GLUCOREGULATORY ACTIONS OF LEPTIN IN RODENT MODELS OF DIABETES

by

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Abstract

The hormone leptin reduces food intake and increases energy expenditure; leptin deficient rodents and humans are severely obese with impaired glucose homeostasis, and leptin therapy reverses these metabolic abnormalities. Based on this, leptin gained significant interest as a potential therapeutic agent to combat obesity; however, it was soon discovered that most obese humans are resistant to its anorectic actions and the initial excitement over the therapeutic applicability of leptin dwindled. It is now evident that leptin regulates glucose metabolism independent of its actions on body weight. Underlining this, leptin displays the remarkable ability to reverse hyperglycemia in rodent models of type 1 diabetes. These findings sparked interest in leptin as a therapy for type 1 diabetes. The overarching goal of this thesis was to perform pre-clinical studies to elucidate the mechanism by which leptin lowers blood glucose levels in insulin deficient mice and assess whether resistance to these effects of leptin can occur. To this end, we assessed the plasma metabolomic profile of leptin-treated insulin deficient mice, which revealed global alterations in amino acid metabolism. Thus, we characterized amino acid utilization in leptin-treated mice, tested the mechanistic role of amino acid catabolism genes, and extensively characterized the metabolic changes in the liver using an 'omics' based approach. In addition, we assessed whether recapitulating leptin-mediated changes in the liver using a small molecule can mimic the glucose lowering actions of leptin. Lastly, we assessed whether hyperleptinemia or high-fat intake, which are reported to cause resistance to the weight reducing actions of leptin, also impede glucose lowering effects of leptin. This thesis revealed that leptin globally alters the liver metabolic profile to suppress the utilization of amino acids for glucose production and recapitulating the transcriptomic profile of leptin therapy with a novel small molecule can lower blood glucose levels in insulin deficient mice. Furthermore, dietary fats, but not hyperleptinemia, causes resistance to the glucose lowering actions of leptin in insulin deficient mice. Collectively, these investigations help elucidate the mechanism by which leptin reverses hyperglycemia in insulin deficient mice and shed insight into the suitability of leptin as a therapy for diabetes.

Lay Summary

Type 1 diabetes is a condition where the pancreas does not produce enough insulin, leading to high blood sugar. These patients take insulin but walk a fine line between lowering blood sugar to avoid damaged tissues and risking low blood sugar, which can be deadly. Another hormone, called leptin, can also lower blood sugar in diabetic animals. The goal of this thesis was to determine how leptin lowers blood sugar and to assess whether having too much leptin or a fat rich diet can cause resistance to leptin. We show that leptin lowers the process of converting amino acids to sugars in the liver and mimicking the effects of leptin on the liver with a drug lowers blood sugar. Also, we show that a high fat diet can prevent leptin from working. These results indicate that leptin may be a useful therapy for diabetes, provided resistance can be avoided.

Preface

All studies presented in this dissertation were conceived, designed, performed, and analyzed by the author MM Kwon, with assistance as described below. This thesis was written by MM Kwon with editing provided by TJ Kieffer, WT Gibson, SM Clee, and SD Covey.

Data from Chapter 3 of this thesis will be submitted for publication. MM Kwon and TJ Kieffer designed the experiments. RK Baker designed and generated the *in vivo* expression plasmids. A McAfee and LJ Foster performed the proteomics experiments and provided raw data for further analysis. RNA-sequencing and mass spectrometry experiments for metabolomics analysis were performed at the Biomedical Research Centre Sequencing Core at the University of British Columbia (Vancouver, BC, Canada) and Metabolon (Durham, NC, USA), respectively, and raw data was provided for further analysis as described in the methods. S Chen and PR Cullis provided lipid nanoparticle encapsulated siRNAs. JNC Toews assisted with experiments and data analysis. MM Kwon performed the experiments and analyzed all data. All authors were involved in the discussion of the data.

Data from Chapter 4 of this thesis will be submitted for publication. MM Kwon and TJ Kieffer designed the experiments. MM Kwon performed the experiments and analyzed the data. MM Kwon and TJ Kieffer are listed as co-inventors in an invention disclosure with the University of British Columbia.

Data from Chapter 5 of this thesis is from the following manuscript, which is currently in revision for publication: MM Kwon, MM Glavas, SD Covey, RK Baker, SM Clee, TJ Kieffer. Dietary fats, but not hyperleptinemia, induce resistance to the blood glucose lowering actions of

leptin in mice with insulin-deficient diabetes. MM Kwon, MM Glavas, and TJ Kieffer designed the experiments. RK Baker designed and generated the *in vivo* expression plasmids. TJ Kieffer, SM Clee, and SD Covey guided interpretation of the results and edited the manuscript. MM Kwon and MM Glavas performed the experiments and analyzed the data, and MM Kwon wrote the manuscript. All authors were involved in the discussion of the data.

In addition to the data presented in this thesis, MM Kwon has published the following first author manuscript on the metabolic actions of fibroblast growth factor 21 (FGF21): MM Kwon, S O'Dwyer, RK Baker, SD Covey, TJ Kieffer. 2015. FGF21-Mediated Improvements In Glucose Clearance Require Uncoupling Protein 1. *Cell Reports* 13(8):1521-7. The following manuscript on FGF21 will be submitted for publication: MM Kwon and TJ Kieffer. Sitagliptin and Rosiglitazone do not enhance FGF21-mediated improvements in body weight and glucose metabolism. Data from these two manuscripts were not included in this thesis.

Animal studies described in this thesis were approved by The University of British Columbia Animal Care Committee (Certificate # A10-0275, A14-0063, A14-0081, and A17-0026).

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List of Abbreviations

α-MSH	α-melanocyte stimulating hormone
1,5AG	1,5-anhydroglucitol
5' UTR	5' untranslated region
AAV	adeno-associated virus
Actb	β-actin
ACTH	adrenocorticotropic hormone
aFP	α-fetoprotein
AgRP	agouti-related protein
ARC	arcuate hypothalamic nucleus
Atg7	autophagy-related gene 7
B2m	β-2-microglobulin
BAT	brown adipose tissue
BBB	blood brain barrier
BBS	Bardet-Biedl syndrome
BMI	body mass index
Bps	basepairs
CAA	2-chloroacetamide
Cas9	CRISPR associated protein 9
CDM	Centre for Disease Modeling
CGM	continuous glucose monitors
CMAP	Connectivity Map
CNS	central nervous system
CREB	cAMP responsive element binding protein 1
CRH	corticotropin releasing hormone
CRISPR	clustered regularly interspaced short palindromic repeats
CRP	C-reactive protein
CSF	cerebral spinal fluid
CTLA4	cytotoxic T-lymphocyte-associated protein 4

Db	diabetes
DEXA	dual energy x-ray absorbance
DLin-MC3-DMA	dilinoleylmethyl-4-dimethylaminobutyrate
DMH	dorsomedial hypothalamic nucleus
DPP4	dipeptidyl peptidase-4
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ESI	electrospray ionization
F7	coagulation factor VII
FA	formic acid
FGF21	fibroblast growth factor 21
G6pc	glucose-6-phosphatase catalytic subunit
GABA	γ-aminobutyric acid
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
Hprt1	hypoxanthine phosphoribosyltransferase 1
GcgR KO	glucagon receptor knockout
GEO	Gene Expression Omnibus
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide-1
Glut2	glucose transporter 2
GLUT4	glucose transporter type 4
GO	gene ontology
Gpd1	glycerol-3-phosphate dehydrogenase
Gpt	glutamic pyruvic transaminase
GRA	glucagon receptor antagonists
GSR	gene score resampling
GWAS	genome wide association studies
HbA1c	hemoglobin A1c

HFD	high fat diet
HILIC	hydrophilic interaction liquid chromatography
HLA	human leukocyte antigen class
HPA	hypothalamic-pituitary-adrenal
HPLC	high-performance liquid chromatography
i.p.	intraperitoneal
i.v.	intravenous
ICV	intracerebroventricular
IGF1	insulin-like growth factor 1
IGFBP2	insulin like growth factor binding protein 2
IKKβ/NF-κB	IκB kinase β/nuclear factor-κB
IL2RA	interleukin 2 receptor α
Ins1	mouse insulin 1
IRS	insulin receptor substrate
JAK2	janus kinase 2
JNK1	c-Jun amino-terminal kinase 1
KCNJ11	potassium voltage-gated channel subfamily J member 11
KLF15	krüppel like factor 15
KPBS	potassium phosphate-buffered saline
KPBSX	Triton X potassium phosphate-buffered saline
Lepr	leptin receptor
LFD	low fat diet
LHA	lateral hypothalamic area
LNP-siRNAs	lipid nanoparticle encapsulated siRNAs
mALB	mouse albumin
MC4R	melanocortin 4 receptor
Metreleptin	methionyl human leptin
MHC	major histocompatibility complex
miRNA	micro RNA
MSEA	metabolite set enrichment analysis

NOD	non-obese diabetic
nPOD	network for pancreatic organ donors with diabetes
NPY	neuropeptide Y
NPY1R	neuropeptide Y receptor Y1
NTS	nucleus tractus solitarius
NZO	New Zealand obese
Ob	obese
ORA	over representation analysis
ORF	open reading frame
Ori	origin of replication
PBS	phosphate buffered saline
Pck1	phosphoenolpyruvate carboxykinase 1
Pdx1	pancreatic duodenal homeobox 1
PEG-DMG	polyethylene glycol-dimyristolglycerol
Pepck	phosphoenolpyruvate carboxykinase
PFPA	perfluoropentanoic acid
Pgk1	phosphoglycerate kinase
PI3K	phosphatidylinositol 3-kinase
PLM	predicted leptin mimetic
PMV	ventral premammillary nucleus
poly(A)	polyadenylation
POMC	pro-opiomelanocortin
PPAR-γ	peroxisome proliferator activated receptor γ
Ppia	peptidylprolyl isomerase A
pSTAT3	phospho-STAT3
PTP1B	protein tyrosine phosphatase 1B
PTPN2	tyrosine phosphatase non-receptor type 2
PVN	paraventricular nucleus
qPCR	real time PCR
Rcf	relative centrifugal force

RER	respiratory exchange ratio
RIP	rat insulin promoter
RNA-seq	RNA sequencing
RP/UPLC-MS/MS	reverse phase ultra-performance liquid chromatography-tandem mass spectrometry
SEM	standard error of mean
SF1	steroidogenic factor-1
SGLT2	sodium-glucose co-transporter 2
SH2	src homology 2
SHP2	SH2-containing protein tyrosine phosphatase 2
SILAM	stable isotope labeling of amino acids in mice
siRNA	small interfering RNA
SNS	sympathetic nervous system
SOCS3	suppressor of cytokine signalling-3
STAT	signal transducer and activator of transcription
STZ	streptozotocin
TCA	tricarboxylic acid
TCEP	tris(2-carboxyethyl)phosphine
TCF7L2	transcription factor 7 like 2
TLR4	toll-like receptor 4
Ucp1	uncoupling protein 1
UHPLC-MS/MS	ultra high-performance liquid chromatography-tandem MS mass spectrometry
VMH	ventromedial hypothalamic nucleus
WAT	white adipose tissue

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Chapter 1: Introduction

1.1 Diabetes Mellitus

Statistics on Diabetes

Diabetes mellitus is a chronic disease characterized by high blood glucose levels due to either an insufficient amount of insulin or the inability to utilize insulin [1]. It is estimated that more than 2.6 million Canadians, 30 million Americans, and 425 million worldwide are living with diabetes [1, 2]. These numbers are growing at an alarming rate; by the year 2045, more than 629 million people may develop diabetes, which is an increase of approximately 48% in 28 years [1]. Additionally, approximately 352 million people have impaired glucose tolerance, putting them at risk for developing diabetes [1]. The costs associated with diabetes to the health care systems are staggering; approximately $17 billion in Canada and $350 billion in the US is being spent yearly on healthcare costs related to diabetes [1]. While there are treatment options for diabetes, it is difficult to achieve stable euglycemia. When not well managed, diabetes can lead to secondary complications including neuropathy, retinopathy, and nephropathy ([1] and reviewed in [3, 4]). These devastating complications can then lead to blindness, lower-limb amputations, cardiovascular disease, and kidney disease, which decreases the quality of life and life expectancy [1]. What's more, half of the people living with diabetes are undiagnosed, leaving them unaware that they may be developing secondary complications [1]. Clearly, given the rapidly growing incidence of diabetes and the heavy burden on health care systems, a better understanding of etiology and therapy are urgently required to curb the disease.

Type 1 Diabetes

Around 10% of all people living with diabetes have type 1 diabetes [1], which was once known as juvenile diabetes or insulin-dependent diabetes. In children, peak in the symptomatic onset for type 1 diabetes occurs between 10 to 14 years of age [5], but diagnosis can occur at any age including in adulthood (reviewed in [6]). The diagnostic hallmarks of type 1 diabetes are hyperglycemia, polydipsia, hyperphagia, polyuria, and ketoacidosis (reviewed in [7]). Once diagnosed, patients require immediate insulin therapy, for which lifelong treatment is needed (reviewed in [7]). A diagnosis of type 1 diabetes can be devastating, especially for children and their families, and management of this chronic disease causes physical, emotional, and financial strain.

Type 1 diabetes is believed to be an autoimmune disease. The generally accepted view for the progression of type 1 diabetes is that lymphocytes infiltrate the pancreatic islets (insulitis) and promote the destruction of insulin-producing β cells (reviewed in [8]). However, insulitis is not observed in all pancreatic specimens from patients with type 1 diabetes [9, 10], suggesting that insulitis is not a hallmark of type 1 diabetes. Furthermore, the pancreas before, at, and after disease onset contains insulin-deficient islets, inflamed islets, and normal islets, although in variable proportions [11]. These findings have led to the notion that islet inflammation may not be the cause of β cell death, but instead a consequence of defective β cell function (reviewed in [12]). Understanding the degree and timeline of islet inflammation and β cell death will shed light into the applicability of therapies seeking type 1 diabetes prevention and reversal. To this end, the network for pancreatic organ donors with diabetes (nPOD) was created to advance collaborative research using tissues from cadaveric organ donors with type 1 diabetes. While the

exact cause of type 1 diabetes is unknown, there is strong evidence that complex genetic and environmental factors may be involved (reviewed in [13]). The average prevalence risk in siblings is 6% compared to 0.4% in the general population, suggesting that there is significant familial clustering of type 1 diabetes [5, 14]. Many genetic loci have been found to be associated with type 1 diabetes, including the genes within the human leukocyte antigen class (*HLA*) cluster which encode components of the major histocompatibility complex (*MHC*; [15]), insulin [16, 17], tyrosine phosphatase non-receptor type 2 (*PTPN2*; [18]), cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*; [19]), and interleukin 2 receptor alpha (*IL2RA*; [20, 21]). In addition to a genetic component, there has been an increase in the rate of type 1 diabetes incidence over time [22], indicating that environmental factors may also play a role. Environmental factors that may trigger type 1 diabetes include viral infections [23, 24], consumption of milk [25], deficiency of vitamin D [26], higher weight gain during infancy [27, 28], and exposure to toxins such as nitrate [29]. Thus, type 1 diabetes is a heterogeneous disease with marked variations in disease progression, genetic susceptibility, and environmental contributions.

Type 2 Diabetes

Type 2 diabetes is the most common form of diabetes, and accounts for approximately 90% of all cases of diabetes [1]. Type 2 diabetes is characterized by hyperglycemia resulting from relative insulin deficiency, insulin resistance, or both [1]. Typically, type 2 diabetes is diagnosed in adults but the incidence is rising in children and adolescents [30]. The symptoms of type 2 diabetes may be similar to type 1 diabetes, including polydipsia, polyuria, fatigue, slow-healing wounds, and recurrent infections [1]. However, the progression of type 2 diabetes is slower and less severe at early stages than seen in type 1 diabetes, and thus many patients remain

undiagnosed, which increases their risk for the development of secondary complications [1]. Both environmental and genetic factors contribute to the development of type 2 diabetes. Type 2 diabetes is modestly correlated with obesity; approximately 58% of adults with type 2 diabetes are also obese [31]. In addition, pollutants [32, 33], dietary vitamin supplementation [34], and chemical contaminants in food such as dioxins [35] are associated with type 2 diabetes. Type 2 diabetes is at least partially hereditary, with a 70% lifetime risk for a child when both parents are affected [36]. Large-scale genome wide association studies (GWAS) have identified genetic variants that increase the risk of type 2 diabetes, including peroxisome proliferator activated receptor gamma (*PPAR-γ*), potassium voltage-gated channel subfamily J member 11 (*KCNJ11*) and transcription factor 7 like 2 (*TCF7L2*) [37-39]. The relative importance of genetics and environment likely varies significantly among patients, underlining the complexity of type 2 diabetes.

There are a variety of treatment options for patients living with type 2 diabetes. Dietary modifications and exercise are recommended to lower blood glucose levels, but pharmacological intervention may also be used [1]. The most commonly prescribed medication is metformin, which lowers hepatic glucose production and increases insulin sensitivity [40-42]. Other medications include sulfonylureas to increase insulin secretion, sodium-glucose co-transporter 2 (SGLT2) inhibitors to increase glucose excretion, dipeptidyl peptidase-4 (DPP4) inhibitors to prevent the inactivation of insulin secretagogues, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), and GLP-1 analogues [43]. Although these medications can be effective at the early stages of type 2 diabetes, β cell destruction can occur at the later stages, for which insulin therapy is required to restore euglycemia [43]. Despite a wide array of treatment options available for type 2 diabetes, optimal glycemic control is difficult to

obtain and many patients battle long-term complications of hyperglycemia [44].

The Complexity in Classifications of Diabetes

Increasingly, it is becoming evident that the canonical classification of diabetes into type 1 and type 2 may be insufficient. In fact, there is considerable overlap between these two conditions. Immune responses associated with type 1 diabetes, such as elevated autoantibodies and cytokines, have also been observed in patients living with type 2 diabetes [45]. Obesity and insulin resistance is also associated with an increased risk of type 1 diabetes [46, 47]. Islet amyloid, which is thought to precipitate β cell dysfunction in type 2 diabetes is present in the pancreas of donors with type 1 diabetes [48, 49]. Lastly, β cell loss appears also in type 2 diabetes [50]. Furthermore, there is a high degree of heterogeneity in disease progression and severity within both type 1 and type 2 diabetes [51, 52]. Combined, these data suggest that the current classification of diabetes may be inadequate. Therefore, a more refined definition based on clinical observations may better guide research and improve the efficacy of therapeutic interventions.

1.2 The Hormone Insulin

The Discovery of Insulin

The discovery of insulin has transformed the treatment of diabetes [53]. Before the discovery of insulin, type 1 diabetes was an acute and fatal disease [53]. In 1921, Fredrick Banting and John Macleod isolated pancreatic extracts at the University of Toronto, which they used to reverse diabetes in animal models [54]. In 1922, these pancreatic extracts were used on humans with type 1 diabetes, which dramatically reversed hyperglycemia, polyuria, and ketoacidosis [54, 55]. These effects on patients, especially children, who until then were bound

to die, was deemed miraculous, and the Nobel prize was awarded to Banting and Macleod in 1923 [56]. Since the discovery of insulin, type 1 diabetes has become a chronic but manageable disease [1]. Technologies surrounding insulin therapy has continued to progress; synthetic insulin analogues with varying onset, peak, and duration of activity are available to control blood glucose levels (reviewed in [57]). In addition to insulin injections, patients can now deliver insulin via a pump, which avoids the need for constant needle injections [58]. Thus, almost a century after its discovery, insulin continues to save the lives of patients worldwide.

Insulin Therapy

As stated by Frederick Banting in his Nobel Lecture for the discovery of insulin, "Insulin is not a cure for diabetes; it is a treatment" [56]. The majority of patients living with type 1 diabetes relies on glucometers to track their blood glucose levels, and must self-administer a dose of insulin via injections or pumps to lower their blood glucose levels (reviewed in [59]). In addition, patients must track their carbohydrate intake and physical activity to carefully adjust their insulin dose. Patients walk a fine line between minimizing glucose excursions to reduce chances of developing complications of the disease and risking hypoglycemia, which can cause seizures, coma, and death [1]. Thus, the management of diabetes is extremely arduous and difficult to maintain, especially for children. Technologies aimed at relieving the burden of diabetes are progressing. For example, continuous glucose monitors (CGM) avoid the need for repeated finger pricks (reviewed in [60]). Recently, artificial pancreas systems, which use closed-loop algorithms to couple CGM to insulin pumps for automated insulin release, have been approved by various regulatory agencies. These devices lessen the burden of diabetes management and are more effective at achieving glycemic control over standard methods of care

[61]; however, current iterations still require some degree of user input and patients using these devices do not reach optimal glycemic control (reviewed in [62]). Islet transplantation has proven to be effective in minimizing glucose fluctuations [63, 64] but is limited by donor shortage and the challenges of lifelong immunosuppression (reviewed in [65]). To address these limitations, stem cell-derived β-like cells have been developed, and show promising results in rodent models of type 1 diabetes [66, 67]. Eventually, cell therapies may be an option for all patients living with diabetes (reviewed in [68]). Currently, despite its major limitations and challenges, insulin replacement is the only effective treatment option for patients living with type 1 diabetes.

Metabolic Actions of Insulin

In response to elevated blood glucose levels, insulin is released to promote glucose uptake in multiple tissues. Insulin is synthesized in the pancreatic β cells, packaged into secretory vesicles, and then released in response to a stimulus such as elevated glucose levels from a meal (reviewed in [69]). In addition to glucose, other nutrients such as amino acids [70] and free fatty acids [71] promote insulin secretion. The gut also senses nutrients such as glucose and amino acids, and then releases hormones such as GLP-1 and GIP to augment insulin secretion [72-76]. In muscle and adipose tissues, circulating insulin binds to the insulin receptor, which triggers the movement of glucose transporter type 4 (GLUT4) from intracellular stores to the plasma membrane [77-80]. It is estimated that approximately 90% of insulin-stimulated glucose uptake occurs in skeletal muscle, while adipose tissue accounts for approximately 10% [81]. Glucose uptake also occurs through insulin-independent mechanisms using alternative glucose transporters in tissues such as the brain, liver, and pancreatic β cells (reviewed in [82]). These

processes must all be precisely orchestrated to restore euglycemia after a meal.

Insulin is a potent inhibitor of glucose production. In the liver, insulin directly inhibits gluconeogenesis and stimulates glycogenesis through transcriptional modulation (reviewed in [83]). Insulin also suppresses hepatic gluconeogenesis through indirect mechanisms; insulin lowers the release of gluconeogenic substrates from the adipose tissue and the skeletal muscle, reduces the release of gluconeogenic activators, and acts on the brain to lower sympathetic tone [83]. In the muscle, insulin stimulates glycogenesis and inhibits proteolysis to reduce plasma levels of amino acids, which are substrates for gluconeogenesis [84, 85]. In addition to insulin, the hormone glucagon released from the pancreatic α cells is also a key regulator of glucose production; glucagon stimulates hepatic gluconeogenesis and glycogenolysis (reviewed in [86]). Insulin inhibits glucagon secretion and thus reduces glucagon-mediated stimulation of hepatic glucose production [87, 88]. In uncontrolled type 1 and type 2 diabetes, diminished insulin signalling increases glucagon levels, which worsens hyperglycemia (reviewed in [89]). Lastly, insulin, but not glucagon, directly inhibits gluconeogenesis in the kidneys [90]. Thus, insulin and glucagon act as key regulators of glucose production to sustain euglycemia between meals.

In addition to stimulating glucose uptake and suppressing glucose production, insulin promotes anabolic processes in lipid and protein metabolism. Insulin promotes lipid synthesis and storage of lipids such as triglycerides (reviewed in [91]). In addition, insulin inhibits lipolysis and lipid oxidation. In the muscle, insulin promotes the synthesis of amino acids and proteins [85]. The shortage of insulin in uncontrolled type 1 diabetes results in exacerbated lipolysis, diminished triglyceride stores, and increased free fatty acids in the circulation (reviewed in [92]). The increase in hepatic fatty acids and lipid oxidation results in elevated

ketone bodies, which can cause acidification of the blood resulting in the life threatening complication of diabetes called diabetic ketoacidosis (reviewed in [93]). Insulin also stimulates the breakdown of lipoproteins rich in triglycerides contributing to an increase in circulating triglyceride levels in uncontrolled type 1 diabetes (reviewed in [94]). Overall, the diminished anabolic actions of insulin on the adipose tissue and muscle reduces fat and lean mass, which contributes to rapid weight loss observed in undiagnosed patients living with type 1 diabetes [1]. In type 2 diabetes, resistance to the glucose lowering actions of insulin develops but it appears that some anabolic actions of insulin, such as hepatic lipogenesis, are selectively enhanced ([95] and reviewed in [96]). While circulating free fatty acids and triglycerides are elevated as also seen in states of insulin-deficiency, there is an increase in lipogenesis and ectopic lipid accumulation (reviewed in [96, 97]). Therefore, insulin regulates lipid and protein anabolism, and these processes, when compromised, account for many of the diagnostic hallmarks of diabetes.

1.3 The Hormone Leptin

The Discovery of Leptin

The road to discovery of leptin began when inbred mouse strains with obesity and diabetes spontaneously arose at the Jackson Laboratory. In the summer of 1948, mice characterized by massive obesity, marked hyperphagia and mild transient diabetes were discovered at the Jackson Laboratory, and were named *obese* or *ob/ob* mice [98]. In 1965, another obesity mutant was discovered [99]. This second mutant strain also had obesity, hyperphagia, but had much more severe, life-shortening diabetes, and thus were named *diabetes* or *db/db* mice [99]. These two strains had near identical phenotypes when crossed onto the same

genetic background and the two mutations were mapped to separate chromosomes [100]; therefore, it was suspected that the mutations may occur in genes in a common metabolic pathway [101]. To test this hypothesis, Coleman performed a series of parabiosis studies, which involves joining the circulation of two mice surgically. First, Coleman found that when a *db/db* mouse was parabiosed with a wildtype mouse, normal partners had severe hypoglycemia, lacked food in their stomachs, and died [102]. Second, parabiosis of *ob/ob* mice with normal mice decreased food intake and body weight in *ob/ob* mice, and the pairs survived for months [103]. Combined with additional parabiosis experiments, Coleman concluded that the *db/db* mice overproduced a satiety factor but could not respond to it, likely due to a defective receptor, whereas the *ob/ob* mice recognized and responded to this satiety factor but could not produce it [101]. These studies challenged the presiding dogma at the time that obesity was entirely behavioral, not physiological, and prompted a hunt for the elusive satiety factor.

More than 20 years after the parabiosis experiments by Coleman, the circulating satiety factor and its receptor were identified. A group led by Jeffrey Friedman identified the protein product of the *Obese* gene through positional cloning [104]. The protein is made in the adipose tissue and has weight reducing effects when administered to wild type and *ob/ob* mice; therefore, this protein product was named leptin, derived from the Greek word leptós, meaning thin [105]. The *ob/ob* mouse harbors a single nonsense mutation in the gene encoding leptin, which leads to an early stop codon at the residue position 105 and the production of a truncated protein product [104]. As a result, *ob/ob* mice have undetectable circulating leptin levels, and exogenous leptin administration improves their metabolic abnormalities including obesity and diabetes [105, 106]. Shortly after the discovery of leptin in mice, humans with homozygous loss of function mutations in leptin, including the mutation identical to the *ob/ob* mouse, were identified [107-

113]. These patients have congenital leptin deficiency or lack bioactive leptin, and as a result, are morbidly obese and hyperphagic from a young age [107, 109-113]. In addition to obesity, both mice and humans with congenital leptin deficiency have a plethora of dysfunctions, including insulin resistance, an impaired immune and reproductive system, and neuroendocrine defects ([114-116] and reviewed in [117]). Remarkably, leptin replacement potently lowers body weight and normalizes metabolic disturbances in both humans and mice [107, 109-113, 118-121]. Thus, leptin therapy is life changing for patients with congenital leptin deficiency.

Leptin and the Leptin Receptor

Since its discovery, the hormone leptin has been studied extensively. Leptin is a member of the type I helical cytokine family, and is related to the growth hormone, prolactin and interleukins [122]. Leptin contains 167 amino acid residues and circulates as a 16 kDa protein [104]. There is an approximately 80% sequence homology between human and rodent forms of leptin [104]. Similar to other class I helical cytokines, leptin has four α helix bundle folds [123]. The N-terminal region of leptin is essential for receptor binding and anorectic actions of leptin, while the C terminal region is important for enhancing these activities [124]. Leptin is expressed in many tissues including the white adipose tissue (WAT; [125]), brown adipose tissue (BAT; [126]), mammary gland [127], placenta [128, 129], skeletal muscle [130], stomach [131], and the pituitary [132]. The WAT is the major source of circulating leptin, and thus these alternative sources of leptin may confer autocrine or paracrine effects [133]. Leptin is secreted from the WAT through a constitutive pathway [125, 134], and circulating leptin levels are typically proportional to fat mass [135, 136]. Leptin levels are also increased acutely in response to food intake [135, 137] and insulin [138]. In contrast, fasting and insulin deficiency dramatically

decrease circulating leptin levels independent of fat mass, which triggers metabolic and behavioral changes associated with hunger [139-141]. Furthermore, starvation causes neuroendocrine and reproductive dysfunction, and exogenous leptin reverses these effects of starvation [139]; thus, it has been proposed that leptin serves as a mediator during adaptation to fasting, and this is the primary function for which leptin evolved [139]. Therefore, the discovery of leptin unveiled the adipose tissue as not merely a fat store but also a dynamic endocrine organ.

Soon after the discovery of leptin, the leptin receptor (*Lepr*) was identified. *Lepr* was cloned from a mouse choroid plexus cDNA library through expression cloning [142]. The *Lepr* gene is a member of the class I cytokine receptor family and encodes a transcript, which produces at least 6 isoforms (Lepr-a to Lepr-f) through alternative splicing [143, 144]. The Lepr-b isoform contains the longest intracellular domain [142-144]. Consistent with Coleman's predictions 20 years earlier [102], *db/db* mice were found to contain an insertion mutation in the *Lepr* gene, which impairs splicing and produces Lepr-b with a truncated intracellular domain [143, 144]. All other isoforms remain intact in *db/db* mice, suggesting that signalling through the Lepr-b isoform is responsible for the key metabolic actions of leptin [143, 144]. The precise roles of the other Lepr isoforms are unclear. The Lepr-a isoform has the most abundant and broad tissue expression [145], and has been suggested to be involved in leptin transport across the blood brain barrier (BBB) [146, 147]. The other leptin receptor isoforms with an intact transmembrane domain (Lepr-c, Lepr-d, and Lepr-f) may modulate leptin binding by forming heteromers with Lepr-b [148]. In mice, the Lepr-e isoform does not contain a transmembrane domain [144], and thus has been suggested to encode the soluble leptin receptor (reviewed in [149]). However, there are no definitive studies confirming that Lepr-e acts as a soluble leptin receptor. Furthermore, Lepr-e expression has not been confirmed in humans [150, 151]. Lepr

isoforms containing the transmembrane domain may be cleaved then shed into the circulation to act as a soluble leptin receptor [152, 153]; however, further studies are required to resolve the precise role of cleaved leptin receptors in humans and in mice. Although the identities of the soluble leptin receptors remains largely unknown, soluble leptin receptors may sequester free leptin from signalling through Lepr-b [154-156]. Thus, determining the precise identity and roles of soluble leptin receptors may shed light into the mechanism by which leptin bioactivity is regulated in the circulation.

The binding of leptin to Lepr-b initiates a number of signalling pathways. Lepr-b homodimers are present on the cell membrane and undergo a conformational change upon binding of leptin [157]. This conformational change induces the tyrosine kinase activity of constitutively associated janus kinase 2 (JAK2) resulting in JAK2 auto-phosphorylation, and JAK2-mediated phosphorylation of Tyr985, Tyr1077, and Tyr1138 on Lepr-b [158-161]. Phosphorylation of each residue recruits distinct intracellular proteins. Phosphorylation at the Tyr985 residue recruits Src homology 2 (SH2)-containing protein tyrosine phosphatase 2 (SHP2), which initiates the extracellular signal-regulated kinase (ERK) signalling pathway [160]. Phosphorylation of Tyr1077 and Tyr1138 in turn phosphorylates signal transducer and activator of transcription (STAT) proteins 5 and 3, respectively, which dimerize and translocate into the nucleus [158, 160-163]. In the nucleus, STATs bind to DNA to modulate the transcription of target genes that mediate the metabolic actions of leptin (reviewed in [164]). Leptin signalling is regulated by a negative feedback mechanism. Activated STAT3 induces the expression of suppressor of cytokine signalling-3 (SOCS3), which can bind to phosphorylated Tyr985 or JAK2 to inhibit leptin signalling [165-167]. In addition, protein tyrosine phosphatase 1B (PTP1B) can dephosphorylate JAK2 to negatively regulate leptin signalling [168]. Phosphorylated JAK2 can

also activate the insulin receptor substrates (IRSs) and subsequently initiate the phosphatidylinositol 3-kinase (PI3K) pathway, suggesting that there is some level of cross-talk between leptin and insulin signalling [169-171]. All isoforms, except for Lepr-e, contain the JAK2 binding domain, but only Lepr-b contains the STAT binding domain (reviewed in [172]). These diverse signal transduction pathways allow for the complex behavioral and metabolic actions of leptin.

Lepr-b is expressed throughout the central nervous system (CNS) and many peripheral tissues [142, 144, 173-175]. The hypothalamus is a key brain region involved in the regulation of feeding, body weight, and neuroendocrine systems (reviewed in [176]). Consistent with the effects of leptin on these processes, Lepr-b expression is highly expressed in the nuclei within the hypothalamus, including the arcuate hypothalamic nucleus (ARC), ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), and lateral hypothalamic area (LHA) [173]. In addition to the hypothalamus, Lepr-b is expressed in the thalamus and cerebellum, suggesting that leptin may act on specific sensory and motor systems [173]. In the brainstem, Lepr-b is expressed in several nuclei, including the dorsal raphe, the lateral parabrachial, the Edinger-Westphal, and the nucleus tractus solitarius (NTS) [177-179]. Leptin receptors are also expressed in non-neuronal cells in the brain, including the meninges, choroid plexus, and blood vessels, which suggests that leptin receptors may be involved in leptin transport into the brain and clearance from the cerebrospinal fluid [173]. Outside the CNS, leptin receptors, including the Lepr-b isoform, are expressed in multiple peripheral tissues, including the endocrine cells of the pancreatic islets [180], skeletal muscle [181], adipose tissue [182], liver [183], immune cells [184], heart [185], intestine [186], and ovaries [187]. Thus, leptin receptor expression is widely distributed throughout the CNS and the periphery.

Central Actions of Leptin

There is strong evidence that the majority of body weight and blood glucose lowering effects of leptin are mediated through the CNS. Low doses of leptin delivered directly into the brain of *ob/ob* or normal mice via intracerebroventricular (ICV) injections decrease body weight and lower blood glucose levels, similar to systemic leptin administration [188, 189]. Moreover, re-expression of leptin receptors specifically in the brain of *db/db* mice attenuates obesity and diabetes [190], while deletion of leptin receptors in the brain of normal mice induces obesity and diabetes [191]. Within the CNS, the hypothalamus seems to be a major target for leptin action, as evident from the concentrated expression of leptin receptors in this region [173]. Even before the discovery of leptin, Coleman and others observed that lesions in the hypothalamus recapitulated obesity and diabetes seen in *ob/ob* and *db/db* mice [192, 193]. Lepr null mice are deficient in leptin receptors except in tissues with Cre expression, and reconstitution of Lepr-b exclusively in the ARC of Lepr null mice decreases body weight and blood glucose levels in rodents [194, 195]. In addition, transplantation of wildtype hypothalamic neural progenitors attenuates obesity and diabetes in *db/db* mice [196]. Combined, these studies suggest that the CNS, particularly the ARC, is a key site for leptin's effects on body weight and glucose metabolism.

The ARC contains distinct populations of neurons that are responsive to leptin. A subset of neurons in the ARC expresses pro-opiomelanocortin (POMC), the polypeptide precursor from which melanocortins such as α-melanocyte stimulating hormone (α-MSH) are derived [197-199]. Activation of POMC neurons stimulates the secretion of α-MSH, which binds to and activates melanocortin 4 receptors (MC4R) on second-order neurons to induce satiety and energy expenditure [200, 201]. While neurons expressing MC4R are distributed throughout the brain,

neurons within the paraventricular nucleus (PVN) express high levels of MC4R [202]. Furthermore, injection of an α-MSH peptide analogue directly into the PVN potently decreases food intake, suggesting that PVN is a key site for α-MSH-mediated regulation of food intake [203]. Adjacent to the POMC neurons, there are neurons that co-express neuropeptide Y (NPY) and agouti-related protein (AgRP). AgRP is an endogenous antagonist against MC4R, and thus opposes the actions of α-MSH [204]. The most common monogenic form of obesity in humans is due to mutations in MC4R, and it is estimated that loss of function mutations in *MC4R* account for ~6% of severe childhood obesity cases [205]. NPY stimulates feeding by acting primarily on neuropeptide Y receptor Y1 (NPY1R) on second-order neurons [206, 207]. Lepr-b is expressed in both POMC and AgRP/NPY neurons, and leptin activates POMC neurons and inhibits AgRP/NPY neurons, thereby inhibiting food intake and increasing energy expenditure [189, 208]. Conversely, when leptin levels fall during periods of fasting or weight loss, the inhibitory signals on AgRP/NPY neurons are reduced, which stimulates food intake and suppresses energy use [204, 207]. Thus, distinct neurons in the ARC are important for leptin-mediated regulation of body weight and energy expenditure.

Although ARC is a key site of leptin action, evidence suggests that other leptin responsive neurons are also involved. Selective deletion of leptin receptor signalling in POMC neurons, AgRP neurons, or both, only results in mild obesity and diabetes [208, 209]. Conversely, mice with expression of leptin receptors exclusively in POMC neurons remain obese [210, 211] and display only mild improvements in symptoms of diabetes. Therefore, neurons in the ARC that do not express POMC or AgRP, or neurons outside of the ARC may also mediate the effects of leptin on body weight and glucose metabolism. Indeed, disruption of leptin receptor signalling in hypothalamic nitric oxide synthase expressing neurons [212], steroidogenic

factor-1 (SF1) expressing neurons of the VMH [213], and the hindbrain [214] leads to weight gain and aberrant glucose metabolism. In addition, redundant neural circuits may mediate the effects of leptin on body weight and glucose homeostasis. Consistent with this notion, there is an additive increase in body weight and insulin levels with a combined deletion of leptin receptors in POMC and AgRP neurons [209], and an additive increase in body weight and glucocorticoid levels with deletion of leptin receptors in in POMC and SF1 neurons [213]. The relative contributions of these neurons and the extent of redundant circuits in leptin action remain largely unknown.

Peripheral Actions of Leptin

Although it is believed that majority of leptin's effects on body weight and glucose metabolism are mediated through the CNS, leptin also acts directly on peripheral tissues. Leptin receptors are highly expressed in key peripheral tissues involved in metabolic homeostasis, including the liver [183], endocrine cells of the pancreas [180], muscle [181], and the adipose tissue [182]. To study direct leptin action on these tissues, mice with tissue specific ablation of leptin signalling have been generated using the Cre-*lox* technology. Although previous studies had suggested that leptin acts through the CNS to increase insulin sensitivity in the liver [215-218], ablation of leptin signalling in the liver enhances hepatic insulin sensitivity [219]. There is conflicting evidence on the role of direct leptin action on β-cells. Reducing leptin signalling in β-cells using the rat insulin promoter (RIP) Cre line increased body weight and insulin levels [220] but when the pancreatic duodenal homeobox 1 (Pdx1) Cre was used, insulin levels increased without changes in body weight [221]. It should be noted that Cre is expressed in the hypothalamus of RIP Cre mice [222] and in the hypothalamus and the duodenum of Pdx1 Cre

mice [223], and off target loss of Lepr signalling may contribute to differences in phenotype observed. Recently, a less promiscuous mouse insulin 1 (Ins1) Cre line was used to reduce leptin signalling in β-cells, and neither insulin levels nor body weight were altered [224]. Partial ablation of leptin receptors in α-cells did not alter body weight, glucose or lipid metabolism, suggesting that direct leptin signalling in α-cells is not important for these metabolic parameters [224, 225]. Central leptin action increases insulin sensitivity in muscle [226] but there is conflicting evidence on the direct actions of leptin in muscle, with some studies showing decreased insulin sensitivity [227, 228], and others reporting that leptin can mimic the effect of insulin on glucose transport and glycogen synthesis [229]. Interestingly, leptin may act in an autocrine or paracrine manner on the adipose tissue. Leptin receptor knockdown in white adipocytes with small interfering RNA (siRNA) leads to increased adiposity, impaired glucose tolerance, and decreased insulin sensitivity [182], but when adiponectin Cre is used to knockdown the leptin receptor, mice are leaner with enhanced glucose stimulated insulin secretion [230]. Overall, these conflicting results may be due to differences in the extent or specificity of leptin receptor knockdown, and should be interpreted with caution. Thus, leptin acts directly on peripheral tissues but the precise effects of peripheral leptin signalling are unresolved.

Body Weight Independent Effects of Leptin on Metabolism

It is well established that adult rodents deficient of leptin (*ob/ob* mice or KiloRat) [98, 103, 106, 231] or leptin receptor signalling (*db/db* mice, Zucker diabetic fatty rats, and JCR:LA-cp or SHR/N-cp rats) [99, 102, 103, 232-235] exhibit symptoms of diabetes, including hyperinsulinemia, insulin resistance, impaired glucose tolerance, and in some cases,

hyperglycemia. Most documented cases of congenital leptin deficiency involve adolescents and children, and in these patients, hyperinsulinemia is common, but impairments in glucose tolerance are milder than in rodents and hyperglycemia is not commonly observed [109-111, 120, 121, 236, 237]. Incidence of hyperglycemia is more commonly reported in adults with congenital leptin deficiency [107, 119, 238], suggesting that symptoms of diabetes may be unveiled with age in these patients. Consistent with this notion, hyperglycemia worsens with age in *ob/ob* and *db/db* mice [239, 240], although these studies only tracked glycemia until 8 and 8 months of age, respectively. With leptin therapy, aberrant metabolic parameters are improved in both rodents and humans with leptin deficiency [110, 119-121, 238].

Initially, it was believed that perturbed glucose metabolism in *ob/ob* or *db/db* mice was secondary to obesity; however, several lines of evidence suggests that leptin regulates glucose metabolism independent of its effects on body weight. First, hyperinsulinemia is observed before the onset of obesity in *ob/ob* mice [239, 241-243] and in rodents with defective leptin receptor signalling, including *db/db* mice, Zucker fatty (*fa/fa*) rats, and obese Koletsky (fa^k/fa^k) rats [234, 244-247]. Furthermore, disruption of leptin signalling in wildtype mice using a leptin antagonist causes hyperinsulinemia within a few days of antagonist treatment, prior to development of obesity [217]. Second, pair feeding *ob/ob* mice such that food intake is comparable to leptin-treated *ob/ob* mice does not lower glucose or insulin levels to the same extent as seen with leptin treatment [248-250]. Third, low dose leptin treatment in *ob/ob* mice can normalize insulin and glucose levels without lowering body weight [106]. When a higher dose is used, leptin lowers circulating insulin and glucose levels in *ob/ob* mice before changes in body weight [251, 252], and these glucose lowering effects outlast the weight reducing effects of leptin [253]. Combined,

these studies clearly establish that leptin regulates glucose metabolism independent of its effects on body weight.

