholesterol [178, 179]. While temporary weight loss in humans is achievable with lifestyle interventions such as diet and exercise [180], the recidivism rates are very high indicating that maintenance of a reduced body weight is the major challenge in obesity treatment [181]. The inability to maintain weight loss is a result of physiological and molecular changes that accompany the weight reduced state (described in an earlier section) which ultimately promote weight regain [99, 102, 103].

These physiological responses during maintenance of reduced body weight are similar but not identical to those seen during dynamic weight loss [182]. Current efforts in obesity treatment are primarily focused on the induction of weight loss, yet the therapies available are less than satisfactory [183]. As discussed earlier, exogenous leptin has limited efficacy in induction of weight loss in lean or obese subjects [108]; however, it is much more effective during the maintenance of reduced weight when given in small doses [110]. This observation suggests that

pharmacological strategies for relieving the physiological adaptations to the weight reduced state could have important applications in the treatment of obesity. The physiology of the weight-reduced state suggests that pharmacologic agents affecting energy homeostasis would have greater efficacy in individuals maintaining weight loss. In the Chapter 6 of this thesis we compare the effects of a novel MC4R agonist on inducing vs. maintaining weight loss to in mice.

Summary

This thesis includes 5 manuscripts investigating aspects of leptin physiology in regulation of body weight. Chapter 2 describes the creation of a transgenic mouse that overexpresses leptin independent of adiposity. Leptin expression can be transiently turned on or off at any age. These mice provide a model to investigate whether hyperleptinemia *per se* drives conditions associated with obesity without the other, related, confounds such as diet, circulating lipids, glucose, insulin, ghrelin, etc. Moreover, this mouse model allows for precise temporal control of hyperleptinemia.

We have used this leptin transgenic mouse to evaluate the physiological effects of elevated leptin during distinct developmental periods on the defense of body weight later in life (Chapter 3). We have shown that exposing adult or "adolescent" mice to chronic hyperleptinemia (via transient dox exposure) does not alter either body composition or weight; however, inducing hyperleptinemia during the nursing period (P0-P22) results in increased body weight gain in these mice when exposed to high fat (60% kcals from fat) diet later in life.

Chapter four investigates energy homeostasis of leptin deficient mice. Mice that lack leptin are still capable of regulating body weight and energy expenditure. This work attempts to determine a signal, independent of leptin, that is produced by adipose tissue and alters energy expenditure

when the mouse's food intake is restricted in. It does not appear that this signal alters food intake, since, when leptin deficient mice are released from restriction, they do not overeat relative to never restricted mice. This is in contrast to leptin competent mice that transiently overeat for several days post-release.

In humans and mice, leptin circulates in proportion to fat mass. However, there is variation among humans in this relationship, with some displaying levels of plasma leptin well below those predicted by fat mass. Dr. Ruth Loos and colleagues at Icahn School of Medicine at Mount Sinai carried out a GWAS on circulating leptin and found associations – controlled for adiposity – in 4 genomic regions. To identify the causal genes in implicated loci, we developed a functional assay to systematically knock down (using siRNA) candidate genes in explanted mouse adipose tissue and measured both leptin expression and secretion in media. With this approach we attempted to find the genes that influence circulating leptin in humans independent of fat mass and demonstrated a technique to determine the genetic signal that was revealed by the GWAS [1].

The final chapter describes experiments which investigate the efficacy of an MC4R agonist, in maintaining reduced body weight in mice. As described in earlier sections, weight loss leads to physiological adjustments that work coordinately to promote weight regain. MC4R is a downstream target of leptin that is central to energy homeostasis. The MC4R agonist was effective at a significantly lower dose when used to maintain reduced body weight than to induce weight loss in *ad libitum* fed mice, supporting the idea that targeting the weight reduced state may be more tractable than inducing weight loss [2].

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Chapter 2

"Creation of a tetracycline-inducible leptin overexpressing transgenic mouse"

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A.A.S., C.A.L., and R.L. designed experiments. A.A.S., C.A.L., K.S.F., and D.E. performed experiments. A.A.S., C.A.L., and R.L. analyzed and interpreted data, and wrote the manuscript.

Introduction

Obesity has been associated with type II diabetes mellitus, hypertension, stroke, coronary heart disease, and certain types of cancer [1]; even moderate weight loss can improve metabolic function and health outcomes [2, 3]. The molecular mechanisms responsible for the adverse effects of obesity are generally unclear, and may vary by phenotype. However, one consequence of increased adiposity is increased circulating leptin. Leptin [4] is a hormone primarily secreted by adipose tissue that acts through the central nervous system to regulate long term food intake and energy expenditure [5]. Circulating leptin concentrations are directly proportional to fat stores in weight stable humans [6] and rodents [7]. The hormone signals through the long form of the leptin receptor (LepRb) expressed in specific areas of the hypothalamus, midbrain and brainstem [8]. Mice or humans lacking either functional leptin [4, 9] or its receptor [10, 11] are

hyperphagic and hypometabolic, resulting in extreme obesity. Administration of exogenous leptin normalizes the physiology of leptin deficient mice and humans [12, 13]; however, obese humans with intact leptin signaling have increased levels of circulating leptin – proportional to increased adiposity – and supplementation with additional leptin conveys minimal effect on body weight [14-16]. In lean rodents, administration of exogenous leptin transiently reduces food intake and body weight [17]. Obese animals display decreased sensitivity to exogenous leptin [18]. Aspects of the neuroanatomic substrates for mediation of regulatory responses to leptin are conveyed by the hormone's effects on the development of these circuits. Congenital absence of leptin reduces brain size [19]; absence of the hormone during early postnatal periods critical to maturation of hypothalamic neuronal circuitry permanently impairs the development and function of these circuits [20-22].

Neurophysiological models of body weight homeostasis posit various "set point" constructs that include variable sensitivity to leptin's access to and actions within the CNS as causes for the defense of adiposity. Some of this variability is attributed to genetic and developmental effects on the molecules and pathways that comprise these complex circuits. An unanswered question is the extent to which the functionality of these circuits can be influenced by "environmental" factors such as intrauterine events, diet, and intercurrent obesity. Specifically, can the set point for adiposity be altered by pre- and/or postnatal factors. Since dietary fat content [23], adiposity [23] and leptin *per se* [20] have been implicated in such effects, and since these elements covary experimentally, it has been difficult to developmentally isolate these factors.

Transgenic mice that congenitally overexpress leptin have been created [24, 25]. These mice initially have reduced body weight and fat stores but, by 33 weeks, are indistinguishable from wild type littermates. When exposed to high fat diet at 9 weeks of age for 20 weeks, these mice

are more prone to diet induced obesity [26]. While transgenic mice that constitutively overexpress leptin are useful in assessing life-long hyperleptinemia, congenital models are limited and do not allow for assessment of the effects of transient hyperleptinemia.

We generated a transgenic mouse that permits non-invasive induction of hyperleptinemia completely dissociated from obesity. A TET-ON system enables transgenic leptin expression that is regulated by exposure to the tetracycline analog, doxycycline (dox), in a dose-responsive manner; the response can be rapidly turned on and off. These mice provide a model with which to investigate whether hyperleptinemia *per se* drives the conditions associated with obesity without the other, related, confounds such as diet.

Methods

Leptin/rtTA double transgenic mice were made using a commercially available KH2 embryonic stem (ES) cell line (Mirimus, Inc). KH2 ES cells contain an FRT-hygro-pA "homing" cassette in the ColA1 locus and a reverse tet-transactivator (rtTA) located in the Rosa26 locus (R26-rtTA; **Figure 2.1A**). The mouse leptin gene (*Lep*) was isolated using PCR with forward primer: 5' GGCGCGAATTCATGTGCTGGAGACCCCTGTGT and reverse primer 5' TCATCAAGACCATTGTCACCAGGAT (primers were designed to introduce EcoR1 sites flanking the gene). PCR reaction was run using cDNA from a wild type C57BL6J mouse; it was then cloned into the EcoRI site of pBS31tetOpgkATGfrt vector (source D. Egli, **Figure 2.1A**). pBS31tetOpgkATGfrt-Lep vector was electroporated into KH2 cells with FlpE Recombinase (pCAGGS-FLPe) as described earlier [27] (**Figure 2.1A**). KH2-Lep cells were selected with hygromycin (Sigma). To confirm integration, leptin transgene expression in KH2-Lep cells was

induced *in vitro* with 4 concentrations of dox: 50, 100, 200, and 400 ng/ml for 48 hours. Media was collected after 48 hours of dox treatment and leptin protein contents measured using leptin ELISA (R&D). Four clones that responded to dox treatment were selected for karyotypic analysis (Cell line genetics, Inc). Cytogenetic analysis was performed on 20 G-banded metaphase cells for each clone. Three out of four clones were karyotypically abnormal; one had an inverted duplication on chromosome 4 from band D1 to band C4, and two had a large metacentric chromosome resulting from fusion of the centromeres of chromosomes 1 and 13. The clone that was karyotypically normal was selected for mouse generation. Chimeric mice were generated by injecting ES cells into non-agouti BDF2 blastocysts followed by transferring embryos into day 2.5 pseudopregnant albino ICR females. Pups were delivered by Caesarian section on embryonic day 19.5 and fostered to an ICR foster mother that had given birth within the previous 4 days.

To secrete leptin in response to dox, mice must segregate for two transgenes (2TG): the dox-responsive leptin transgene (tetracycline-responsive elements-CMV-Lep, TRE-Lep), and a reverse transactivator rtTA (rtTA is driven by the Rosa26 promoter; R26-rtTA) [28]. Mice that segregate for both transgenes are denominated: 2TG. Single transgenic littermates (1TG) carry either the dox responsive leptin transgene or the reverse transactivator rtTA and do not secrete leptin when exposed to dox were used as controls.

Genotypes in 1TG and 2TG mice were confirmed with primers described by Jackson Laboratories for *Rosa26-rtTA::Col1a1-tetO-H2B-mCherry* mice which contain the same alleles as our mice (the same Rosa26-rtTA transgene, while H2B-mCherry transgene was generated using the same KH2 ES cells) [29]. Following are the genotyping primers:

Rosa26-rtTA locus:

Mutant Reverse 5’ GCG AAG AGT TTG TCC TCA ACC

Common Forward 5’ AAA GTC GCT CTG AGT TGT TAT

Wild type Reverse 5’ GGA GCG GGA GAA ATG GAT ATG

ColA1-TRE-Lep locus:

Mutant Forward 5’ GCA GAA GCG CGG CCG TCT GG

Common Reverse 5’ CCC TCC ATG TGT GAC CAA GG

Wild type Forward 5’ GCA CAG CAT TGC GGA CAT GC

Animals: Throughout the study, mice were maintained at room ambient 22-24°C with a 12-h dark-light cycle (lights on at 0700h) in a pathogen-free barrier facility. The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

Induction of leptin in transgenic mice: 36 male mice (1TG, n=18 and 2TG, n=18) at 3 weeks of age were exposed to 50 µg/ml of dox in drinking water for 7 days. Body composition by EchoMRI and concomitant blood were obtained at baseline and after 7 days of dox exposure.

Acute induction of leptin: Five 12 week old male 2TG mice were treated with *ad libitum* access to 200 µg/ml of dox in drinking water for 24 hours to induce transgenic leptin expression. Blood was collected at baseline (prior to dox administration) and at 24 hours of dox exposure. Dox-free water was provided for the following 24 hours. This 24-hour on and off dox treatment

was repeated. Blood was collected every morning to determine both how quickly leptin induction can be turned on and off, and to show that there is no residual alteration of endogenous leptin production.

Dox gavage: Five 8 week old male 2TG mice were gavaged with 400 µg of doxycycline (suspended in 400 µl of water). Blood was collected at baseline (immediately prior to gavage) and at 2h, 4h, 6h, 8h, 10h, 12h, 14h, and 24h using heparinized tubes.

Leptin secretion from ex-vivo tissues: 14 2TG mice were treated with 300 µg/ml of dox in 5% sucrose water or with 5% sucrose water only (*ad libitum* access) for 48 hours to induce leptin expression. Mice were then sacrificed and tissues harvested for culture and RNA isolation: subcutaneous adipose tissue (SCAT), perigonadal adipose tissue (PGAT), brown adipose tissue (BAT), liver (LIV), kidney (KID), stomach (STO), duodenum (DUO), jejunum (JEJ), ileum (ILE), spleen (SPL), lung, hypothalamus (HYPO). Approximately 100 mg of each tissue (except for HYPO which was ~20 mg) was minced into 4-5 pieces and cultured for 4 h in 0.5 ml of M199 media with 7 nM insulin and 25 nM dexamethasone [30]. Leptin secreted into the media was normalized by tissue mass. After 4 h in culture, media were collected for determination of leptin concentration.

RNA extraction, cDNA, qPCR: Total RNA was isolated using TRIzol reagent (Invitrogen). One µg of RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) using both OligoDT and random hexamer primers. Lightcycler 480 SYBR Green I Master was used for quantitative PCR assays (Roche). Relative gene expression of *rtTA* and *Lep* in perigonadal (PGAT), subcutaneous (SCAT) and brown (BAT) adipose tissues, liver (LIV), jejunum (JEJ), and whole hypothalamus (HYPO) was calculated by Lightcycler 480 software

(Roche) based on the Second Derivative Maximum method using a standard curve generated from a serially diluted pool of cDNA. Gene expression was normalized to the geometric mean of the housekeeping genes – *Actβ* and *36b4*. Primers used are listed below:

Actb Forward 5'-CGGGCTGTATTCCCCTCCAT

Actb Reverse 5'-GGGCCTCGTCACCCACATAG

36b4 Forward 5'-ACCTCCTTCTTCCAGGCTGG

36b4 Reverse 5'-CGAAGGAGAAGGGGGAGGTT

Lep Forward 5'-CGAGGAATCGTTCTGCAAATCC

Lep Reverse 5'-GCCAGGTTAAGTGCAGCTATCACA

rtTA Forward 5'-AGTCATTCCGCTGTGCTCTC

rtTA Reverse 5'-GCTCCTGTTCCTCCAATACG

Bioactivity of leptin: Fourteen 10 week old male mice (7 - 1TG, 7 - 2TG) were housed 2-3 per cage and given *ad libitum* access to 200 µg/ml dox water for 10 days. Dox concentration was then increased by 5 fold to 1 mg/ml for another 4 days. Body weight and food intake were measured daily. Body composition was measured using EchoMRI at baseline, 7, 10, and 14 days of exposure to dox. Blood was collected immediately after body composition measurement at baseline, 7 and 14 days of exposure to dox.

Dox dose response: Thirty 10 week old male mice (12 - 1TG, 18 - 2TG) were exposed to increasing concentrations of dox in 5% sucrose water every 2 weeks. Baseline blood was collected 1 week prior to dox exposure. The dox concentrations were then provided in the following order: 10, 15, 20 ug/ml, increasing every 2 weeks.

Plasma collection and assays: Blood was collected into heparinized tubes (Fisherbrand) by submandibular bleed at 9am from mice in a fed state (unless otherwise stated) and immediately placed on ice; plasma was isolated by centrifugation for 20 min at 2,000 x g at 4°C, aliquoted, and frozen at −80°C until time of assay. Circulating leptin concentrations were measured using mouse leptin ELISA (R&D). Leptin in media from the explanted tissue cultures was measured using mouse leptin AlphaLISA (PerkinElmer).

Plasma Leptin concentrations during in utero and postnatal dox exposure: Pregnant 1TG dams (carrying 1TG and 2TG fetuses) were exposed to 200 ug/ml of dox in drinking water starting at conception through pregnancy. The pregnant females were euthanized by cervical dislocation, at day 16-19 post conception, had blood collected, and the fetuses removed. Blood was collected from individual fetuses along with tissue for genotyping. Nursing 1TG mothers were exposed to dox (200 ug/ml) or drug-free water from parturition until weaning. Mice were bred so that the offspring were 1:1 1TG and 2TG. At postnatal day 15 (P15) while the pups were exclusively breastfeeding, pups (both 1TG and 2TG and both males and females) and mothers were bled for plasma leptin determination.

Results

Generation and validation of leptin overexpressing ES cells

Leptin-overexpressing mouse embryonic stem cells were generated using a previously described system [27]. Pre-engineered KH2 ES cells that carry a reverse tet-transactivator (rtTA) in the Rosa26 locus and an FRT-hygro-pA cassette in the ColA1 locus were co-electroporated with a targeting vector containing leptin transgene and FlpE Recombinase expressing vector to allow

for site directed recombination. The resulting KH2-Lep cell line contains the R26-rtTA and a leptin transgene downstream of the tetracycline-responsive elements (TRE) and the CMV promoter (located in the ColA1 locus, **Figure 2.1A**). In the absence of dox the rtTA present in the cell cannot bind to the TRE; when dox is present it binds to rtTA changing its confirmation and allowing it to bind to the TRE which in turn activates the CMV promoter and drives the expression of transgenic leptin (**Figure 2.1B**). The expression of leptin is controlled by dox concentration. To confirm proper integration of the targeting vector and to validate the effectiveness of dox in inducing lep expression, KH2-Lep ES cells were treated with increasing doses of dox (**Figure 2.2A**). In the basal state, prior to dox treatment, KH2-Lep cells did not secrete leptin. However, administration of increasing concentration of dox (50, 100, 200, and 400 ng/ml) in the culture media resulted in KH2-Lep cells secreting leptin into media in a dose-responsive manner (0.35, 0.92, 2.29, 2.94 ng/ml, respectively, **Figure 2.2A**) with deviation from linearity at the highest concentration, suggesting that the amount of secreted leptin is limited by the availability of rtTA or by the rate of production and/or secretion of leptin. Four leptin secreting cell lines were selected for cytogenetic analysis of G-banded metaphase cells. One cell line was karyotypically normal (**Figure 2.2B**) and was selected for subsequent generation of a transgenic mouse.

Leptin overexpressing mice

KH2-Lep ES cells were used to generate leptin transgenic mice via tetraploid blastocyst injection [31]. Similar to the KH2-Lep ES cells described above, the mouse reported here requires – for leptin expression in response to doxycycline – 2 transgenes: (1) the rtTA in the Rosa26 locus (R26-rtTA) and (2) the leptin transgene in ColA1 locus controlled by the TRE and the CMV promoter (TRE-Lep). These mice are referred to here as double transgenic or "2TG". Single

transgenic littermates (segregating for either R26-rtTA OR the TRE-Lep) do not respond to dox and are used as controls; they are denoted as "1TG".

Prior to treatment with dox, circulating leptin levels were directly proportional to fat mass in both the 1TG and 2TG male mice (**Figure 2.3A**) and there was no difference in either fat mass or circulating leptin between the two groups (**Figure 2.3A, C-D**). 1TG and 2TG mice were then given *ad libitum* access to drinking water containing 50 µg/ml of dox for 7 days. Average daily water intake for these mice is ~6 ml [32]; with dox at 50 ug/ml water, the estimated 24h dox intake was ~300 ug. At this dox concentration, the amount of circulating leptin increased 25 fold in the 2TG mice (2.5 ± 0.28 ng/ml at baseline compared to 60.5 ± 7.1 ng/ml; **Figure 2.3B, D**). This increase was independent of the amount of both baseline circulating leptin and body fat (**Figure 2.3B-D**). After 7 days of dox exposure, 2TG mice lost 27% of their initial body fat (1.2 ± 0.05 g to 0.91 ± 0.06 g; **Figure 2.3C**); whereas 1TG mice showed a mild gain in fat mass over a week as expected in mice at 3 weeks of age. Dox-induced circulating leptin concentration in 2TG mice was 40 times greater than the amount of endogenous leptin that would be predicted from this new level of adiposity [7, 33]. As predicted, circulating leptin concentrations in the 1TG mice were unchanged by the addition of dox to the water (2.9 ± 0.33 ng/ml vs 2.2 ± 0.22 ng/ml; **Figure 2.3D**).

The induction of leptin with dox in 2TG mice ceased rapidly upon withdrawal of dox (**Figure 2.3E**). Twelve week old male 2TG mice were exposed for successive 24 hour intervals to *ad libitum* access to 200 µg/ml of dox in drinking water or drug-free water. Plasma was obtained at the same time each day for five consecutive days. Within 24h of dox exposure, circulating leptin concentrations in 2TG mice increased from 18.06 ± 5.78 ng/ml to 98.61 ± 11.49 ng/ml (**Figure 2.3E**). Circulating leptin concentration returned to baseline within 24h of dox removal (16.23 ±

6.08 ng/ml; **Figure 2.3E**). The results of serial exposures of the same mice are shown in **Figure 2.3E**. After two cycles of 24h dox exposure, circulating leptin in 2TG mice returned to concentrations seen in dox-free water but these levels were significantly below their concentrations at baseline (13.12 ± 5.54 ng/ml vs 18.06 ± 5.78 ng/ml, $p<0.01$; **Figure 2.3C**) suggesting that overexpression of leptin for 48 hours reduced endogenous leptin production in adipose tissue likely due to fat loss.

To determine the kinetics of leptin overexpression, 2TG mice were gavaged with 400 µg of dox (in 400 ul of water; **Figure 2.3F**); this amount represents roughly the daily amount of dox that a mouse allowed *ad libitum* water intake would receive when exposed to 50 µg/ml dox in water assuming 6ml of water intake per 24 hours [32]. Plasma leptin concentration began to increase approximately 4 hours after gavage and peaked after 6-8 hours (83.62 ± 18.68 ng/ml; **Figure 2.3F**). Within 24 hours after the single dox gavage, plasma leptin concentrations returned to baseline, consistent with the sequential dox exposure experiment. The elimination half-life of doxycycline administered to mice by gavage is approximately 170 min [34], and the half-life of IP-administered leptin is ~40 minutes [35]. This timing implies that dox has to be physically present in the cell to promote leptin production; there is no persistence of expression after dox exposure is stopped.

To demonstrate that the 2TG mice respond to dox in a dose-dependent manner, we exposed them to escalating concentrations of dox in water. Leptin concentration in 2TG mice increased significantly at the lowest dox dose of 10 µg/ml (1.9 ± 0.4 ng/ml to 30.6 ± 5.4 ng/ml; **Figure 2.4A**). 1TG control circulating leptin concentration maintained proportionality to fat mass (**Figure 2.4A**). Inter-mouse variability in response was ~60% at any dose of dox. Such differences are probably due primarily to differences in water intake.

The transgenic strategy in this experiment did not explicitly restrict leptin expression to a specific cell type (see Discussion). To determine which tissues in the 2TG mice contributed to the dox-induced increases in circulating leptin, we isolated organs from 2TG mice exposed to dox (300 µg/ml *ad libitum*) water for 48 hours and 2TG mice given drug-free water as controls. Organs were isolated, washed in PBS, minced, and cultured for 4 hours in M199 medium supplemented with insulin and dexamethasone. Leptin expression in the isolated tissues and secreted leptin in the culture medium were determined (**Figure 2.4B, C**).

Adipose tissue: perigonadal (PGAT), subcutaneous (SCAT), and brown (BAT) were isolated. There was no change in either expression or secretion of leptin in any of the adipose depots studied (**Figure 2.4B, C**). *rtTA* was expressed in all tissues (**Figure 2.4D**) so either the dox did not reach the fat depots in sufficient quantities to statistically increase the already large production of leptin (likely), and/or the adipose tissue was already secreting and expressing at the maximum amount possible for the tissue.

Gastrointestinal tract (GI): The stomach, duodenum, jejunum, and ileum are the first tissues exposed to ingested dox. All had little or no endogenous leptin production and all showed large increases in both leptin expression and secretion upon dox exposure (**Figure 2.4B, C**). This suggests that the GI tract is the primary source of the increased plasma leptin in the dox-induced 2TG mice; this is likely due to both the route of dox administration and the capacity of the gut to secrete peptides.

Liver, lungs and spleen: All of these organs increased expression of leptin in response to dox exposure, but only the lungs and spleen increased secretion of leptin (**Figure 2.4B, C**).

Hypothalamus: The hypothalamus from 2TG mice showed no increase in leptin expression. However, there was a significant increase in low levels (0.07 ng/ml/hr) of apparent secretion of leptin in 2TG compared to 1TG mice (0.009 ng/ml/hr, **Figure 2.4B, C**). This apparent effect could be due to contamination with blood and/or cerebrospinal fluid (CSF), or could be caused by trace amounts of dox crossing the blood brain barrier. Dox concentration in the CSF of mice exposed to dox in food was found to be ~30 fold lower than that in plasma [36]. Exposing 2TG mice to a high concentration of dox (1 ug/ml which is at least 3.33 times higher dose than in our experiments) resulted in significantly higher expression of the leptin transgene in the hypothalami of 2TG mice compared to 1TG mice (**Figure 2.4E**). Such doses are not required to generate plasma leptin concentrations in a physiological range.

In these transgenic animals, the majority of circulating leptin is apparently secreted from the digestive tract. If mice were injected with dox IP or IV, the organs contribution to circulating leptin could be different. Although basal expression of the *rtTA* transgene is variable by organ, expression of *rtTA* is not altered in any tissue by exposure to dox (**Figure 2.4D**). *rtTA* expression in subcutaneous adipose tissue (SCAT), perigonadal adipose tissue (PGAT), and jejunum (JEJ) is lower than in brown adipose tissue (BAT) and liver (LIV), but leptin expression in response to dox is higher in those tissues, indicating that the *rtTA* presence in the cell is not a limiting factor for expression of transgenic leptin.

1TG and 2TG male mice were housed 2-3 per cage and provided *ad libitum* access to 200 µg/ml of dox in drinking water for 10 days. The dox dose was then increased to 1 mg/ml to assess whether a maximal leptin response was achieved. Body weight and food intake were measured daily while plasma leptin concentration and body composition were determined at the beginning of the study (baseline), 7, 10 and 14 days of dox exposure. The 2TG mice, when given *ad libitum*

access to 200 µl/ml dox, had significantly lower food intake than the 1TG controls on days 2 and 3 of dox exposure (*day 2*: 2TG, 9.12 ± 0.32 kcal/day, 1TG, 12.85 ± 1.0 kcal/day, *p<0.05*; *day 3*: 2TG, 8.68 ±0.03 kcal/day, 1TG, 12.65 ± 1.20 kcal/day, *p<0.05*; **Figure 2.5B**). After 14 days of dox exposure, 2TG mice lost 2.65 ± 0.35 g of body weight compared to a weight gain of 0.09 ± 0.28 g in 1TG controls (p<0.001, **Figure 2.5A**). In 2TG animals both fat mass (-1.52 ± 0.24 g vs a gain of 0.34 ± 0.08 g in 1TG, p<0.001, **Figure 2.5C**) and lean mass (-0.90 ± 0.17 g vs a gain of 0.13 ± 0.17 g, p<0.001, **Figure 2.5D**) were reduced. These results indicate that the dox-induced leptin is bioactive and is capable of causing anticipated physiological responses [12, 37, 38]. Interestingly, increasing the dox dose by 5 fold doubled the amount of circulating leptin but no additional weight loss occurred (**Figure 2.5A, E**).

1TG female mice homozygous for the TRE-Lep gene and wild type for the R26-rtTA gene (TRE-Lep/TRE-Lep) were crossed to 1TG male mice that were heterozygous for R26-rtTA (rtTA/+). This resulted in all offspring being TRE-Lep/+ and half being rtTA/+. Therefore, the offspring were predicted to be 1:1 1TG and 2TG. Animals in the breeding cages were provided *ad libitum* access to 200 ug/ml dox or drug-free (controls) water when the crosses were set. Exposure of pregnant females to dox or control water was continued throughout gestation; these animals were sacrificed between day E16 and E19 to assess plasma leptin concentrations in the fetuses. There were no differences in plasma leptin concentrations between the 1TG pregnant females exposed to dox and 1TG controls given drug-free water (**Figure 2.6A**). Leptin concentration in the E16-19 fetuses was significantly elevated only in the 2TG offspring conceived by 1TG females exposed to dox water (34.2 ± 13.5 ng/ml vs 0.7 ± 0.1 ng/ml; p<0.01; **Figure 2.6B**); elevation of plasma leptin was not seen in 1TG or 2TG fetuses from females given

drug-free water. Therefore, dox is transferred from maternal circulation to the fetuses through the placenta.

1TG females (either TRE-Lep/TRE-Lep or rtTA/+) were exposed to 200 ug/ml of dox water or drug-free water (controls) at parturition and continued exposure postnatally throughout the nursing period. The offspring were either 1TG or 2TG in equal numbers (1TG, n= 38, 2TG, n=40). On postnatal day 15, blood was collected on both dams and pups to assess plasma leptin concentrations. As predicted, 1TG nursing females had plasma leptin concentrations that showed no difference between dox and drug-free water exposure (**Figure 2.6C**). 2TG P15 pups born to 1TG mothers exposed to dox, had significantly higher plasma leptin concentrations compared to their 1TG littermates (202.5 ± 16.9 ng/ml vs. 7.3 ± 0.8 ng/ml; $p<0.001$; **Figure 2.6D**) and compared to P15 pups born to mothers maintained on drug-free water. Thus, bioactive dox is transferred in breast milk.

Discussion

We have generated a transgenic mouse with doxycycline-titratable (by time and degree) overexpression of bioactive leptin. In wild type mice and humans, the major site of leptin synthesis and release is adipose tissue. Low levels of leptin expression are detected in brown adipose tissue, stomach [39], placenta [40], mammary tissue [41] and ovaries [42]. The transgenic mouse described here over-expresses leptin in multiple tissues when exposed to dox. The major site of leptin production and release in these animals is the small intestine. Interestingly, adipose tissue leptin expression is not increased in these animals. We attempted to create an adipose tissue-only overexpressing animal by replacing the rtTA in the Rosa26 locus

with the rtTA under the adiponectin promoter; however, exposure to high dox concentrations raised circulating leptin modestly and failed to elevate circulating leptin concentrations to those seen in DIO mice.

Leptin produced by the transgene is bioactive and appears to emanate primarily from secretory cells in the gut. The transient weight loss of the animals in response to dox appears to be proportional to circulating concentrations of leptin, indicating that conventional leptin physiology is intact in these animals. At very high levels of dox, ectopic leptin production in the hypothalamus could contribute to the functional consequences of dox exposure. In some experimental circumstances this effect – if it occurs – might constitute an experimental confound. At doses of dox that produce circulating leptin concentrations that are within the physiological range, this potential hypothalamic effect is likely not significant. Production of the leptin in the liver of dox-treated animals could possibly influence vagal signaling by paracrine effects. Our data do not suggest that this effect is large if it occurs at all.

A mouse model overexpressing leptin in response to oral doxycycline offers benefits over administration of exogenous leptin. When osmotic mini pumps are used to elevate circulating leptin in mice there are limitations to the amount of leptin that can be administered because of limited leptin solubility, variability in efficiency of delivery among individual mice, and weight loss from surgical recovery [15, 17]. Harris *et al.* were able to elevate circulating leptin concentrations to a maximum of approximately five times baseline [17]. The concentrations of plasma leptin in lean mice administered 10 µg/day of exogenous leptin via osmotic pump only reached 6.8 ng/ml [17] – much lower than that occurring in DIO mice (~75 ng/ml after 16 weeks of HFD feeding) [33]. Achieving a circulating leptin concentration similar to that characterizing the DIO mouse is important to being able to distinguish the effects of diet and obesity, from

those conveyed by leptin *per se*. Ravussin *et al.* administered leptin to mice via osmotic mini pumps with maximum exposure limited by the solubility of leptin in the mini-pump [15]. The maximum leptin dose deliverable by the pump was 25 µg/day, elevating plasma leptin concentration to approximately 50 ng/ml with large variability among animals [15].

Daily intraperitoneal (IP) injections are commonly used to deliver leptin to mice. Repeated injections cause stress and injecting a bolus of leptin once per day does not recapitulate normal circadian physiology. Cole-Burnett *et al.* estimated that the half-life of injected exogenous leptin was 40 minutes: at 30 minutes post IP injections of 3 mg/kg of leptin, plasma leptin concentration was 170-fold of baseline and leptin was cleared from the circulation within 4 hours [35]. This suggests that, in studies using injections to manipulate circulating leptin concentrations, large transients of non-physiological concentration are created followed by normal concentrations of leptin for the majority of the 24 hour period [35]. Freely fed male mice naturally vary their circulating leptin concentration with an increase of about 60% from the nadir at 8AM to 10AM to the peak at 10PM to 2AM [43]. While the leptin-overexpressing mouse reported here does not fully recapitulate normal circadian physiology either, the mouse has *ad libitum* access to dox-supplemented water throughout the 24h period. Since mice drink and eat more in the dark cycle, there is some periodicity to variation in leptin concentration with the mice producing more leptin at night.

The leptin-overexpressing mouse described here can be used in studies of the role of leptin in various stages of development. Exposing 1TG females to dox in drinking water during pregnancy results in overexpression of leptin in the 2TG offspring due to transfer of dox from maternal to fetal circulation. Similarly, when nursing 1TG females are exposed to dox in

drinking water, dox is transferred in milk to their pups and activates the leptin transgene in the 2TG pups. This effect enables non-invasive delivery of leptin at all points in development.

High dose administration of leptin for 24 weeks to human subjects resulted in the development of non-neutralizing antibodies in 71% of these individuals [14]. In more recent studies of lipodystrophic or obese subjects [44, 45] administration of exogenous *metreleptin* resulted in the development of neutralizing anti-leptin antibodies in the majority of recipients. Chronic (20 week) exogenous leptin infusion in mice via an osmotic mini-pump resulted in elevated leptin concentrations, adjusted for fat mass, 20 weeks after the leptin infusion cessation [15]. Anti-leptin antibodies were not evaluated in that study; however, it is possible that the mice developed neutralizing antibodies to exogenous leptin as has been reported in human studies. We did not detect any increases in circulating leptin concentrations in our transgenic mice after cessation of a long term dox exposure; leptin concentration returned to amounts predicted by the fat mass within 24 hours of dox cessation.

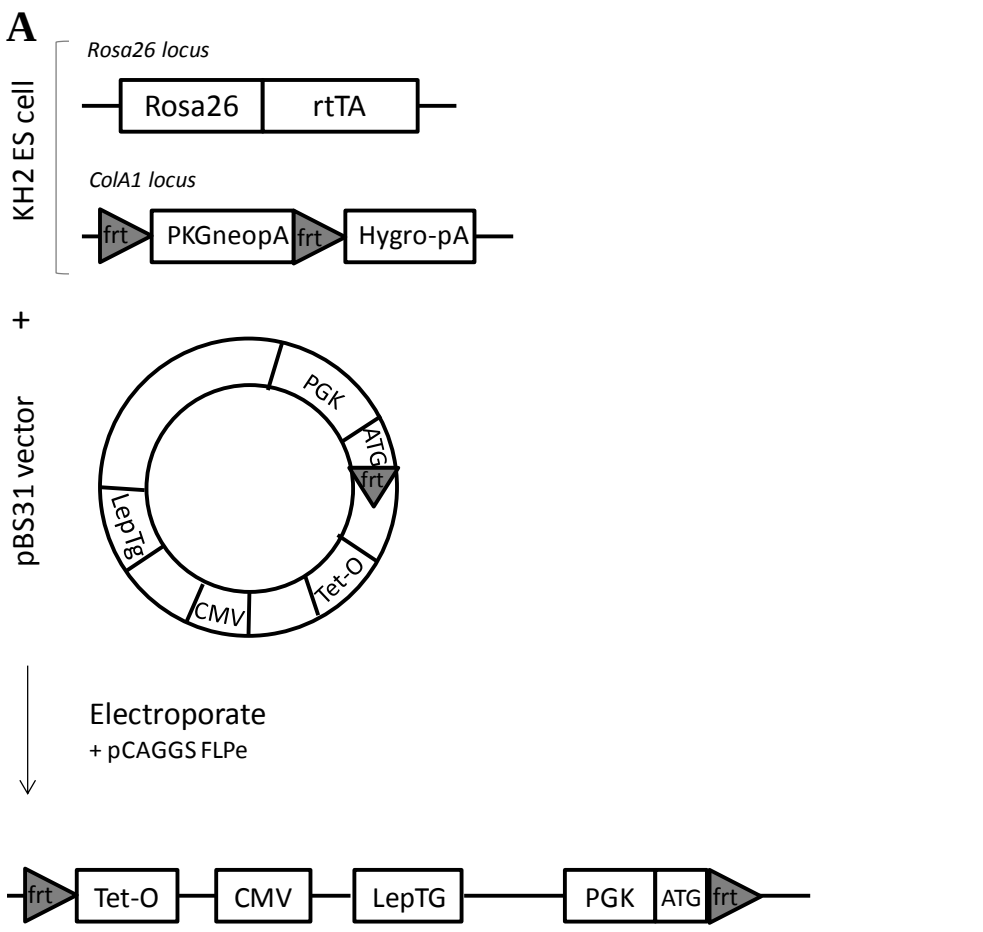
Qui *et al.* transgenically overexpressed human leptin in fat cells [24]; Ogawa *et al.* did the same for mouse leptin in hepatocytes [25]. The adipose tissue-specific AP2 promoter was used to drive human leptin expression; secondary reductions in fat mass limited the elevations in plasma leptin in these mice [24]. In the hepatocyte overexpressors, leptin plasma concentrations reached 60 ng/ml – consistent with a DIO animal – but could not be turned on or off [25]. In our transgenic mouse, leptin was expressed in the liver, however we did not detect any protein in the liver culture media from the *ex vivo* leptin secretion assay. It is possible that the liver secretes leptin *in vivo*, but perhaps the blood flow is necessary to observe this secretion.

No change in expression of leptin from the endogenous locus in control compared to hyperleptinemic mice indicates that elevated circulating leptin does not down regulate the production of endogenous leptin within the adipocytes.

Another model of hyperleptinemia is the diet induced obese (DIO) mouse. However, this mouse does not disentangle the metabolic effects of excess adiposity from the effects of excess leptin *per se*. Additionally, DIO leads to attenuated leptin signaling in many brain areas previously identified to have a role in feeding circuits [23]; however, the mechanism of this decreased leptin sensing is unclear. One possibility is that hyperleptinemia *per se* – the direct consequence of increased adiposity – drives the attenuation of the cellular response to leptin. The leptin transgenic mouse reported here will allow for testing this hypothesis.

The leptin 2TG mouse provides the capability to reversibly increase circulating leptin concentration to virtually any level at any point in development. The ability to titrate the amount of excess leptin and the ability to rapidly terminate exposure are key benefits. These mice will enable studies on the effects of time-limited elevations of circulating leptin *per se* (in the absence of other circulating cofactors accompanying in obesity), on brain development, "set point" physiology, food intake, and specific metabolic phenotypes such as insulin homeostasis.

Figures and Figure Legends



Resulting KH2-LepTg cell line

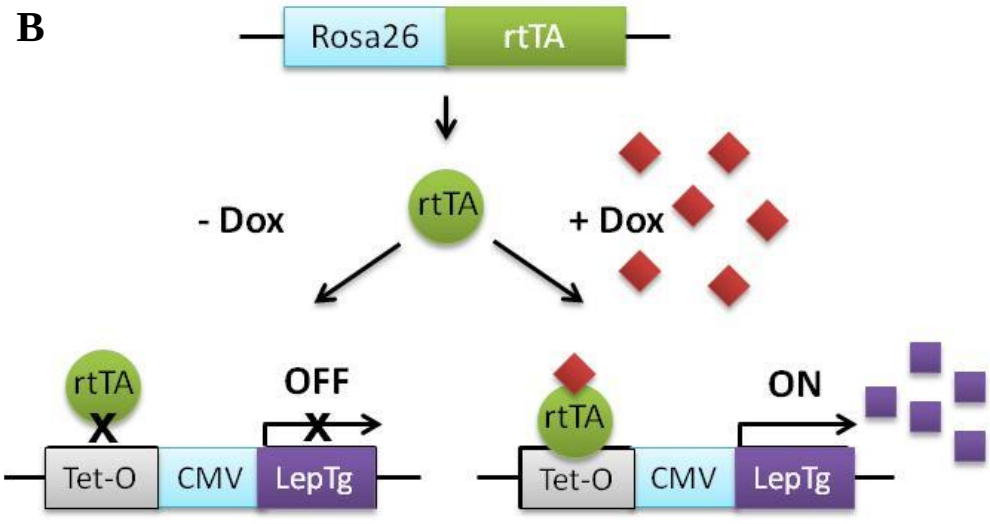


Figure 2-1 Schematic of Creation of a tetracycline-inducible leptin overexpressing transgenic mouse.

(A) Construct schematic. KH2 embryonic stem cells, containing a reverse tet-transactivator (rtTA) located in the Rosa26 locus and an FRT-hygro-pA "homing" cassette in the ColA1 locus, were electroporated with pBS31tetOpgkATGfrt-Lep vector and FlpE Recombinase (pCAGGS-FLPe) to generate the dox-inducible leptin overexpressing mouse. (B) Tet-ON schematic. In the absence of dox the rtTA present in the cell cannot bind to the TRE, thus transgenic leptin is not expressed; when dox is present it binds to rtTA changing its confirmation and allowing it to bind to the TRE which in turn activates the CMV promoter and drives the expression of transgenic leptin.

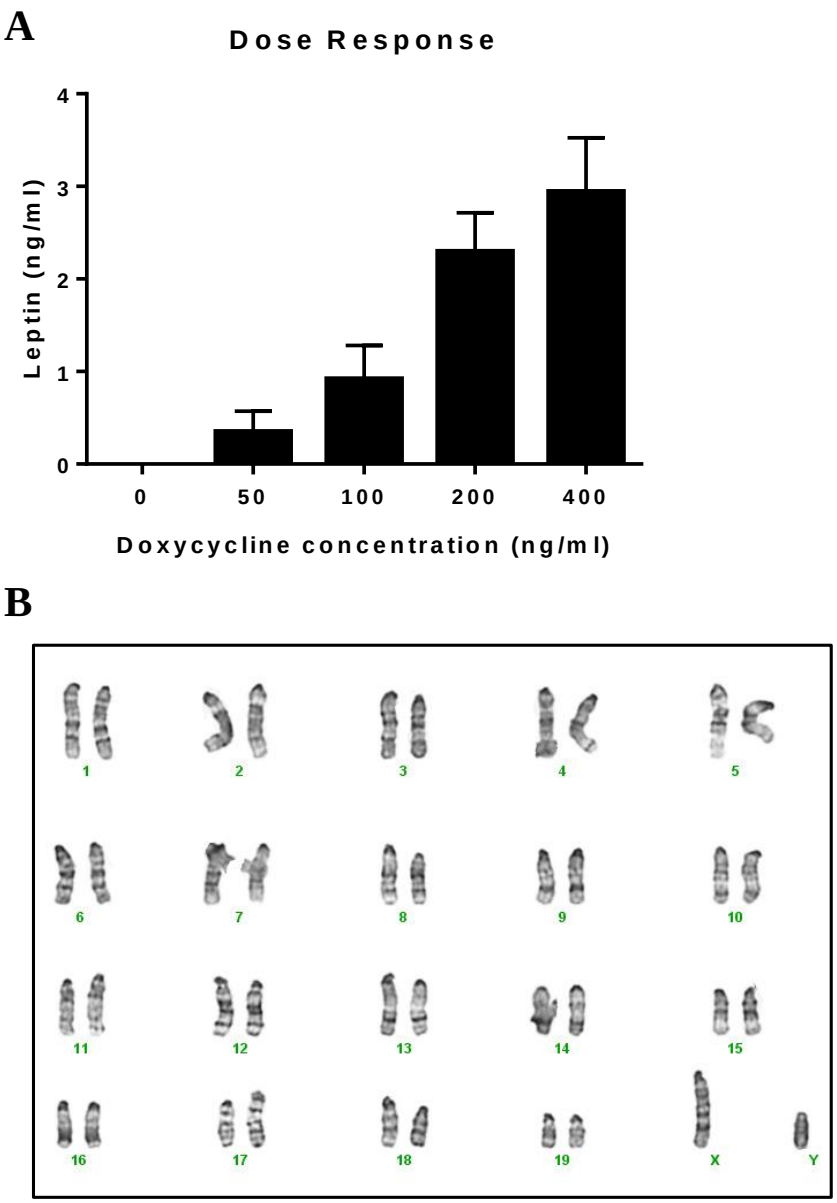


Figure 2-2 Validation of leptin overexpressing ES cells.

(A) Dose dependent doxycycline induced secretion of leptin in the leptin transgenic mouse embryonic stem cell line, KH2-lep, into culture media (n=4). (B) Cytogenetic analysis of G-banded metaphase cells from leptin transgenic mouse embryonic stem cell line, KH2-lep. The ES cell clone selected for blastocyst injection was karyotypically normal.

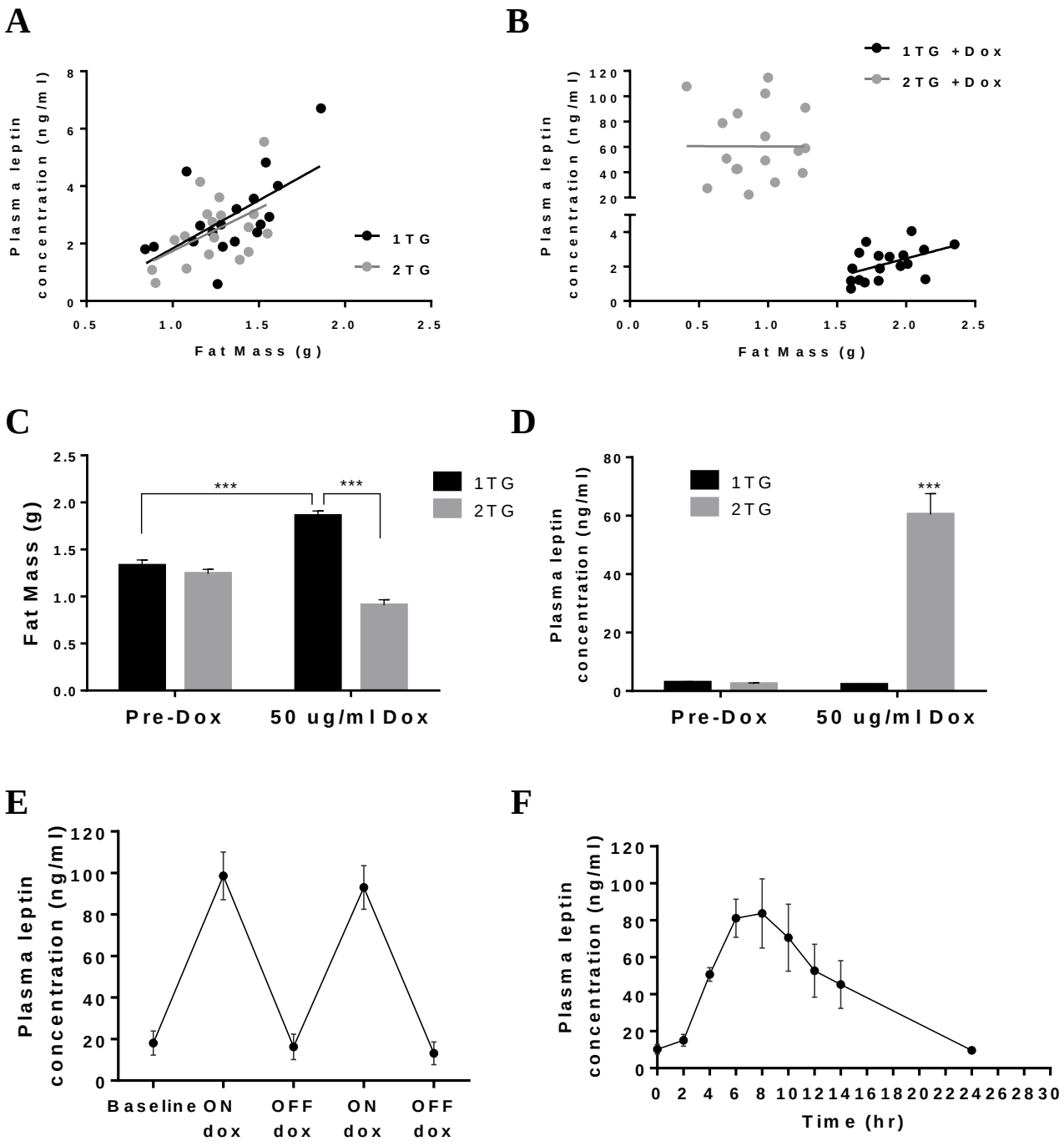
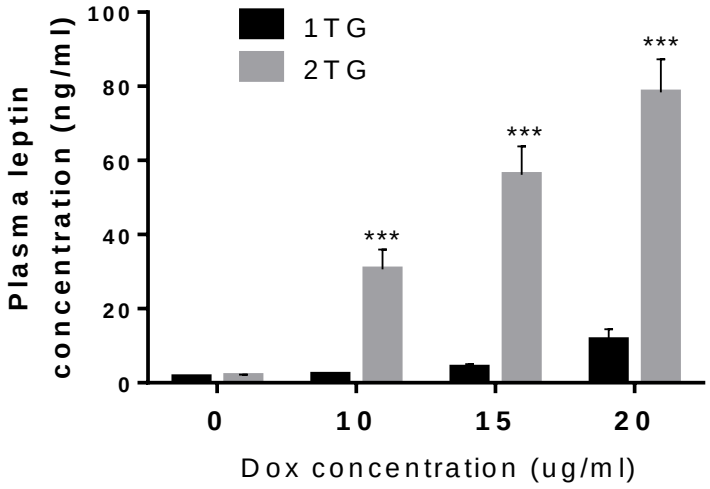


Figure 2-3 Induction of leptin with doxycycline exposure in leptin transgenic mice.