There are conditions of leptin deficiency without increased adiposity, which further illustrate the importance of leptin in glucose and lipid metabolism. In rodents and humans with lipodystrophy, there is a partial or near complete loss of adipose tissue, which results in extremely low leptin levels (reviewed in [254]). These patients exhibit signs of diabetes, including mild to severe hyperglycemia, hyperinsulinemia, insulin resistance, and dyslipidemia [255-257]. These metabolic aberrations are largely corrected by leptin therapy in both rodents and patients with lipodystrophy [255-257], demonstrating that leptin deficiency leads to severe metabolic aberrations even in the absence of obesity. Insulin deficient rodents have severely depleted WAT [258], and recapitulate symptoms of type 1 diabetes including hyperglycemia, insulin resistance, impaired glucose tolerance, and dyslipidemia (reviewed in [259]). Administration of leptin to insulin-deficient rodents largely normalizes these metabolic abnormalities without detectable changes in adiposity [260-268]. Thus, leptin deficiency, even in the absence of extreme adiposity, leads to perturbed glucose and lipid metabolism.

1.4 Leptin Resistance

The discovery of leptin led to initial excitement over its potential as a treatment for obesity and type 2 diabetes. While there are rare cases of congenital leptin deficiency in humans, a deficit of leptin does not underlie most cases of obesity [269, 270]. In fact, circulating leptin levels are highly correlated with body mass index (BMI), and *ob* mRNA in adipocytes and circulating leptin levels are elevated in both obese humans [271-273] and diet-induced obese mice [135, 137]. Thus, high endogenous leptin levels fail to suppress feeding and increase

energy expenditure in obesity, and the concept of leptin resistance emerged to explain the seemingly paradoxical elevation of leptin levels in obesity. Soon after reports of hyperleptinemia in common obesity, it was found that diet-induced obese rodents are resistant to the body weight reducing effects of exogenous leptin therapy [274]. Leptin action is impaired in the brain, as evidenced by decreased STAT3 phosphorylation and neuropeptide release [275-278]. In obese humans, leptin therapy showed limited and variable efficacy in reducing body weight at low [279] and high doses, respectively [280]. Leptin therapy in obese patients living with type 2 diabetes did not improve insulin sensitivity and only marginally lowered glycemia [281, 282]. However, leptin therapy appears to be effective in preventing weight regain and decreasing appetite in individuals who have undergone ~10% weight loss through diet [283-286] or gastric bypass [287, 288]. In addition, obese individuals with lower baseline leptin levels exhibit greater weight loss with leptin therapy [289]. Thus, leptin resistance prevents the widespread use of leptin as a therapy for obesity and type 2 diabetes, but leptin therapy may be effective in a selective subset of these individuals.

The precise mechanism of leptin resistance is unknown but several models have been proposed. It has been postulated that despite hyperleptinemia, there may be less bioactive leptin available in the brain due to leptin binding proteins [290, 291]. The exact identities of these leptin binding proteins are unknown but the soluble leptin receptor and C-reactive protein (CRP) have been implicated [151, 292]. In diet-induced obese mice, leptin transport across the blood brain barrier is significantly decreased [293], and plasma to cerebral spinal fluid (CSF) leptin ratio is increased in obese mice and humans [294, 295]. Consistent with the notion that leptin transport is impaired in obesity, diet-induced obese mice and New Zealand obese mice (NZO) display resistance to peripherally but not centrally administered leptin [188, 274]. However, diet-

induced obese mice progressively develop resistance to leptin delivered directly into the brain [277], suggesting that there may eventually be defects in central leptin signalling. One possible mechanism for impaired leptin signalling is chronic activation of negative feedback regulators SOCS3 and PTP1B. In diet-induced obese mice, SOCS3 and PTP1B expression is increased in the hypothalamus [165, 278, 296]. Brain specific deletion of SOCS3 or PTP1B increases the amplitude of leptin signalling, resulting in animals that are leaner than wildtype animals and resistant to diet-induced obesity [297-299]. In contrast, overexpression of SOCS3 in POMC neurons causes leptin resistance and obesity [300]. In addition to leptin, other cytokines promote SOCS3 accumulation (reviewed in [301]), suggesting that multiple inflammatory pathways have the potential to impair leptin signalling. Leptin receptor trafficking to the cell surface is mediated by Bardet-Biedl syndrome (BBS) proteins [302], and impaired leptin receptor signalling due to BBS deficiency has been implicated in leptin resistance [303]. Interestingly, cellular leptin resistance is prominently detected in the ARC relative to other hypothalamic sites [275, 278]. Therefore, impaired leptin signalling in the ARC may cause resistance to the anorectic actions of leptin. Alternatively, maintenance of leptin signalling in other areas of the brain, including regions of the hypothalamus, may contribute to weight gain and perturbed metabolism in obesity. Indeed, leptin signalling in the DMH has been implicated in hypertension and chronic activation of the sympathetic nervous system (SNS) in obesity [275, 304]. It seems plausible that these proposed mechanisms contribute collectively to leptin resistance in obesity.

Many factors have been reported to cause leptin resistance in obesity. Chronic hyperleptinemia has been suggested to cause leptin resistance [305], potentially due to over activation of negative feedback regulators SOCS3 or PTP1B. In agreement with this idea, expression of a constitutively active form of STAT3 in POMC neurons induces leptin resistance

and obesity [306]. Conversely, preventing the development of high fat diet-induced hyperleptinemia by stably infusing leptin to *ob/ob* mice from birth improves leptin sensitivity, although these mice still become obese [305]. Moreover, decreasing leptin expression in adipocytes or increasing leptin clearance in the kidneys lower body weight and improve leptin sensitivity in diet-induced obese mice [307]. Growing evidence suggests that high fat diet feeding activates proinflammatory pathways in the hypothalamus, which contributes to leptin resistance [308, 309]. Neuron specific deletion of c-Jun amino-terminal kinase 1 (JNK1), a key regulator of inflammation, protects mice from diet-induced obesity [310, 311]. Conversely, activation of the proinflammatory IκB kinase β/nuclear factor-κB (IKKβ/NF-κB) pathway induces leptin resistance [309]. Excess lipids may contribute to hypothalamic inflammation. For example, fatty acids can bind and activate proinflammatory toll-like receptor 4 (TLR4) signalling in the hypothalamus [312], and reducing TLR4 signalling attenuates hypothalamic inflammation, weight gain, and leptin resistance [313, 314]. Hypothalamic endoplasmic reticulum (ER) stress is observed in diet-induced obese mice, and thus has been implicated in leptin resistance [315]. Deletion of ER chaperones in the brain or central administration of pharmacological ER stress inducers causes leptin resistance [315-317]. Defective autophagy in the hypothalamus has also been implicated in the development of leptin resistance, since deletion of autophagy-related gene 7 (Atg7), an essential autophagy gene, in POMC neurons leads to leptin resistance [318]. In summary, multiple factors, including hyperleptinemia, inflammation, ER stress and autophagy, have been implicated to cause leptin resistance, but the relative contributions of these factors have not been tested.

1.5 Leptin Therapy for Type 1 Diabetes

Leptin Deficiency in Type 1 Diabetes

Leptin levels are depleted in type 1 diabetes. Rodents rendered insulin deficient with streptozotocin (STZ, a β cell toxin) have reduced leptin levels [141]. This may be partly due to a reduction in adipose tissue; however, insulin replacement by injection normalizes leptin levels, before any detectable changes in fat weight, suggesting hypoleptinemia in type 1 diabetes is mainly due to insufficient leptin synthesis or secretion [141]. Humans with newly diagnosed type 1 diabetes who have not received insulin treatment also have low circulating leptin levels [319, 320], and leptin levels are normal in patients living with type 1 diabetes receiving insulin therapy [319]. These observations open up the possibility that some of the metabolic aberrations in type 1 diabetes may be due to hypoleptinemia as opposed to hypoinsulinemia. Indeed, insulin therapy in STZ-mice with genetic or pharmacological blockade in leptin signalling blunts the glucose lowering effects of insulin [321]. These data suggest that normalization of leptin levels contribute to the glucose lowering effect of insulin therapy in insulin-deficient diabetes [321]. Furthermore, restoring leptin to non-diabetic levels in STZ-diabetic rats improves some metabolic disturbances, including insulin resistance, hyperglucagonemia, and hypercorticosteronemia [267]. Leptin replacement only modestly lowers blood glucose levels in rats with STZ-induced diabetes, suggesting that hyperglycemia in type 1 diabetes is not due to leptin deficiency [267]. Given that hypoleptinemia contributes to some of the metabolic disturbances in insulin-deficient diabetes, type 1 diabetes is another disease that could benefit from leptin therapy.

Leptin as a Therapy for Type 1 Diabetes

There is substantial evidence from independent sources that administration of leptin to rodent models of type 1 diabetes can dramatically improve glycemia. Beneficial effects of leptin in rodent models of type 1 diabetes were first reported by Chinookoswong *et al.*, who administered leptin to STZ-rats to test whether leptin's effects on glucose disposal and glucose production were dependent on insulin [322]. Remarkably, leptin at high doses completely normalized blood glucose levels, without increasing levels of circulating insulin [322]. In addition, leptin reduced plasma lipids and ketones, improved insulin sensitivity, and ameliorated hyperphagia caused by insulin deficient diabetes [322]. Thus, this study revealed that leptin alone could normalize blood glucose levels and metabolic disturbances in rodent models of type 1 diabetes. These metabolic actions of leptin are highly reproducible; in STZ-diabetic rodents [260-263, 265, 323, 324], non-obese diabetic (NOD) mice [265, 266], virus induced diabetic (BioBreeding) rats [325], and insulinopenic Akita mice [326], leptin delivered through either subcutaneous infusion, adenoviral leptin gene delivery, or transgenic leptin overexpression can have considerable beneficial effects on both glucose and lipid metabolism. Combined, these studies have shown that leptin therapy establishes euglycemia [260, 262-266, 322-324], stabilizes body weight [260, 264, 322, 327], decreases hyperphagia [261-266, 322-324], normalizes water intake [261, 264], eliminates polyuria and glucosuria [261, 262, 264-266], lowers plasma lipids and ketones [260, 262, 264-266, 322], increases insulin sensitivity [260, 267, 322], and extends lifespan [262, 263, 266, 324, 328]. Despite significant interest, the precise mechanism by which leptin improves metabolism in rodent models of type 1 diabetes remains unknown.

Both *ob/ob* and insulin deficient mice are leptin deficient, but there are key differences in the effects of leptin therapy in these models. In *ob/ob* mice, leptin potently inhibits food intake and decreases body weight mostly in the form of fat mass [105, 106]. In insulin deficient mice, leptin therapy lowers food intake [261-266, 322-324] but does not cause weight loss relative to un-treated controls [260, 264, 322, 327]. In fact, un-treated insulin deficient mice that are pair-fed to leptin-treated insulin deficient mice lose body weight [322], suggesting that leptin is preventing weight loss in insulin deficient mice. Since leptin administration in insulin deficient mice reverses polyuria and glucosuria [261, 262, 264-266], less calories may be lost through the urine thus preventing dramatic weight loss. In addition, insulin deficient rats have increased energy expenditure potentially due to inappropriately high rates of gluconeogenesis and futile cycling, and a counter-intuitive reduction in energy expenditure with leptin treatment may account for its ability to stabilize body weight in these rats [267]. The lack of significant body weight loss with leptin treatment of insulin deficient rodents suggest that the site of leptin action may be distinct from the neuronal populations involved in the anorectic actions of leptin. Furthermore, leptin action at these sites may counter the anorectic effects of leptin to prevent weight loss in leptin-treated insulin deficient animals. Alternatively, since leptin-induced weight loss in *ob/ob* mice is largely due to a reduction in fat mass and insulin deficiency severely depletes fat stores [105, 188], insulin deficient mice may not have substantial fat mass to lose upon leptin therapy. Another explanation is that body weight reducing effects of leptin require insulin. In summary, leptin does not reduce body weight in insulin deficient mice in contrast to the potent weight reducing effects of leptin in *ob/ob* mice.

The precise target cells and signalling pathways that mediate the glucose lowering actions of leptin in insulin deficient mice are unknown, but there is mounting evidence that the CNS

plays a major role. Administration of leptin or adeno-associated virus (AAV) encoding leptin directly into the brain via ICV injection reverses hyperglycemia, hyperketonemia, and dyslipidemia in insulin deficient mice and rats [261, 262, 323, 329]. Importantly, in these studies, leptin is undetectable in the circulation after ICV administration of leptin or AAV encoding leptin [261, 262, 323, 329]. In addition, low doses of leptin delivered via ICV injection to STZ-rats can lower blood glucose levels, but these doses are insufficient to lower glucose levels when delivered peripherally [261]. Combined, these data suggest that leptin likely acts through the CNS to lower blood glucose levels in insulin deficient rodents. Interestingly, when leptin is delivered peripherally to insulin deficient mice, supraphysiological levels (beyond levels in non-diabetic mice) of leptin are required to restore euglycemia [267, 327]. A possible explanation for this may be that a sufficiently high level of leptin is required to cross the blood brain barrier. It should be noted there might be redundant central and peripheral mechanisms for glucose lowering actions of leptin; therefore, the contributions of peripheral leptin signalling should be tested further.

To identify the precise regions of the brain mediating leptin action, studies have used microinjections of leptin or Cre-*lox* technology for region specific deletion of the leptin receptor. Leptin lowers blood glucose levels in insulin deficient mice with disrupted leptin signalling in the LHA and ventral premammillary nucleus (PMV) [330], suggesting that these regions of the brain are not responsible for glucose lowering actions of leptin in these mice. Leptin injection into the VMH is sufficient to lower blood glucose levels; however, mice lacking leptin signalling in the VMH still respond to leptin therapy [331]. Thus, VMH may be responsible for glucose lowering actions of leptin in insulin deficient rodents but redundant mechanisms may exist, which involves brain regions outside of the VMH. In addition to determining brain regions

driving the glucose lowering actions of leptin in insulin deficient animals, there has been significant interest in identifying the precise neuronal populations involved. Recently, Xu *et al.* reported the generation of mice with adult-onset disrupted leptin signalling in POMC or AgRP neurons within the ARC using clustered regularly interspaced short palindromic repeats (CRISPR) coupled with CRISPR associated protein 9 (Cas9) technologies, which allows temporal and spatial specific deletion of genes [332]. These mice were rendered insulin deficient with STZ then treated with leptin. Leptin reversed hyperglycemia in mice with disrupted leptin signalling in POMC neurons. Conversely, glucose lowering actions of leptin were blocked in mice with disrupted leptin signalling in AgRP neurons. These data suggest that leptin signalling in AgRP, but not POMC, neurons is required for the glucose lowering actions of leptin in insulin deficient mice. Consistent with these data, insulin deficient mice with leptin signalling exclusively in γ-aminobutyric acid (GABA)-ergic neurons, which include AgRP but not POMC neurons, remain responsive to glucose lowering actions of leptin [263]. Combined, these studies suggest that AgRP neurons within the VMH and ARC may be important for glucose lowering actions of leptin in insulin deficient rodents but redundant neural circuits or peripheral targets may exist.

The precise mechanisms by which leptin lowers blood glucose levels in insulin deficient rodents are unknown but several possibilities have been ruled out. Leptin does lower food intake in insulin deficient rodents; however, STZ-rodents that are pair-fed to leptin-treated STZ-rodents remain hyperglycemic [265, 322, 326]. Furthermore, low-dose leptin therapy, which does not improve glycemia in insulin deficient mice, also lowers food intake [267]. Combined, these data indicate that decreased food intake cannot account for the glucose lowering effects of leptin in insulin deficient rodents. In addition, leptin lowers glucose levels in the urine and total urine

output in insulin deficient rodents [261, 262, 265, 266], suggesting that glucose excretion likely does not account for the blood glucose lowering effects of leptin. Several studies using rodent models of type 1 diabetes have reported that circulating insulin levels remain low or undetectable with leptin therapy [265, 266, 322], and β cell area is unaffected [260, 265]. Most rodent models of type 1 diabetes do not have 100% destruction of β cells and some residual insulin remains in the circulation [263, 265]. Interestingly, insulin sensitivity is increased with leptin therapy in mice with STZ-induced diabetes, suggesting that leptin may enhance the activity of residual insulin [260, 267]. However, leptin prolongs lifespan and lowers fasting blood glucose levels in insulin knockout mice, that otherwise do not survive for more than a day due to their extreme diabetes [328], demonstrating that leptin can act completely independent of insulin. The possibility that leptin itself may have insulin-like actions cannot be ruled out since there is some level of cross-talk between leptin and insulin signalling pathways [169-171]. It has also been suggested that leptin may increase levels of hormones that have insulin-like actions. Suggested candidates include insulin like growth factor binding protein 2 (IGFBP2) and fibroblast growth factor 21 (FGF21), which were shown to mimic glucose lowering actions of leptin in insulin deficient mice [333, 334]. However, IGFBP2 levels are already elevated in insulin deficient mice compared to non-diabetic controls [267, 327] and FGF21 levels are unchanged with leptin therapy compared to diabetic controls [268], suggesting that these hormones likely do not mediate glucose lowering actions of leptin in insulin deficient mice. Another possibility is that leptin may enhance glucose utilization in peripheral tissues. Glucose uptake in the soleus muscle is comparable between leptin-treated mice and diabetic controls [260]. WAT depots are severely depleted in leptin-treated mice, and thus likely do not contribute to glucose utilization in leptin-treated mice [264]. Since leptin increases glucose uptake in BAT [261, 263], which can utilize

glucose for non-shivering thermogenesis, it was postulated that the BAT might act as a 'glucose sink' in leptin-treated mice. Indeed, leptin therapy increases BAT temperature and expression of uncoupling protein 1 (*Ucp1*), a key mediator of non-shivering thermogenesis [323, 335]. However, leptin therapy restores euglycemia in *Ucp1* knockout mice with insulin deficient diabetes [335], suggesting that the mechanism of leptin action is UCP1 independent. Although an extensive list of possibilities have been ruled out, the mechanism of leptin action remains unknown.

A potential mechanism by which leptin lowers blood glucose levels is through reduced glucagon action. Circulating glucagon levels are elevated in insulin deficient animals and patients living with uncontrolled type 1 diabetes [336-338]. Reducing glucagon action is sufficient to lower blood glucose levels in rodents with insulin deficient diabetes. For instance, immunoneutralization of endogenous glucagon [339], antagonism of glucagon receptor signalling [340-342], knockdown of hepatic glucagon receptors [343], or genetic deletion of the glucagon receptor [266, 343, 344], lower blood glucose levels in rodents with STZ-induced diabetes. Interestingly, circulating glucagon is reduced to non-diabetic levels in leptin-treated STZ-diabetic rodents [260, 263, 265, 266] and NOD mice [265]. Consistent with decreased glucagon action in the liver of leptin-treated mice, phosphorylated cAMP responsive element binding protein 1 (CREB) levels are significantly reduced [265]. Given the beneficial effects of suppressed glucagon action in insulin deficient diabetes, it was postulated that reduced glucagon levels might drive the glucose lowering actions of leptin. However, a low dose of leptin administered to STZ-diabetic mice normalizes glucagon levels without restoring euglycemia [267], suggesting that suppression of glucagon action may not be sufficient for the glucose lowering actions of leptin. It remains unknown whether suppression of glucagon action is

required for leptin action in insulin deficient mice. Given the beneficial effects of glucagon suppression in diabetes, the role of glucagon in leptin action should be directly tested by preventing the reversal of hyperglucagonemia in leptin-treated mice.

It has been proposed that reduced glucose production is responsible for the glucose lowering actions of leptin in insulin deficient mice. Initially, leptin-treated STZ-mice appeared to be euglycemic when blood glucose levels were measured after 4 hours of fasting [260, 264]. However, leptin-treated STZ-mice are hyperglycemic without fasting, and these mice fall into life threatening hypoglycemia with 8 hours of fasting [264]. Glucose production from the liver occurs through glycogenolysis and gluconeogenesis (reviewed in [345]). Hepatic glycogen levels are depleted in leptin-treated STZ-mice before blood glucose levels are lowered [264], suggesting that depleted glycogen levels cannot fully account for the glucose lowering actions of leptin in STZ-mice. The effects of leptin on gluconeogenic genes in the liver of insulin deficient mice are unclear. For instance, phosphoenolpyruvate carboxykinase (*Pepck*) expression was decreased in some studies [266] but not others [264]. Interestingly, injection of pyruvate, a key substrate for gluconeogenesis, temporarily increases blood glucose levels in leptin-treated STZ-mice [264], suggesting that gluconeogenic pathways remain intact in these mice. Thus, instead of suppressing gluconeogenic capacity through alterations in gene expression, it has been suggested that leptin may deplete gluconeogenic substrates in insulin deficient rodents. In states of hypoglycemia, the main substrates for gluconeogenesis are glycerol, lactate, and amino acids [346]. Indeed, leptin therapy reduces plasma glycerol levels [264] and decreases glycerol turnover in the liver of STZ-rodents [268]. To determine whether depletion of glycerol mediates the glucose lowering actions of leptin in STZ-rodents, glycerol levels were increased with an acute injection of glycerol [264] or an infusion of a lipid emulsion with heparin [268] in leptin-

treated STZ-rodents. Acute injection of glycerol increased blood glucose levels but not to the level of diabetic controls [264], and the infusion of a lipid emulsion increased blood glucose levels to the diabetic range [268]. Combined, these studies illustrate that depletion of glycerol contributes to the glucose lowering actions of leptin in STZ-rodents. It is unknown whether depletion of plasma lactate and alanine contribute to the glucose lowering effects of leptin, and given that these substrates are more preferred than glycerol [347], their roles should be assessed.

It has been proposed that leptin-mediated reduction in the activity of the hypothalamic-pituitary-adrenal (HPA) axis lowers gluconeogenesis. There is excessive HPA activity in states of insulin deficiency; both adrenocorticotropic hormone (ACTH) and corticosterone levels are increased in the circulation of insulin deficient mice [348] and humans [349-352]. As a result of increased corticosterone levels, uncontrolled lipolysis elevates glycerol, which is a substrate for gluconeogenesis [353], and increases free fatty acids and its breakdown product hepatic acetyl-CoA, which is a positive regulator for gluconeogenesis [354]. Indeed, whole body lipolysis is suppressed with leptin treatment in insulin deficient rats, as reflected by a 60% reduction in whole-body glycerol and palmitic acid turnover [268]. However, the role of the HPA axis in leptin action remains controversial, as leptin therapy has been reported to have no effect [355] or cause a reduction in ACTH levels [268, 356], and to have no effect [260] or cause a reduction in corticosterone levels [268, 355, 356]. Perry *et al.* reported that infusion of corticosterone blocked the glucose lowering effects of leptin in STZ-diabetic rats [268, 356], suggesting that glucose lowering actions of leptin requires reduced corticosterone levels. In contrast, Meek *et al.* observed that neither adrenalectomy induced corticosterone deficiency nor pharmacological corticosterone receptor blockade lowered blood glucose levels in STZ-diabetic rats [355]. Moreover, German *et al*. reported that low dose leptin therapy, which is insufficient to lower

blood glucose levels, lowered corticosterone levels in STZ-diabetic rats [267]. Combined, findings of Meek *et al*. and German *et al.* suggest that reduced corticosterone action is neither sufficient nor required for the glucose lowering actions of leptin. Therefore, although suppression of lipolysis may contribute to decreased gluconeogenesis, the precise upstream mechanisms remain unknown.

1.6 Clinical Trials for Leptin Therapy in Type 1 Diabetes

The promising results of leptin therapy in insulin deficient rodents propelled leptin into clinical trials for the treatment of type 1 diabetes. To test whether leptin may lower insulin requirements and establish better glycemic control, leptin was given as an adjunct to insulin for adults living with type 1 diabetes (clinicaltrials.gov; NCT01268644). Patients received twice-daily subcutaneous injections of 0.04-0.08 mg/kg/day recombinant methionyl human leptin (metreleptin) for 20 weeks. Interestingly, total body weight and body fat were decreased in patients receiving leptin and insulin co-therapy [357], which is also observed with co-administration of leptin and insulin to STZ-mice [358]. Leptin mono-therapy does not cause weight loss in insulin deficient rodents [260, 264, 327]. Thus, leptin-mediated reduction in body weight and fat mass may be insulin dependent. Consistent with this notion, co-infusion of insulin with leptin into the brain of rats potentiates leptin signalling through the JAK/STAT pathway in the hypothalamus [169]. Total daily insulin dose was significantly reduced in patients receiving leptin and insulin co-therapy [357], which is consistent with the insulin sensitizing abilities of leptin in rodent models of type 1 diabetes [260, 322]. Surprisingly, hemoglobin A1c (HbA1c) and continuous 24 hour plasma glucose profiles were not changed in patients after 12 and 20 weeks of leptin and insulin co-therapy compared to baseline levels before therapy [357].

Furthermore, TAG, fatty acids, and glucagon levels were unaltered [357]. This clinical trial has been discontinued and it is unclear whether leptin therapy will continue to be tested for the treatment of type 1 diabetes. It is worth noting that twice daily injections of metreleptin only increased median serum leptin levels by 1.7 and 2.2 fold after 12 and 20 weeks of treatment, respectively [357]. Consistent with these clinical findings, increasing plasma leptin levels by 2 fold in insulin deficient mice is insufficient to lower blood glucose levels [327]. Instead, leptin levels within the supraphysiological range (~7 fold increase) are required in insulin deficient mice to restore euglycemia [327]. Thus, higher doses of leptin may also be required in patients living with type 1 diabetes to reduce HbA1c and blood glucose levels. In mice, administration of leptin at excessively high doses reduced leptin immunoreactivity in the plasma and glucose lowering effects of leptin eventually waned [327]. This may be due to a failure in pumps delivering leptin or may be due to the development of leptin resistance in these mice [327]. Despite interests in leptin as a potential therapy for type 1 diabetes, it is unknown whether resistance to the glucose lowering actions of leptin can occur. In summary, achieving higher levels of leptin may prove to be more effective in patients living with type 1 diabetes but leptin resistance should be carefully assessed for the glucose lowering actions of leptin.

1.7 Thesis Investigation

Leptin has profound beneficial effects on glucose metabolism in rodent models of type 1 diabetes but the mechanism of leptin therapy remains unknown. Furthermore, while leptin resistance is extensively characterized for body weight reducing actions of leptin, it is unknown whether resistance to the glucose lowering actions of leptin also occurs. We hypothesized that leptin induces global changes in metabolic pathways of the liver to lower blood glucose levels in

insulin deficient mice, but resistance to these effects of leptin can develop. To test this hypothesis, we utilized metabolomics for a non-biased and global assessment of the plasma from leptin-treated STZ-mice (Chapter 3). We found that amino acids, which are key substrates for hepatic gluconeogenesis, were significantly altered. Thus, we characterized amino acid utilization in leptin-treated mice, and tested the mechanistic role of amino acid catabolism genes. Furthermore, we extensively characterized the metabolic changes in the liver of leptin-treated mice using a triple omics approach, which combined transcriptomics, proteomics, and metabolomics. Next, we assessed whether recapitulating leptin-mediated changes in the liver using small molecules can mimic the glucose lowering actions of leptin (Chapter 4). Lastly, we assessed whether hyperleptinemia or high-fat intake, which are reported to cause resistance to the weight reducing actions of leptin, also impede glucose lowering effects of leptin (Chapter 5). Collectively, these investigations help elucidate the mechanism of leptin action in insulin deficient diabetes and shed insight into the suitability of leptin as a therapy for diabetes.

Chapter 2: Materials and Methods

2.1 Animals

Animal Housing and Facilities

All experiments were approved by the University of British Columbia Animal Care Committee and performed in accordance with the Canadian Council on Animal Care guidelines. All experiments using animals were performed at the Centre for Disease Modeling (CDM) at the University of British Columbia. Upon arrival at the CDM, mice were allowed to acclimatize for a minimum of 1 week before experiments began. At the CDM, animals were tested every 3 months during the study to ensure the absence of pathogens and opportunistic organisms excluded from the maximum barrier facilities at the Jackson Laboratory. Majority of mice were group housed (4-5 mice/cage) except if mice had to be separated due to fighting, had cage mates reach humane endpoint due to long-term diabetes, or were singly housed during metabolic cage testing. All mice were housed in filter-top cages containing enrichment (mouse huts and nesting material) and were housed at standard housing temperatures (21-22°C) on a 12:12 hour light-dark cycle throughout the studies. Mice had ad libitum access to irradiated chow diet (Envigo, Huntingdon, UK; Cat# 2918) or irradiated specialty diets where indicated, and autoclaved water.

C57Bl/6 Mice

Nine-week-old male C57BL/6J mice were obtained from the maximum barrier facility at the Jackson Laboratory (Bar Harbor, USA; Stock# 000664).

Generation of STZ-Diabetic Mice

STZ (Sigma-Aldrich, Oakville, ON, Canada; Cat# S0130) was freshly prepared in a pH 4.5 acetate buffer (118 mM $C_2H_3NaO_2$, 38.5 mM NaCl) and administered by intraperitoneal (i.p.) injection at a dose of 180 mg/kg for all mice except for high fat diet fed obese mice, which were given a dose of 100 mg/kg. STZ-induced diabetes was defined as two consecutive blood glucose measurements >16 mM. Non-diabetic mice, when included as controls, received an i.p. injection of acetate buffer alone.

Generation of High Fat Diet Fed Obese Mice

Nine-week-old male C57BL6J mice were switched from a chow diet to a high fat diet (HFD) containing 60% fat caloric content and control mice were fed a low fat diet (LFD) containing 10% fat caloric content (Research Diets, New Brunswick, NJ, USA; Cat# D12492 and Cat# D12450K, respectively). Composition of the diets can be found in Table 1. For high fat diet mice that were injected with STZ, mice were fed a high fat diet for ~12 weeks then injected i.p. with STZ at a dose of 100 mg/kg. After the introduction of a HFD or a LFD, mice remained on the special diet until the end of the study.

Survival Curves and Definition of Humane Endpoint

Most mice with long-term diabetes reached humane endpoint during the study. Humane endpoint was defined as mice displaying sign of pain such as a hunched back or piloerection, were lethargic for extended time periods, or were non-responsive.

Table 1. Composition of 60% and 10% fat diets

	D12492 (60% Fat)		D12450B (10% Fat)	
Component	grams (%)	kcal (%)	grams (%)	kcal (%)
Protein	26.2	20	19.2	20
Carbohydrate	26.3	20	67.3	70
Fat	34.9	60	4.3	10
kcal/g	5.24		3.85	
Ingredient	**grams**	**kcal**	**grams**	**kcal**
Casein, 30 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	0	0	315	1260
Maltodextrin 10	125	500	35	140
Sucrose	68.8	275.2	350	1400
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	245	2205	20	180
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H_2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Blue Dye #1	0.05	0	0	0
FD&C Yellow Dye #5	0	0	0.05	0
Total	**773.85**	**4057**	**1055.05**	**4057**

2.2 Blood and Plasma Analytes

Blood Collection, Glucose Measurements, and HbA1c Assays

Blood was collected through the saphenous vein from restrained and conscious mice using heparin-coated microhematocrit capillary tubes unless otherwise stated (ThermoFisher, Ottawa, Ontario, Canada; Cat# 22-362566). Plasma was separated by centrifugation of samples at 4500 rcf (relative centrifugal force) for 9 minutes at 4°C and stored at -20°C until the assay was performed. Blood glucose levels were measured using blood taken from the saphenous vein and a OneTouch Ultra 2 Glucometer (LifeScan) with a detection limit of 1.1 – 33.3 mM. When glucometer readings were <1.1 mM or >33.3 mM, we assigned a value of 1.1 mM or 33.3 mM, respectively, and statistical analysis was not performed. For HbA1c measurements, whole blood was collected with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and assayed with a Siemens DCA 2000 Vantage Analyzer (Siemens Healthcare Diagnostics Inc, Los Angeles, CA, USA; Cat# 5035C).

Plasma Analyte Measurements

Plasma leptin (Mouse Leptin ELISA; Crystal Chem, Downers Grove, IL, USA; Cat# 90030), glucagon (Glucagon ELISA; Mercodia, Salem, NC, USA; Cat# 10-1281-01), insulin (Insulin Rodent Chemiluminescence ELISA; Alpco Diagnostics, Salem, NH, USA; Cat# 80-INSMR-CH01), β-hydroxybutyrate (β-Hydroxybutyrate LiquiColor Test; Stanbio, Boerne, TX, USA; Cat# 2440058), triglycerides and glycerol (Serum Triglyceride Determination Kit; Sigma-Aldrich, Oakville, ON, Canada; Cat# TR0100-1KT), and free fatty acids (HR Series NEFA HR2 Reagents and Solvents; Wako Diagnostics, Richmond, VA, USA; Cat# 999-34691; Cat# 995-34791; Cat# 991-34891; Cat# 993-35191) were measured from plasma samples according to

manufacturer instructions or by scaling down the assay to a 96-well format. When samples fell above the standard curve of the assay, samples were diluted then re-assayed. When samples fell below the standard curve of the assay, samples were given the minimum value on the standard curve and analysis was not performed to assess statistical differences between groups.

2.3 Experimental Manipulations

Leptin and Insulin Delivery With Mini-Osmotic Pump Implantation

Recombinant mouse leptin (Peprotech, Rocky Hill, NJ, USA; Cat# 450-31) was solubilized in sterile water to a concentration of 1.6 μg/μl. Recombinant human insulin (Novolin ge Toronto; Novo Nordisk, Mississauga, ON, Canada) was diluted from 100 IU/ml to 10 IU/ml in Dulbecco's phosphate buffered saline (PBS) without calcium chloride and magnesium chloride (Sigma-Aldrich, Oakville, ON, Canada; Cat# D8537). Both insulin and leptin were loaded into mini-osmotic pumps (DURECT Corporation, Cupertino, CA, USA; Cat# 1007D) then primed by incubating in sterile PBS at 37°C according to the manufacturer's instructions. Water (vehicle) filled pumps were also prepared in parallel, which were used for controls. Mice were first anesthetized with isoflurane, given s.c. injections of ketoprofen at a dose of 5 mg/kg (Merial, Baie d'Urfé, QC, Canada), bupivicaine at a dose of 6 mg/kg (Hospira Healthcare Corporation; Montreal, QC, Canada), and 200 μl saline (0.9% NaCl; Hospira Healthcare Corporation; Montreal, QC, Canada; Cat# 7983-02). A small subcutaneous incision (~1 cm) was made laterally between scapula and spine, a small pocket was made under the skin, then a mini-osmotic pump was implanted in the subcutaneous pocket. Surgical incisions were closed with sutures and Gluture Tissue Adhesive (World Precision Instruments, Sarasota, FL, USA; Cat# 503763). Non-diabetic controls received sham surgery.

Generation and Delivery of Plasmids for Hydrodynamic Gene Delivery

For plasmids encoding leptin, the mouse leptin open reading frame (ORF) was amplified from C57BL/6J mouse adipose tissue cDNA using primers that introduce unique *XbaI* and *XhoI* sites to flank the ORF (Figure 1A). After restriction digestion, the amplicons were ligated into *NheI* and *XhoI* sites of pLIVE (Mirus Bio; Madison, WI, USA; Cat# MIR5420), downstream of a murine albumin promoter. For plasmids encoding glutamic pyruvic transaminase (*Gpt*), the ORF of mouse *Gpt* was amplified from C57BL/6J mouse liver tissue cDNA using primes that introduced flanking *XbaI* and *SalI* cut sites, digested, then ligated into pLIVE as described above (Figure 1B). Primers used to clone *Ob* and *Gpt* ORF are reported in Table 2. Both cloned ORFs were sequence validated, amplified with endotoxin-free gigapreps (QIAgen, Toronto, ON, Canada; Cat# 12391), and re-suspended in sterile water. The empty pLIVE vector was used as a vehicle control. For hydrodynamic gene delivery, 2.0-2.5 ml of PBS (equivalent to ~8-9% lean mass) containing 5-50 μg of plasmid DNA was infused intravenously (i.v.) via tail vein over 5-8 seconds.

Table 2. Primers used to amplify cDNA for ligation into pLIVE expression plasmids

Target	**Sequence**
Leptin (*Lep*)	
Forward Primer (5’ - 3’)	CACACATCTAGAATGTGCTGGAGACCCT
Reverse Primer (5’ - 3’)	CACACACTCGAGTCAGCATTCAGGGCTAACATC
Glutamic pyruvic transaminase (*Gpt*)	
Forward Primer (5’ - 3’)	CACACATCTAGAATGGCCTCACAAAGGAATGACC
Reverse Primer (5’ - 3’)	CACACAGTCGACTCAGGAGTACTCATGAGTGAATTTAGC

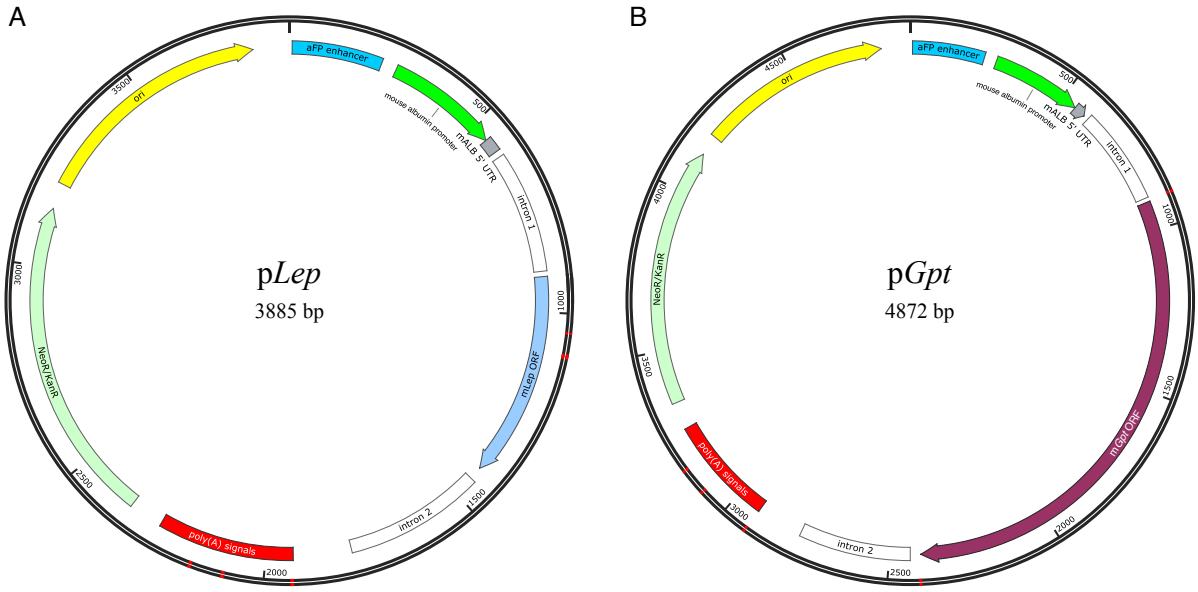

Figure 1. Maps of plasmids used for overexpression of leptin (pLep) and glutamic pyruvic transaminase (pGpt) in the liver. The mouse albumin promoter drives expression of mouse leptin (mLep; A) or mouse glutamic pyruvic transaminase (mGpt; B) open reading frame (ORF) cDNA. The NeoR/KanR gene confers resistance to the antibiotic kanamycin used for colony selection and origin of replication (ori) allows bacterial amplification of plasmid. An alpha fetoprotein (aFP) moiety enhances expression in the liver. Mouse albumin (mALB) 5' untranslated region (5' UTR), pLIVE introns 1 and 2, and polyadenylation (poly(A)) signals stabilize the mRNA transcript. The sizes of plasmids are indicated in basepairs (bp).

Preparation of Lipid Nanoparticle siRNA Systems and In Vivo Delivery

Lipid nanoparticles siRNA systems were prepared by Dr. Sam Chen at the laboratory of Dr. Pieter Cullis in the University of British Columbia. *Gpt* siRNAs and coagulation factor VII (*F7*) siRNAs were purchased from Integrated DNA Technologies (Coralville, IA, USA). Sequences of all siRNAs are outlined in Table 3.

Table 3. Sequences of siRNA used to knockdown gene expression in the liver

Target	Sequence
Gpt siRNA 1[1]	
Sense (5' - 3')	cuGaGgCACUUcCaUgCuAAAUUcA
Antisense (5' - 3')	UGaAUUUAgCaUgGAAGUGCCUcAguu
Gpt siRNA 2[1]	
Sense (5' - 3')	cuGaGgUUAUCcGuGcCaAUAUUcG
Antisense (5' - 3')	CCaAUAUUgGcAcGGAUAACCUcAgua
Gpt siRNA 3[1]	
Sense (5' - 3')	cuAcCcCAAUCuUcUgAgCAGUCcG
Antisense (5' - 3')	CGgACUGCuCaGaAGAUUGGGGuAgac
F7 siRNA 3[2]	
Sense (5' - 3')	GGAucAucucAAGucuuAcT*T
Antisense (5' - 3')	GuAAGAcuuGAGAuGAuccT1*T

[1]Lower case letters 2' O-methyl modifications

[2]Lowercase letters indicate 2′-fluoro modifications and the asterisks indicate phosphothioate linkages

The lipid 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and cholesterol was obtained from Millipore-Sigma (Burlington, MA, USA). The ionizable amino-lipid dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA) was synthesized by BioFine International (Vancouver, BC, Canada) and polyethylene glycol-dimyristolglycerol (PEG-DMG) was synthesized as previously described [359]. Lipid nanoparticles were prepared by rapid mixing of lipid components with RNA through a T-junction mixer [360]. Briefly, DLin-MC3-DMA, DSPC, cholesterol and PEG-DMG were dissolved in ethanol at a molar ratio of 50:10:39:1, respectively. RNA was dissolved in 25 mM acetate buffer at pH 4.0 such that the final mixture would have a nucleic acid to lipid (wt/wt) ratio of 0.056. Lipids in ethanol and RNA in acetate buffer were mixed together at a ratio of 1:3,

respectively, at total flow rates of >10 mL/minute at room temperature. Resulting mixtures were dialyzed against PBS overnight to remove residual ethanol. Particle size was determined by dynamic light scattering (Malvern Zetasizer NanoZS; Malvern Panalytical, Malvern, Worcestershire, UK). RNA concentration and entrapment efficiency was determined using the Quant-iT RiboGreen RNA Assay according to manufacturer's protocols (ThermoFisher, Ottawa, Ontario, Canada). Lipid concentrations were determined by measuring the cholesterol content (Cholesterol E Assay; Wako Chemicals, Richmond, VA, USA). All LNP used for experimentation had particle diameters of 40-50 nm and > 85% RNA entrapment. After preparation, the lipid nanoparticle encapsulated siRNAs were stored at 4°C overnight then injected i.v. through the tail vein, which allows delivery to the liver.

2.4 In Vivo Assays

Glucose Tolerance Test

Mice were fasted for 4 hours and 50% dextrose solution (Vetoquinol, Lavaltrie, Québec, Canada; Cat# 1150625) was administered via i.p. injection at a dose of 1.5 g/kg. Blood glucose was measured via the saphenous vein at the indicated time points after injection. There were some instances where blood glucose levels were above the detectable range of 33.3 mM. In these cases, a value of 33.3 mM was assigned and statistical analysis was not performed for the group.

Insulin Tolerance Test

For insulin tolerance tests, mice were fasted for 4 hours and 0.2 IU/ml of insulin solution (Novolin ge Toronto; Novo Nordisk, Mississauga, ON, Canada; Cat# 02024233) was

administered via i.p. injection at a dose of 0.65 IU/kg. Blood glucose levels were measured via the saphenous vein at the indicated time points after injection.

Fasting Tolerance Test

For the fasting tolerance test, food was removed at 8 AM (1 hour after initiation of light phase) and blood glucose levels were measured via the saphenous vein at indicated times following food removal. For blood samples taken during the fasting tolerance test for analyte measurements, blood was collected through the saphenous vein from restrained and conscious mice using heparin-coated microhematocrit capillary tubes (ThermoFisher, Ottawa, Ontario, Canada; Cat# 22-362566). In some instances, mice became dangerously hypoglycemic during the fasting tolerance test (blood glucose <1.1 mM and/or unresponsive) and were given glucose immediately via i.p. injections.

Alanine and Pyruvate Tolerance Tests

All mice were fasted to mild hypoglycemia with comparable starting blood glucose levels (~5-6 mM), which was 24 hours and 8 hours of fasting for non-diabetic and leptin-treated STZ- mice, respectively. The fasting time was shorter for leptin-treated mice since these mice reach lethal hypoglycemia with prolonged fasting [264]. In both experiments, half of the mice in each group received either pyruvate (2 g/kg dissolved in saline) or L-alanine (1.6 g/kg dissolved in sterile water), or corresponding vehicle injection i.p. at time 0, and blood glucose was measured at the indicated times from the saphenous vein.

2.5 Metabolic Cages and Body Composition Analysis

Indirect calorimetry, activity, food intake, and drink consumption were measured using the PhenoMaster metabolic cages (TSE Systems, Chesterfield, MO, USA). Mice were first acclimatized to the metabolic cages for 4 days and the measurements began immediately after with data collected every 15-16 minutes for ~48 hours. Some mice were excluded from analysis because some diabetic mice would not eat from the food hopper or drink from the water bottle, and had to have food and hydrogel placed on the bottom of the cage. Total activity was calculated as the total beam breaks in the XY plane per day, and both ambulatory and fine motor movement were included as total activity. Respiratory exchange ratio (RER) is expressed as a function of time or expressed as the average RER over the dark cycle, light cycle, or both. Indirect calorimetry, activity, food intake, and drink consumption were measured in collaboration with Dr. Susanne M. Clee and with assistance from Shuba Karunakaran. Body composition (fat mass, lean mass, and bone mineral density) was measured in mice under isoflurane anesthesia by dual energy x-ray absorbance (DEXA) using a Lunar PIXImus 2.0 Densitometer (Inside Outside Sales LLC, Fitchburg WI, USA). Body length was measured from head to base of tail.

2.6 Analysis of Mouse Tissues

Collection of Mouse Liver, Hepatic RNA isolation and cDNA Synthesis

After 4 hours of fasting, mice were anesthetized with isoflurane then euthanized via cervical dislocation. The liver was dissected, flash frozen in liquid nitrogen, then stored at -80°C. To extract RNA, liver was homogenized using a TissueLyser II (Qiagen, Hilden, Germany; Cat# 85300) in TRIzol Reagent (Invitrogen, Carlsbad, California, United States; Cat# 15596026)

according to manufacturer instructions. Next, samples were centrifuged at 12,000 rcf for 10 minutes at 4°C to remove polysaccharides. Following centrifugation, supernatant was transferred to a new tube, combined with chloroform, mixed, then centrifuged at 6800 rcf for 15 minutes at 4°C to separate the organic and aqueous phases. The aqueous phase was transferred to a new tube then the chloroform wash was repeated twice to ensure removal of phenol contaminants. The aqueous phase from the final wash was transferred to a new tube, combined with isopropanol, then incubated overnight at -80°C for RNA precipitation. The samples were centrifuged at 6800 rcf for 15 minutes at 4°C to pellet the RNA and the pellet was washed 3 times in 70% ethanol. Finally, the ethanol was removed and the pellet was dried at room temperature for ~5 minutes before being solubilized in RNase free water. RNA concentration was measured using a nanodrop (NanoDrop 2000; ThermoFisher, Ottawa, Ontario, Canada; Cat# ND-2000) and cDNA was generated using the iScript cDNA synthesis kit, which contains a mix of oligo(dT) and random hexamer primers (Bio-Rad, Mississauga, ON, Canada).

Quantification of Hepatic Transcripts Using qPCR

RNA was isolated and cDNA was synthesized as described above. For real time PCR (qPCR), the SYBR green method was used using the SsoFast EvaGreen supermix with Low ROX (Bio-Rad, Mississauga, ON, Canada). For every batch of cDNA that was synthesized, we assayed for 5 different reference genes (beta-actin (*Actb*), β-2-microglobulin (*B2m*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), phosphoglycerate kinase (*Pgk1*), and peptidylprolyl isomerase A (*Ppia*); Table 4) then chose the best reference gene as assessed by the geNorm software [361], which selects the most stable reference gene. For every qPCR experiment, a standard curve was measured to determine

the PCR efficiency and define the limit of detection. Relative transcript levels were calculated using the Pfaffl method [362], which takes into account the efficiency of the reaction. All primer sequences can be found in Table 4.

Table 4. Sequences of primers used for qPCR experiments

Target	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
Actb	AAGAGCTATGAGCTGCCTGA	TACGGATGTCAACGTCACAC
B2m	GGCCTGTATGCTATCCAGAA	GAAAGACCAGTCCTTGCTGA
Gapdh	CTGGAGAAACCTGCCAAGTA	TGTTGCTGTAGCCGTATTCA
Gpt	CAAGCAGTTTCAAGCAGAGAGGCAG	GGTTGTGTCCACCAGTGCC
Hprt1	GCTGACCTGCTGGATTACAT	TTGGGGCTGTACTGCTTAAC
Pgk1	GCAGATTGTTTGGAATGGTC	TGCTCACATGGCTGACTTTA
Ppia	AGCTCTGAGCACTGGAGAGA	GCCAGGACCTGTATGCTTTA

Transcriptomics Analysis Using RNA sequencing

For transcriptomics analysis, 8 week-old mice were rendered insulin deficient with STZ as described above. Upon confirming STZ-induced diabetes, mice were implanted with osmotic pumps delivering either vehicle, leptin, or insulin as described above. Non-diabetic mice were included as controls. Four days post pump implantation, liver was harvested from all mice as described above. RNA was isolated as described above and RNA sequencing (RNA-seq) libraries were prepared with the Illumina NeoPrep automated nanofluidic system using the Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA; Cat# 20020594). Samples were sequenced on the Illumina NextSeq 500 at a read length of 81 base pairs (paired end reads; 81bp x 81bp) and 10-20 million reads per sample. Reads were aligned to the University of California Santa Cruz (https://genome.ucsc.edu/) mm9/NCBI37 genome and

raw library sizes were scaled using the DGEList and calcNormFactors functions, respectively, which are offered in the edgeR package in R [363, 364]. Raw read counts per gene were normalized using the Voom-Limma pipeline in R which uses the voom and lmFit functions in the limma package [364-367]. Briefly, voom transforms count data to log2-counts per million, estimates the mean-variance relationship and uses this to compute appropriate observation-level weights [367]. Limma applies linear modeling to voom-transformed read counts [367]. Upon linear modeling, eBayes function in the limma package was used to calculate the empirical Bayes moderated t-statistic for contrast significance and the topTable function in the limma package was used to calculate multiple hypothesis adjusted p-values which implemented the Benjamini-Hochberg procedure with the false discovery rate set at 5% [367]. For differential expression analysis, an adjusted p-value less than 0.05 was deemed significant. For gene ontology analysis using RNA-seq data, the over representation analysis (ORA) tool of ErmineJ (version 3.1.2) was employed to identify significantly enriched biological processes [368-370] using significantly down-regulated and up-regulated genes.

Proteomics Analysis Using Stable Isotope Labeling of Amino Acids in Mice (SILAM) Tissue

Mouse liver tissue was stable isotopically-labelled as previously described using a method called stable isotope labeling of amino acids in mice (SILAM) [371]. Briefly, eight-week-old mice (F0) were fed a chow diet with isotopically labelled lysine (8 g $^{13}C_6$-Lysine/kg; Cambridge Isotope Laboratories, Andover, MA, USA) for ten weeks, then mated to obtain litters (F1). F1 mice were fed isotopically labelled lysine chow for eight weeks and mated to obtain the F2 litter. F2 mice were fed SILAM chow for eight weeks then the tissue was harvested. By the F2 generation, 97% incorporation efficiency of the heavy lysine label was achieved. Dr.

Nichollas E Scott (Department of Microbiology and Immunology at the University of Melbourne) generated the SILAM mice and prepared the SILAM samples for further analysis [371].

For proteomics analysis, STZ-induced diabetic mice were implanted with osmotic pumps delivering either vehicle, leptin, or insulin as described above and non-diabetic mice were included as controls. Separate cohorts of mice were used for transcriptomics and proteomics analysis. Four days post pump implantation, livers were harvested from all mice as described above for transcriptomics analysis.

Liver samples were lysed by grinding in liquid nitrogen followed by suspending in cold solubilization buffer (150 mg tissue in 1.5 ml buffer; 6 M guanidinium chloride, 100 mM tris pH 8.5, 10 mM tris(2-carboxyethyl)phosphine (TCEP), 40 mM 2-Chloroacetamide (CAA)). Suspended samples were forced through a 25 gauge hypodermic needle 10 times to shear remaining cells and DNA, then heated for 2 minutes at 95°C. Lysates were cooled, centrifuged at a speed of 3,500 rcf for 30 minutes at 4°C. Resulting supernatant was transferred to a new tube and diluted with 1:1 in dH_2O. Protein was precipitated by adding 4 volumes of cold 100% acetone, incubated at -20°C overnight, then precipitated protein was pelleted by centrifuging at a speed of 2000 rcf for 15 minutes at 4°C. The protein pellets were washed twice with 1 ml of cold 80% acetone, the supernatant was discarded, and the pellet was allowed to air dry for 15 minutes. Protein was then solubilized in digestion buffer (50 mM ammonium bicarbonate, 1% deoxycholate) and sample quality was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined by a bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit; ThermoFisher, Ottawa, Ontario, Canada; Cat# 23225).

For protein digestion, 100 µg of SILAM liver protein was spiked into 200 µg of experimental sample liver protein and digested with 6 µg of endoproteinase Lys-C (Wako Chemicals, Chuo-ku, Osaka, Japan; Cat# 127-06621), a protease that cleaves proteins on the C-terminal side of lysine residues. Samples were acidified with one volume of 1% trifluoroacetic acid and the precipitated deoxycholate was removed by centrifuging through a 0.6 µm filter (Sartorius Stedim Biotech, Göttingen, Germany) prior to desalting on a high-capacity stage tip as previously described [372]. Desalted peptides were fractionated using basic reverse-phase high-performance liquid chromatography as previously described [373]. Briefly, an Agilent 1100 high-performance liquid chromatography (HPLC) system and Agilent Zorbax Extend column (1.0 x 50 mm, 3.5 um particles) were used with a gradient ranging from 6% Buffer B (5mM NH_4HCO_2, 90% acetonitrile), 94% Buffer A (5mM NH_4HCO_2, 2% acetonitrile) to 60% Buffer B over 60 minutes. Every tenth fraction was pooled and these pooled fractions were dried via centrifugation under vacuum. Dried samples were re-suspended in 100 µl of 0.1% formic acid.

For mass spectrometry, 5 µl (approximately 1.5 µg of peptides) of each sample was injected on an EASY-nLC 1000 liquid chromatography system (ThermoFisher, Ottawa, Ontario, Canada; Cat# LC120) coupled to an Impact II Q-TOF mass spectrometer (Bruker, Billerica, MA, USA). The liquid chromatography system included a fused-silica (5 µm Aqua C18 particles; Phenomenex, Torrance, CA, USA; Cat# 00G-4299-E0) fritted 2 cm trap column connected to a 50 cm analytical column packed with ReproSil C18 (ReproSil-Pur 120 C18-AQ 3 µm; Dr. Maisch, Ammerbuch, Germany; Cat# r13.00.). The separation gradient ran from 5% to 35% Buffer B (80% acetonitrile, 0.1% formic acid) over 90 minutes, followed by a 15 minute wash with 95% Buffer B (flow rate: 250 µL/min). The instrument parameters were the following: scan from 150 to 2200 m/z, 100 µs transient time, 10 µs prepulse storage, 7 eV collision energy, 1500

Vpp Collision RF, a +2 default charge state, 18 Hz spectral acquisition rate, 3.0 second cycle time, and the intensity threshold was 250 counts. Dr. Allison McAfee (Department of Biochemistry and Molecular Biology at the University of British Columbia) processed the liver samples, fractionated samples, and performed mass spectrometry experiments for proteomics analysis.

Mass spectrometry data were searched using the MaxQuant software (v1.6.1.0) with Lys-C cleavage and two quantitative channels specified (no label and Lys6 label), re-quantify enabled, and match between runs enabled, and all other settings using default parameters [374]. The protein search database was the Uniprot mouse representative proteome (downloaded on November 29, 2018 which had 54,189 entries). Data were analyzed using the Perseus software (v1.6.1.1) as previously described [375]. Differentially expressed proteins were identified using an ANOVA followed by post-hoc t-tests with the false discovery rates at 10% using the Benjamini-Hochberg correction method. For gene ontology analysis using proteomics data, the gene score resampling (GSR) tool of ErmineJ (version 3.1.2) was employed, which identifies significantly enriched biological processes based on p-values of all measured proteins obtained from differential expression analysis.

Metabolomics Analysis Using Ultra-Performance Liquid Chromatography-Tandem Mass Spectroscopy

For metabolomics analysis, STZ-mice were implanted with osmotic pumps delivering either vehicle, leptin, or insulin as described above with non-diabetic mice included as controls. Samples used for proteomics and metabolomics analysis were from the same cohort of mice. Four days post pump implantation, mice were anesthetized with isoflurane then cardiac puncture

was performed to collect blood in EDTA coated tubes (S-Monovette® 1.2ml K2E; Sarstedt, Nümbrecht, Germany; Cat# 06.1660.100). Blood was centrifuged at 4500 rcf for 9 minutes at 4°C to collect the plasma. The liver was collected as described above for transcriptomics analysis.

Metabolic profiling, peak identification, and curation were performed by Metabolon (Durham, NC, USA). First, methanol was added to samples, shaken for 2 minutes using the Geno/Grinder 2000 (SPEX SamplePrep, Metuchen, NJ, USA), then centrifuged to remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites. After removal of proteins, the resulting extract was divided into 5 different aliquots: 2 aliquots were analyzed by two separate reverse phase ultra-performance liquid chromatography-tandem mass spectrometry (RP/UPLC-MS/MS) methods with positive ion mode electrospray ionization (ESI), 1 aliquot was analyzed by RP/UPLC-MS/MS with negative ion mode ESI, 1 aliquot was analyzed by hydrophilic interaction liquid chromatography ultra-performance liquid chromatography-tandem mass spectrometry (HILIC/UPLC-MS/MS) with negative ion mode ESI, and the last aliquot was stored as backup. Organic solvent was removed from samples by vaporization using a TurboVap (Zymark, Hopkinton, MA, USA) then stored overnight under nitrogen before further analysis.

The dried samples were reconstituted in solvents compatible for 4 different methods of analysis. The first aliquot was analyzed using acidic positive ion conditions, which is chromatographically optimized for more hydrophilic compounds. For this method, water and methanol containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA) was used to gradient elute the extract with a C18 column (ACQUITY UPLC BEH C18 Column, 130

Å, 1.7 µm, 2.1 mm x 100 mm; Waters, Mississauga, ON, Canada; Cat# 186002352). The second aliquot was also analyzed using acidic positive ion conditions chromatographically optimized for more hydrophobic compounds. For this method, the extract was gradient eluted from a C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA operated at an overall higher organic content. The third aliquot was analyzed using basic negative ion optimized conditions. For this method, the extracts were gradient eluted from the C18 column using methanol, water, and ammonium bicarbonate (6.5 mM, pH 8). A fourth aliquot was analyzed using negative ionization after elution from a HILIC column (ACQUITY UPLC BEH Amide Column, 130 Å, 1.7 µm, 2.1 mm x 150 mm; Waters, Mississauga, ON, Canada; Cat# 186004802) using a gradient consisting of water, acetonitrile, ammonium formate (10 mM, pH 10.8). Metabolon utilized a mass spectrometer (Q-Exactive mass spectrometer; Thermo Scientific, Ottawa, Ontario, Canada) interfaced with a heated electrospray ionization source and Orbitrap mass analyzer operated at 35,000 mass resolution. The MS analysis alternated between MS and data-dependent tandem mass spectrometry scans using dynamic exclusion, and the scan range varied slighted between methods but covered 70-1000 m/z.

Raw data was processed to identify peaks using Metabolon's custom software. Briefly, compounds were identified by comparison to library entries of purified standards; Metabolon maintains a library of standards, which contains the retention time/index, mass to charge ratio, and chromatographic data (including tandem mass spectrometry spectral data). The ions in the experimental samples were identified by comparison to the ions in the library. Upon identification, peaks were quantified using area-under-the-curve. The Welch's two-sample *t*-test was used to test for significance with a false discovery rate correction of 10%.

Phospho-STAT3 Immunostaining

Mice were perfused with 4% borate-buffered paraformaldehyde (pH 9.5) for 12 minutes. Perfused brains were sectioned coronally on a sliding microtome at 30 μm (one-in-six series) through the rostrocaudal extent of the hypothalamus and brainstem, and stored in cryoprotectant (0.1 M sodium phosphate buffer, 0.9 M sucrose, 5 M ethylene glycol) at -20°C. Every 6th section was immunostained for phospho-STAT3 (pSTAT3). Sections were washed in potassium phosphate-buffered saline (KPBS), blocked in 0.4% Triton X/KPBS (KPBSX) with 2% donkey serum, and incubated in 1:2000 rabbit anti-pSTAT3 antibody (Cell Signalling Technology, Danvers, MA, USA; Cat# 9145; Lot#26) in KPBSX/2% donkey serum (2 days). Sections were then incubated with 1:600 biotinylated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Cat# 711-066-152), followed by VECTASTAIN ABC Reagent (Vector Laboratories, Burlingame, CA, USA; Cat# PK-6100). Sections were then washed in KPBS, 0.175 M sodium acetate, and then incubated for 20 minutes in nickel-intensified diaminobenzidine chromagen solution (2.5% nickel (II) sulphate, 0.02% 3,3'-diaminobenzidine tetrahydrochloride, 0.002% hydrogen peroxide, 0.175 M sodium acetate) and then washed 0.175 M sodium acetate and KPBS. Sections were mounted onto gelatin-coated slides, dried and then hard-mounted. The number of pSTAT3 positive nuclei was manually counted using a brightfield microscope by an investigator blinded to the treatment group and totaled over 6 anatomically matched hypothalamic sections per mouse, within the ARC, VMH, DMH, and LHA. No staining was observed in other regions of the hypothalamus or in the brainstem, including the NTS.

2.7 Data Analysis

Data and statistics were analyzed using GraphPad Prism (Version 8; GraphPad Software, San Diego, CA, USA) except in Chapter 3, where all statistics for transcriptomics, proteomics, and metabolomics were analyzed using R [364]. The precise statistical analysis performed for these techniques are outlined above in sections describing each method. For line graphs (except survival graphs), data are presented as mean ± standard error of mean (SEM) where mean is the dark line and SEM is the lighter shading. For box and whisker plots, top and bottom boundaries indicate max and min values and line indicates the median. Unless otherwise stated in the figure legends, data were analyzed using an unpaired student's t-test for comparison between 2 groups, an ordinary 1-way ANOVA with Tukey post-hoc testing for multiple comparison between three or more groups, a repeated measures 2-way ANOVA with Tukey post-hoc testing for line graphs, or a log rank (Mantel-Cox) test for survival graphs. Additional details on statistics including number of mice in each experiment can be found in the figure legends.

Chapter 3: The Role of Amino Acid Catabolism in the Beneficial Effects of Leptin Therapy

3.1 Introduction

Since the discovery of insulin, it has been thought that insulin is the only hormone that can reverse hyperglycemia in type 1 diabetes; thus, it was surprising when the hormone leptin was shown to completely reverse hyperglycemia in rodent models of type 1 diabetes [260-262, 264-266, 268]. These glucose lowering actions of leptin are independent of changes in insulin levels [328] and food intake [265, 267, 322, 326]. In addition to lowering glycemia, leptin can reverse a plethora of symptoms associated with uncontrolled diabetes. For example, leptin improves hyperphagia and polydipsia [261-266, 322-324], decreases polyuria and glucosuria [261, 262, 264-266], lowers levels of plasma lipids and ketones [260, 262, 264-266, 322], increases insulin sensitivity and glucose uptake in peripheral tissues [260, 263, 267], and extends lifespan [262, 263, 266, 324, 328]. Given these beneficial effects of leptin therapy, there is tremendous interest in determining the precise molecular mechanisms that drive glucose lowering actions of leptin.

There is increasing evidence that decreased glucose production is responsible for leptin-mediated lowering of blood glucose levels in insulin deficient rodents. Although leptin-treated mice appear to be euglycemic when blood glucose levels are measured after 4 hours of fasting [260], they quickly fall into life threatening hypoglycemia during a fasting tolerance test within 8 hours of fasting [264]. These data suggest that leptin-treated mice have defective glucose production, and it is likely that the mechanism driving leptin-mediated hypoglycemia during fasting is also responsible for reversal of hyperglycemia in these rodents. Glucose is made from

both glycogenolysis and *de novo* synthesis of glucose from non-carbohydrate substrates, such as glycerol, lactate, and amino acids (reviewed in [376]). Glycogen levels are depleted in the liver of leptin-treated mice before blood glucose levels are lowered [264], suggesting that depletion of glycogen alone cannot account for the glucose lowering actions of leptin. There is very limited and conflicting evidence on whether genes involved in gluconeogenesis are altered. For example, some studies report down-regulated hepatic *Pepck* [266] and glucose transporter 2 (*Glut2*) expression [264, 323] in leptin-treated mice compared to diabetic controls, while others report *Pepck* [264, 323] expression is unaltered. Furthermore, mRNA expression does not always faithfully reflect protein levels or activity [377, 378]. Thus, to definitively assess the role of gluconeogenic genes in leptin action, a more comprehensive survey of gene expression is needed, and metabolites should be measured in parallel to determine the consequences of altered gene expression.

There is growing evidence that glucose lowering actions of leptin are mediated by depletion of substrates. Despite reports that leptin alters the expression of gluconeogenic genes in the liver [264, 266, 323], pyruvate injections increase blood glucose levels in leptin-treated mice [264]. Moreover, the relative contributions of hepatic and renal gluconeogenesis are not changed in leptin-treated mice compared to diabetic controls [268]. Combined, these data suggest that the gluconeogenic pathway remains intact in leptin-treated mice. Glycerol is a key substrate for gluconeogenesis, and circulating glycerol levels and rates of whole body glycerol turnover are reduced in leptin-treated mice compared to diabetic controls [264, 268]. Increasing glycerol levels through acute injection [264] or intralipid infusion [268] modestly increases blood glucose levels and completely reversed the glucose lowering actions of leptin, respectively. It is thought that suppression of lipolysis is responsible for depletion of glycerol [268], but the upstream

mechanisms leading to decreased lipolysis remain controversial [268, 355, 356]. Thus, the depletion of glycerol may partially contribute to decreased glucose production in leptin-treated mice.

In addition to glycerol, lactate and amino acids are also substrates for gluconeogenesis [379]. Lactate is derived from the reduction of pyruvate in muscle and red blood cells, and amino acids are obtained from the diet and breakdown of proteins in the muscle (reviewed in [380, 381]). These substrates are released into the circulation, taken up by hepatocytes, and then converted into glucose [379]. It is estimated that lactate and amino acids (alanine and glutamate) contribute up to 60% and 20% of glucose production from hepatocytes, respectively [379]. Increased utilization of these substrates is associated with hyperglycemia in patients with type 2 diabetes [382, 383]. Furthermore, there is some indirect evidence that blocking the utilization of alanine and lactate lowers glucose levels. For example, Krüppel-like factor 15 knockout mice, which have defects in breakdown of alanine, are more glucose tolerant and exhibit severe hypoglycemia with fasting [384]. Decreased utilization of lactate in gluconeogenesis is thought to contribute to the mechanism by which metformin lowers blood glucose levels in rodents with diabetes [385]. Unlike glycerol, circulating lactate and alanine levels are not depleted in leptin-treated mice compared to diabetic controls [264]. However, the effects of leptin on utilization of these substrates and their roles in glucose lowering actions of leptin have not been assessed.

To better characterize leptin-mediated alterations in metabolism, we first performed a comprehensive survey of metabolites in the plasma of leptin-treated STZ-mice and controls using quantitative metabolomics. These studies revealed that leptin profoundly alters the plasma metabolic profile, as evidenced by alterations in all major classes of metabolites and diverse

effects of leptin therapy within each major class. We focused on amino acids and their derivatives, which were strikingly altered in the plasma of leptin-treated mice. We performed a rigorous analysis of amino acid utilization in leptin-treated mice and extensively characterized the liver of leptin-treated mice using a triple omics approach, which combined transcriptomics, proteomics, and metabolomics analysis. This chapter reveals the novel finding that leptin-treated mice cannot utilize alanine for glucose production, and presents evidence that leptin therapy reduces amino acid utilization by globally altering amino acid catabolism pathways in the liver.

3.2 Results

Leptin Lowers 4 Hour Fasted Blood Glucose Levels in Mice With STZ-Induced Diabetes

We first confirmed the glucose lowering effects of leptin in STZ-diabetic mice. Nine-week-old C57BL/6J mice were injected with STZ, while non-diabetic controls received acetate buffer injections instead of STZ (Figure 2A). STZ injection severely depleted circulating insulin levels, lowered plasma leptin levels, induced hyperglycemia, and caused weight loss relative to non-diabetic controls (Figure 2B-E). Six days following the onset of STZ-induced hyperglycemia, we implanted osmotic pumps in STZ mice to deliver leptin (STZ-Leptin), insulin (STZ-Insulin), or vehicle (STZ-Vehicle), and non-diabetic mice underwent sham surgeries. Leptin pump implantation resulted in a ~19 fold increase in plasma leptin levels compared to non-diabetic controls (0.46 ± 0.04 ng/ml STZ-Vehicle, 55.3 ± 9.7 ng/ml STZ-Leptin, 2.91 ± 0.69 ng/ml Non-diabetic; Figure 2C), and thus we defined this as a supraphysiological dose of leptin. As previously reported [141], insulin therapy normalized plasma leptin levels in STZ-mice (2.82 ± 0.68 ng/ml STZ-Insulin, 0.46 ± 0.04 ng/ml STZ-Vehicle, 2.91 ± 0.69 ng/ml non-diabetic; Figure 2C). Insulin pump implantation in STZ-mice increased plasma insulin levels compared to

diabetic controls (1.6 ± 0.62 ng/ml STZ-Insulin, 0.15 ± 0.02 ng/ml STZ-Vehicle; Figure 2B), and these levels were ~2 fold greater than levels observed in non-diabetic mice (0.66 ± 0.10 ng/ml). By day 3 post pump implantation, 4 hour fasted blood glucose in STZ-Leptin mice were completely normalized to non-diabetic levels (23.2 ± 1.9 mM STZ-Vehicle, 7.8 ± 1.8 mM STZ-Leptin, 7.7 ± 0.2 mM Non-diabetic; Figure 2D), despite maintenance of severe insulin deficiency (Figure 2B). Insulin therapy also lowered blood glucose levels to the non-diabetic range (8.5 ± 2.0 mM STZ-Insulin; Figure 2D). Since a high dose of leptin reduces body weight in *ob/ob* and wildtype mice [106], we assessed whether leptin therapy worsened weight loss observed in STZ-mice. Body weight was comparable between STZ-Leptin and STZ-Vehicle mice (Figure 2E), indicating that leptin does not exacerbate weight loss caused by insulin deficiency. These data confirm that leptin displays the remarkable ability to lower blood glucose levels in STZ-mice without raising circulating insulin, and the therapeutic efficacy of leptin is comparable to insulin therapy.

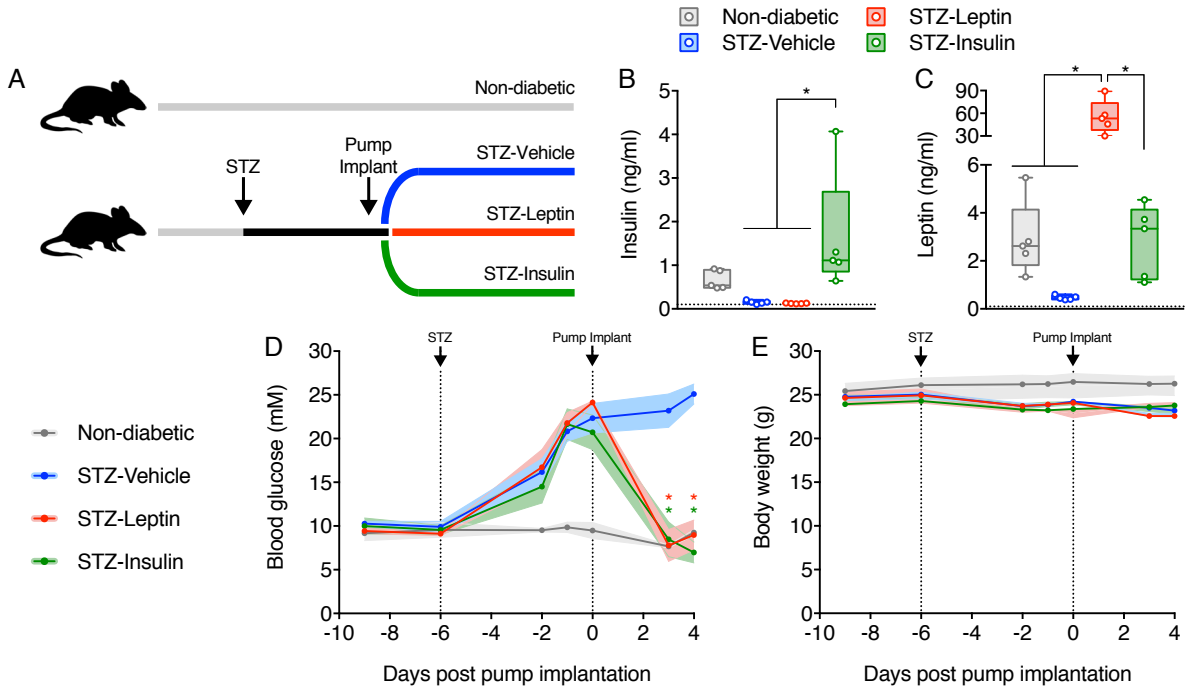

Figure 2. Leptin lowers blood glucose levels in STZ-mice without raising circulating insulin levels. Nine-week-old C57Bl6/J mice were rendered diabetic with STZ on day -6 then implanted with mini-osmotic pumps delivering vehicle (STZ-Vehicle), leptin (STZ-Leptin), or insulin (STZ-Insulin) on day 0 (A). Non-diabetic mice were included as controls. Plasma insulin (B) and plasma leptin (C) levels were measured on day 4 post pump implantation. Blood glucose levels (D) and body weight (E) were tracked throughout the study. B and C are shown as min to max whisker plots and statistical analysis was performed by one-way ANOVA (*, $P<0.05$). D and E are line graphs where the solid lines are the averages with shading representing the ± SEM, and statistical analysis was performed by two-way ANOVA (*, $P<0.05$ for comparison against STZ-Vehicle where colour indicates the group being compared). N=5 for all groups.

Leptin Therapy Dramatically Alters the Plasma Metabolomic Profile in STZ-Mice

To further characterize the metabolic effects of leptin therapy, we examined the plasma metabolomic profile in leptin-treated STZ-mice. Nine-week-old C57Bl/6J mice were rendered diabetic with STZ, and then implanted with osmotic pumps delivering either leptin (STZ-Leptin) or vehicle (STZ-Vehicle). Hyperglycemia was ameliorated by day 3 post pump implantation in

STZ-Leptin mice (24.2 ± 1.4 mM and 8.3 ± 0.9 mM on days -1 and 3, respectively), while STZ-Vehicle mice remained severely diabetic (22.8 ± 1.5 mM on day 3). Reversal of hyperglycemia itself may have secondary effects on the plasma metabolomic profile. Thus, we reasoned that the primary effects of leptin therapy might be better assessed earlier (few days), as opposed to after long-term leptin treatment (weeks or months). Four days after pump implantation, plasma was collected from 4 hour fasted STZ-Leptin and STZ-Vehicle mice. Using targeted quantitative metabolomics, 547 different metabolites were measured in the plasma, and levels of 234 metabolites (43%) were significantly altered in leptin-treated mice compared to STZ-Vehicle controls.

For a more detailed look at the plasma metabolomic profile, we categorized the metabolites into major classes (lipids, carbohydrates, nucleotides, xenobiotics, amino acid, peptide, cofactors and vitamins, and energy), which were further branched into metabolite subclasses (Figure 3). For almost half of all lipids measured (137 out of 269; blue nodes), levels were significantly altered in STZ-Leptin mice compared to STZ-Vehicle controls. Out of all lipid sub classes, leptin had the most dramatic effect on glycerides and fatty acids, which are used as an energy source; leptin therapy significantly diminished almost all metabolites within the diacylglycerol (14 out of 15), monoacylglycerol, (11 out of 12), polyunsaturated fatty acid (13 out of 14), long chain fatty acid (12 out of 14), monohydroxy fatty acid (9 out of 13), and fatty acyl carnitine subclasses (12 out of 17). Leptin significantly altered levels of lipids within phospholipid metabolism, lysolipid, plasmalogen, and sphingolipid metabolism subclasses, which are important in biological processes such as membrane integrity [386], cell signalling [387], and cell to cell recognition [388]. Thus, the effects of leptin on lipid metabolism extend beyond altering levels of energy yielding lipids. Interestingly, not all lipids within each subclass

were altered similarly with leptin therapy. For example, leptin significantly diminished sphingomyelin d18:1/24:0 but elevated sphingomyelin d18:1/24:1, which only differs by a double bond in the fatty acyl chain. Levels of metabolites within the carbohydrate major class were significantly altered with leptin therapy (orange nodes). Consistent with blood glucose levels measured using a glucometer, glucose levels were significantly reduced in the plasma of leptin-treated mice. The metabolically inactive monosaccharide 1,5-anhydroglucitol (1,5AG) is a validated marker of glycemic control (reviewed in [389]). STZ-Leptin mice had elevated 1,5AG levels compared to STZ-Vehicle controls, suggesting that leptin therapy improves short-term glycemic control in diabetic mice. Leptin administration significantly altered metabolites within the nucleotide (dark grey nodes), xenobiotics (dark blue nodes), and cofactors/vitamins (green nodes) major classes, which indicates that the metabolic effects of leptin therapy extend beyond energy storing carbohydrates and lipids. Leptin extensively altered levels of metabolites in the amino acid major class (red nodes). In fact, metabolite levels were altered in majority of the amino acid subclasses (12 out of 14), and altered metabolites included amino acids such as arginine and glycine, and amino acid derivatives such as dimethylarginine and N-acetylglycine. Combined, these data indicate that leptin therapy in STZ-mice globally alters the plasma metabolomic profile, and these profound changes extend beyond energy yielding substrates.

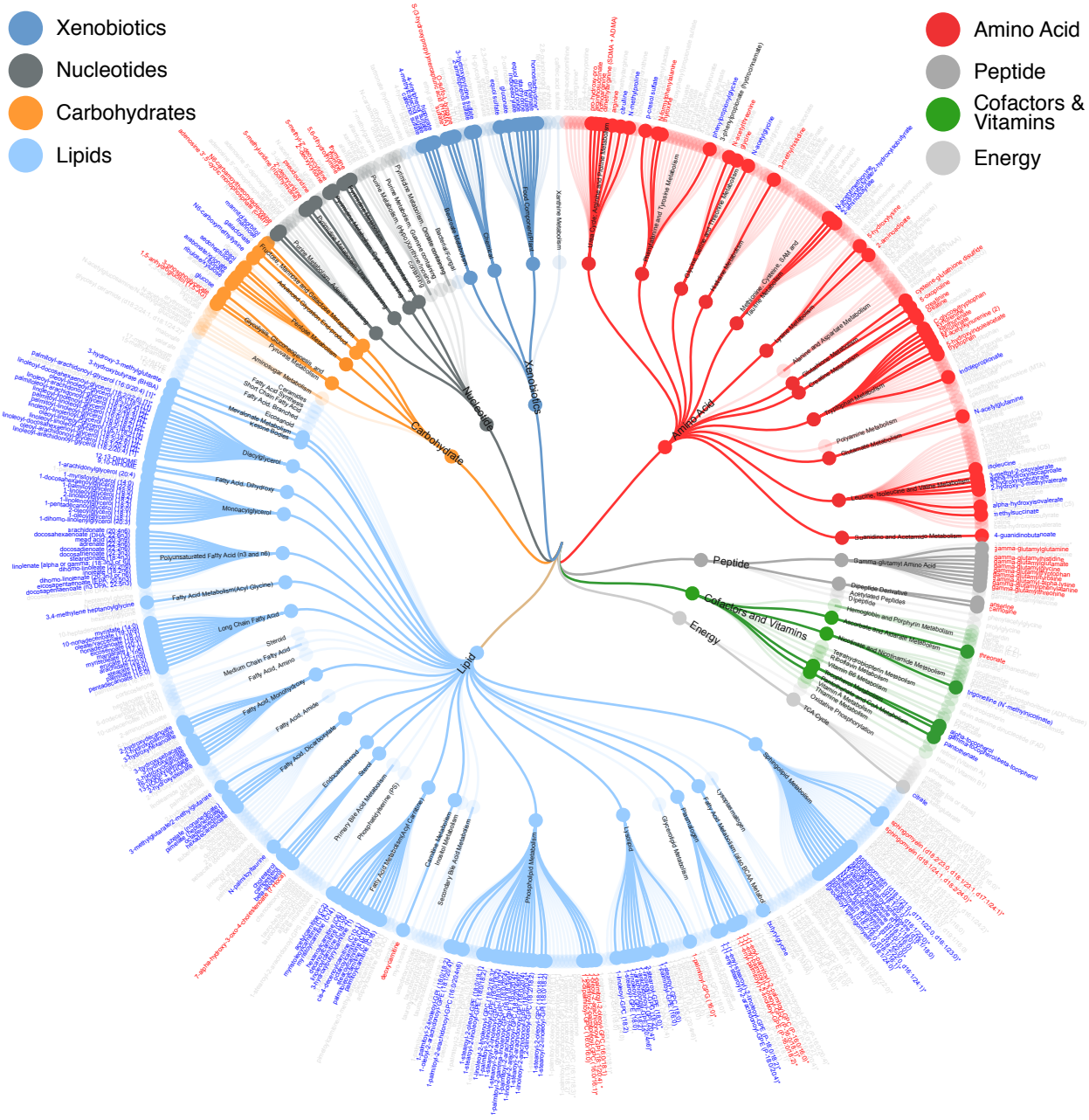

Figure 3. Leptin therapy significantly alters the plasma metabolic profile in STZ-mice. Nine-week-old mice were rendered diabetic with STZ on day -6 then implanted with mini-osmotic pumps delivering either leptin (STZ-Leptin) or vehicle (STZ-Vehicle) on day 0. Plasma was collected on day 4 for metabolomics analysis, which measured 547 different metabolites. These metabolites were grouped into major classes (indicated by different colours; lipids, carbohydrates, nucleotides, xenobiotics, amino acid, peptide, cofactors and vitamins, and energy) then further divided into subclasses (indicated by branching nodes). Darker shading indicates that these metabolites were significantly changed in the plasma of STZ-Leptin compared to STZ-Vehicle, where significantly elevated and diminished metabolites are labeled in red and blue text, respectively. N=5 for all groups.

Leptin Blocks the Utilization of Alanine for Glucose Production in STZ-Mice

Suppressed glucose production has been proposed to be the mechanism by which leptin lowers blood glucose levels in STZ-mice [264, 268]. Our plasma metabolomic analysis revealed that levels of amino acids and their derivatives are globally altered by leptin, and since amino acids are key substrates for gluconeogenesis ([379]), we chose to further assess amino acid metabolism. For this, we examined amino acid utilization in leptin-treated STZ-mice and non-diabetic controls by performing alanine tolerance tests and pyruvate tolerance tests as a comparison. STZ-mice were treated with leptin, which normalized blood glucose levels (22.1±0.5 vs 6.7±0.7 mM day -1 and day 3). After 5 days of leptin therapy, STZ-leptin and non-diabetic control mice were fasted to the point of mild hypoglycemia and injected with alanine. In non-diabetic controls with mild hypoglycemia, blood glucose levels increased with injection of alanine (4.7±0.1 vs 6.9±0.4 mM at 0 and 30 minutes; Figure 4A), indicating that normal mice escape hypoglycemia by utilizing the amino acid alanine to produce glucose. In contrast, alanine did not increase blood glucose levels in leptin-treated STZ-mice (6.0±0.5 vs 6.4±0.4 mM at 0 and 30 minutes; Figure 4B), and hypoglycemia worsened (3.9±0.6 mM at 90 minutes). We next sought to assess the utilization of pyruvate, a product of alanine breakdown, in leptin-treated mice and non-diabetic controls. Pyruvate increased blood glucose levels in both non-diabetic mice (4.5±0.2 vs 11.1±0.3 mM at 0 and 30 minutes; Figure 4C) and leptin-treated STZ-mice (6.5±0.5 vs 9.9±1.8 mM at 0 and 30 minutes; Figure 4D), although the degree of glucose excursion with pyruvate injection was more pronounced in non-diabetic mice. Combined, these data suggest that leptin-treated STZ-mice cannot utilize alanine to produce glucose but their ability to utilize pyruvate, a product of alanine breakdown, remains intact.