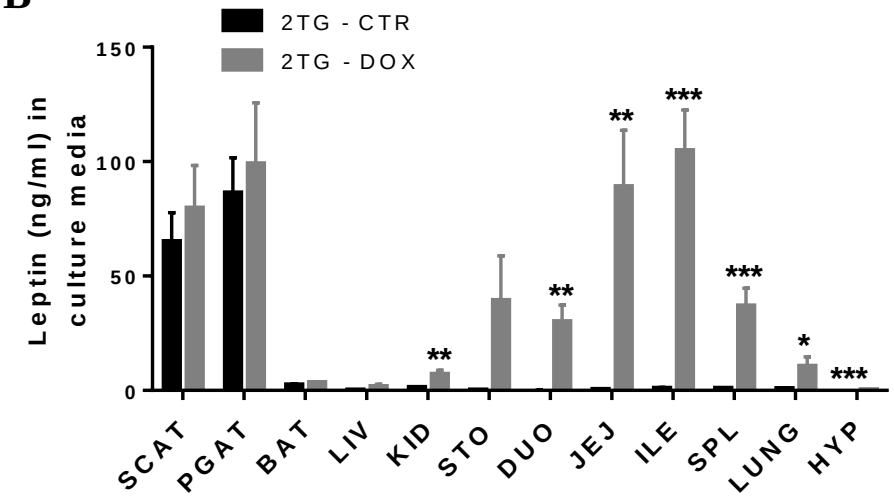
(A) Baseline and (B) dox-induced circulating leptin in 1TG (Ctr, n=18) and 2TG (n=18) mice as a function of fat mass. At baseline plasma leptin concentrations are correlated with fat mass

[1TG: R^2=0.38; 2TG: R^2=0.25]. After dox exposure leptin concentrations are independent of fat mass in 2TG mice [1TG: R^2=0.24; 2TG: R^2=1.2e^{-5}]. (C) Mean fat mass and (D) mean circulating leptin in 1TG (n=18) and 2TG (n=18) mice at baseline and after 7 days of 50 ug/ml dox exposure. (E) Rapid control of leptin expression with two 24h dox exposures in 2TG mice (n=5). Baseline circulating leptin, followed by 24h on 200 ug/ml dox levels, then off dox for 24h and the cycle repeated. (F): Dynamic induction of circulating leptin after a gavage of 400 ug of dox to 2TG mice (n=5). Plasma leptin concentration fully restored to baseline within 24h of gavage. All values are means with SEM. Significant difference calculated with Student t-test, ***$p<0.001$.

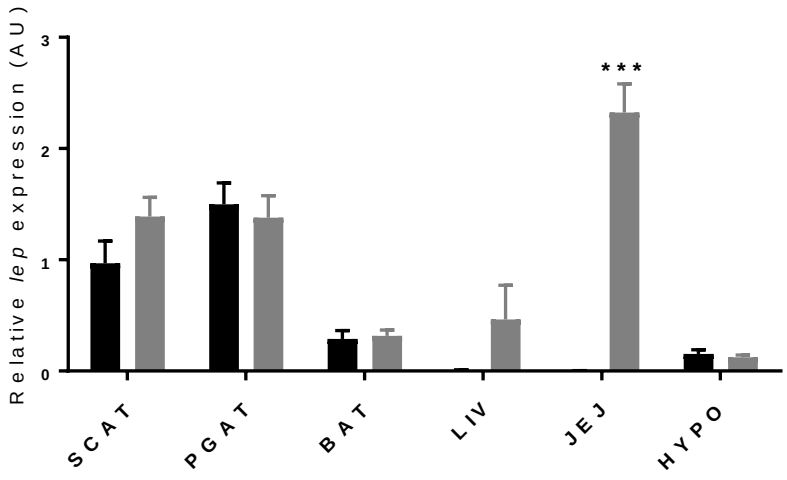
A


1TG
2TG
Plasma leptin concentration (ng/ml)
100
80
60
40
20
0
***
***
***
0
10
15
20
Dox concentration (ug/ml)

B


2TG - CTR
2TG - DOX
Leptin (ng/ml) in culture media
150
100
50
0
**
**
**
***
***
*
***
SCAT
PGAT
BAT
LIV
KID
STO
DUO
JEJ
ILE
SPL
LUNG
HYP

C


Relative lep expression (AU)
3
2
1
0
***
SCAT
PGAT
BAT
LIV
JEJ
HYPO

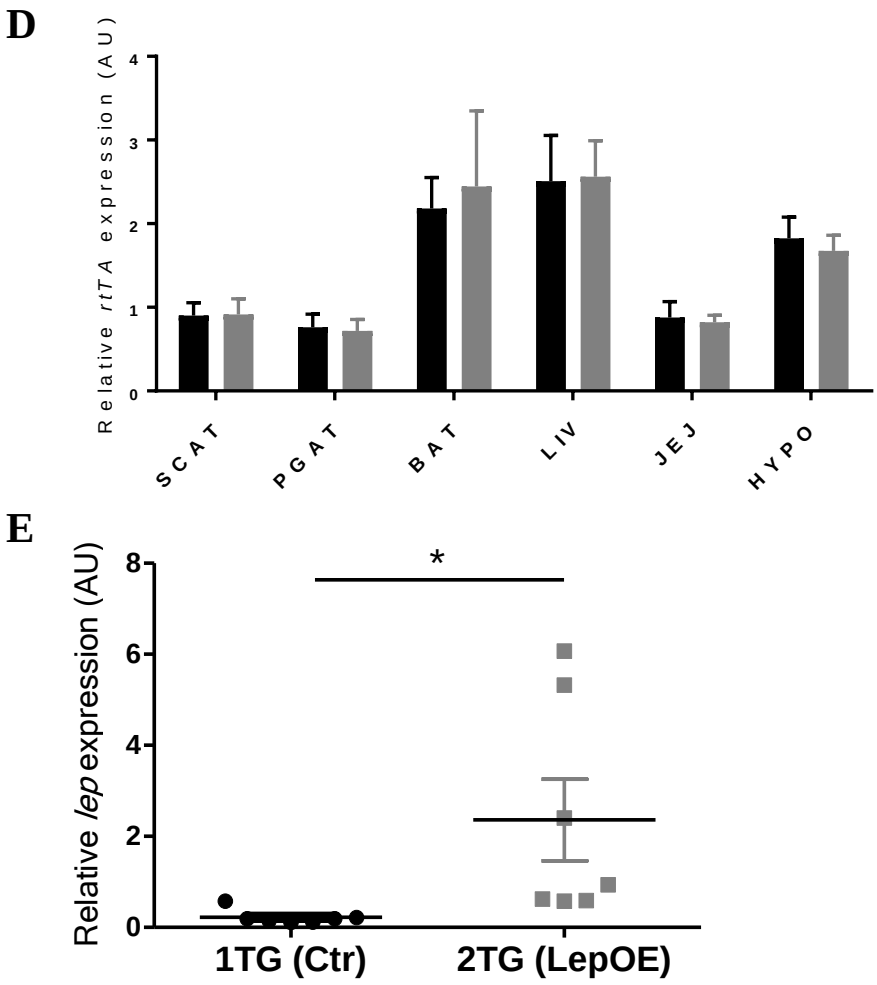
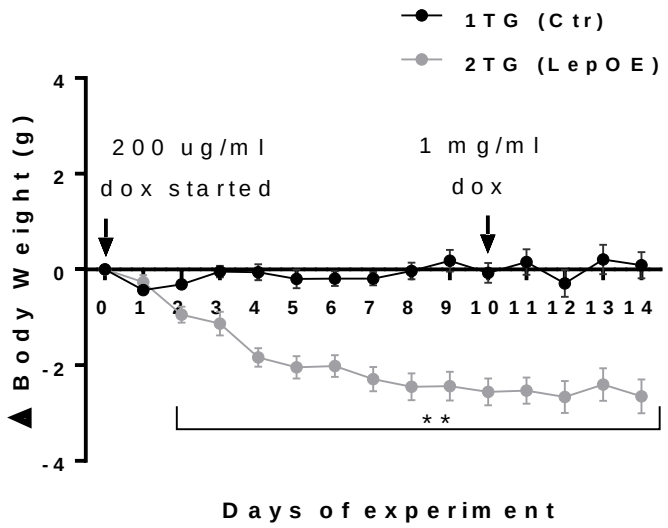


Figure 2-4 Tissue contribution to circulating leptin in transgenic mice.

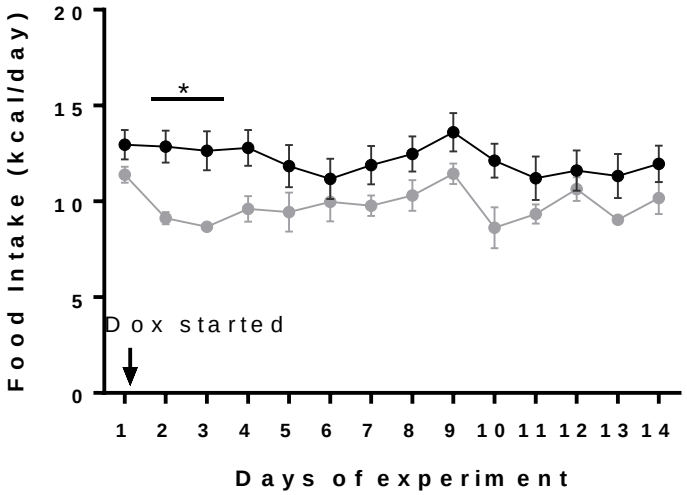
(A) Dose response to increasing amount of dox in water in 1TG (n=12) and 2TG (n=18) mice. Dox concentration was increased every 2 weeks. (B) Secretion of leptin into culture media from various tissues (SCAT – subcutaneous adipose tissue, PGAT – perigonadal adipose tissue, BAT – brown adipose tissue, LIV – liver, KID – kidney, STO – stomach, DUO – duodenum, JEJ – jejunum, ILE – ileum, SPL – spleen, LUNG, HYPO – hypothalamus), and relative expression of (C) *lep* and (D) *rtTA* in cultured tissues from 2TG mice exposed to sucrose (n=7) or doxycycline (n=7). (E) Relative *Lep* expression in the hypothalami of 1TG (n=7) and 2TG (n=7) mice

exposed to high dose of dox (1000 ug/ml). Expression was normalized to housekeeping genes, *Actb* and *36b4*. All values are means with SEM. Significant difference calculated with Student t-test, *p<0.05, **p<0.01. ***p<0.001.

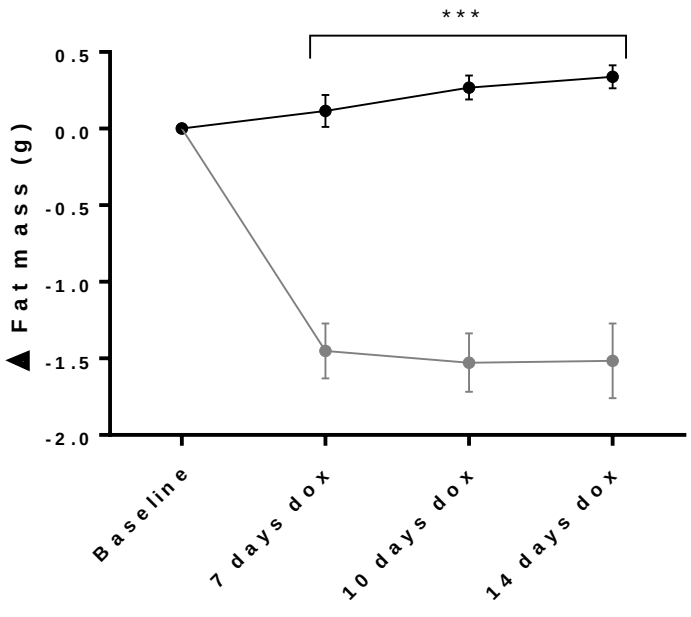
A


1TG (Ctr)
2TG (LepOE)
200 ug/ml
dox started
1 mg/ml
dox
Body Weight (g)
4
2
0
-2
-4
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
**
Days of experiment

B


Food Intake (kcal/day)
20
15
10
5
0
*
Dox started
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days of experiment

C


***
Fat mass (g)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
Baseline
7 days dox
10 days dox
14 days dox

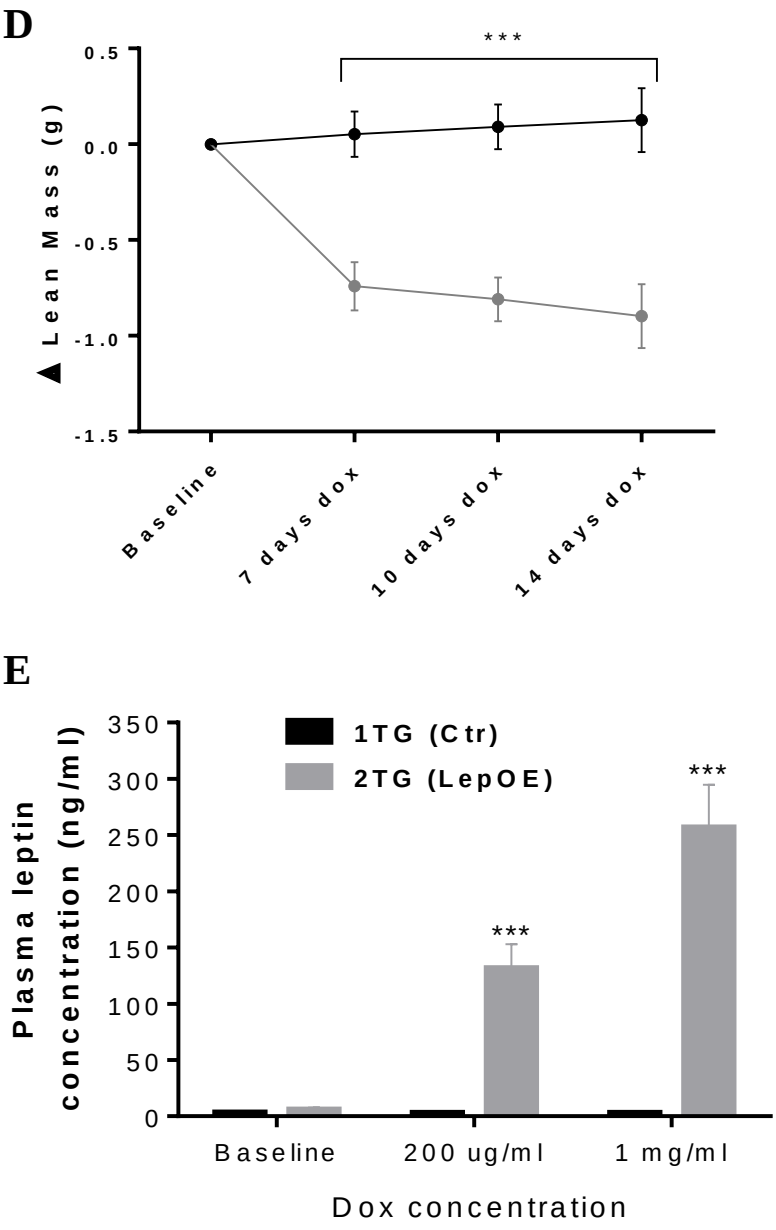


Figure 2-5 Bioactivity of leptin.

(A) Daily body weight, (B) daily regular chow intake, change in (C) fat mass, (D) lean mass, and (E) circulating leptin at baseline (before start of dox exposure), at 7 days and 10 days of 200 ug/ml dox exposure, and additional 4 days of 1mg/ml of dox in single (1TG controls, n=7) and double (2TG leptin overexpressing, n=7) transgenic mice. All values are means with SEM. Significant difference calculated with Student t-test, *p<0.05, **p<0.01. ***p<0.001.

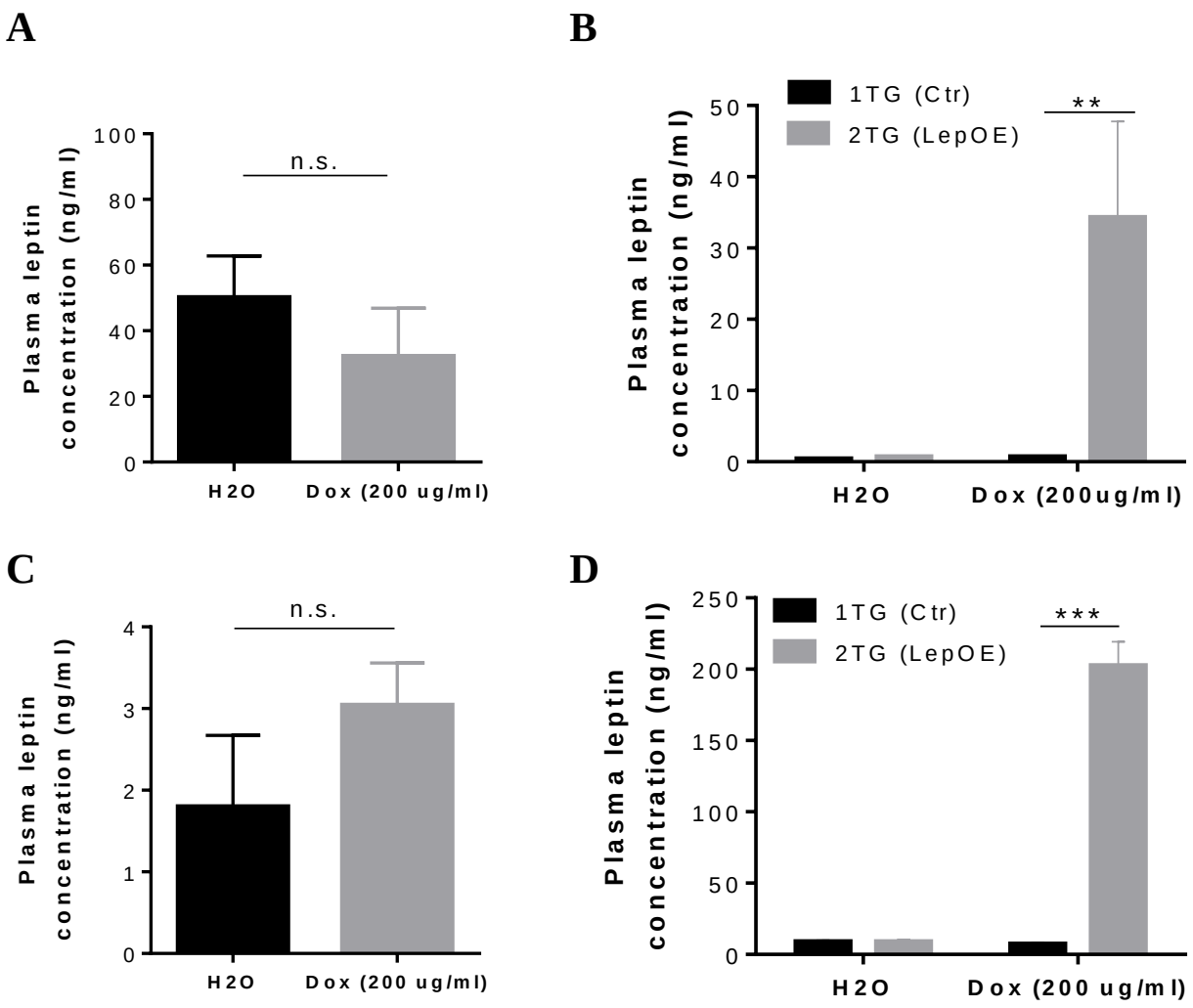


Figure 2-6 Circulating leptin during in utero and postnatal dox exposure.

Plasma leptin concentrations in (A) pregnant 1TG females (H_2O, n=3; Dox, n=5), (B) 1TG and 2TG fetuses (n=11-23), (C) nursing 1TG females (H_2O, n=4; Dox, n=7), (D) 1TG and 2TG suckling pups (n=9-25) on day P15 during *in utero* and postnatal exposure to 200 ug/ml dox or water (control). All values are means with SEM. Significant difference calculated with Student t-test, ** $p<0.01$. *** $p<0.001$.

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Chapter 3

"Physiological consequences of transient hyperleptinemia during discrete developmental periods on body weight in mice"

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Author contributions:

A.A.S., C.A.L., and R.L. designed experiments. A.A.S., C.A.L., and L.C.B. performed experiments. A.A.S., C.A.L., and R.L. analyzed and interpreted data, and wrote the manuscript.

Introduction

Obesity is a major international public health concern [1]. Prevalence of obesity is at an alarming level in both adults [2] and the pediatric population [3]. Childhood obesity is highly correlated with future adult obesity [4].

Leptin is a hormone produced and secreted primarily by the adipose tissue [5]. Under weight-stable conditions leptin circulates in direct proportion to fat stores in both humans [6] and rodents [7] to signal peripheral energy availability to the central nervous system (CNS). Weight loss is associated with molecular and physiological leptin-mediated adaptations that promote weight regain [8-10]; when circulating leptin in this state is restored to pre-weight loss levels, most of the changes associated with weight reduction are alleviated [11], but exogenous leptin

administration to lean and obese humans at their normal weights has minimal effects on energy intake and expenditure [12, 13]. Conversely, administration of leptin to animals or humans at reduced body weight reverses many of the bioenergetics and behavioral consequences of this state [11]. In rodents at usual body weight, leptin infusion transiently suppresses body weight gain but ultimately body weight is not altered with chronic leptin exposure [14] thus, adiposity in mice is inversely related to exogenous leptin response [15]. Dietary fat and adiposity attenuate leptin signaling in the CNS [16]; some of the hypothesized factors mediating this effect include elevated free fatty acids in the hypothalamus[17, 18], diet-induced hypothalamic inflammation [via increased interleukins -1β and -6 (IL-1β and IL-6) and tumor-necrosis factor-α (TNFα)] [17, 19-21], hypothalamic ER stress [22], decreased transport of leptin from periphery to the CNS [23, 24], and increase in circulatory proteins that bind leptin (such as soluble leptin receptor) [25].

There appears to be a CNS "set point" or threshold for minimum adiposity that is influenced by genetic and developmental factors [26]. Circulating leptin provides a signal regarding adiposity to the cells/circuits mediating this threshold. When leptin falls below this threshold a robust physiologic response including reduced energy expenditure and increased hunger is induced to oppose the fat loss; however, responses triggered by leptin concentration above this set-point are mild and transient [26]. The extent to which the molecular (leptin homeostasis) and neuroanatomic (neuronal circuit alterations) processes involved in the establishment of the adiposity set point can be influenced by "environmental" factors such as adequacy and quality of intrauterine metabolic fuels and endocrine events, postnatal diet, and intercurrent obesity is of importance to understanding the causes of obesity. Specifically, can the set point for adiposity be altered by perinatal factors, and what are the mechanisms underlying such alterations. Dietary fat

content [16], adiposity [16], overfeeding [27], and leptin *per se* [28] have been implicated in such effects, but since these co-vary experimentally, it has been challenging to isolate these factors during development.

In 1990, an English epidemiologist – David Barker – hypothesized that programming by the metabolic environment in fetal and infant life has consequences later in life for adult onset metabolic disease [29]. His hypothesis was based on historical cohort studies in which he noted a strong association between low birth weight or pre-term birth and an increased incidence of coronary heart disease in adulthood [29]. Barker's studies of developmental origins of adult disease focused research on the relationship of early development and adult disease.

The prevalence of obesity in the offspring of obese mothers who undergo bariatric surgery decreases by ~50% compared to siblings born pre-surgery [30]. Epidemiological studies indicate that maternal obesity increases the risk of the offspring to develop obesity [31-33]. Rodent studies have investigated the role of obesity during gestation and/or postnatally and suggest that maternal high fat diet feeding during the perinatal period as well as overnutrition during nursing (by decreasing litter size) results in obesity and metabolic abnormalities (such as insulin resistance or hepatic steatosis) in adult offspring [34-39]. The underlying cellular and molecular mechanisms for maternal programming of adult obesity remain unclear.

Simerly and colleagues identified a role of leptin as a neurotrophic factor that plays a critical role in the development of feeding circuits [40]. They showed that a lack of leptin during development impairs the formation of projections from the arcuate nucleus of hypothalamus (ARH) to other brain regions involved in energy homeostasis [the paraventricular nucleus (PVH), the dorsomedial hypothalamic nucleus (DMH), and the lateral hypothalamic area (LHA)] [40]. Excess leptin during development could also impair the formation of projections in these

circuits [27, 41]. There is precedence for such effects based on the U-shaped association between birth weight and BMI later in life.

Here we investigate the physiological consequences of developmentally timed transient hyperleptinemia on apparent set point for adiposity. We generated and used a leptin-overexpressing transgenic mouse model (Thesis Chapter 2). This transgenic mouse permits non-invasive induction of hyperleptinemia isolated from obesity *per se* and its resulting metabolic consequences such as elevated FFA and glucose, insulin insensitivity, fatty liver, etc. A TET-ON system enables transgenic leptin expression that is regulated by exposure to doxycycline (dox) in a dose-responsive manner and can be rapidly turned on and off. Specifically, we evaluated the physiological effects of elevated leptin during (1) adulthood, (2) adolescence and (3) postnatal period on the defense of body weight (adiposity) later in life and on the susceptibility to gain weight when offered a highly palatable diet *ad libitum*.

Methods

Animals

Experiments were carried out with doxycycline (dox)-inducible leptin overexpressing transgenic mice generated and described in Chapter 2. Briefly, these transgenic mice use the TET-ON system to express leptin in response to dox. Two transgenes are required for leptin expression: (1) a leptin transgene (TRE-Lep) downstream of the tet-O responsive elements (TRE) and the CMV promoter; and (2) a reverse transactivator rtTA in the Rosa26 locus (R26-rtTA) [42]. These mice are denominated: 2TG throughout the manuscript. Mice that segregate for only one

of the transgenes (denominated: 1TG with either TRE-Lep or R26-rtTA) do not produce excess leptin in response to dox, and serve as controls. Dox was administered to mice in drinking water.

Throughout the study, animals were maintained at room ambient 22-24°C with a 12-h dark-light cycle (lights on at 0700h) in a pathogen-free barrier facility. The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

Experiment 1: Hyperleptinemia in adult mice

Three groups of mice were studied in this experiment: (1) Double transgenic (2TG) control group given 5% sucrose in drinking water but no dox (2TG no dox), (2) single transgenic (1TG) control group given dox in 5% sucrose drinking water (1TG +dox), and (3) 2TG experimental group given dox in 5% sucrose drinking water (2TG +Dox). Controls and experimental mice were raised in the same litters. After weaning, mice were group-housed (3 per cage) with *ad libitum* access to chow (Purina LabDiet 5058) and water. Dox (Doxycycline hyclate, Sigma D9891) exposure began at 9 weeks of age and all mice were fed chow (22% calories as fat) during this period. Mice were exposed to increasing concentrations of dox in 5% sucrose water every 2 weeks. Dox water was changed twice weekly. Baseline blood was collected 1 week prior to dox exposure. The dox concentrations in water were: 5, 7.5, 12.5, 17.5, 20, 22.5, 27.5, 35, 40, 45 ug/ml, increasing every 2 weeks. Circulating leptin concentration was measured from plasma isolated from submanibular bleeding every two weeks, 1 week after each dox dose escalation. Glucose was measured in submandibular whole blood with a Freestyle Lite glucose meter (Abbott) at the same time as blood collection for leptin assay. Dox degrades over time; this was observed on week 22 (age of mice) when circulating leptin concentrations were lower than the previous period despite higher nominal concentration of dox in the drinking water. A new batch of dox was purchased for the 24 week exposure.

Body weight and body composition (using an EchoMRI Body Composition Analyzer) was measured weekly throughout the experiment. Food was placed on the wire cage tops and food intake was measured twice a week for all mice throughout the study (food intake was measured on a per cage basis). During dox exposure, water intake was measured twice a week to ensure that there was no dox (taste) effect on the amount of water consumed.

Dox-free water was provided after 20-weeks of dox exposure and mice immediately reverted to leptin concentrations of untreated animals. Monitoring of body weight, composition and food intake continued weekly after dox exposure was ceased. 5 weeks after release from dox exposure, mice were switched from regular chow to high fat diet (HFD; Research Diets, Inc. D12492i, 60% kcal from fat). Mice were sacrificed 16 weeks after release from hyperleptinemia.

Experiment 2: Hyperleptinemia and concurrent high fat diet feeding in adult mice

As in experiment 1, three groups of mice were studied in the 2nd experiment: (1) 2TG no dox, (2) 1TG +Dox, and (3) 2TG +Dox. Experiment 2 was designed similarly to experiment 1; mice were group-housed (3 per cage) with *ad libitum* access to chow and dox exposure started when mice were 9 weeks old. The concentration of dox in 5% sucrose water was increased every 2 weeks in the following order: 10, 15, 17.5, 20, 22.5, 25, 27.5, 30, 35, 45 ug/ml. The modification to this protocol was that after the initial 7 weeks of dox exposure mice were switched from regular chow diet to 60% HFD. Plasma Leptin, glucose, body weight and composition, food and water intake were measured at the same time points in the same way as experiment 1. Mice were released from hyperleptinemia after 20 weeks of dox exposure; at the same time they were switched back to regular chow diet. Post release, body weight, composition and food intake (on a per cage basis, 3 mice per cage) were monitored weekly. Mice were sacrificed 12 weeks after release from hyperleptinemia.

Experiment 3: Hyperleptinemia in adolescent mice

1TG (controls, n=18) and 2TG (dox-inducible leptin overexpressors, n=18) littermates were weaned on postnatal day 22 (P22). At weaning pups were separated by genotype and group-housed (3 mice per cage) with *ad libitum* access to chow (Purina LabDiet 5058). At the same time mice began a 5-week exposure to 50 ug/ml of dox in drinking water. Based on the first two experiments described here, addition of dox did not affect water intake in mice, therefore water was not supplemented with sucrose. Blood was collected at P15 and at weaning (P22) for baseline plasma leptin measurement and every 2 weeks during dox exposure (week 4, 6 and 8 of age). At 8 weeks of age, mice were released from dox exposure and continued *ad libitum* access to chow. At 14 weeks (6 weeks post release from dox) both groups, 1TG and 2TG, were switched to *ad libitum* 60% HFD. Body weight and food intake were recorded twice per week. Body composition (by EchoMRI) was measured biweekly throughout the experiment. Leptin was measured every 2 weeks during dox exposure and every 4 weeks during the post-dox period.

Experiment 4: Hyperleptinemia in postnatal mice

1TG females homozygous for the tetracycline responsive elements (TRE)-Lep gene (TRE-Lep/TRE-Lep; Chapter 2) but non-carriers for the R26-rtTA allele were crossed to 1TG males that were heterozygous for R26-rtTA (rtTA/+) but non-carriers for the TRE-Lep insert. This strategy resulted in all offspring carrying the TRE-Lep/+ gene and ~half segregating for the rtTA/+. The offspring were born with the expected 1:1 ratio of 1TG and 2TG. At parturition, mothers were exposed to 200 ug/ml of dox water and exposure continued postnatally throughout the nursing period. They were given *ad libitum* access to chow. While the pups were exclusively breastfeeding (P15), and at weaning (P22), pups were bled to measure plasma leptin. At weaning, mice were separated by genotype into home cages (group housed, 2-3 mice per cage)

with *ad libitum* access to chow and dox-free water; mice were maintained under these conditions until 10 weeks of age. At 10 weeks, mice were switched to *ad libitum* access to 60% HFD and maintained on this diet until the end of the study.

Plasma collection and assays

Blood was obtained by submandibular bleed at 9am from mice in a fed state. Plasma was collected on ice using heparinized tubes (Fisherbrand) and was isolated by centrifugation for 20 min at 2,000 x g at 4°C and aliquoted and frozen at −80°C until time of assay. Plasma leptin concentration was measured using mouse leptin ELISA (R&D).

Results

We have generated a dox-inducible leptin overexpressing transgenic mouse (2TG) (Chapter 2) which produces leptin in proportion to dox concentration and can be rapidly turned on (within 8h) or off (within 24h) with no lasting effects on plasma leptin concentration. These mice were used throughout this manuscript.

Experiment 1: Hyperleptinemia in adult mice

To investigate whether chronic hyperleptinemia affects body weight set point, we exposed adult 2TG mice fed *ad libitum* chow to increasing concentrations of dox in drinking water supplemented with 5% sucrose (2TG +Dox group). Two control groups were used: 2TG mice exposed to 5% sucrose water without any dox (2TG no dox) and 1TG mice exposed to dox in 5% sucrose water (1TG +dox). Dox exposure started when mice were sexually mature at 9 weeks old and was continued for 20 weeks. The initial dox dose was very low and was escalated on a bi-weekly basis to mimic the leptin concentration profile seen in a mouse that is exposed to HFD

[43]. Circulating leptin concentration in 2TG +dox group was significantly higher than both control groups starting at 7.5 ug/ml and 12.5 ug/ml dox dose, respectively, and remained significantly elevated for 20 weeks until the end of dox exposure (Figure 3.1A).

There were no significant differences in body weight among the groups at the start of dox exposure or throughout the 20 weeks of hyperleptinemia (Figure 3.1B). Body weights at the highest dose of dox (45 ug/ml) were similar in all groups (Figure 3.1B). No difference was seen in body composition, fat mass, or lean mass throughout the duration of hyperleptinemia (Figure 3.1C, D). Food and water intake were also not different in any of the groups throughout dox exposure (Figure 3.1E, F). Plasma glucose concentrations did not differ between any of the groups (Figure 3.1G).

Following a 20-week period of gradual elevation of circulating leptin concentrations, dox was removed from the drinking water; the leptin concentrations of 2TG +dox mice declined to levels appropriately proportional to body fat. Leptin was measured 5 weeks post dox release and declined in 2TG +dox mice to control concentrations (Figure 3.1A). Body weight, composition and food intake for 5 weeks after the cessation of hyperleptinemia were the same in all three groups (Figure 3.2A-D). Mice were then challenged with 60% HFD to determine if formerly hyperleptinemic mice were more sensitive to highly palatable food. Mice were monitored for additional 11 weeks but did not show any differences in food intake body weight, or body composition (Figure 3.2A-D).

Experiment 2: Hyperleptinemia and concurrent high fat diet feeding in adult mice

In the second experiment we investigated whether hyperleptinemia combined with HFD feeding has distinct consequences for resetting defended body weight/fat. We used the same groups of mice as experiment 1: 2TG +dox, 2TG no dox, and 1TG +dox. Mice were exposed to the first

dose of dox at 9 weeks of age. In this cohort, 2TG +dox mice significantly elevated circulating leptin after the first dose of dox and it remained significantly higher than both controls throughout the 20 week dox exposure period.

In this cohort, mice significantly elevated circulating leptin concentrations more rapidly than in the first experiment. As a consequence, the 2TG +dox mice initially lost fat mass and lean mass. Fat mass for the 2TG mice +dox group was significantly lower than both control groups for 3 weeks (week 11-13). Body weight and lean mass for the 2TG mice +dox mice were significantly lower than the 1TG +dox control group only. Body weight in the 2TG mice +dox group remained slightly lower from week 11 to 15. Food intake was slightly but significantly suppressed in 2TG +dox at week 10, a likely result of anorexigenic effects of leptin.

The 2TG +dox mice caught up in body weight and fat with the controls at 16 weeks of age. At this point, mice were switched from regular chow to 60% HFD. All three groups responded similarly to the HFD, displaying a similar level of hyperphagia upon exposure to HFD; each group initially increased caloric intake by ~50%. Hyperphagia subsided over a few weeks but HFD caloric intake remained elevated compared to caloric intake when fed regular chow.

Following a chronic, gradual, elevation of circulating leptin and concurrent feeding with 60% HFD, dox was removed from the drinking water and, at the same time, mice were switched from HFD back to regular chow. Body weight was monitored for 11 weeks after the cessation of dox-induced hyperleptinemia. All mice lost weight after they were switched from HFD to regular chow diet and there was no effect of chronic hyperleptinemia on body weight after dox was removed (Figure 3.4A). Fat and lean mass were measured weekly for 4 weeks after dox cessation; lean mass remained unchanged, but fat mass was decreased in all mice due to the diet

switch (Figure 3.4B, C). No difference in caloric intake was detected among any of the groups (Figure 3.4D).

Experiment 3: Hyperleptinemia during adolescence (P22-P56)

We investigated the effects on body weight of hyperleptinemia in "adolescent" mice. Immediately after weaning, mice were separated by genotype and placed in home cages (3 mice per cage) with *ad libitum* access to regular chow. At the same time, 1TG and 2TG mice, began a 5-week exposure to 50 ug/ml of dox in drinking water. In response to dox, 2TG mice increased circulating leptin concentrations by ~25 fold compared to 1TG controls on dox. Leptin remained significantly elevated throughout the duration of the exposure (Figure 3.5 A). Food intake was significantly reduced in 2TG mice compared to 1TG controls for 2 weeks after the switch to dox water and remained slightly, but not significantly, suppressed throughout the 5-week period of hyperleptinemia (Figure 3.5F). Consequently, body weight of 2TG mice was significantly lower than 1TG from 3 to 8 weeks of age (Figure 3.5B). Fat mass and lean mass were also reduced during dox exposure (Figure 3.5D, E).

At 8 weeks of age, mice were released from dox-induced hyperleptinemia; leptin concentrations measured 1 week after dox removal, returned to fat mass proportional levels in 2TG (Figure 3.1A). In response to the sharp drop in circulating leptin, 2TG mice increased food intake significantly for 1 week until their body weights increased to the level of 1TG controls (Figure 3.5F). Similarly, fat mass and lean mass were restored to the levels of 1TG mice within 1 week (Figure 3.5D, E). Once 2TG mice caught up with the controls, body weight, body composition and food intake were not different from 1TG controls for the following 6 weeks while mice were maintained on chow.

Both groups, 1TG and 2TG, were switched from chow to 60% HFD at 14 weeks (6 weeks post dox exposure). During the first 2 weeks, the hyperphagia induced by HFD feeding was significantly greater in 2TG compared to 1TG mice and their caloric intake remained slightly, but not significantly, elevated for the following two weeks. After 4 weeks of HFD feeding, caloric intake was identical in both groups and remained the same until the end of the study. No statistically significant difference was detected in body weight between 1TG and 2TG mice throughout the study but, as expected from their increased caloric intake, 2TG mice gained significantly more weight than the 1TG controls in the first 3 weeks of HFD feeding (Figure 3.5C).

Experiment 4: Hyperleptinemia during postnatal period (P0-P22)

We evaluated effects of dox-induced hyperleptinemia in postnatal mice on body weight later in life. 1TG dams were given dox in drinking water immediately following parturition until weaning of their progeny at P22. Leptin is present in breast milk in small quantities (about 5 fold lower concentration than in circulation of a nursing dam and is absorbed by the gastrointestinal tract of suckling pups [44, 45]. We previously showed that 1TG mothers do not elevate their circulating leptin when exposed to dox, but nursing 2TG pups have significantly higher circulating leptin concentrations than 1TG littermates, indicating that dox crosses to the pups through mother's milk and induces expression of transgenic leptin in 2TG pups (Thesis Chapter 2).

Plasma leptin concentration in 2TG pups at P15 and P22 was elevated versus 1TG littermates (Figure 3.6A) and was higher on P22 than on P15 possibly as a result of mice ingesting dox through drinking water directly at P22 as opposed to dox transfer exclusively through mother's milk at P15.

Fat mass was lower in 2TG compared to 1TG controls at 3 weeks of age; at this age mice have little body fat but 2TG animals had ~50% the amount of fat of 1TG mice (Figure 3.6E). Lean mass was the same in both groups (Figure 3.6F). The small difference in fat mass was not detected in body weight at 3 week of age (Figure 3.6C). At P22, pups were caged by genotype (3 mice per cage) with *ad libitum* access to regular chow and dox-free water. One week after release from hyperleptinemia, circulating leptin concentrations were not different in 2TG mice compared to 1TG (Figure 3.6A). Fat mass also recovered in 2TG mice and at 4 weeks old was not different than 1TG littermates (Figure 3.6E).

Until 10 weeks of age (7 weeks after release from dox exposure) mice were fed *ad libitum* chow and no group differences in body weight, composition and food intake were seen. Mice were then switched to 60% HFD. Both groups of mice increased food intake by ~50% in the first 3 days. No significant difference in caloric intake was detected between the two groups (Figure 3.6G); however, the relative hyperphagia in 2TG mice (defined as caloric intake after switch to HFD divided by caloric intake on the last week of regular chow before the switch) was significantly higher in the first 3 days and the following 4 days (Figure 3.6H). Within the first 3 days of HFD feeding, 2TG mice gained more weight than controls (2TG: 2.6 ±0.2g, 1TG: 1.6 ±0.2g, $p<0.01$) and continued to gain body weight at a velocity greater than 1TG; at the end of the study (mice were 38 weeks old) the difference in body weight gain was 5.7g (Figure 3.6D). Absolute body weight difference reached significance 6 weeks after the start of HFD feeding (Figure 3.6C). 2TG animals remained significantly heavier than 1TG until the end of the study at 38 weeks (1TG: 49.9 ±1.3 g; 2TG: 55.4 ±1.5 g; p-value<0.05) (Figure 3.6C). Lean mass did not statistically differ between 1TG and 2TG mice throughout the study (Figure 3.6F). Fat mass was significantly higher in 2TG mice 2 weeks after diet switch and remained relatively higher until

the end of the study (Figure 3.6E). Circulating leptin was measured 4 and 18 weeks after the start of HFD exposure and was significantly higher in 2TG mice after 18 weeks of HFD feeding (1TG: 122.9 ±8.0 ng/ml, 2TG: 172.6 ±9.5 ng/ml; $p<.001$). After 18 week HFD exposure, 2TG mice (dox-induced hyperleptinemic postnatally from 0-3 weeks of age) have a higher fat mass than 1TG mice, therefore they secrete more leptin, but leptin secretion is still in proportion to fat mass (Figure 3.6B).

Discussion

With large increases in obesity prevalence, maternal obesity has become more common and potentially could have a long lasting impact on future generations. Genetic predispositions are important contributors to the risk of obesity. Heritability studies suggest that ~40-70% of the variation in adiposity among individuals reflects genetic factors [45, 46]. However, genetics cannot explain the rapid increase in obesity prevalence within the past 4 decades. It is likely that interactions between our genes and energy-dense, palatable food work in synergy to increase individual risk of obesity. Rodent models that evaluate maternal obesity and HFD feeding during gestation and/or lactation provide insight into the physiological effects on offspring but are not well suited to investigate the mechanisms that mediate these effects. One of the signals associated with increased obesity and HFD feeding is increased circulating leptin – which may contribute *per se* to the phenotypes observed in the offspring. Animal models of exogenous leptin administration are confounded because postnatal interventions are highly disruptive to the environment of the pups (daily handling for injections, maternal stress, etc.). The transgenic model of dox-controlled leptin overexposure we utilized circumvents these problems and

provides close control over neonatal leptin exposure without disturbing the neonatal environment.

We evaluated the effects of hyperleptinemia on body weight and subsequent responses to highly palatable food at 3 distinct developmental periods. We found that (1) inducing chronic hyperleptinemia in adult mice, with or without concurrent HFD exposure, does not increase the set point of defended body weight when excess leptin is removed; (2) hyperleptinemia during "adolescence" transiently increases the hyperphagic response to a HFD; but ultimately does not alter body weight thereafter; and (3) transient elevation of circulating leptin in the immediate postnatal period increases the hyperphagic response to a highly palatable diet and renders animals more susceptible to obesity as adults. These results emphasize the importance of the timing of exposure to hyperleptinemia on the phenotypes of progeny. We identify the immediate postnatal period as a critical time window during which exposure to elevated leptin *per se* increases the body weight in adult offspring under conditions of *ad libitum* access to highly palatable food.

Knight *et al.* attempted to distinguish the effects of HFD from the effects of hyperleptinemia as a result of HFD feeding in the development of apparent hypothalamic leptin resistance [47]. Exogenous leptin was chronically infused with subcutaneous Alzet mini-pumps into leptin-deficient ($Lep^{ob/ob}$) mice to generate plasma concentrations comparable to those of a wild type (WT) mouse fed regular chow. These mice were then switched from low to high fat diet but the leptin infusion rate was held constant [47]. Over the ensuing 20 weeks of HFD feeding, WT and leptin-infused $Lep^{ob/ob}$ mice gained weight at the same rate, despite circulating leptin concentrations in the HFD fed WT mice continuously increasing [47]. At the same level of adiposity as the WT mice (but lower leptin concentrations), leptin-infused $Lep^{ob/ob}$ displayed

greater leptin sensitivity – assessed by leptin induced hypothalamic pSTAT3 – compared to HFD fed WT mice, indicating that hyperleptinemia in HFD-fed mice is necessary to reduce hypothalamic cellular responses to leptin [47].

The current study demonstrates that extended elevation of plasma leptin in adult mice – by itself or in combination with HFD feeding – does not alter body weight set point. Upon initial dox-induced leptin elevation in Experiment 2, 2TG mice transiently decreased food intake and body weight, but, within 4-6 weeks, food intake and body weight in these mice were not different from control mice (1TG +Dox and 2TG no dox). The response to increased circulating leptin that was only seen in the 2nd cohort of adult mice exposed to hyperleptinemia is possibly due to (1) initial leptin induction in experiment 2 was much higher than seen in experiment 1; it is likely that with a more gradual elevation of leptin, mice accommodate to higher leptin without physiological response; and (2) mice in experiment 1 were slightly fatter than mice in experiment 2 at the start of dox exposure; fatter mice are less sensitive to leptin [15].

Experiment 1 confirms previous chronic hyperleptinemia experiments in adult WT mice which showed no changes in body weight after mouse release from elevated leptin [28]. In that study, exogenous leptin or saline was chronically infused via Alzet mini-pumps in adult WT mice for 18 weeks. Following cessation of leptin administration, body weight, composition and food intake were monitored [28]. Chronic elevation of circulating leptin concentrations, *per se*, did not lead to metabolic or behavioral "defense" of a higher body weight [28]. Some limitations of this study included: (1) the amount of exogenous leptin delivered via a mini-pump is limited due to low leptin solubility at high concentrations, (2) a large variation in delivery efficiency of exogenous leptin in individual mice, and (3) weight loss caused by repeated surgeries to replace mini-pumps. All of these issues are avoided with the transgenic mouse model used in this study.

Experiment 3 demonstrated that inducing hyperleptinemia during the "adolescent period" in mice does not affect body weight set point after leptin concentration is normalized. As reported in experiment 2, dox exposure in 2TG mice at 5 weeks of age triggered an anorectic response. Mice decreased food intake compared to 1TG controls and consequently lost both fat and lean mass. Circulating leptin during dox exposure was significantly higher in 2TG than in 1TG group, the 2TG leptin concentrations fell within the typical physiological range induced with HFD feeding. When challenged with HFD at 14 weeks, 2TG mice transiently increased food intake and gained more weight than 1TG controls within the first 4 weeks of HFD exposure but, ultimately, no difference in long term body weight was detected between the 1TG and 2TG groups. Increased hyperphagia in response to highly palatable food in the 2TG mice suggests that exposure to hyperleptinemia during adolescence may have altered leptin responsive neural circuitry.