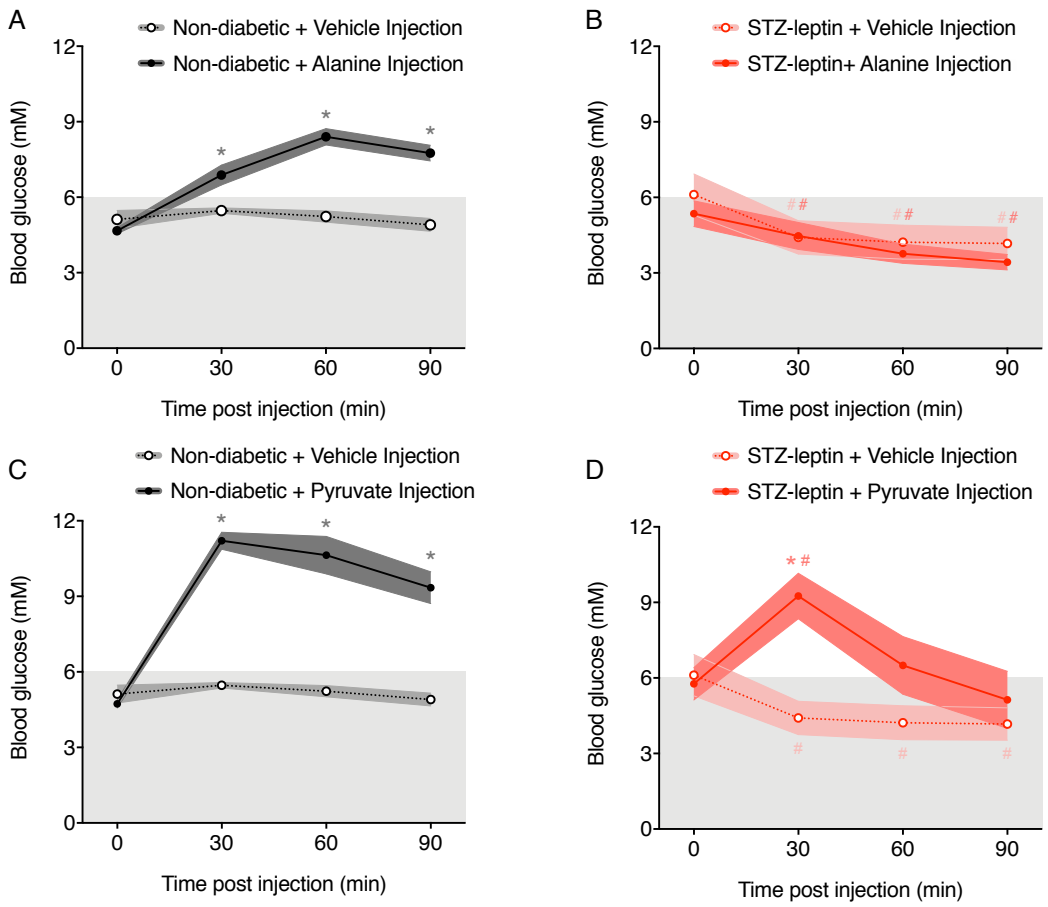

Figure 4. Leptin-treated STZ-mice cannot utilize alanine for glucose production, while pyruvate utilization remains intact. Nine-week-old mice were rendered diabetic with STZ on day -7 then implanted with mini-osmotic pumps delivering leptin (STZ-Leptin) on day 0 and non-diabetic mice were included as controls. On day 5 post pump implantation, alanine tolerance tests (A and B) were performed with pyruvate tolerance tests as a comparison (C and D), since pyruvate is the breakdown product of alanine. All panels are line graphs where the solid lines are the averages with shading representing ± SEM, and statistical analysis was performed by two-way ANOVA (*, $P<0.05$ vehicle vs pyruvate or alanine injection). N=6-11 for all groups.

Gpt Alone Is Not Responsible for the Glucose Lowering Actions of Leptin

Since leptin-treated STZ-mice cannot utilize alanine for glucose production, we sought to identify potential mechanisms for this defect. Alanine enters hepatocytes, where it is broken down to pyruvate in a single enzymatic reaction by glutamic pyruvate transaminase (*Gpt*), and

pyruvate subsequently feeds into gluconeogenesis (reviewed in [390]). *Gpt* transcript levels were increased in the liver of STZ-Vehicle mice compared to non-diabetic controls (Figure 5A). In leptin-treated STZ-mice, *Gpt* transcript levels were down-regulated by ~2 fold compared to STZ-Vehicle controls ($p<0.0001$), such that *Gpt* transcript levels became comparable between STZ-Leptin mice and non-diabetic controls (Figure 5A). There was a strong positive correlation between hepatic *Gpt* transcript and blood glucose levels in non-diabetic, STZ-Vehicle, and STZ-Leptin mice (Figure 5B).

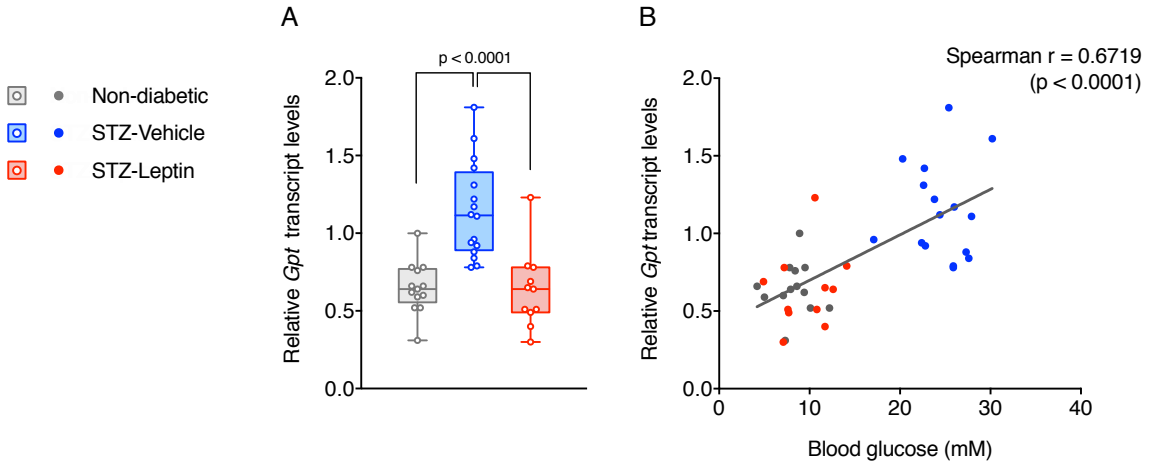

Figure 5. Leptin therapy lowers hepatic glutamic pyruvic transaminase (*Gpt*) in the liver of STZ-mice. Nine-week-old mice were rendered diabetic with STZ on day -7 then implanted with mini-osmotic pumps delivering either vehicle (STZ-Vehicle) or leptin (STZ-Leptin) on day 0 and non-diabetic mice were included as controls. Liver was collected on day 7 post pump implantation then hepatic *Gpt* transcript levels were measured (A). Blood glucose levels on the day 7 post pump implantation were plotted against *Gpt* transcript levels on day 7 post pump implantation (B). A is a min to max whisker plot and statistical analysis was performed by one-way ANOVA (*, $P<0.05$). Linear regression was used in B to obtain a Spearman's correlation coefficient. N=13-16 for all graphs.

Given that leptin lowers hepatic *Gpt* levels and these levels correlate to glycemia in leptin-treated STZ-mice, we hypothesized that lowering of *Gpt* contributes to the glucose lowering effects of leptin. To test this hypothesis, we sought to prevent leptin mediated lowering

of *Gpt* by overexpressing *Gpt* in leptin-treated STZ-mice, which we predicted would attenuate the glucose lowering actions of leptin. Our approach for overexpression of *Gpt* was to deliver a plasmid encoding *Gpt* via hydrodynamic gene transfer, a method that establishes sustained and often supraphysiological expression of target genes in the liver [391-393]. We first validated our approach by injecting non-diabetic mice with plasmids encoding *Gpt* (p*Gpt*) at 3 different doses (1, 10, or 50 µg/mouse for low, medium, and high doses, respectively) or empty plasmids as controls (pEmpty). Low or medium doses of p*Gpt* did not increase hepatic *Gpt* transcript levels compared to pEmpty controls, but there was a trend towards increased hepatic *Gpt* transcript levels in mice injected with a high dose of p*Gpt* (p = 0.10; Figure 6A). In non-diabetic mice, injection of p*Gpt* did not alter body weight (Figure 6B) or blood glucose levels (Figure 6C). Thus, we confirmed that injection of high doses of p*Gpt* via hydrodynamic gene transfer increases hepatic *Gpt* expression.

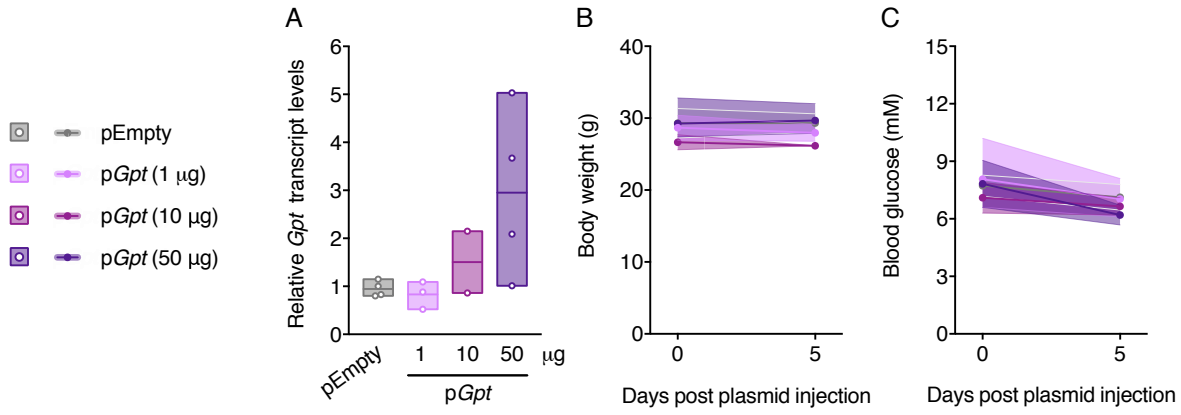

Figure 6. Administration of plasmids encoding glutamic pyruvic transaminase (*Gpt*) increases *Gpt* transcript levels in the liver. Nine-week-old C57Bl6/J mice were injected with empty plasmid (pEmpty) as a control or plasmid encoding GPT (p*Gpt*) on day 0 at 3 different doses (1, 10 or 50 µg plasmid/mouse). Liver was harvested on day 5 post plasmid injection then *Gpt* transcript levels were measured (A). Body weight (B) and blood glucose (C) levels were measured before (day 0) and after (day 5) plasmid injection. A is a min to max whisker plot. B and C are line graphs where solid lines are the averages and shading represents the ± SEM. N=2-4 for each group.

Upon validation, we utilized our *Gpt* plasmids to assess whether overexpression of hepatic *Gpt* blocks the glucose lowering actions of leptin. For this, nine-week-old C57Bl6/J mice were injected with STZ to induce diabetes, then injected with p*Gpt* or empty plasmids (Figure 7A). Four days after plasmid injection, mice injected with empty plasmids received either pumps delivering leptin (STZ-pEmpty-Leptin) or vehicle (STZ-pEmpty-Vehicle), and all mice injected with p*Gpt* received leptin pumps (STZ-p*Gpt*-Leptin). Consistent with our initial observations, hepatic *Gpt* transcript levels were increased in STZ-pEmpty-Vehicle mice compared to non-diabetic controls, then normalized to non-diabetic levels in STZ-pEmpty-Leptin mice (Figure 7B). Hepatic *Gpt* expression was increased by ~4 fold in STZ-p*Gpt*-Leptin mice compared to STZ-pEmpty-Leptin controls, indicating that we successfully blocked leptin mediated reduction in hepatic *Gpt* expression (Figure 7B). STZ caused weight loss, which was not exacerbated with plasmid injection or leptin therapy (Figure 7C). As expected, administration of leptin reversed STZ-induced hyperglycemia in STZ-pEmpty-Leptin mice (Figure 7D). Despite overexpression of hepatic *Gpt*, leptin lowered blood glucose levels in STZ-p*Gpt*-Leptin mice to the same extent and with comparable kinetics as STZ-pEmpty-Leptin controls (8.4 ± 0.7 vs 7.5 ± 0.9 mM, respectively; Figure 7D). Furthermore, leptin-treated mice with and without overexpression of *Gpt* eventually succumbed to fasting hypoglycemia within 12 hours of fasting (Figure 7E), suggesting that the reduction in *Gpt* does not cause fasting induced hypoglycemia in leptin-treated mice. Thus, overexpression of *Gpt* is insufficient to the block glucose lowering actions of leptin, which implies that leptin-induced down-regulation of *Gpt* is not required for these effects of leptin therapy.

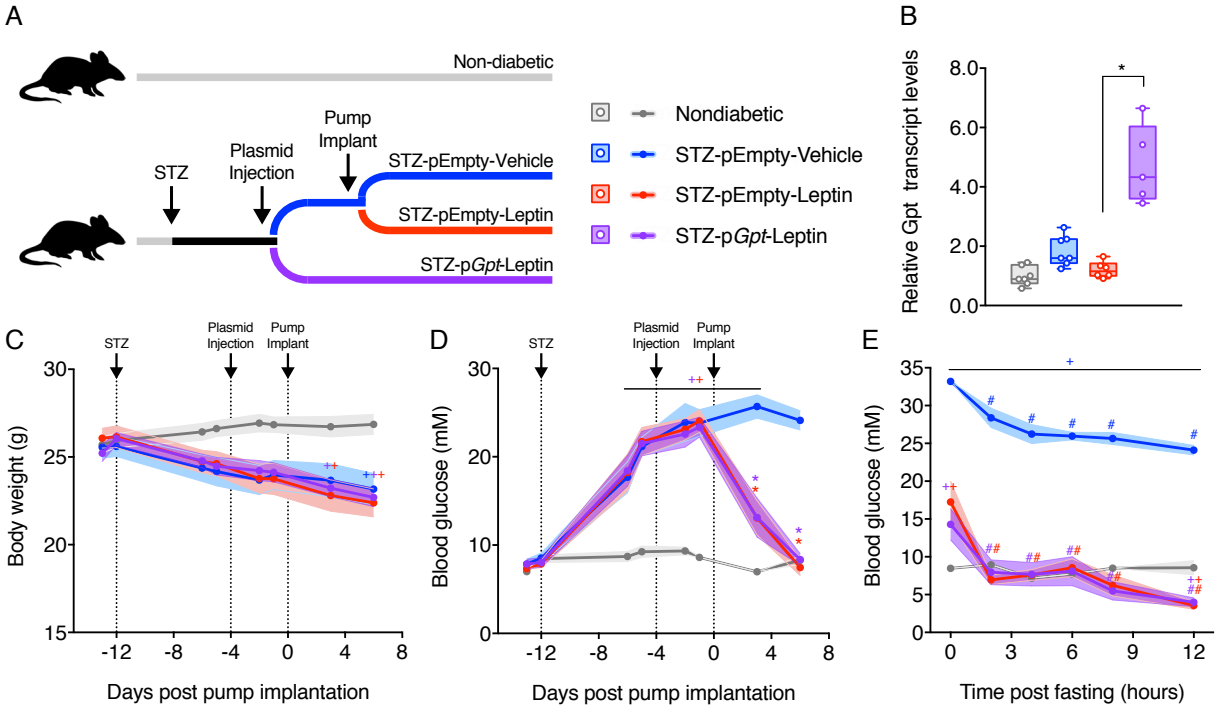

Figure 7. Reduced expression of hepatic glutamic pyruvic transaminase (*Gpt*) is not required for the glucose lowering actions of leptin. Nine-week-old C57Bl6/J mice were rendered diabetic with STZ on day -12 then injected with empty plasmid (pEmpty) as a control or plasmid encoding GPT (p*Gpt*) on day -4 (A). Four days after plasmid injection (day 0), mice that received pEmpty were implanted with mini osmotic pumps delivering either vehicle (STZ-pEmpty-Vehicle) or leptin (STZ-pEmpty-Leptin), and all mice that received p*Gpt* were implanted with mini osmotic pumps delivering leptin (STZ-p*Gpt*-Leptin). Non-diabetic mice were included as controls. On day 6 post pump implantation, livers were harvested and hepatic *Gpt* transcript levels were measured (B). Body weight (C) and blood glucose levels (D) were tracked throughout the study. On day 6 post pump implantation, a fasting tolerance test was performed and blood glucose levels were during the test (E). B is a min to max whisker plot and statistical analysis was performed by one-way ANOVA (*, P<0.05). For C-E, solid line is the average with shading as the ± SEM, and statistical analysis was performed by two-way ANOVA (*, P<0.05 for comparison against STZ-pEmpty-Vehicle group; +, P<0.05 for comparison against non-diabetic group; #, P<0.05 for comparison against 0 hours post fasting). N=5-7 for each group.

Next, we examined whether knockdown of hepatic *Gpt* alone would recapitulate glucose lowering actions of leptin in insulin deficient mice. In order to knockdown *Gpt* in the liver, we used lipid nanoparticles to deliver siRNAs against *Gpt*, which is a system previously shown to

efficiently transport siRNAs to the liver *in vivo* [394, 395]. We first tested the knockdown efficiency of 3 different *Gpt* siRNAs relative to coagulation factor VII (*F7*) siRNA, and tested 3 varying doses for each siRNA sequence (0.5, 1, and 5 mg/kg for low, medium, and high, respectively; Figure 8A). Lipid nanoparticle encapsulated siRNAs (LNP-siRNAs) were administered to wildtype mice via tail-vein injections, and we also included mice injected with PBS as an additional control. By day 7 post siRNA injection, *Gpt* siRNA 1 significantly lowered *Gpt* transcript levels in the liver by ~5 fold at the low dose, by ~10 fold at the medium dose, and by ~15 fold at the high dose (Figure 8B). *Gpt* siRNA 2 also effectively knocked down hepatic *Gpt* transcript levels at all 3 doses, but we observed higher variability at the medium dose for *Gpt* siRNA 2 compared to *Gpt* siRNA 1. *Gpt* siRNA 3 did not significantly reduce hepatic *Gpt* transcript levels at the low or medium doses, and knockdown by *Gpt* siRNA 3 at the high dose was not as efficient as *Gpt* siRNA 1 and 2. Injection of *Gpt* siRNAs in non-diabetic mice did not alter body weight (Figure 9A-C) or blood glucose levels (Figure 9D-F). We concluded that *Gpt* siRNA 1 most effectively and reliably reduced hepatic *Gpt* transcript levels in non-diabetic mice, and found that low and medium doses of this siRNA was sufficient for efficient knockdown.

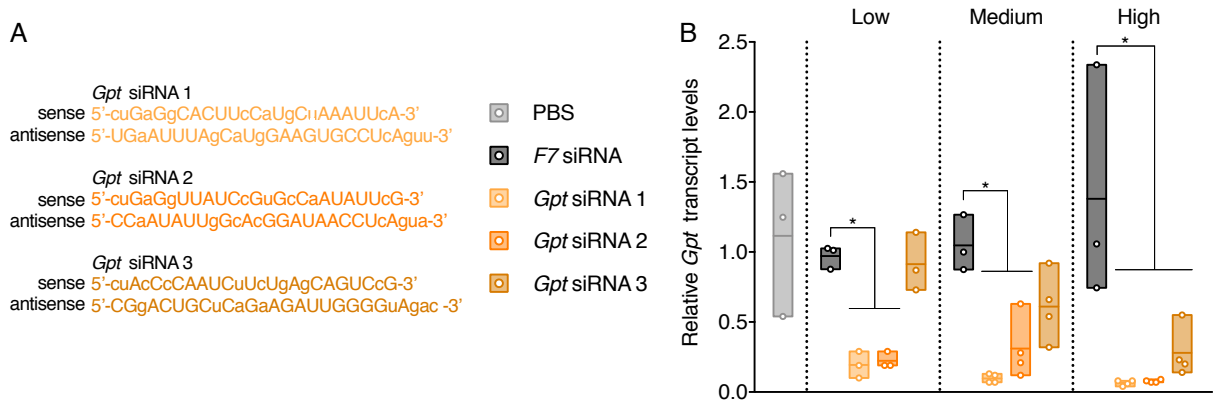

Figure 8. Glutamic pyruvic transaminase (*Gpt*) siRNA administration to non-diabetic mice reduces *Gpt* transcript levels in the liver. Nine-week-old mice were injected with siRNA against coagulation factor VII as a control (*F7* siRNA) or injected with 3 different sequences of siRNA against *Gpt* (A; *Gpt* siRNA1-3) on day 0 at 3 different doses for all sequences of siRNA (0.5, 1, and 5 mg/kg for low, medium, and high, respectively). Mice injected with PBS were included as controls. On day 7 post siRNA injection, livers were harvested from all mice and hepatic *Gpt* transcript levels were measured (B). B is a min to max whisker plot and statistical analysis was performed by one-way ANOVA (*, P<0.05). N=3-4 for each group.

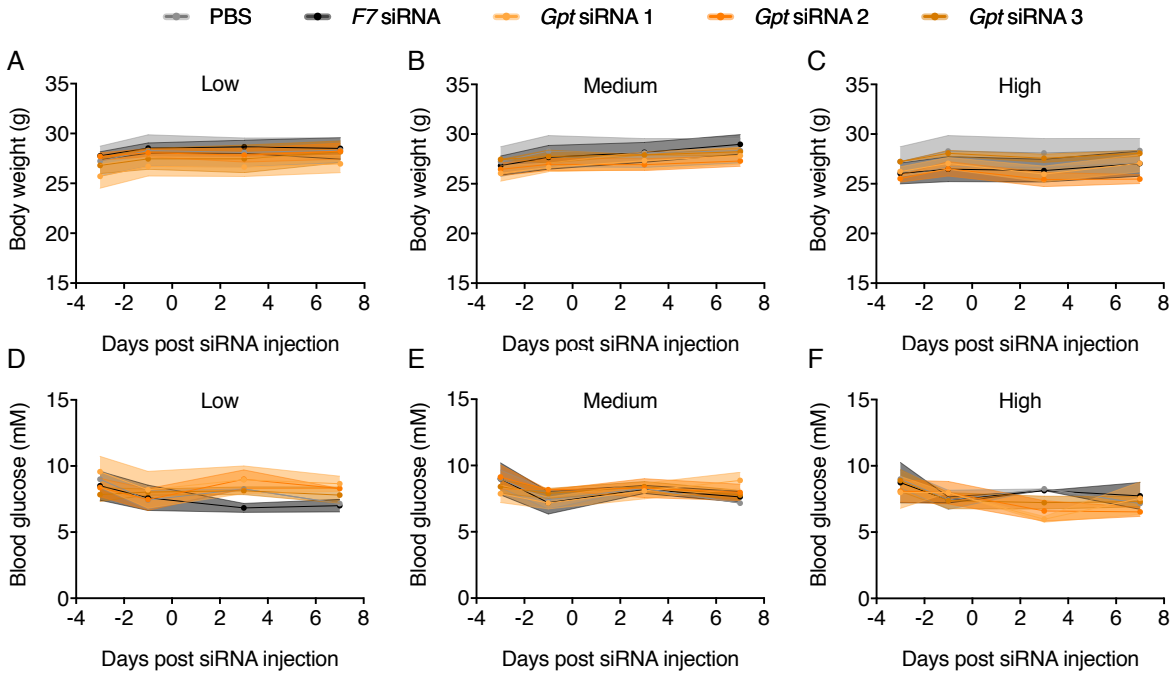

Figure 9. Glutamic pyruvic transaminase (*Gpt*) siRNA administration to non-diabetic mice does not alter body weight or blood glucose levels. Nine-week-old mice were injected with siRNA against F7 as a control (*F7* siRNA) or injected with 3 different sequences of siRNA against GPT (*Gpt* siRNA1-3) on day 0 at 3 different doses for all sequences of siRNA (0.5, 1, and 5 mg/kg for low, medium, and high, respectively). Mice injected with PBS were included as controls. Body weight (A-C) and blood glucose (D-F) levels were tracked for mice receiving low (A and D), medium (B and E), and high (C and F) doses of siRNA. For all panels, solid line is the average with shading as the ± SEM and statistical analysis was performed by two-way ANOVA, which did not reveal any statistical differences between groups. N=3-4 for each group.

After validation, we utilized *Gpt* siRNA 1 to examine whether knockdown of hepatic *Gpt* in STZ-mice could recapitulate the blood glucose lowering actions of leptin. Nine-week-old C57Bl6/J mice were rendered diabetic with STZ, then injected with lipid nanoparticle encapsulated *Gpt* siRNA or *F7* siRNA at 2 different doses (0.5 and 1 mg/kg for low and medium doses, respectively). Non-diabetic mice were included as a reference for normal glycemia. STZ induced weight loss, which was not exacerbated with LNP-siRNA injections at low or medium doses (Figure 10A&C). Administration of *Gpt* siRNA to STZ-mice at low or medium doses did not reverse hyperglycemia, and blood glucose levels remained comparable between STZ-mice injected with *F7* and *Gpt* siRNAs throughout the study (Figure 10B&D). Thus, these data, when combined with our *Gpt* overexpression studies, reveal that the reduction in hepatic *Gpt* expression is neither required nor sufficient for the glucose lowering actions of leptin

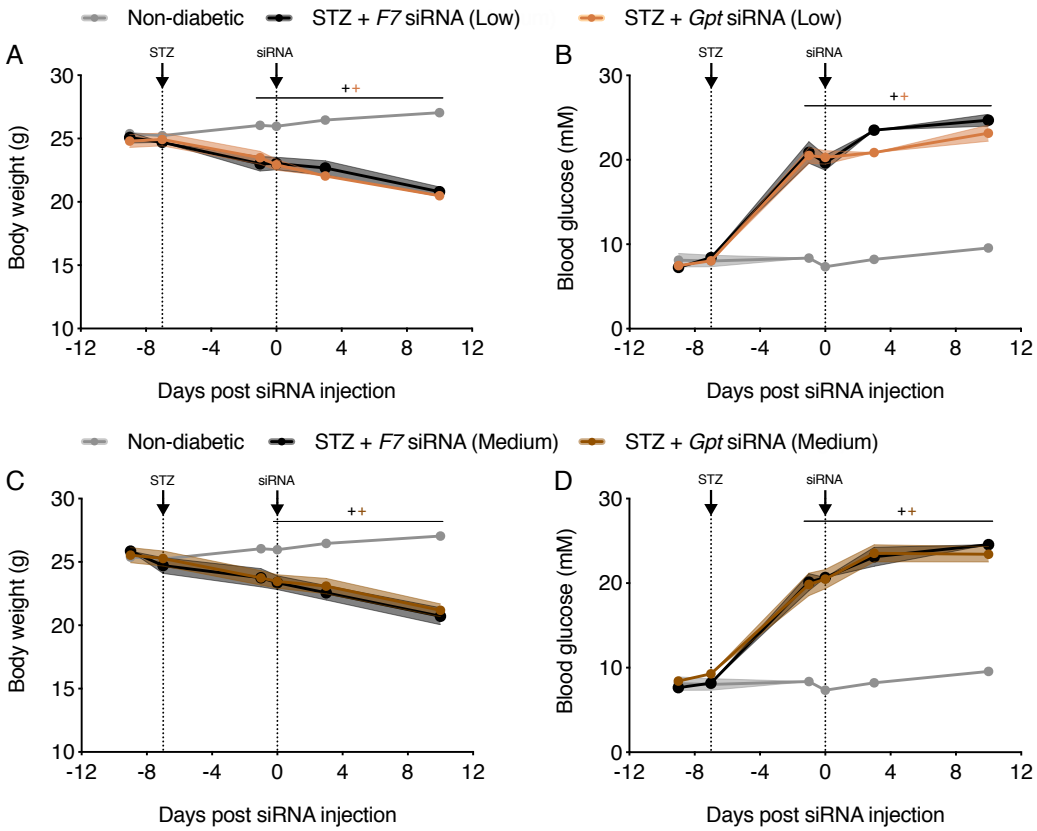

Figure 10. Glutamic pyruvic transaminase (*Gpt*) reduction is not sufficient for the glucose lowering actions of leptin in STZ-mice. Mice were rendered diabetic with STZ were injected with lipid nanoparticle encapsulated siRNA against F7 as a control (*F7* siRNA) or siRNA against *Gpt* (*Gpt* siRNA) on day 0 at 2 different doses (0.5 and 1 mg/kg for low and medium doses, respectively). Non-diabetic mice were included as controls. Body weight (A and C) and blood glucose (B and D) levels were tracked for mice receiving low (A and B) and medium (C and D) doses of siRNA. For all panels, solid line is the average with shading as the ± SEM and statistical analysis was performed by two-way ANOVA (+, $P<0.05$ for comparison against non-diabetic group). N=6-7 for each group.

We next assessed whether knockdown of hepatic *Gpt* in STZ-mice impairs fasting tolerance or utilization of alanine for glucose production. We postulated that if leptin-mediated lowering of *Gpt* in STZ-mice is the cause of fasting intolerance and defective alanine utilization, then *Gpt* knockdown alone should recapitulate these effects in STZ-mice. STZ-mice were injected with *Gpt* siRNA or *F7* siRNA as a control, and then challenged with a fasting tolerance test immediately followed by an alanine tolerance test on day 5 post siRNA injection. In STZ-mice injected with low or medium doses of *Gpt* siRNA, blood glucose levels decreased with 3

hours of fasting but glucose levels stabilized by 6 hours of fasting (Figure 11A&C). Blood glucose tracking during the fasting tolerance test was near superimposable between STZ-mice injected with *Gpt* siRNA and *F7* siRNA at both doses (Figure 11A&C). These data suggest that knockdown of hepatic *Gpt* does not cause fasting intolerance in STZ mice, and implies that the reduction of *Gpt* expression in leptin-treated STZ-mice is not the cause of fasting intolerance. In STZ-mice injected with low or medium doses of *Gpt* siRNA, alanine administration led to an increase in blood glucose levels and alanine-induced glucose excursions in these mice were comparable to *F7* siRNA and non-diabetic controls (Figure 11B&D). These data suggest that administration of *Gpt* siRNA, despite efficient knockdown in the liver, does not block the utilization of alanine for glucose production, and leptin-induced reduction in hepatic *Gpt* alone is not the cause of defective alanine utilization for the production of glucose.

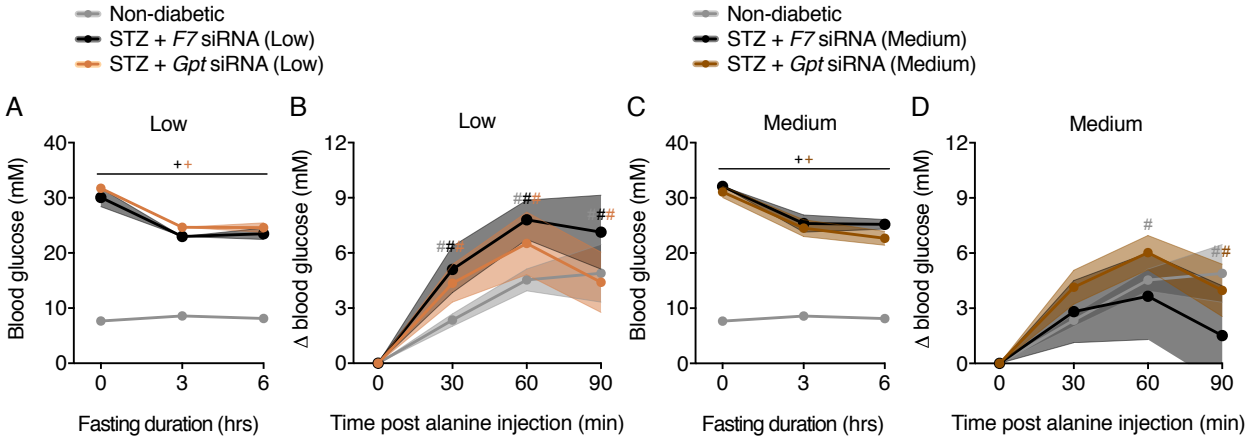

Figure 11. Glutamic pyruvic transaminase (*Gpt*) knockdown does not promote fasting induced hypoglycemia and does not suppress utilization of alanine for the production of glucose. Nine-week-old C57Bl6/J mice were rendered diabetic with STZ on day -7 then injected with LNP-siRNA against F7 (*F7* siRNA) or *Gpt* (*Gpt* siRNA) on day 0 at 2 different doses (0.5 and 1 mg/kg for low and medium doses, respectively). Non-diabetic mice were included as controls. On day 5 post siRNA injection, a fasting tolerance test was performed (A and C) followed by an alanine tolerance test (B and D). For all panels, solid line is the average with shading as the ± SEM. Statistical analysis was performed by two-way ANOVA (+, P<0.05 for comparison against non-diabetic group; #, P<0.05 for

comparison against 0 minutes post alanine injection). N=6-7 for each group.

Triple Omics Analysis Reveals a Global Suppression of Amino Acid Catabolism in Leptin-Treated STZ-Mice

Given that hepatic *Gpt* alone is not responsible for the glucose lowering actions of leptin in STZ-mice, we moved to a more global and un-biased approach to identify the precise metabolic pathways altered in leptin-treated mice. For this, we surveyed the liver of leptin-treated mice using a triple omics approach, which combined transcriptomics, proteomics, and metabolomics. Each approach has advantages and limitations, and these approaches are highly complementary when combined. We employed RNA sequencing for transcriptomics analysis, which is a well-validated and highly sensitive technique, and thus allows us to detect small changes in transcript levels for nearly all annotated genes within the genome (reviewed in [396]). On the other hand, changes in transcript levels do not always correlate to alterations in protein expression (reviewed in [397]). Proteomics analysis allows for the direct assessment of protein levels. We chose to utilize a highly innovative proteomics technique called stable isotope labeling of amino acids in mice (SILAM), which is more quantitatively accurate and reproducible than chemical labeling or label-free quantification strategies ([398] and reviewed in [399]). However, proteomics analysis often does not cover the whole proteome and there can be more technical variability than RNA sequencing (reviewed in [400]). Moreover, changes in protein levels do not always reflect alterations in activity [378]. Metabolomics analysis provides a biochemical snapshot of metabolic pathways, which is more reflective of the functional phenotype than transcriptomics and proteomics analysis (reviewed in [401]). For our metabolomics analysis, we chose to employ ultra high-performance liquid chromatography-

tandem mass spectrometry (UHPLC-MS/MS), which allows high mass accuracy and resolution capabilities, combined with an extensive biochemical reference library, which allowed for a more quantitative metabolomics analysis [402]. On the other hand, standard metabolomics analysis does not reveal alterations in the flux of metabolites (reviewed in [403]). Moreover, current technologies used for metabolomics analysis cannot measure the entire metabolome and is vulnerable to more technical variability compared to RNA sequencing technologies (reviewed in [403]). Combining these omics technologies can harness the advantages and address some of the limitations for each layer of omics analysis, and thus we used a triple omics approach for a more comprehensive and detailed view of the metabolic pathways altered in the liver of leptin-treated STZ-mice.

For our triple omics analysis, we followed our usual experimental scheme for leptin treatment. We administered leptin or vehicle by pumps to STZ-diabetic mice (STZ-Leptin and STZ-Vehicle, respectively), and tracked blood glucose levels and body weight to ensure that leptin effectively reversed hyperglycemia in STZ-mice. To compare the effects of leptin to insulin therapy on metabolic pathways in the liver, we also included STZ-mice that were administered insulin by pumps. Furthermore, we included non-diabetic mice in all our omics analysis for comparison of leptin-treated STZ-mice to healthy controls. Transcriptomics analysis was performed on a separate cohort of mice than proteomics and liver metabolomics analysis, but the same experimental scheme was repeated for both studies. On day 4 post pump implantation, mice were fasted for 4 hours, then the livers were harvested and immediately frozen in liquid nitrogen for preservation of transcripts, proteins, and metabolites. Our aim was to assess the primary effects of leptin therapy in the liver of STZ-mice but we suspected that leptin-mediated reversal of hyperglycemia might also have secondary effects. To address this, we

chose to assess the liver immediately after the reversal of hyperglycemia (after 4 days) as opposed to after long-term leptin therapy (after weeks or months). Thus, our reproducible experimental design provided comparable samples for different layers of omics analysis, and we tailored our experimental design to better assess the primary effects of leptin therapy.

Using our transcriptomics analysis, we found that leptin drastically alters the hepatic transcriptome in leptin-treated STZ-mice. Using RNA sequencing, we measured expression levels of ~17,000 genes in the liver. Leptin therapy in STZ-mice altered levels of 1851 genes in the liver, and of those genes, 887 (48%) were up-regulated and 964 (52%) were down-regulated (Figure 12A-C). In contrast, insulin therapy altered 172 genes in the liver of STZ-mice, where 61 (35%) were up-regulated and 111 (65%) were down-regulated (Figure 12A-C). Thus, leptin therapy in STZ-mice alters the hepatic transcriptome more drastically than insulin therapy. Only 129 out of 1851 (7%) genes altered by leptin therapy were also changed with insulin therapy, and there was more overlap for down-regulated genes (9%) than up-regulated genes (5%; Figure 12A-C). These data suggest that leptin-mediated alterations in the hepatic transcriptome are largely distinct from the effects of insulin. A total of 2988 genes were differentially expressed in the liver of non-diabetic mice compared to STZ-Vehicle controls (Figure 12A), which is consistent with the well-characterized effects of hepatic insulin signalling on gene expression. Of these genes, a surprisingly small proportion was also altered in STZ-Insulin mice compared to STZ-Vehicle controls (4% of genes altered in non-diabetic mice vs STZ-Vehicle; Figure 12A). These data suggest that effects of STZ on the hepatic transcriptome are not reversed after 4 days of human recombinant insulin delivered by pumps. Combined, our data reveal that leptin globally alters the hepatic transcriptome in STZ-mice, and these effects of leptin are more dramatic than insulin therapy.

Since leptin altered the expression of such a large number of genes, we categorized these genes into biological processes to gain insight into the mechanism of leptin therapy. For this, we selected genes that are differentially expressed in the liver of STZ-Leptin mice compared to STZ-Vehicle controls, then performed a gene ontology (GO) analysis, which assigns genes to predefined categories based on their biological functions and assesses whether some categories are over-represented in a list of differentially expressed genes [368]. For genes up-regulated in the liver of STZ-Leptin compared to STZ-Vehicle controls, the most represented categories were related to translation and ribosome assembly (Figure 12D). Interestingly, many genes that were down-regulated in the liver of STZ-Leptin mice compared to STZ-Vehicle controls were related to the breakdown of amino acids (Figure 12D). This was particularly interesting to us because as we reported in a section above, leptin-treated STZ-mice cannot utilize the amino acid alanine for glucose production despite fasting hypoglycemia (Figure 3B). In addition to amino acid catabolism, many of the down-regulated genes were involved in lipid oxidation (Figure 12D), which is consistent with reports of decreased ketone levels in leptin-treated STZ-mice [264]. In summary, many of the down-regulated genes were involved in the breakdown of amino acids and lipids, consistent with a reduction in utilization of these substrates for the production of glucose and ketones.

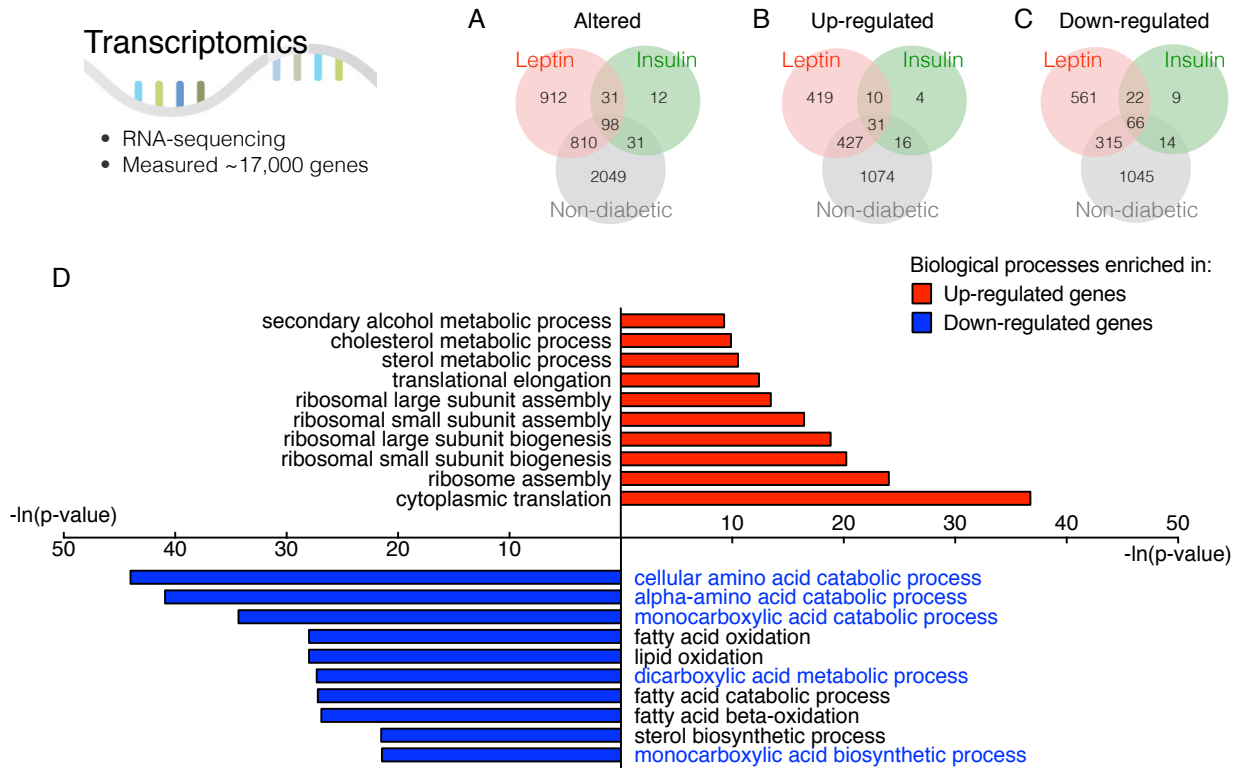

Figure 12. Genes involved in amino acid catabolism are globally down-regulated in the liver of STZ-Leptin mice. Nine-week-old C57Bl6/J mice were rendered diabetic with STZ on day -6 then implanted with pumps delivering vehicle (STZ-Vehicle), leptin (STZ-Leptin), or insulin (STZ-Insulin) on day 0. Non-diabetic mice were included as controls. Livers were collected on day 4 for RNA-sequencing. The number of genes that were significantly altered in STZ-Leptin, STZ-Insulin, and non-diabetic mice compared to STZ-Vehicle controls were determined ($q<0.05$), and Venn diagram shows the common genes that were significantly altered (A; up-regulated or down-regulated), up-regulated (B), and down-regulated (C). Genes that were differentially expressed in STZ-Leptin group compared to STZ-Vehicle controls were determined, then gene ontology (GO) analysis (D) was performed to determine the biological processes enriched in significantly up-regulated (red) and down-regulated (blue) genes. The p-value associated with the biological process was determined and negative natural log of the p-value is shown in the x-axis. The biological processes related to amino acid catabolism are highlighted in blue text. N=5 for all groups.

Proteomics analysis independently converged on a conclusion similar to transcriptomics analysis. The SILAM technique enabled detection of ~5000 proteins in our liver samples, and the relative levels of these proteins were compared between groups using an ANOVA followed by post-hoc t-tests with the false discovery rates at 10% using the Benjamini-Hochberg correction

method. Similar to the transcriptomics analysis, we found that leptin therapy in STZ-mice globally altered the proteome in the liver; levels of 736 proteins were altered in the liver of STZ-Leptin mice compared to STZ-Vehicle controls, and of these proteins, 317 were up-regulated and 419 were down-regulated (Figure 13A-C). Surprisingly, we did not detect differentially expressed proteins in the liver of insulin-treated STZ-mice compared to STZ-Vehicle controls (Figure 13A), using our parameters for statistical analysis. These data are consistent with the low number of transcripts that were differentially expressed in insulin-treated STZ-mice compared to diabetic controls (Figure 12A). These findings reveal that administration of leptin to STZ-mice has a more profound effect on the hepatic proteome than insulin therapy. To examine the roles of proteins that are differentially expressed in the liver of STZ-Leptin mice compared to STZ-Vehicle controls, we performed a GO analysis. We found that many of the up-regulated proteins were related to ribosome assembly (Figure 13D), which is consistent with the transcriptomics analysis (Figure 12D), and we also found that there was an enrichment of genes in categories related to cell growth (Figure 13D). For down-regulated proteins, we found that many of these proteins were involved in the breakdown of amino acids (Figure 13D), which is consistent with the findings of GO analysis using down-regulated transcripts (Figure 12D). Therefore, both transcriptomics and proteomics analysis independently pointed towards down-regulation of genes involved amino acid catabolism.

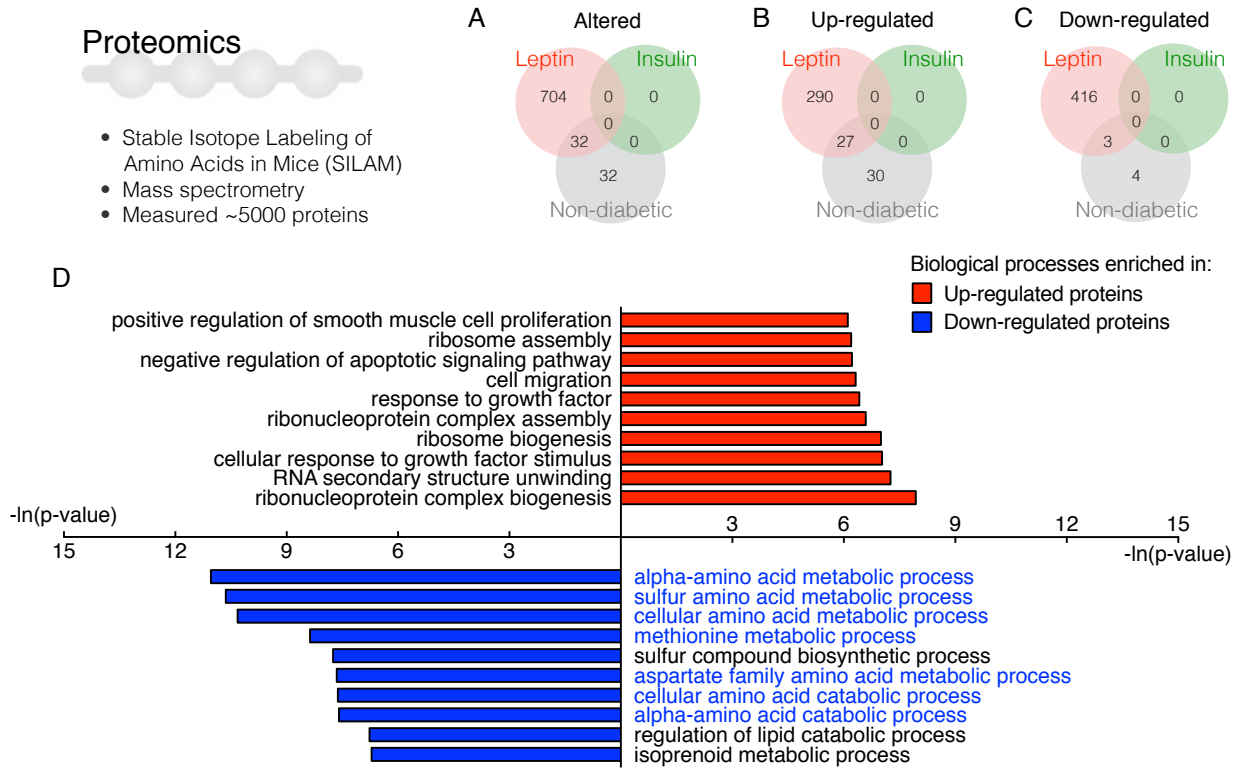

Figure 13. Many of the proteins down-regulated in the liver of leptin-treated mice are involved in amino acid catabolism. Nine-week-old C57Bl6/J mice were rendered diabetic with STZ on day -6 then implanted with mini-osmotic pumps delivering either vehicle (STZ-Vehicle), leptin (STZ-Leptin), or insulin (STZ-Insulin) on day 0. Non-diabetic mice were included as controls. Livers were harvested on day 4 post pump implantation for proteomics analysis. Proteins that were significantly altered in STZ-Leptin, STZ-Insulin, and non-diabetic mice compared to STZ-Vehicle controls were determined ($q<0.1$), and Venn diagram shows the common proteins that were significantly altered (A; up-regulated or down-regulated), up-regulated (B), and down-regulated (C). Gene ontology analysis (D) was performed to determine the biological processes enriched in significantly up-regulated (red) and down-regulated (blue) proteins in STZ-Leptin mice compared to STZ-Vehicle controls. The p-value associated with the biological process was determined and negative natural log of the p-value is shown in the x-axis. The biological processes related to amino acid catabolism are highlighted in blue text. N=5 for all groups.

Next, we assessed leptin-induced changes in the hepatic metabolome. Using a highly sensitive UHPLC-MS/MS platform, ~600 different metabolites were detected in our liver samples. Leptin dramatically altered the hepatic metabolome in STZ-mice; levels of 301 metabolites were significantly altered in livers of STZ-Leptin mice compared STZ-Vehicle

controls, and of these metabolites, 162 (54%) were elevated and 139 (46%) were diminished (Figure 13A-C). Although we did not detect differentially expressed proteins in livers of STZ-Insulin mice compared to STZ-Vehicle controls, metabolite levels were altered by insulin therapy (Figure 13A). These discrepancies underscore the need to evaluate the functional outcome of altered gene expression, such as measuring metabolite levels. Insulin therapy changed levels of 237 different metabolites in STZ-mice, and of these metabolites, 144 (61%) were elevated and 93 (39%) were diminished (Figure 14A-C). Thus, the hepatic transcriptome and proteome were more dramatically altered by leptin than insulin therapy in STZ-mice, but the alterations in the metabolome were more comparable. In line with our transcriptomics analysis, we found that there was some level of overlap between metabolites that were elevated or diminished with leptin and insulin therapies, but a significant number of metabolites were exclusively elevated (59 out of 162) or diminished (74 out of 139) by leptin therapy (Figure 14A-C). In summary, the hepatic metabolome was globally altered by leptin therapy in STZ-mice, and levels of many metabolites were altered by leptin, but not insulin, therapy.

After identifying metabolites whose levels were altered in leptin-treated STZ-mice, we next determined the metabolic pathways associated with these metabolites. For this, we performed a metabolite set enrichment analysis (MSEA), which uses predefined metabolic pathways to assess whether some pathways are significantly enriched in a list of metabolites [404]. For metabolites that were diminished in the livers of STZ-Leptin mice compared to STZ-Vehicle controls, enriched pathways were related to glucose and lipid metabolism, but we did not observe a biological function that was clearly over-represented over others (Figure 14D). In contrast, for metabolites that accumulated in livers of STZ-Leptin mice compared to STZ-Vehicle controls, most significant enriched pathways were related to amino acid metabolism

(Figure 14D). Thus, transcriptomics and proteomics analysis independently pointed towards down-regulated genes involved in amino acid metabolism, while metabolomics analysis revealed an accumulation of metabolites related to amino acid metabolism. Combined, these data suggest that amino acid metabolism is altered in leptin-treated STZ-mice.

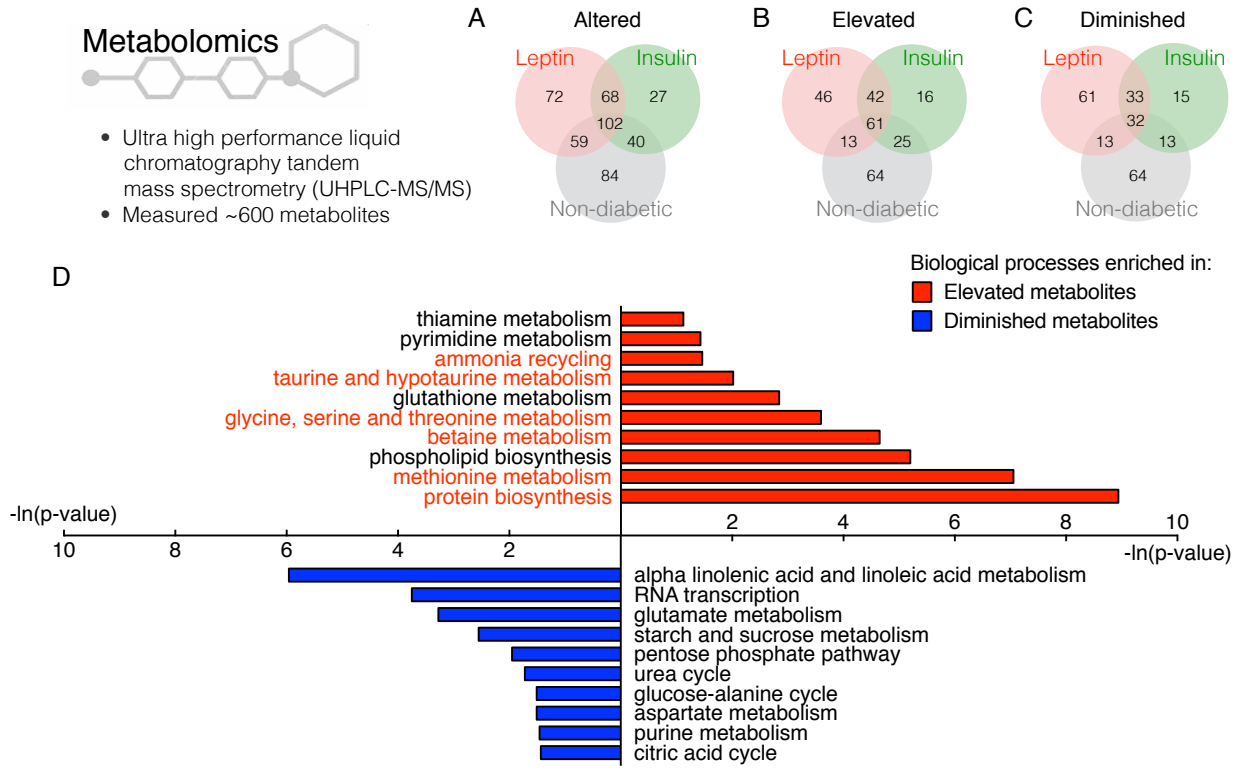

Figure 14. Amino acids and amino acid derivatives are enriched in the liver of leptin-treated mice. Nine-week-old mice were rendered diabetic with STZ on day -6 then implanted with mini-osmotic pumps delivering either vehicle (STZ-Vehicle), leptin (STZ-Leptin), or insulin (STZ-Insulin) on day 0, and non-diabetic mice were included as controls. Livers were collected on day 4 post pump implantation for metabolomics analysis. Metabolites with significantly altered levels in STZ-Leptin, STZ-Insulin, and non-diabetic mice compared to STZ-Vehicle controls were determined ($q<0.1$), and Venn diagram shows the common metabolites between groups (A; elevated or diminished), elevated (B), and diminished (C). Metabolite set enrichment analysis (MSEA) was performed to determine the biological processes enriched in significantly elevated (red) and diminished (blue) metabolites for STZ-Leptin group compared to STZ-Vehicle controls (D). The p-value associated with the biological process was determined and negative natural log of the p-value is shown in the x-axis. Biological processes related to amino acid metabolism are highlighted in red. N=5 for all groups.

Next, we examined the types of amino acids elevated in the liver of leptin-treated mice using our metabolomics data (Figure 15). In the liver of STZ-Leptin mice compared to STZ-Vehicle controls, we observed an increase in amino acids that are exclusively gluconeogenic, such as asparagine, cysteine, glycine, proline, serine, and methionine. In addition, amino acids that are both gluconeogenic and ketogenic, such as tyrosine, tryptophan, phenylalanine, and threonine, were elevated in STZ-Leptin mice compared to STZ-Vehicle controls. In contrast, amino acids that are exclusively ketogenic were not elevated in leptin-treated STZ-mice. Thus, leptin therapy increased levels of amino acids that are utilized for glucose, but not ketone, production. Both non-essential and essential amino acids were elevated in the liver of leptin-treated mice compared to STZ-diabetic controls. The elevation of essential amino acids in leptin-treated STZ-mice suggest that accumulation is not due to enhanced *de novo* synthesis, but instead points to a defect in the breakdown of amino acids. For all amino acids that were elevated with leptin treatment, the levels were comparable between non-diabetic and STZ-Vehicle mice, suggesting that amino acid accumulation in leptin-treated STZ-mice is not reversing the effects of insulin deficiency. All amino acid levels were comparable between STZ-Vehicle and STZ-Insulin groups, indicating that amino acid accumulation is unique to leptin, but not insulin therapy. In summary, leptin therapy in STZ-mice elevates gluconeogenic, but not ketogenic, amino acids in the liver, and this state of amino acid abundance is observed in leptin-treated STZ-mice but not in non-diabetic or insulin-treated STZ-mice.

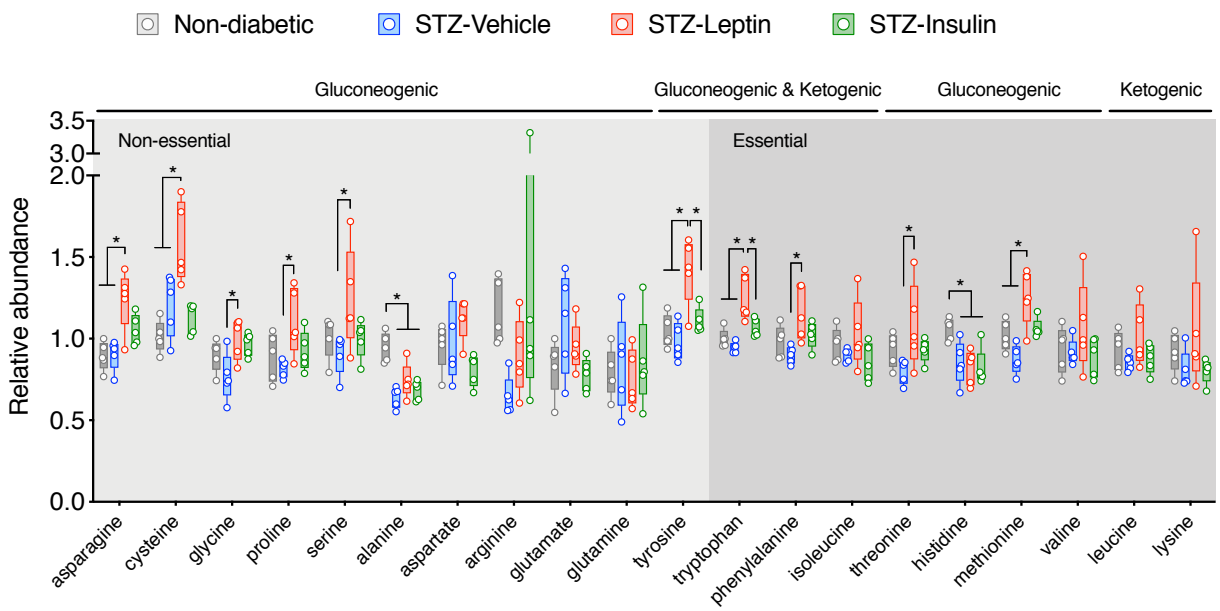

Figure 15. Both essential and non-essential gluconeogenic amino acids are enriched in the liver of leptin-treated STZ-mice. STZ-mice were implanted with mini-osmotic pumps delivering either vehicle (STZ-Vehicle), leptin (STZ-Leptin), or insulin (STZ-Insulin) then livers were collected on day 4 post pump implantation for metabolomics analysis (see also Figure 13). Amino acids were grouped into non-essential (left; light grey boxes) and essential (right; dark grey boxes), then further categorized into exclusively gluconeogenic, both gluconeogenic and ketogenic, and exclusively ketogenic amino acids. Graph is min to max whisker plot and statistical analysis was performed by one-way ANOVA (*, $P<0.05$). N=5 for each group.

We then directly examined the pathways involved in the breakdown of amino acids using our transcriptomics and metabolomics data sets. Amino acids are broken down and fed into the tricarboxylic acid (TCA) cycle to be eventually converted to oxaloacetate, which is a substrate for the production of glucose via gluconeogenesis [405]. As also described above, there was an accumulation of gluconeogenic amino acids in the liver of leptin-treated STZ-mice compared to STZ-diabetic controls (Figure 15 and Figure 16). Most of the genes involved in the breakdown of these amino acids were down-regulated by leptin in STZ-mice (Figure 16). In fact, all gluconeogenic amino acids with the exception of aspartate and asparagine had at least one gene

within its catabolic pathway that was down-regulated compared to diabetic controls. For some amino acids such as alanine and proline, all catabolic genes required for conversion to a TCA cycle intermediate were down-regulated. Within the TCA cycle, most genes were down-regulated by leptin therapy. Consistent with decreased expression of genes involved in the breakdown of amino acids and the accumulation of amino acids themselves, the TCA cycle intermediates were diminished in leptin-treated STZ-mice. Combined, these data suggest that there is a global suppression of amino acid breakdown in leptin-treated STZ-mice.

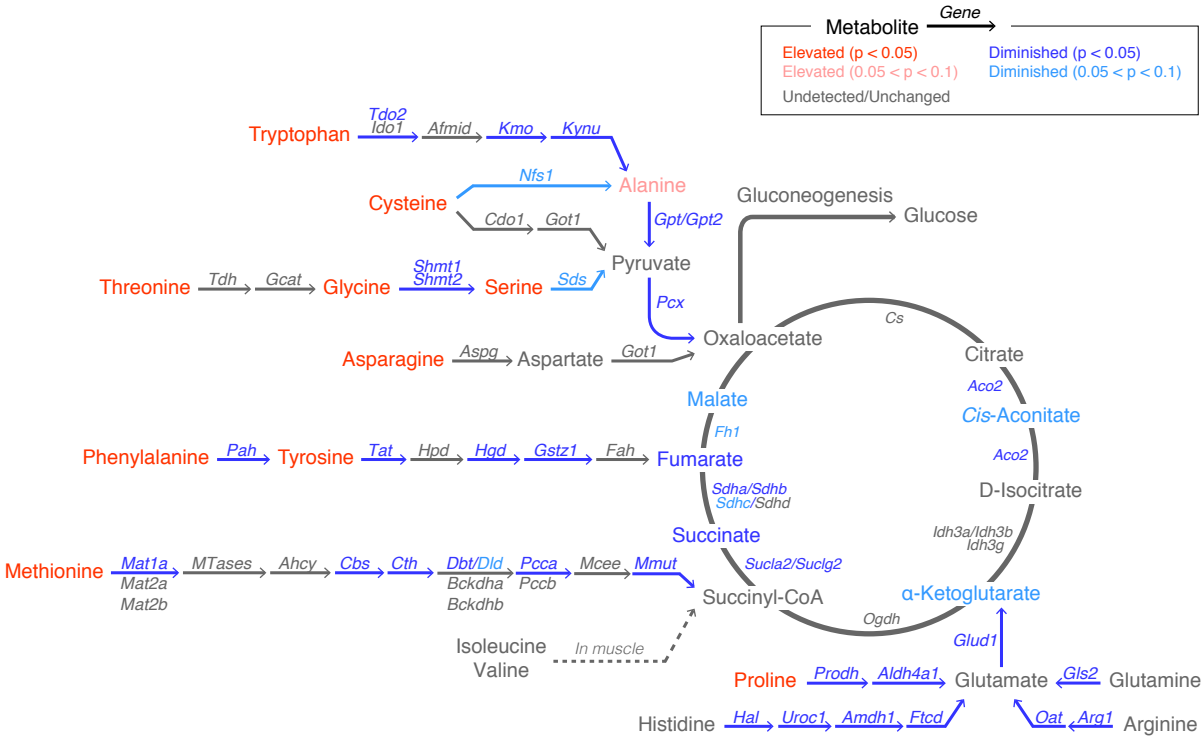

Figure 16. Pathways for amino acid catabolism are globally altered in the liver of leptin-treated STZ-mice. Mini-osmotic pumps delivering either vehicle (STZ-Vehicle) or leptin (STZ-Leptin) were implanted in STZ-mice. Liver was collected on day 4 post pump implantation for transcriptomics and metabolomics analysis to identify significantly altered genes and metabolites, respectively within the amino acid catabolism pathway (see also Figures 11 and 13). Genes and metabolites that were significantly elevated are shown in red ($p<0.05$) or pink ($0.05<p<0.1$), while genes and metabolites that were significantly diminished are shown in blue ($p<0.05$) or light blue ($0.05<p<0.1$). Genes and metabolites that were undetected or unchanged are shown in grey.

3.3 **Discussion**

In this chapter, we examined the effect of leptin therapy on amino acid metabolism in STZ-diabetic mice. We found that high dose leptin therapy normalized 4 hour fasted blood glucose levels in STZ-mice, without raising insulin levels, consistent with previous reports in STZ-mice and other rodent models of type 1 diabetes [260, 261, 264-266, 268]. Leptin globally altered the plasma metabolome; levels of metabolites within all major classes were changed, revealing that the leptin-induced changes in the plasma extend beyond glucose and lipid metabolism. We focused on amino acid metabolism since decreased glucose production is suspected to drive glucose lowering by leptin and amino acids are a key substrate for glucose production. Remarkably, leptin-treated STZ-mice cannot utilize alanine to produce glucose but their ability to utilize pyruvate, a product of alanine breakdown, remains intact. *Gpt*, the gene responsible for alanine breakdown is down-regulated by leptin but this is neither required nor sufficient for the glucose lowering actions of leptin. Our triple omics analysis of the liver revealed that leptin globally alters the hepatic transcriptome, proteome, and metabolome, and enrichment analysis independently pointed towards alterations in amino acid metabolism. A more detailed examination revealed that genes responsible for amino acid breakdown are globally down-regulated in the liver of leptin-treated mice, which likely contributed to amino acid accumulation and diminished TCA cycle intermediates. Thus, suppressed utilization of amino acids for the production of glucose may contribute to the glucose lowering effects of leptin therapy in insulin deficient mice.

Our results shed light into key differences in mechanisms by which insulin and leptin lower blood glucose levels. Upon administration of insulin, blood glucose levels decrease within

minutes through the translocation of glucose transporters to the plasma membrane. In contrast, it takes several days for recombinant leptin to normalize blood glucose levels when delivered by pumps or multiple daily injections [264]. Moreover, glucose lowering effects of insulin are reversed within minutes while hyperglycemia returns several days after stopping leptin administration [260]. We suspect that leptin therapy remodels the liver transcriptional profile to lower blood glucose levels, which would require more time (days) than translocation of glucose transporters by insulin (minutes). Indeed, we observed a more dramatic alteration in the liver transcriptome with leptin therapy than insulin in STZ-mice. The observation that leptin therapy takes several days to improve glycemia and is longer-acting than insulin should be taken into consideration in future clinical trials testing leptin in patients living with type 1 diabetes.

The results of this study reveal an important and relatively uncharacterized link between leptin and amino acid metabolism. While our studies were performed in leptin-treated STZ-mice, leptin deficient *ob/ob* mice given leptin by daily subcutaneous injections also have decreased expression of genes involved in the breakdown of amino acids [406]. Leptin levels were not measured in these leptin-treated *ob/ob* mice, and thus it is unclear whether these effects of leptin were achieved by physiological or supraphysiological levels of leptin. Interestingly, administration of leptin directly into the brain via ICV injections also decreased genes involved in amino acid catabolism [406], suggesting that these effects of leptin are mediated through leptin signalling in the brain. Indeed, leptin mediated glucose lowering remains effective in STZ-mice with disrupted leptin signalling in the liver [260], which indicates that leptin does not act directly on the liver to lower blood glucose levels in STZ-mice. The precise neural targets that mediate these effects of leptin remain elusive but hypothalamic insulin signalling modulates amino acid metabolism in the liver [407], and thus hypothalamic leptin signalling may suppress

amino acid catabolism in leptin-treated STZ-mice. Overall, while leptin action on glucose and lipid metabolism has been extensively studied in numerous rodent models of diabetes, the actions of leptin on amino acid metabolism are less defined.

The findings in this chapter present a novel aspect of leptin therapy, namely the suppressed utilization of amino acids, which could contribute to leptin-mediated glucose lowering in STZ-mice. Previous reports have shown that administration of leptin to insulin deficient rodents depletes glycerol and free fatty acids, which are substrates and positive regulators of gluconeogenesis, respectively, and the depletion of these substrates contribute to the glucose lowering actions of leptin [264, 268]. Although leptin lowers the utilization of both glycerol and alanine, there are key differences in the mechanisms by which leptin achieves these effects. Leptin appears to deplete plasma glycerol levels by reducing whole body lipolysis, which lowers glycerol turnover in the liver [268]. Importantly, glycerol administration raises blood glucose levels, suggesting that genes involved in the conversion of glycerol to glucose remain intact [264]. In contrast, we observed that there is an accumulation of amino acids in the liver of leptin-treated STZ-mice, which is likely due to the down-regulation of genes involved in amino acid breakdown; therefore, leptin appears to block the use of amino acids for glucose production. In future studies, downregulation of hepatic genes involved in amino acid breakdown and accumulation of amino acids in the liver and should be confirmed using more precise methods such as qPCR and enzyme-based assays for amino acid quantification, respectively. Despite differences in the mechanisms by which leptin lowers utilization of glycerol and amino acids, it is likely that the combination these mechanisms drive the glucose lowering actions of leptin.

To date, all studies, including ours, investigating the effects of leptin on glucose production have focused on the liver. It is estimated that glucose production in the kidneys using alanine, glutamine, glycerol and lactate can account for ~20% of all glucose released into the circulation in humans during the fasted state [408]. In addition, the intestine can perform gluconeogenesis, and intestinal gluconeogenesis is essential to maintain fasting euglycemia in mice with liver specific deletion of G6P, which lack glucose production in the liver [409]. Thus, the kidneys and the intestine are contributors of glucose production during fasting conditions. Future studies should assess renal and intestinal transcriptional profiles of leptin-treated STZ-mice in order to determine whether genes involved in glucose production are also downregulated in these tissues.

We directly tested the role of alanine utilization, which is a small component of the amino acid catabolism pathway. Since leptin-treated mice cannot utilize alanine for the production of glucose, we investigated the role of *Gpt*, the gene responsible for alanine breakdown, and found that *Gpt* was neither required nor sufficient for the glucose lowering actions of leptin. A caveat in our studies is that we measured *Gpt* transcript levels in our overexpression and knockdown studies and did not directly measure GPT activity. However, hepatic *Gpt* transcript levels correlate to hepatic GPT activity in mice [410], and thus GPT activity was likely altered accordingly in our overexpression and knockdown studies. Interestingly, mice with knockdown of *Gpt* in the liver can still utilize alanine for the production of glucose. We speculate that this may be due to the utilization of alanine for the production of glucose in extra-hepatic tissues, such as the kidneys [411] and the intestine [412]. Nonetheless, leptin lowered blood glucose levels in STZ-mice with hepatic overexpression of *Gpt*, which argues against the mechanistic role of *Gpt* in leptin action. To determine whether leptin treated

STZ-mice with overexpression of hepatic *Gpt* can utilize alanine for glucose production, we will perform alanine tolerance tests in these mice. Our triple omics analysis revealed that leptin lowers the expression of genes in all branches of amino acid catabolism. Down-regulation of these genes likely lowers glycemia in leptin-treated mice, as opposed to down-regulation of *Gpt* alone, which is a small branch within amino acid catabolism.

How leptin therapy suppresses amino acid catabolism in insulin-deficient mice remains elusive. Evidence from our studies suggest that suppressed utilization of amino acids for glucose production in leptin-treated mice is not a secondary effect due to the restoration of euglycemia. First, insulin therapy in STZ-mice does not lead to an accumulation of amino acids in the liver, despite restoration of euglycemia. Second, non-diabetic mice are able to utilize the amino acid alanine for the production of glucose during an alanine tolerance test. Thus, suppressed amino acid utilization for the production of glucose is likely a primary effect of leptin therapy. Given that leptin globally alters pathways for amino acid catabolism, it is likely that the upstream mechanism involves a master regulator, such as transcription factors or micro RNAs (miRNAs). One possible candidate may be the transcription factor krüppel like factor 15 (KLF15) since whole body or liver-specific deletion of KLF15 globally down-regulates genes involved in amino acid catabolism and causes fasting hypoglycemia [384, 413], which recapitulates the effects of leptin-therapy in STZ-mice. Like many other transcription factors, KLF15 modulates transcriptional activity by forming complexes with coactivators and corepressors [414], and thus measuring transcript or protein levels may be insufficient to assess activity. Further supporting the idea that blood glucose lowering actions of leptin require transcriptional remodeling in the liver, it takes several days for blood glucose levels to be normalized when leptin is delivered by osmotic pumps and multiple daily injections [264]. Thus, while it is clear that leptin suppresses

genes involved in amino acid utilization for glucose production, the upstream mediators of this effect remains unknown and warrants further investigation.

Regardless of the mechanism mediating leptin-induced suppression of amino acid utilization, this chapter highlights that amino acid catabolism pathways may be therapeutic target for diabetes. Supporting this idea, exposing cultured hepatocytes to metformin lowers key genes involved in amino acid catabolism [413], which suggests that decreased amino utilization may in part contribute to the glucose lowering actions of metformin. Interestingly, overexpression of KLF15 attenuates metformin's ability to down-regulate amino acid catabolism genes [413], suggesting that KLF15 may be the transcription factor that globally alters gene expression in this model. In addition, small molecule glucagon receptor antagonists (GRA) lower blood glucose levels in rodents and monkeys, and hepatic gene-expression profiling in monkeys treated with GRA revealed down-regulation of numerous genes involved in amino acid catabolism [415]. These data link the glucose lowering effects of glucagon receptor antagonism to suppressed utilization of amino acids. Thus, suppressing amino acid catabolism may be a viable strategy to lower blood glucose levels in diabetes.

Based on observations from our studies and work of others, leptin may prove to be a viable therapy for type 1 or late stages of type 2 diabetes. Indeed, low dose leptin therapy in combination with insulin was shown to modestly decrease insulin requirements in humans with type 1 diabetes [357]. However, there are major pitfalls to leptin therapy. Insulin sensitivity is enhanced with high dose leptin therapy in rodent models of type 1 diabetes [260], and thus leptin at high doses in conjunction with insulin may increase the risk of hypoglycemia in patients living with type 1 diabetes. In line with this, hypoglycemia is reported in lipodystrophic patients

receiving leptin and insulin co-therapy [416]. Furthermore, high dose leptin mono-therapy leads to severe hypoglycemia in insulin deficient rodents [264]. While other studies claim that leptin does not induce hypoglycemia [263], these studies were conducted in 2 hour fasted mice, which does not mimic typical fasting durations in humans. It has previously been reported that leptin therapy reduces the gluconeogenic substrate glycerol [264, 268], and we have shown decreased utilization of amino acids in leptin-treated STZ-mice. Leptin-induced fasting intolerance is likely due to impairments in all pathways towards glucose production. Although leptin therapy itself may pose a risk of hypoglycemia, therapies that target distinct pathways in glucose production such as amino acid catabolism, without effecting the utilization of alternative substrates, may lower blood glucose levels while reducing the risk of hypoglycemia. In summary, while high doses of leptin alone or in combination with insulin may dramatically lower glycemia in rodent models of type 1 diabetes, further studies must more rigorously assess the risk of hypoglycemia before leptin is considered a viable therapy for patients with type 1 diabetes.

Chapter 4: Recapitulating the Effects of Leptin on the Hepatic Transcriptome With A Small Molecule Confers Glucose Lowering Effects in STZ-mice

4.1 Introduction

Administration of leptin to insulin deficient mice causes profound changes in the liver, a major hub for glucose and lipid metabolism. Leptin treatment in STZ-diabetic rodents suppresses gluconeogenesis through several distinct mechanisms. In these rodents, whole body lipolysis is decreased, which depletes the gluconeogenic substrate glycerol [264, 268]. Increasing glycerol levels in leptin-treated STZ-diabetic rodents through injections [264] or infusion of a lipid emulsion with heparin [268] raises blood glucose levels, suggesting that depletion of glycerol in part contributes to the glucose lowering actions of leptin. Reduced lipolysis also lowers acetyl-CoA levels in the liver [264, 356], which is an allosteric activator of the gluconeogenic enzyme pyruvate carboxylase [417-420] and a substrate for ketone production (reviewed in [421]). Infusion of acetate to increase hepatic acetyl-CoA levels raises blood glucose levels in leptin in STZ-diabetic rats [356], suggesting that depletion of acetyl-CoA contributes to leptin mediated glucose lowering in insulin-deficient diabetes. Furthermore, administration of leptin to STZ-rats reduces ketogenesis by 50% within hours [356], which illustrates that the effects of leptin in the liver extend beyond glucose metabolism. In Chapter 3 of this thesis, we showed that leptin-treated mice cannot use exogenous alanine for the production of glucose. In the liver, we showed a global down-regulation of genes involved in the breakdown of amino acids and an accumulation of amino acids, suggesting that wide-spread changes in gene expression may prevent the utilization of amino acids for gluconeogenesis. The effect of leptin on liver glycogen stores is unclear, with some reports showing depletion [261, 264] and others showing an increase

in liver glycogen [266] in leptin-treated mice compared to untreated diabetic controls. The observation that liver glycogen is increased in the latter study indicates that a reduction in glycogen stores is not necessary for the glucose lowering by leptin. Thus, leptin profoundly alters metabolic processes in the liver through mechanisms involving substrate depletion and global alterations in hepatic gene expression. It is likely that these mechanisms all contribute to leptin mediated improvements in hyperglycemia, hyperketonemia, and dyslipidemia in insulin deficient rodents.

The changes in the liver that occur with leptin therapy mimic the effects of insulin and oppose the actions of glucagon. Insulin also suppresses gluconeogenesis in the liver through direct and indirect mechanisms. Insulin signalling in the liver reduces the expression of genes involved in gluconeogenesis, including phosphoenolpyruvate carboxykinase 1 (*Pck1*) and glucose-6-phosphatase catalytic subunit (*G6pc*) (reviewed in [83]), although we showed in Chapter 3 that the effects of leptin on the hepatic transcriptome are more dramatic than insulin in STZ-mice. Furthermore, insulin reduces gluconeogenic substrate release from adipose tissue and skeletal muscle by inhibiting lipolysis and proteolysis, respectively (reviewed in [83]). Both leptin and insulin reduce the production of glucose from glycogen, although leptin appears to deplete total glycogen stores [264] while insulin stimulates glycogen synthesis (reviewed by [422]). On the other hand, glucagon increases the expression of gluconeogenic genes, promotes lipolysis and uptake of gluconeogenic substrates in the liver, and enhances the production of glucose from glycogen (reviewed in [86]). Indeed, whole body and liver specific glucagon receptor knockout mice have lower blood glucose levels and improved glucose tolerance [423, 424]. Furthermore, glucagon stimulates fatty acid oxidation and decreases amino acid catabolism in the liver (reviewed in [86]). Remarkably, whole body glucagon receptor knockout mice that

have been injected with STZ to deplete insulin display normal glucose levels, improved glucose tolerance, and reduced levels of circulating lipids and ketones [344]. Thus, leptin action in the liver of STZ-mice is similar to the effects of insulin and glucagon receptor antagonism.

Given the profound effects of leptin on the liver, we hypothesized that recapitulating the effects of leptin on hepatic gene expression may have beneficial effects on glucose metabolism. To test this hypothesis, we utilized the expression profiles from the liver of leptin-treated STZ-mice and searched for a small molecule that has the ability to create a similar expression profile. As described above, the effects of leptin in the liver mimic the net downstream effects of insulin and oppose the effects of glucagon. Therefore, we used the gene expression signatures from the liver of insulin-treated STZ-mice and glucagon receptor knockout STZ-mice, then looked for small molecules that can create an expression profile similar to the hepatic signatures of these animal models. Using these methods, we identified a novel small molecule which produces a hepatic gene signature similar to the transcriptional profiles of all 3 animal models. Moreover, we tested the glucose lowering abilities of this small molecule in insulin deficient mice, and found that the small molecule displayed modest but reproducible glucose lowering effects in STZ-mice.

4.2 Results

Identification of a Small Molecule Which Produces a Transcriptional Profile Similar to the Hepatic Expression Profile of Leptin-Treated STZ-Mice

We first obtained the hepatic gene expression profile of leptin-treated STZ-mice. To do this, C57Bl6/J mice were rendered diabetic with STZ then implanted with osmotic pumps delivering either leptin (STZ-Leptin) or Vehicle (STZ-Vehicle). Blood glucose levels were

normalized in STZ-Leptin mice (24.1 ± 0.2 mM and 9.0 ± 1.7 mM on days -1 and 4 post pump implant), while STZ-Vehicle mice remained diabetic (22.3 ± 1.7 mM and 25.1 ± 1.1 mM on days -1 and 4). Four days after pump implantation, the liver was harvested for RNA sequencing. We performed a differential expression analysis using LIMMA to compare expression levels of genes in STZ-Leptin mice to STZ-Vehicle controls. We used the transcriptomics data from Chapter 3 of this thesis, where we found that 887 genes were significantly up-regulated and 964 genes were significantly down-regulated in the liver of STZ-Leptin mice compared to STZ-Vehicle controls (Figure 12B-C). We then identified the top 100 significantly up-regulated and top 100 significantly down-regulated genes with the greatest fold change in STZ-Leptin mice compared to STZ-Vehicle controls, and used this list of genes for further analysis (Figure 17A).

We then compared the hepatic gene expression profile of leptin-treated STZ-mice to ~1400 different transcriptional profiles created with small molecule treatment of cultured human epithelial cells. For comparison of transcriptional profiles, we used the Connectivity Map (CMAP), which is a database and analysis software provided by the Broad Institute (https://www.broadinstitute.org/connectivity-map-cmap). CMAP contains whole-genome expression profiles derived from human cell lines that were-treated with more than ~1400 different small molecules [425-427]. The CMAP software uses algorithms to compare a set of genes that are up-regulated and down-regulated in a specific condition to ~1400 different small molecule transcriptional profiles, in order to identify small molecules that create similar gene expression patterns. Similar pipelines have been used by others, including the studies by Liu *et al*., in which CMAP was used to compare the hepatic transcriptional profile of transgenic rodents with increased leptin sensitivity to small molecule expression signatures [428]. In their studies,

Celastrol, identified through CMAP, increased leptin sensitivity in diet-induced obese mice, and thus establishing proof-of-concept for this pipeline. Since CMAP contains data obtained from human cells using the Affymetrix microarray, we first converted the genes in our expression profile to the human homolog, and then further converted the NCBI gene numbers to Affymetrix probe identifiers (Figure 17A). We obtained enrichment scores for ~1400 different small molecules, which reports the degree of similarity between the query transcriptional profile and the profile created with small molecule treatment of cultured cells. Enrichment scores range between -1 to 1, which indicates low to high degrees of similarity, respectively. We identified the small molecule with an enrichment score of 0.96, which indicates that treatment of cultured cells with this small molecule created a transcriptional profile resembling the hepatic expression signature of STZ-Leptin mice, and we called this molecule the predicted leptin mimetic (PLM; Figure 17B). Interestingly, the molecule with the second highest enrichment score of 0.93 was phenyl biguanide (Figure 17B), which is a member of the biguanide family of small molecules [429]. Metformin, the most commonly prescribed drug for type 2 diabetes, is also a member of this family, and suppression of glucose production is thought to be a key mechanism by which metformin lowers blood glucose levels [430]. These data suggested to us that our pipeline may be identifying small molecules that lower glucose production, similar to leptin therapy in STZ-mice, and that these molecules might be able to lower blood glucose levels.

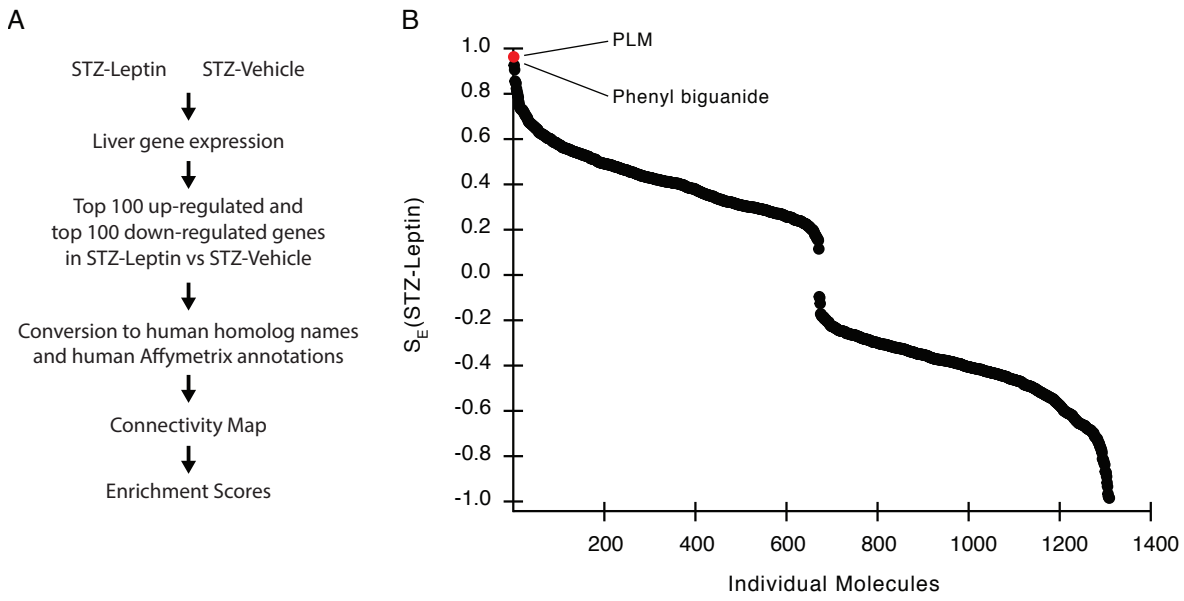

Figure 17. Predicted leptin mimetic (PLM) treatment of cultured cells produces a transcriptional profile similar to leptin therapy in STZ-mice. Nine-week-old mice were rendered diabetic with STZ on day -6 then implanted with mini-osmotic pumps delivering vehicle (STZ-Vehicle) or leptin (STZ-Leptin; same cohort of mice as Figure 12; N=5 for all groups). The liver was collected on day 4 post pump implantation for transcriptomics analysis. The liver transcriptional profile, which we defined as the top 100 significantly up-regulated and top 100 significantly down-regulated genes with the greatest fold change, was determined (A). The liver transcriptional profile of STZ-Leptin mice was compared to ~1400 different transcriptional profiles created with treatment of human cultured cells with small molecules (data available from CMAP; [425, 426]). Comparisons of transcriptional profiles provide enrichment scores (S_E(STZ-Leptin)), where a high S_E suggests a large degree of similarity. The S_E were plotted and each data point represents a small molecule being compared to STZ-Leptin mice (B). PLM and phenyl biguanide, which had the highest S_E, are labeled.

To avoid false positive identification of small molecules, we repeated the CMAP analysis with other rodent models with improved glucose metabolism. Using transcriptomics data from Chapter 3 of this thesis, we found that 61 genes were significantly up-regulated and 134 genes were significantly down-regulated in the liver of STZ-Insulin mice compared to STZ-Vehicle controls, and thus we used the 61 significantly up-regulated and top 100 significantly down-regulated genes with the greatest fold change for further analysis. In addition, we identified

genes that were differentially expressed in the livers of whole body glucagon receptor knockout (*GcgR* KO) mice compared to wildtype controls using microarray data deposited by Solloway *et al.* in Gene Expression Omnibus (GEO) [431]. We used the top 100 up-regulated and top 100 down-regulated genes with the greatest fold change in the liver of *GcgR* KO mice compared to wildtype controls. We then used the hepatic transcriptional profiles of STZ-Insulin and *GcgR* KO to obtain enrichment scores for ~1400 small molecules (Figure 18A). Upon obtaining the small molecule enrichment scores for all 3 animal models, we collapsed the enrichment scores down to a single dimensional metric by multiplying the absolute value of 3 enrichment scores for a given small molecule, which we called the absolute product score. PLM had the second highest absolute enrichment score of 0.85 (Figure 18B), which indicated that treatment of cultured cells with PLM created a transcriptional profile resembling the liver profile of leptin-treated STZ-mice, insulin-treated STZ-mice, and *GcgR* KO mice. Phenyl biguanide also had a high absolute enrichment score of 0.88, which suggests that our pipeline was identifying therapeutically relevant molecules.