In mice, a physiologic leptin surge (5-10 fold increase in circulating leptin concentrations) occurs between P8-P12 independent of pup fat mass and food intake [48]. This surge – the mechanism of which is unclear – is a major developmental signal which affects the outgrowth of neuronal projections from the ARH to PVH, DMH, and LHA involved in feeding circuits [40]. Maternal HFD feeding during nursing causes the surge to start earlier with higher intensity and to last for a longer period of time [41]. Casabiell *et al*. reported that leptin is transferred from maternal circulation to breast milk and absorbed intestinally by neonatal pups [44]. In a separate experiment, orally administered leptin was detected in the circulation of suckling pups [44]. The amount of leptin administered orally was 10 ug – well above physiological levels (leptin in human milk has a concentration of 1.35 ug/L) [44]. In humans, some studies have detected a correlation between leptin concentration in maternal circulation and breast milk, however, leptin

concentrations in breast milk are significantly lower than the concentrations reported in either maternal or infant circulation [49-52]. Therefore, even if ingested leptin enters the offspring's circulation, it is unlikely that the direct contribution to circulating infant leptin is physiologically significant. While the concentration of leptin in the breast milk of obese post-partum women is significantly higher than in lean women [53], rodent data suggest that elevated leptin in postnatal pups born to DIO dams is due, at least partially, to increased leptin production from the adipose tissue of the pups [41]. This finding suggests that the HFD and/or maternal obesity *per se* conveys leptin-trophic molecules. Other physiological and molecular changes are induced in dams as a consequence of obesity and/or HFD feeding such as increased circulating free fatty acids, elevated glucose, decreased insulin sensitivity, increased circulating insulin, fatty liver, etc. The transgenic mouse model used in this study isolates hyperleptinemia *per se* in the pup from other confounds of HFD feeding and/or maternal obesity. Another advantage of this model is that by breeding 1TG dams homozygous for the leptin transgene but no rtTA to 1TG males that are heterozygous for the rtTA gene, the resulting progeny have a ~1:1 ratio of 1TG to 2TG where all mice have one copy of the leptin transgene and ~ half also have one copy of the rtTA gene. This produces ideal littermate controls. When the 1TG dam is exposed to dox in drinking water immediately post parturition – the mother herself does not elevate leptin – dox is transferred to the offspring in breast milk, therefore 2TG pups increase leptin expression, while 1TG littermates do not.

In experiment 4 we show that isolated hyperleptinemia during the postnatal period predisposes mice to be more hyperphagic when exposed to highly palatable food later in life resulting in increased weight gain when given access to HFD. At 3 weeks of age, while 2TG mice are still producing excess leptin, 2TG mice had half the amount of somatic fat of the 1TG mice.

Exogenous leptin in mice does not suppress food intake during the first 3 weeks of life [54, 55], but is an important neurotrophic factor affecting the development of ARH projections to downstream hypothalamic targets [40]. Although no difference in body weight was detected at 3 weeks, decreased fat mass in hyperleptinemic 2TG compared to 1TG mice may be a confound with regard to leptin-specific effects in the CNS, since postnatal undernutrition in rodents causes growth retardation and decreases body weight throughout life even when animals are subsequently exposed to high fat diet [56, 57]. 2TG mice in this study had the same body weights as 1TG controls while maintained on chow diet, hence it is unlikely that they had experienced postnatal undernutrition. It is possible that, at P22, leptin is just beginning to acquire its function as an anorectic signal [54, 55] and that some of these effects are apparent as decreased fat mass at the time of weaning. One week after release from hyperleptinemia, 1TG and 2TG mice were indistinguishable in body weight and composition; they remained identical in this regard until 10 weeks of age when they were switched to 60% HFD. Immediately following HFD exposure, 2TG mice gained significantly more weight than 1TG littermates. The hyperphagia (caloric intake relative to food intake on chow) in 2TG mice was 17% greater compared to the 1TG mice during the first week. By the end of the study, 2TG mice had increased body weight (11%) and fat mass (19%) relative to the 1TG mice. The fat difference is approximately 4 grams per mouse after 14 weeks on HFD when most of the fat difference between 1TG and 2TG groups has been accrued, and it represents a delta of only 38 kcals out of approximately 1300 kcals ingested over this same period or an imbalance of 3%. We do not have the resolution to determine if this is from food intake or energy expenditure.

Increased fat mass in 2TG mice after 18 weeks of *ad libitum* HFD access resulted in increased circulating leptin but there was no effect on leptin secreted per unit of fat mass. There is no

indication that mice produce anti-leptin antibodies or that there is any impact on leptin produced per unit of fat mass after dox cessation. In an earlier study in which exogenous leptin was infused in adult mice by Alzet mini-pump, several months following cessation of exogenous leptin exposure, some mice exhibited disproportionately increased (relative to fat mass) circulating leptin concentrations [28]. This finding suggested the possibility that mice may have generated anti-leptin antibodies. In the present study, dox-induced hyperleptinemic mice returned to their fat mass-appropriate circulating concentrations of leptin at the first post-dox measurement (1-5 weeks post dox release) and leptin concentration remained proportional to fat mass at every time point following cessation of dox.

Three other groups have reported on the physiological consequences on body weight of postnatal exogenous leptin supplementation in rats; these studies varied in the timing and route of leptin administration and reported conflicting effects on body weight depending on the route of leptin administration. Pico *et al.* administered leptin orally to rats from P0 to P20. At weaning, rats were switched to HFD; at 6 months the leptin fed rats had lower body weight than vehicle-treated control rats [58]. Plasma leptin concentrations in leptin-supplemented pups were not reported, therefore, it is unclear whether oral leptin elevated circulating leptin. Maternal HFD feeding increases leptin expression in neonatal adipose tissue [41] and this effect was possibly absent with the oral leptin administration. Another group administered leptin to neonatal rats via IP injections either during the first or last 10 days of nursing and showed that, by 16 weeks of age, leptin injected rats had higher body weight than controls while maintained on normal chow [59]. Vickers *et al.* injected suckling rats with IP leptin from P3 to P13; leptin supplemented rats gained more weight than controls after they were switched to high fat diet [60]. The latter two studies are consistent with results reported in this manuscript.

By what mechanisms might elevated circulating leptin during the postnatal period affect developmental programming of body weight? Simerly and colleagues demonstrated leptin's neurotrophic role in the development of feeding circuitry [40]. In *Lep*$^{ob/ob}$ mice, ARH projections to other hypothalamic regions are greatly reduced compared to the WT mouse, and the formation of these pathways is temporally delayed (i.e. in WT mice ARH innervate the PVH on P12, whereas in *Lep*$^{ob/ob}$ littermates no axons from the ARH are detected at this time) [40]. Postnatal (P4-P12) administration of leptin to *Lep*$^{ob/ob}$ mice restores the density of the innervation from the ARH to the PVH to the levels of a WT control. However, administering leptin to adult *Lep*$^{ob/ob}$ mice does not rescue the density of AgRP and α-MSH immunoreactive innervation in the PVH to those seen in WT mice, indicating that there is a restricted neonatal time window in which ambient leptin impacts the formation of ARH connections [40]. Kirk *et al.* showed that the offspring of dams exposed to HFD during gestation and lactation gain more weight than controls, and display decreased hypothalamic leptin sensitivity (determined by leptin-induced pSTAT3 in the ARH and VMH) at P30 and P90. The density of AgRP immunoreactive fibers in the PVH was decreased in the offspring of HFD dams compared to chow fed dams [41]. The authors hypothesized that early hyperleptinemia induces leptin resistance thereby attenuating leptin signaling and impairing the development of hypothalamic projections [41]. Vogt *et al.* showed that the most critical period for the effects of maternal HFD feeding on offspring body weights is during postnatal period (birth to weaning) and that the fiber density of AgRP and α-MSH from ARH to three downstream hypothalamic areas: PVH, DMH and LHA is reduced [27]; these alterations in hypothalamic circuits are similar to those reported in *Lep*$^{ob/ob}$ mice [40]. We found the pre-weaning period to be critical for the effects of hyperleptinemia on body weight later in life. It is plausible that the neurobiological changes seen in the offspring of HFD-fed dams result,

at least in part, from hyperleptinemia associated with the obese state. Thus, investigating the density of AgRP and α-MSH fibers from the ARH to the PVH, DMH and LHA in our transient hyperleptinemic mouse model is critical to decipher whether these circuits are affected by elevated leptin *per se*.

Metabolic signals other than leptin (in particular insulin and ghrelin) have also been suggested to act as a neurotrophic factor during pre-weaning. Insulin injected directly into the hypothalamus of P8 rats causes a decrease in neuronal density of the VMH in pups at P15 and results in elevated body weight gain in adulthood [61]. Carmody *et al.* demonstrated that HFD feeding, in addition to maternal insulin resistance (dams were heterozygous for a null allele of the insulin receptor, $Insr^{+/-}$), increases the number of POMC-expressing cells by 20% in the ARH of P9 offspring [39]. This suggests that insulin can influence the specification of POMC neurons in response to maternal HFD feeding. Vogt *et al.* showed that maternal HFD-induced hyperinsulinemia disrupts the projections from the ARH POMC neurons to the preautonomic compartment of the PVH [27]. Deletion of the insulin receptor specifically in POMC neurons in the offspring, under conditions of maternal HFD feeding, restores normal development of POMC projections in this circuit and corrects glucose intolerance [27], implicating insulin in the development of hypothalamic circuits involved in glucose homeostasis.

Ghrelin's role has also been studied during mouse postnatal development. Ghrelin is an appetite stimulating circulating hormone, synthesized primarily by the stomach, which signals through the growth hormone secretagogue receptor (GHSR) [62, 63]. GHSR is expressed in the hypothalamus and administration of ghrelin activates neurons in the ARH, VMH, and PVH – nuclei known to control feeding [64]. Steculorum *et al*. investigated physiological and neurobiological effects of excess and deficiency of ghrelin during pre-weaning period [65].

Blocking ghrelin signaling from P4 to P22 results in increased density of AgRP and α-MSH immunoreactive fibers in the PVH; whereas supplementing exogenous ghrelin from P4 to P12 has opposite effects – innervation in the PVH is reduced [65]. Surprisingly, both interventions result in increased body weight, fat, and blood glucose. While the overall density of AgRP and αMSH fibers in the PVH are affected in the opposite way by ghrelin and anti-ghrelin treatment, the ratio of orexigenic AgRP to anorexigenic α-MSH is increased in both conditions which could explain the increase in body weight due to both manipulations [66]. These data emphasize that appropriate timing and magnitude of ghrelin's action is important in proper formation of hypothalamic neurocircuits and in control of body weight in adulthood [65].

Other potential mechanisms linking hyperleptinemia during the postnatal period to developmental programming include epigenetic modifications, hypothalamic inflammation and neuroanatomic changes in the hedonic or reward circuits. Over-nutrition of weanling rats (by decreasing litter size) during the perinatal period results in hypermethylation of the hypothalamic POMC promoter [67] which is negatively correlated with hypothalamic POMC expression adjusted for circulating leptin. When mice are exposed to highly palatable food they massively overeat in the first 1-2 weeks. This response is mediated, at least in part, by hedonic, reward-based mechanisms that can override homeostatic regulation during abundant food availability, and drive the increased desire to consume highly palatable foods in the absence of hunger [68]. One of the most critical brain reward pathways is the mesolimbic dopamine (DA) system. The anatomic core of this system is located in a subset of DA neurons in the ventral tegmental area (VTA)/substantia nigra (SN) which project to the striatum (nucleus accumbens (NAc), caudate/putamen), amygdala, and prefrontal cortex [69]. Maternal HFD feeding decreases the function of the mesolimbic DA system [70]. Specifically, adult offspring born to HFD-fed

mothers have decreased DA release in NAc and locomotor responses when administered amphetamine (which acts via the DA transporter, dopamine active transporter, DAT, to promote DA efflux) [71, 72]. Ong *et al.* demonstrated that maternal high-fat, high-sugar diet from before pregnancy through weaning increases high-fat and high-sugar foods preference in the offspring. This increased preference was associated with expression changes in molecules critical in the mesolimbic reward pathway [including tyrosine hydroxylase (TH, enzyme which catalyzes the rate-limiting step in DA synthesis), dopamine receptors, DAT, and μ-opioid receptor] [73]. Other studies demonstrate that maternal HFD feeding alters food preference in the offspring toward highly palatable foods [34, 74] and is associated with DNA hypomethylation of DAT (transports DA from the synapse back to the nerve terminal, hence decreasing dopamine activity) promoter which subsequently increases expression of Dat in the mesolimbic dopamine system, specifically in the VTA, NAc and prefrontal cortex [34, 74].

Leptin receptors (LepRb) are expressed in the VTA and the SN [75] and leptin suppresses the reward value of food (controlled by the mesolimbic DA system) [76]. TH expression and DA content in the VTA and NAc is decreased in $Lep^{ob/ob}$ mice [77, 78]. Administration of amphetamine to $Lep^{ob/ob}$ mice does not induce normal locomotor activity, treatment with exogenous leptin restores $Lep^{ob/ob}$ mice responsiveness to amphetamine [77]. Perry *et al.* demonstrated that a single ip injection of leptin to wt mice does not change expression but acutely increases DAT and TH activity [79]. Infusion of leptin directly into the VTA decreases food intake and aspects of food reward such as motivation to work for a food reward [80-82]. Additionally, LepRbs are expressed in the lateral hypothalamic area (LHA) and these LHA LepRb GABAergic/inhibitory neurons were shown to innervate the VTA [78]. Direct LHA leptin injection confirms leptin action on the LHA LepRb neurons by decreasing food intake and

body weight over a period of 24 hours [78]. Morabito *et al.* reported changes in leptin sensitivity (by leptin-induced pSTAT3 immunohistochemistry) in the NAc and SN in response to weight loss by diet switch or caloric restriction [16]. Similar to hypothalamic feeding circuit development, the central reward circuitry – projections from the VTA to the NAc – in rodents mature postnatally by the 3rd week of life [83, 84]. These findings suggest that DIO during gestation and/or lactation could influence the anatomy and cellular function of elements of both homeostatic and hedonic circuits in the offspring and that leptin may be involved in this maternal programming.

Finally, studies by Schwartz and colleagues implicate a role of hypothalamic inflammation in diet- induced obesity. Thaler *et al.* demonstrated that within 1-3 days of HFD feeding, prior to substantial weight gain, mice develop hypothalamic inflammation characterized by activation of local microglia and astrocytes (also referred to as gliosis) [85]. Microglia and astrocytes are subtypes of glial cells – the most abundant cell type in the CNS involved in various processes that support the function of neurons [86]. Interventions that decrease inflammation in neurons and glia reduce food intake and weight gain associated with HFD exposure [87]. In rodents, glia cell number in the brain at birth is relatively small, and markedly increases postnatally during the second and third week of life, coinciding with the leptin surge [88]. *Lep*$^{ob/ob}$ mice become severely obese on chow diets, however, they display a decrease in activated microglia compared to WT mice on chow or on HFD [89] indicating that leptin is at least a partial mediator of gliosis. Astrocytes express LepRb [90, 91]. Administration of leptin between P8-P12 increases proliferation of astrocytes in the hypothalamus; whereas deletion of LepRb from astrocytes decreases astrogenesis [92]. Conditional deletion of LepRb in astrocytes in adult mice results in altered astrocyte morphology and increased synaptic inputs onto hypothalamic POMC and AgRP

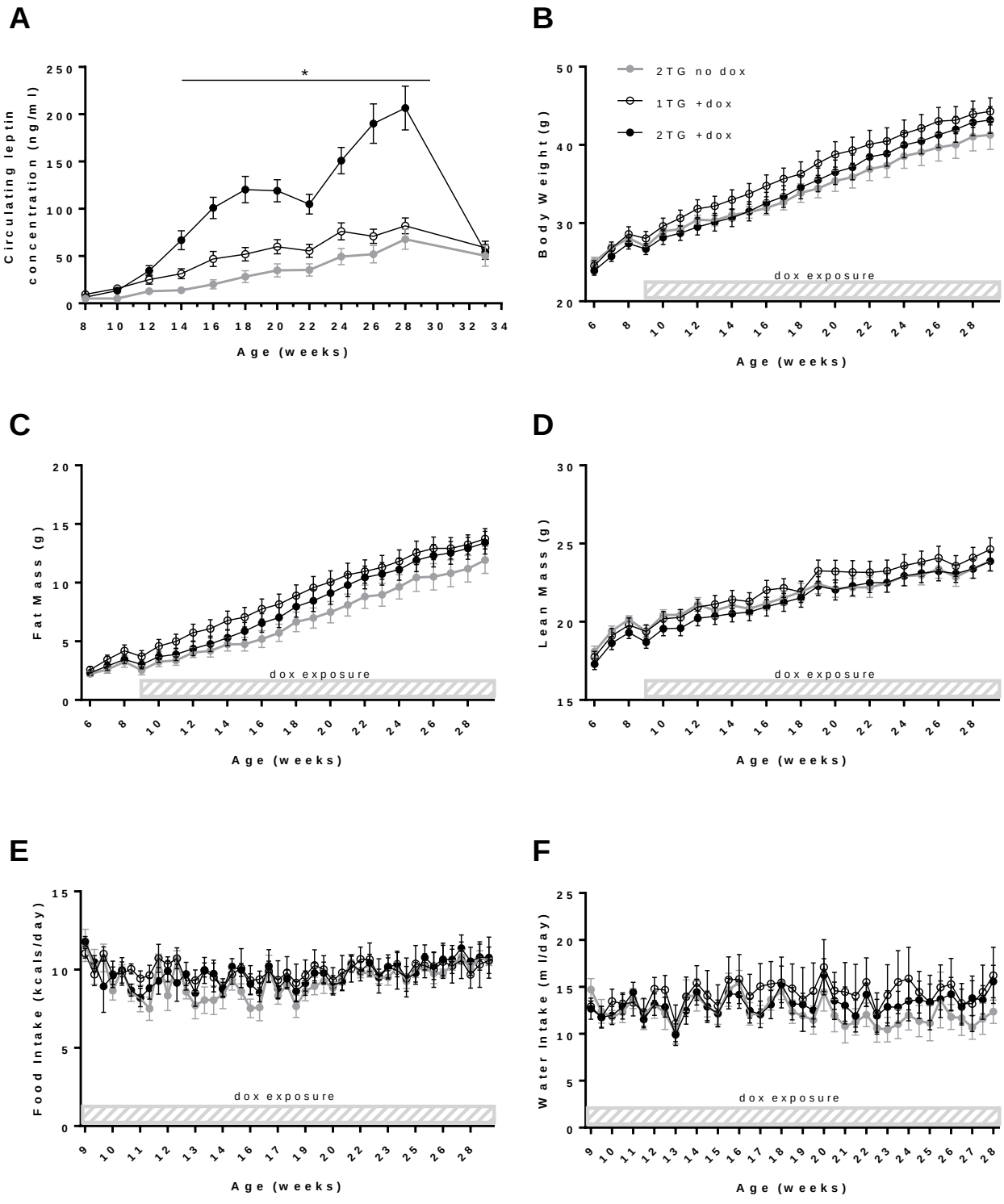
neurons [90]. Additionally, leptin-induced suppression of food intake is reduced in the astrocyte-specific LepRb adult knockout mice [90]. Martin Myers and colleagues showed that 10-days of continuous systemic leptin infusion in WT mice fed a chow diet was sufficient to induce microglial and astrocyte activation in the hypothalamus (personal communication). These data suggest that high ambient hypothalamic leptin during development may influence the susceptibility to HFD later in life via its effects on proliferation and activation of astrocytes.

Rodents experience a transient peak in plasma leptin concentrations during the 2nd week of life [48] which impacts the development and function of the feeding circuits [40]. In humans, maternal circulating leptin reaches its maximum at around the 36th week of pregnancy and decreases postpartum [93]. From a developmental standpoint, the postnatal period in mice is more analogous to the 3rd trimester of human (or primate) gestation (i.e. in rodents the hypothalamic circuitry is immature until the 3rd week of life whereas in primates it matures functionally *in utero*) [40, 94]. From this perspective, studies investigating the effects of HFD feeding and obesity in postnatal rodents may be more relevant to maternal obesity/metabolic status during human gestation.

Nutritional changes during critical developmental periods can have lasting effects on energy homeostasis. The hypothalamus is particularly sensitive to changes in the hormonal milieu (as described earlier, changes in circulating leptin [40], insulin [27, 39] and ghrelin [65] alter the architecture of key hypothalamic centers involved in energy and glucose homeostasis); these developmental mechanisms remain unclear. Many physiological and molecular changes are triggered by obesity and HFD feeding; however, the direct contributors to phenotypes observed in the offspring are largely unknown. Leptin is increased as a consequence of obesity/HFD and plays a role in determining the feeding circuitry of a developing rodent brain. In this study we

isolated and investigated the effects – in mice – of transient hyperleptinemia *per se* on the regulation of body weight during 3 distinct time periods. We identified the immediate postnatal period as a critical time window where exposure to hyperleptinemia alters the future response to highly palatable food. Even 12 weeks after leptin exposure had ended, mice that were previously exposed to elevated circulating leptin in the immediate postnatal period gained more fat and body weight than control mice when allowed *ad libitum* access to HFD. Further studies are needed to determine the mechanism(s) by which hyperleptinemia *per se* during the postnatal period mediates the developmental programming of the body weight in offspring. We hypothesize that elevated leptin during development permanently alters hypothalamic neuronal projections in the feeding circuits including AgRP and POMC innervation from the ARH to PVH, DMH and LHA. Other alterations may include epigenetic changes in genes relevant to energy balance, differences in proliferation of astrocytes and/or microglia during postnatal period which may affect hypothalamic inflammation in response to HFD feeding, and fiber density changes in the leptin responsive hedonic circuits (e.g. LHA to VTA and VTA to NAc).

Figures and Figure Legends



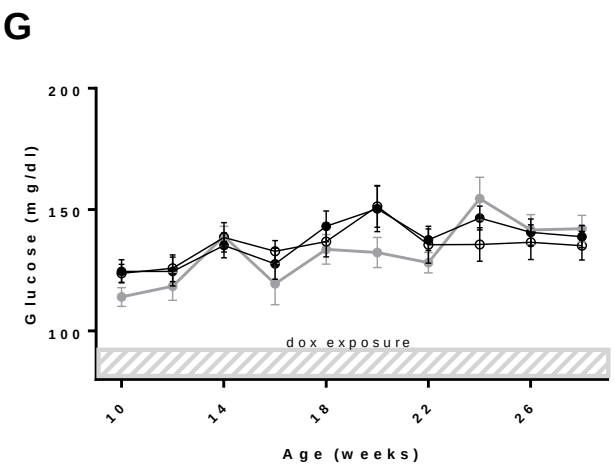


Figure 3-1 Experiment 1: Dox-induced chronic hyperleptinemia in adult mice.

(A) Circulating leptin concentrations, (B) body weight, (C) fat mass, (D) lean mass, (E) daily food intake in kcals per mouse, (F) daily water intake per mouse, and (G) glucose in 2TG controls given 5% sucrose (gray), 1TG controls given dox in 5% sucrose (black open circles), and 2TG leptin overexpressors given dox in 5% sucrose (black closed circles) during 20 weeks of escalating dose of dox exposure. All values are means ± SEM. N per group in A-D and G: 2TG no dox, n=12; 1TG +Dox, n=19; 2TG +Dox, n=24. N per group in E and F: 2TG no dox, n=4; 1TG +Dox, n=4; 2TG +Dox, n=6. No significant differences between any of the groups were detected with a two-way ANOVA in any of the parameters except for circulating leptin. *P < 0.05 between 2TG +dox and each control group by Student's t-test.

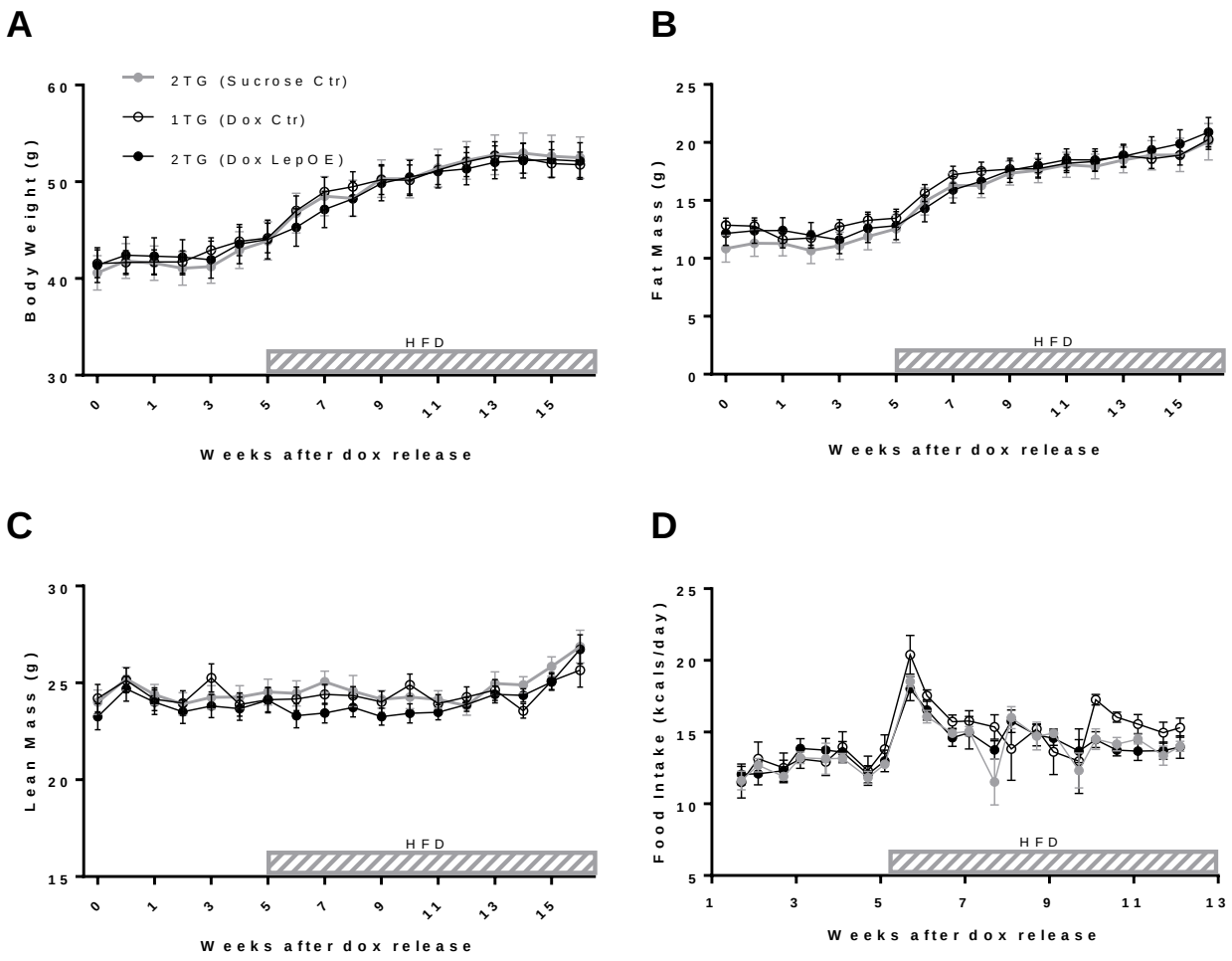
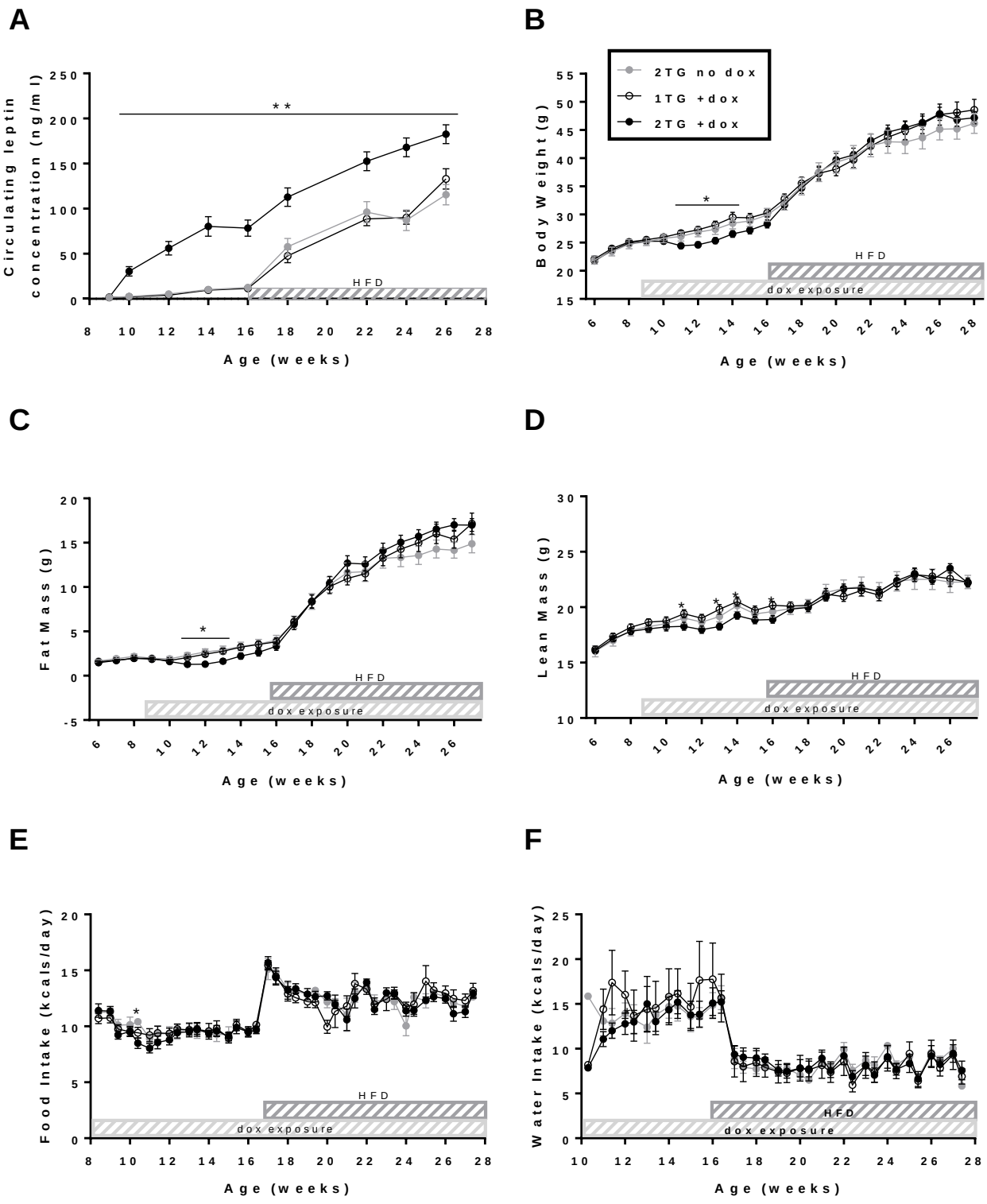


Figure 3-2 Experiment 1: Body weight, body composition and food intake after release from dox.

(A) Body weight, (B) fat mass, (C) lean mass, (D) daily caloric food intake per mouse after mice were released from hyperleptinemia. Five weeks after dox cessation mice were switch from chow to 60% HFD. All values are means ± SEM. N per group in A-C: 2TG no dox, n=9; 1TG +Dox, n=14; 2TG +Dox, n=20. No significant differences between any of the groups were detected with a two-way ANOVA in any of the parameters.


A
Circulating leptin concentration (ng/ml)
**
HFD
Age (weeks)
B
2TG no dox
1TG +dox
2TG +dox
Body Weight (g)
*
HFD
dox exposure
Age (weeks)
C
Fat Mass (g)
*
HFD
dox exposure
Age (weeks)
D
Lean Mass (g)
HFD
dox exposure
Age (weeks)
E
Food Intake (kcals/day)
*
HFD
dox exposure
Age (weeks)
F
Water Intake (kcals/day)
HFD
dox exposure
Age (weeks)

G

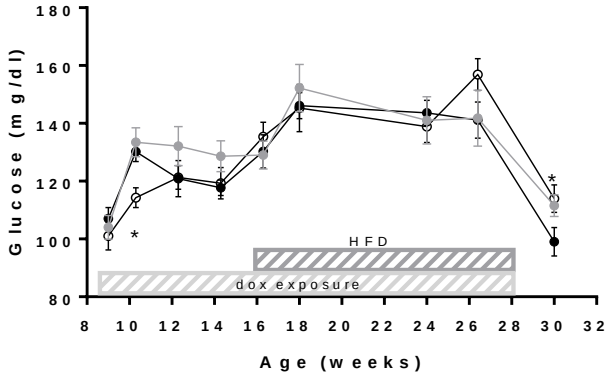


Figure 3-3 Experiment 2: Dox-induced chronic hyperleptinemia with concurrent HFD feeding in adult mice.

(A) Circulating leptin concentrations, (B) body weight, (C) fat mass, (D) lean mass, (E) daily food intake in kcals per mouse, (F) daily water intake per mouse, and (G) glucose in 2TG controls given 5% sucrose (gray), 1TG controls given dox in 5% sucrose (black open circles), and 2TG leptin overexpressors given dox in 5% sucrose (black closed circles) during 20 weeks of escalating dose of dox exposure. All values are means ± SEM. N per group in A-D and G: 2TG no dox, n=9; 1TG +Dox, n=12; 2TG +Dox, n=18. N per group in E and F: 2TG no dox, n=3; 1TG +Dox, n=4; 2TG +Dox, n=6. Significant difference calculated with Student t-test, *$p < 0.05$ between 2TG +dox and at least one of the control groups.

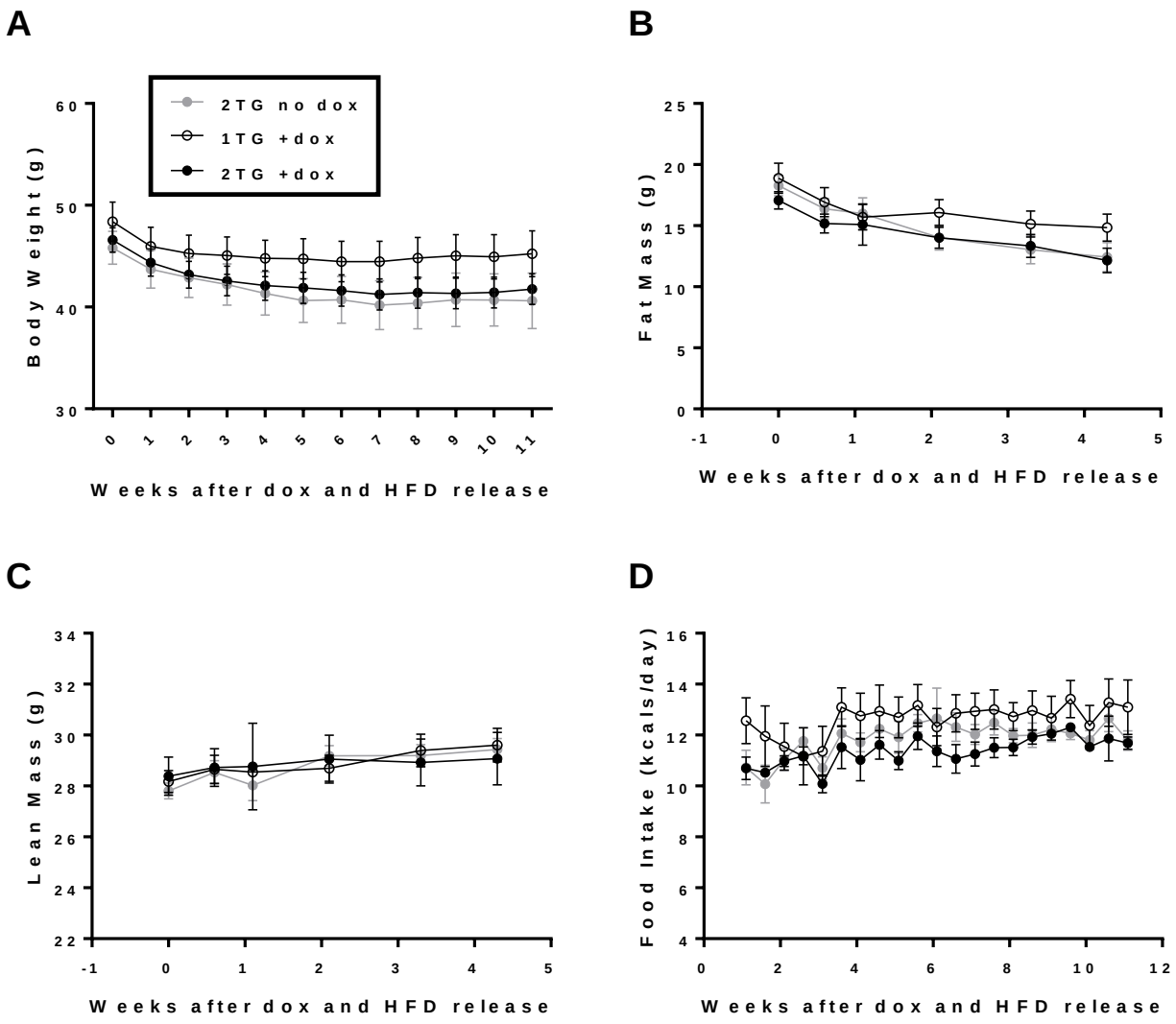


Figure 3-4 Experiment 2: Body weight, body composition and food intake after release from dox.

(A) Body weight, (B) fat mass, (C) lean mass, and (D) daily caloric food intake per mouse after mice were released from hyperleptinemia and HFD feeding (switched back to regular chow). All values are means ± SEM. N per group in A-C: 2TG no dox, n=9; 1TG +Dox, n=12; 2TG +Dox, n=18. No significant differences between any of the groups were detected with a two-way ANOVA in any of the parameters.

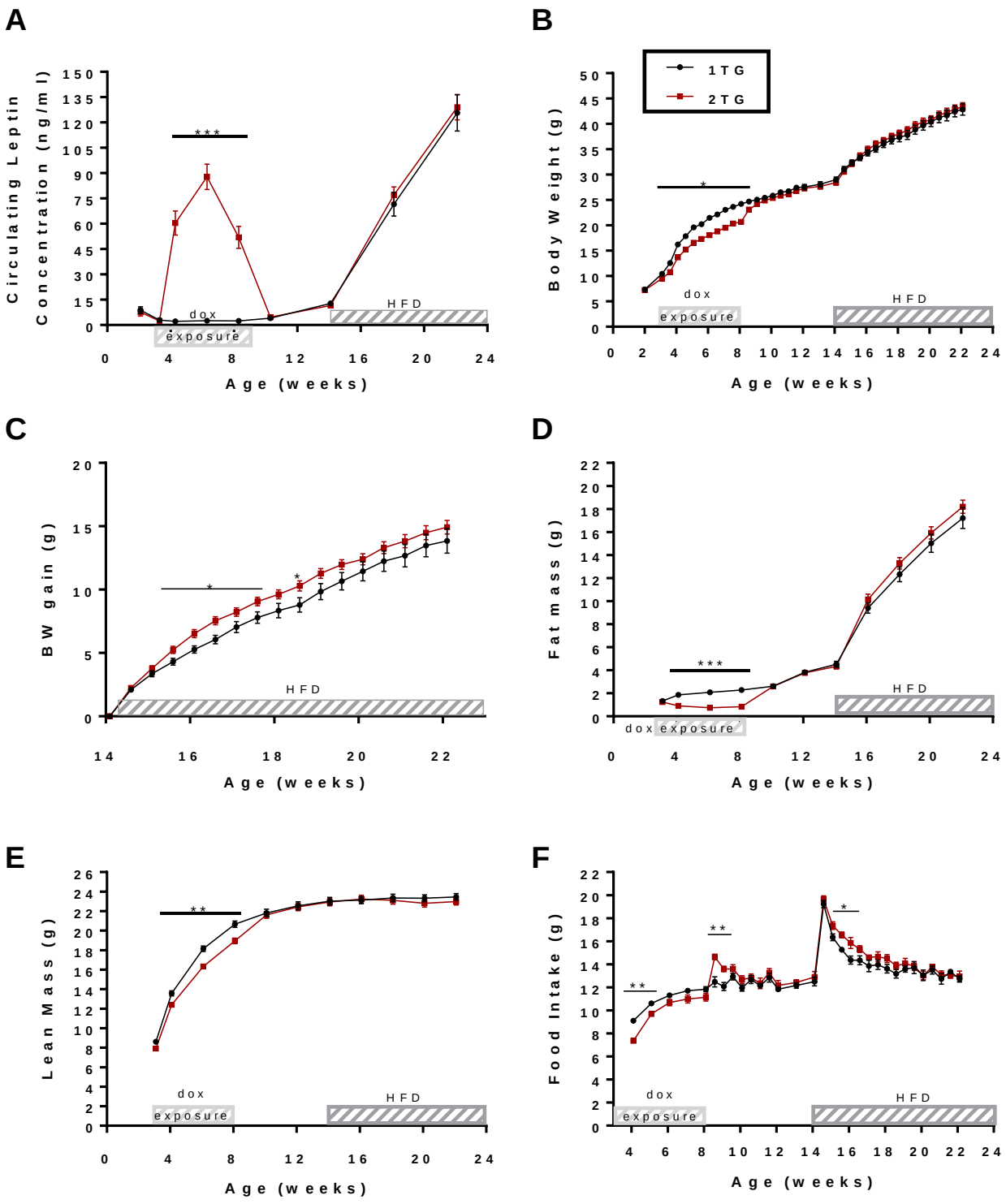
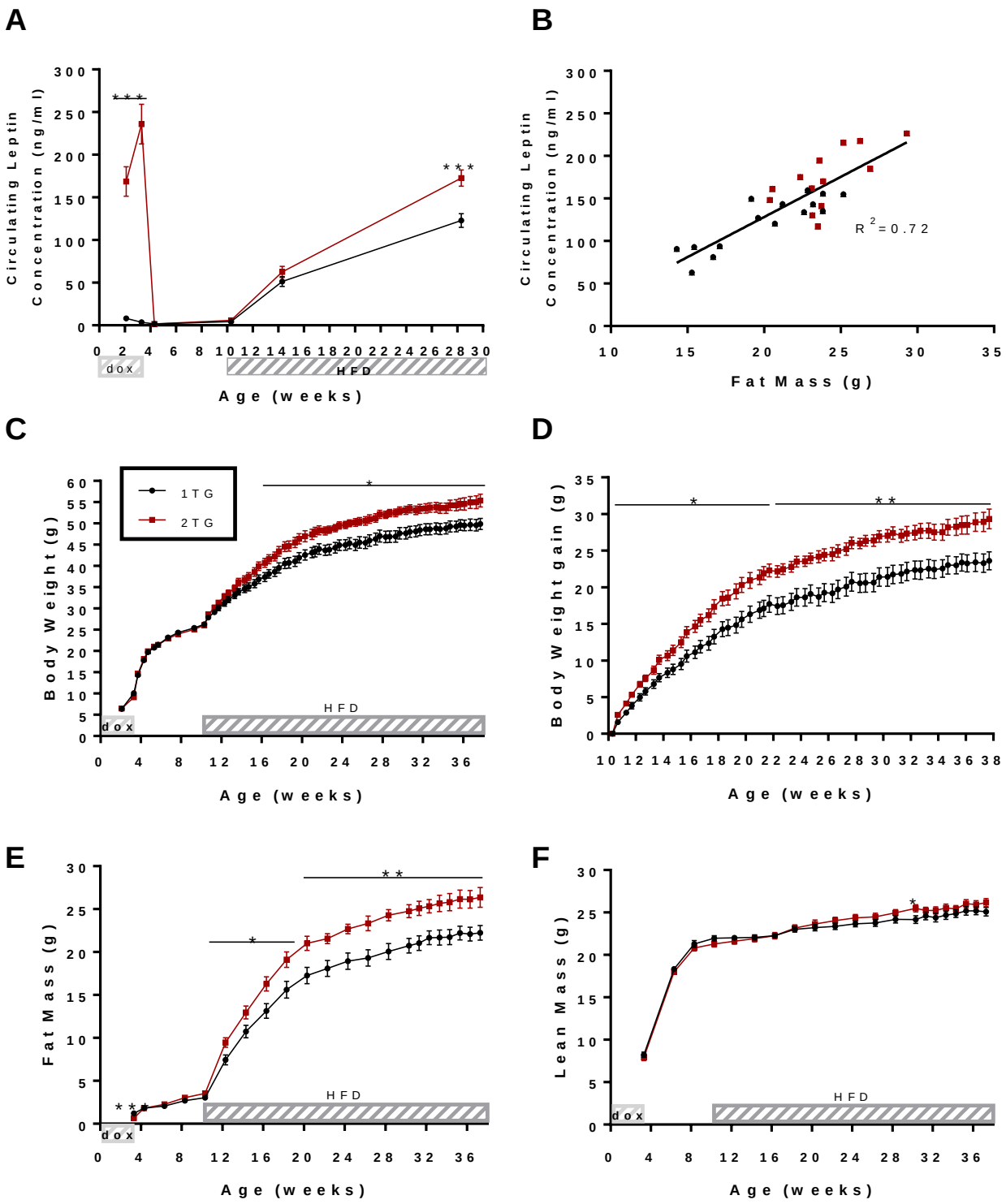

A
Circulating Leptin Concentration (ng/ml)
***
dox
exposure
HFD
Age (weeks)
B
1TG
2TG
Body Weight (g)
*
dox
exposure
HFD
Age (weeks)
C
BW gain (g)
*
*
HFD
Age (weeks)
D
Fat mass (g)
***
HFD
dox
exposure
Age (weeks)
E
Lean Mass (g)
**
dox
exposure
HFD
Age (weeks)
F
Food Intake (g)
**
**
*
dox
exposure
HFD
Age (weeks)

Figure 3-5 Experiment 3: Dox-induced hyperleptinemia during "adolescent" period (P22-P56) followed by 60% HFD exposure at 14 weeks.

(A) Circulating leptin concentrations, (B) body weight (C) body weight gain, (D) fat mass, (E) lean mass, (n=18 per group in A-E) and (F) daily caloric food intake (n=6 per group) of 1TG controls (black) and 2TG leptin overexpressing (red) mice throughout the study. All values are means ± SEM. Significant difference calculated between 1TG and 2TG groups with Student t-test, *$p < 0.05$, **$p < 0.01$, ***$p<0.001$.