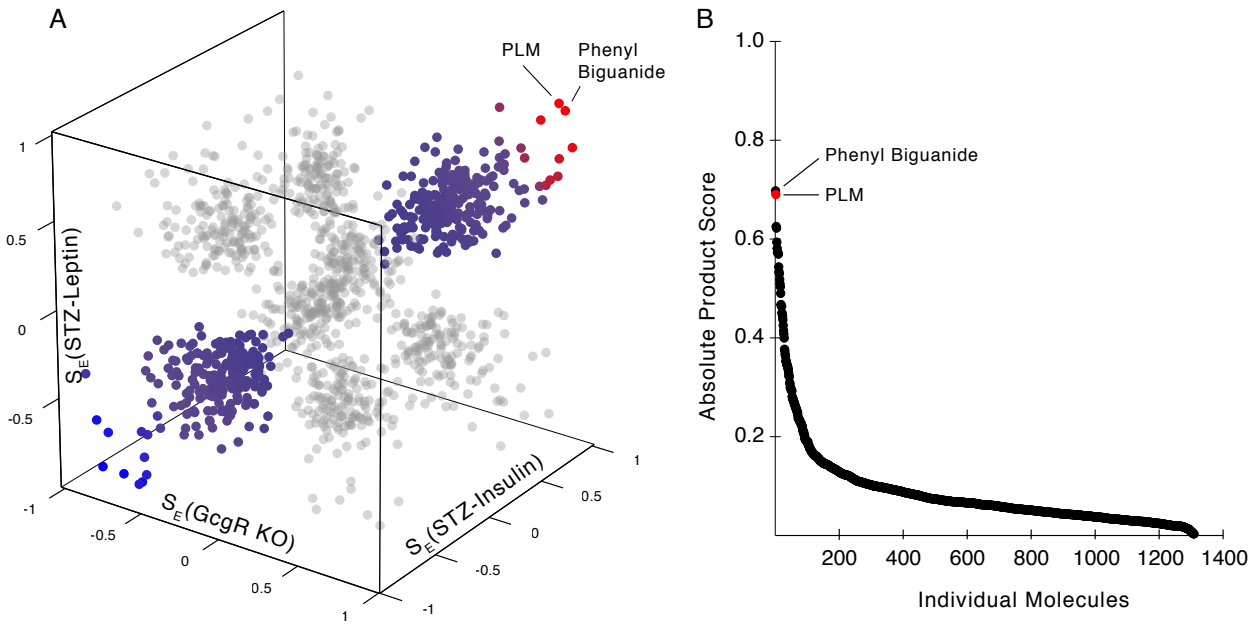

Figure 18. The hepatic transcriptional profile after leptin therapy, insulin therapy, or glucagon signalling suppression, resembles transcriptional profile of cultured cells after PLM treatment. Nine-week-old mice were rendered diabetic with STZ on day -6 then implanted with mini-osmotic pumps delivering vehicle (STZ-Vehicle), insulin (STZ-Insulin), or leptin (STZ-Leptin; same cohort of mice as Figure 12; N=5 for all groups). The liver was collected on day 4 post pump implantation for transcriptomics analysis. The transcriptional profile, which we defined as the top 100 significantly up-regulated and top 100 significantly down-regulated genes with the greatest fold change, was determined in STZ-Leptin and STZ-Insulin mice compared to STZ-Vehicle controls ($q<0.05$). For STZ-Insulin mice, top 61 significantly up-regulated (maximum number) and top 100 significantly down-regulated genes with the greatest fold change were used. Top 100 significantly up-regulated and top 100 significantly down-regulated genes with the greatest fold change in the liver of glucagon receptor knockout mice (*GcgR* KO) were determined (raw data from Gene Expression Omnibus; accession #GSE68143; [431]). The hepatic transcriptional profiles of STZ-Leptin, STZ-Insulin, and *GcgR* KO mice were compared to ~1400 transcriptional profiles created with treatment of human cultured cells with small molecules (data available from the CMAP [425-427]). Comparisons of transcriptional profiles provide enrichment scores (S_E(STZ-Leptin), S_E(STZ-Insulin), S_E(*GcgR* KO)), where high S_E suggests a high degree of similarity between the liver and cell culture transcriptional profiles. Enrichment scores for STZ-Leptin, STZ-Insulin, and *GcgR* KO mice were plotted in (A). The absolute product score (B) was calculated by multiplying absolute values of S_E(STZ-Leptin), S_E(STZ-Insulin), and S_E(*GcgR* KO). PLM and phenyl biguanide, which had the highest absolute product scores, are labeled.

PLM Modestly Lowers Blood Glucose Levels in Mice With STZ-Induced Diabetes

We next assessed whether PLM can mimic the glucose lowering actions of leptin in insulin deficient mice. For this, we injected 9-week-old C57Bl6/J mice with STZ to deplete insulin and induce hyperglycemia (Figure 19A). We then administered PLM or Vehicle to STZ mice via daily i.p. injections (STZ-PLM and STZ-Vehicle, respectively). Initially, we administered PLM to STZ-mice at a dose of 150 μg/day, which did not lower blood glucose levels compared to vehicle controls (23.4 ± 3.0 mM STZ-PLM vs. 22.6 ± 2.7 mM STZ-Vehicle on day 9 of injections). However, we were injecting the maximum volume that could be injected safely in mice and found that we were reaching the solubility limit of PLM, as evidenced by the appearance of small particles in the PLM solution (data not presented). Thus, we lowered the dose starting on day 10 to 100 μg/day, which allowed us to prepare PLM at a lower concentration. At this dose, PLM modestly lowered blood glucose levels in STZ-mice; on day 15, blood glucose levels were significantly lower in STZ-PLM mice compared to STZ-Vehicle controls (15.7 ± 1.8 mM STZ-PLM vs. 22.9 ± 1.8 mM STZ-Vehicle on day 15; Figure 19B), and these glucose lowering effects were maintained on day 26 of PLM injections, although the differences on day 26 did not reach statistical significance (16.1 ± 3.9 mM STZ-PLM vs. 25.8 ± 2.1 mM STZ-Vehicle on day 26; $p = 0.0763$; Figure 19B). Thus, PLM has modest glucose lowering effects in STZ-mice, and these effects are maintained over weeks of PLM administration.

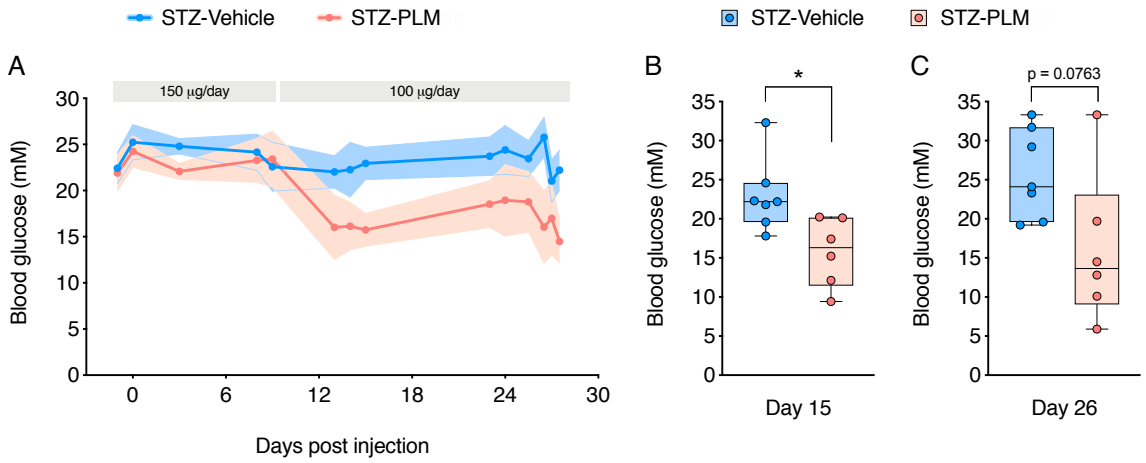

Figure 19. Predicted leptin mimetic (PLM) modestly lowers blood glucose levels in STZ-induced diabetic mice. Nine-week-old mice were rendered diabetic with STZ then given daily i.p. injections of vehicle (STZ-Vehicle) or PLM (STZ-PLM). Blood glucose levels were tracked throughout the study (A-C), where the dose of PLM delivered is indicated in grey bars. A is a line graph where the solid lines are the averages with shading as the ± SEM, and statistical analysis was performed by two-way ANOVA. B and C are min to max whisker plots and statistical analysis was performed by one-way ANOVA (*, P<0.05). N=6-7 per group.

We tested the reproducibility of PLM mediated glucose lowering by repeating our studies in a new cohort of STZ-mice. To determine the therapeutic range of PLM, we started PLM administration at a lower dose then gradually increased the dose. PLM at a dose of 1 µg/day for 7 days followed by 10 µg/day for 7 days did not lower blood glucose levels in STZ-mice (Figure 20A). We then increased the dose to 100 µg/day and found that STZ-PLM mice had significantly lower blood glucose levels compared to STZ-Vehicle controls on day 12 post initiation of high dose PLM (26 days after starting injections; 16.1 ± 1.8 mM STZ-PLM vs. 23.0 ± 2.6 mM STZ-Vehicle), but blood glucose levels were not further lowered on day 15 (29 days after starting injections; 17.7 ± 2.3 mM STZ-PLM vs. 23.8 ± 0.7 mM STZ-Vehicle). At this time, we began solubilizing PLM by incubating overnight with gentle shaking at room temperature, and we found that PLM stayed in solution at a higher concentration (data not shown). Thus, we were

able to increase the dose to 150 µg/day, which further lowered blood glucose levels in STZ-PLM mice compared to STZ-Vehicle controls after 5 and 7 days of treatment (34 and 36 days after starting injections, respectively; Figure 20A&B). Combined, these studies suggest that PLM confers modest but reproducible glucose lowering effects in STZ-mice.

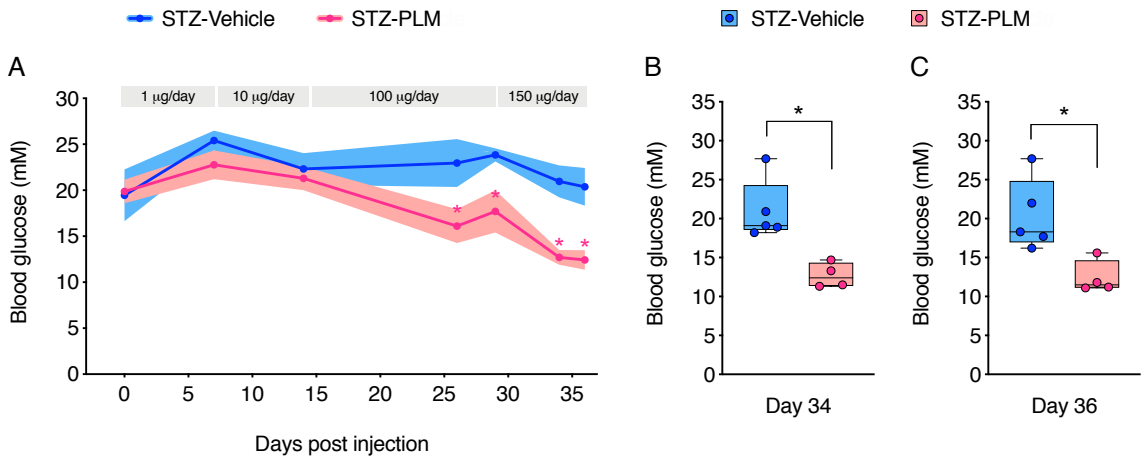

Figure 20. Glucose lowering effects of predicted leptin mimetic (PLM) treatment in STZ-mice is reproducible. A cohort of STZ-mice (distinct from mice in Figure 19) received daily i.p. injections of vehicle (STZ-Vehicle) or PLM (STZ-PLM). The dose of PLM delivered was gradually changed throughout the study (noted in grey bars in A), and blood glucose levels were tracked (A-C). A is a line graph where the solid lines are the averages with shading as the ± SEM, and statistical analysis was performed by two-way ANOVA. B and C are min to max whisker plots and statistical analysis was performed by one-way ANOVA (*, P<0.05). N=6-7 per group.

4.3 Discussion

In this chapter, we assessed whether recapitulating the effects of leptin on the hepatic transcriptome may confer glucose lowering properties in rodent models of diabetes. To do this, we took the top 100 up-regulated and top 100 down-regulated genes with the greatest fold change in the liver of STZ-Leptin mice compared to STZ-Vehicle controls, and compared it to expression profiles created with small molecule treatment of cultured cells. Out of ~1400

different small molecules, PLM had the highest enrichment score, which indicates that treatment of cultured cells with PLM produced a transcriptional profile similar to the liver expression profile of STZ-Leptin mice. Furthermore, PLM also had high enrichment scores for STZ-Insulin mice and *GcgR* KO mice, suggesting that PLM may produce a transcriptional profile which mimics the hepatic expression signature of STZ-Insulin mice and *GcgR* KO mice. Remarkably, administration of PLM modestly but reproducibly lowered blood glucose levels in STZ-mice. These data suggest that recapitulating the effects of leptin on hepatic gene expression may have glucose lowering effects in insulin deficient mice.

Although leptin reverses hyperglycemia in insulin deficient rodents, there are some aspects of leptin that may potentially limit its use as a therapy for diabetes. First, replacing diminished leptin levels in STZ-mice through low dose leptin administration does not reverse hyperglycemia [267, 327]. Instead, supraphysiological levels of leptin are required to reverse hyperglycemia in insulin deficient rodents [327]. Consistent with these rodent studies, clinical trials testing low dose leptin as an adjunct to insulin therapy reported that leptin does not lower blood glucose or HbA1c levels in patients living with type 1 diabetes [289]. These data suggest that high levels of leptin may also be required in patients to improve glycemia. Given well-known effects of leptin on reproduction, neuroendocrine function, and the immune system, chronic hyperleptinemia may elicit unwanted side effects related to these pleiotropic actions of leptin (reviewed in [128, 432, 433]). Thus, studies that assess these aspects of leptin action in rodent models of type 1 diabetes are warranted to evaluate the therapeutic applicability of leptin. Second, high leptin levels must be sustained in STZ-mice to maintain euglycemia [264] and leptin has a short half life of ~40 minutes [434], suggesting that patients will likely need numerous daily injections of recombinant leptin to control their blood glucose levels. Lastly,

leptin-treated STZ-mice cannot tolerate fasting and fall into life-threatening hypoglycemia [264]. While other studies do not report hypoglycemia in leptin-treated STZ-mice, it should be noted that these studies were conducted in 2 hour fasted mice [263], which likely does not recapitulate typical fasting durations in humans. Leptin therapy in STZ-rodents lowers many counter regulatory hormones, including glucagon, growth hormone, and corticosterone [260, 268, 355]. Thus, we suspect that most branches of the counter regulatory response are blunted in leptin-treated STZ-mice, which contributes to the severe fasting intolerance.

Recapitulating the effects of leptin on hepatic gene expression with small molecules may bypass some limitations of leptin therapy in type 1 diabetes. Small molecules may confer glucose lowering effects potentially without the need for high doses, and its potency may be more easily altered through chemical modifications. Identifying small molecules that do not act through the leptin receptor may be a viable strategy to mimic the effects of leptin in certain tissues such as the liver but not in others, and thus avoid the pleiotropic effects of leptin. It should be noted that in our studies, we did not identify the target tissues of PLM; although PLM was identified using the hepatic transcriptional profile of STZ-Leptin mice, it is possible that PLM may have effects in other tissues such as the brain. If small molecules that exclusively mimic the effects of leptin in hepatic gene expression can be identified, then it is possible that some components of the counter regulatory response can be spared using these molecules. For example, it has been shown that leptin-treated mice have depleted glycerol levels but retain the ability to utilize glycerol for gluconeogenesis [264, 268]. In contrast, we showed in Chapter 3 of this thesis that leptin globally down-regulates expression of amino acid catabolism genes in the liver, which may reduce amino acid utilization for glucose production, and there is an accumulation of gluconeogenic amino acids in the liver. Thus, leptin appears to reduce glucose production

through mechanisms that are distinct between substrates, and if utilization of some substrates can be selectively inhibited while sparing others, then the severity of fasting hypoglycemia may be reduced. Furthermore, leptin may act via distinct signalling pathways to regulate glycerol and amino acid metabolism, and thus small molecules may be useful to selectively modulate these branches of the leptin signalling pathway. In agreement with the idea that selectively reducing substrate utilization can avoid fasting hypoglycemia, glycerol-3-phosphate dehydrogenase (*Gpd1*) knockout mice, which cannot utilize glycerol for gluconeogenesis have lower fed blood glucose levels but are not prone to fasting hypoglycemia [435]. Future studies should assess whether PLM causes fasting hypoglycemia in STZ-mice. While PLM displays some modest but promising glucose lowering effects in STZ-mice, further studies are required to determine its target tissues and its mechanism of glucose lowering.

Beyond identification of PLM, using CMAP or similar databases will be a useful strategy to identify other small molecules with glucose lowering abilities. In our studies, we only tested the glucose lowering abilities of PLM; however, there are many other candidates with high similarity indexes that should be investigated. It is possible that despite having lower similarity indexes than PLM, these other small molecules may be more potent and perhaps easier to solubilize. Recently, the CMAP database was expanded and significantly improved [425]. While the previous CMAP only contained ~1400 transcriptional profiles that were created with treatment of cultured cells with small molecules [426], the improved CMAP now contains more than 1.3 million profiles. This expanded database was created with treatment of cultured cells with small molecules as well as siRNAs and plasmids to knockdown and overexpress genes, respectively [425]. Comparing the transcriptional profile of leptin treated STZ-mice using the expanded CMAP database may reveal other small molecules with glucose lowering abilities.

Furthermore, this new database may identify genes, like transcription factors or siRNAs, that may be responsible for creating the transcriptional profile of leptin treated STZ-mice. In our studies, we identified a small molecule that creates a similar transcriptional profile to leptin treated STZ-mice (positive similarity index), but CMAP also has the ability to identify opposite transcriptional profiles (negative similarity index). If we compare STZ-Vehicle mice to non-diabetic controls, we can obtain a liver transcriptional profile of diabetic mice. It would be interesting to use CMAP to identify small molecules which can create an opposite transcriptional profile to that of STZ-Vehicle mice then assess whether these small molecules may confer beneficial effects on glycemia. Thus, comparing transcriptional profiles using CMAP or a similar resource may reveal novel and promising small molecules for the treatment of diabetes.

Chapter 5: Dietary Fats, But Not Hyperleptinemia, Induce Resistance to the Blood Glucose Lowering Actions of Leptin in STZ-Diabetic Mice

5.1 Introduction

Leptin is well known for its role in regulation of food intake and energy expenditure. Leptin deficient rodents and humans have increased food intake and reduced energy expenditure, which leads to morbid obesity, and leptin replacement robustly reverses these metabolic aberrations [104, 105, 109, 121]. Leptin acts primarily on the POMC and AgRP neurons in the arcuate nucleus of the hypothalamus, although leptin signalling in other hypothalamic areas and non-hypothalamic regions are also implicated in leptin action (reviewed in [436, 437]). In addition to its effects on energy homeostasis, leptin also regulates glucose metabolism. Indeed, leptin deficient *ob/ob* mice and leptin receptor deficient *db/db* mice are diabetic with fasting hyperglycemia, glucose intolerance, hyperinsulinemia, and insulin resistance [99, 106, 239, 241, 242, 247, 438]. Thus, leptin is an important regulator of body weight and glucose homeostasis.

Some aspects of leptin action on glucose metabolism are independent from its action on body weight. Perhaps the most compelling evidence for this is the glucose lowering effect of leptin in insulin deficient rodents, which has been demonstrated by others [260, 261, 264-266, 268, 328] and confirmed in Chapter 3 of this thesis. Remarkably, leptin lowers blood glucose levels in STZ-mice without raising insulin levels [260, 264-266, 322, 325, 326] or significant changes in body weight [260, 264, 322], and independent from changes in food intake [265, 322, 326]. These glucose lowering effects of leptin in STZ-mice are highly reproducible, and have also been confirmed across different models of type 1 diabetes, including STZ-diabetic rats [261, 268], NOD mice [265, 266], BB rats with virally induced diabetes [325], and insulinopenic Akita

mice [326]. In addition to reversing hyperglycemia, leptin improves a plethora of metabolic defects associated with insulin deficiency; leptin monotherapy in insulin deficient rodents decreases hyperphagia and polydipsia [261-266, 322-324], stabilizes body weight [260, 264, 322], increases insulin sensitivity [260, 267, 322], reduces glucosuria and polyuria [261, 262, 264-266], and extends lifespan [262, 263, 266, 324, 328]. Furthermore, we showed in Chapter 3 that leptin dramatically alters levels of energy yielding substrates in STZ-mice. Administration of leptin to STZ-mice lowers levels of circulating lipids that are used as an energy source, especially glycerides and fatty acids, and dramatically alters levels of circulating amino acids and their derivatives. In the liver of these mice, there is an accumulation of amino acids, which may be due to a global down-regulation of genes involved in amino acid breakdown. Previous reports have shown that leptin therapy in STZ-rodents suppresses lipolysis [268], which lowers circulating glycerol levels [264, 268]. Overall, reduced utilization of glycerol and amino acids which are substrates for glucose production, and lowered levels of hepatic acetyl-coA which is a product of lipid oxidation and a substrate for ketone production, may drive the glucose and ketone lowering actions of leptin in insulin deficient rodents. Thus, leptin therapy profoundly alters glucose, lipid, and amino acid metabolism in rodent models of type 1 diabetes.

Leptin is a promising therapeutic option for the treatment of various forms of diabetes. The profound glucose lowering effects of leptin in rodents with diabetes propelled leptin into clinical trials as an adjunct to insulin therapy in patients living with type 1 diabetes [357]. In these patients, low dose leptin reduced total insulin requirements but did not lower HbA1c or fasting blood glucose levels compared to baseline before therapy initiation [357]. To lower blood glucose levels in humans, a higher dose of leptin may be required, since the glucose lowering effects of leptin in rodent studies require supraphysiological levels of leptin achieved with high

dose leptin therapy [267, 327]. In addition to type 1 diabetes, leptin has been used to treat lipodystrophy, which is a heterogeneous group of disorders characterized by partial or near-total lack of adipose tissue (reviewed in [439]). Lipodystrophy can cause symptoms of diabetes, including hyperinsulinemia, insulin resistance, hyperglycemia, glucose intolerance, and dyslipidemia (reviewed in [254]). Remarkably, leptin therapy improved these symptoms of diabetes in rodent models of lipodystrophy [257] and during clinical trials in patients living with lipodystrophy [255, 256]. Following positive outcomes in clinical trials, leptin was recently approved for the treatment of lipodystrophy by regulatory agencies around the world (reviewed in [440]). In rodent models of type 2 diabetes, leptin modestly improved metabolic parameters, including insulin resistance, hyperglycemia, and dyslipidemia [194, 210, 441, 442]. Results of clinical trials indicated that leptin therapy was ineffective at improving insulin resistance, and only marginally effective at lowering body weight and blood glucose levels in obese patients living with type 2 diabetes [281, 282]. Interestingly, weight-reducing effects of leptin are conserved in patients with type 2 diabetes that have lower baseline leptin levels [289], suggesting that the beneficial effects of leptin on glucose metabolism may also be intact in these patients. Thus, leptin therapy may be better suited as a glucose lowering agent in non-obese type 2 diabetes, which is more common in European and Asian populations (reviewed in [443]). Given the heterogeneous nature of lipodystrophy, as well as type 1 and type 2 diabetes, efforts to characterize the beneficial effects of leptin in the spectrum of diabetes are ongoing.

Prior to interest in leptin as a glucose lowering agent, there was excitement regarding its potential as a therapeutic agent to combat obesity, which later faded with the challenges of leptin resistance. Unlike obese humans and mice with congenital leptin deficiency, most obese humans [271-273] and diet-induced obese mice [135, 137] have elevated leptin levels. Thus, the concept

of leptin resistance emerged to explain the seemingly paradoxical elevation of leptin levels in common obesity. This concept of leptin resistance was further bolstered by the observation that leptin therapy in obese humans showed limited and variable efficacy in reducing body weight at low [279] and high doses [280], respectively, and showed minimal effects on body weight in mice with chronic high fat diet-induced obesity [274, 444]. Many factors have been reported to cause leptin resistance in obesity. Chronic hyperleptinemia has been suggested to cause leptin resistance. Indeed, preventing diet-induced hyperleptinemia by decreasing leptin expression in adipocytes [307] or increasing leptin clearance in the kidneys [307] increases leptin sensitivity. A potential mechanism by which hyperleptinemia may induce leptin resistance is through over activation of negative feedback regulators SOCS3 or PTP1B. Consistent with this idea, diet-induced obese mice have increased expression of SOCS3 and PTP1B in the hypothalamus [165, 278, 296], and transgenic mice with expression of a constitutively active form of STAT3 in POMC neurons are leptin resistant and obese [306]. Another factor that may contribute to leptin resistance is excess dietary fats. In support of this idea, direct infusion of dietary fats to the hypothalamus causes central leptin resistance [445]. Proposed mechanisms by which dietary fats cause leptin resistance in the hypothalamus include inflammation [308, 309], ER stress [315-317], and defects in autophagy [318]. In high fat diet-fed obese rodents, consumption of excessive dietary fats is closely linked to hyperleptinemia [136, 137, 271] and therefore their respective roles in the development of leptin resistance cannot be easily dissected. Currently, the individual contributions of dietary fats and hyperleptinemia to leptin resistance remain unclear.

Most studies that characterize the development of leptin resistance focus on measuring body weight and food intake as outputs of leptin bioactivity. Given that leptin also regulates glucose metabolism independent from its action on body weight, characterization of leptin

resistance in the context of glucose metabolism is needed. Furthermore, with interest in leptin as a potential therapeutic agent to lower blood glucose levels in patients with lipodystrophy as well as type 1 and type 2 diabetes, an understanding of leptin resistance is relevant to the long-term applicability of leptin as a glucose lowering agent. In this chapter, we examined whether hyperleptinemia or excessive dietary fats causes resistance to the blood glucose lowering actions of leptin in 4 independent studies. We utilized STZ-induced diabetic mice as a model because the blood glucose lowering by leptin is substantial (change of ~20 mM) [260, 264], easy to measure, and highly reproducible [260, 261, 264-266, 268]. Moreover, leptin does not significantly alter body weight in STZ-induced diabetic mice [260, 264], allowing us to assess blood glucose lowering actions of leptin independent from its action on body weight. STZ-induced diabetic mice also allow us to assess glucose lowering actions of leptin independent from effects of insulin [263, 328]. First, we introduced chronic hyperleptinemia in STZ-induced diabetic mice to assess the ability of leptin to lower blood glucose levels long-term. Second, we assessed whether pre-existing hyperleptinemia before the onset of STZ-induced diabetes may confer resistance to the glucose lowering effects of leptin therapy. Third, we assessed whether excessive high-fat intake before the onset of STZ-induced diabetes may prevent the glucose lowering actions of leptin. Lastly, we tested whether excessive dietary fats may reverse the blood glucose lowering actions of leptin in STZ-diabetic mice.

5.2 Results

Chronic Hyperleptinemia Does Not Lead to Resistance in the Glucose Lowering Actions of Leptin

First, we assessed whether chronic leptin delivery induces resistance to the blood glucose lowering actions of leptin. Male C57BL/6J mice were rendered insulin-deficient with STZ, then administered a vehicle or plasmid encoding the mouse leptin gene (p*Lep*) by hydrodynamic gene delivery to STZ-diabetic mice at 3 different doses (0.5, 5, and 50 μg plasmid/mouse). We have previously utilized this method for long-term overexpression of hormones [391]. Thus, we reasoned that hydrodynamic gene delivery would enables us to evaluate the effectiveness of leptin when circulating levels were elevated over many months, beyond the few days or weeks that are typically examined following leptin therapy.

As expected, STZ-diabetic mice receiving empty plasmids had severely depleted plasma leptin levels compared to non-diabetic controls (Figure 21A-C). Administration of low dose plasmid (0.5 μg/mouse) restored plasma leptin levels comparable to non-diabetic mice by day 2 post plasmid injection (3.3±0.4 ng/ml vs 3.7±0.4 ng/ml, respectively; Figure 21A). Delivery of medium (5 μg/mouse) and high doses (50 μg/mouse) of leptin plasmid to STZ-diabetic mice increased plasma leptin levels to the supraphysiological range by day 2 (51.5±11.6 ng/ml and 511.8±104.9 ng/ml, respectively; Figure 21B&C). Restoring physiological levels of leptin with a low dose of leptin plasmid did not significantly lower blood glucose compared to STZ-Vehicle controls (Figure 22A), while supraphysiological levels of leptin achieved with medium and high doses of leptin plasmid completely reversed hyperglycemia by day 2 (Figure 22B&C). These data confirm previous observations that supraphysiological levels of leptin are required for the glucose lowering actions of leptin [267, 327].

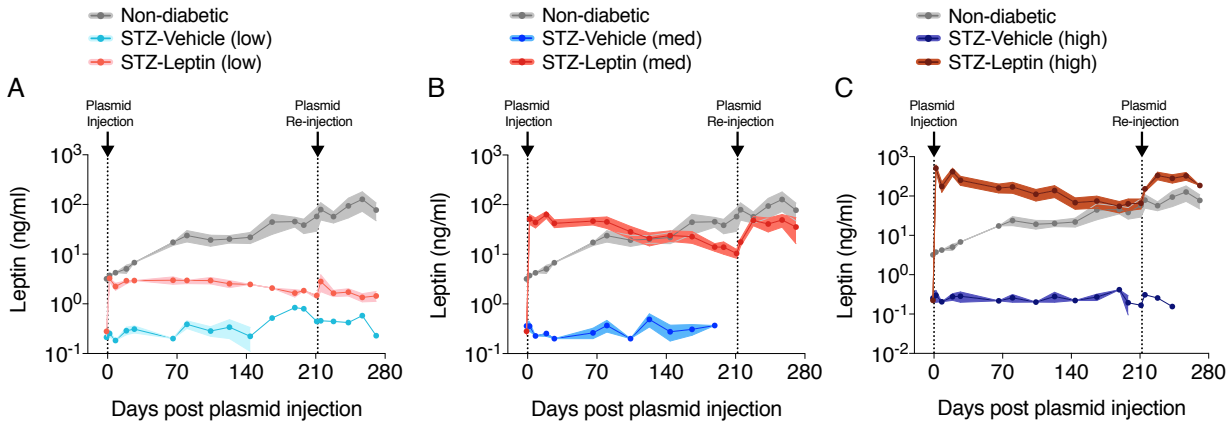

Figure 21. Administration of plasmid encoding leptin increases plasma leptin levels in mice with STZ-induced diabetes. STZ-mice were injected with empty plasmid (STZ-Vehicle) or plasmid encoding leptin (STZ-Leptin) on day 0 (indicated by dotted line) via hydrodynamic gene delivery at 3 different doses (0.5, 5 or 50 µg plasmid/mouse for low, medium, and high doses, respectively) and re-injected at the same dose on day 210 (indicated by dotted line). Plasma leptin levels were tracked throughout the study for low (A), medium (B), and high dose (C) plasmid groups. Statistical analysis was not performed since mice in the vehicle groups reached humane endpoints at various time-points and numbers of mice were progressively reduced throughout the study (see also survival graphs in Figure 26A-C). The solid lines are the averages and shading represents the ± SEM. On day 0, N=6-9 for each group.

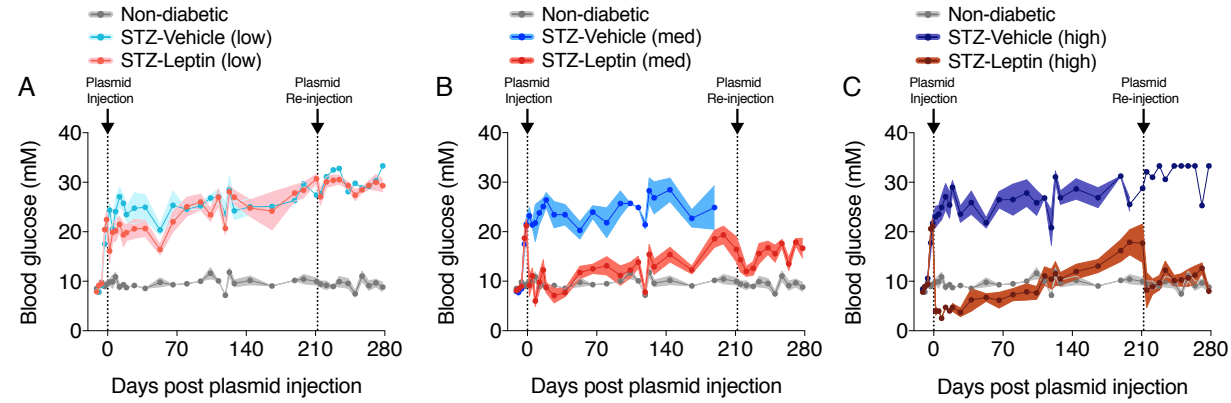

Figure 22. Chronic leptin administration does not lead to resistance in the glucose lowering actions of leptin. STZ-mice were injected with empty plasmid (STZ-Vehicle) or plasmid encoding leptin (STZ-Leptin) on day 0 (indicated by dotted line) via hydrodynamic gene delivery at 3 different doses (low, medium, and) and re-injected at the same dose on day 210 (indicated by dotted line). Four hour fasted blood glucose levels were tracked throughout the study for mice receiving low (A), medium (B), and high (C) doses of plasmid. Non-diabetic mice were included as controls. Statistical analysis was not performed since mice in the vehicle groups reached humane endpoints at various time-points and numbers of mice were progressively reduced throughout the study (see survival graphs in Figure 26A-C). The solid lines are the averages and shading represents the ± SEM. On day 0, N=6-9 for each group.

We assessed glucose metabolism over the next ~280 days to determine whether chronic hyperleptinemia would eventually cause resistance to the glucose lowering actions of leptin. Medium and high dose STZ-Leptin groups had significantly lower blood glucose levels compared to STZ-vehicle controls throughout the study (Figure 22B&C), despite maintenance of chronic hyperleptinemia (Figure 21B&C) and insulin deficiency (Figure 23A). Furthermore, STZ-mice given leptin plasmids at the medium and high doses had significantly lowered HbA1c levels on day 198 post plasmid injection compared to vehicle controls (Figure 23B). Medium dose leptin plasmid significantly improved glucose tolerance compared to STZ-Vehicle controls on day 112 post plasmid injection (Figure 24B), and high dose leptin plasmid completely normalized glucose tolerance (Figure 24C). Consistent with 4 hour fasted blood glucose tracking, low dose leptin therapy did not lower HbA1c levels (Figure 23B) or improve glucose tolerance compared to STZ-Vehicle controls (Figure 24A). Blood glucose levels began to climb into the mild hyperglycemic range in STZ-Leptin medium and high dose groups starting on day 112 and 189 respectively; however, upon reinjection of plasmid encoding leptin on day 210, blood glucose levels were again lowered in the STZ-Leptin medium dose group and normalized to the non-diabetic range in the STZ-Leptin high dose group for the remainder of the study (Figure 22B&C). Combined, these data suggest that chronic leptin administration in STZ-diabetic mice does not elicit resistance to the blood glucose lowering actions of leptin.

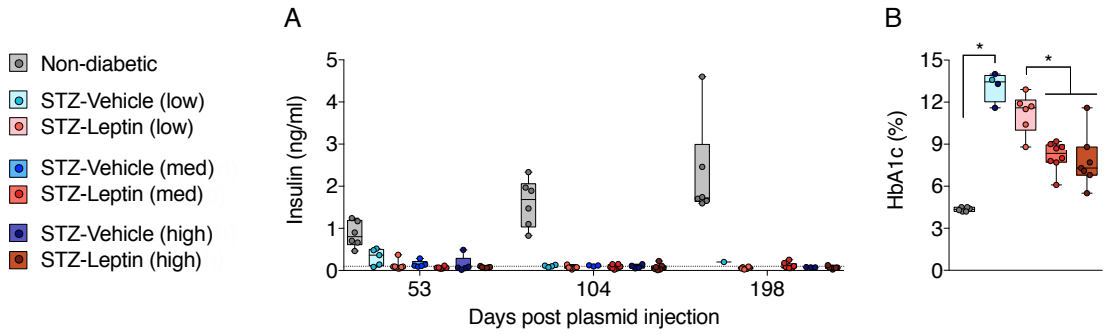

Figure 23. Chronic hyperleptinemia remains effective at improving glycemia, despite maintenance of insulin deficiency. STZ-mice were injected with empty plasmid (STZ-Vehicle) or plasmid encoding leptin (STZ-Leptin) at 3 different doses (0.5, 5 or 50 µg plasmid/mouse for low, medium, and high doses, respectively). Plasma insulin levels (A) were measured on days 53, 104, and 198-post plasmid injection. Statistical analysis was not performed for plasma insulin measurements since many of the mice injected with STZ had plasma insulin levels below the limit of detection (0.1 ng/ml indicated by the dotted line). HbA1c levels (B) were measured on day 198-post plasmid injection. Vehicle groups for the 3 doses were combined for HbA1c measurements since most mice within all 3 vehicle groups reached humane endpoint. Statistical analysis was performed by one-way ANOVA (*, P<0.05). Both graphs are min to max whisker plots.

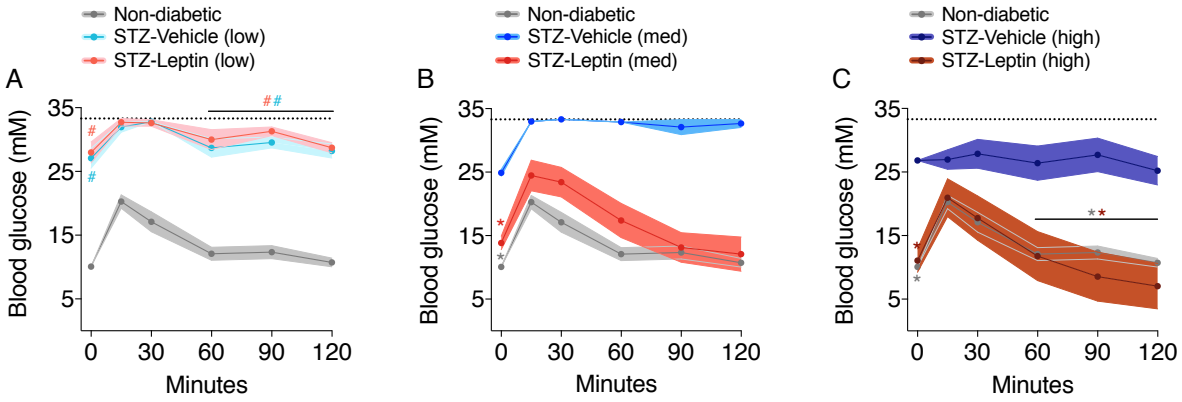

Figure 24. Leptin-mediated improvements in glucose tolerance remain intact despite chronic hyperleptinemia. STZ-mice injected with empty plasmid (STZ-Vehicle) or plasmid encoding leptin (STZ-Leptin) at 3 different doses (0.5, 5 or 50 µg plasmid/mouse for low, medium, and high doses, respectively) and an i.p. glucose tolerance test was performed on day 112 post plasmid injection for mice receiving low (A), medium (B), and high (C) doses of plasmid. Statistical analysis was performed by two-way ANOVA (*, P<0.05 for comparison against STZ-Vehicle; #, P<0.05 for comparison against non-diabetic mice, where colour indicates the group being compared). Statistical analysis was not performed 15 and 30 minute time points for low dose groups and at all timepoints except 0 minutes for medium dose groups since some STZ-Vehicle mice had blood glucose levels above the detection limit of 33.3 mM. For all line graphs, the solid lines are the averages and shading represents the ± SEM. N=3-7 for each group.

Next, we assessed whether chronic and supraphysiological doses of leptin improve parameters associated with long-term complications of diabetes. Body weight remained stable throughout the study with all three doses of leptin (Figure 25A-C). Interestingly, more mice survived with low dose leptin therapy (7 out of 9 mice by day 200) compared to vehicle controls (1 out of 6 mice by day 200), although these differences did not reach statistical significance as assessed by the log rank (Mantel-Cox) test ($p = 0.09$; Figure 26A). Lifespan was significantly prolonged in the medium dose leptin group compared to their STZ-Vehicle controls (Figure 26B), and modestly improved in the high dose compared to their vehicle controls (Figure 26C). Notably, while all STZ-Leptin mice receiving the medium dose of leptin survived past day 198, all mice in the vehicle control group reached humane endpoint by day 198 (Figure 26B). Others have reported that fat mass is not changed with short-term leptin therapy in insulin deficient mice [264], and we observed that long-term hyperleptinemia does not eventually reduce fat mass in STZ-mice (Figure 27A). Interestingly, there was a trend towards increased lean mass and body length for medium and high doses of leptin compared to STZ-Vehicle controls on day 209, although only the increase in lean mass at the medium dose leptin group reached statistical significance (Figure 27B&C). Bone mineral density was not altered at all doses of leptin tested compared to STZ-Vehicle and non-diabetic controls (Figure 27D). It has previously been reported that leptin therapy rapidly reverses diabetic hyperphagia, glycosuria, and increases activity 3 days after treatment initiation [264]. To test whether these metabolic improvements are maintained with chronic hyperleptinemia, we performed metabolic cage studies on day 205 post leptin treatment initiation. Due to the constraints in the number of metabolic cages available, we included non-diabetic mice, surviving STZ-Vehicle mice, and high dose STZ-Leptin mice. During the study, STZ-Vehicle mice had to be provided with wet food pellets outside of the food

hopper and water in the form of gels due to their long-term diabetes, and thus these mice were excluded from food intake and drink consumption measurements. We found that total activity of high dose leptin recipients was increased compare to STZ-Vehicle controls such that activity levels were now comparable to non-diabetic controls (Figure 27E). Furthermore, food intake and drink consumption in STZ-Leptin mice was comparable to non-diabetic controls (Figure 27F&G). Although we did not measure these metabolic parameters in low and medium dose groups, it has previously been reported that physiological doses of leptin do not normalize activity or food intake [267], suggesting that leptin may improve these metabolic parameters in a dose dependent manner. Combined, these data indicate that leptin improves parameters associated with long-term diabetes and resistance does not develop for these long-term beneficial effects of leptin despite chronic hyperleptinemia.