A
Circulating Leptin Concentration (ng/ml)
***
***
dox
HFD
Age (weeks)
B
Circulating Leptin Concentration (ng/ml)
R 2 = 0.72
Fat Mass (g)
C
1TG
2TG
*
Body Weight (g)
HFD
dox
Age (weeks)
D
*
**
Body Weight gain (g)
Age (weeks)
E
**
*
**
Fat Mass (g)
HFD
dox
Age (weeks)
F
*
Lean Mass (g)
HFD
dox
Age (weeks)

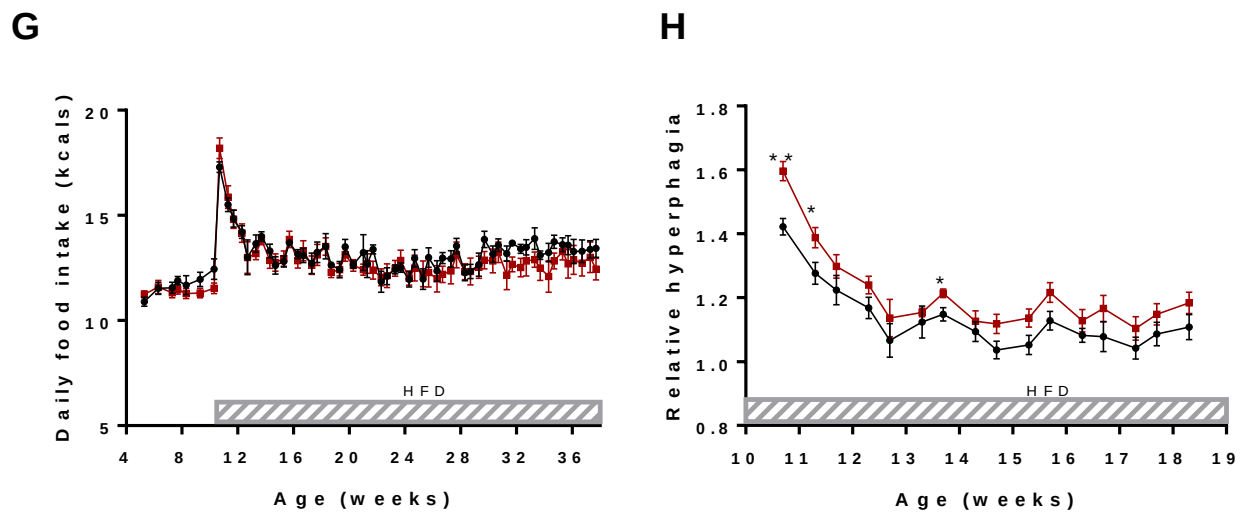


Figure 3-6 Experiment 4: Dox-induced hyperleptinemia during postnatal period (P0-P22) followed by 60% HFD exposure at 10 weeks.

(A) Circulating leptin concentrations of 1TG controls (black) and 2TG leptin overexpressing (red) mice throughout the study. (B) Regression of fat mass vs. circulating leptin concentrations of 1TG and 2TG mice at 28 weeks of age, after 18 weeks of HFD feeding. (C) Body weight (D) body weight gain, (E) fat mass, (F) lean mass, (G) daily caloric food intake and (H) fold increase in caloric intake after the initiation of HFD feeding of 1TG and 2TG mice. All values are means ± SEM. N per group in A-F: 1TG, n=15; 2TG, n=13; in G and H, n=5 per group. Significant difference between 1TG and 2TG groups calculated with Student t-test, *$p < 0.05$, **$p < 0.01$, ***$p<0.001$.

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Chapter 4

"Energy homeostasis in leptin deficient *Lep*$^{ob/ob}$ mice"

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Author contributions:

A.A.S., Y.R., R.L.L., and C.A.L. designed experiments. A.A.S., Y.R., and C.A.L. performed experiments. A.A.S., C.A.L., and R.L.L. analyzed and interpreted data, and wrote the manuscript.

Introduction

In rodents and humans with an intact leptin axis, changes in body weight imposed by either overfeeding or dietary restriction are rapidly reversed when *ad libitum* feeding is resumed by coordinate reduced energy expenditure and hyperphagia [1, 2]. In addition, when non-obese individuals undergo liposuction, adipose tissue is redistributed to other depots, leading to the same overall level of adiposity within a year [3]. These observations support the concept that individuals regulate their body weight and adiposity at a level ("set point") influenced by genetics, developmental factors, and the environment. In a weight-reduced state, both humans [4, 5] and mice [6, 7] become hyperphagic and their energy expenditure decreases more than predicted by their smaller body size; both phenotypes are largely reversed by physiological doses of exogenous leptin [8]. These observations suggest that reduction of circulating leptin is a major

signal responsible for the metabolic and behavioral responses that lead to regain of lost weight. However, despite their congenital absence of leptin, *Lep*$^{ob/ob}$ mice reduce their energy expenditure when calorically restricted [9]. Following surgical excision of fat, *ad libitum*-fed *Lep*$^{ob/ob}$ mice regain lost fat in other depots and eventually achieve the same level of adiposity as sham-operated *Lep*$^{ob/ob}$ mice [10]. In the current study we found that *Lep*$^{ob/ob}$ mice, weight reduced by transient caloric restriction, regained lost weight to the level of *ad libitum* fed controls; unlike wild type mice that overeat for a period of time after release from restriction, the *Lep*$^{ob/ob}$ mice did this without any transient overeating compared to *ad libitum* controls. These observations suggest that *Lep*$^{ob/ob}$ mice regulate adiposity via a leptin-independent pathway. The striking resistance to dietary weight loss reported in rare humans homozygous for inactivating leptin mutations is consistent with this inference [11].

Methods

Animals

48 *Lep*$^{ob/ob}$ six week old male mice fed *ad libitum* on (Purina LabDiet 5058) chow diet were obtained from Jackson Laboratory (Bar Harbor, ME). Upon arrival, mice were group-housed (3 mice per cage) with *ad libitum* access to chow and water for a two week acclimation period. Throughout the study, animals were maintained at room ambient 22-24°C with a 12-h dark-light cycle (lights on at 0700h) in a pathogen-free barrier facility. The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

Pilot Experiment

6 *Lep*$^{ob/ob}$ male mice at 8 weeks of age were either weight reduced by 20% (CR, n=3) via caloric restriction, or fed chow *ad libitum* (AL, n=3). Body weight and food intake were monitored daily. Upon reaching 80% of their initial body weight, CR mice were released to *ad libitum* feeding.

Large cohort - study design

Study design is outlined in **Figure 4.1**. After the two week acclimation period, at 8 weeks of age, half of the mice were assigned to the control group and fed *ad libitum* chow (AL group; n=21); the other half were calorie restricted to achieve 80% of initial body weight (CR group, n=21). Cages were assigned so that the two groups had no differences in starting weight, adiposity, or variance of those variables. After achieving 20% weight loss (approximately 2 weeks), the CR *Lep*$^{ob/ob}$ mice were stabilized at 80% of initial body weight, by titrated feeding, for two weeks and then released from food restriction.

Twenty percent weight reduction was achieved by feeding mice 1g of chow daily. During the weight maintenance phase, food intake was increased to 2-3g per day per mouse (the amount of food was adjusted daily when % of initial body weight of a mouse deviated from 80% by more than 2%). Calorically-restricted mice were provided with food twice daily, 1/3 of the total daily calories in the morning (09:00-9:30h) and 2/3 in the evening (18:00-18:30h). Body weight and food intake were monitored daily. During the second week of weight maintenance, mice were placed individually in metabolic cages to assess their energy expenditure (EE; TSE calorimetry system). They were then released to *ad libitum* feeding and EE was measured for another week.

Mice were monitored for eight weeks until the body weight of the previously calorie restricted group reached that of with the never-restricted controls at which point mice were sacrificed.

Body weight, body composition, food intake, and body temperature

BW was measured (± 0.1 g) daily in all mice throughout the experiment using an Ohaus Scout Pro 200g scale (Nänikon Switzerland, between 09:00-09:30h). Body composition [fat mass (FM), fat-free mass (FFM), and extracellular fluid] was measured by time-domain-NMR (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany) [12] once per week. Food was placed on the top of the cages and food intake was recorded daily for all mice throughout the study. Since mice were housed 3 per cage, food intake data was monitored on a per cage basis. Group housing the *Lep*$^{ob/ob}$ mice (3 per cage) reduced stress and the individual mice lost body weight at the same rate. 24 hour core body temperature of mice was measured during the first week of the weight maintenance phase (**Figure 4.1**). Rectal core body temperature was measured every 3 h for 24 h using a Thermalert Monitoring Thermometer starting at 1500.

Plasma assays

Blood was collected on ice using heparinized tubes (Fisherbrand). Plasma was isolated by centrifugation for 20 min at 2,000 x g at 4°C and frozen at −80°C until assay. Mice were bled at 11 weeks of age prior to transfer to the calorimeters (**Figure 4.1**). Blood from CR mice was collected before feeding (fasted overnight) while AL group was bled in a fed state. Insulin was assayed using Rat/Mouse ELISA kit (Mercodia) and glucose using Autokit Glucose (Wako).

Energy expenditure

Energy expenditure was measured individually with a LabMaster-CaloSys-Calorimetry System (TSE Systems, Bad Homburg, Germany) from 1 week before the release of the CR group through 1 week of the body weight regain phase (**Figure 4.1**). Concentrations of cage oxygen (O_2) and carbon dioxide (CO_2) were measured from every mouse every 17 minutes during the two weeks of calorimetry. To mitigate the effects of stress associated with exposure of mice to a new environment, the first 24 hours of data were excluded. Resting energy expenditure (REE; kcal/24hr) was defined as the lowest one hour period of energy expenditure during the day; this value was extrapolated to 24 hours. This value was taken instead of the lowest period in 24 hours because, for the CR mice, the 24 hour nadir occurred during nocturnal torpor [9] which depressed the REE. Torpor suppression of energy expenditure in CR mice was calculated by subtracting the lowest one hour period of energy expenditure during the night from the REE and multiplying it by the percent of time the mouse spent in torpor during any given 24 hour period. Non-resting energy expenditure (NREE; kcal/24hr) was calculated by subtracting REE from TEE and adding torpor suppression (for mice that entered torpor) ($NREE_{CR}$ = TEE – REE + Torpor suppression; NREE=TEE-REE when no torpor).

Physical activity was determined with an infrared beam system integrated with the TSE LabMaster system. Total activity (number of infrared beam breaks) in X, Y, and Z axis was recorded in 17 minute time intervals and a corresponding TEE was measured at the end of each interval. To assess the instantaneous TEE as a function of activity, every reading for every mouse was categorized as occurring during the day (between 7am and 7pm) or night (between 7pm and 7am) and then combined by group (AL, CR, or POST CR). To determine if energy expenditure was conserved at a given activity level in the groups, each group was sorted by day

[0700-1900] and night [1900-0700] from lowest to highest activity [beam breaks] with contemporaneously measured TEE. Measured TEE and beam breaks were assigned to bins of 100 beam breaks. Means and standard deviations of both activity and TEE were determined for each bin.

Average weekly energy expenditure after release from calorie restriction until the body weight of CR mice recovered to AL levels was calculated for individual mice per mean value for each cage using the energy balance equation: TEE = FI − (Δ somatic Fat Energy + Δ somatic Fat−Free Energy) [13]. Weekly food intake and weekly change in fat and lean mass were used in the calculation.

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using GraphPad PRISM software. Student t-tests (2-tailed) were conducted to compare AL and CR groups. P alpha < 0.05 was taken as significant. To determine whether the increased energy expenditure efficiency in the CR and post-CR state occurred in the dark or light cycle and if they were due to differences in physical activity, plots of TEE as a function of movement were made. Regression of instantaneous TEE as a function of movement was analyzed by first smoothing the data with a Lowess curve (with 5 points per smoothing window) then calculating the difference in TEE between the groups followed by a repeated measure one way ANOVA and post hoc Bonferroni's multiple comparisons test to determine the difference between the AL and CR (during and post CR) curves.

Several mice were excluded from the analysis. Two mice died during body composition measurement (one from each group). One mouse was found dead in its cage (CR group). Four

mice (two from each group) were removed from the study because they were not gaining weight and dropped below the 3rd standard deviation for weight of the group.

Results

Pilot Experiment

At baseline (7 week of age) *Lep*$^{ob/ob}$ mice were similar in body weight (**Figure 4.2A**) and consumed the same number of calories per day (**Figure 4.2B**). One cage of three mice was restricted to 1 g of food per day per mouse and lost weight at an average rate of 0.6 g per day, these CR mice achieved 20% weight loss in 2 weeks. In parallel, AL mice gained body weight at a steady rate of approximately 0.3 g per day, for a total weight gain of 4 g over 2 weeks. CR and AL groups had significantly different body weights throughout the calorie restriction phase (**Figure 4.2A**). After release from caloric restriction, CR mice immediately returned to their pre-CR food intake and never overate relative to the never-restricted control mice (**Figure 4.2B**). The CR group re-gained their lost body weight within 8 days of *ad libitum* feeding (**Figure 4.2A**). Despite the re-gain of body weight, the CR group had a significantly lower BW compared to AL group since the *ad libitum* mice continued to increase body weight throughout the experiment (**Figure 4.2A**). Mice were allowed to continue eating *ad libitum* for another 4 weeks. By the end of the study, CR mice fully caught up with AL group and did so without any detectable difference in food intake. Neither energy expenditure nor body temperature were monitored in this pilot study but body weight and food intake data suggest that weight reduced mice had decreased energy expenditure.

Large Cohort Experiment

Body weight and composition: Upon arrival from JAX at 6 weeks of age the average weight of the *Lep*$^{ob/ob}$ male mice was 34.72±0.38 g. At 8 weeks of age, *Lep*$^{ob/ob}$ mice were assigned to either a calorie restriction group (CR, n=15) or *ad libitum* fed group (AL, n=15) so that the groups did not differ in mean body weight (AL: 42.19±0.6 g; CR: 43.30±0.54 g; p = 0.19; **Table 4.1**, **Figure 4.3A**) or body composition (fat mass, AL: 18.54±0.40 g; CR: 19.5±0.30 g, p=0.07; and lean mass, AL: 19.69±0.27 g; CR: 20.03±0.22 g, p=0.35; **Table 4.1, Figure 4.3C-D**). Mice were group-housed (3 per cage) therefore food intake was measured on a per cage basis. The mean baseline 24h chow intake (3.45 kcal per g) per mouse during the week prior to the start of CR was 7.30±0.18 g and 7.51±0.36 g in AL and CR groups, respectively (**Table 4.1, Figure 4.4A**). Mice in the CR group were calorie restricted to 1 g of chow per mouse per day and lost weight at an average rate of 0.7 g per day; mean weight loss per animal by cage was the same as for individual mice. All mice in the CR group reached 20% weight loss within 12 days of CR and lost a total of 8.5 g on average. At the same time, AL mice steadily gained body weight at an average rate of approximately 0.4 g per day, with a total 4.5 g weight gain over 12 days. After the first day of CR, body weight was significantly lower in the CR mice compared to AL mice (AL: 42.70±0.62 g; CR: 40.23±0.47 g; p = 0.0047; **Figure 4.3A**) and remained significantly lower throughout the calorie restriction phase. Upon reaching 20% weight loss, body weight of CR group was 34.75±0.43 g compared to 46.73±0.80 g in AL group (**Table 4.1,** p <0.001). Both fat mass and lean mass were significantly decreased in CR mice compared to AL (fat mass, AL: 22.03±0.48 g; CR: 15.37±0.23 g, p<0.001; lean mass, AL: 20.87±0.24 g; CR: 15.37±0.32 g, **Table 4.1,** p<0.001). At 10 weeks of age, CR mice entered the body weight stabilization phase to maintain reduced body weight for two weeks. Food intake in the CR group during this phase was

titrated up from 1 g per day per mouse to 1.5-2.5 g per day with individual body weight and trio food intake monitored daily and appropriate food intake adjustments made to maintain the body weight of CR mice at 80% of their initial weight. Average 24h food intake of AL group mice at 10 weeks of age was 6.58±0.27 g (**Table 4.1, Figure 4.4A**).

At 11 weeks (**Figure 4.1**) of age *ad libitum* fed controls and CR mice were placed individually in metabolic cages. At 12 weeks of age CR mice were released from calorie restriction and allowed to eat *ad libitum*. Mice in the CR group re-gained their lost body weight within 8 days of *ad libitum* feeding (CR: 43.19±0.71 g; **Figure 4.3A**). Despite the re-gain of their body weight, CR group had a significantly lower BW compared to AL group since the AL mice continued increasing body weight throughout time the CR mice were calorie restricted (AL: 50.60±1.04 g; $p<0.001$; **Figure 4.3A**). It took CR mice another 5 weeks of *ad libitum* feeding to achieve body weights not significantly different from AL mice (age 18 weeks; AL: 60.18±0.95 g; CR: 57.55±0.91 g; $p = 0.058$; **Table 4.1, Figure 4.3A**). Lean mass in CR group was regained sooner than body weight and fat mass. One week after release from caloric restriction, CR group mice regained most of their lean mass (AL: 20.36±0.26 g; CR: 19.33±0.41 g, $p=0.04$) but were still significantly lower in lean mass than AL group. At 16 weeks of age the difference in lean mass between CR and AL groups was no longer significant (lean mass, AL: 24.07±0.20 g; CR: 24.66±0.27 g; $p = 0.087$; **Figure 4.3D**). Fat mass in CR group did not differ from AL group 6 weeks after release from calorie restriction (age 18 weeks; fat mass, AL: 29.52±1.04 g; CR: 27.87±0.47 g; $p = 0.12$; **Table 4.1, Figure 4.3C**).

Mice were allowed to continue eating *ad libitum* for another 2.5 weeks before they were sacrificed at 21 weeks of age, reaching final weights of 62.54±0.97 g and 60.64±0.92 g in AL and CR groups ($p=0.17$), respectively.

Food intake: The mean baseline 24h food intake per mouse at 8 weeks of age (prior to start of CR) was 7.30±0.18 g and 7.51±0.36 g in AL and CR groups, respectively. During caloric restriction phase mice in CR group were provided with an average of 1 g of chow per day until at target weight and then food intake was titrated up to an average of 1.5-2.5 g per day per animal during reduced weight maintenance phase. At 11 weeks of age, during the last week of the weight maintenance phase, all mice were placed individually in metabolic cages to assess their energy expenditure for two weeks. The mean 24 hour food intake during the first week after release from caloric restriction was 5.10±0.36 g and 5.46±0.35 g in AL and CR groups, respectively (p=0.50; **Figure 4.4A**). Food intake was monitored daily until the end of the study (when mice reached 21 weeks of age) and was not different between AL and CR groups (**Figure 4.4A-B**). Over the 9 week period following the release of CR mice to *ad libitum* feeding (until the end of the study) cumulative food intake calculated for each group was 338.75±9.99 g and 335.86±7.40 g in AL and CR groups, respectively (p=0.83; **Figure 4.4B**). Despite the difference in weight gained over this 9 week period (14.10 g in AL vs 26.92 g in POST CR), formerly calorie restricted mice did not increase their food intake relative to the control animals, suggesting that the CR animals re-gained weight solely as a result of their reduced energy expenditure in part due to their smaller size. Interestingly, for both groups, the amount of food ingested was inversely correlated to their age and weight. The mean 24h food intake at the beginning of the study, when mice were 8 weeks of age, was 7.41±0.20 g compared to 5.05±0.14 g by the end of the study at 21 weeks of age (p<.001) despite weighing almost 50% more (~62 g vs ~43 g) .

Plasma glucose and insulin concentrations: plasma was obtained from AL and CR mice at 11 weeks of age, while CR group was weight stable at 80% of initial body weight. Blood was

collected in the morning before CR mice were fed (hence an overnight fast) but AL were in the fed state. Despite a 14g lower body weight, plasma insulin in CR mice did not differ from the AL group (AL: 20.95±5.62 ng/ml; CR: 38.53±7.46 ng/ml; p = 0.07, **Figure 4.4C**). Plasma glucose was significantly lower in CR compared to AL group (AL: 332.6±39.21 mg/dl; CR: 149.5±26.50 mg/dl; p <0.001) – consistent with their feeding status (**Figure 4.4D**). Unlike mice with intact leptin axis [14, 15], leptin-deficient mice showed no correlation between fat mass and plasma insulin concentration in both the reduced weight and *ad libitum* fed states (**Figure 4.4E**).

Body temperature: 24 hour core body temperature was measured every 3 hours for 24 hours during the initial weight maintenance phase. AL mice maintained their body temperature between 32.8°C and 36.8°C with the lowest temperatures measured between 9am and 12pm. CR group maintained their body temperature within normal range during the day between 9 am and 9 pm. Between 9 pm and 6 am CR mice dropped their body temperature significantly; the lowest temperature recorded was 28.2°C at 6 am a few hours before the morning feeding (compared to 34.8°C in AL group, p<0.001; **Figure 4.5**).

Energy expenditure: Mice were placed in metabolic cages during reduced weight maintenance phase to measure their energy expenditure for one week while still calorically restricted. They were then released to *ad libitum* feeding to compare energy expenditure during the body weight regain phase. During the reduced weight maintenance phase, absolute mean 24h total energy expenditure (TEE) was 36% lower in CR group compared to AL (AL: 10.10±0.20 kcal/24h; CR: 6.42±0.31 kcal/24h; p < 0.001; **Figure 4.6A**). Decreased TEE was accounted for by nocturnal periods of torpor in addition to decreased resting and non-resting energy expenditure (**Figure 4.6A**). In wild type mice, we have defined resting energy expenditure (REE) as the lowest one hour period of energy expenditure during each 24h period [6]. However, periods of torpor should

not be included in estimates of REE. To address this problem, in calorie-restricted *Lep*$^{ob/ob}$ mice, we defined REE as the lowest one hour period of energy expenditure during the day (mice did not enter torpor during daytime). Absolute resting energy expenditure (REE) during reduced weight maintenance phase was decreased by 39% in CR compared to AL group (AL: 6.94±0.12 kcal/24h; CR: 4.26±0.17 kcal/24h; $p < 0.001$; **Figure 4.6A**); NREE was 18% lower in CR mice than in AL (AL: 3.16±0.15 kcal/24h; CR: 2.59±0.17 kcal/24h; $p < 0.05$; **Figure 4.6A**); additionally, CR mice conserved 0.43 kcal/24h by entering torpor. NREE was decreased in CR mice despite significantly higher total activity compared to AL mice (AL: 26,433±3,358 beam breaks per 24 hours; CR: 66,844±8,754 beam breaks per 24 hours; $p < 0.001$; **Figure 4.6B**). The regression of instantaneous TEE as a function of movement (individual mouse total activity during the period that was used for the TEE calculation) shows that this movement was achieved at lower energy cost (*i.e.* mice were more energetically efficient) during calorie restriction than in the AL group; this was true during both day and night (**Figure 4.6C-D**). CR mice were more active than AL mice during the day but were comparably active at night. The decreased body temperature and total activity at night suggests that CR mice entered torpor but had increased activity during the day, likely due to increased food seeking behavior.

Energy expenditure was also measured for 6 days after the CR group was released to *ad libitum* feeding and entered the body weight re-gain phase. The TSE indirect calorimetry system uses the Weir's equation to calculate the heat production from the volume of O_2 consumed and CO_2 produced which assumes that the respiratory exchange ratio (RER) of animals is between 0.7 and 1.0 [16]. During the body weight re-gain phase, the CR mice were actively gaining muscle and fat; their RER at that time was above 1, consistent with their anabolic state (1.03 on average but 1.05 at night). As a result of RER being outside of range for the Weir equations heat

estimate, the TEE obtained from the TSE system is inaccurate. Therefore, we calculated the mean total energy expenditure for individual mice using energy balance equation [TEE= FI − (Δ somatic Fat Energy + Δ somatic Fat−Free Energy)] [13] in AL and CR mice in the 5 weeks following release from caloric restriction until the CR group regained lost weight (**Figure 4.7A**). Using this method, TEE in CR mice was significantly lower than in the AL group during the 2nd week post release from CR (**Figure 4.7A**). Results were similar when average energy expenditure was calculated on a per cage basis (average of 3 mice per cage).

In addition, the regression of TEE as a function of movement (total activity) shows that, after CR mice were released to *ad libitum* feeding, they were still significantly more efficient with regard to the energy cost of motion than AL mice but this increased efficiency was apparent only during the dark phase (**Figure 4.6C-D**). The total activity increase found in the CR compared to the AL mice persisted despite *ad libitum* access to food (AL: 327.2±47.0 beam breaks; CR: 622.6±46.0 beam breaks; $p < 0.001$; **Figure 4.6C**).

Respiratory exchange ratio (RER or RQ) fluctuates throughout the day but the amplitude was more pronounced in the CR state compared to AL (**Figure 4.7B**). The 24 hour RER of the weight stable CR mice was equal to the diet quotient of the chow (both 0.89). At night, when AL mice primarily eat, the RER was elevated and it fell significantly during the day when mice eat less. In the CR state, mice ingest their food rapidly after the twice-daily feeding. This pattern of feeding imposes on these animals extended periods of fasting, leading to reduced RER both during the day and the night compared to the AL mice (**Figure 4.7B-D**). RER increased dramatically when the CR mice were released from caloric restriction; the POST CR mice had consistently higher RER at every time point (**Figure 4.7B-D**).

In the *Lep*$^{ob/ob}$ mice at reduced weight and during *ad libitum* re-feeding there was no correlation between total or resting energy expenditure and lean mass, fat mass, or both combined over the ranges observed (**Figure 4.8)**. In humans and rodents with an intact leptin axis, energy expenditure is highly correlated with metabolic mass [6].

Discussion

In humans and rodents with intact leptin signaling, weight reduction imposed by caloric restriction reduces energy expenditure more than predicted by remaining metabolic mass [4, 6, 8, 9, 17]. We find that this response is significantly amplified in leptin-deficient mice. The TEE of weight-reduced *Lep*$^{ob/ob}$ mice in this study was almost 40% lower than the *ad libitum* fed mice. These effects were conveyed by reductions in REE (approximately 73% of the reduction), enhanced efficiency of motion (about 16%), and periods of torpor (about 12%). About 50 % of energy expenditure of a mouse maintained at room temperature of 22-24°C is used to maintain body temperature [9]. Under conditions of restricted access to food, *Lep*$^{ob/ob}$ mice dramatically lower their body temperature at night and enter torpor; energy expenditure in CR mice was 39% less than in *ad libitum* fed controls. In comparison, diet induced obese wild type mice reduce resting energy expenditure by 25% when calorie restricted [9]. In wild type mice, circulating leptin follows a circadian rhythm that peaks in the middle of the night [18] and this peak coincides with the trough in body temperature that was detected in the CR *Lep*$^{ob/ob}$ mouse.

Like *Lep*$^{ob/ob}$ mice, the A-ZIP/F1 mice have greatly reduced circulating leptin and enter torpor when fasted [19]. But, unlike *Lep*$^{ob/ob}$ mice, the leptin deficiency of A-ZIP/F1 mice is due to absence of white adipose tissue. Administration of leptin prevents fasting- induced torpor and

hypothermia in *Lep*$^{ob/ob}$ mice [20], but not in A-ZIP/F1 mice, suggesting that, in addition to low leptin, torpor in mice may be dependent on adipose signal(s) that are independent of leptin[19].

In *Lep*$^{ob/ob}$ mice, REE was calculated as the lowest energy expenditure during the day (*Lep*$^{ob/ob}$ mice did not enter torpor with the lights on) and torpor suppression is the amount of energy that is conserved at night beyond the REE due to torpor-related lowering of body temperature. Defining NREE as TEE minus REE plus torpor suppression, the restricted *Lep*$^{ob/ob}$ mice have approximately the same NREE as the AL mice, despite 2.5 times greater movement (beam breaks). The increased physical activity persists in the CR mice after they are released to *ad libitum* feeding; we speculate that this persistence is due to increased food seeking behavior.

In AL mice, physical activity is highest at night and reduced during the day. CR mice display an inverted pattern in which highest activity is during the day while at night they enter torpor and show almost no physical activity. Compared to wild type mice, *Lep*$^{ob/ob}$ mice exhibit less physical activity. The same TSE system was used to measure physical activity in these studies. *Lep*$^{ob/ob}$ consistently moved less than wild type mice fed regular chow *ad libitum*. As reported here, caloric restriction resulted in a significant 2.5 fold increase in physical activity in *Lep*$^{ob/ob}$ animals. An increase in movement is also apparent in wild type mice that are food restricted but the magnitude of the effect is much smaller (~20%) [6].

Data in Figure 4.6C-D demonstrate that the CR mice, prior to release, are more energy efficient in spontaneous physical activity. Once released from caloric restriction, this increased efficiency is restricted to the lights-off period despite the released mice no longer going into torpor.

The *ad libitum* fed previously CR *Lep*$^{ob/ob}$ mice gained weight at a faster rate than the AL mice despite no detectable difference in energy intake. The TEE in the formerly restricted mice

(estimated using changes in somatic energy content over a 7 day period [21]) was significantly lower in CR animals during the second week of *ad libitum* feeding, but not thereafter.

Wild type mice are hyperphagic following release from caloric restriction [7, 22]. On the first day post restriction – while wild type calorie restricted mice are significantly lighter (-25% body weight) than *ad libitum* fed controls – food intake is almost doubled [7]. This relative hyperphagia decreases over the ensuing 7 days, at which point the intake of the weight-reduced animals equals that of the controls [7]. In study of $Lep^{ob/ob}$ mice reported here, food intake in the immediate post restriction period was identical in the released mice and the never-restricted AL groups despite the increased food seeking behavior of the formerly restricted mice. Figure 4.4 shows that total food intake does not increase as L$ep^{ob/ob}$ mice age and gain weight. Additionally, Figure 4.8 demonstrates that there is no correlation between somatic mass and energy expenditure in either the CR or the AL state in $Lep^{ob/ob}$ animals over the range of this study. Since there is no relationship between mass and energy expenditure in $Lep^{ob/ob}$ animals, CR mice, released to *ad libitum* feeding, should have gained weight at the same rate as the never-restricted mice unless there is an increase in energy efficiency that persists after release. The absence of post-restriction hyperphagia is not due to mice reaching their physical (e.g. stomach capacity) maximum daily energy intake since the same mice ate greater amounts earlier in the study (7.51 ± 0.36 g per day at 8 weeks of age vs. 5.46 ± 0.35 g per day at 13 weeks of age after release from restriction; $p<0.01$). Another argument against $Lep^{ob/ob}$ mice having reached a physical maximum of food intake is that $Lep^{ob/ob}$ mice are capable of further increasing food intake when suitably provoked. For example, the FAT-ATTAC transgenic mouse segregates for a myristoylated caspase 8-FKBP fusion protein enabling adipocyte apoptosis to be induced by administration of a chemical dimerizer for 1-2 weeks [23]. $Lep^{ob/ob}$ mice segregating for this

transgene, following administration of the dimerizer, increase daily food intake of chow from 5 g to 9 g (6 weeks of treatment) [23].

As extensively discussed in literature, expressing food intake in terms of calories per gram of body weight is problematic especially when the animals being compared differ significantly in body mass. For example, normalizing food intake of a *Lep*$^{ob/ob}$ mouse to body weight would suggest that such mouse is hypophagic relative to a wild type mouse [24]. Others have recommended that energy intake be analyzed by multivariate regression [25, 26]; or that both energy intake and expenditure be normalized using the same method [27, 28]. In the AL *Lep*$^{ob/ob}$ group, food intake at 8, 9, and 10 weeks of age was significantly higher than food intake at the end of the study when mice were 21 weeks old and 20 grams heavier. This observation further supports the inference that food intake should not be normalized to body weight in these mice over the body weight ranges in this study. Energy intake and expenditure in the AL group decreased gradually from 8 to 12 weeks of age and appeared to stabilize after week 12; higher energy expenditure in *Lep*$^{ob/ob}$ mice during this time may be related to maximal growth rate during this time. Despite the mice having 35% more mass at 21 weeks, there was no increase in calorie intake after 12 weeks of age; there was no correlation between energy expenditure and fat, lean, or total mass in any of the groups of leptin deficient mice over the range measured. In aggregate, these data suggest that leptin is required for the regulation of energy intake, but is not essential for regulation of energy expenditure.

Lep$^{ob/ob}$ mice develop without exposure to leptin leading to congenital neuronal alterations [29, 30]. Compensatory pathways could develop as a result of congenital leptin deficiency and this leads to an important caveat that the responses to the weight perturbations reported here may be unique to these animals.

Kaiyala *et al.* reported that *Lep*$^{ob/ob}$ mice adjust energy expenditure but not food intake in response to changes in ambient temperature [31]. In contrast, wild type mice increase food intake when ambient temperature is decreased and reduce energy intake under thermoneutral conditions. In addition, wild type mice display a strong inverse relationship between ambient temperature and energy expenditure [31]. These data are consistent with our finding that *Lep*$^{ob/ob}$ mice reduce energy expenditure but do not increase food intake in response to imposed weight reduction.

The CR *Lep*$^{ob/ob}$ regained most of their lean mass within one week of release to *ad libitum* feeding, probably due to rehydrating of the muscle, whereas their fat mass took several weeks to reach the level of AL group. Mice regained lost lean mass quickly but did not exceed the amount of lean mass in the AL mice. Hambly *et al.* reported body composition of wild type mice post release from caloric restriction; like our *Lep*$^{ob/ob}$, following release wild type mice regained lean mass more rapidly than the fat mass [7]. Both of these rapid recoveries in lean mass are likely due to both the rehydrating of muscle and the lower energy demand to gain 1 g of lean mass compared to gaining 1 g of adipose tissue.

The CR mice displayed a decline in RER reflecting an appropriate reliance on fat stores for provision of metabolic need, and indicating that leptin is not required for this important metabolic accommodation.

Lep$^{ob/ob}$ mice utilize energy in an age dependent but an apparent/composition mass (lean, fat or both) independent manner over the range of body mass/composition studied here. Unlike humans [4] or mice with intact leptin axis [6] figure 4.8 indicates there is no apparent relationship between energy expenditure and fat, lean, or a combination of both in our *Lep*$^{ob/ob}$

mice. Energy expenditure in *Lep*$^{ob/ob}$ mice is only dependent on the feeding status of the mice; CR mice use significantly fewer calories than the AL controls but within either the CR or AL groups, in the size range that we observed, they use a non-detectably different number of calories regardless of size. Max Kleiber showed that across a wide range of body sizes, energy expenditure scales to the 3/4th power of body mass [32]. In *Lep*^{obob} mice, over the size range studied, this relationship is not apparent due to methodological limitations. Using a multivariate regression model for 24h energy expenditure, Kaiyala *et al.* found that in leptin-deficient mice lean and fat mass were not significant contributors to variation in energy expenditure [20]. However, when leptin was administered to *Lep*$^{ob/ob}$ mice, lean mass became an independent predictor of EE, suggesting that leptin is necessary to render the positive relationship between lean mass and EE detectable. Weight-reduced humans and rodents exhibit hypometabolism resulting from – at least in part – relative leptin insufficiency due to lost body fat [4, 6, 8]. This decrease in energy expenditure is due in part to increased skeletal muscle work efficiency and can be reversed with administration of physiological ("replacement") doses of leptin [8, 33], further suggesting a role for leptin in the slope of the correlation between lean mass and EE. In the studies reported here, the lower energy expenditure in calorically restricted *Lep*$^{ob/ob}$ mice is driven by a combination of torpor, a dramatic decrease in REE, and a decrease in NREE. In weight- reduced leptin-deficient mice the increased energy efficiency is more pronounced than in a weight reduced wild type mouse.

Insulin, in addition to its critical role in glucose homeostasis, is a known regulator of food intake and adiposity [34]. In leptin competent humans and mice, circulating insulin concentrations correlate directly with body weight and adiposity. However, in mice that lack leptin, this relationship is interrupted. [35-37]. Several experiments provide evidence of a direct regulation

of insulin secretion by leptin. Leptin administration suppresses beta cell insulin release, and reduces preproinsulin mRNA in multiple models including islets isolated from *Lep*$^{ob/ob}$ mice [38, 39], rats [40] and humans [40, 41] and a rat pancreatic β-cell line [42]. Administration of exogenous leptin to *Lep*$^{ob/ob}$ mice decreases circulating insulin and fasting plasma glucose concentrations even at doses that do not induce reductions in body weight [43]. Similarly, in mouse models of lipodystrophy – characterized by severe hypoleptinemia and hyperinsulinemia – treatment with low doses of recombinant leptin significantly improved insulin sensitivity [44, 45]; chronic food restriction does not normalize circulating insulin concentrations in these mice [44, 45]. These data suggest that in lipodystrophic mice leptin modulates insulin sensitivity and glucose homeostasis independently of either food intake or body weight. Evidence from mouse models of Congenital Generalized Lipodystrophy (CGL) support the role of leptin deficiency in the insulin resistance found in this condition [44, 45]. As reported here, (and in leptin receptor-deficient fa/fa rats [46]), *ad libitum* fed *Lep*$^{ob/ob}$ mice have elevated circulating insulin concentrations that are not normalized by caloric restriction. Hyperinsulinemia in AL *Lep*$^{ob/ob}$ mice is not corrected by reducing fat mass (**Figure 4.4D**). The absence of circulating leptin in *Lep*$^{ob/ob}$ mice is accompanied by a loss of the normal (**Figure 4.4E**) relationship between fat mass and circulating insulin.

In this study, *Lep*$^{ob/ob}$ mice weight reduced by transient caloric restriction regained lost weight to the level of *ad libitum* controls. They did so by reduced energy expenditure; unlike wild type mice, there was no compensatory relative hyperphagia. Their reacquisition of lost body fat suggests that factors other than leptin are involved in regulating body weight of the *Lep*$^{ob/ob}$ mouse. The adiposity "set point" in the *Lep*$^{ob/ob}$ mouse is apparently higher than that of a wild type mouse, and this higher level is defended when the mouse is challenged with caloric

restriction. Other circulating and/or neural factors inform the CNS about aspects of energy intake and somatic energy stores. Some of these have their predominant effects on either energy intake (insulin, ghrelin, CCK, GLP-1, PYY) or expenditure (thyroid hormone). Others almost certainly exist [47]. For example, when a wild type mouse is parabiosed to a leptin receptor deficient mouse ($Lep^{db/db}$) with high (but adipose mass-appropriate) circulating concentrations of leptin the wild type mouse starves to death [48, 49]. However, administering a similar amount of leptin to a wild type mouse only transiently reduces food intake and the animal does not starve [50]. These results support the possible existence of another circulating factor – a starvation signal – that is present in a $Lep^{db/db}$ because the mouse is unable to properly sense its energy stores. Such a signal might account for the hypometabolic response of the $Lep^{ob/ob}$ mouse to weight reduction.

Figures, Figure Legends, and Tables

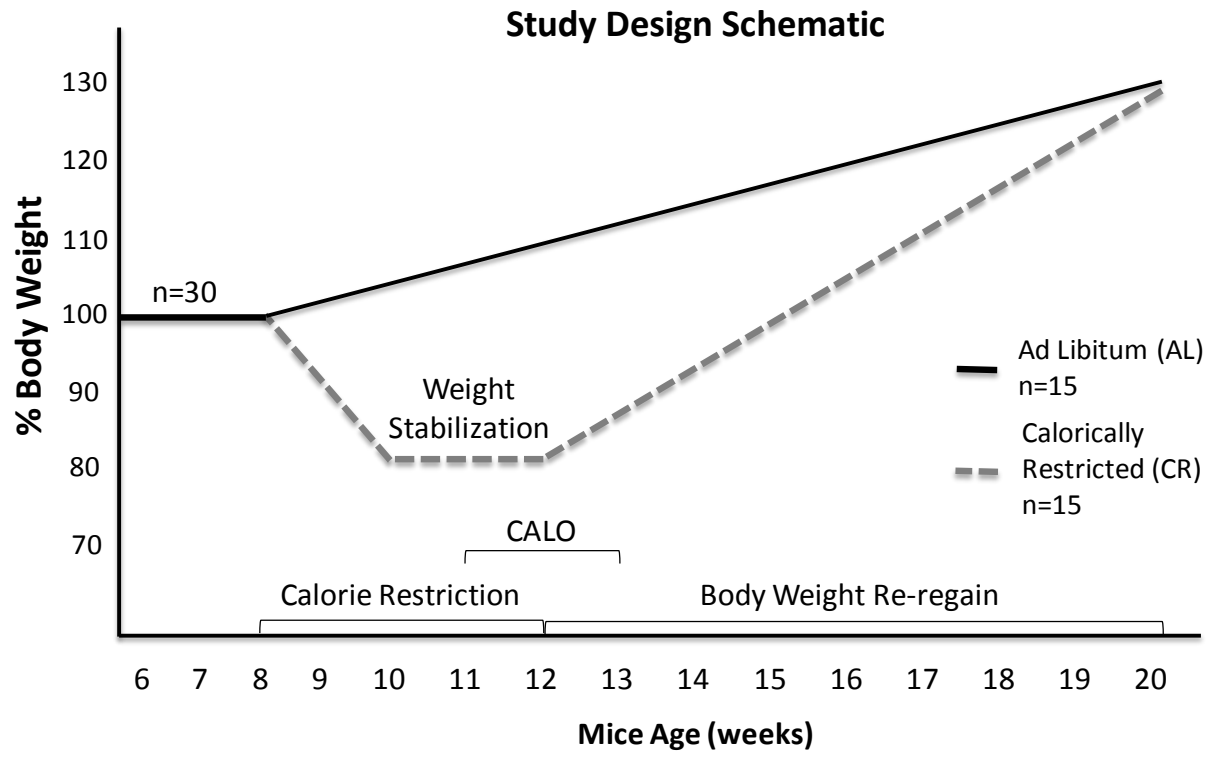

Figure 4-1 Study Design Schematic.

After a two week acclimation period, at 8 weeks of age, half of the mice were assigned to the control group and fed *ad libitum* chow; the other half were calorie restricted to achieve 80% of initial body weight. After achieving 20% weight loss (approximately 2 weeks), the CR *Lep*$^{ob/ob}$ mice were stabilized at 80% of initial body weight for two weeks and then released from food restriction during the body weight re-gain phase.

	Baseline (8 wks old)			During CR (10 wks old)			BW regained (18-18.5 wks old)		
	AL	CR	p-value	AL	CR	p-value	AL	CR	p-value
Body weight (g)	42.19±0.60	43.3±0.54	0.19	46.73±0.80	34.75±0.43	*<0.001*	60.18±0.95	57.55±0.91	0.06
Fat mass (g)	18.54±0.40	19.50±0.30	0.07	22.03±0.48	15.37±0.23	*<0.001*	29.52±1.04	27.87±0.47	0.12
Lean mass (g)	19.69±0.27	20.03±0.22	0.35	20.87±0.24	15.37±0.32	*<0.001*	26.13±1.19	26.16±0.32	0.98
Food intake (g)	7.30±0.18	7.51±0.36	0.61	6.58±0.27	1.40±0.08	*<0.001*	5.45±0.29	5.26±0.15	0.61

Table 4-1 Body weight, body composition, and food intake of AL and CR mice.

Mean body weight, fat mass, lean mass and food intake with SEM at baseline (8 weeks of age), during calorie restriction (10 weeks of age) and after CR group re-gained body weight (18.5 weeks of age; fat mass, lean mass and food intake measured at 18 weeks of age) in mice fed *ad libitum* throughout the study (AL) and mice calorically restricted to 80% of initial body weight then released to *ad libitum* feeding (CR).

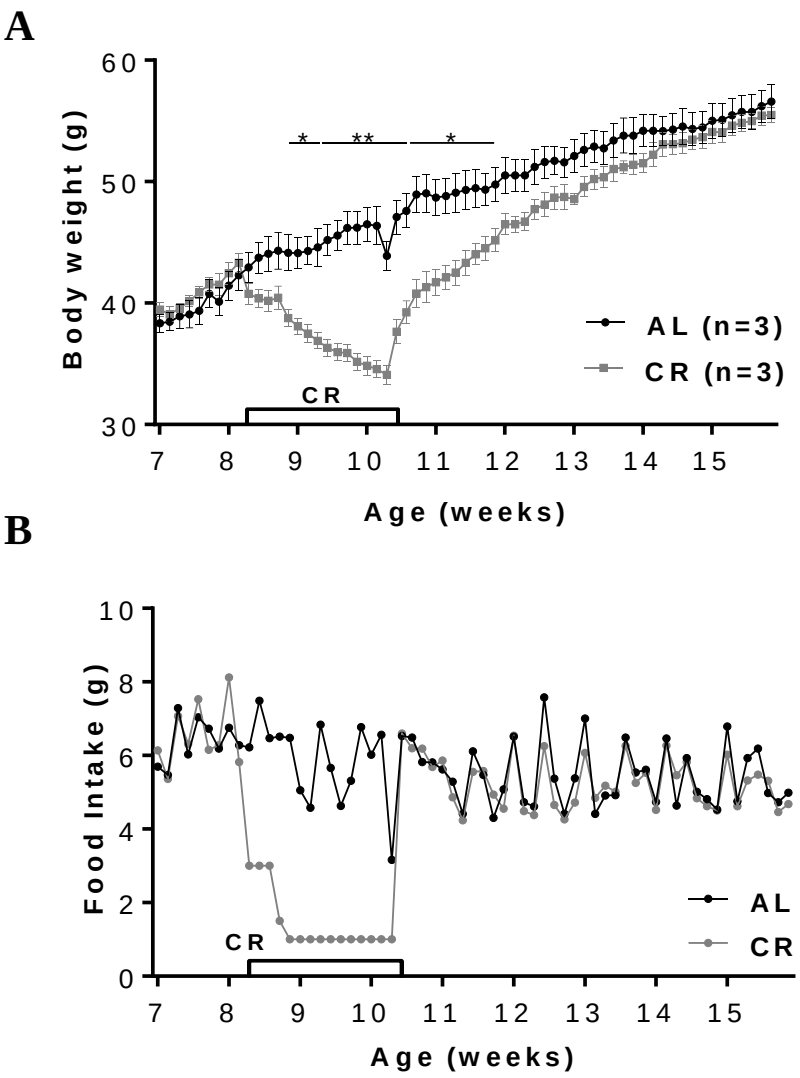

Figure 4-2 Body weight and food intake of AL and CR mice in a pilot study.

(A) Mean body weight ±SEM (g) and (B) Mean 24h food intake in mice fed *ad libitum* throughout the study (AL) and mice calorically restricted to 80% of initial body weight then released to *ad libitum* feeding. P values: *<0.05, **<0.01.

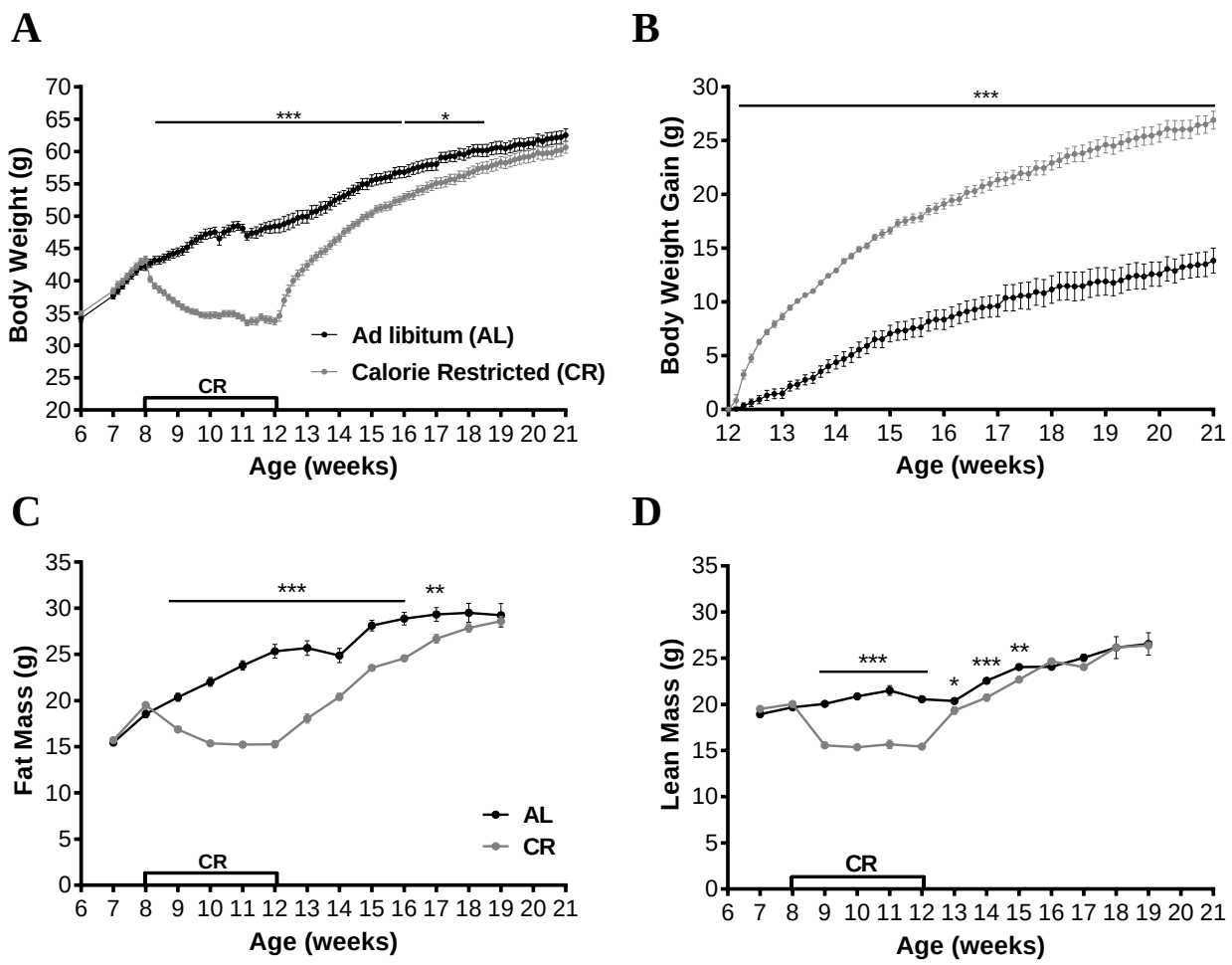

Figure 4-3 Body weight and composition of AL and CR mice.

(A) Mean body weight, (B) body weight gain, (C) fat mass, (D) lean mass ±SEM (g) in mice fed *ad libitum* throughout the study (AL, n=13) and mice calorically restricted (CR, n=13) to 80% of initial body weight then released to *ad libitum* feeding. P values: *<0.05, **<0.01, ***<0.001.

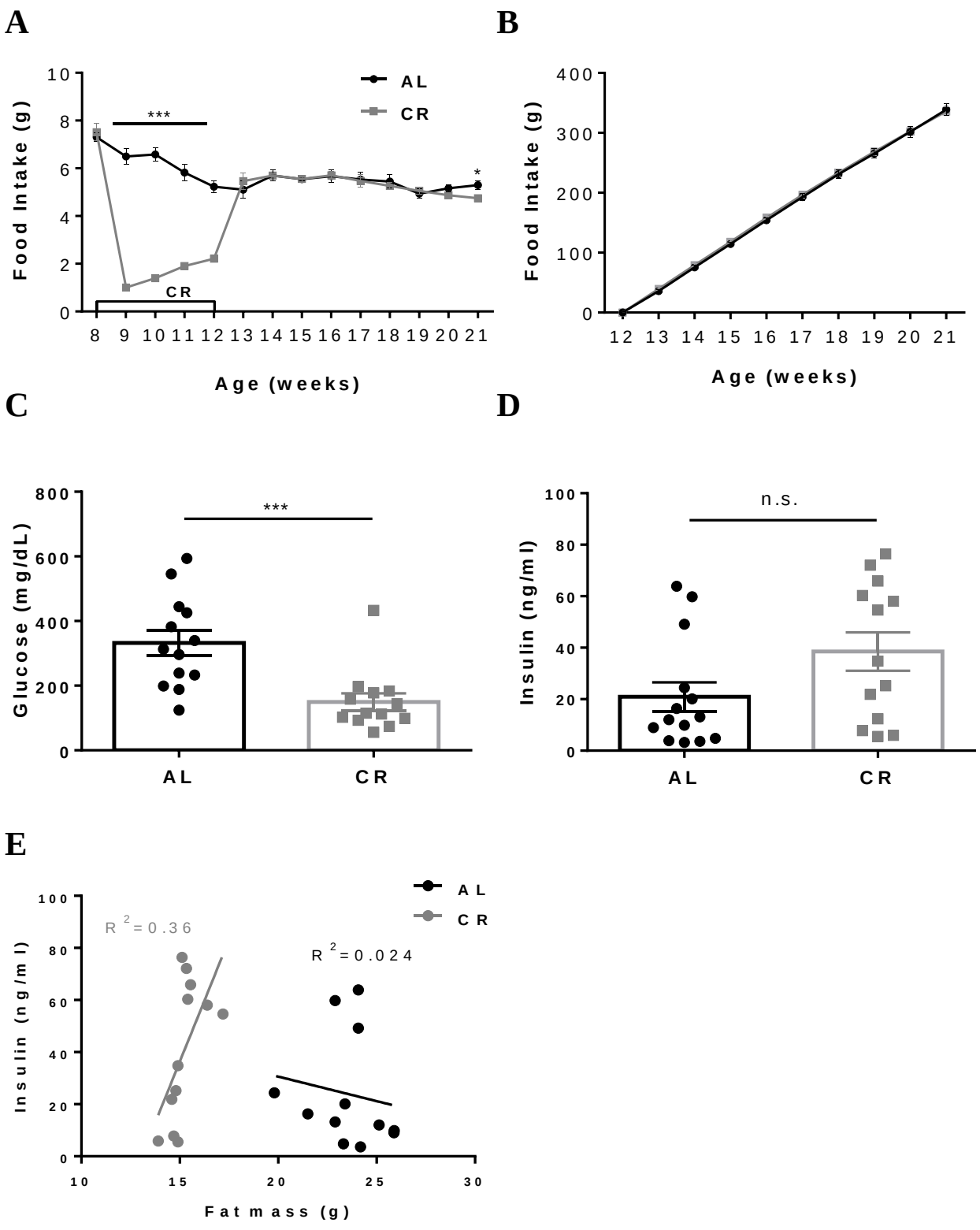
A
B
C
D
E
AL
CR
***
*
Food Intake (g)
Age (weeks)
Glucose (mg/dL)
n.s.
Insulin (ng/ml)
R 2 = 0.36
R 2 = 0.024
Fat mass (g)

Figure 4-4 Food intake, plasma glucose and insulin in CR and AL mice.

(A) Mean 24h food intake ±SEM (g) and (B) Cumulative food intake over 8 weeks of body weight re-gain in mice fed *ad libitum* chow throughout the study (AL, n=5) and mice calorically restricted (n=5) to 80% of initial body weight then released to *ad libitum* feeding. (C) Mean glucose and (D) insulin ±SEM in *ad libitum* fed (AL, n=13) or calorically restricted (CR, n=12) mice measured at 12 weeks of age while CR mice were calorically restricted to maintain 80% of initial body weight. (E) Regression of circulating insulin concentrations against fat mass in the AL (n=13) and CR (n=12) groups of mice at 11 weeks of age while CR were weight stable at the reduced body weight. P values: ***<0.001.

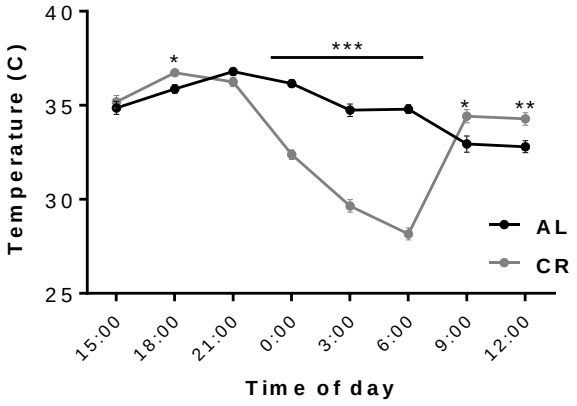

Figure 4-5 Body temperature of AL and CR mice.

Body temperature of mice fed *ad libitum* chow throughout the study (AL, n=10) and mice calorically restricted to 80% of initial body weight (CR, n=13) measured during the weight maintenance segment of the CR phase.

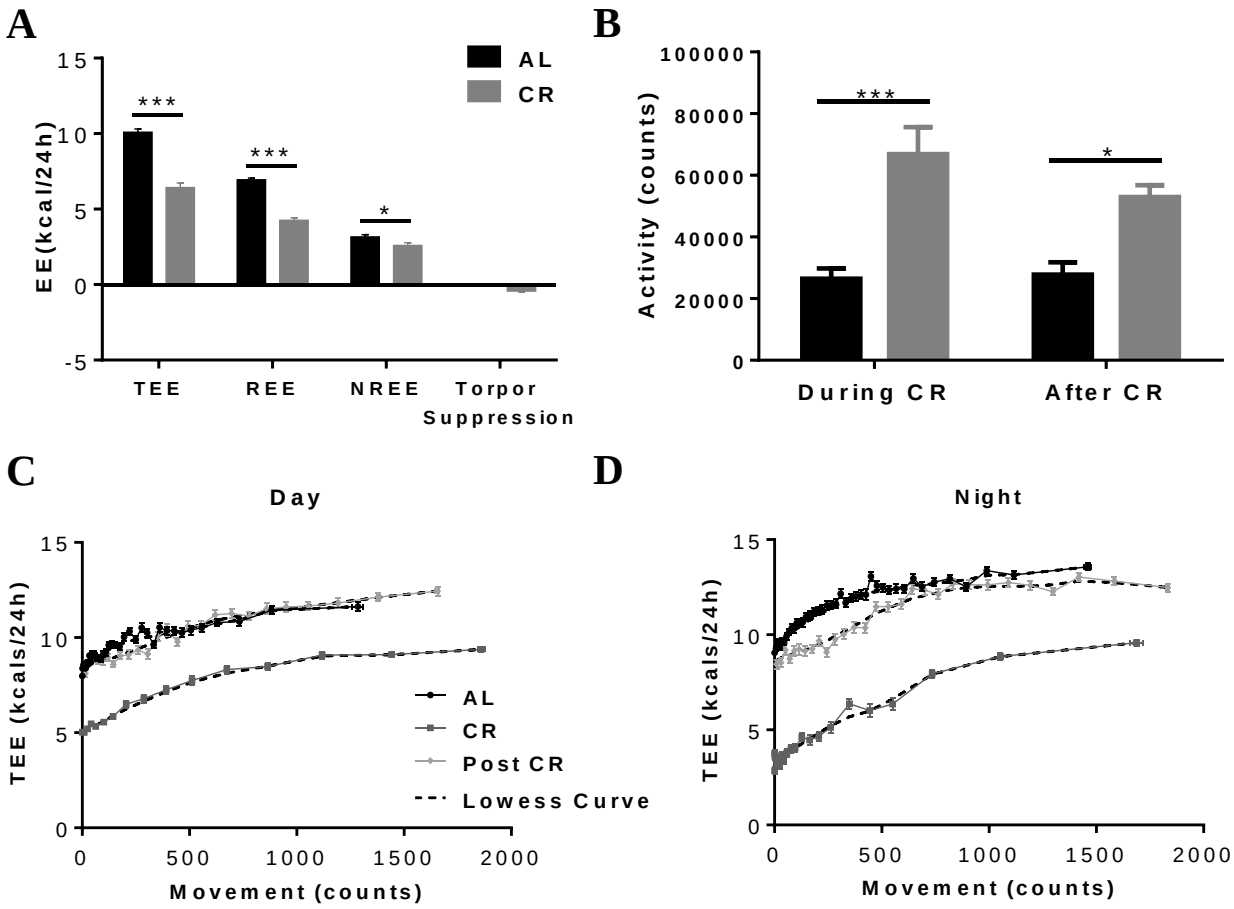

Figure 4-6 Energy expenditure and activity of AL and CR mice.

(A) Energy expenditure during calorie restriction in mice fed *ad libitum* chow throughout the study (AL) and mice calorically restricted to 80% of initial body weight (CR). Energy expenditure during calorie restriction was measured in the TSE metabolic chambers. Included are the following: TEE – total energy expenditure, REE – resting energy expenditure, NREE – non resting energy expenditure and torpor suppression. (B) Physical activity in AL and CR mice during CR and after release to *ad libitum* feeding. Activity was measured in the TSE system. Regression of instantaneous TEE as a function of movement (C) during the day and (D) at night in mice fed *ad libitum* chow throughout the study (AL), mice calorically restricted to 80% of initial body weight (CR) and the CR group after release to *ad libitum* feeding. N in each group: AL, n= 10; CR, n=12. P values: *<0.05, ***<0.001.

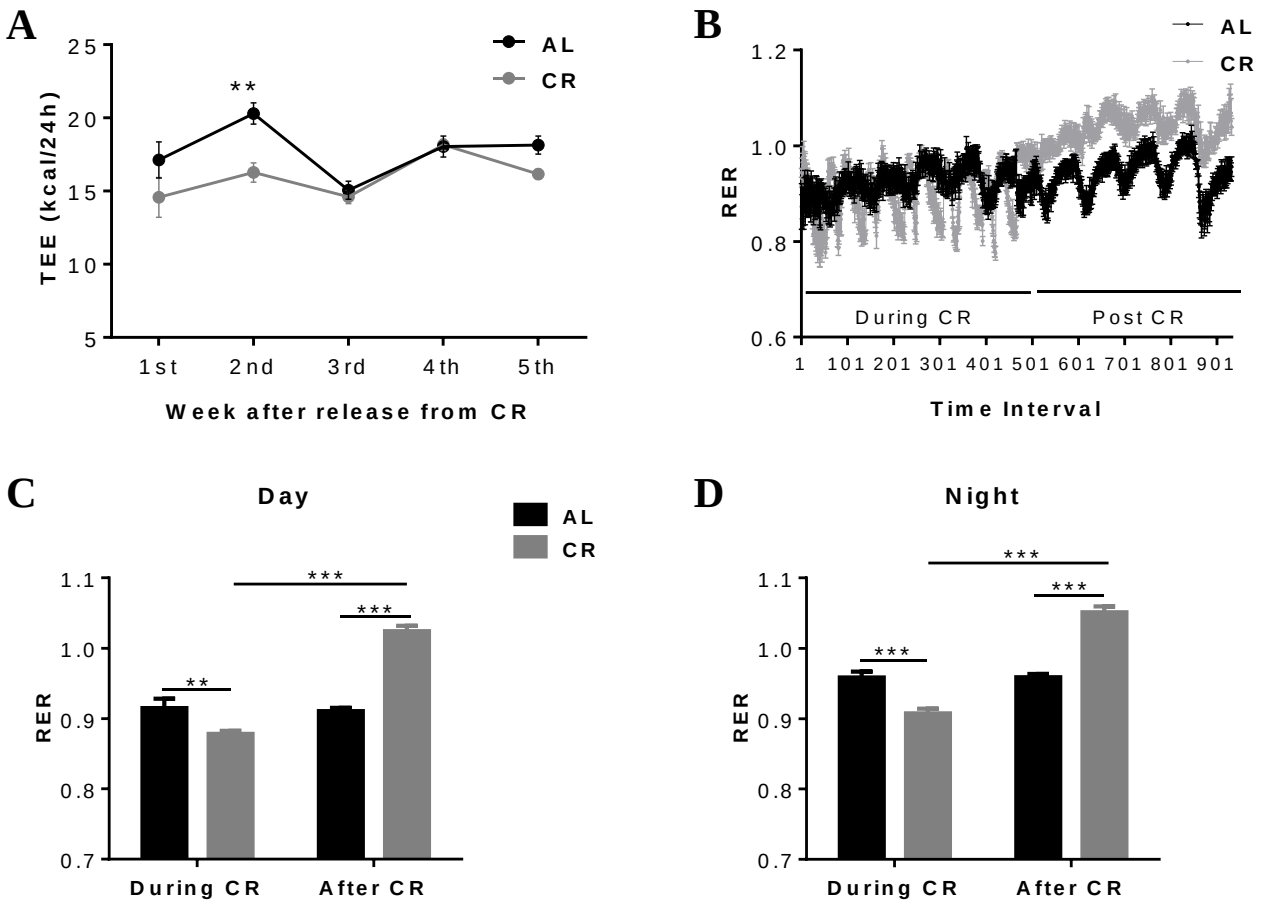

Figure 4-7 Total energy expenditure and respiratory exchange ratio.

(A) Total energy expenditure after release from calorie restriction in mice fed *ad libitum* chow throughout the study (AL) and calorically restricted (CR) mice. TEE post-restriction was calculated using the energy balance equation: TEE= FI − (Δ somatic Fat Energy + Δ somatic Fat−Free Energy). (B) average respiratory exchange ratio (RER) measured at each time interval and (C) average 24-hour RER during the day and (D) and at night during and post calorie restriction in mice fed *ad libitum* chow throughout the study (AL) and mice calorically restricted then released to *ad libitum* feeding. N in each group: AL, n= 10; CR, n=12. P values: **<0.01, ***<0.001.

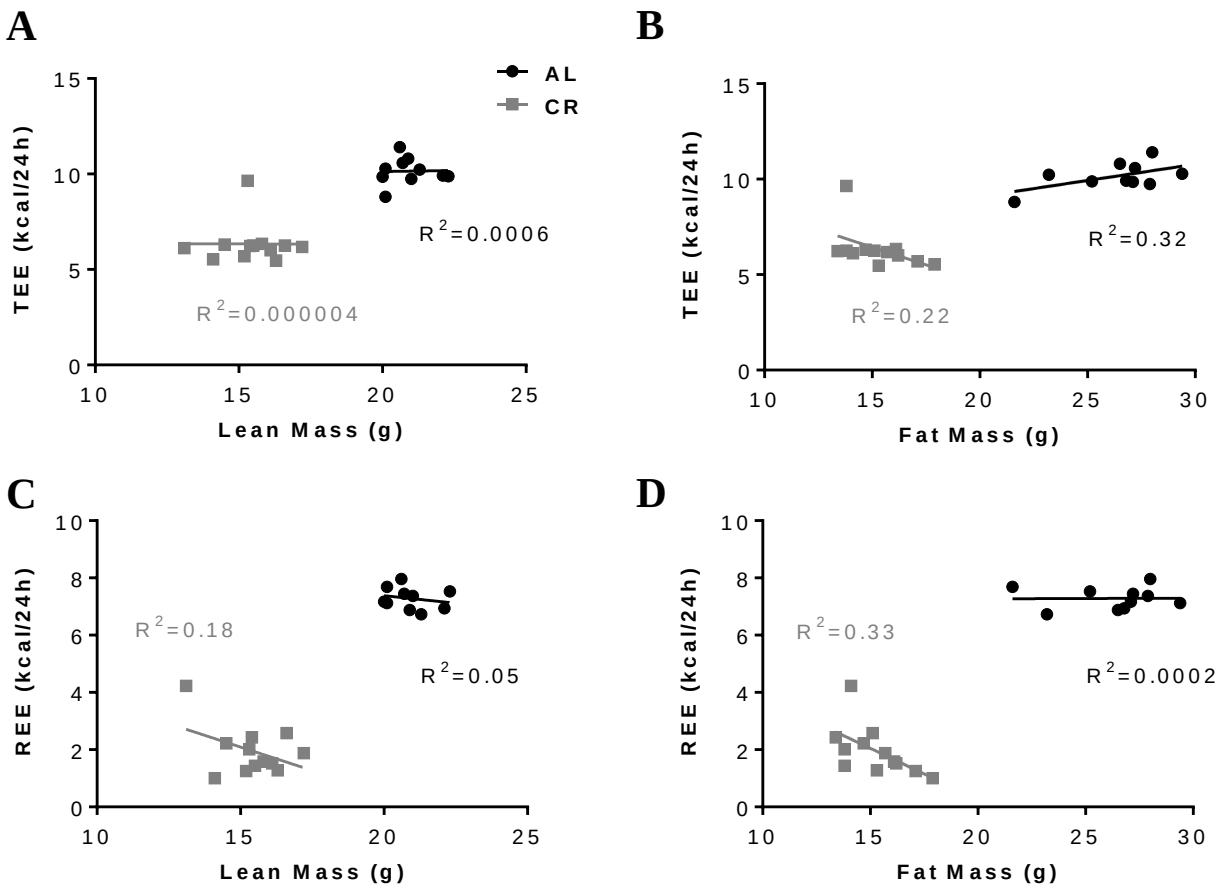

Figure 4-8 Correlations of energy expenditure with body composition in AL and CR mice.

Regression of (A,C) lean mass and (B, D) fat mass against (A, B) total and (C, D) resting energy expenditure in the AL and CR groups of mice during the weight maintenance segment of the CR phase. N in each group: AL, n= 10; CR, n=12.

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Chapter 5

"Genome-wide meta-analysis uncovers novel loci influencing circulating leptin levels"

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Author contributions:

* These authors contributed equally to this work.

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https://www.nature.com/articles/ncomms10494#author-information

T.O.K. and R.J.F.L. conceived and designed the study. T.O.K. and Q.S. performed the meta-analyses. J.F.M.C., A.A.S., C.A.L., Y.Z. and R.L.L. carried out the knockdown studies in mouse adipose tissue explants. T.O.K., J.F.M.C., A.A.S., Q.S., J.K., M.F.F., P.W.F., C.M.L., R.L.L. and R.J.F.L. wrote the manuscript.

Abstract

Leptin is an adipocyte-secreted hormone, the circulating levels of which correlate closely with overall adiposity. Although rare mutations in the leptin (*LEP*) gene are well known to cause leptin deficiency and severe obesity, no common loci regulating circulating leptin levels have been uncovered. Therefore, we performed a genome-wide association study (GWAS) of circulating leptin levels from 32,161 individuals and followed up loci reaching $P<10^{-6}$ in 19,979 additional individuals. We identify five loci robustly associated ($P<5 \times 10^{-8}$) with leptin levels

in/near *LEP*, *SLC32A1*, *GCKR*, *CCNL1* and *FTO*. Although the association of the *FTO* obesity locus with leptin levels is abolished by adjustment for BMI, associations of the four other loci are independent of adiposity. The *GCKR* locus was found associated with multiple metabolic traits in previous GWAS and the *CCNL1* locus with birth weight. Knockdown experiments in mouse adipose tissue explants show convincing evidence for *adipogenin*, a regulator of adipocyte differentiation, as the novel causal gene in the *SLC32A1* locus influencing leptin levels. Our findings provide novel insights into the regulation of leptin production by adipose tissue and open new avenues for examining the influence of variation in leptin levels on adiposity and metabolic health.

Introduction

Leptin is an adipocyte-secreted hormone that influences long-term regulation of energy homeostasis by informing the brain about the amount of stored body fat[1,2]. Circulating leptin levels correlate closely with measures of adiposity, such as body fat mass and body mass index (BMI)[3]. Yet, at any given level of adiposity, there is substantial variation in circulating leptin levels[4], of which estimated 30–50% is explained by genetic factors[5,6,7].

Rare homozygous loss-of-function mutations in the leptin-encoding gene (*LEP*) cause leptin deficiency that leads to hyperphagia and severe obesity, which can be corrected by exogenous leptin administration[8]. Leptin-deficient children are born with a normal birth weight but exhibit rapid weight gain in the first few months of life. They show marked abnormalities of T-cell number and function, and have high rates of childhood infection[9]. Hypothalamic hypothyroidism is present, characterized by a low free thyroxine and high serum thyroid-stimulating hormone

levels[10]. Pubertal development generally does not occur due to hypogonadotropic hypogonadism[10]. Individuals heterozygous for leptin mutations exhibit a partial leptin deficiency with higher body fat than control individuals[11].

Candidate gene studies, typically small in size, have reported associations of two common variants (A19G (rs2167270, minor allele frequency (MAF) 35%) and G2548A (rs7799039, MAF 49%)) in the promoter or 5′-untranslated region of *LEP* with circulating leptin levels in the general population, but these results are inconclusive[12,13,14,15,16]. The same *LEP* variants have been studied for association with obesity, but a meta-analysis of the published results (n_{A19G}=918 and n_{G2548A}=2,174) found no evidence of such association[17]. Candidate gene studies of *LEP* were published before the human genome sequence was extensively characterized and are therefore restricted to the variants known at that time. Furthermore, although *LEP* is an obvious candidate, variants in other genes may also influence circulating leptin levels by regulating leptin production, secretion, clearance or response. Identification of such leptin-regulating genes could provide novel insights into mechanisms that regulate energy homeostasis and neuro-endocrine function[1,2].

In this study, we sought to identify genetic loci associated with circulating leptin levels by a genome-wide meta-analysis. Given the strong correlation between leptin and adiposity, we also examined genome-wide associations with circulating leptin levels adjusted for BMI, to identify loci associated with leptin levels independent of BMI.

Methods

Main analyses

Study design. We conducted a two-stage meta-analysis to identify leptin-associated loci in adults of European ancestry. In stage 1, we performed a meta-analysis of 23 GWAS (*n*=32,161) (Supplementary Table 5.1) for BMI-unadjusted and BMI-adjusted circulating levels of leptin. Stage 2 included 13 additional studies (*n*=19,979), which provided either *de novo* or *in silico* data for the lead SNPs of the independent loci reaching $P<1 \times 10^{-6}$ in Stage 1 (Supplementary Table 5.5). Secondary meta-analyses were conducted in men (*n*=13,363) and women (*n*=18,698) separately, and with adjustment for body fat percentage (assessed by dual-energy X-ray absorptiometry or bioimpedance analysis) instead of BMI (*n*=18,980). The study-specific descriptive statistics are presented in Supplementary Table 5.23.

Stage 1 genome-wide association analyses. Following study-specific quality control measures, the genotype data were imputed using the HapMap Phase II CEU reference panel (Supplementary Table 5.24). Directly genotyped and imputed variants were then tested for association with logarithmically transformed leptin (ng ml^{-1}), adjusting for age, age^2 and any necessary study-specific covariates (for example, genotype-derived principal components) in a linear regression model. The analyses were performed with and without additional adjustment for BMI. In studies that had assessed body fat percentage with bioimpedance analysis or dual-energy X-ray absorptiometry, additional analyses were performed with adjustment for body fat percentage. The analyses were performed in men and women separately. In studies that included closely related individuals, regression coefficients were also estimated in the context of a variance component model that modeled relatedness in men and women combined, with sex as a covariate.

Before performing meta-analyses on the data from individual studies, SNPs with poor imputation quality scores (r^2-hat <0.3 in MACH, proper-info <0.4 in IMPUTE, INFO <0.8 in PLINK) or with a minor allele count <6 were excluded for each study (Supplementary Table 5.24). The genotype data for the leptin-associated lead SNPs was of high quality with a median imputation score of ≥0.94 (Supplementary Table 5.26). The fifth percentile for all SNPs was ≥0.80, except for the previously established rs900400 SNP near *CCNL1*.

All individual GWAS were genomic control corrected before meta-analyses. Individual study-specific genomic control values ranged from 0.977 to 1.051. Fixed effects meta-analyses were then conducted using the inverse variance-weighted method implemented in METAL. The genomic control values for the meta-analysed results were 1.050, 1.026 and 1.022 in the BMI-unadjusted meta-analyses of all individuals, men and women, and 1.046, 1.022 and 1.015 in the BMI-adjusted meta-analyses, respectively. Using the LD score regression method[67] in the Stage 1 meta-analyses suggests that the observed inflation is not due to population substructure. The regression intercept, which estimates inflation after removing polygenic signals, was 0.994 for BMI-unadjusted and 1.004 for BMI-adjusted meta-analyses of men and women combined.

Selection of SNPs for follow-up. We used a pairwise distance criterion of ±500 kb and r^2<0.1 between SNPs that reached $P<10^{-6}$in the meta-analysis of BMI-adjusted or -unadjusted meta-analysis of leptin levels in Stage 1 in men and women combined or separately, to select loci forward for follow-up in Stage 2. We tested the association of the lead SNPs in up to 19,929 adults of white European ancestry in Stage 2.

Stage 2 follow-up of the loci reaching $P<10^{-6}$ in Stage 1. Association results were obtained from 13 studies that had not been included in the Stage 1 meta-analyses (Supplementary Table 5.5). Samples and SNPs that did not meet the quality control criteria defined by each individual study

were excluded. Minimum genotyping quality control criteria were defined as Hardy–Weinberg equilibrium $P>10^{-7}$, call rate >90% and concordance >99% in duplicate samples in each of the follow-up studies.

We tested the association between the SNPs and leptin in each Stage 2 study using approaches similar to those described for the Stage 1 studies. We subsequently performed a meta-analysis of β-coefficients and s.e. from Stage 2 using the inverse variance fixed effects method. The final meta-analysis combined GWAS results from Stage 1 with the Stage 2 results. The conventional *P*-value threshold of $<5 \times 10^{-8}$ in the combined Stage 1 and Stage 2 meta-analysis was used to determine genome-wide significance.

Identifying genes and biological pathways at associated loci

Cross-trait look-ups. To further examine the relationship between the leptin-associated loci and anthropometric and metabolic parameters, we acquired association results for the loci in or near *LEP*, *SLC32A1*, *GCKR*, *CCNL1* and *COBLL1* from nine GWAS meta-analysis consortia: ADIPOGen (BMI-adjusted adiponectin), BCGC (body fat percentage), DIAGRAM (type 2 diabetes), Early growth genetics (birth weight, early-onset obesity), ICBP (systolic and diastolic blood pressure), GIANT (height, BMI, waist–hip ratio adjusted for BMI), GLGC (circulating levels of high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides and total cholesterol), MAGIC (fasting glucose, fasting insulin) and ReproGen (age at menarche) (Supplementary Table 5.7).

National Human Genome Research Institute GWAS Catalog look-ups. To identify the associations of the leptin-associated loci in published GWAS, we extracted previously reported GWAS associations within 500 kb and $r^2>0.7$ with any of the lead leptin-associated SNPs, from

the GWAS Catalog of the National Human Genome Research Institute (www.genome.gov/gwastudies) (Supplementary Table 5.11).

Overlap with functional regulatory elements. We used the Uncovering Enrichment Through Simulation method to combine the leptin association data with the Roadmap Epigenomics Project segmentation data[54]. The pipeline chose 10,000 sets of random SNPs among HapMap2 SNPs with a MAF>0.05 and that matched the original input SNPs based on proximity to a transcription start site and the number of LD partners (r^2>0.8 in individuals of European ancestry in the 1000 Genomes Project). The LD partners were combined with their original lead SNP to create 10,000 sets of matched random SNPs and their respective LD partners. These sets were intersected with the 15-state ChromHMM data from the Roadmap Epigenomics Project and resultant co-localizations were collapsed from total SNPs down to loci, which were then used to calculate an empirical *P*-value when comparing the original SNPs with the random sets. In addition to examining overall enrichment for all leptin-associated loci combined, we examined the variant-specific overlap with regulatory elements for each of the leptin-associated index SNPs and variants in strong LD (r^2>0.8).

Expression quantitative trait loci. We examined the *cis*-associations of the leptin-associated loci with the expression of nearby genes in the lymphocytes, skin, liver, omental fat, subcutaneous fat and brain tissue (Supplementary Table 5.8). Conditional analyses were performed by including both the leptin-associated SNP and the most significant *cis*-associated SNP in the association model for a given transcript. To minimize the potential for false positives, we only considered associations that reached study-specific Bonferroni-corrected significance threshold (P<0.05/(total number of transcripts tested)).

Pathway analyses

GRAIL analyses: We used GRAIL to identify genes near the leptin-associated loci having similarities in the published scientific text using PubMed abstracts as of December 2006 (ref. [53]). The leptin loci were queried against HapMap release 22 for the European panel and we controlled for gene size.

DEPICT analyses: We used DEPICT to identify the most probable causal gene at a given associated locus, reconstituted gene sets enriched for BMI associations, and tissues and cell types in which genes from associated loci are highly expressed[52]. We clumped GWAS-based meta-analysis summary statistics using 500 kb flanking regions, LD r^2>0.1 and excluded SNPs with $P \geq 1 \times 10^{-5}$. HapMap Project Phase II CEU genotype data were used to compute LD and genomic coordinates were defined by genome build GRCh37.

Knockdown of genes in mouse adipose tissue explants

Materials. Expression analyses were performed on PGAT and SCAT from 4-month-old C57BL/6J mice (derived from Jackson, Stock number 000664) fed chow (Purina PicoLab 5058) or high-fat diet (Research Diets, Inc., D12492i, 60% kcal from fat), to increase adiposity and circulating leptin levels. We measured expression of genes located ±100 kb of each lead variant or genes including SNPs with r^2>0.4 with the lead variant. *Tiparp* was included after its identification by eQTL analysis, and *Ucn* and *Mpv17* were included based on their proximity to variants with r^2>0.4 with the lead variant.

For knockdown experiments, 15-week-old male C57BL/6J mice fed high-fat diet *ad libitum* starting at 6 weeks of age were purchased from Jackson Laboratory (Stock Number 380050, Bar Harbor, ME). Animals were maintained at Columbia University animal facility for up to an additional 5 weeks until they reached ~30% fat mass as determined by time-domain NMR (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany).

Mice were maintained at an ambient temperature of 22 °C–24 °C with a 12-h dark–light cycle (lights on at 0700, h) in a pathogen-free barrier facility. The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

Electroporation and culture of adipose tissue explants. Non-fasted mice were killed at around 20 weeks of age at ~1000, h. PGAT was dissected and minced into 1- to 2-mm fragments. These fragments were evenly distributed into three replicates per control or knockdown condition. Approximately 7–11 fragments were added per well (for a total amount of ~80 mg tissue) in 12-well culture dishes containing 1 ml M199 with Antibiotic-Antimycotic (Anti-Anti, 5 × ; Invitrogen). Following a 20-min incubation in 5 × Anti-Anti media, tissue fragments were washed twice with 1 ml PBS and then transferred to 4 mm Gene Pulser cuvettes (Bio-Rad) and electroporated in 400 µl PBS with 1 nmol siRNA
against *Lep*, *Adig*, *Ift172*, *Mpv17*, *Tiparp* or *Cobll1* (Stealth siRNA, Invitrogen). Non-targeting sequences were used as negative controls (Invitrogen). Electroporation was performed with a Gene Pulser XceII (Bio-Rad) using 50 V, 10^2 wave pulses, with a pulse length of 30 ms and 0.1 ms between pulses[68]. The tissue fragments were subsequently cultured at 37 °C in 5% CO_2 in 12-well plates for 20 h in basal media consisting of M199 media with 10% fetal bovine serum (Invitrogen) plus 1 × Anti-Anti before stimulation for 12 h with basal media plus 7 nM insulin and 25 nM dexamethasone (both from Sigma), to maintain leptin expression in the explants at levels comparable to those of *in vivo* tissues[69]. Knockdown was considered successful if candidate expression was decreased by ≥30%. The effect of insulin and dexamethasone on expression of candidate genes was determined using the same mincing and culturing strategy without electroporation.

Measuring mRNA levels and leptin and adiponectin secretion. Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) using both OligoDT and random hexamer primers. Lightcycler 480 SYBR Green I Master was used for quantitative PCR assays (Roche). Expression of murine homologues of candidate genes in PGAT and SCAT was determined using the $2(-\Delta\Delta C(T))$ method[70]. Gene expression in the knockdown experiments was calculated by Lightcycler 480 software (Roche) based on a standard curve. Primers used are listed in Supplementary Table 5.25. Culture media was collected from the same samples used for RNA analyses. Following the 12 h insulin/dexamethasone stimulation, secreted leptin and adiponectin were measured using the Perkin-Elmer AlphaLISA kits for mouse leptin and adiponectin (according to the manufacturer's protocol). Not all samples were included for adiponectin measurement due to the discontinuation of the AlphaLISA kit by Perkin-Elmer.

Statistics. Each gene knockdown was tested on tissue from 5 to 13 different mice, as indicated. Control and knockdown samples from each mouse were treated as matched pairs. Each data point represents the mean of three replicates from a single mouse. Differences between control and knockdown conditions were calculated by two-way repeated measures analysis of variance using GraphPad Prism 6. *P*-values <0.05 were considered significant.

Results

Stage 1 genome-wide meta-analysis in 32,161 individuals

We first performed a meta-analysis of the results from genome-wide associations between ~2.5 million genotyped and HapMap-imputed single-nucleotide polymorphisms (SNPs) and

circulating leptin levels, including up to 32,161 individuals of European descent from 23 studies (Supplementary Table 5.1). After logarithmic transformation that normalized the distribution of leptin levels and adjusting for age and sex, we carried out association analyses within each study and subsequently meta-analysed the study-specific results. To identify loci associated with circulating leptin levels independently of adiposity, we performed a meta-analysis of genome-wide associations in which we additionally adjusted for BMI. We also performed secondary genome-wide meta-analyses in men (n=13,363) and women (n=18,698) separately, as women generally have higher leptin levels than men, primarily due to larger percentage of body fat and greater subcutaneous fat storage[18].

Two loci, near the *LEP* and *SLC32A1* genes, reached genome-wide significance ($P<5 \times 10^{-8}$) in the BMI-adjusted meta-analysis of men and women combined (Table 5.1). To confirm these associations and to identify additional leptin-associated loci, we took forward all independent (pairwise distance >500 kb and $r^2<0.1$) SNPs reaching$P<10^{-6}$ with leptin levels with or without adjustment for BMI in meta-analyses of all individuals combined, men only or women only, for follow-up in stage 2 (Supplementary Tables 2–4).

Stage 2 follow-up in 19,979 individuals identifies five loci

We examined the associations of the loci taken forward from stage 1 in up to 19,979 additional individuals of European descent from 13 studies (Supplementary Table 5.5). All studies performed the same association analyses as described in Stage 1; that is, with and without adjustment for BMI and in men and women combined, as well as separately. Finally, after performing a joint meta-analysis of the stage 1 and stage 2 results, five independent SNPs reached genome-wide significance ($P<5 \times 10^{-8}$) in the combined meta-analyses of men and women (Table 5.1). In the BMI-adjusted meta-analysis, we confirmed genome-wide significant associations for the loci near *LEP* and *SLC32A1*, and identified an additional locus in *GCKR*. In the BMI-unadjusted meta-analysis, we identified two additional loci near *CCNL1* and in *FTO*. A locus in *COBLL1*, previously identified for association with BMI-adjusted waist–hip ratio (WHR_{adjBMI})[19], blood triglycerides[20] and risk of type 2 diabetes[21], reached $P=1 \times 10^{-6}$ with BMI-unadjusted leptin and $P=2 \times 10^{-6}$ with BMI-adjusted leptin levels, with the leptin-increasing allele being associated with lower WHR_{adjBMI}, triglycerides and risk of type 2 diabetes.

The estimated effects of five of the six loci (in/near *LEP*, *SLC32A1*, *GCKR*, *CCNL1* or *COBLL1*) on leptin levels did not markedly differ in magnitude between the BMI-unadjusted and BMI-adjusted models, suggesting that these associations are not mediated by adiposity *per se* (Figure 5.1). In contrast, the association between the *FTO* locus and leptin levels was completely abolished after adjusting for BMI, indicating that the association with leptin is entirely mediated by the well-established association between *FTO* and BMI[22] (Figure 5.1).

BMI is the most commonly used index of adiposity, but it is not a direct measure of adiposity and it does not distinguish between lean and fat body mass. To assess whether adjustment for a more direct measure of adiposity could enhance our ability to identify adiposity-independent

loci, we performed secondary analyses in 13 studies that had data on both BMI and body fat percentage assessed by dual-energy X-ray absorptiometry or bioimpedance analysis (*n*=18,980 or 59% of stage 1 sample). The analysis showed no marked differences in the effect sizes between the BMI and body fat percentage-adjusted results for the leptin-associated *LEP*, *SLC32A1*, *CCNL1*, *GCKR*, *COBLL1* and *FTO* loci (Supplementary Table 5.6), suggesting that adjustment for BMI as compared with a more direct measure of adiposity did not compromise our ability to identify adiposity-independent leptin-associated loci.

Effects on other traits and potential functional roles

We took forward the genome-wide significant leptin loci near *LEP*, near *SLC32A1*, in *GCKR* and near *CCNL1*, to examine their associations with obesity-related and metabolic traits and to more directly assess their putative roles in the control of circulating leptin. We also took forward the locus near *COBLL1*, given its robust association with WHR_{adjBMI}[19], even though it just missed the genome-wide significance threshold for association with BMI-adjusted and BMI-unadjusted leptin levels (Table 5.1). As the *FTO*-leptin association was completely accounted for by *FTO*'s association with BMI (Figure 5.1), extensively described in the literature[23], we did not include this locus in our follow-up analyses.

To examine the associations of the identified loci with obesity-related and metabolic traits, we performed look-ups in the data from relevant genetic consortia (Supplementary Table 5.7). To study the associations of the leptin-associated loci with the expression of nearby genes, we performed *cis*-expression quantitative trait locus (eQTL) analyses in several human tissues, including the subcutaneous (*n*=776) and omental fat (*n*=742), liver (*n*=567), lymphocytes (*n*=778), brain (*n*=193) and skin (*n*=667) (Supplementary Table 5.8). We also examined the regulatory functions of these loci by studying their enrichment with functional genomic elements

in data from the Roadmap Epigenomics Project[24]. Finally, to identify the causal genes in the leptin-associated loci, we performed *ex vivo* knockdown studies of adipocyte-expressed genes using small interfering RNA (siRNA) in explanted mouse adipose tissue.

Common variation near *LEP* regulates leptin levels

The rs10487505 variant (MAF 49%) is located 21 kb from *LEP* (Figure 5.2a) and is in modest linkage disequilibrium (LD) (r^2=0.4,D'=0.8) with the A19G (rs2167270, MAF 35%) variant that has been extensively studied in candidate gene studies but whose associations with increased levels of leptin and obesity have been inconclusive[13,16]. The leptin-increasing allele of the rs10487505 variant has been nominally associated with weight regain after bariatric surgery in a candidate gene-based analysis of 1,443 patients[25]. Look-ups in consortium data showed a nominally significant association for the leptin-decreasing allele of rs10487505 with higher BMI in the GIANT Consortium (*P*=0.03, *N*=221,677), as well as with increased risk of early-onset obesity (*P*=0.04, *N*=13,848) and higher birth weight (*P*=0.02, *N*=26,836) in the EGG Consortium (Supplementary Table 5.7). Although *LEP* is an obvious candidate gene to account for the association with circulating leptin levels, the rs10487505 variant was not associated with *LEP* messenger RNA expression in the omental or subcutaneous adipose tissue (SCAT), liver, lymphocytes, brain or skin (Supplementary Tables 8 and 9).

A variant in strong LD with rs10487505 (rs6979832, r^2=0.98) overlapped with predicted enhancer elements in all three adipose cell lines of the Roadmap Epigenomics Project[24]. Further, a previous study identified a 465-bp adipocyte-specific enhancer region 4.5 kb upstream from the *LEP* transcription start site by using luciferase assays and chromatin state mapping[26]. This region harbours rs10249476 that is in modest LD with rs10487505 (r^2=0.4,D'=0.8) and reached

the second most significant association with BMI-adjusted leptin levels in stage 1 meta-analysis (P=3 × 10^{-10};n=30,810) (Figure 5.2a).

Collectively, although the locus near *LEP* overlaps with predicted enhancer elements, the lack of association with *LEP* transcript expression in the fasting state suggests that other mechanisms may be involved in mediating the association of this locus with leptin levels, such as an effect on *LEP* expression in the fed state[27] or an effect on leptin protein secretion.

To validate our knockdown strategy for subsequent analyses of candidate genes in loci other than the locus near *LEP*, we used siRNA against *Lep* in mouse adipose tissue explants. Electroporation of the perigonadal adipose tissue (PGAT) explants with siRNA against *Lep* resulted in a 92% decrease in *Lep* mRNA (P<1 × 10^{-4}) and a 92% decrease in secreted leptin (P=4 × 10^{-4}) (Figure 5.4a,b, Supplementary Figure 5.1C,D and Supplementary Table 5.10). In addition, to determine whether electroporation with siRNA altered other secretory function(s) of the perigonadal explants, we measured secretion of adiponectin and found no changes associated with *Lep* knockdown (Figure 5.4d and Supplementary Figure 5.1E).

ADIG may regulate leptin expression

The intergenic rs6071166 variant, ~20 kb from the *SLC32A1* gene (Figure 5.2c), reached genome-wide significance for association with BMI-adjusted leptin levels and has not been previously identified for association with any other traits. In look-ups of genome-wide association study (GWAS) consortium data, we did not find significant association with other obesity-related or metabolic traits (Supplementary Table 5.7). The rs6071166 variant was not associated with the mRNA expression of nearby genes in the adipose tissue, liver, lymphocytes, brain or skin (Supplementary Tables 5.8 and 5.9).

To identify the potential causal gene in this locus using the mouse PGAT explant model described above, we first measured the expression levels of murine homologues of genes surrounding the lead variant associated with circulating leptin levels. We tested PGAT and SCAT of 4-month-old C57BL/6J mice fed chow or high-fat diet (Figure 5.3c,d). In addition, we analyzed candidate gene expression in other tissues (liver and hypothalamus) that we predicted could play a role in circulating leptin levels via effects on leptin clearance or response (Supplementary Figure 5.7). Genes were considered strong candidates if they were highly expressed in adipose tissue and/or if they were regulated by high-fat diet feeding in a manner similar to *Lep*. This analysis identified *adipogenin* (*Adig*) as a candidate gene in the *SLC32A1* locus; *Adig* is highly expressed in the adipose tissue, in contrast to other nearby genes. To test whether *Adig* affected *Lep* expression, we performed *ex vivo* knockdown studies using siRNA against *Adig* in mouse PGAT explants. We found that knockdown of *Adig* decreased *Lep* expression by 26% ($P=4 \times 10^{-4}$) and leptin secretion by 23% ($P=0.003$), consistent with a causal role for *ADIG* in control of circulating leptin levels (Figure 5.4a,b and Supplementary Figure 5.2C,D).

ADIG is located ~116 kb from the rs6071166 variant and encodes a cytoplasmic adipocyte protein adipogenin, that is, similar to leptin, highly and specifically expressed in the adipose tissue[28,29,30] and upregulated by treatment with insulin and glucose[30]. *Adig* expression is also strongly upregulated in murine 3T3-L1 preadipocytes during *in vitro* differentiation into adipocytes[28,29]. Two studies have investigated the effect of *Adig* knockdown on the differentiation of 3T3-L1 cells and expression of *Pparγ2*, a master regulator of adipocyte differentiation, but with conflicting results; whereas the first study found *Adig* knockdown to block adipocyte differentiation and decrease *Pparγ2* expression[28], a later study found no similar

changes[30]. When we measured *Pparγ2* expression following *Adig* knockdown in PGAT explants containing mature adipocytes, we did not see a change as compared with controls (Supplementary Figure 5.2F).

Common variation in *GCKR* regulates leptin levels

Variants ($r^2 \geq 0.9$ with our lead SNP rs780093) of the leptin-associated locus in *GCKR* have previously shown genome-wide significant associations with more than 25 metabolic traits; the leptin-increasing allele has been associated with increased fasting glucose and fasting insulin but decreased 2-h glucose and higher high-density lipoprotein cholesterol, and lower total cholesterol, low-density lipoprotein cholesterol, triglycerides, C-reactive protein and circulating uric acid levels, among others (Supplementary Table 5.11). The *GCKR* gene encodes a regulatory protein in the liver that inhibits the activity of glucokinase, the enzyme responsible for regulating the uptake, metabolism and storage of circulating glucose[31]. A putative causal variant in this gene is the common nonsynonymous Pro446Leu variant (rs1260326), for which rs780093 acts as a good proxy (r^2=0.9). Carriers of the glucose-lowering Leu allele have a reduced ability to sequester and inhibit glucokinase and a blunted response to fructose 6-phosphate, both of which favour the generation of free and active cytoplasmic glucokinase[32].

The mechanisms that might link changes in *GCKR* function to leptin levels are not known. As insulin increases leptin secretion from adipocytes[33] and the *GCKR* locus is strongly associated with circulating levels of insulin[34], the association of the *GCKR* locus with leptin levels could be the consequence of the GCKR locus' effect on insulin levels. The leptin-increasing allele of the rs780093 variant was significantly associated with higher levels of fasting insulin in studies included in our stage 2 meta-analyses ($P=2 \times 10^{-5}$, N=8,953). To test whether insulin mediated the association of rs780093 with circulating leptin levels, we analysed the association of

rs780093 with leptin, while adjusting for fasting insulin levels. Although the effect size was somewhat attenuated, the association of rs780093 with BMI-adjusted leptin levels remained significant after adjustment for fasting insulin (β=0.047, P=2 × 10^{-4} versusβ=0.034, P=0.004 before and after the adjustment, respectively), suggesting that the association of the *GCKR* locus with leptin is at least in part independent of effects on insulin levels.

Although *GCKR*'s function renders it a potential candidate among the genes in this region, *cis*-eQTL analyses showed association of the leptin-increasing allele of rs780093 with increased expression of the nearby *IFT172* in the liver (P=7 × 10^{-30}), omental fat (P=6 × 10^{-64}) and subcutaneous fat (P=3 × 10^{-52}) (Supplementary Table 5.9). The rs780093 variant is, however, only in moderate LD (r^2=0.4) with the peak SNP influencing *IFT172* expression in the region and the peak SNP remained significantly associated with *IFT172*expression after adjustment for rs780093, whereas the association of rs780093 was abolished after adjustment for the peak SNP (Supplementary Table 5.9).