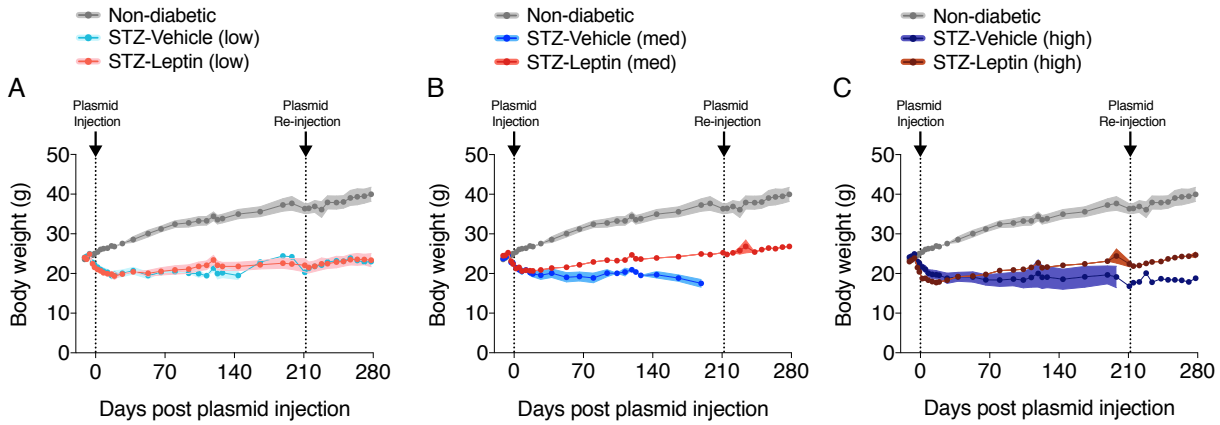

Figure 25. Chronic leptin therapy stabilizes body weight loss associated with insulin deficient diabetes. STZ- mice were injected with empty plasmid (STZ-Vehicle) or plasmid encoding leptin (STZ-Leptin) on day 0 (indicated by dotted line) via hydrodynamic gene delivery at 3 different doses (0.5, 5 or 50 μg plasmid/mouse for low, medium, and high doses, respectively) and re-injected at the same dose on day 210 (indicated by dotted line). Body weight was tracked for mice receiving low (A), medium (B), and high (C) doses of plasmids. Statistical analysis was not performed since STZ-Vehicle groups reached humane endpoints. Non-diabetic mice were included as controls. For all graphs, the solid lines are the averages and shading represents the ± SEM. On day 0, N=6-9 for each group.

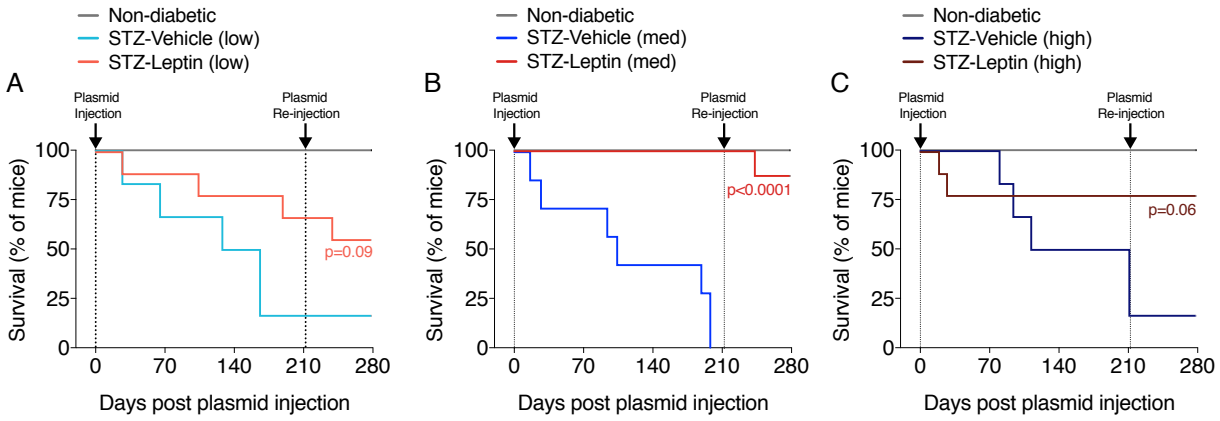

Figure 26. Chronic leptin therapy prolongs lifespan in STZ-diabetic mice. STZ-induced diabetic mice were injected with low (A), medium (B), and high (C) doses of leptin plasmid then survival was tracked throughout the study (A-C). For statistical analysis, a log rank (Mantel-Cox) test was performed to compare STZ-Vehicle and STZ-Leptin groups for each dose and the p-value is shown on each graph.

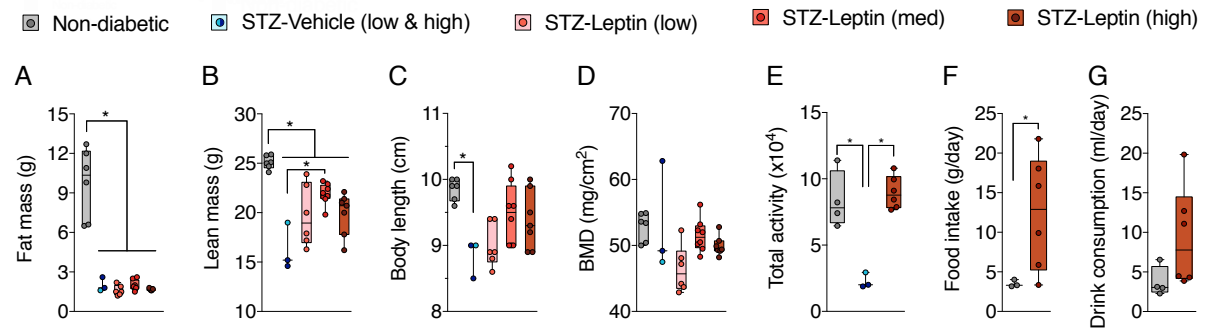

Figure 27. Leptin therapy improves long-term complications of diabetes in STZ-mice without eliciting resistance. STZ-induced diabetic mice were injected with low, medium, and high doses of leptin plasmid then fat mass (A), lean mass (B), body length (C), and bone mineral density (BMD; D) were measured on day 209 post plasmid injection. Between days 205 and 207 post plasmid injection, total activity (E), food intake (F), and drink consumption (G) were measured. STZ-Vehicle groups for 3 doses were combined for analysis since most mice within all 3 vehicle groups reached humane endpoint by these timepoints (see also survival graphs in Figure 26A-C). All graphs are min to max whisker plots and statistical analysis was performed by one-way ANOVA (*, P<0.05).

Short-term leptin therapy has been reported to improve hyperglucagonemia and hyperketonemia in STZ-mice [264, 268], and thus we assessed whether these parameters remain improved with chronic hyperleptinemia in STZ-mice. Administration of leptin at the low dose

did not lower circulating glucagon levels on days 144 and 189 post plasmid injection (Figure 28A). Leptin at the medium dose did not lower glucagon levels on day 144 compared to STZ-Vehicle controls but we observed a trend towards improvements in hyperglucagonemia on day 189 (Figure 28A). Notably, blood glucose levels were significantly lower on day 144 (Figure 22B), suggesting that improvements in hyperglucagonemia are not required for the glucose lowering actions of leptin in STZ-mice. At the high dose, glucagon levels were modestly lowered on day 144 ($p = 0.019$) and significantly lowered on day 189 compared to STZ-Vehicle controls (Figure 28A), suggesting that chronic hyperleptinemia does not necessarily lead to resistance in the glucagon lowering actions of leptin. With short-term leptin therapy, β-hydroxybutyrate levels are lowered in STZ-mice [264], which has been attributed to decreased circulating lipid levels and β-oxidation in the liver [268, 356]. β-hydroxybutyrate levels trended towards being lower on days 53 and 104 for all 3 doses of leptin therapy compared to STZ-vehicle controls (Figure 28B), although we could not perform statistical tests for these data since some STZ-vehicle control values were above the limit of detection. β-hydroxybutyrate levels were significantly lower for all 3 doses of leptin therapy on day 189 compared to STZ-vehicle controls (Figure 28B), suggesting that chronic hyperleptinemia does not cause resistance to the ketone lowering actions of leptin in STZ-mice. Respiratory exchange ratio (RER) was increased in STZ-vehicle controls on day 205 (Figure 29A-D), such that the 24 hour RER was 1.1±0.04, which is above 1.0 indicative of glucose oxidation. It has previously been reported that rises in RER above 1.0 can be due acidosis, since shifts in the bicarbonate buffer system produces excess CO_2. Thus, the increased RER in STZ-mice may be indicative of ketoacidosis associated with long-term diabetes [446]. Consistent with decreased ketone levels, RER was significantly lowered in STZ-mice given chronic high-dose leptin therapy (Figure 29A-D).

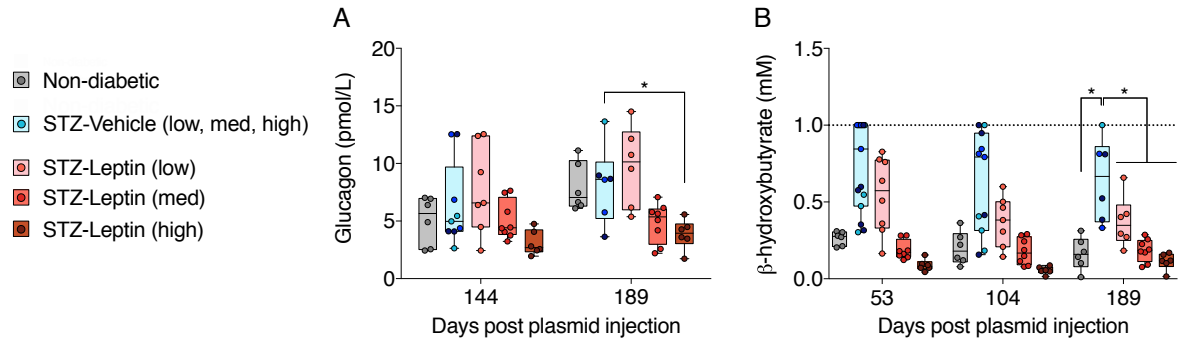

Figure 28. Leptin-mediated lowering of plasma glucagon and ketones levels remains intact despite chronic hyperleptinemia. STZ-induced diabetic mice were injected with low, medium, and high doses of empty (STZ-Vehicle) or leptin plasmid (STZ-Leptin) then plasma glucagon levels were measured on days 144 and 189 (A) and β-hydroxybutyrate levels were measured on days 53, 104, and 189 (B). STZ-Vehicle groups for 3 doses were combined for analysis since many of the mice within all 3 STZ-Vehicle groups reached humane endpoint by these timepoints (see also survival graphs in Figure 26A-C). All graphs are min to max whisker plots and statistical analysis was performed by two-way ANOVA (*, P<0.05).

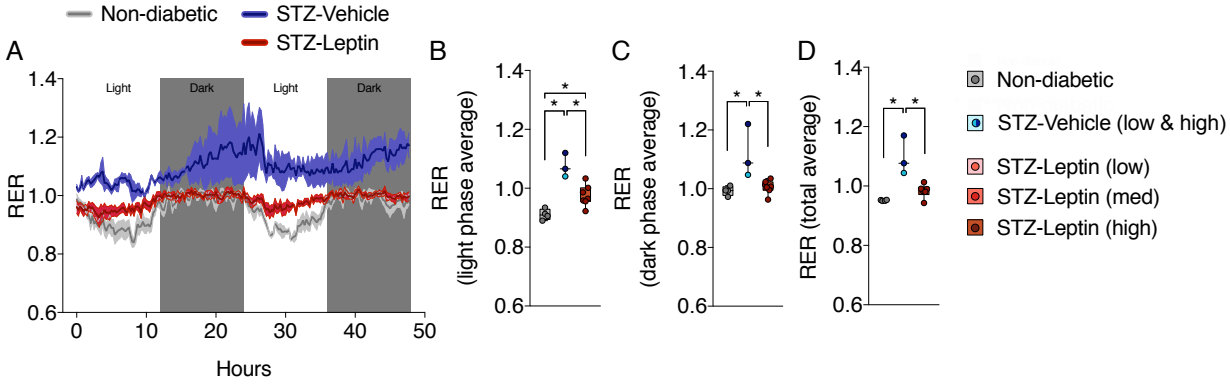

Figure 29. Chronic leptin administration to STZ-diabetic mice leads to long-term changes in the respiratory exchange ratio. STZ-induced diabetic mice were injected with low, medium, and high doses of leptin plasmid then the respiratory exchange ratios (RER) were measured between days 205 and 207 post plasmid injection. Non-diabetic mice were included as controls. RER for two mice from the high dose STZ-Vehicle group and 1 mouse from the low dose STZ-Vehicle group were measured. RER for all mice from the high dose STZ-Leptin group were measured. Four non-diabetic mice were selected randomly for RER measurements. For A, the solid lines are the averages and shading represents the ± SEM. B-D are min to max whisker plots and statistical analysis was performed by one-way ANOVA (*, P<0.05). N=3-7 for each group.

Pre-Existing Hyperleptinemia Does Not Block the Blood Glucose Lowering Actions of Leptin

We next sought to determine whether pre-existing hyperleptinemia before the onset of diabetes may prevent the blood glucose lowering actions of leptin. We developed a mouse model with adult-onset hyperleptinemia without obesity by injecting 9-week-old C57BL/6J male mice with a plasmid encoding leptin, which avoids the confounding effects of introducing hyperleptinemia during development such as with transgenic leptin overexpression [447]. Since a non-obese adult onset hyperleptinemic mouse model has not been described previously, we first characterized metabolic parameters in these mice before the onset of diabetes. Hydrodynamic delivery of plasmid encoding leptin at a high dose (50 μg/mouse) increased leptin levels to the supraphysiological range (155.3±14.7 ng/ml on day 5 post plasmid injection) compared to non-injected (1.7±0.3 ng/ml) and vehicle plasmid injected mice (1.9±0.3 ng/ml), and these high leptin levels were sustained for ~15 weeks (Figure 30A). Importantly, these levels are comparable to leptin levels observed after 12-20 weeks of HFD feeding in our studies (later presented in Figure 34A) and by others [305, 448]. Initially, leptin lowered body weight and blood glucose levels (Figure 30B&C). Eventually, these parameters returned to levels comparable to non-injected and vehicle plasmid controls within ~40 days for body weight and ~15 days for blood glucose levels (Figure 30B&C). The transient lowering of body weight with hyperleptinemia was due to a reduction in both fat and lean mass (Figure 31A&B) but not bone mineral density or body length (Figure 31C&D). The reduction in lean mass appear to contradict an early and broadly held view that leptin decreases fat mass while sparing lean mass in both wildtype and *ob*/*ob* mice [105]. Combined, our data suggest that body weight and blood glucose levels are initially altered in mice with adult onset hyperleptinemia without high fat diet-feeding but these changes are

eventually normalized; therefore, plasmid induced hyperleptinemia is a model to establish hyperleptinemia in adulthood without obesity.

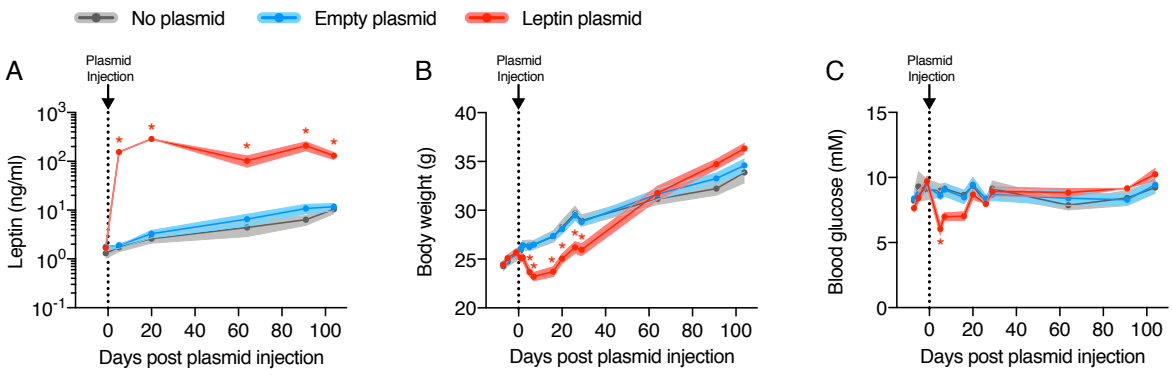

Figure 30. Adult onset hyperleptinemia transiently lowers body weight and blood glucose levels. Nine-week-old C57Bl6/J mice were injected with vehicle plasmid (empty plasmid) or plasmid encoding leptin (leptin plasmid) on day 0 via hydrodynamic gene delivery at a dose of 50 µg plasmid/mouse. Non-injected mice were included as controls (no plasmid). Plasma leptin (A), body weight (B), and blood glucose levels (C) were tracked for ~15 weeks. For all graphs, solid line is the average with shading as the ± SEM, and statistical analysis was performed by two-way ANOVA (*, P<0.05 Leptin plasmid vs Empty plasmid).

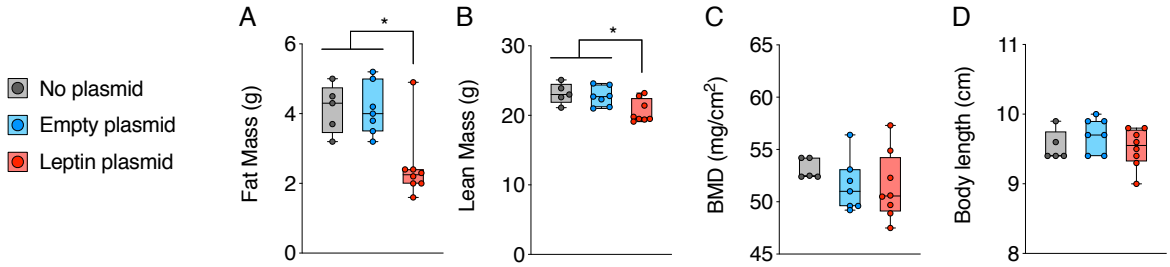

Figure 31. Adult onset hyperleptinemia in non-diabetic mice transiently alters body composition. Nine-week-old C57Bl6/J mice were injected with vehicle plasmid (empty plasmid) or plasmid encoding leptin (leptin plasmid) on day 0 via hydrodynamic gene delivery at a dose of 50 µg plasmid/mouse. Non-injected mice were included as controls (no plasmid). Fat mass (A), lean mass (B), bone mineral density (BMD; C), and body length (D) were measured on day 22 post plasmid injection when there was a transient decrease in body weight and blood glucose levels (see also Figure 30A-C). All graphs are min to max whisker plots and statistical analysis was performed by one-way ANOVA. For all groups, representative mice were selected for DEXA measurements. N=5-8 per group.

Leptin-treated STZ-mice cannot tolerate fasting and fall into life threatening hypoglycemia, which is thought to occur due to the depletion of substrates and positive regulators of gluconeogenesis [264, 268]. We used our unique adult-onset hyperleptinemic mice to assess whether these effects of hyperleptinemia are also observed in non-diabetic mice. Despite lower starting blood glucose levels, the hyperleptinemic mice were not prone to fasting-induced hypoglycemia on day 16 post plasmid injection (Figure 32A). The expected changes in plasma analyte levels with fasting were conserved in hyperleptinemic mice; fasting decreased plasma triglycerides and increased plasma non-esterified fatty acids (NEFA), glycerol, and ketone levels in hyperleptinemic mice to levels comparable to non-injected and vehicle plasmid injected controls (Figure 32B-E). Notably, levels of these metabolites before and after 9 hours of fasting were comparable between no plasmid, empty plasmid, and leptin plasmid injected mice (Figure 32B-E). Combined these data suggest that unlike leptin treatment in STZ-mice, hyperleptinemia does not cause fasting intolerance and does not lead to statistically significant changes in triglycerides, NEFA, glycerol, or ketones in non-diabetic mice.

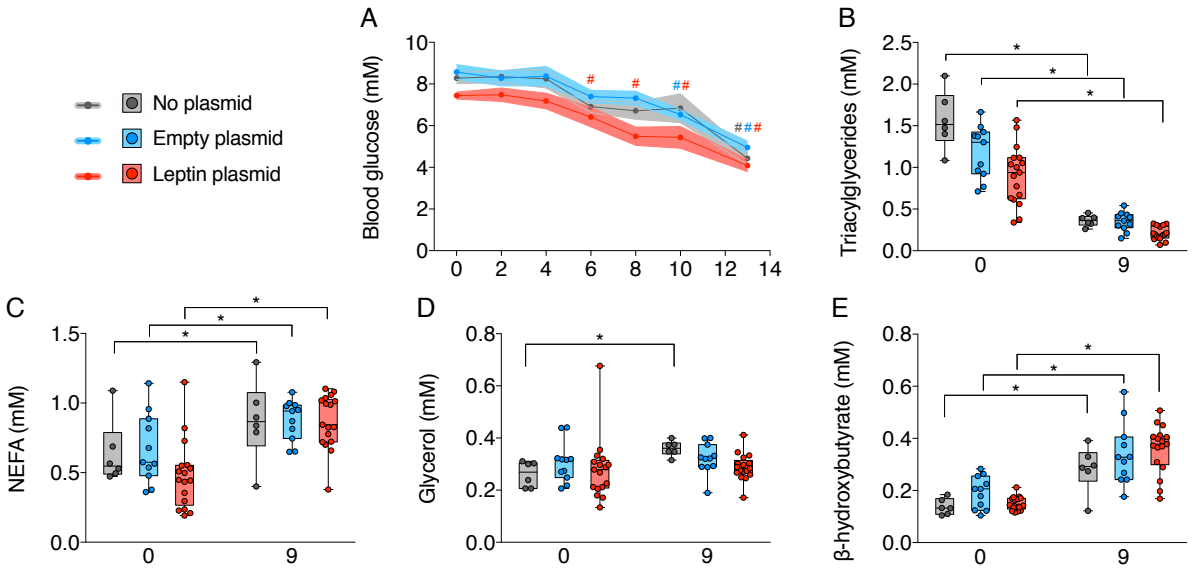

Figure 32. Fasting induced changes in blood glucose and plasma analyte levels are intact in mice with adult onset hyperleptinemia. Nine-week-old C57Bl6/J mice were injected with vehicle plasmid (empty plasmid) or plasmid encoding leptin (leptin plasmid) on day 0 via hydrodynamic gene delivery at a dose of 50 µg plasmid/mouse. Non-injected mice were included as controls (no plasmid). Fasting tolerance test (A) was performed 16 days post plasmid injection. Plasma triglycerides (B), non-esterified fatty acids (NEFA; C), glycerol (D), and β-hydroxybutyrate (E) levels were measured before and after a 9 hour fast on day 26 post plasmid injection. For A, solid line is the average with shading as the ± SEM, and statistical analysis was performed by two-way ANOVA (#, $P<0.05$ for each time-point post fasting vs 0 minutes where colour indicates group). B-E are min to max whisker plots and statistical analysis was performed by two-way ANOVA (*, $P<0.05$). N=6-17 per group for all graphs.

We next sought to determine whether pre-existing hyperleptinemia causes resistance to the glucose lowering actions of leptin in insulin deficient STZ-mice. After ~15 weeks of hyperleptinemia, when body weight was comparable between plasmid-induced hyperleptinemic mice and non-injected and vehicle plasmid controls (Figure 30B and Figure 33C), we administered STZ to all groups, which dramatically depleted insulin levels (Figure 33A). We predicted that if hyperleptinemia does not elicit resistance to the anti-diabetic actions of leptin, the high leptin levels would sustain euglycemia despite insulin depletion. Upon STZ injection, leptin levels decreased in all groups; however, leptin levels remained within the

supraphysiological range in leptin plasmid mice after STZ (96.9±14.2 ng/ml on day 9 post STZ injection; Figure 33B). Body weight decreased in all mice with STZ injection but remained comparable between hyperleptinemic mice and controls following STZ (Figure 33C). Initially, the leptin plasmid group had blood glucose levels comparable to non-injected and vehicle plasmid groups on days 6 and 9 post STZ injection (Figure 33D). Eventually, after 39 days post STZ injection, the leptin plasmid group had decreased blood glucose levels (15.0±0.8 mM), while vehicle and non-injected groups remained diabetic (25.6±1.0 mM and 25.5±1.9 mM, respectively; Figure 33D). These data suggest that that pre-existing hyperleptinemia may partially slow the rate at which leptin lowers blood glucose levels. Others have previously reported that supraphysiological leptin levels are required to improve glycemia in STZ-mice [267, 327]. We postulated that pre-existing hyperleptinemia may have induced a higher physiological threshold for leptin to lower blood glucose levels and therefore re-injected mice with leptin plasmids on day 40 post STZ injection. Control groups that were previously not injected or received empty plasmids also received leptin plasmids during the second round of plasmid injections. Re-injection of leptin plasmids increased leptin levels ~3 fold in mice with pre-existing hyperleptinemia (280.0±37.1 ng/ml; Figure 33B), which completely normalized blood glucose levels to the euglycemic range comparable to controls by day 53 post STZ injection (Figure 33D). Therefore, although mice with pre-existing hyperleptinemia initially remained mildly hyperglycemic after depletion of insulin, a boost in leptin levels robustly lowered blood glucose levels comparable to non-diabetic mice. These data suggest that pre-existing hyperleptinemia may raise the minimum therapeutic threshold for blood glucose lowering actions of leptin.

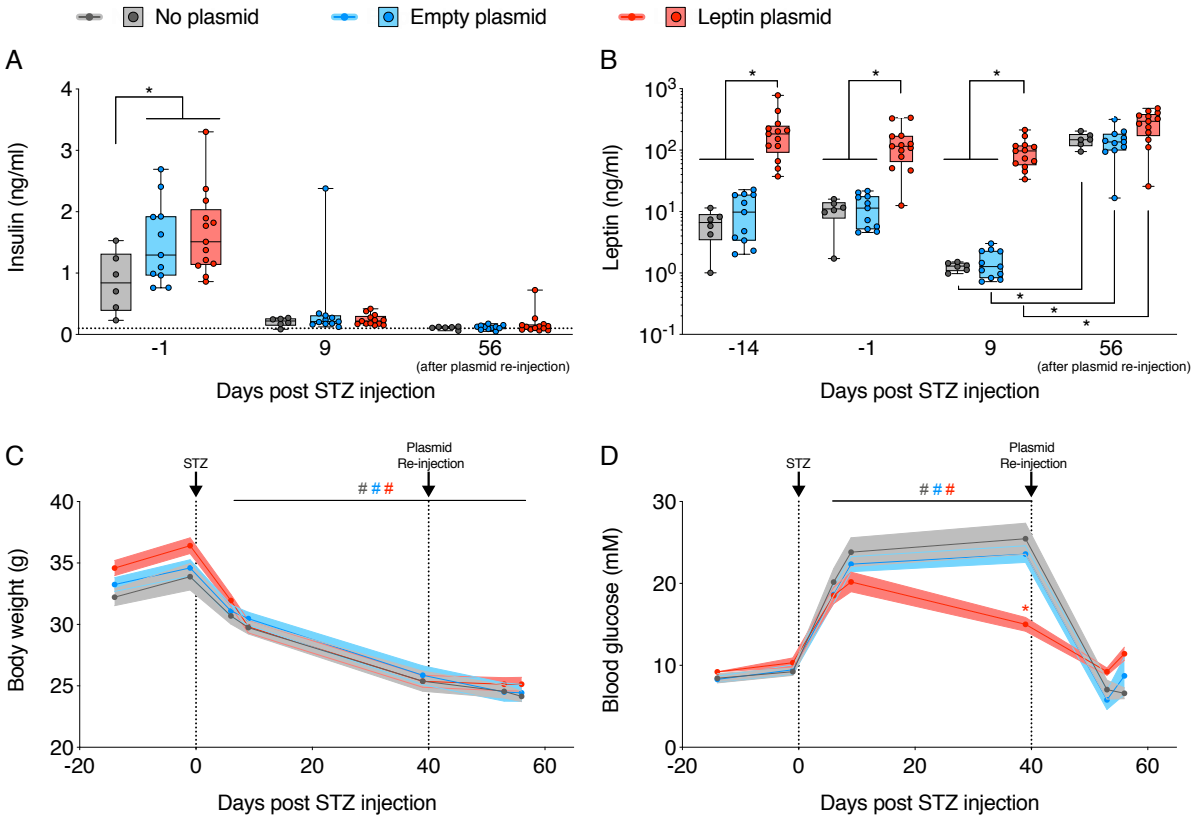

Figure 33. Leptin-mediated glucose lowering is delayed in mice with pre-existing hyperleptinemia but leptin remains efficacious. Nine-week-old mice were injected with an empty plasmid (empty plasmid) or plasmid encoding leptin (leptin plasmid), and non-injected mice were included as controls (no plasmid). After ~15 weeks of hyperleptinemia in leptin plasmid injected mice, all mice were injected with a single high dose of STZ. On day 40 post STZ injection, all groups received an injection of the leptin plasmid too boost their circulating leptin levels. Plasma insulin levels (A), blood glucose levels (B), plasma leptin levels (C), and body weight (D) were measured before and after STZ injection. A and B are min to max whisker plots and statistical analysis was performed by two-way ANOVA (*, P<0.05). C and D are line graphs where solid line is the average with shading as the ± SEM, and statistical analysis was performed by two-way ANOVA (#, P<0.05 for each time-point vs day -1 where colour indicates the group being compared; *, P<0.05 Leptin plasmid vs Empty plasmid). N=6-17 per group.

Chronic HFD Feeding Prevents the Blood Glucose Lowering Actions of Leptin in STZ-Mice

It has been suggested that high-fat intake induces resistance to the body weight reducing actions of leptin. We sought to determine whether high-fat intake might also cause resistance to

the blood glucose lowering actions of leptin in STZ-diabetic mice. We fed mice a HFD for ~12 weeks, which led to hyperleptinemia (114.5±7.1 ng/ml) and obesity (49.9±0.9 g). Next, we injected these mice with STZ to induce insulin-deficient diabetes, which also decreased plasma leptin levels (32.4±3.4 ng/ml; Figure 34A). In these HFD fed STZ-diabetic mice, we administered an empty plasmid as a vehicle control (HFD-STZ-Vehicle) or plasmid encoding leptin (HFD-STZ-Leptin) via hydrodynamic gene delivery. Upon plasmid injection, HFD-STZ-Leptin mice had increased plasma leptin levels compared to vehicle plasmid controls (204.5±38.1 ng/ml HFD-STZ-Leptin vs. 17.1±8.6 ng/ml HFD-STZ-Vehicle on day 19 post plasmid injection; Figure 34A). As expected, HFD-STZ mice were resistant to the body weight reducing actions of leptin therapy (Figure 34B). Despite reaching levels within the therapeutic range, leptin administration did not lower blood glucose levels in HFD-STZ-Leptin mice compared to vehicle controls (25.6±0.9 HFD-STZ-Leptin and 21.8±1.5 mM HFD-STZ-Vehicle on day 19 post plasmid injection; Figure 35A). In addition, leptin did not improve insulin sensitivity or glucose tolerance in HFD-STZ-Leptin mice compared to controls (Figure 35B&C). Thus, exposure to excess dietary fats prior to the development of diabetes causes resistance to the glucose lowering actions of leptin.

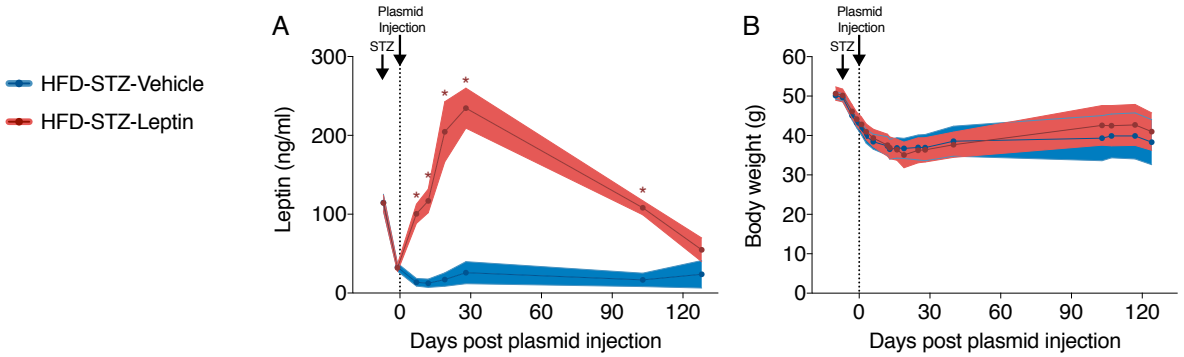

Figure 34. Delivery of leptin plasmids to HFD-fed mice rendered insulin deficient increases plasma leptin levels. Nine-week-old C57Bl6/J mice were fed a HFD for ~12 weeks, rendered diabetic with STZ on day -7 then injected with an empty plasmid (HFD-STZ-Vehicle) or plasmid encoding leptin (HFD-STZ-Leptin) on day 0 via hydrodynamic gene delivery (indicated by dotted line) at a dose of 50 µg plasmid/mouse. Plasma leptin levels (A) and body weight (B) were tracked throughout the study. For both graphs, the solid lines are the averages with the shading as the ± SEM, and statistical analysis was performed by two-way ANOVA (*, P<0.05 HFD-STZ-Vehicle vs HFD-STZ-Leptin). N=6-8 for each group.

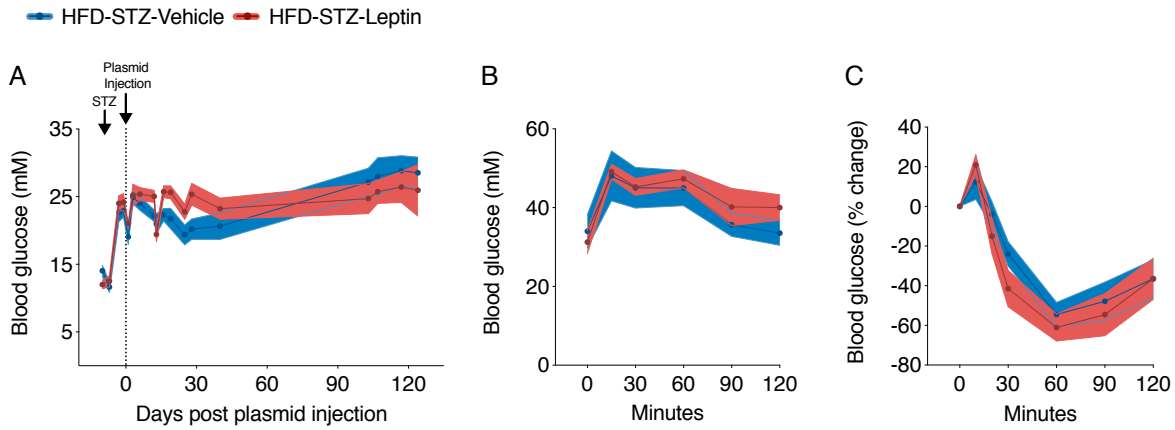

Figure 35. HFD-fed mice rendered insulin deficient are resistant to the glucose lowering actions of leptin. Nine-week-old C57Bl6/J mice were fed a HFD for ~12 weeks, rendered diabetic with STZ on day -7 then injected with an empty plasmid (HFD-STZ-Vehicle) or plasmid encoding leptin (HFD-STZ-Leptin) on day 0 via hydrodynamic gene delivery (indicated by dotted line in A). Blood glucose levels (A) were tracked throughout the study. An i.p. glucose tolerance test (B) and an i.p. insulin tolerance test (C) were performed on days 44 and 107, respectively. For all line graphs, the solid lines are the averages, the shading is the ± SEM, and statistical analysis was performed by two-way ANOVA, which did not reveal significant differences. N=6-8 for each group.

It has been reported that leptin therapy lowers levels of circulating metabolites, including plasma triglycerides, free fatty acids, and glycerol [264, 268]. We examined whether chronic exposure to dietary fats prior to the development of diabetes impairs the ability of leptin to lower these metabolites. After short-term (16 days) and long-term leptin therapy (103 days), plasma triglycerides, NEFA, and plasma glycerol levels were comparable between HFD-STZ-Leptin mice and HFD-STZ-Vehicle controls (Figure 36A-C). These data suggest that excessive exposure to dietary fats prior to the development of diabetes impairs leptin's ability to improve lipid metabolism.

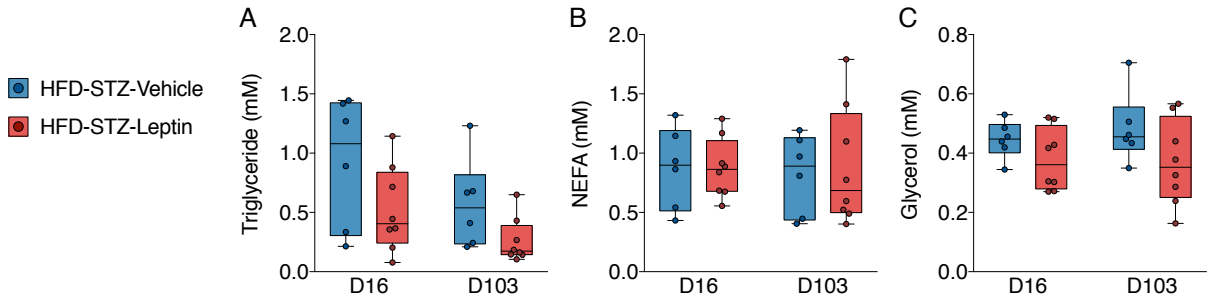

Figure 36. Leptin administration to HFD-fed mice rendered insulin deficient does not lower plasma lipid levels. Nine-week-old C57Bl6/J mice were fed a HFD for ~12 weeks, rendered diabetic with STZ on day -7 then injected with an empty plasmid (HFD-STZ-Vehicle) or plasmid encoding leptin (HFD-STZ-Leptin) on day 0 via hydrodynamic gene delivery at a dose of 50 µg plasmid/mouse. Plasma triglycerides (A), non-esterified fatty acids (NEFA; B), and glycerol levels (C) were measured on days 16 and 103 post plasmid injection. Graphs are min to max whisker plots and statistical analysis was performed by one-way ANOVA, which did not reveal statistically significant differences. N=6-8 for each group.

High-Fat Intake Rapidly Reverses the Blood Glucose Lowering Actions of Leptin in STZ-Mice

HFD feeding before the onset of STZ-diabetes also caused pre-existing hyperleptinemia; therefore, we next sought to determine whether high fat intake alone may cause resistance to the blood glucose lowering actions of leptin, in the absence of pre-existing hyperleptinemia. C57BL/6J mice were rendered insulin deficient with STZ, then injected with plasmid encoding

leptin by hydrodynamic delivery (STZ-Leptin) or vehicle plasmid as a control (STZ-Vehicle). As expected, STZ lowered plasma leptin levels (Figure 37A), caused weight loss (Figure 37B), and induced hyperglycemia (Figure 37C) in all groups. Injection of plasmid encoding leptin increased plasma leptin levels (Figure 37A) and reversed STZ-induced diabetes by day 4 post plasmid injection (Figure 37C). On day 6 post plasmid injection, we introduced a HFD to both vehicle and leptin-treated STZ-mice (STZ-Vehicle-HFD and STZ-Leptin-HFD, respectively). We also included vehicle and leptin-treated STZ-mice fed a LFD as controls (STZ-Vehicle-LFD and STZ-Leptin-LFD, respectively). Plasma leptin levels were not significantly altered with HFD feeding in STZ-leptin mice (Figure 37A). Yet, HFD fed mice rapidly developed resistance to the blood glucose lowering actions of leptin; STZ-Leptin-HFD mice were hyperglycemic after only 4 days of HFD feeding while STZ-Leptin-LFD controls remained euglycemic (Figure 37C). Hyperglycemia in HFD fed STZ-leptin mice worsened over the duration of the study (Figure 37C), and these mice had increased fed blood glucose and HbA1c levels on day 94 post plasmid injection compared to LFD-fed controls (Figure 37D&E). These data suggest that excessive exposure to dietary fats rapidly reverses the glucose lowering actions of leptin. Notably, on day 12 post plasmid injection when hyperglycemia returned in STZ-Leptin-HFD mice, body weight was comparable between STZ-Leptin-HFD and STZ-Leptin-LFD controls (Figure 37B), suggesting that HFD rapidly reverses the glucose lowering actions of leptin, independent of changes in body weight. In addition, fat mass, lean mass, body length, and bone mineral density were comparable between HFD and LFD fed STZ-leptin groups on day 12 post plasmid injection (Figure 38A-D). Eventually, HFD fed STZ-Leptin mice had increased body weight compared to LFD-fed STZ-Leptin mice by day 41 post plasmid injection (Figure 37B). Combined, these data indicate that excessive dietary fats can blunt the glucose lowering actions of leptin in STZ-mice.

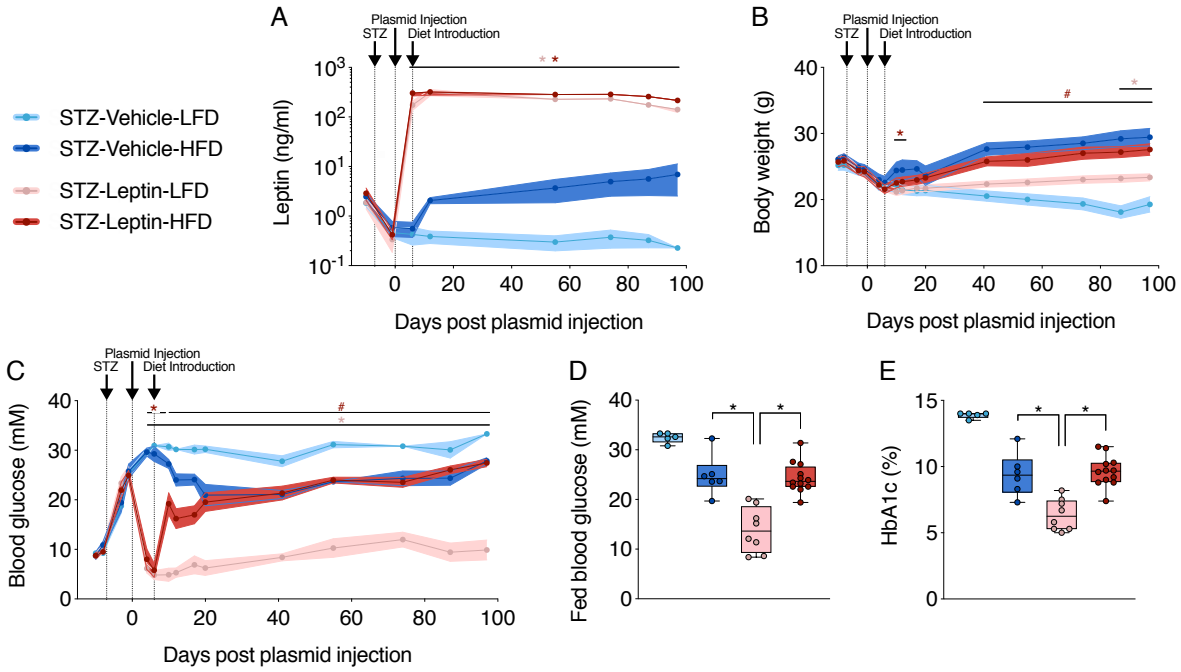

Figure 37. HFD potently reverses the glucose lowering actions of leptin in STZ-mice. Nine-week-old C57Bl6/J mice were rendered diabetic with STZ on day -8 and injected with empty plasmid (STZ-Vehicle) or plasmid encoding leptin (STZ-Leptin) on day 0 (indicated by the left dotted line in A-C). On day 6 post plasmid injection (indicated by second dotted line in A-C), mice were kept on a low fat diet (LFD) as a control (STZ-Vehicle-LFD and STZ-Leptin-LFD) or switched to a high fat diet (HFD; STZ-Vehicle-HFD and STZ-Leptin-HFD). Plasma leptin (A), body weight (B), and blood glucose levels (C) were tracked throughout the study. Fed blood glucose (D) and HbA1c levels (E) were measured on day 94 post plasmid injection. A-C are line graphs where the solid lines are the averages with shading representing the ± SEM, and statistical analysis was performed by two-way ANOVA (*, $P<0.05$ STZ-Vehicle vs STZ-Leptin where colour indicates LFD or HFD comparisons; #, $P<0.05$ STZ-Leptin-LFD vs STZ-Leptin-HFD). D and E are min to max whisker plots and statistical analysis was performed by one-way ANOVA (*, $P<0.05$). N=7 in each group.