Because of the observations in human tissues, we examined *Ift172* in the mouse explant model. *Ift172* was not highly expressed in mouse PGAT or SCAT and levels were not upregulated by high-fat diet (Figure 5.3e,f). *Ift172* was, however, upregulated in the liver under high-fat diet feeding (Supplementary Figure 5.7E). Knockdown of *Ift172* in PGAT explants decreased *Lep* mRNA expression by 22% (P=0.02), but did not decrease leptin protein secretion (P=0.6) (Figure 5.4a,b and Supplementary Figure 5.3C,D). *IFT172* is known to play a major role in assembly and maintenance of primary cilia that act as critical signaling hubs for cellular pathways during development[35]. Knockout of *Ift* genes in central neurons causes obesity in mice[36]and obesity is a clinical feature in two human ciliopathic syndromes, the Alström and Bardet–Biedl syndromes[37,38]. In the hypothalamus, alterations in the function of the primary

cilium lead to impaired leptin signalling[39]. Therefore, we cannot exclude a role for *IFT172* in the regulation of circulating leptin levels.

Another nearby gene, *MpV17* mitochondrial inner membrane protein, is a potential candidate in the region based on its expression in mice fed chow or high-fat diet; *Mpv17* expression was increased by high-fat diet, in a manner similar to *Lep* (Figure 5.3c,d). However, knockdown of *Mpv17* did not change *Lep* mRNA expression (P=0.2) or leptin secretion (P=0.2) by PGAT explants (Figure 5.4a,b and Supplementary Figure 5.4C,D), suggesting that the involvement of *MPV17* in leptin regulation is unlikely.

Locus near *CCNL1* regulates leptin levels and birth weight

The leptin-decreasing allele of rs900400, located 67 kb upstream from *CCNL1* (Figure 5.2d), was previously reported for its association with lower birth weight[40]. This cross-phenotype association could indicate a mechanism that is shared between birth weight and leptin levels in adulthood. Fetal adipose tissue is capable of producing leptin[41] and fetal leptin levels are correlated with fetal fat mass[42,43]. Placenta provides an additional source of leptin for the fetus, however, and it has been suggested that leptin could mediate fetal growth[44,45]. Assuming that leptin levels track from birth through adulthood, increased leptin levels could drive the association of the *CCNL1* locus with birth weight. Other studies suggest that leptin production is decreased in cultured adipocytes from men born with a low birth weight[46]. Therefore, the association of the *CCNL1* locus with leptin levels in adulthood could be mediated by its association with birth weight.

Although *CCNL1* is the nearest gene to rs900400, our *cis*-eQTL analyses identified rs900400 as the variant most significantly associated with the expression of another nearby gene, *TIPARP* (Supplementary Table 5.9). The *TIPARP* gene encodes a poly (ADP-ribose)

polymerase involved in DNA repair. The leptin-increasing allele of rs900400 was associated with lower *TIPARP* expression in omental fat (3×10^{-30}) and subcutaneous fat ($P=7 \times 10^{-58}$) (Supplementary Table 5.9). *Tiparp* was also implicated as a causal gene by our expression analysis of mouse adipose tissue and its expression was increased in SCAT and liver in mice fed with high-fat diet (Figure 5.3h and Supplementary Figure 5.7G). Knockdown of *Tiparp*in mouse PGAT explants did not, however, significantly alter the expression of *Lep* mRNA (P=0.7) or leptin secretion (P=0.8) (Figure 5.4a,b and Supplementary Figure 5.5D,E). Although we attempted to use SCAT for explant knockdown studies, high intra-depot variability compromised this approach. Interestingly, stimulation of the explants with insulin and dexamethasone increased explant expression of *Tiparp* by 50% (P=0.003) over incubation in basal media alone, in a manner similar to *Lep* expression (Figure 5.4c and Supplementary Figure 5.5A). Collectively, although *TIPARP* remains a putative causal gene within the locus near *CCNL1*, further evidence is required to confirm its role in the regulation of circulating leptin levels.

COBLL1 or *GRB14* may regulate leptin levels

The intronic rs6738627 variant in *COBLL1* (Figure 5.2e) did not reach genome-wide significance for the association with leptin levels (Figure 5.1 and Table 5.1). However, as previous GWAS have shown robust associations of the leptin-increasing allele with a lower WHR_{adjBMI}[19], we chose to take it forward for follow-up analyses, to examine the role of leptin levels in the previous associations.

Look-ups in data from genetic consortia showed a strong association of the leptin-increasing allele of rs6738627 with higher body fat percentage ($P=2 \times 10^{-8}$, n=76,338; Supplementary Table 5.7). As reported previously, the rs6738627 variant was also strongly associated with decreased WHR_{adjBMI} ($P=2 \times 10^{-8}$, n=174,672; Supplementary Table 5.7), suggestive of a

preferential gluteal rather than abdominal fat storage, which may contribute to the association of rs6738627 with increased leptin levels[47].

In PGAT and SCAT expression analyses in mice, we found an upregulation of *Cobll1* in high-fat diet-fed mice in both depots (Figure 5.3i,j). Although knockdown of *Cobll1* in the perigonadal explants did not influence *Lep* mRNA expression (P=0.2), it did decrease leptin protein secretion by 16% (P=3 × 10^{-4}, Figure 5.4a,b), suggesting a potential causal role for *Cobll1*. In addition, stimulation of explants with insulin and dexamethasone increased explant expression of *Cobll1* by 78% (P=0.004) over incubation in basal media alone (Figure 5.4c and Supplementary Figure 5.6A). *COBLL1* is known to be involved in neural tube formation[48], but its possible functions in adipose tissue are unknown.

In human eQTL analyses, the leptin-increasing allele of the *COBLL1* locus showed an association with lower expression of *GRB14* in omental fat (P=5 × 10^{-12}) and subcutaneous fat (P=3 × 10^{-5}) (Supplementary Table 5.9). We did not, however, find high expression of *Grb14* in PGAT or SCAT explants from mice and the levels were not regulated by high-fat diet feeding (Figure 5.3i,j). The protein product of *GRB14* is the growth factor receptor-bound protein 14 that binds directly to the insulin receptor and inhibits insulin signalling[49]. The adipose tissue expression of *GRB14* may play a role in regulating insulin sensitivity[50]. Grb14-deficient mice exhibit improved glucose tolerance, lower circulating insulin levels and increased incorporation of glucose into glycogen in the liver and skeletal muscle[51]. Both *COBLL1* and *GRB14* are thus possible candidates to account for the association of the *COBLL1* locus with leptin levels.

Enrichment with pathways and regulatory elements

We used the Data-driven Expression Prioritized Integration for Complex Traits (DEPICT) software[52] to identify enrichment of gene sets and pathways across loci reaching P<1 × 10^{-5} for

association with leptin levels. However, none of our findings reached the false discovery rate threshold of 5% (Supplementary Tables 12–17). Next, we used the Gene Relationships Across Implicated traits (GRAIL) tool[53] to identify genes near the leptin-associated loci having similarities in the text describing them within published article abstracts. However, no statistically significant results were found in these analyses either (Supplementary Tables 18 and 19). Finally, we used the Uncovering Enrichment Through Simulation method[54] to test for the overall enrichment of leptin-associated loci reaching $P<10^{-5}$ with ChromHMM annotations for adipose and brain tissues available from the Roadmap Epigenomics Project[24]. However, we did not find significant enrichment of our leptin-associated loci in any chromatin states once corrected for multiple testing (Supplementary Table 5.20). The lack of significant findings may be due to the small number of loci identified and the limited knowledge available on leptin-regulating pathways in adipose tissue.

Established adiposity loci and leptin

Circulating leptin levels correlate closely with BMI and other measures of adiposity[3]. The most recent GWAS meta-analysis for BMI, including nearly 340,000 individuals, identified 97 loci that reached genome-wide significance[22]. Of the 97 BMI-increasing loci, 89 showed a directionally concordant association with increased BMI-unadjusted leptin levels ($P_{binomal}=2 \times 10^{-18}$), of which 25 reached nominal significance (Supplementary Table 5.21). Previous GWAS of extreme and early-onset obesity have identified 12 genome-wide significant loci[55,56,57,58]. Of these, ten showed a directionally consistent association with increased BMI-unadjusted leptin levels ($P_{binomal}=0.04$), of which five reached nominal significance (Supplementary Table 5.21).

We also examined leptin associations for 49 loci identified in GWAS for WHR_{adjBMI}, a measure of body fat distribution independent of overall adiposity[19]. Of the 49 WHR_{adjBMI}-increasing loci,

only 24 showed a directionally concordant association with increased BMI-adjusted leptin levels (Supplementary Table 5.22). As the distribution of body fat differs between men and women, we also examined the leptin associations for the 49 WHR_{adjBMI} loci in men and women separately. There was no enrichment of leptin associations in either of the sexes, with 27 loci showing a directionally concordant association with increased leptin levels in men and 20 loci in women (Supplementary Table 5.22).

Discussion

In a meta-analysis of genetic association data in up to 52,126 individuals, we identified 5 common loci associated with circulating leptin levels. In addition, a locus near *COBLL1*, previously identified for association with a lower WHR_{adjBMI}[19], reached $P=1 \times 10^{-6}$ for association with increased leptin levels. Even though leptin correlates strongly with adiposity, we did not identify loci previously associated with BMI, other than *FTO*, despite having a sample size similar to early GWAS meta-analyses of BMI that identified multiple loci[59]. On the contrary, five of the six loci we identified were associated with leptin independently of BMI or body fat percentage. Our findings indicate that genetic mechanisms not influencing adiposity may have an important role in the regulation of circulating leptin levels.

Our strongest adiposity-independent leptin signal was near *LEP*, but we also identified leptin-associated variants in four other genomic loci, providing evidence that mechanisms other than those that involve *LEP per se* may regulate leptin production and release from adipose tissue. In one of these loci, near *SLC32A1*, our knockdown studies indicated a role for *adipogenin*, a gene involved in the regulation of adipocyte differentiation[28,29]. Although adipogenin was identified as

a potent regulator of adipogenesis a decade ago[28,29], our results provide the first evidence linking this function to leptin regulation. We anticipate that our findings will motivate and inform eventual testing of *Adig* by transgenic manipulation in mice.

No clear effect on leptin production was seen following knockdown of candidate genes in the *GCKR* and *CCNL1* loci, which may indicate that the gene implicated by position plays no role in the phenotype, or that the effect was undetectable in our experimental conditions. Alternatively, the association with leptin levels may be explained by effects of non-coding elements on other genes outside the implicated genetic interval, or by inter-species differences. Furthermore, although adipose tissue is the most direct contributor to circulating leptin levels, the effect of the causal gene may be conveyed by another tissue; leptin production and secretion are influenced by insulin, catecholamines and other hormones, as well as paracrine effects of local inflammatory cells on adipocytes[60].

Although the locus near *SLC32A1* had not been identified previously for association with other traits, the leptin-associated loci in/near *GCKR*, *CCNL1* and *COBLL1* have been associated with multiple obesity-related and metabolic traits[19,20,34,40,61]. These cross-phenotype associations may either reflect pleiotropy, where a gene product influences multiple traits, and/or mediation effects, where one phenotype is causally related to a second phenotype. For example, the association of the pleiotropic *GCKR* locus with leptin levels may be partly mediated through *GCKR*'s role in the regulation of glucose homeostasis and insulin levels[34,61], which may influence leptin production and secretion in adipose tissue[33]. The *COBLL1* locus is strongly associated with decreased WHR_{adjBMI}, indicative of a preferential accumulation of gluteal subcutaneous fat, which may contribute to the observed association with circulating leptin levels[47]. The identification of the birth weight locus, *CCNL1*, as a leptin-regulating locus may

provide an intriguing link between leptin regulation and fetal growth, albeit such a link remains to be more firmly established[45].

Unraveling the polygenic basis of leptin production could provide opportunities for targeted leptin supplementation in obese individuals. Although leptin therapy is an efficient weight-loss treatment for obese individuals with congenital leptin deficiency, the beneficial effects of leptin supplementation do not translate to all obese patients[62]. Sensitivity to changes in circulating concentration of leptin may be enhanced at very low values[11] where a relatively small increase in leptin production may be sensed by the homeostatic feedback system that controls energy balance. As a substantial minority of individuals with common forms of obesity, not associated with leptin mutations, have relatively low levels of circulating leptin[63], augmenting leptin levels in this subgroup could be therapeutically worthwhile. Identification of leptin-regulating loci may provide new tools for identifying obese individuals with susceptibility to low leptin levels and who may benefit of leptin treatment.

In 2010, Sun *et al.*[64] identified two common non-synonymous SNPs in the leptin receptor (*LEPR*) gene associated with leptin receptor levels. Leptin receptor plays an essential role in mediating the physiological effects of leptin. Although some studies have described abnormally high circulating leptin levels in carriers of rare *LEPR* mutations[65], others have not[66]. We did not find association between *LEPR* variants and circulating leptin levels, suggesting that common variants in *LEPR* are not important regulators of circulating leptin levels.

Our meta-analyses were limited by the number of available studies with leptin data, imputation by HapMap reference panel for autosomal chromosomes and the fact that we examined additive effects only. In addition, we corrected for adiposity by adjusting for BMI, which is a heterogeneous measure of adiposity as it does not account for individual differences in body fat

and lean mass. Future discovery efforts in extended sample sizes based on genome-wide imputation of 1000 Genomes reference panels, which include X and Y chromosomes and which also test for recessive and dominant inheritance, will allow for the discovery of more and lower-frequency variants, and for refining association signatures of already established leptin-associated loci.

In summary, we identified six genetic loci associated with circulating leptin levels, of which five showed associations independently of adiposity. Our findings represent a step forward in the understanding of biological mechanisms regulating leptin production in adipose tissue and open new avenues for examining the influence of variation in leptin levels on adiposity and metabolic health.

Tables

SNP	Nearest gene	Chr:Position	Effect/ Other allele	EAF	Leptin unadjusted for BMI								Leptin adjusted for BMI							
					Stage 1		Stage 2		Stage 1+2				Stage 1		Stage 2		Stage 1+2			
					P	N	P	N	Beta	SE	P	N	P	N	P	N	Beta	SE	P	N
rs10487505	*LEP*	7:127647399	G/C	0.50	4.0E-06	29470	2.0E-01	17110	0.023	0.005	9.0E-06	46580	2.7E-11	29255	5.2E-03	16781	0.029	0.004	2.0E-12	46036
rs780093	*GCKR*	2:27596107	C/T	0.61	1.8E-07	32147	3.6E-04	19979	0.032	0.005	2.3E-10	52126	6.3E-07	31802	1.4E-04	19648	0.024	0.004	3.8E-10	51450
rs900400	*CCNL1*	3:158281469	T/C	0.60	7.3E-07	32128	1.7E-03	19685	0.030	0.005	5.6E-09	51813	9.8E-07	31785	1.7E-02	19354	0.021	0.004	1.2E-07	51139
rs6071166	*SLC32A1*	20:36766426	C/A	0.37	2.0E-07	29471	1.6E-01	17007	0.027	0.006	6.6E-07	46478	2.9E-08	29256	3.9E-02	16678	0.024	0.004	1.8E-08	45934
rs6738627	*COBLL1*	2:165252696	A/G	0.37	8.3E-07	25573	8.6E-02	19573	0.027	0.006	1.4E-06	45146	4.6E-06	25229	4.5E-02	19242	0.020	0.004	1.9E-06	44471
rs8043757	*FTO*	16:52370951	T/A	0.40	8.8E-08	32120	2.7E-03	19919	0.030	0.005	1.1E-09	52039	6.1E-01	31776	7.5E-01	19588	0.001	0.004	8.4E-01	51364

Leptin (µg/ml) was logarithmically transformed. The study-specific analyses were performed with linear regression models while accounting for sex, age and age ^{2}. Meta-analyses of the study-specific results were performed using fixed effects meta-analysis. Beta refers to the change in logarithmically transformed leptin per each copy of the effect allele. EAF, effect allele frequency

Table 5-1 Meta-analysis results in men and women combined for the genome-wide significant leptin-associated loci and for the locus in COBLL1.

Figures and Figure Legends

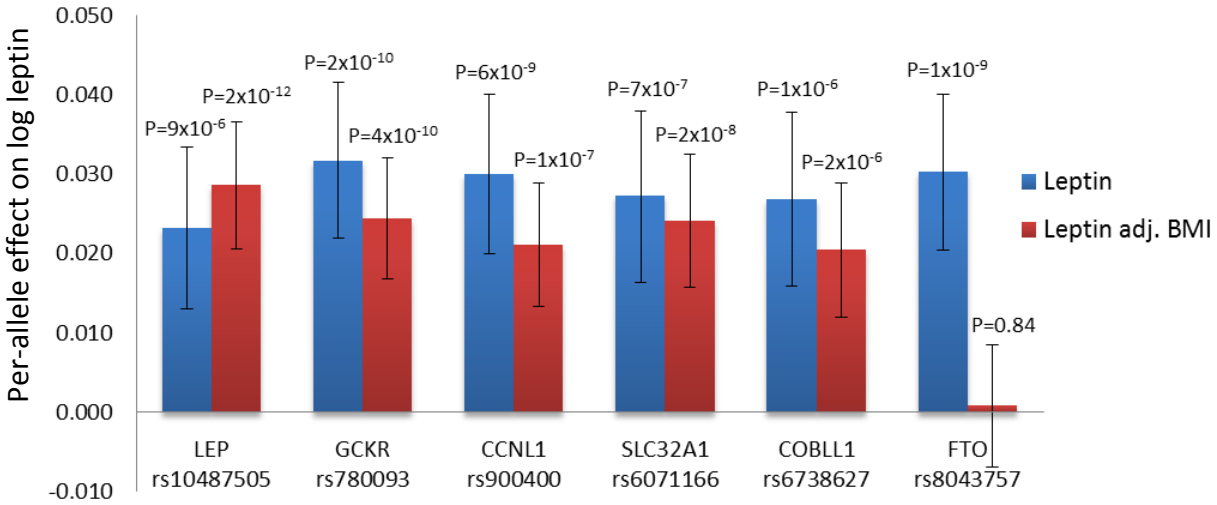


Figure 5-1 Association of genome-wide significant loci.

Association of genome-wide significant loci and the locus in COBLL1 with BMI-adjusted and unadjusted leptin levels in a meta-analysis of Stage 1 and Stage 2 combined.

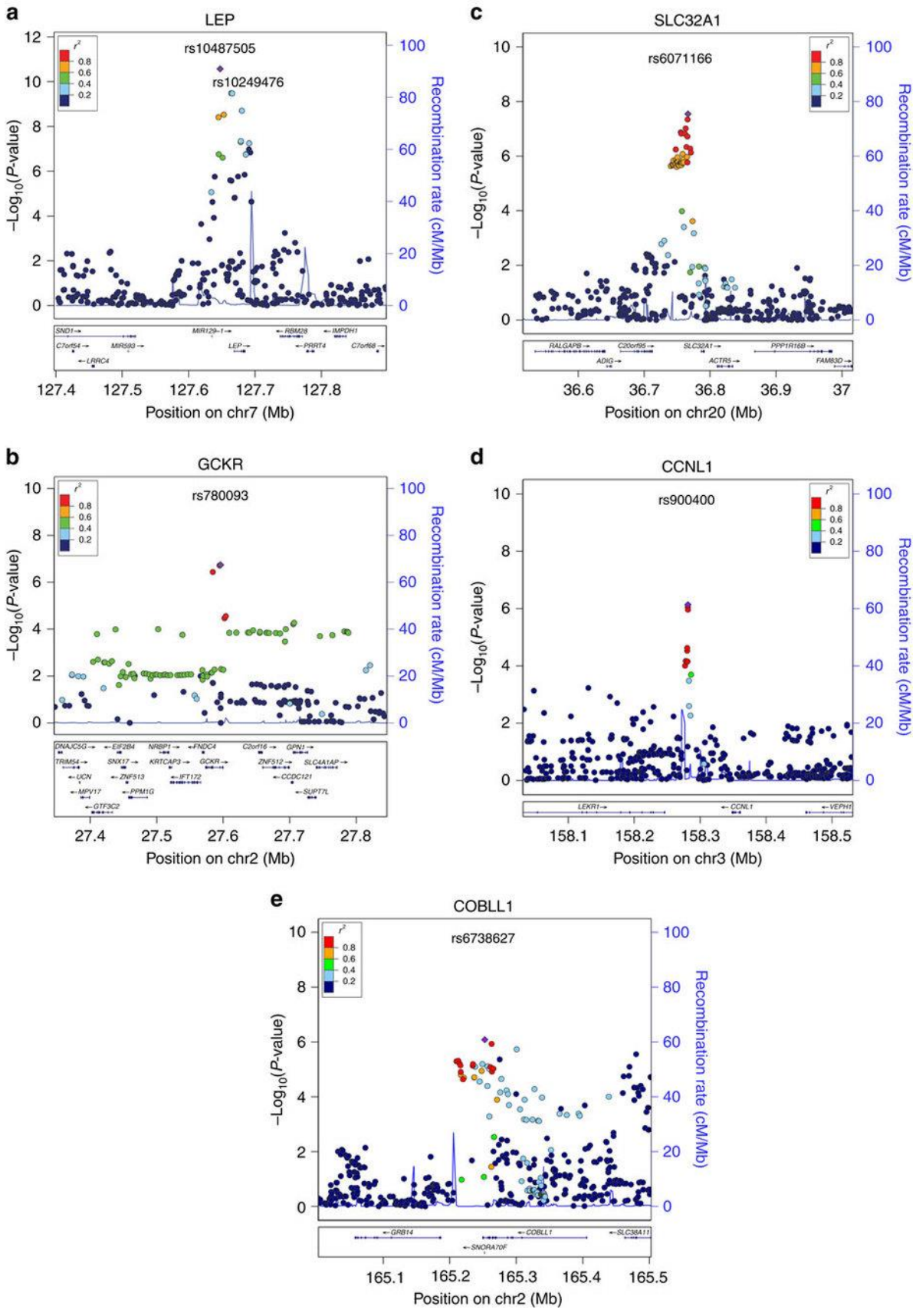

a
LEP
rs10487505
rs10249476
−Log10(P-value)
Recombination rate (cM/Mb)
Position on chr7 (Mb)
b
GCKR
rs780093
Position on chr2 (Mb)
c
SLC32A1
rs6071166
Position on chr20 (Mb)
d
CCNL1
rs900400
Position on chr3 (Mb)
e
COBLL1
rs6738627
Position on chr2 (Mb)

Figure 5-2 Regional plots for the loci associated with circulating leptin concentrations.

Regional plots for the loci in or near *LEP* (**a**), *GCKR* (**b**), *SLC32A1* (**c**) and *CCNL1* (**d**), which reached genome-wide significance in the combined meta-analysis of Stage 1 and Stage 2 for BMI-unadjusted or BMI-adjusted leptin levels. The *COBLL1* locus (**e**) that reached $P=1 \times 10^{-6}$ with BMI-unadjusted and $P=2 \times 10^{-6}$ with BMI-adjusted leptin levels is also shown. For the locus near *LEP* (A), the rs10249476 SNP, located in a previously identified adipocyte-specific enhancer region[26], is indicated.

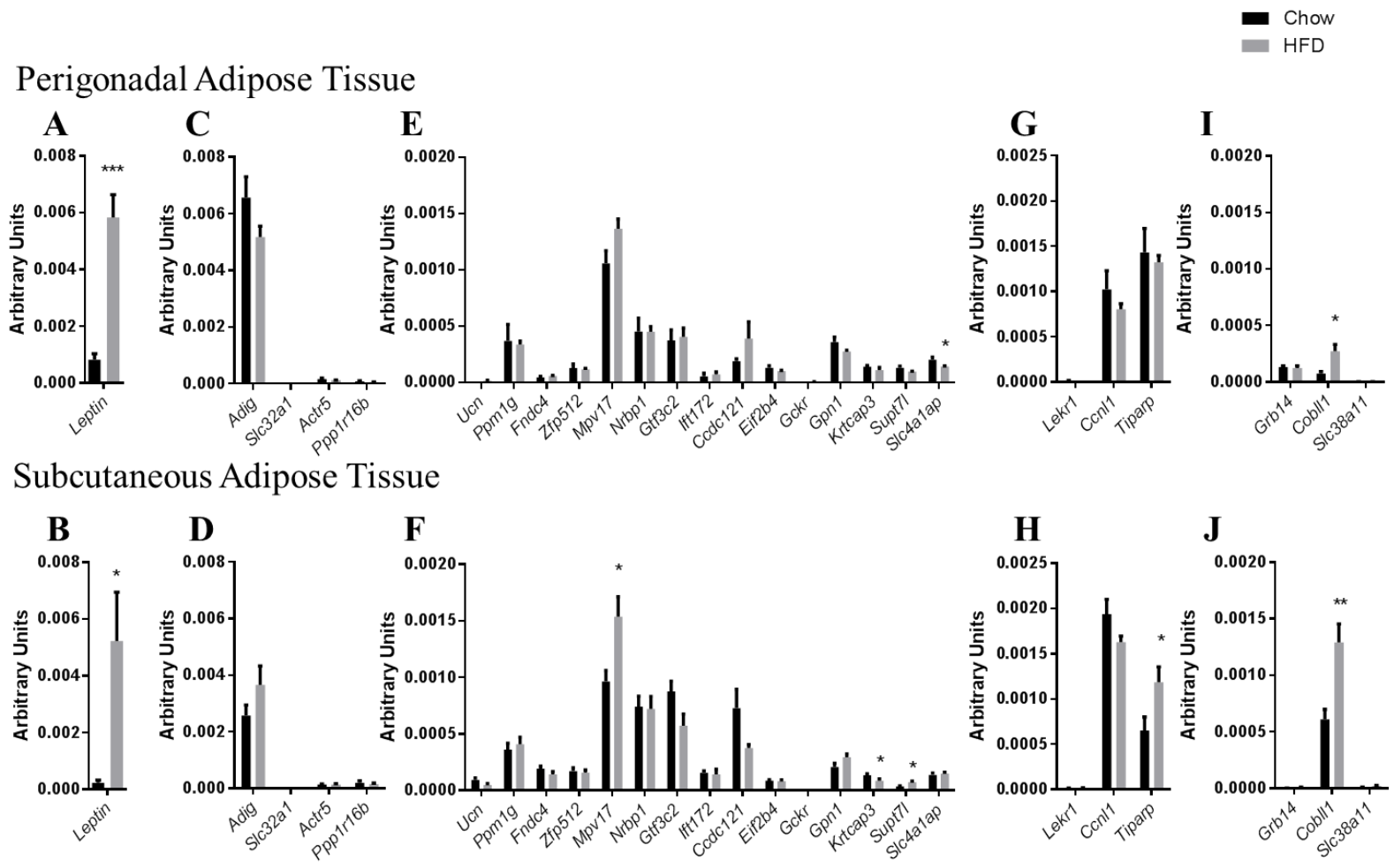


Figure 5-3 Expression of murine homologues of candidate genes.

Expression of murine homologues of genes located within *Lep* (**a**,**b**), *Slc32a1* (**c**,**d**), *Gckr* (**e**,**f**), *Ccnl1* (**g**,**h**) and *Cobll1* loci (**i**,**j**) in PGAT and SCAT from 4-month-old mice fed chow (black bars) or high-fat diet (HFD; grey bars). Quantitative PCR (qPCR) transcripts were normalized using *ActB, Rplp0, Gapdh* and *Ppia* as housekeeping genes. *N*=5 mice per group. T-test. **P*<0.05, ***P*<0.01 and ****P*<0.001.

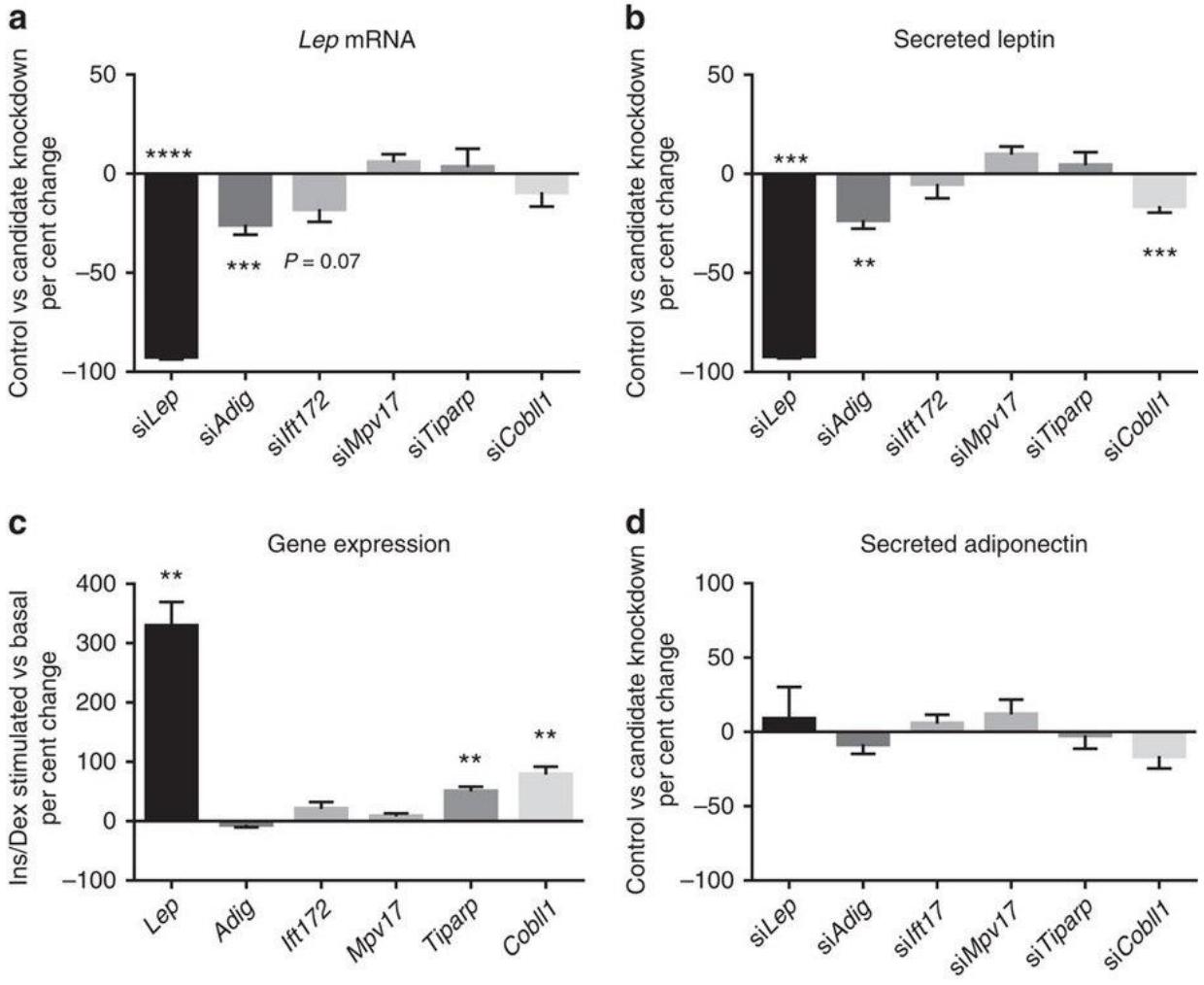


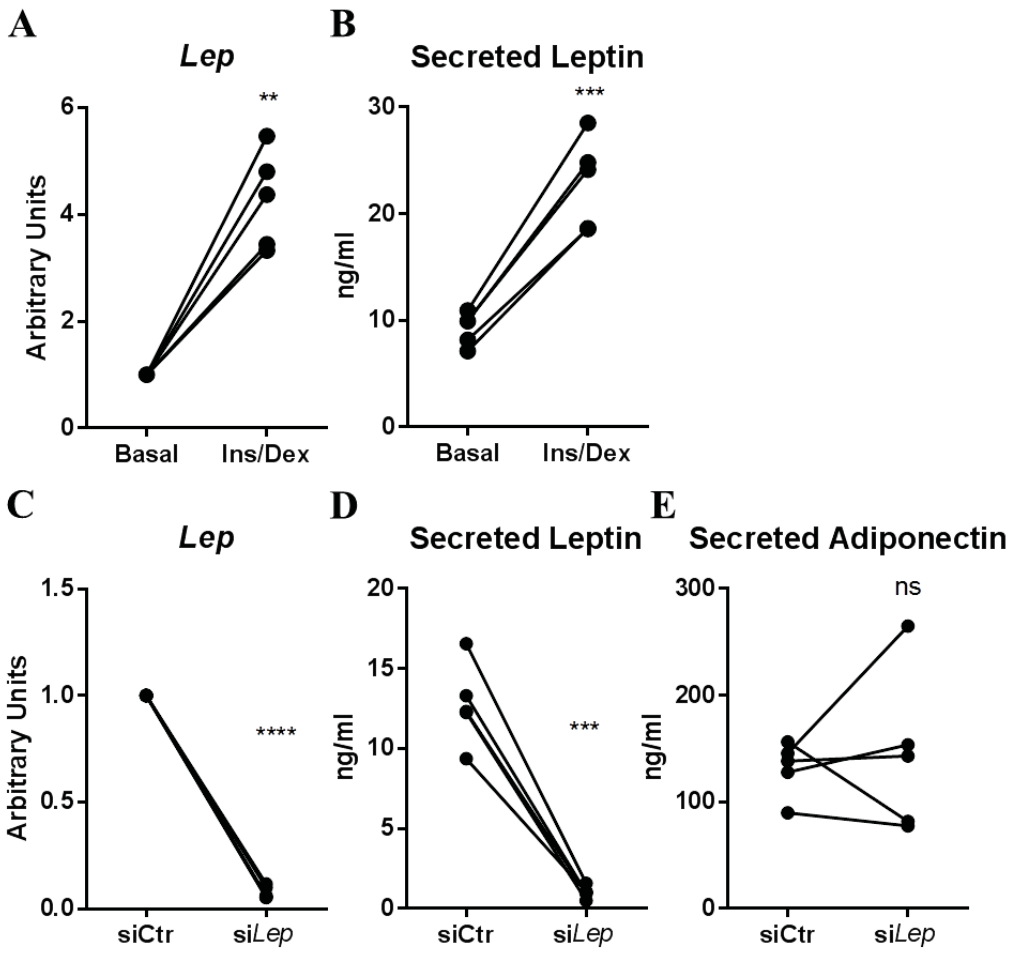
Figure 5-4 Candidate gene knockdown studies in PGAT explants.

Changes in *Lep* mRNA expression (**a**) and secretion into media (**b**) following candidate gene knockdown in PGAT explants following stimulation with insulin and dexamethasone for 12 h. Gene expression induced by stimulation with insulin and dexamethasone (**c**) *N*=5–13 mice per group (3 replicates/condition/mouse). Secreted adiponectin was measured as a control for non-leptin secretory function (**d**) *N*=5 mice per group. Two-way repeated measures analysis of variance (ANOVA). **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001.

Supplementary Figures

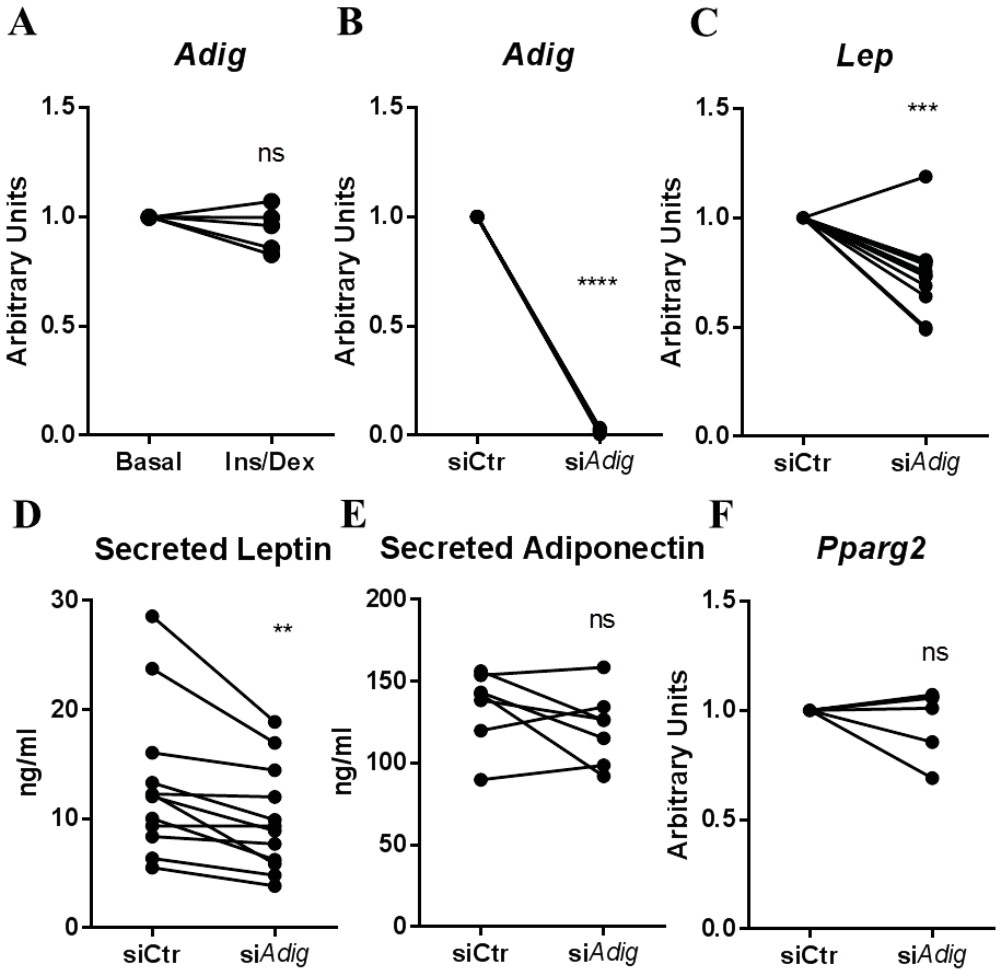
(For complete supplementary material please refer to:

https://www.nature.com/articles/ncomms10494#supplementary-information)



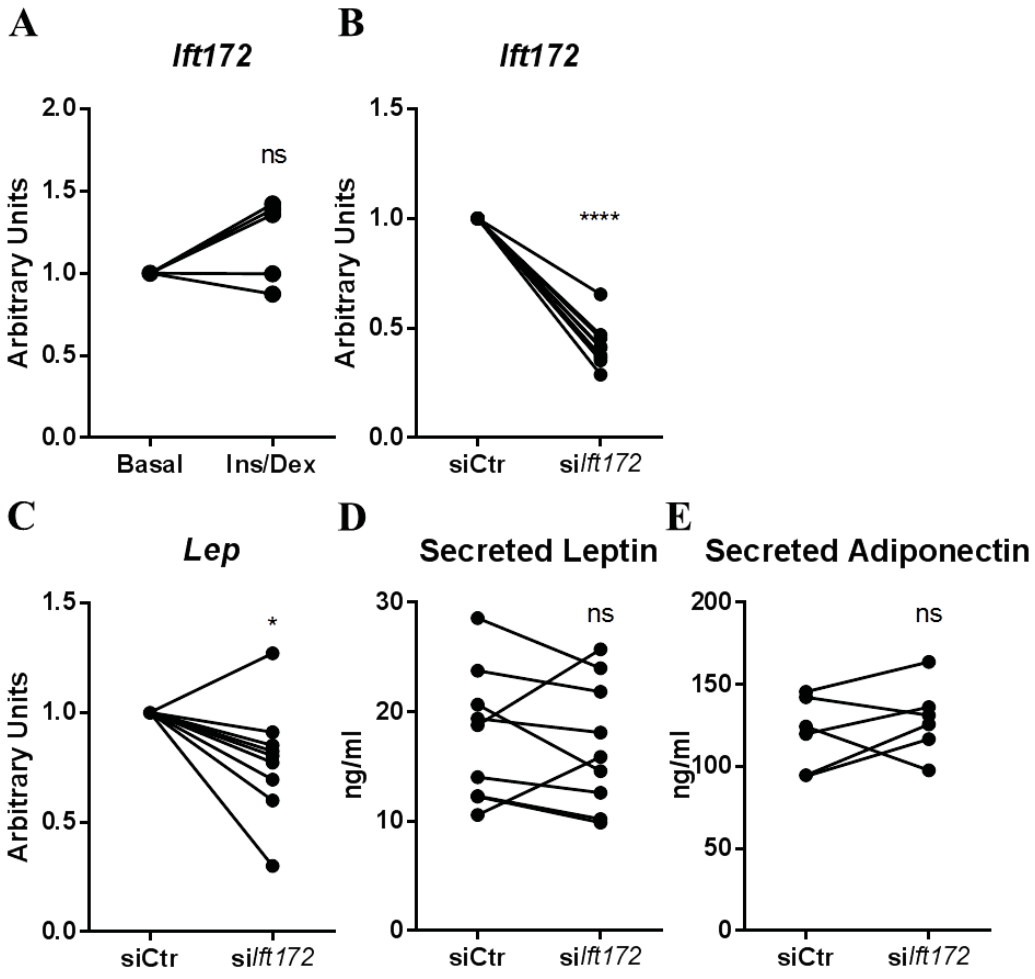
Supplementary Figure 5-1 Effects of Lep knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.

Lep transcription (A) and secretion (B) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Lep* knockdown (C) decreased LEP secretion (D) following stimulation with insulin and dexamethasone for 12 hours. Adiponectin secretion (E) remained unchanged. N=5 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



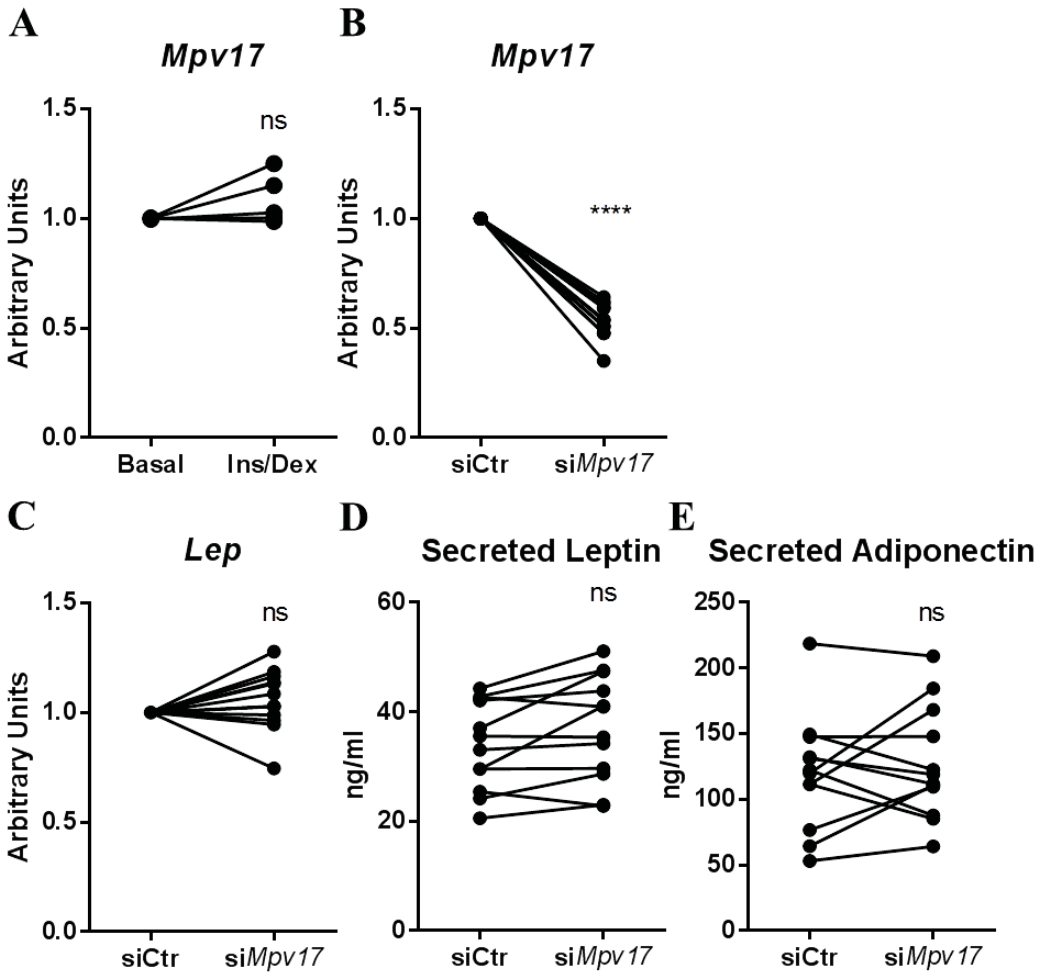
Supplementary Figure 5-2 Effects of Adig knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.

Adig expression (A) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Adig* knockdown (B) decreased both *Lep* expression (C) and leptin secretion (D) following stimulation with insulin and dexamethasone for 12 hours. N=12 mice per group. Adiponectin secretion (E) remained unchanged. N=7 mice per group. *Pparg2* expression (F) was unchanged as well. N=6 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



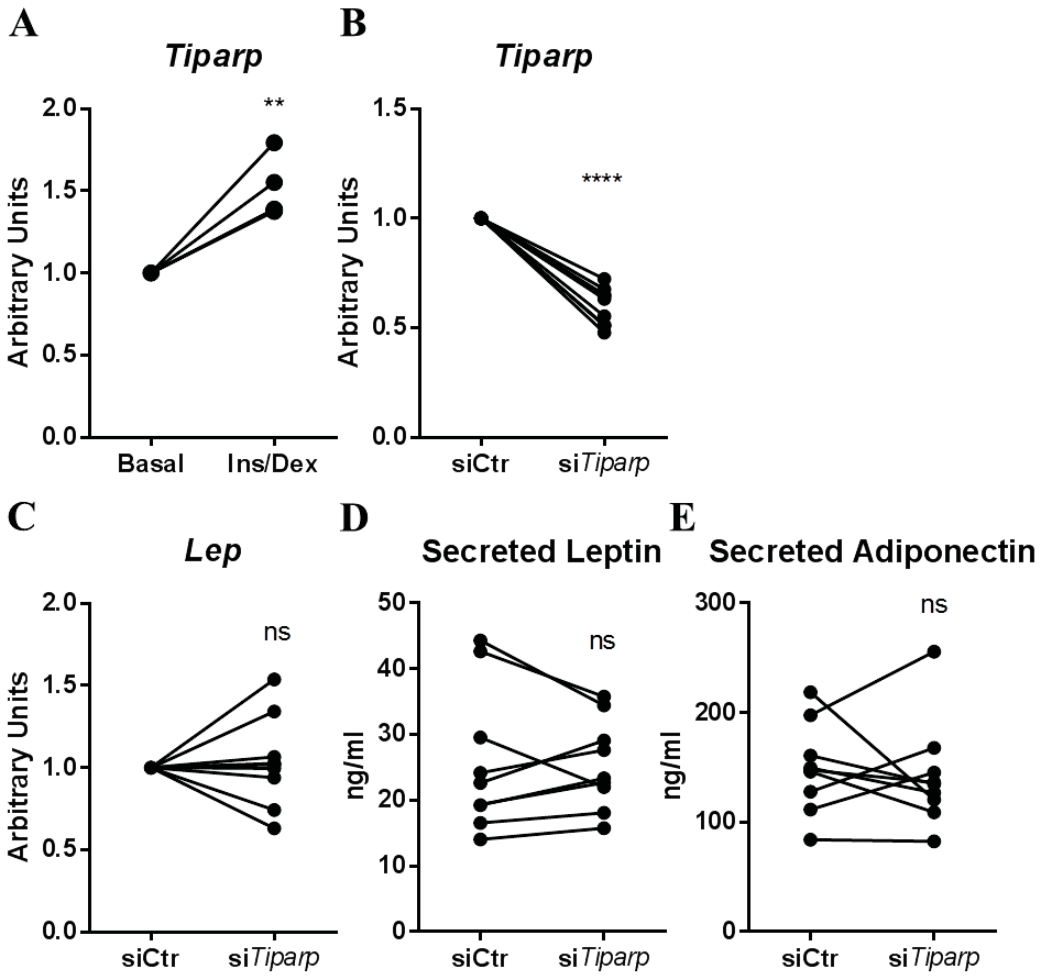
Supplementary Figure 5-3 Effects of Ift172 knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.