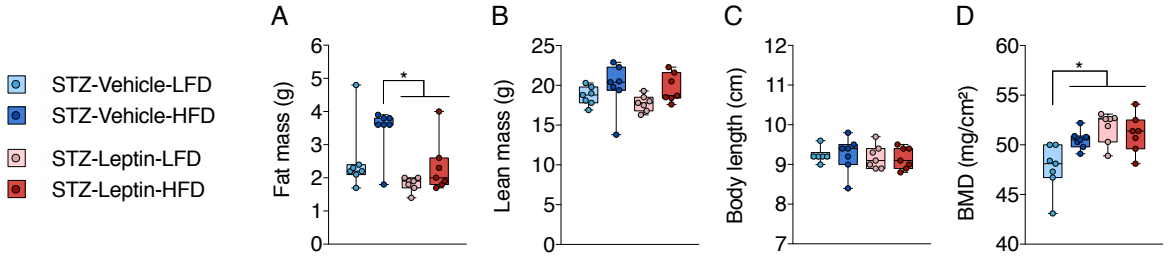

Figure 38. High fat diet does not significantly alter body weight composition in leptin-treated STZ-mice. Nine-week-old C57Bl6/J mice were rendered diabetic with STZ on day -8 then injected with empty plasmid (STZ-Vehicle) or plasmid encoding leptin (STZ-Leptin) on day 0. On day 6-post plasmid injection, mice were kept on a low fat diet (LFD) as a control (STZ-Vehicle-LFD and STZ-Leptin-LFD) or switched to a high fat diet (HFD; STZ-Vehicle-HFD and STZ-Leptin-HFD). Fat mass (A), lean mass (B), body length (C), and bone mineral density (BMD; D) were measured on day 12 post plasmid injection (6 days post diet switch) when HFD had reversed the glucose lowering actions of leptin in STZ-Leptin mice (see also Figure 37). All graphs are min to max whisker plots and statistical analysis was performed by one-way ANOVA (*, $P<0.05$). N=7 in each group.

It has been reported that increased circulating triglycerides with HFD feeding causes resistance to the body weight reducing actions of leptin by interfering with leptin transport across the blood brain barrier [293, 449], which ultimately reduces leptin signalling in the brain. Thus, we assessed whether HFD may reverse the blood glucose lowering actions of leptin by reducing leptin signalling in the hypothalamus, as assessed by phosphorylated STAT3 (pSTAT3) immunoreactivity. We found that the number of cells with pSTAT3 immunoreactivity in the VMH and ARC increased with leptin administration (Figure 39A-C). Notably, the number of cells with pSTAT3 immunoreactivity was comparable with between HFD fed and LFD-fed leptin groups (Figure 39A-C), suggesting that impaired leptin signalling cannot explain HFD-induced resistance to the glucose lowering actions of leptin. There were no statistical differences in the number of cells with pSTAT3 immunoreactivity in the dorsomedial hypothalamus (DMH) and LHA with leptin-treatment (Figure 39A&B). Thus, we conclude that leptin transport into the

brain and leptin signalling in the hypothalamus remains intact with HFD feeding, and HFD may prevent leptin action downstream of leptin signalling in the brain.

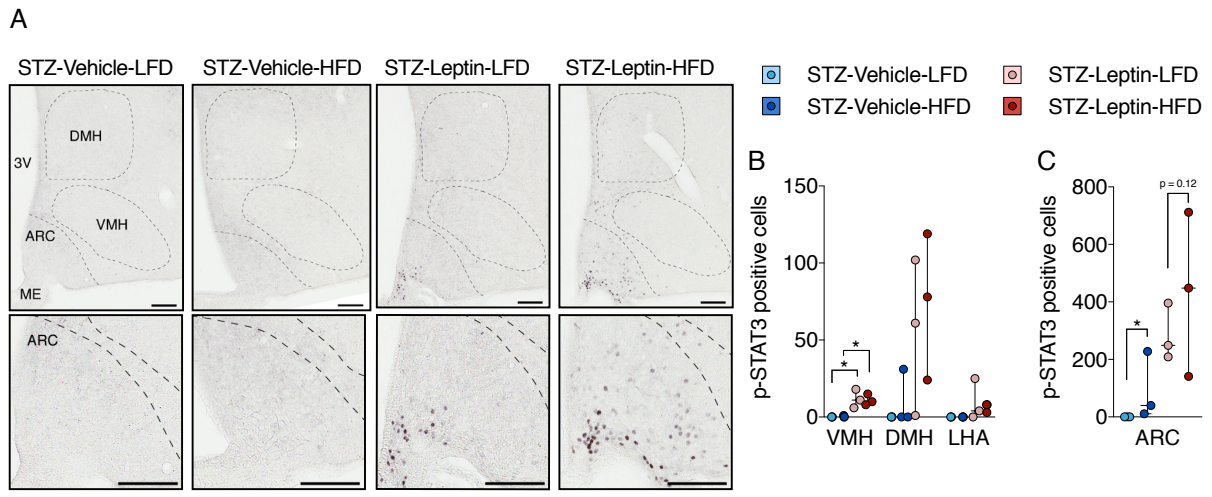

Figure 39. High fat diet reverses the glucose lowering actions of leptin but leptin signalling in the hypothalamus remains intact. Nine-week-old C57Bl6/J mice were rendered diabetic with STZ on day -8 and injected with empty plasmid (STZ-Vehicle) or plasmid encoding leptin (STZ-Leptin) on day 0. On day 6-post plasmid injection, mice were kept on a low fat diet (LFD) as a control (STZ-Vehicle-LFD and STZ-Leptin-LFD) or switched to a high fat diet (HFD; STZ-Vehicle-HFD and STZ-Leptin-HFD). On day 97-post plasmid injection, all mice were euthanized and the hypothalamus was stained for phosphorylated STAT3 (pSTAT3) immunoreactivity. Panel A shows 1 representative image from each group where upper images show the whole hypothalamus and lower images are a magnification showing the arcuate nucleus. DMH is dorsomedial hypothalamus, VMH is ventromedial hypothalamus, LHA is lateral hypothalamic area, and ARC is arcuate nucleus, 3V is 3rd ventricle, and ME is median eminence. Scale bar is 100 μm for all images. Representative mice from each group were used to quantify pSTAT3 positive cells in regions of the hypothalamus (B-C). B and C are min to max whisker plots and statistical analysis was performed by one-way ANOVA (*, $P<0.05$).

We next examined whether HFD reverses the ability of leptin to improve lipid metabolism in STZ-mice. It has been previously reported that leptin administration lowers circulating levels of triglycerides, and reduces its breakdown products glycerol and free fatty acids. Glycerol is a substrate for gluconeogenesis, and its depletion may contribute in part to the

glucose lowering effects of leptin [264, 268]. Furthermore, administration of leptin has been reported to decrease free fatty acid levels and suppress β-oxidation in insulin deficient rodents [260, 264, 268], which may lead to a reduction in ketone production. In order to examine whether leptin-mediated reduction in these metabolites is also impaired with HFD feeding, we measured these metabolites before leptin therapy (day -1), after leptin therapy but before HFD introduction (day 6), and after HFD had reversed the blood glucose lowering actions of leptin (day 12). We found that leptin administration significantly lowered plasma triglyceride levels on day 6 but these levels were not significantly different after diet introduction in HFD and LFD fed STZ-leptin groups on day 12 (Figure 40A). Glycerol and NEFA levels were also significantly lower in STZ-leptin groups on day 6 compared to STZ-vehicle controls (Figure 40B&C). Interestingly, after HFD feeding, glycerol and NEFA levels were increased in the STZ-leptin mice (Figure 40B). Notably, glycerol levels were comparable between STZ-leptin-HFD group and hyperglycemic STZ-vehicle-LFD controls on day 12 (Figure 40B) while the increase in NEFA levels did not reach STZ-vehicle-LFD control levels (Figure 40C). Consistent with the increase in NEFA levels in the HFD fed STZ-leptin group, plasma ketone levels were modestly increased on day 12 in the HFD fed STZ-leptin group compared to LFD-fed controls, although these differences did not reach statistical significance (Figure 40D). Collectively, these data suggest that leptin therapy in STZ-mice lowers triglycerides, NEFA, glycerol, and ketones; however, high fat diet feeding completely reverses the glycerol lowering effects and partially attenuates the free fatty acid and ketone lowering effects of leptin.

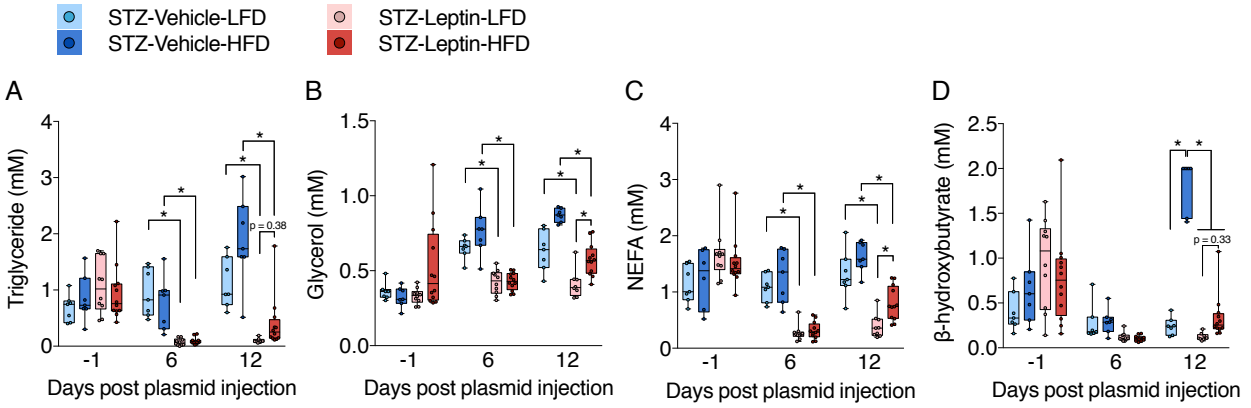

Figure 40. Leptin therapy lowers plasma analytes involved in gluconeogenesis, and these changes are reversed with high fat diet. Vehicle plasmid injected STZ-mice (STZ-Vehicle) and leptin plasmid injected STZ-mice (STZ-Leptin) were kept on a low fat diet (STZ-Vehicle-LFD and STZ-Leptin-LFD) or switched to a high fat diet (STZ-Vehicle-HFD and STZ-Leptin-HFD) on day 6-post plasmid injection. Plasma analytes were measured before leptin therapy (day -1), after leptin therapy and before diet switch (day 6), and after the diet switch (day 12). Plasma triglycerides (A), glycerol (B), non-esterified fatty acids (NEFA; C), and β-hydroxybutyrate levels (D) were measured at the indicated time-points. All graphs are min to max whisker plots and statistical analysis was performed by two-way ANOVA (*, $P<0.05$). N=7-11 per group.

5.3 Discussion

In this chapter, we examined whether hyperleptinemia or excessive dietary fats, which are reported to cause resistance to the weight reducing actions of leptin, also cause resistance to the blood glucose lowering effects of leptin. To test this, we generated a novel mouse model with chronic hyperleptinemia independent of high-fat intake using plasmids to stably overexpress leptin. Remarkably, chronic leptin administration did not elicit resistance to the blood glucose lowering actions of leptin in STZ-diabetic mice. Similarly, pre-existing hyperleptinemia did not prevent the beneficial effects of leptin on glycemia, although glucose lowering was delayed and the therapeutic threshold for leptin was modestly increased. In contrast, leptin was completely ineffective at improving glucose metabolism in mice that were given excessive dietary fats

before the onset of diabetes, and this diet also completely reversed the therapeutic action of leptin in STZ-diabetic mice that were previously fed a regular diet. These effects of high-fat intake appear to be independent of body weight, defects in leptin transport to the brain, or leptin signalling in the hypothalamus but may involve increased metabolites, which act as gluconeogenic substrates and regulators.

In our experiments, we tested the long-term efficacy of low dose leptin therapy in STZ-mice. Low dose leptin did not lower blood glucose levels, HbA1c levels, or improve glucose tolerance in STZ-mice but there were some beneficial effects worth noting. Despite unresolved hyperglycemia, more STZ-mice receiving low-dose leptin survived by day 200 post plasmid injection (7 out of 9 mice) compared to STZ-vehicle controls (1 out of 6 mice). Thus, leptin replacement may extend lifespan in STZ-mice. Diabetic ketoacidosis is one of the major complications of diabetes which can have long-lasting detrimental effects, and in some cases, be deadly (reviewed in [93]). Since we observed a significant reduction in ketone levels with low dose leptin therapy in STZ-mice, we suspect that reduced ketoacidosis may be responsible for prolonging lifespan in mice given low dose leptin therapy. In agreement with this idea, it has been previously reported that leptin replacement in a rat model of diabetic ketoacidosis reduces hepatic ketogenesis by 50% within 6 hours [356], and our studies suggest that these effects can persist long-term. During clinical trials which tested leptin as an adjunct to insulin therapy in patients living with type 1 diabetes, leptin was only administered at a low dose [289]. In fact, twice daily injections of metreleptin only increased median serum leptin levels by 1.7 and 2.2 fold after 12 and 20 weeks of treatment, respectively [289]. Consistent with rodent studies [267, 327], low dose leptin therapy did not lower blood glucose levels or HbA1c levels in patients [289]. Ketone levels were not measured and only patients with suboptimal control of glycemia

were recruited [289], who are likely not at risk for diabetic ketoacidosis. Our findings reveal that leptin replacement may be useful to prevent ketoacidosis in patients with brittle diabetes that are at risk for diabetic ketoacidosis. Furthermore, leptin replacement may be more preferred than insulin to prevent diabetic ketoacidosis in these patients since dangers of sudden hypoglycemia can be avoided with a low dose of leptin.

Our findings suggest that resistance to the blood glucose lowering actions of leptin in STZ-diabetic mice are unlike reported cases of resistance to the body weight reducing actions of leptin in HFD fed obese rodents. Previous reports have shown that preventing diet-induced hyperleptinemia by decreasing leptin expression in adipocytes [307] or increasing leptin clearance in the kidneys [307] increases leptin sensitivity. These studies illustrate that in obesity, hyperleptinemia causes resistance to the body weight reducing actions of leptin. In contrast, we showed that for insulin deficient diabetes, chronic hyperleptinemia before or after the onset of diabetes does not cause resistance to the blood glucose lowering actions of leptin. These differences may be a hint that the body weight and blood glucose lowering actions of leptin may be through distinct mechanisms. For example, leptin may target distinct neurons for these effects, and these neurons may have different levels of resilience to chronic hyperleptinemia. High-fat intake interferes with both glucose lowering actions of leptin in STZ-diabetic mice and body weight-reducing actions of leptin in obese mice. In obese mice, excess dietary fats interfere with leptin transport to the brain [293, 449-451] and leptin signalling in the hypothalamus [277]. Proposed mechanisms by which dietary fats impair leptin signalling in the hypothalamus include inflammation [308, 309], ER stress [315-317], and defects in autophagy [318]. In contrast, we showed that HFD fed STZ-mice are resistant to the glucose lowering effects of leptin but leptin transport and signalling in the hypothalamus remains intact. It is possible that HFD may disrupt

signalling of neuropeptides within the CNS that is downstream of STAT3 phosphorylation such as POMC or corticotropin releasing hormone (CRH), which are implicated in the glucose lowering actions of leptin in STZ-mice [263, 268]. Furthermore, we did not directly assess inflammation, ER stress, or autophagy in our mice; however, we suspect that the defects in these pathways would be upstream of STAT3 phosphorylation. Clearly, there are key differences in leptin resistance, which depends on the metabolic actions of leptin and the disease model. Leptin resistance in STZ-diabetic mice may be distinct from other metabolic states in which leptin improves glucose metabolism such as lipodystrophy [255-257]. There is a growing interest in leptin as a therapeutic agent to combat symptoms of diabetes (reviewed in [439]); therefore, the effects of chronic hyperleptinemia and high-fat intake on the glucose lowering abilities of leptin should be assessed across the spectrum of diabetes.

Our data reveal important aspects of pre-existing hyperleptinemia that may be relevant to the therapeutic application of leptin. Upon depletion of circulating insulin, mice with pre-existing hyperleptinemia became hyperglycemic despite maintenance of high leptin levels. Eventually, mice with pre-existing hyperleptinemia had reduced blood glucose levels, although they still remained mildly hyperglycemic. Leptin levels in these mice were comparable to the medium dose leptin group in our dose response studies, which had reduced blood glucose levels by day 2-post leptin administration. Thus, pre-existing hyperleptinemia delays leptin-mediated lowering of blood glucose levels. When we increased plasma leptin levels ~3 fold with a second plasmid injection, blood glucose levels were completely normalized to the non-diabetic range. Similarly, in our dose response studies, STZ depleted leptin and to confer glucose lowering effects, leptin levels beyond pre-diabetic levels were required. Therefore, to improve glycemia, it appears leptin levels must exceed baseline levels before the onset of diabetes. Consistent with this

observation, transgenic mice with life-long overexpression of leptin eventually stop responding to the anorexic effects of excess endogenous leptin but boosting leptin levels even further with exogenous leptin therapy can induce weight loss [452]. Our findings indicate that blood glucose lowering effects of leptin for type 1 or advanced type 2 diabetes may be delayed if patients displayed obesity driven hyperleptinemia prior to the onset of diabetes. Furthermore, high leptin levels may be required for these patients, which may not be pharmacologically achievable nor desirable. It is unknown how pre-existing hyperleptinemia sets a therapeutic threshold in STZ-mice. It is possible that despite high circulating leptin levels in mice with pre-existing hyperleptinemia, leptin bioactivity is decreased. In mice and humans with obesity, hyperleptinemia alters the ratio of free to bound leptin [290]. In our studies, we used a leptin ELISA to measure leptin levels, which likely cannot distinguish between free and bound leptin. In future studies, a leptin bioassay may be used to assess the bioactivity of leptin in our mice with pre-existing hyperleptinemia. Thus, pre-existing leptin levels set a baseline, which is also the therapeutic threshold for glucose lowering actions of leptin, and further studies are warranted to determine the mechanisms by which this threshold is set.

Since hypothalamic leptin signalling remains intact with high-fat intake in leptin-treated STZ-mice, we postulated that high-fat intake interferes with leptin action downstream of leptin signalling in the brain. It has been reported that depletion of glycerol, a key substrate for gluconeogenesis, contributes to the glucose lowering actions of leptin [264, 268]. Interestingly, glycerol levels were increased with HFD feeding in leptin-treated STZ-mice. Therefore, we suspect that high-fat intake may increase glycerol levels in leptin-treated STZ-mice to prevent blood glucose lowering actions of leptin. In our dose response studies, we observed that STZ severely depletes fat mass. In addition, fat mass and body weight were not different between

HFD fed and LFD-fed STZ-leptin mice when hyperglycemia returned in the HFD fed STZ-leptin group. Therefore, increased lipolysis in the adipose tissue is unlikely to be the cause of increased plasma glycerol levels with HFD feeding. Instead, lipoprotein lipase mediated breakdown of triglycerides in chylomicrons may contribute to increased plasma glycerol levels. It has also been reported that leptin lowers circulating free-fatty acids in STZ-diabetic rats, which lowers acetyl-CoA levels in the liver [264, 268] and reduced gluconeogenesis via suppression of pyruvate carboxylate flux [268]. Free fatty acid levels were increased in HFD fed STZ-leptin mice compared to LFD-fed STZ-leptin controls, albeit not to levels observed in diabetic STZ-vehicle controls. Therefore, increased free-fatty acids may partially contribute to HFD induced resistance to the glucose lowering actions of leptin in STZ-diabetic mice. In chapter 3 of this thesis, we showed that leptin-treated STZ-mice cannot utilize amino acids for the production of glucose. Thus, future studies should assess whether excessive utilization of amino acids for glucose production contributes to HFD-induced leptin resistance in STZ-mice. Regardless of the mechanism, this chapter reveals that excess consumption of dietary fats can interfere with the glucose lowering effects of leptin therapy. In light of this, future clinical studies should measure fat intake as a potential factor that could help predict the efficacy of leptin as a glucose lowering agent.

Chapter 6: Conclusions and Future Directions

Since its discovery in 1994, the hormone leptin has challenged numerous long-standing dogmas in the field of obesity and diabetes. The identification of a mutated gene that can cause morbid obesity and symptoms of type 2 diabetes provided an alternative to the widely held view that obesity results purely from a lack of willpower or as a consequence of modernized environments (reviewed in [453]). Indeed, it is estimated that more than 10% of morbid obesity in humans is a result of genetic defects, with the largest contribution from mutations in *MC4R*, which is downstream of leptin signalling (reviewed in [454]). The discovery of leptin precipitated research into the molecular mechanisms of body weight regulation, and we now know that a complex web of hormonal signals control food intake, energy expenditure, and feeding behavior. In addition, the findings that leptin is produced and secreted from adipocytes as a signal for overall nutritional state unveiled the adipose tissue as not just a fat store but also a dynamic endocrine organ (reviewed in [455]). There is now substantial evidence that leptin regulates glucose metabolism, independent of its actions on body weight (reviewed in [456, 457]). Perhaps the most striking evidence for this may be its ability to lower blood glucose levels in rodent models of type 1 diabetes. For almost a century, insulin has been assumed to be the only hormone that can reverse the catabolic consequences of type 1 diabetes, and thus beneficial effects of leptin in rodent models of type 1 diabetes challenged this long-held dogma.

A growing number of studies have demonstrated that leptin reverses hyperglycemia in rodent models of type 1 diabetes. The glucose lowering effects of leptin are robust; in STZ-diabetic rodents [260-263, 265, 323, 324], NOD mice [265, 266], virus induced diabetic rats [325], and insulinopenic Akita mice [326], leptin delivered through either subcutaneous infusion,

adenoviral leptin gene delivery, or transgenic leptin overexpression can have considerable beneficial effects on both glucose and lipid metabolism. In this thesis, we showed that administration of leptin using plasmids is another method of delivery, which is especially useful for sustaining chronic hyperleptinemia in rodents without the need for repeated injections or surgical procedures. In addition to reversing hyperglycemia [260, 262-266, 322-324], leptin improves a plethora of deleterious symptoms associated with type 1 diabetes; leptin therapy increases insulin sensitivity [260, 267, 322], stabilizes body weight [260, 264, 327], decreases hyperphagia [261-266, 322-324], normalizes water intake [261, 264], eliminates polyuria and glucosuria [261, 262, 264-266], lowers plasma lipids and ketones [260, 262, 264-266, 322], and extends lifespan [262, 263, 266, 324, 328]. Remarkably, leptin corrects these metabolic disturbances in rodent models of type 1 diabetes without raising circulating insulin levels and independent from alterations in food intake [260, 264-266, 326].

The overarching goal of this thesis was to further characterize leptin therapy in insulin deficient STZ-mice. First, we sought to gain insight into the mechanism by which leptin lowers blood glucose levels in insulin deficient mice. For this, we rigorously characterized the liver of leptin-treated STZ-mice using a triple omics approach. Second, we assessed whether recapitulating the effects of leptin on the hepatic transcriptome may confer glucose lowering effects in insulin deficient mice. We compared the hepatic transcriptome of leptin-treated STZ-mice to a library of expression profiles created by exposing cultured human epithelial cells with ~1400 different small molecules. Lastly, we examined whether chronic hyperleptinemia or excessive dietary fats, which are factors known to cause resistance to the body weight reducing actions of leptin, interfere with the ability of leptin to improve glycemia in insulin deficient mice.

There is strong evidence that suppressed glucose production, as opposed to enhanced glucose uptake, may underlie the glucose lowering actions of leptin in insulin deficient rodents. Administration of leptin to STZ-rodents lowers whole body [322] and hepatic glucose production [261, 331] during basal conditions. This reduction in glucose production manifests as severe fasting intolerance, as demonstrated by their descent into life-threatening hypoglycemia within 8 hours of fasting [264]. Furthermore, leptin-treated STZ-mice are hyperglycemic during fed conditions [260]. Thus, although leptin has been reported to achieve normoglycemia in insulin deficient rodents [260, 261, 264-266, 268, 327, 355, 356], this phenomenon is likely to depend on fasting before blood glucose measurements are taken. On the other hand, whether leptin enhances glucose uptake in rodent models remains controversial; leptin therapy in STZ-rodents has been reported to increase [261] or have no effect [260] on radiolabeled glucose uptake in muscle, and to decrease [261] or have no effect [322] on whole body glucose disappearance under basal conditions. In these studies, leptin treated insulin deficient rodents were compared to diabetic controls. Since diabetic mice exhibit polyuria and glucosuria, it is highly likely that radiolabeled glucose is excreted at higher rates in these animals, and thus these studies should be interpreted with caution. Since leptin-treated STZ-rodents have markedly enhanced insulin sensitivity, the discrepancy in whether leptin enhances glucose uptake during basal conditions may be due to differences in residual insulin levels for various rodent models of insulin deficiency. In summary, mounting evidence suggests that leptin-treated mice have defective glucose production, and it is likely that the mechanism driving leptin-mediated hypoglycemia during fasting is also responsible for reversal of hyperglycemia in insulin deficient animals.

Leptin appears to suppress glucose production in insulin deficient rodents through several distinct mechanisms. In leptin-treated STZ-rodents, whole body lipolysis is reduced compared to

diabetic controls, which depletes circulating glycerol levels [264, 268] and reduces whole body glycerol turnover by ~60% [268]. In addition, replenishing glycerol levels with injections [264] or infusion of a lipid emulsion [268], increases blood glucose levels in leptin-treated STZ-rodents. These data suggest that depletion of glycerol in part contributes to the glucose lowering actions of leptin, but the pathways involved in the conversion of glycerol to glucose remain intact. What this thesis now shows is that leptin therapy also suppresses the utilization of amino acids for glucose production. More specifically, we showed that leptin-treated STZ-mice cannot utilize the amino acid alanine for the production of glucose, while their ability to utilize pyruvate for gluconeogenesis remains intact. Hepatic *Gpt*, the enzyme responsible for the breakdown of alanine to pyruvate, is down-regulated in leptin-treated STZ-mice but this down-regulation is neither required nor sufficient for the glucose lowering actions of leptin. Using a triple omics based approach, we showed that there is a global down-regulation of genes within the amino acid catabolism pathway and an accumulation of gluconeogenic amino acids. Thus, these data suggest that suppressed utilization of amino acids for glucose production in part contributes to the glucose lowering actions of leptin in STZ-mice. Notably, although leptin suppresses the utilization of both glycerol and amino acids, the mechanisms driving these effects are distinct; in leptin-treated STZ-mice, glycerol levels are depleted but the pathways for glycerol utilization remain intact [264, 268], while amino acid utilization is blunted with an accumulation of gluconeogenic amino acids. In addition to glycerol and amino acids, lactate is another major gluconeogenic substrate [458]. Previous studies have reported unchanged circulating lactate levels in leptin-treated STZ-mice compared to diabetic controls [264]. Thus, while lactate depletion is unlikely to be a mechanism for glucose lowering in leptin-treated mice, lactate catabolism and transport should be assessed. Although we focused on characterization of the

liver, gluconeogenesis in the kidneys and intestines also contributes to the maintenance of normoglycemia during fasting [412]. Severe fasting intolerance observed in leptin-treated STZ-mice indicates that renal and intestinal gluconeogenesis may also be suppressed [264]. Thus, leptin suppresses the utilization of gluconeogenic substrates glycerol and amino acids, albeit the mechanisms of suppression appear to be distinct for these substrates.

The upstream signals that mediate the effects of leptin on glucose production remain unknown. Studies have demonstrated that glucagon levels are increased in insulin deficient rodents, and leptin therapy lowers glucagon levels to the non-diabetic range [260, 264, 265, 268]. Indeed, reducing glucagon action through immunoneutralization of endogenous glucagon [339], antagonism of glucagon receptor signalling [340-342], knockdown of hepatic glucagon receptors using siRNAs [343], or genetic deletion of the glucagon receptor [266, 343, 344], lowers blood glucose levels in rodents with insulin deficient diabetes. It should be noted that reducing glucagon receptor signalling is not sufficient to lower blood glucose levels in cases of severe insulin deficiency; decreased glucagon receptor signalling only modestly lowers blood glucose levels in insulin knockout mice or mice with near complete ablation of β-cells using diptheria toxin [459, 460]. Nevertheless, these studies suggest decreased glucagon signalling may be responsible for the glucose lowering actions of leptin in insulin deficient rodents. In this thesis, we showed that STZ-mice treated with chronic hyperleptinemia are hyperglucagonemic, despite having normal blood glucose levels. These data suggest that reduced glucagon levels are not required for the glucose lowering actions of leptin. To further test the role of glucagon in leptin action, glucagon levels could be elevated in leptin-treated STZ-mice towards the diabetic range using recombinant glucagon or plasmids encoding glucagon (similar to leptin plasmids used in this thesis). If blood glucose levels increase in these hyperglucagonemic leptin-treated STZ-

mice, then this would provide more direct evidence to support the notion that leptin mediated lowering of glucagon levels contributes to improvements in glycemia.

In addition to hyperglucagonemia, excessive HPA activity is reported in states of insulin deficiency, as evidenced by increased ACTH and cortisol/corticosterone levels in insulin deficient humans [349-352] and mice [348]. However, the role of the HPA axis in leptin action remains controversial. Leptin therapy has been reported to have no effect [355] or reduce ACTH levels [268, 356], and to have no effect [260] or reduce corticosterone levels [268, 355, 356]. Perry *et al.* found that infusion of corticosterone blocked the glucose lowering effects of leptin in STZ-diabetic rats [268, 356], suggesting that glucose lowering actions of leptin requires reduced corticosterone levels. However, Meek *et al.* reported that neither adrenalectomy induced corticosterone deficiency nor pharmacological corticosterone receptor blockade lowered blood glucose levels in STZ-diabetic rats [355], which suggests that reduced levels in corticosterone is not sufficient for the glucose lowering actions of leptin. Growth hormone levels are reduced by ~28 fold in leptin-treated STZ-mice compared to diabetic controls [260], but the significance of this reduction has not been tested directly. The severe fasting hypoglycemia observed in leptin-treated STZ-mice suggest that many branches of the counter regulatory response may be defective in these mice [264]. Thus, we suspect that the reduction in glucagon, corticosterone, and growth hormone may all contribute to suppressed glucose production in leptin-treated insulin deficient mice. It has also been suggested that leptin may increase levels of hormones that have insulin-like actions. Candidates include IGFBP2, insulin-like growth factor 1 (IGF1), and FGF21. However, IGFBP2 levels are already elevated in insulin deficient mice compared to non-diabetic controls [267, 327], and both IGF1 and FGF21 levels are unchanged with leptin therapy compared to diabetic controls [268], suggesting that these hormones likely do not play a role in

leptin mediated reversal of suppression of glucose production. Therefore, although suppression of glucose production may drive the glucose lowering actions of leptin, the precise upstream mechanisms remain unknown.

In addition to reversing hyperglycemia, leptin also appears to profoundly alter lipid metabolism in insulin deficient mice. Previous studies have shown that leptin lowers circulating free fatty acids and triglycerides in the plasma of STZ-rodents [262, 264-266, 268]. Using metabolomics analysis, we identified the precise lipid species that are altered by leptin in STZ-mice. Notably, leptin had the most dramatic effect on lipids classified as glycerides and fatty acids, which are used as an energy source; most metabolites categorized as diacylglycerol, monoacylglycerol, polyunsaturated fatty acid, long chain fatty acid, monohydroxy fatty acid, and fatty acyl carnitine subclasses were diminished in STZ-Leptin mice compared to diabetic controls. In the liver, β-oxidation of fatty acids produces acetyl-CoA, which is a substrate for ketogenesis (reviewed in [421]). In this thesis, we showed that ketogenic amino acid levels are not altered in the liver of leptin-treated STZ-mice. Thus, depletion of lipids, but not amino acids, likely drives leptin mediated improvements in hyperketonemia. Hepatic acetyl-CoA also acts as a potent allosteric activator of the rate limiting enzyme pyruvate carboxylase, which converts pyruvate to oxaloacetate during gluconeogenesis [417-420]. Consistent with the reduction in circulating fatty acids, acetyl-CoA levels are reduced in the liver of leptin-treated STZ-mice [264, 268]. It has been proposed that reduction in hepatic acetyl-CoA reduces pyruvate carboxylase flux, and this contributes to suppressed gluconeogenesis in leptin-treated STZ-rodents [268]. However, we showed in this thesis that injection of pyruvate increases blood glucose levels in leptin-treated mice, although this response was modestly blunted compared non-diabetic controls. Similarly, previous reports show that the change in blood glucose levels

with pyruvate injections are comparable between leptin-treated STZ-mice and diabetic controls [264]. Combined, these data do not support the idea of decreased pyruvate carboxylase activity in leptin-treated STZ-rodents compared to diabetic controls. In this thesis, we showed the effects of leptin on lipid metabolism extend beyond diminishing levels of energy yielding lipids; leptin significantly altered levels of phospholipids lysolipid, plasmalogen, and sphingolipids, which are important in biological processes such as membrane integrity [386], cell signalling [387], and cell to cell recognition [388]. It is possible that for some lipids, the changes to circulating levels are a consequence of restored euglycemia as opposed to direct leptin action. To address this, it would be interesting to compare the metabolomic profiles of STZ-Leptin mice to STZ-mice treated with another glucose lowering agent, such as SGLT2 inhibitors. In summary, leptin therapy has profound effects on lipid metabolism in insulin deficient rodents, and these changes may contribute to the reversal of hyperketonemia.

This thesis highlights the beneficial effects of low dose leptin therapy in insulin deficient mice. By administering leptin plasmids at a low dose to STZ-mice, we showed that supraphysiological levels of leptin are required to reverse hyperglycemia in insulin deficient rodents. Consistent with these results, clinical trials testing low dose leptin as an adjunct to insulin therapy reported that leptin does not lower blood glucose or HbA1c levels in patients living with type 1 diabetes compared to baseline, although total insulin requirements were modestly reduced in these patients [289]. Although low dose leptin does not improve glycemia in rodent models of type 1 diabetes, we showed that there were some beneficial effects. STZ-mice treated with low dose leptin had reduced circulating ketone levels and extended lifespan compared to diabetic controls. Consistent with these data, it has been previously reported that leptin replacement in a rat model of diabetic ketoacidosis reduces hepatic ketogenesis by 50%

within 6 hours [356], and our results show that these effects can persist long-term. Since prolonged and severe hyperketonemia is one of the life threatening complications of type 1 diabetes (reviewed in [93]), we suspect that reduced ketoacidosis may be responsible for prolonging lifespan in STZ-mice treated with a low dose of leptin. Thus, low dose leptin therapy may be useful to prevent diabetic ketoacidosis, especially in patients with brittle type 1 diabetes.

The requirement of hyperleptinemia for lowering glucose levels in rodent models of type 1 diabetes has raised some concern regarding leptin resistance. Obese humans and rodents are resistant to the anorectic effects of exogenous leptin therapy [274, 279, 280, 444], and pre-existing hyperleptinemia is thought to cause this resistance. Indeed, preventing diet-induced hyperleptinemia by decreasing leptin expression in adipocytes [307] or increasing leptin clearance in the kidneys [307] increases sensitivity to weight reducing actions of leptin. Thus, initial excitement of leptin as a potential weight reducing agent has dwindled. In this thesis, we showed for insulin deficient diabetes, chronic hyperleptinemia before or after the onset of diabetes does not cause resistance to the glucose lowering actions of leptin. Therefore, resistance to the glucose lowering actions of leptin in STZ-diabetic mice are unlike reported cases of resistance to the body weight reducing actions of leptin in HFD-fed obese rodents. Furthermore, our studies suggest that high dose leptin therapy may be a viable strategy to achieve long-term euglycemia in patients living with type 1 diabetes. In our studies, we assessed parameters relevant for symptoms of type 1 diabetes, such as glucose and lipid metabolism. Given well-known effects of leptin on reproduction and the immune system, chronic hyperleptinemia may elicit unwanted side effects related to these pleiotropic actions of leptin (reviewed in [128, 432, 433]). It should be noted that our studies were conducted in male mice. Since female humans have higher leptin levels than males [461] and our studies show that baseline leptin levels set the

therapeutic threshold, the kinetics of glucose lowering or the dose of leptin required to lower glycemia may be different in females than males. However, unlike in humans, female adult mice have lower leptin levels than males [462], and thus mice may not recapitulate sex differences in the therapeutic efficacy of leptin for humans. Future studies should assess these aspects of leptin action in insulin deficient rodents to further evaluate the therapeutic applicability of leptin.

In addition to hyperleptinemia, we assessed whether dietary fats can interfere with the glucose lowering effects of leptin. Several studies indicate that dietary fats cause resistance to the body weight reducing actions of leptin; in diet-induced obese mice, leptin transport across the blood brain barrier [293] and the CSF to plasma leptin ratio is reduced [294, 295], and dietary fats have been shown to directly interfere with leptin transport to the brain [293, 449-451]. In addition, direct administration of lipids to the brain via ICV injections dampen leptin signalling in the hypothalamus [445], suggesting that lipids may directly cause hypothalamic leptin resistance. Proposed mechanisms by which dietary fats impair leptin signalling in the hypothalamus include inflammation [308, 309], ER stress [315-317], and defects in autophagy [318]. In this thesis, we showed that excess dietary fats before or after the onset of insulin deficient diabetes can cause resistance to the blood glucose lowering actions of leptin. Unlike resistance to weight reducing actions of leptin, we showed that STZ-mice resistant to glucose lowering actions of leptin have intact leptin transport and signalling in the hypothalamus. We did not definitively identify the mechanism by which excess dietary fats cause resistance to the glucose lowering actions of leptin. However, we observed a correlation between blood glucose levels and levels of circulating glycerol and free fatty acids; leptin therapy initially lowered circulating glycerol and free fatty acid levels in STZ-mice, but these effects were reversed by HFD feeding. From these data, we speculate that the repletion of these substrates may have

restored gluconeogenic capacity in these mice. In Chapter 3, we showed that reduced amino acid utilization for glucose production in part contributes to the glucose lowering actions of leptin. Thus, future studies should assess whether dietary fats cause resistance to the glucose lowering effects of leptin by enhancing amino acid catabolism. In light of our findings, future clinical studies testing leptin for the treatment of type 1 diabetes should measure fat intake as a potential predictive factor for the efficacy of leptin therapy. Our studies were only performed in STZ-mice, which is a rodent model of type 1 diabetes. Given the growing interest in leptin as a therapy for other forms of diabetes, including lipodystrophy and type 2 diabetes (reviewed in [439]), the effects of chronic hyperleptinemia and high-fat intake on the glucose lowering abilities of leptin should be assessed across the spectrum of diabetes.

There are several caveats of leptin therapy for type 1 diabetes. First, we demonstrated that supraphysiological levels of leptin are required to reverse hyperglycemia in insulin deficient rodents, which may elicit deleterious effects associated with the pleiotropic actions of leptin. Second, supraphysiological levels must be sustained [264] and leptin has a short half life of ~40 minutes [434], suggesting that patients will likely need constant injections of recombinant leptin to maintain normoglycemia. In fact, during clinical trials testing leptin and insulin co-therapies, twice daily injections of metreleptin only increased median serum leptin levels by 1.7 and 2.2 fold after 12 and 20 weeks of treatment, respectively [289], and the variation in leptin levels throughout the trial was not reported. Lastly, severe fasting intolerance observed in leptin-treated STZ-mice is a major concern [264]. Indeed, a clinical trial testing leptin therapy for lipodystrophy reported hypoglycemic events in 11% of patients [416]. For clinical trials testing leptin as an adjunct to insulin, blood glucose levels were tracked for 24 hours at weeks 12 and 20 post treatment initiation and hypoglycemia was not observed [357]; however, we suspect that the

lack of hypoglycemia may be due to the low dose of leptin used in this study. This clinical trial has ended and it is unclear whether leptin therapy will continue to be tested for type 1 diabetes. If testing continues, a higher dose of leptin will likely be needed to lower blood glucose levels in patients living with type 1 diabetes and the risks of hypoglycemia should be carefully assessed.

Some of the caveats associated with leptin may be avoided with small molecules that can recapitulate the effects of leptin in the liver. Small molecules can potentially be more easily modified to increase the half life and potency. Identifying small molecules that do not act through the leptin receptor may be a viable strategy to recapitulate the effects of leptin in certain tissues like the liver but not in others. Furthermore, while leptin treatment appears to blunt most branches of the counter regulatory response, small molecules may be more selective, which might reduce the risk of hypoglyemia. In agreement with this idea, glucagon receptor knockout mice with STZ-induced diabetes have lowered blood glucose levels with fasting but they can maintain stable blood glucose levels over 24 hours [344]. The liver is a major metabolic hub which may mediate the glucose lowering effects of leptin; leptin-treated STZ-rodents have reduced hepatic glucose production [261, 331], and we showed in Chapter 3 that the hepatic transcriptome is globally altered with leptin therapy. Thus, we postulated that recapitulating the effects of leptin on the hepatic transcriptome with small molecules may confer beneficial effects on glycemia. We used the hepatic gene signature of leptin-treated STZ mice and compared it to ~1400 different expression profiles obtained from cultured cells exposed to small molecules. We identified a novel small molecule, PLM, which displays modest but reproducible glucose lowering effects in STZ-mice. Although PLM shows some promising results, our results are preliminary and there are several remaining questions. First, although PLM was identified using the hepatic transcriptional profile of STZ-Leptin mice, it is possible that PLM may have effects

in other tissues. Thus, we cannot definitively conclude that the modest glucose lowering effects of PLM are through metabolic alterations in the liver. Second, the mechanism by which PLM lowers blood glucose levels was not directly examined in our studies. Since leptin suppresses the utilization of glycerol and amino acids, it is possible that PLM may also curb the use of these substrates for glucose production. Third, it is unknown whether treatment of STZ-mice with PLM causes fasting hypoglycemia. Lastly, it would be interesting to modify the chemical moieties of PLM to assess whether its glucose lowering effects can be enhanced. In summary, we showed that PLM displays modest but promising glucose lowering effects in STZ-mice, but further studies are required before its therapeutic applicability can be assessed.

The discovery of leptin has challenged the notion that insulin is the only hormone that can reverse hyperglycemia and life threatening complications in type 1 diabetes. Indeed, leptin displays the truly remarkable ability to reverse hyperglycemia and prolong lifespan in rodent models of type 1 diabetes. However, similar to insulin, leptin will never be a cure for diabetes but merely a potential treatment. Hopefully, the pre-clinical studies in this thesis have contributed to the understanding of both the benefits and pitfalls of leptin as a potential treatment for diabetes. Whether or not leptin is adopted as a therapy for type 1 diabetes, it is hoped that this thesis can contribute basic scientific knowledge in the field of diabetes, in order to improve treatment of and prognosis for patients living with diabetes.

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