Ift172 expression (A) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Ift172* knockdown (B) decreased *Lep* expression (C) but did not change leptin secretion (D) following stimulation with insulin and dexamethasone for 12 hours. N=9 mice per group. Adiponectin secretion (E) remained unchanged. N=6 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



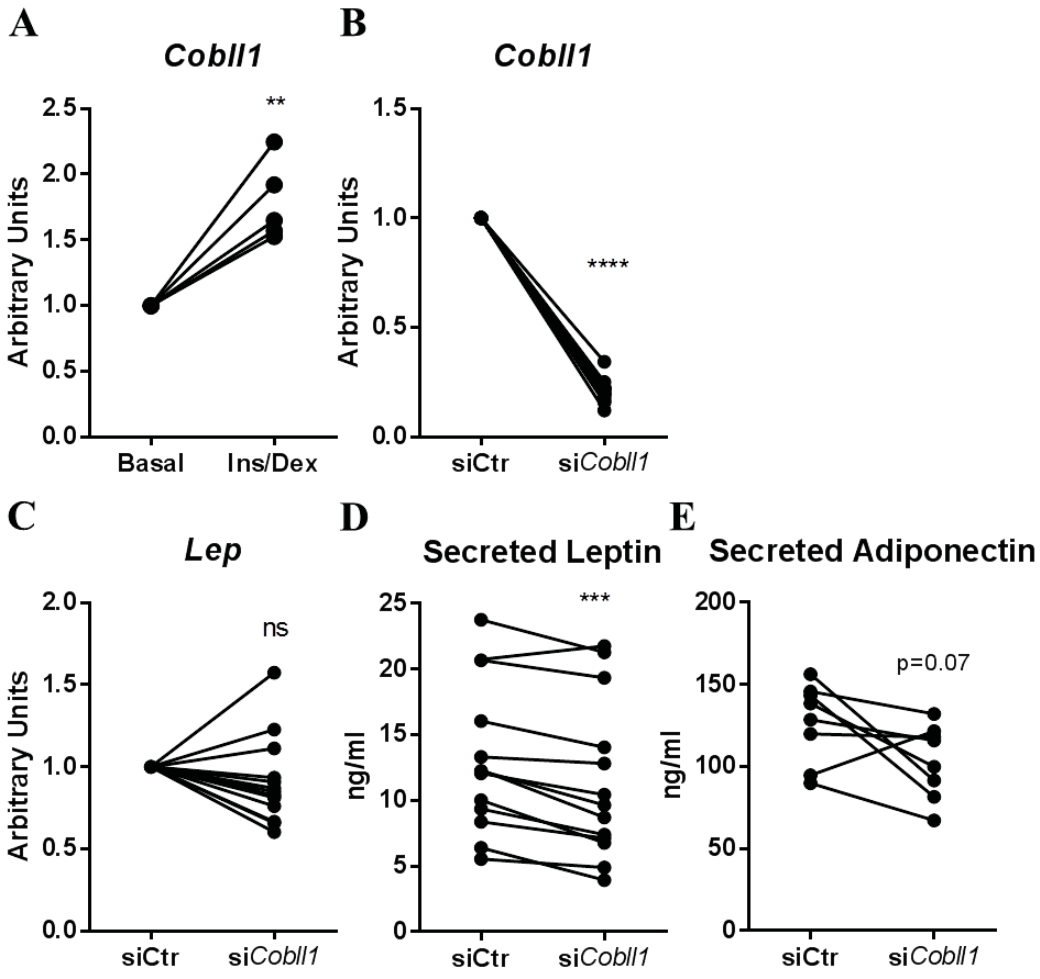
Supplementary Figure 5-4 Effects of Mpv17 knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.

Mpv17 expression (A) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Mpv17* knockdown (B) did not change *Lep* expression (C) or secretion (D) following stimulation with insulin and dexamethasone for 12 hours. Adiponectin secretion (E) remained unchanged. N=12 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplementary Figure 5-5 Effects of Tiparp knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.

Tiparp expression (A) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Tiparp* knockdown (B) did not change *Lep* expression (C) or secretion (D) following stimulation with insulin and dexamethasone for 12 hours. Adiponectin secretion (E) remained unchanged. N=9 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplementary Figure 5-6 Effects of Cobll1 knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.

Cobll1 expression (A) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Cobll1* knockdown (B) did not change *Lep* expression (C) but decreased Lep secretion (D) following stimulation with insulin and dexamethasone for 12 hours. N=13 mice per group. Adiponectin secretion (E) remained unchanged. N=8 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

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Chapter 6

"Effects of a Novel MC4R Agonist on Maintenance of Reduced Body Weight in Diet-Induced Obese Mice"

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Abstract

Objective: The physiology of the weight-reduced (WR) state suggests that pharmacologic agents affecting energy homeostasis may have greater efficacy in WR individuals. Our aim was to establish a protocol that allows for evaluation of efficacy of weight maintenance agents and to

assess the effectiveness of AZD2820, a novel melanocortin 4 receptor (MC4R) agonist in such a paradigm.

Design and Methods: MC4R agonist was administered in stratified doses to mice who were either fed high-fat diet ad libitum (AL) throughout the study; or stabilized at a 20% reduced body weight (BW), administered the drug for 4 weeks, and thereafter released from caloric restriction while continuing to receive the drug (WR).

Results: After release of WR mice to AL feeding, the high-dose group (53.4 nmol/day) regained 12.4% less BW than their vehicle-treated controls since the beginning of drug treatment. In WR mice, 10.8 nmol/day of the agonist was sufficient to maintain these animals at 95.1% of initial BW versus 53.4 nmol/day required to maintain the BW of AL animals (94.5%).

Conclusions: In the WR state, the MC4R agonist was comparably efficacious to a five-fold higher dose in the AL state. This protocol provides a model for evaluating the mechanisms and quantitative efficacy of weight-maintenance strategies and agents.

Introduction

Using diet and exercise many obese individuals are capable of losing weight but are unable to maintain this reduced body weight (BW) for extended periods of time [1]. Resistance to the maintenance of a weight reduced (WR) state results from the coordinate action of systems regulating energy intake and output to favor weight regain. WR individuals and rodents are hyperphagic and their energy expenditure is decreased beyond what would be predicted for their lower body mass and composition [2-4]. Changes in neuroendocrine axis, decreased circulating concentrations of leptin and bioactive thyroid hormones, and decreased sympathetic nervous

system tone and increased parasympathetic nervous system tone promote the BW regain [2]. In rodents, weight reduction decreases the proportion of excitatory synapses on anorexigenic POMC neurons in the arcuate nucleus of the hypothalamus [3]. Behavioral studies indicate that WR humans are hungrier, have decreased perception of how many calories they have eaten and have reduced satiation [5]. These phenotypes are associated with characteristic changes in fMRI activity in regions of the brain mediating homeostatic and hedonic responses to food [6, 7].

Most of the physiological and neuro-functional changes that accompany maintenance of a reduced BW in either lean or obese individuals can be reversed with low doses of exogenous leptin sufficient to return the circulating leptin levels to those present at the usual BW [6-9]. These responses are similar to those seen in patients with congenital leptin deficiency [10-12]. Administration of exogenous leptin to obese or never-obese humans at their usual weights - even at doses sufficient to raise circulating leptin concentrations to supraphysiological levels - has modest or no effect on BW [13]. Similar to the effects of leptin, WR individuals may have increased sensitivity to other relevant molecules, influencing strategies for the design and application of weight-maintaining pharmacologic agents.

The melanocortin (MC) system is a downstream target of leptin [14]. The MC4 receptor (MC4R) is a G-protein-coupled receptor expressed primarily in paraventricular hypothalamus (PVH), dorsal motor nucleus of the vagus (DMV), thalamus and hippocampus [15, 16]. In addition, MC4Rs are also found outside of the CNS including in the nodose and dorsal root ganglia [17, 18]. Two major neuronal populations in the hypothalamus, the anorexigenic proopiomelanocortin (POMC) and orexigenic Agouti-related protein (AgRP)/Neuropeptide Y (NPY)/GABA neurons, are activated and inhibited by leptin, respectively, to reciprocally mediate energy balance through the MC4 receptors [14]. α-MSH, one of the peptides released

from POMC neurons, is an agonist of MC4R whereas the AgRP is an inverse agonist of this receptor. *Mc4r*-null mice are obese due to both increased food intake and reduced energy expenditure [19]. Mice overexpressing α-MSH display increased energy expenditure [20]. Administration of MC4R agonists to mice or rats promotes weight loss by decreasing food intake and/or increasing energy expenditure, identifying MC4R as a potential pharmacological target [21, 22].

Physiologically, the weight-reduced state is one of relative leptin deficiency in which the expression of hypothalamic AgRP and NPY neuropeptides is increased [23, 24] and Pomc expression is decreased [25]. These changes can be largely normalized with administration of exogenous leptin [26]. In the WR state, treatment with MC4R agonist might be efficacious as well. Leptin has little or no efficacy when administered at a usual BW [13]; MC4R agonist might also be expected to have greater efficacy in the weight-reduced state.

In this study we examined the long term effects of a potent and selective cyclic peptide MC4R partial agonist (AZD2820; MC4R: EC50 1nM in cAMP generation assay, 38% efficacy vs. NDP-α-MSH, and MC3R: binding Ki 9nM, no agonist efficacy detected (AstraZeneca data on file) [27] on induction of weight loss and on maintenance of reduced BW in diet induced obese (DIO) mice. We found that, in the weight-reduced state, the dose required for equal efficacy of AZD2820 with regard to body weight maintenance was decreased nearly 5 fold.

Methods

Animals

18 week old male mice C57BL/6J fed high fat diet (HFD; Research Diets, Inc. D12492i, 60% kcal from fat) starting at six weeks of age were obtained from Jackson Laboratory (Bar Harbor, ME). Throughout the study, animals were maintained at room ambient 22-24°C with a 12-h dark-light cycle (lights on at 0700h) in a pathogen-free barrier facility. The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

Study Design (schematized in Figure 1)

After the 5-week acclimatization period, mice were randomly assigned to one of the following groups: 1.) Maintained on HFD *ad lib* (AL group; n=40) throughout the study or 2.) Weight reduced (WR group, n=40) to 80% of their initial weight by caloric restriction (CR). Eight weeks after the WR group entered the CR phase, both AL and WR groups were further randomized to receive either low (2.64 nmol/day), intermediate (10.8 nmol/day) or high (53.4 nmol/day) dose of AZD2820 dissolved in 5% mannitol solution or vehicle (5% mannitol solution; n=20 per treatment; n=10 WR and n=10 AL) via implanted subcutaneous mini-osmotic pumps (Alzet Model 2004) to allow for delivery at a steady infusion flow rate (0.25 µl/h). The pumps were replaced at 4 week intervals throughout the experiment for a total of 12 weeks of treatment. Four weeks following the initiation of drug administration (week 17; **Figure 6.1**), WR mice were released from CR and returned to *ad lib* food access (weight re-gain phase). AL group continued *ad lib* HFD feeding. During the last week of drug administration (11-12 weeks following the onset of drug administration) mice were placed in metabolic cages for 72 hours to assess their energy expenditure (EE) and were then sacrificed immediately following removal from metabolic chambers.

Food intake was measured over 48 hours every 2-3 weeks in all AL mice throughout the study. The first week after release of WR mice from CR, food intake was measured daily (see Supplementary Methods).

Mini-pump implantation

Three mini-osmotic pump implantations (at 4 week intervals) were performed in each mouse over the 12 weeks of AZD2820 administration according to the manufacturer's recommendations (see Supplementary Methods).

Body weight, body composition, and food intake

BW was measured (± 0.1 g) weekly in AL groups throughout the experiment and daily in the WR groups during CR phase using an Ohaus Scout Pro 200g scale (Nänikon Switzerland, 08:00-08:30h). Body composition was measured by time-domain-NMR (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany) [28] prior to the initiation of CR (week 5), before the insertion of each mini-pump (weeks 13, 17, and 21) and at the time of sacrifice (week 25). Food intake was recorded daily for the WR mice during the CR phase (week 5-17) and every 2-3 weeks for AL mice over 48 hours by weighing feeding baskets that were designed specifically to reduce the food spillage.

Serum or plasma assays

Blood was obtained by retro-orbital bleed following a 4.5-h fast at several time points [weeks 5, 13, 17, 24 and 25 (**Figure 6.1**)]. The plasma concentrations of AZD2820 were determined after protein precipitation, by liquid chromatography with mass spectrometric detection (LLOQ 2 nmoles/L). Leptin was assayed using Quantikine ELISA kit (R&D Systems and insulin using the Crystal Chem Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem). Glucose was measured

at the same time as blood collection using the FreeStyle Lite Blood Glucose Monitoring System (Abbott Laboratories; see Supplementary Methods).

Indirect calorimetry

Energy expenditure and physical activity were measured with a LabMaster-CaloSys-Calorimetry System (TSE Systems, Bad Homburg, Germany) at the end of the study (week 25, immediately prior to sacrifice; see Supplementary Methods).

Results

Body weight and body composition. Prior to food restriction, BW of all groups were equal (by design). 40 mice were maintained on HFD *ad lib* throughout the study (AL group) and their weights are reported as percent initial BW - defined as BW (g) on any given day divided by BW (g) on day 0 of the experiment (when the first mini-pump was implanted; **Figure 6.2A**). 40 mice fed a HFD were calorically restricted to reduce their weights by 20%. Weights are reported as percent initial BW which in the case of WR mice is defined as BW (g) on any given day divided by BW on the day prior to start of the calorie restriction (CR) phase (on day 0 when the first mini-pump is implanted; **Figure 6.2B**).

Mice maintained on the *ad lib* feeding regimen for the duration of the study lost weight when treated with high drug dose (53.4 nmol/day) compared to their vehicle-treated controls (5% mannitol). The high dose-AL lost 5.5 ± 4.2% *vs.* control-AL which gained 3.5 ± 1.8% (p= 0.042; **Figure 6.2A**). Intermediate drug dose (medium; 10.8 nmol/day) treated mice also lost weight (1.0 ± 2.5%) but were not significantly different from the vehicle treated controls (**Figure 6.2A**). There was a dose-response relationship regarding drug efficacy in WR mice (**Figure 6.2B**).

Eight weeks after WR mice were released from CR and allowed AL access to HFD, the high dose group regained 10.0 ± 2.6% of their BW since the beginning of the drug treatment compared to 22.4 ± 2.2% in the vehicle-treated control group (p= 0.0027; **Figure 6.2B**). In WR mice, an intermediate dose of the agonist was sufficient to maintain these animals at 95.1 ± 3.7% of initial BW *vs.* 94.5 ± 4.2% in the high dose-AL mice (**Figure 6.2C**). Similarly, low dose AZD2820 (2.64 nmol/day) in WR mice kept them at 98.4 ± 3.0% of initial BW compared to 99.0 ± 2.5% in the medium dose-AL mice (neither group was significantly lower than their respective controls, but both show a trend towards lower % initial BW; **Figure 6.2D**). Whereas weight-reduced animals are sensitive to the agonist even in the lowest concentration rate (**Figure 6.3B)**, mice fed AL require an agonist concentration of >10 nM to produce weight loss (**Figure 6.3A**). Thus, mice in the WR state were more sensitive to the MC4R agonist and required an ~5-fold lower dose of the agonist to achieve a response comparable to that required in the AL state (**Figure 6.2C-D**).

The change in BW in response to MC4R agonist administration was primarily accounted for by changes in fat mass. Following the MC4R agonist treatment, fat mass stratified according to changes in BW (**Figure 6.4A-B**). The WR group treated with high drug dose had a significantly lower body fat mass after 8 weeks of drug administration compared to their vehicle-treated controls (at 8 weeks of drug: high-WR: 11.9 ± 0.6 g *vs.* control-WR: 15.8 ± 1.5 g; p= 0.030; data not shown; at 12 weeks of drug: high-WR: 12.2 ± 0.5 g *vs.* control-WR: 16.5 ± 1.6 g; p= 0.015; **Figure 6.4B**). Mice fed AL and given high dose, as well as medium dose-treated mice (in both AL and formerly WR state), showed a trend towards reduced fat mass at 8 and 12 weeks of drug treatment (**Figure 6.4A**). Lean mass of WR mice was lower than AL mice only during the CR

phase; no difference was noted among any of the groups in response to AZD2820 treatment after the release of WR mice to AL feeding (**Figure 6.4C-D**).

Plasma hormones and glucose. As expected, fasting plasma leptin and insulin levels maintained proportionality to fat mass following weight loss (**Figures 6.5-6**). By the end of the study both AL and formerly WR mice treated with high-dose MC4R agonist had significantly lower circulating leptin levels compared to their respective vehicle-treated controls (**Figure 6.5A-B**). Circulating insulin concentrations were significantly reduced in high dose-WR mice compared to WR controls (**Figure 6.6B**). Medium dose-treated mice showed a trend towards a decrease in circulating leptin and insulin levels (**Figure 6.5A-B, 6.6A-B**). At 4 weeks of drug treatment, while WR mice were still calorie restricted, their plasma leptin concentrations were increased relative to fat mass when compared to a regression of leptin *vs.* fat mass in the vehicle treated AL fed mice (**Figure 6.5C**). This increase in leptin levels was not correlated with MC4R agonist dose, occurred only in WR animals, and was reversed after formerly WR mice had returned to AL feeding for 8 weeks (**Figure 6.5D**). Fasting blood glucose levels were not different among groups throughout the study (**Figure 6.6C-D**).

Food Intake. No difference was found between any of the groups in the 24hr food intake measurements taken one week after the first mini-pump implantation and every 2-3 weeks thereafter (data not shown). Food intake was also measured more precisely at the end of the study when mice were housed in metabolic chambers for 48hr (**Figure 6.7**). Similarly, there were no differences detected in food intake.

Energy Expenditure. Immediately prior to sacrifice, mice were placed in metabolic chambers for assessment of energy expenditure. Absolute total and resting EE did not differ among treatment groups which did not differ significantly in lean body mass; fat mass was lower in the WR high

dose treated mice compared to the controls (**Figure 6.8D**) but their TEE and REE was the same (REE in high-WR: 10.1 ± 0.3 kcal/24h *vs.* control-WR: 10.4 ± 0.3 kcal/24h; TEE in high-WR 13.3 ± 0.4 kcal/24h *vs.* control-WR: 13.3 ± 0.3 kcal/24h; **Figure 6.8A-B**). The limited number of mice in the control group (n=10) and the low variation in lean mass among all mice limited power to build a significant regression model to calculate the residual EE values for the treatment groups. Ratio of TEE and REE (**Figure 6.8E**) to lean mass was not different among the treatment groups. However, the ratios of TEE and REE to BW were higher in high dose (WR and AL combined) treated animals (REE in high dose: 0.226 ± 0.005 kcal/g/24h *vs.* control: 0.209 ± 0.005 kcal/g/24h; p=0.02; TEE in high dose 0.294 ± 0.006 kcal/g/24h *vs.* control: 0.272 ± 0.006 kcal/g/24h; p=0.012; data not shown). Respiratory exchange ratio (RER or respiratory quotient, RQ) and physical activity were not different among treatment groups (data not shown).

Drug delivery verification: AZD2820 plasma concentrations measured after 4, 11, and 12-13 weeks of drug treatment were indistinguishable between AL and WR groups. AZD2820 treatment caused penile erection in all mice regardless of drug dose but was not observed in vehicle-treated mice. Every mouse was assessed visually three times throughout the study. There was no sign of drug tolerance as the penile erection persisted until the end of the study.

Discussion

The purpose of this study was to establish a study protocol to enable evaluation of the efficacy of weight maintenance agents, and to assess the effectiveness of a novel MC4R partial agonist (AZD2820) [27] in the maintenance of reduced BW. We found that in the WR state a 5x lower drug exposure is sufficient to maintain mice at a reduced weight versus the dose required

to induce that degree of weight loss. Mice in the WR state are consequently more sensitive to the MC4R partial agonist.

We were unable to detect quantitatively significant differences in food intake or energy expenditure related to the AZD2820 administration. However, these studies were conducted relatively late in the treatment (11-12 weeks post start of agent). Also, subtle differences in food intake too small to measure (especially in mice) can accumulate into significant weight change. The ultimate differences in body mass and composition indicate that differences in energy balance must have occurred.

The effects of MC4R agonists on energy homeostasis have been assessed in animal studies [21, 29-31]. Food intake is initially suppressed - most strongly in the first day of treatment, and lasting for about a week - but then returns to control levels. In longer term studies [21, 22, 32], weight loss occurs initially as a result of decreased food intake but persistence of weight loss throughout the drug treatment period suggests that relative elevation of energy expenditure plays a role in sustaining the reduced BW. Hamilton, et al. showed that, in DIO rats, FI was significantly suppressed during the first week of continuous 28-day MTII (MC4R and MC3R full agonist) treatment, whereas EE was increased 3 days after the start of agonist administration [22]. An eight-week treatment with a novel MC4R agonist in DIO Rhesus macaques caused weight loss (13.5%), due to decreased food intake (~25-35%) for the first two weeks. EE was measured at baseline and at 8 weeks after drug treatment and showed a 14% increase suggesting that reduced food intake is an initial transient response, and that elevated EE may explain the persistence of reduced BW and contribute to the weight loss as well [32].

Using a paradigm similar to the one used here, DIO rats were either fed *ad libitum* throughout the study or fed calories reduced (CR) by 30% or 60% (versus *ad lib*) for 2 weeks,

followed by 4 weeks of MTII treatment (at either low [0.3 mg/kg/day] or 10 fold higher dose) [33]. In the AL state, both MTII doses were equally efficacious in inducing weight loss; but in the CR groups (both 30% and 60% CR), the effectiveness of MTII in maintaining reduced BW was greater with the higher dose; the drug was more dynamically efficacious in the WR state. This is similar to the current study where AZD2820 was more efficacious in the WR state. The lack of difference in efficacy of low or high doses of the MTII in AL rats suggests that the low dose induced maximal weight loss in the AL state [33].

Circulating leptin concentrations were, as expected, significantly lower in all WR groups compared to control AL mice when measured at 4 weeks of AZD2820 treatment, while WR mice were still calorie restricted. Interestingly, leptin concentration per fat mass was increased in WR mice compared to AL control group, independent of drug dose, found only in WR animals, and reversed after mice had returned to *ad lib* feeding for 8 weeks. High dose-treated mice showed decreases in leptin and insulin plasma concentrations; these changes are likely a reflection of weight loss and not drug itself. Blood glucose concentration was only lower during the CR phase while WR mice were maintaining 80% of their initial BW. It was not affected by the MC4R agonist *per se*.

Therapies for obesity are focused primarily on inducing weight loss. Since weight regain, and not the inability to lose weight, is what defeats most efforts at obesity treatment [1], sustaining the weight-reduced state deserves more attention. Induction of weight loss is physiologically different than maintenance of a reduced BW [2, 8, 34]. The weight-reduced state in both humans and mice is characterized by hyperphagia and reduction in energy expenditure greater than predicted by lower body size [2, 4, 8], phenotypes that are largely reversed by administration of low “replacement” doses of leptin [6, 7]. These observations, congruent with

those described in this report, suggest that the pharmacology of the weight-reduced state deserves greater attention as a therapeutic target in obesity treatment. Similarly to effects seen in WR individuals treated with low levels of leptin, MC4R agonists might be efficacious in maintenance of weight loss. However, MC4R agonists would be expected to be less effective due to increased levels of endogenous MC4R antagonist/inverse agonist (AgRP) which could potentially interfere with MC4R agonism.

Drugs found to be modestly efficacious in the induction of weight loss, may be more useful in the maintenance of reduced BW; those agents effective in producing weight loss, but problematic by virtue of off-target effects, may be helpful in maintaining reduced BW at much lower doses. This protocol could be used to examine agents for efficacy in maintenance of reduced BW.

Conflicts of Interest

The authors AL and SH are or were employees of AstraZeneca and may hold stock in that company.

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Supplementary Methods

Animals and study design:

Upon receipt of mice from Jackson Laboratory, they were separated into individual cages and fed the same HFD *ad-libitum* with free access to water for a five week acclimatization period. During this period, body weight (BW) was monitored on a weekly basis.

20% weight reduction was achieved by feeding mice 50% of their *ad-libitum* daily caloric intake until they reached 80% of initial value (defined as BW immediately before the start of CR phase). Subsequently, mice were switched to ~80% of their *ad libitum* daily food intake and the number of calories provided were adjusted (when % of initial BW of a mouse deviated from 80% by more than 1%, the daily amount of food intake was adjusted by 0.1g = 0.524 kcal) on a daily basis in order to weight stabilize each mouse at 80% ±1% of their original weight. WR mice were provided with food twice daily, 1/3 of the total daily calories in the morning (08:00-8:30h) and 2/3 in the evening (18:00-18:30h). BW was monitored daily or weekly in WR and AL groups, respectively. WR mice were determined to be weight stable at 80% of their initial weight when their BW did not deviate by more than ±1% of the value reported on the previous day (with no changes in food given) for 4 consecutive days at which point the value for their daily provision of HFD was fixed for the remaining of the CR phase.

The doses of the MC4R agonist in this study were chosen based on previous studies in DIO mice showing a ~10% BW loss with the drug *vs*. vehicle over a 2-w infusion period (AstraZeneca data on file).

During the course of the experiment, some of the mice developed skin lesions which were treated with topical antibiotics. Skin lesions were primarily found in the AL group of mice as a likely

consequence of *ad libitum* access to the HFD. To reduce the progression of skin lesions and development of new ones, all mice were washed in warm water with a pet shampoo (Ferret Glow) twice during the drug treatment.

Mini-pump implantation

The mini-pumps (ALZET Model 2004) held 200 μL and release the solution at a nominal rate of 0.25 μl/hr over 28 days. Mini-pumps were filled with solution sterilely and soaked in PBS at 37°C for 40 hrs prior to surgery according to the manufacturer's recommendations. Animals were anesthetized with inhaled isoflurane (2-3.5%) in oxygen. Skin was shaved and wiped with betadine and alcohol pads, followed by a small incision (0.5-1 cm) in the interscapular region. The pump was inserted into a small pocket that was formed by spreading the subcutaneous connective tissue apart with a hemostat. The pump was positioned with the flow moderator facing caudally. Care was taken not to disrupt BAT tissue. The incision was closed with two wound clips followed with application of triple antibiotic to minimize the risk of infections. During the second and third mini-pump surgeries (weeks 17 and 21), the existing pumps were explanted and replaced with a fresh pump.

Serum and plasma collection

Blood was obtained by retro-orbital bleed following a 4.5-h fast at several time points. Blood from the first two bleeds was allowed to clot for 2 h at room temperature, centrifuged at 4°C for 20 min at 2,000 x g, and serum was collected and frozen at −80°C until time of assay. For the remaining three bleeds, plasma was collected on ice using heparinized capillary tubes (Fisherbrand) in order to protect the integrity of the MC4R agonist. A protease inhibitor (Protease inhibitor cocktail set 1, Calbiochem, USA) was added to the aliquots (1 part to10 parts

plasma of the 50x solution) used for assaying the drug concentration. Plasma was obtained by centrifugation for 10 min at 1,500 x g at 4°C and frozen at −80°C until time of assay.

Indirect calorimetry

Measurements of oxygen (O_2) and carbon dioxide (CO_2) concentrations were taken every 26 minutes during a 72 hour period while mice had AL access to HFD and water. To diminish the effects of stress associated with exposure of mice to new environment data points collected during the last 48 hours were used to calculate the average total 24-hour energy expenditure (TEE; kcal/24hr) and respiratory exchange ratio (RER = VCO_2/VO_2) [3]. Resting energy expenditure (REE; kcal/24hr) was defined as the lowest one hour period of energy expenditure, coinciding with the lowest one hour of total ambulatory activity during the 48-hour period; this value was extrapolated to 24 hours. Non-resting energy expenditure (NREE; kcal/24hr) was calculated as the difference between TEE and REE over the 48 hour period.

Physical activity was measured by an infrared beam system integrated with the LabMaster system. Total activity (infrared beam breaks) in X, Y, and Z axis was recorded every 26 minutes. The system is designed to differentiate between fine motor movement (a single X or Y axis beam break), ambulatory movement (simultaneous breaking of two adjacent X or Y beams), and rearing (Z axis beam break).

Sacrifice

After a 5-6 hour fast on the day of sacrifice, mice were anesthetized with isoflurane, the last mini-pump was removed, and the body composition was measured immediately prior to sacrifice. Mice were sacrificed by carbon dioxide asphyxiation and had their blood collected by cardiac puncture.

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using JMP and Statistica software. Where appropriate, two-way ANOVAs were performed using drug treatment and state as grouping variables. Student t-tests (2-tailed) were conducted to compare treatment effects between different groups. P alpha < 0.05 was taken as significant.

Figures and Figure Legends

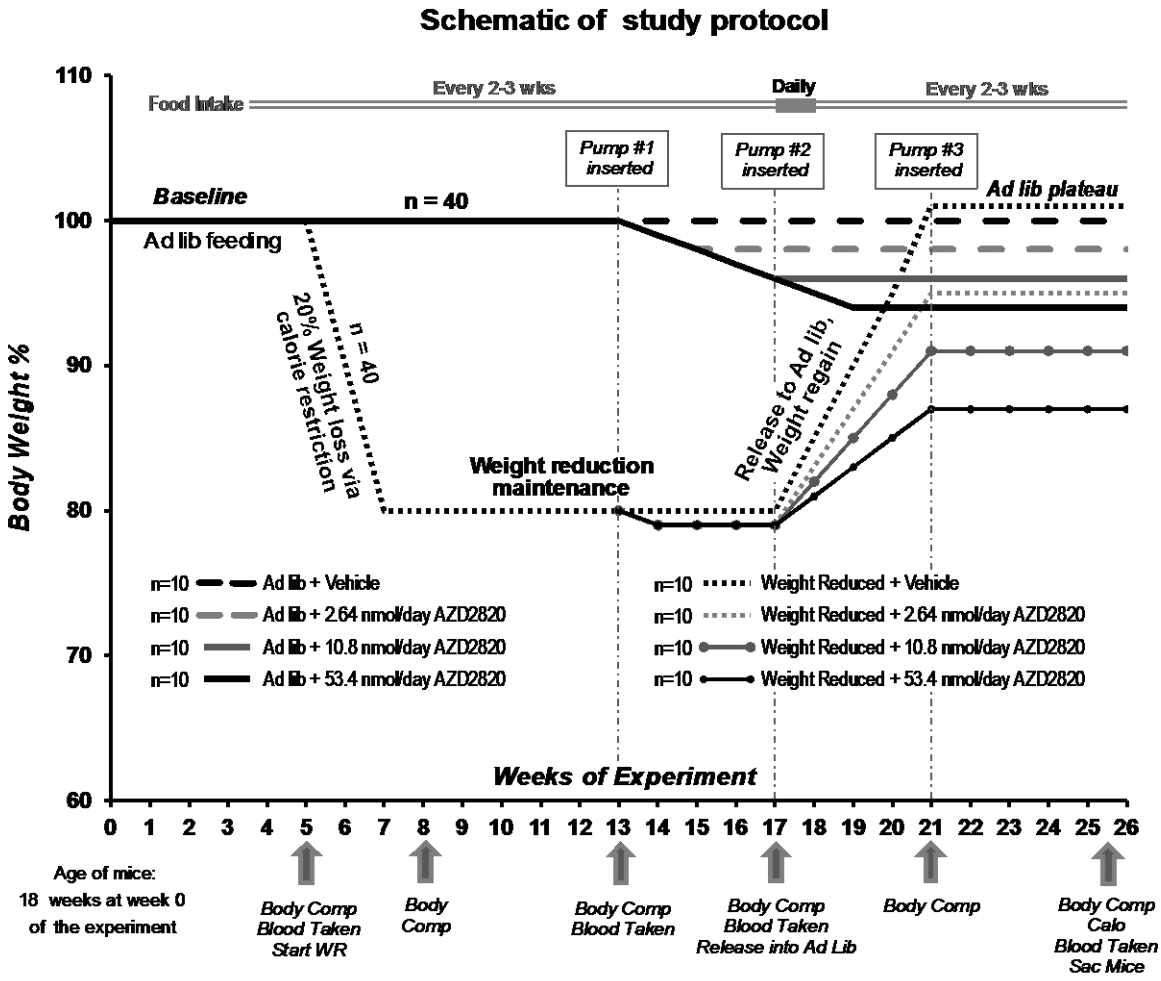
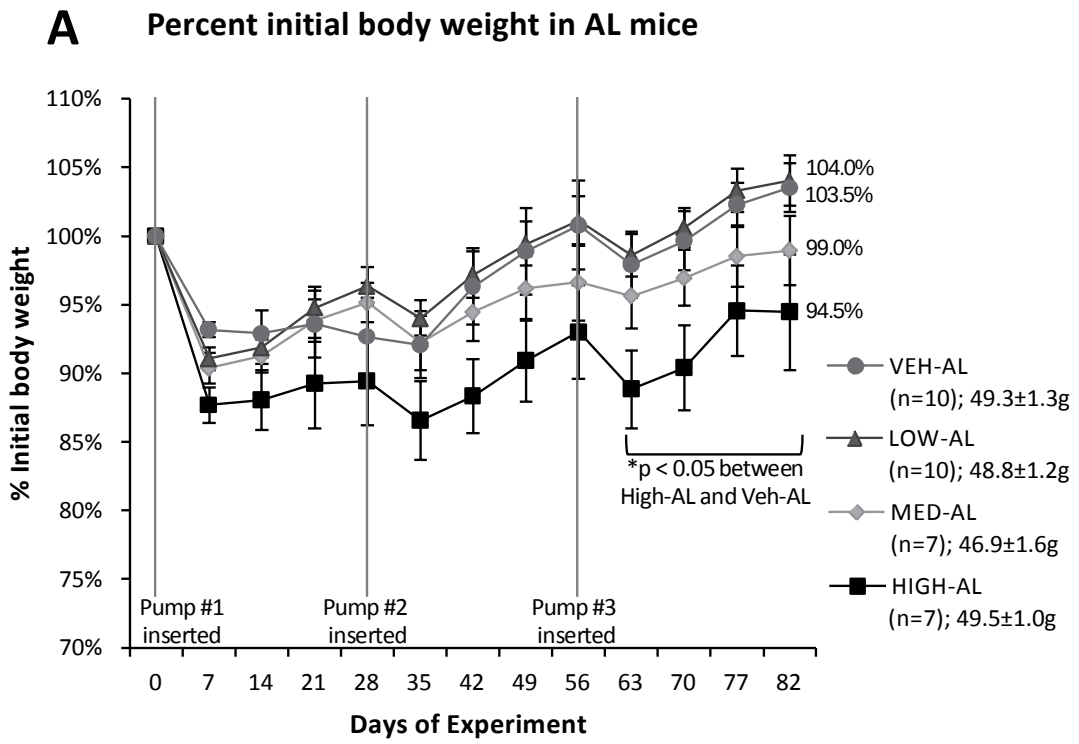
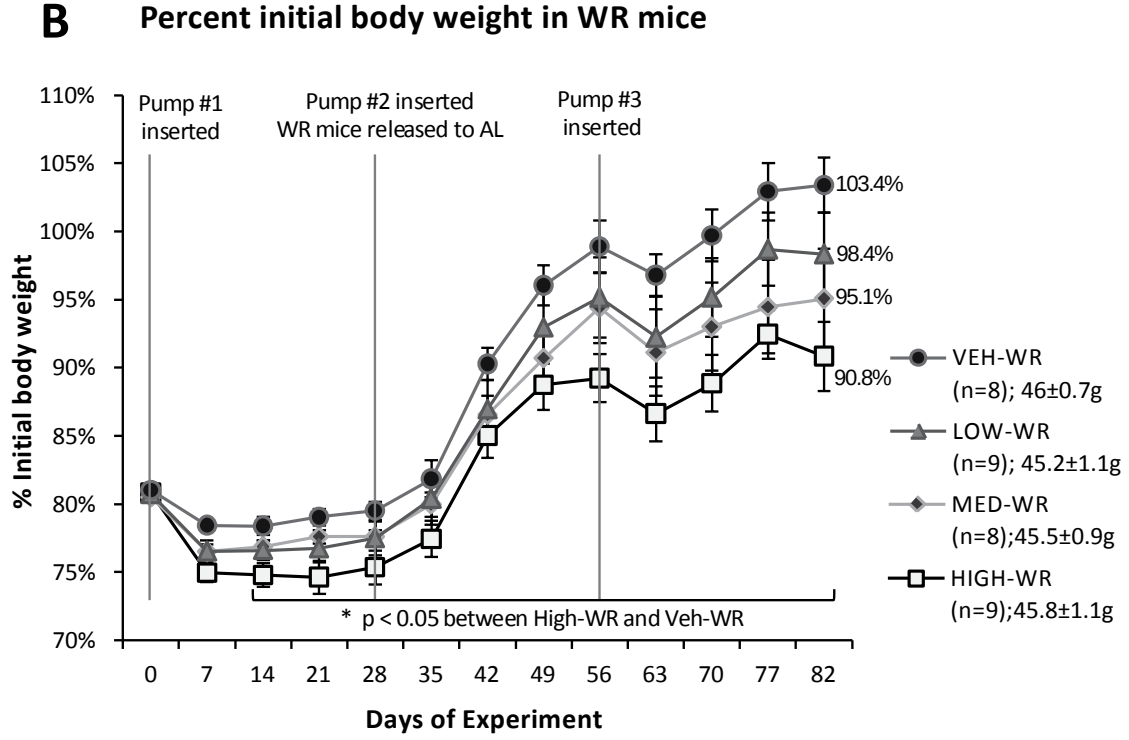


Figure 6-1 Schematic of protocol.

Schematic of protocol to assess the effects of a novel MC4R agonist (AZD2820) on maintenance of reduced body weight in DIO mice. Timepoints of physiological measurements and pharmacological treatment are indicated by arrows. WR - weight reduction, Body Comp - Body Composition, Calo - Calorimetry, Sac – Sacrifice, Time 0 – mice arrived at the Columbia facility and began the 5-week acclimatization period.


A
Percent initial body weight in AL mice
% Initial body weight
110%
105%
100%
95%
90%
85%
80%
75%
70%
Pump #1 inserted
Pump #2 inserted
Pump #3 inserted
0 7 14 21 28 35 42 49 56 63 70 77 82
Days of Experiment
104.0%
103.5%
99.0%
94.5%
*p < 0.05 between High-AL and Veh-AL
VEH-AL (n=10); 49.3±1.3g
LOW-AL (n=10); 48.8±1.2g
MED-AL (n=7); 46.9±1.6g
HIGH-AL (n=7); 49.5±1.0g


B
Percent initial body weight in WR mice
% Initial body weight
110%
105%
100%
95%
90%
85%
80%
75%
70%
Pump #1 inserted
Pump #2 inserted WR mice released to AL
Pump #3 inserted
0 7 14 21 28 35 42 49 56 63 70 77 82
Days of Experiment
103.4%
98.4%
95.1%
90.8%
* p < 0.05 between High-WR and Veh-WR
VEH-WR (n=8); 46±0.7g
LOW-WR (n=9); 45.2±1.1g
MED-WR (n=8);45.5±0.9g
HIGH-WR (n=9);45.8±1.1g

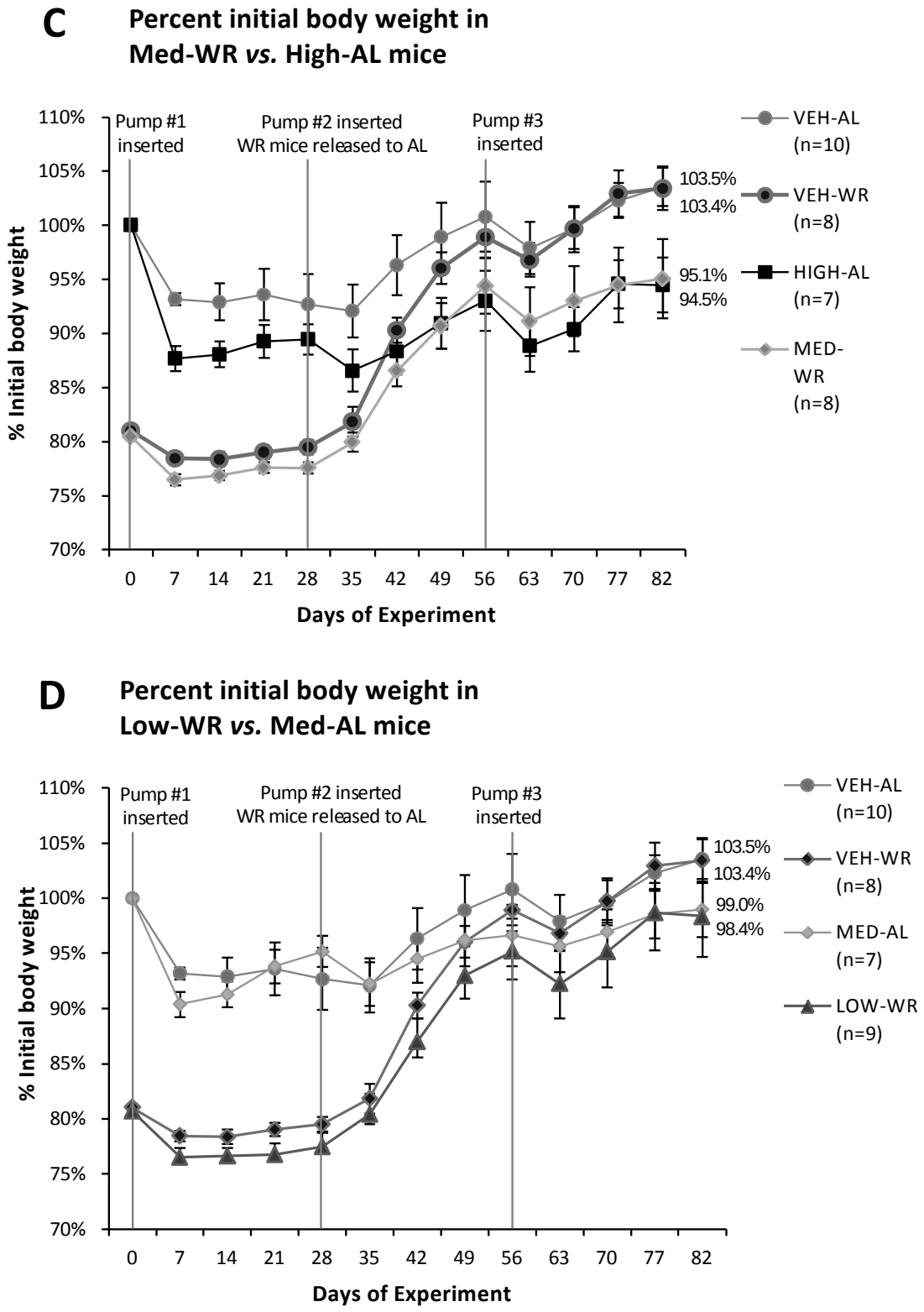


Figure 6-2 Percent initial body weight post pump #1 implantation.

Percent initial body weight post pump #1 implantation of mice fed a HFD throughout the study (A) and treated with MC4R agonist or vehicle and weight reduced by 20% prior to administration of the drug (B). Percent initial body weight in medium-WR mice compared with high-AL group (C) and in low-WR mice compared with medium-AL group (D). Medium and low dose in WR mice is sufficient to maintain them at the same percentage initial body weight as AL mice treated with a high and medium dose of MC4R agonist, respectively. Mean initial body weight (g) (SEM) is given for each group in the figure legend (A, B). Nominal doses of AZD2820—High: 53.4 nmol/day; medium: 10.8 nmol/day; low: 2.64 nmol/day. Each data point represents mean percent body weight for each group with SEM.

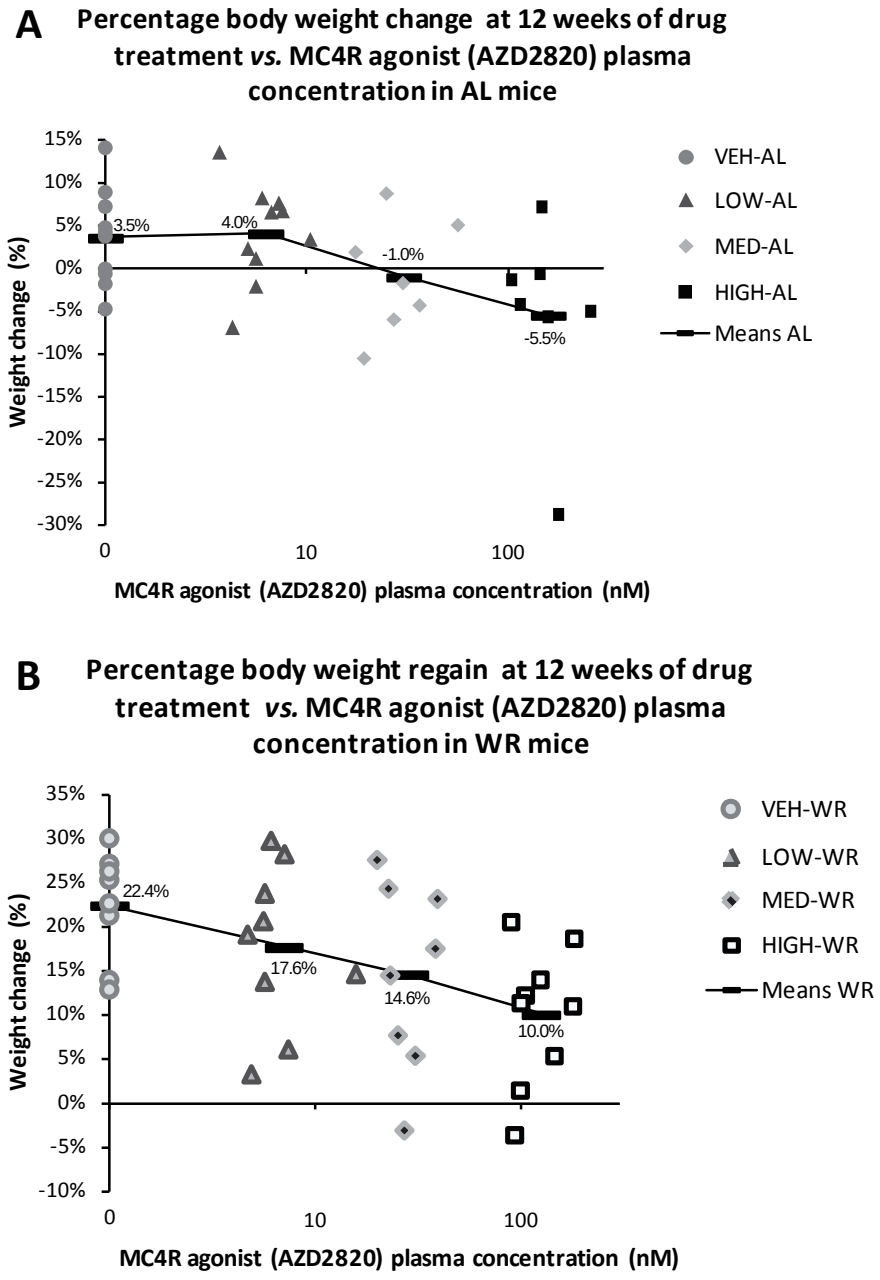
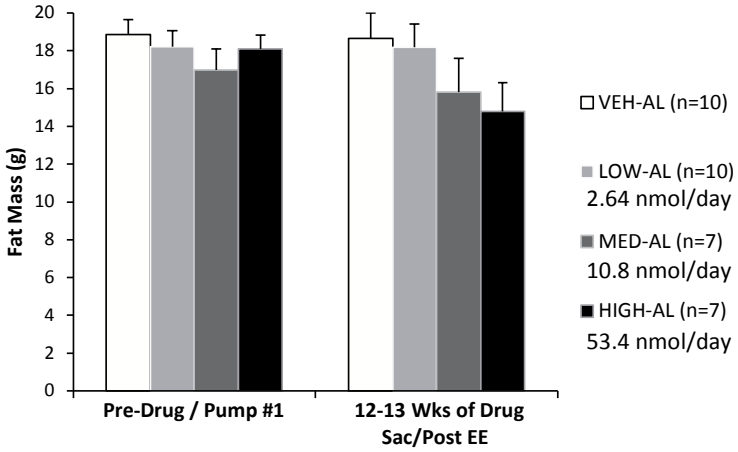


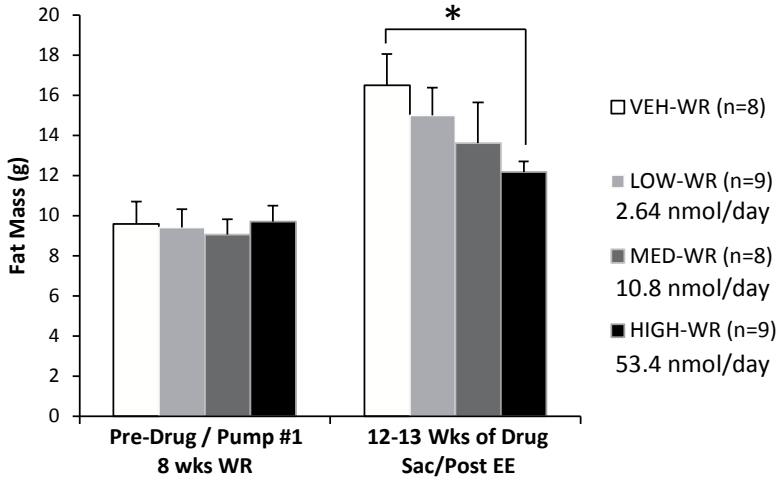
Figure 6-3 Change in percent body weight.

Percent body weight change (**A**) or regain (**B**) at 12 weeks of drug treatment plotted individually for each mouse against plasma concentration of MC4R agonist. AL (**A**) and WR (**B**) mice are plotted separately. Black bars are % BW change means for each group. Note, the drug concentration (x-axis) is plotted on a log scale.

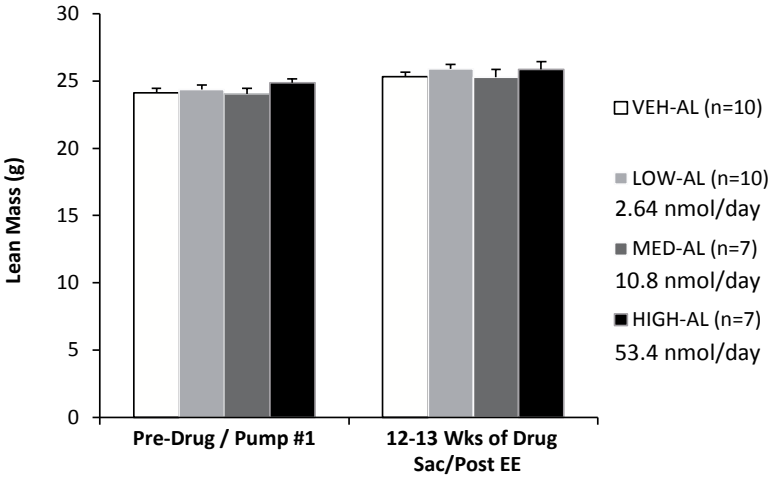
A **Fat Mass (g) in AL mice**



B **Fat Mass (g) in WR mice**



C **Lean Mass (g) in AL mice**



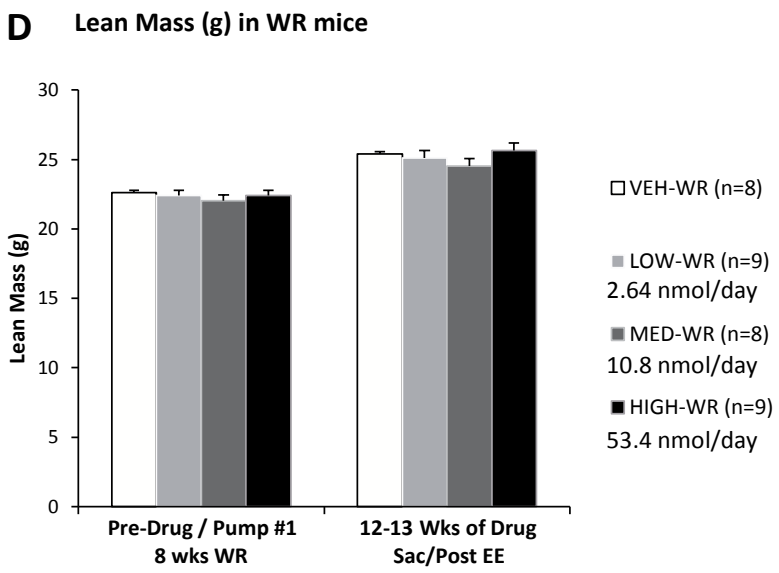
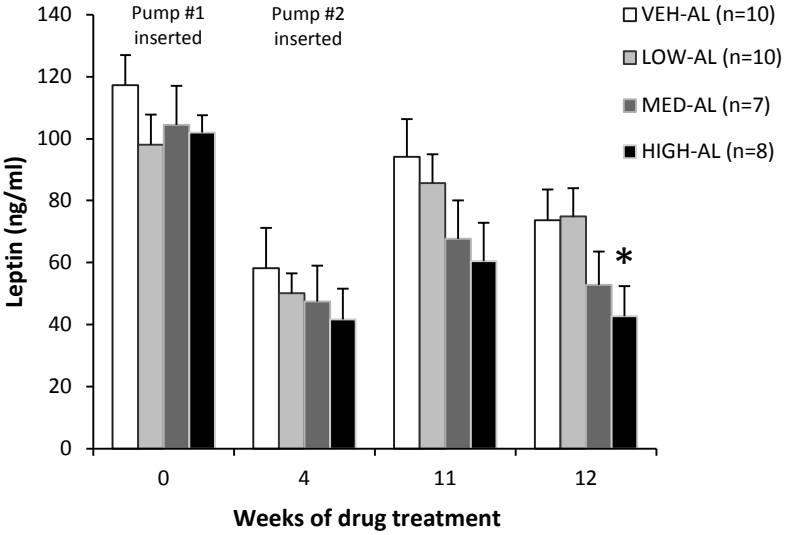


Figure 6-4 Body Composition in AL and WR mice throughout the study.

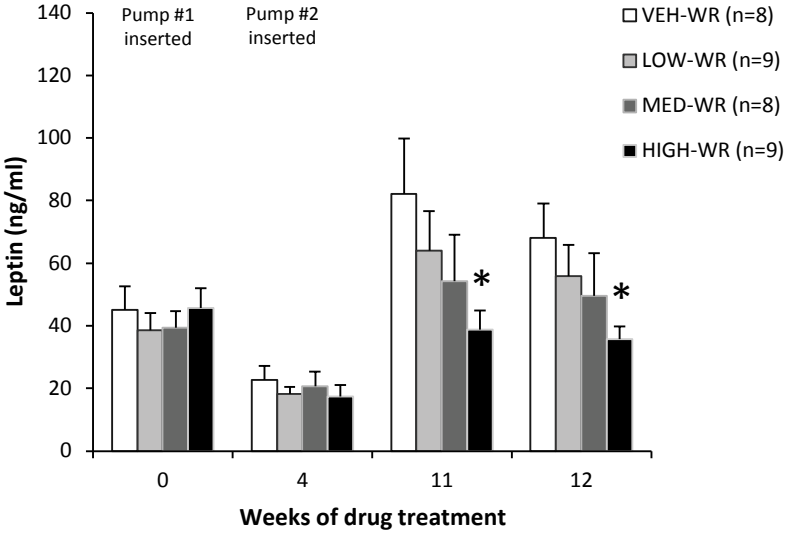
Fat Mass (**A, B**) and lean mass (**C, D**) in mice maintained on HFD *ad lib* throughout the study (**A, C**) or weight reduced by 20% prior to drug administration (**B, D**) measured prior to and 12 weeks after MC4R agonist treatment. Formerly WR mice given high nominal drug dose (53.4 nmol/day) achieved a significantly lower body fat than the controls after an 8 week body weight regain period.

Nominal doses of AZD2820 - High : 53.4 nmol/day; Medium : 10.8 nmol/day; Low : 2.64 nmol/day. Each data point represents mean fat mass or lean (fat free mass) for each group with SEM. *P < 0.05 between vehicle and high dose treated groups by Student's t-test.

A **Plasma leptin concentration in AL groups**



B **Plasma leptin concentration WR groups**



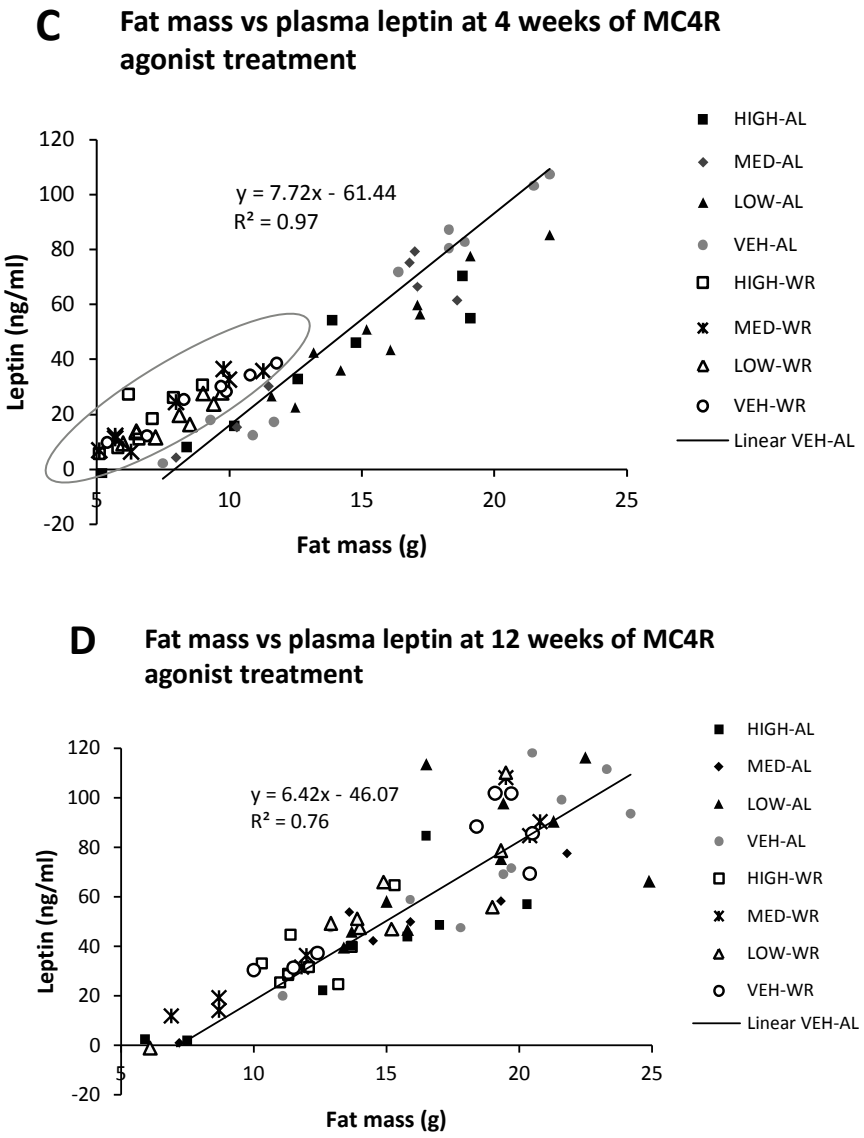


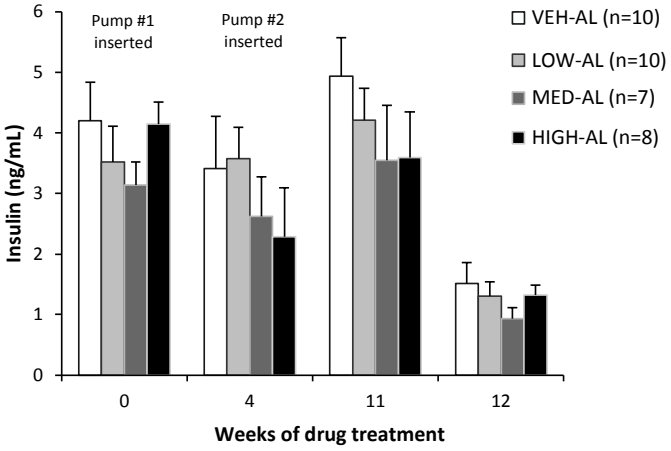
Figure 6-5 Circulating leptin concentrations in AL and WR mice throughout the study.

Circulating leptin concentrations (ng/ml) in mice maintained on HFD *ad lib* throughout the study (**A**) or weight reduced by 20% prior to drug administration (**B**) measured at different timepoints during the study. As expected from the fat mass data, circulating leptin levels are lower in high dose treated animals (both AL and WR) after at least 11 weeks of treatment. Regression of circulating leptin (ng/ml) against fat mass (g) in all mice at 4 weeks (**C**) and 12 weeks (**D**) of

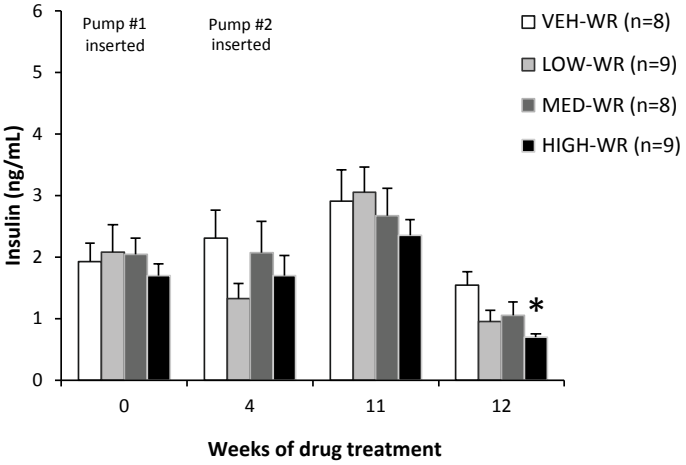
MC4R agonist treatment. The circle denotes WR mice which have an increased plasma leptin concentrations relative to fat mass compared to the vehicle treated AL mice. This effect is independent of MC4R agonist treatment and is reversed after formerly WR mice had returned to AL feeding for 8 weeks (**D**).

Nominal doses of AZD2820 - High : 53.4 nmol/day; Medium : 10.8 nmol/day; Low : 2.64 nmol/day. Each data point represents mean value for each group with SEM. *P < 0.05 between vehicle and high dose treated groups by Student's t-test.

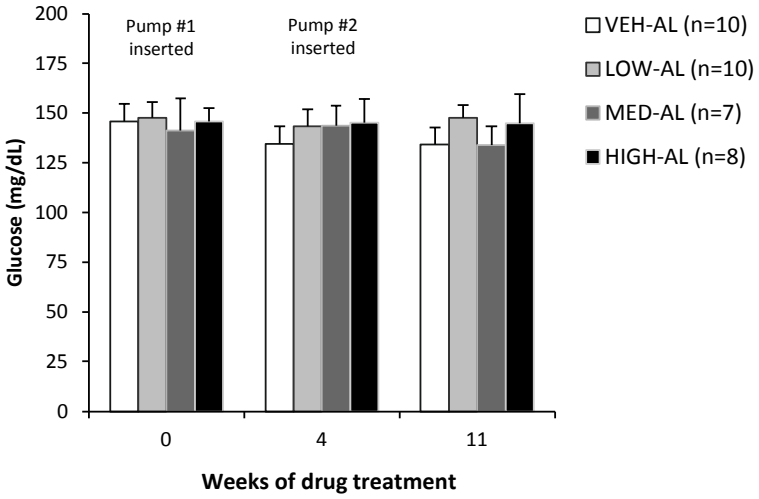
A **Plasma insulin concentration in AL groups**



B **Plasma insulin concentration in WR groups**



C **Blood glucose concentration in AL mice**



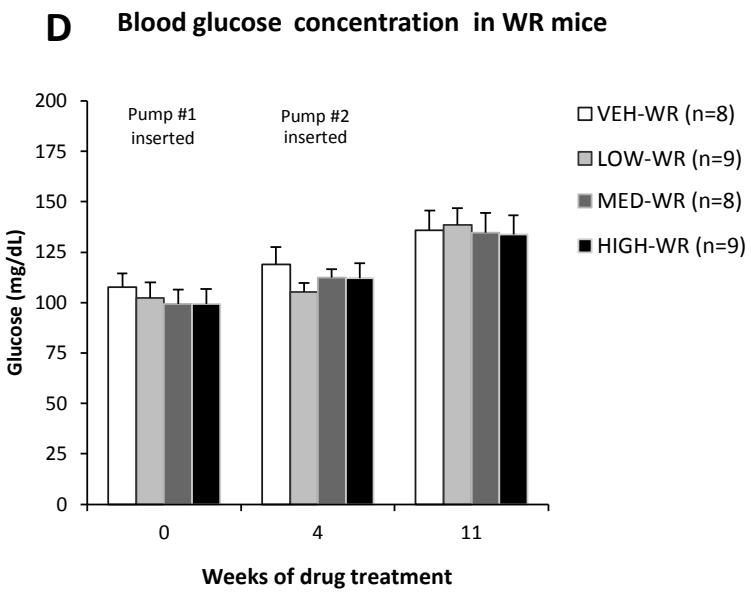


Figure 6-6 Circulating insulin and glucose concentrations in AL and WR mice throughout the study.

Circulating insulin (from plasma; **A-B**) and glucose (from venous tail blood; **C-D**) concentrations in mice maintained on HFD *ad lib* throughout the study (**A, C**) or weight reduced by 20% prior to drug administration (**B, D**) measured at different timepoints during the study. Nominal doses of AZD2820 - High : 53.4 nmol/day; Medium : 10.8 nmol/day; Low : 2.64 nmol/day. Each data point represents mean value for each group with SEM. *P < 0.05 between vehicle and high dose treated groups by Student's t-test.

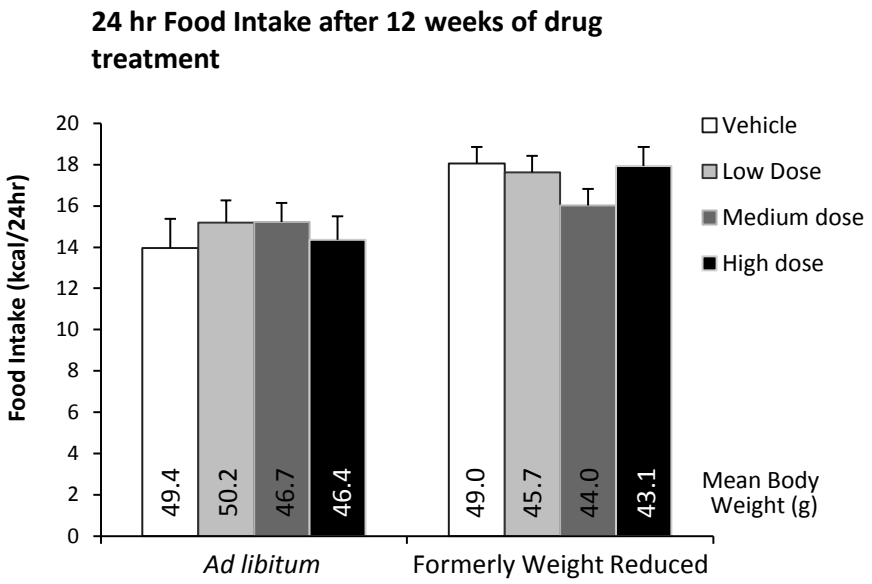
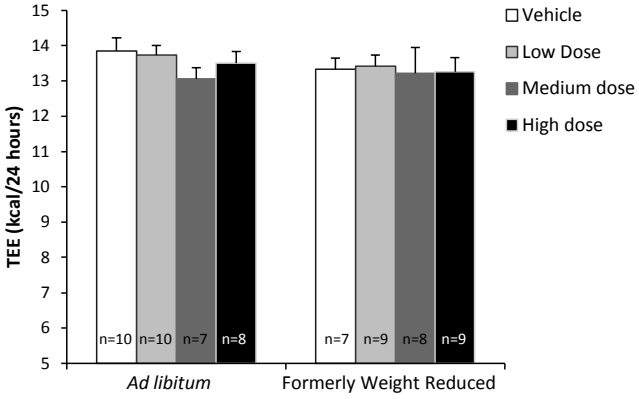


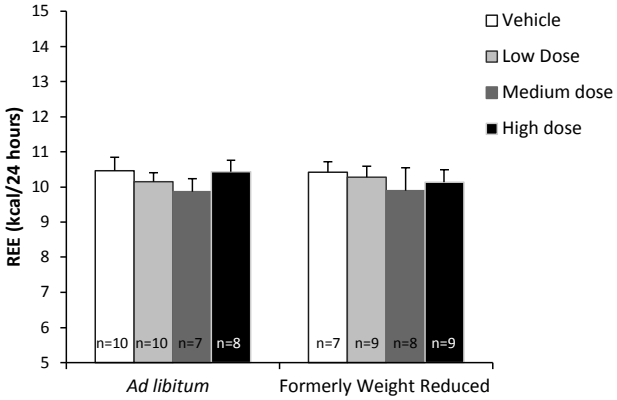
Figure 6-7 Food intake after 12 weeks of drug treatment in AL and WR mice.

24 hour food intake in mice maintained on HFD diet *ad lib* throughout the study or weight reduced by 20% prior to drug administration measured at 12-13 weeks of drug treatment during calorimetry assessment (TSE LabMaster system). Mean body weight is provided for each group. Nominal doses of AZD2820 - High : 53.4 nmol/day; Medium : 10.8 nmol/day; Low : 2.64 nmol/day. Each data point represents mean 24 hr food intake for each group with SEM.

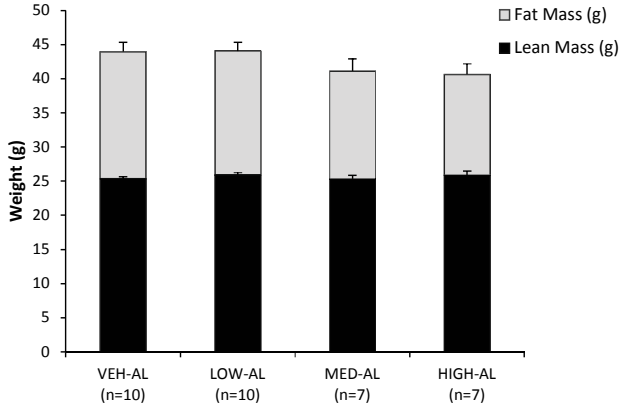
A **Total Energy Expenditure**



B **Resting Energy Expenditure**



C **Body Composition of AL mice at 12 weeks of drug treatment**



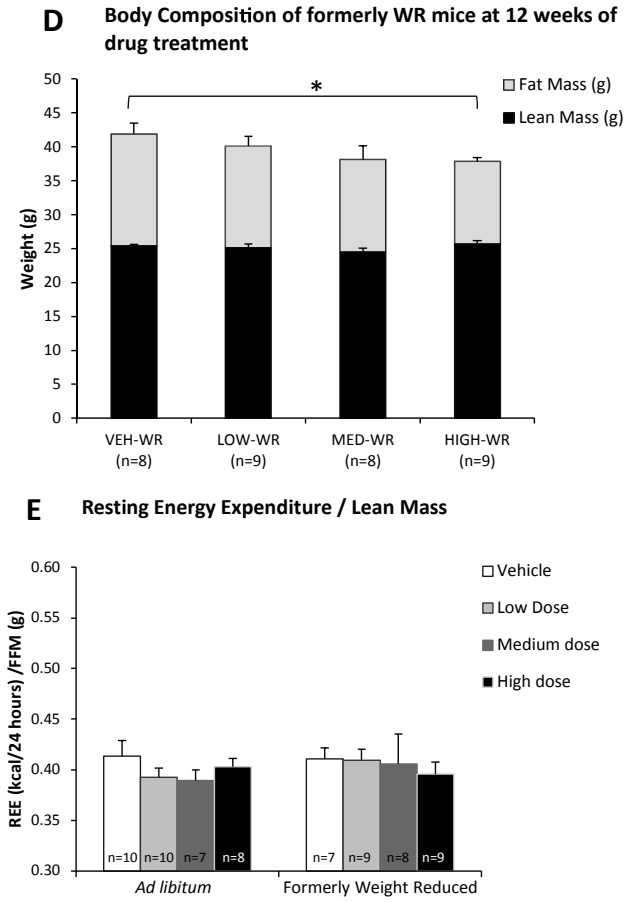


Figure 6-8 Energy expenditure and body composition at 12 weeks of drug treatment.

Absolute total (**A**) and resting (**B**) energy expenditure (kcal/24h) measured at 12-13 weeks of drug treatment and immediately prior to sacrifice by indirect calorimetry (TSE LabMaster system). No significant difference was found in absolute total and resting EE among treatment groups. Body Composition in AL mice (**C**) and formerly WR mice (**D**) at 12 weeks of drug treatment. Formerly WR mice treated with high dose of MC4R agonist have a significantly lower BW and fat mass but show the same TEE and REE. (**E**) Resting energy expenditure (kcal/24h) per unit of lean mass (g) measured at 12-13 weeks of drug treatment and immediately prior to sacrifice by indirect calorimetry (TSE LabMaster system). Nominal doses of AZD2820 - High : 53.4 nmol/day; Medium : 10.8 nmol/day; Low : 2.64 nmol/day. *P < 0.05 between vehicle and high dose treated groups by Student's t-test.

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Chapter 7

"Concluding Remarks and Future Directions"

Concluding Remarks

Obesity is a physical state resulting from an imbalance of energy intake and energy expenditure in which excess fat is stored in peripheral tissues, primarily in the white adipose tissue; when adipose tissue capacity is exceeded, fat is also stored in other tissues including the liver and muscle, resulting in insulin resistance in both organs. Obesity has a range of metabolic, mechanical and psychological consequences which negatively influence health outcomes. Obesity and associated metabolic diseases have increased markedly over the last 4 decades. Genetic factors are important contributors to body weight regulation (they have been estimated to account for 30-60% of risk variance for human obesity); however, human epidemiological studies as well as rodent data suggest that perinatal environment can have substantial effects on body weight in adulthood. Genetic factors can of course interact with environmental factors to promote obesity. Pharmacological approaches and behavioral interventions have not been successful in producing long term weight loss in obese individuals.

Leptin is a critical component of the central circuit that defends against reductions in body fat. The CNS is informed of reduced energy stores due to weight loss by rapid reduction in the concentration of circulating leptin [1, 2]. Weight reduction lowers energy expenditure 15-20% below what would be predicted from changes in body mass and composition, and induces other endocrine and behavioral responses that promote restoration of previous body weight [3, 4].

Administration of small doses of leptin, sufficient to restore circulating leptin to pre-weight loss concentrations, reverses much of the metabolic and behavioral response triggered by the weight reduced state [5-7]. Leptin is also an important neurotrophic factor and, as such, it promotes both the outgrowth of hypothalamic feeding pathways during the postnatal period [8] and neurogenesis during gestation [9, 10].

The general theme of my graduate work has been the role of leptin in body weight regulation. To investigate the physiological consequences of transient hyperleptinemia on the defense of body weight later in life, we generated a transgenic mouse model of inducible (by time and degree) leptin overexpression. Hyperleptinemia is a surrogate for adiposity without the "confounds" of the secondary effects of diet, obesity *per se* and its associated dysmetabolism. We showed that exposing mice to elevated ambient leptin during the "adolescent" period or in adulthood does not affect body weight or composition later in life; however, inducing hyperleptinemia during the pre-weaning period (without permanent consequence to subsequent body weight) causes these mice to gain more weight when exposed to HFD. My future goal is to identify long-term neurobiological consequences of transient elevations in body weight – for example, the impact of obesity in infancy on susceptibility to obesity in adulthood. This mouse can mimic the high circulating leptin seen in obese pregnant and/or nursing women. Determining neuroanatomical alterations in progeny caused by perinatal exposure to high ambient leptin will help in parsing the proximal mechanisms for apparent effects of maternal obesity on risk of obesity in offspring.

In Chapter 4 we found that $Lep^{ob/ob}$ mice, weight reduced by transient caloric restriction, regain lost weight to the level of *ad libitum* fed controls; unlike wild type mice that overeat for up to a week after release from restriction, the $Lep^{ob/ob}$ mice regained the lost weight without any apparent increase in caloric intake compared to *ad libitum*-fed $Lep^{ob/ob}$ controls. These

observations suggest that *Lep*$^{ob/ob}$ mice may sense adiposity via a leptin-independent pathway and regulate body weight through adjustments in energy expenditure and not food intake. Alternatively, congenital leptin deficiency which results in impairments of the hypothalamic circuitry may cause these mice to ingest a fixed number of calories regardless of metabolic status.

The striking resistance to dietary weight loss reported in rare humans homozygous for inactivating leptin mutations is consistent with this inference [11]. The RNAseq results on adipose tissue of weight-reduced and *ad libitum* fed *Lep*$^{ob/ob}$ mice identified an increase (when animals were calorie restricted) in *Ptgrf* – prostaglandin 2Fα receptor; this observation led us to identify significantly lower concentrations of prostaglandin 2Fα (PG2Fα) in the circulation of the calorie-restricted mice. PG2Fα has previously been identified as a potent anti-adipogenic factor in cultured preadipocytes and 3T3-L1 preadipocyte cell line [12-14]. Mice deficient in Akr1b7 (*Akr1b7*$^{-/-}$) enzyme which catalyzes the synthesis of PG2Fα have increased fat mass and are more susceptible to diet-induced obesity due to reduced circulating concentrations of PG2Fα [15]. Interestingly, on chow diet, the *Akr1b7*$^{-/-}$ mice gain more weight by decreasing energy expenditure while eating the same number of calories as WT mice [15]. We intend to further investigate these findings by treating weight-reduced *Lep*$^{ob/ob}$ mice with an inhibitor of prostaglandin transporter (PGT), causing increases in circulating prostaglandin E2 [16] and 2Fα and determining whether these mice increase energy expenditure. The results of this study could add to our understanding of systems governing body weight regulation and they could potentially provide another target for therapeutic weight loss and/or maintenance of reduced weight.

Identifying the causal gene(s) within each associated interval has been a major challenge in making use of the results of genome wide association studies (GWAS). GWAS-identified loci

typically harbor several/many genes that are in linkage disequilibrium (LD) surrounding the signal. Since GWAS are intentionally biased to find signals at >5% population frequency, the associated SNP is typically just a "sign post" in LD to the causative gene/variant. The identification of the causal gene within a locus is a critical step in translating GWAS findings into further biological insights.

In a GWAS of circulating leptin concentrations adjusted for adiposity, Dr. Ruth Loos and colleagues at Icahn School of Medicine at Mount Sinai identified loci putatively modulating leptin production per unit of fat mass. Low production might predispose to obesity. The loci included those in/near *LEP*, *SLC32A1*, *GCKR*, and *CCNL1* [17]. We used a mouse *in vitro* adipose tissue explant model to examine directly the role of implicated candidate genes on leptin synthesis and secretion. Using siRNAs, we knocked down expression of the candidate genes in perigonadal adipose tissue explants from mice then measured leptin expression and secretion into the culture media 12 hours after knockdown. Using this technique, Adipogenin (*Adig*), implicated in the *SLC32A1* locus identified by this GWAS, was found to modify production of leptin in adipose tissue (Chapter 5) [17]. These studies provide a prototype for the functional deconvolution of LD regions identified by GWAS in which a specific phenotype in a cell type can be implicated.

Finally, Chapter 6 addresses an important issue in obesity treatment – the unsuccessful maintenance of reduced weight after otherwise successful weight loss. Since leptin is capable of rescuing neuroendocrine and behavioral compensations associated with maintenance of reduced weight, we hypothesized that a downstream mediator of leptin signaling, a mimetic of αMSH – the physiological agonist of MC4R – could mitigate these compensations by activating the melanocortin circuit, thereby assisting in the maintenance of weight loss. The treatment of diet-

induced obese mice with this novel peptide MC4R agonist in either *ad libitum* fed mice to induce weight loss or to weight-reduced mice showed that this class of drugs can do both, promote weight loss and maintain reduced weight; however 5 x lower dose in a weight-reduced state of the same drug is comparably efficacious to the higher dose in the *ad libitum* state (Chapter 6) [18]. These results suggest that the pharmacology of the weight-reduced state differs from that of individual at usual body weight. Similar to the effects seen in weight-reduced individuals treated with low doses of leptin, MC4R agonists might be effective in the maintenance of weight loss. Major factors limiting approval of new anti-obesity drugs are low drug efficacy in inducing weight loss and unacceptable side effects (i.e. increases in blood pressure). Often the dose of the agent must be relatively high in order to induce substantial weight loss; this high dosing increases off-target effects. This study suggests that a lower dose may be sufficient to maintain reduced body weight versus the dose required to induce the same degree of weight loss (Chapter 6) [18]. Mice in the weight-reduced state were more sensitive to the MC4R agonist. This may be true for other classes of drugs as well.

Future Directions

Good research projects are never truly complete; from that perspective, all of the manuscripts in this thesis represent progress reports on ongoing projects. The mouse that expresses leptin in response to dox treatment is being used to determine both the developmental influences of leptin on body weight regulation and on the type and location of neuronal projections that may be mediated by gestational and postnatal leptin exposure (as a surrogate for maternal adiposity).

In Chapter 4 we show that the *Lep*$^{ob/ob}$ mice reduce energy expenditure when restricted in weight and that they regain fat without increasing food intake when allowed *ad libitum* feeding. RNAseq on fat pad of calorie-restricted *Lep*$^{ob/ob}$ mice and *ad libitum* fed controls – at maximal difference in body weight – identified a transcript signal in prostaglandin F2α receptor. We measured serum prostaglandin F2α concentrations in these mice and found that it was decreased (2 fold) in the weight-reduced animals. We have initiated a collaboration with Dr. Victor Schuster – professor of Physiology & Biophysics at Albert Einstein College of Medicine – who is an expert in prostaglandin signaling. We intend to investigate whether PG2Fα acts as another signaling molecule for the status of fat stores. The details on this follow-up project are described in the section below.

The functional assay that we developed to directly test a knockdown of any gene on production and secretion of leptin from adipose tissue can be used by others to identify new genes involved in regulation of leptin synthesis (Chapter 5) [17].

Rhythm Biopharmaceutics has developed an MC4R agonist that is currently being tested in human patients with monogenic mutations upstream of the MC4R pathway. Dynamic weight loss is physiologically different than sustaining reduced body weight. During maintenance of reduced weight the main purpose is to correct the physiological adaptations that are present as a result of decreased body weight/fat; therefore, interventions designed specifically to improve long-term body weight maintenance are critical. The protocol we developed to test pharmacological agents for efficacy in maintenance of reduced body weight can be used to develop new strategies in obesity treatment (Chapter 6) [18].

In the following section I describe follow up projects that I intend to pursue.

Determine the physiological consequences of transient hyperleptinemia during the gestational period on the apparent set point for adiposity.

Chapter 2 of this thesis describes a transgenic mouse model of inducible hyperleptinemia created as a tool to evaluate the effects of transient elevations of leptin during adulthood, "adolescence" and the postnatal period on the defense of body fat later in life (Chapter 3). We identified the immediate postnatal period as a critical time window during which transient exposure to elevated leptin, *per se*, increases the body weight in adult offspring when given *ad libitum* access to highly palatable food. The postnatal period has previously been described as a critical time for the structural development of the hypothalamus. Using Di-I crystal labeling, Simerly and colleagues reported that in the mouse axonal extensions from the ARH to the PVH DMH, and LHA occur primarily during postnatal week 2 and that neuronal outgrowth is fully mature by P18 [8]. When present at sub-optimal concentrations, hormones such as leptin, insulin, and ghrelin are capable of inducing neuroanatomical changes within the hypothalamic feeding circuits (described in more detail in Chapter 3) [19-22]. Another critical period for hypothalamic development is gestation; hypothalamic neuronal proliferation occurs primarily during mid-gestation in rodents [23]. LepR [9] and POMC [24] expression are detected as early as E10.5, whereas NPY is first expressed between E13.5-14.5 [24]. MC4R mRNA is first expressed on E14 and by E19 is widely expressed across the brain [25].

In non-human primates the development of hypothalamic neural projections (in particular AgRP/NPY neurons originating from the ARH) is evident as early as gestational day 100 (late second trimester), and the density of these projections is substantially increased by day 130 and day 170 of gestation [26]. This is in contrast to the formation of hypothalamic projections in rodents which occur mostly after birth [19] indicating that the critical period for the

hypothalamic neuronal outgrowth differs in rodents and humans; postnatal period in rodents is comparable to the second half of gestation in humans. A study in human fetuses demonstrated that some hypothalamic nuclei such as the lateral hypothalamus differentiate as early as week 9-10 of gestation suggesting that the hypothalamic cell proliferation is initiated during the first trimester in humans [27]. The first appearance of NPY positive neurons in the ARH was detected at 21 weeks of gestation [27]. These data suggest that proliferation and differentiation of hypothalamic neurons begins earlier in human gestation compared to rodents.

Lep$^{ob/ob}$ mice display reduced proliferation of neurons starting on E14 and decreased brain size and weight compared to WT mice throughout their lives [10, 28]. Administration of exogenous leptin to *Lep*$^{ob/ob}$ on E14 increases the number of neurons, indicating a role for leptin in neurogenesis [10]. It is therefore conceivable that exposure to elevated leptin concentrations during gestation may influence developmental neurogenesis in the hypothalamus with effects on the formation and ultimate function of the feeding circuits. Chang *et al*. found that maternal HFD feeding during pregnancy results in increased proliferation, differentiation and migration of neuronal progenitors towards hypothalamic regions in the offspring, leading to higher proportions of putative orexigenic neurons in the PVH of neonates [29]. Offspring of HFD fed dams displayed increased food intake and body weight in adulthood [29]. These findings suggest that maternal HFD and/or obesity affect neurogenesis during gestation and may influence the 'wiring' of the feeding circuits.

It is unclear how maternal HFD/obesity affect leptin concentrations in the fetus. Over the course of pregnancy, leptin concentrations rise by ~65% in maternal circulation in mid to late gestation [30-32]. Interestingly, while obese gravida have higher circulating leptin than lean pregnant women throughout pregnancy, the relative increase in plasma leptin concentration from first to

third trimester is markedly reduced with obesity [31]. The placenta is a site of leptin synthesis and there are studies supporting placental leptin as a source of leptin in fetal circulation in rodents and humans [33, 34]; there is also evidence of transplacental leptin transport from maternal to fetal circulation [35], however, the relative contribution of these two sources is unknown. Additionally, studies have reported leptin expression in fetal tissues, other than placenta, such as fetal cartilage/bone, and hair follicles [33]; our preliminary data also suggest that the mouse fetus may produce leptin from its own tissues.

The leptin transgenic mouse that we generated (Chapter 2) is a suitable model to investigate whether gestational hyperleptinemia *per se* influences future body fat set point. Dox crosses to the fetal circulation through the placenta and elevates leptin in 2TG fetuses while 1TG fetuses do not increase circulating leptin (Chapter 2). Thus, we can induce transient hyperleptinemia in 2TG offspring *in utero*, and include normal leptin 1TG controls that are exposed to the identical intrauterine environment. Dams will be exposed to dox from conception until parturition. After birth, 2TG pups will be released to physiologically normal endogenous circulating leptin and their body weight, composition and food intake will be followed. At 10 weeks of age, mice will be exposed to HFD to evaluate whether gestational hyperleptinemia has influenced the susceptibility of mice to gain weight on a highly palatable diet. We anticipate that hyperleptinemia during gestation will affect hypothalamic neurogenesis and result in altered proportions of orexigenic to anorexigenic neurons in the hypothalamus. Additionally, we will evaluate axonal projections from the ARH to its hypothalamic targets, PVH, DMH, and LHA (described in the following section).

Determine how exposure to elevated leptin during different perinatal periods impacts development of hypothalamic pathways known to regulate energy balance.

In Chapter 3 we showed that hyperleptinemia during the postnatal period results in increased body weight gain when mice are exposed to HFD. However, the mechanism of this apparent postnatal programming is unclear. We have recently initiated a collaboration with Dr. Richard Simerly – professor of Molecular Physiology & Biophysics at Vanderbilt University – an internationally recognized expert on endocrine influences on hypothalamic development. He previously identified a specific role of leptin in the maturation of hypothalamic circuits critical to the regulation of energy homeostasis (work referred to throughout this thesis) [19]. Neuronal projections from the ARH to the PVH, DMH, and LHA are formed during a critical postnatal period (P5 to P16) but, if the mouse lacks leptin, these projections are not fully formed or functional [19]. Brains of mice transiently exposed to hyperleptinemia during the postnatal period - described in Chapter 3 – will be analyzed by immunohistochemical visualization of axons derived from AgRP and POMC neurons in the ARH, and both the distribution and density of neural projections to key hypothalamic targets will be quantified. Specifically, the AgRP- and αMSH-immunoreactive fibers in the PVH, DMH and LHA, which derive from the ARH neurons, will be quantified by visualizing the brain sections with confocal microscopy. Leptin sensitivity will be assessed by immunostaining brains for leptin-induced pSTAT3 and c-Fos.

Presumably, if there are neuroanatomical changes in the feeding circuits, they should precede any change in body weight. Therefore, we are planning to repeat this study and to analyze the brains of postnatally hyperleptinemic mice prior to HFD exposure and immediately after exposure to this diet.

We anticipate that hyperleptinemia during gestation and during lactation will result in a decreased density of projections to the PVH, DMH, and LHA from AgRP- and aMSH-containing neurons in the ARH. Similarly, the impact of elevated leptin in isolation has not been evaluated previously, and may alter cell number, which would alter innervation density in certain hypothalamic targets. We also anticipate that mice exposed to hyperleptinemia will display reduced leptin sensitivity (as determined by leptin induced pSTAT3 staining) before differences in body weight/fat are evident; due, at least in part, to the failure of ARH projections to develop normally. Although at present it is difficult to predict the precise outcomes of the proposed experiments, they will likely provide new insight into the developmental actions of leptin and inform future molecular-mechanistic studies.

Determine whether administration of prostaglandin receptor inhibitor (PGT) to weight-reduced Lep$^{ob/ob}$ mice alters energy expenditure

RNAseq was completed on the subcutaneous (SCAT) and perigonadal (PGAT) adipose tissue depots in both *ad libitum* and weight-reduced *Lep$^{ob/ob}$* mice. Mice were sacrificed at the end of the weight stabilization phase when the difference between the *ad libitum* fed and weight reduced mice was maximal; SCAT and PGAT were collected at sacrifice for RNAseq analysis and serum was collected for further analysis of potential circulating factors. Analysis of RNAseq data revealed 94 significantly (adjusted P-value<0.05) and differentially (|log2 fold change|>1.0) expressed genes for the SCAT and 84 using the same criteria for the PGAT (26 genes overlapped between the depots). The single most significant difference in gene expression was for *Ptgfr* (significant in both adipose depots) with an adjusted P-value for difference of 10^{-35} in the SCAT (**Figure 7.1A**) and 10^{-4} in the PGAT. *Ptgfr* encodes the prostaglandin F2α receptor. Circulating prostaglandin F2α was measured in serum by ELISA on these same mice. The circulating

prostaglandin F2α in the weight reduced mice was twofold lower than in the *ad libitum* fed mice (p-value = 0.018, **Figure 7.2B**). Dr. Victor Schuster – professor of Physiology & Biophysics at Albert Einstein College of Medicine – and colleagues identified the prostaglandin transporter (PGT) which transports extracellular prostaglandins into the cytoplasm for enzymatic inactivation [36]. PGT-deficient mice display elevated extracellular prostaglandin E2 and F2α. Dr. Shuster reports (personal communication) a preliminary phenotype related to body weight and energy expenditure in both the PGT knockout mice and mice treated with a PGT inhibitor that his group has developed. These mice eat double the calories of WT mice but expand more energy resulting in the net reduction of body weight (decreased fat mass and increased lean mass) compared to WT (personal communication). The increase in energy expenditure appears to be due to beiging of adipose tissue that is independent of UCP1 activation (personal communication with Dr. Schuster). We have initiated a collaboration with Dr. Schuster to test the efficacy of this agent in increasing PGF2α in the serum of in weight- reduced $Lep^{ob/ob}$ mice. Our hypothesis is that prostaglandin E2 and/or F2α provide a non-leptin signal of the status of somatic fat stores. The proposed pharmacological elevation of these specific prostaglandins will provide a signal of sufficiency of stores that will increase energy expenditure. $Lep^{ob/ob}$ mice will be injected IP once daily with the PGT inhibitor or vehicle in the weight-reduced state and in the *ad libitum* fed state. Core body temperature (24 hour), body weight, body composition and daily food intake will be measured during drug treatment. Energy expenditure will be measured using indirect calorimetry; the demonstration that this drug increases the energy expenditure of the weight reduced $Lep^{ob/ob}$ mice could nominate PGF2α as another signaling molecule for the status of fat stores and open a new area of research. We hypothesize that any increase in energy expenditure will be due to increased beiging of the adipose tissue.

The mice that I developed as part of this thesis allow experimental alteration of leptin in ways that have never been technically feasible before. By breeding these mice to leptin deficient, *Lep*$^{ob/ob}$ mice, I will be able to turn leptin on and off at any point in development, and I can do this with congenitally leptin deficient littermates, in identical environment, that will not be exposed to excess leptin. I will be able to investigate the neuronal alterations in projections and epigenetic changes that occur with transient hyperleptinemia, and this will allow me to answer questions about maternal environment that could not be previously tested.

Figures and Figure Legends

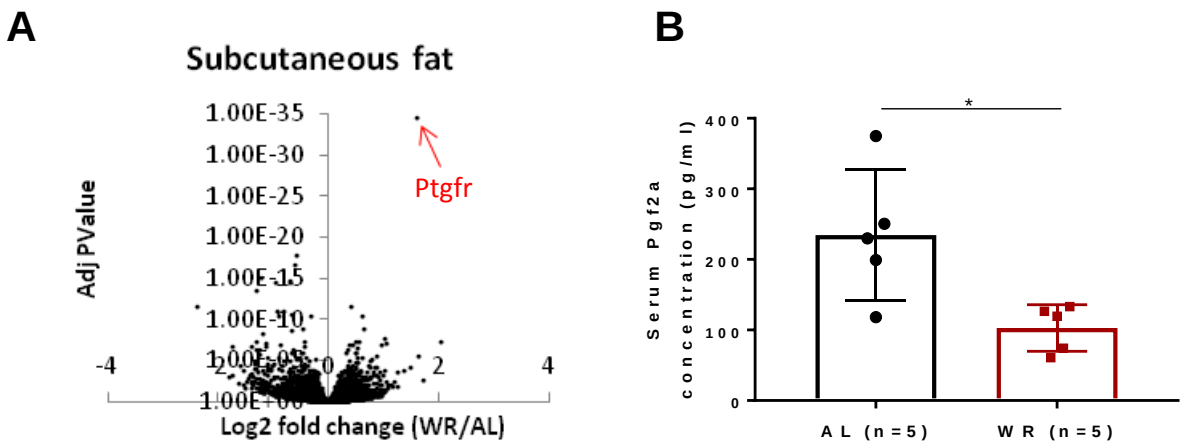

Figure 7-1 SCAT RNAseq data and serum PGF2α concentration in AL and CR Lepob/ob mice.

(A) Volcano plot of RNAseq data from subcutaneous adipose tissue showing adjusted p-value vs log of fold difference in weight reduced to *ad libitum* $Lep^{ob/ob}$ mice. (B) Serum prostaglandin F2α concentration in *ad libitum* compared to weight reduced mice. All values are means ± SEM. N = 5 per group. Significant difference calculated with Student t-test, *p-value < 0.05.

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