THE EFFECTS OF LEPTIN KNOCKOUT AND INSULIN SUPPRESSION ON GLUCOSE METABOLISM IN RODENTS

by

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ABSTRACT

Background: The adipose-derived hormone leptin is well known for its effects on metabolic homeostasis, and in particular its role in suppressing appetite and regulating fat mass. Loss of leptin results in a phenotype characterized by obesity, insulin resistance, hyperinsulinemia and glucose intolerance. Treating leptin deficient mice with leptin is able to lower blood glucose and insulin levels without reducing body weight, indicating that leptin has independent effects on glucose homeostasis. While there have been many studies exploring the role of leptin in metabolism, the glucoregulatory actions of leptin remain to be fully elucidated.

Scope of Thesis: The overall goal of this thesis was to understand the role of leptin in glucose regulation. To do so, we utilized a number of *in vivo* models in which leptin signalling was disrupted. First, we investigated the effects of reducing insulin gene dosage on glucose homeostasis and body mass in leptin deficient *ob/ob* mice. Next we explored the significance of leptin signalling in pancreatic β cells. To determine whether the glucoregulatory actions of leptin are conserved across rodent species, we explored the effects of leptin deficiency in rats, and used this model to investigate the hierarchy of metabolic defects that arise as a result of impaired leptin action. Finally, we describe a novel mouse model to selectively knock out leptin in a temporal and tissue-specific manner.

Conclusions*:* We show that reduction of circulating insulin levels prevents obesity, but results in hyperglycemia in *ob/ob* mice. Furthermore, we show that direct leptin

signalling in β cells is insufficient to restore euglycemia. In addition, we demonstrate that leptin deficiency produces a similar metabolic phenotype in rats as in mice. Finally, we suggest that loss of insulin sensitivity may be a primary metabolic defect that results from leptin deficiency in rats. Together, these results provide further insight into the glucoregulatory mechanisms of leptin that may be used to develop therapeutic strategies to treat obesity and diabetes.

LAY SUMMARY

Obesity is defined as a state of excess fat mass. Many obese individuals also develop insulin resistance, chronically elevated insulin levels, and in some cases, blood glucose levels exceeding the normal range (4-6 mM). Leptin, a hormone produced predominantly from adipose tissue, is best known for its effects on appetite suppression to regulate body weight. However, leptin may also play a pivotal role in regulating blood glucose. In human and rodent populations lacking leptin, obesity, insulin resistance and chronically elevated insulin levels are observed. The main objective of this thesis was to determine the mechanisms by which leptin regulates blood glucose. The role of leptin in regulating insulin secretion was examined, in addition to the hierarchy of metabolic defects that develop in the absence of leptin. Through increased understanding of the effects of leptin on blood glucose regulation, novel therapeutics may be developed to overcome obesity and diabetes.

PREFACE

The studies presented in this dissertation were conceived and designed by AM D'souza and TJ Kieffer. Portions of chapter 1 are from the following review: D'souza AM*, Neumann UH*, Glavas MM, and Kieffer TJ (2017). The gluco-regulatory actions of leptin. *Molecular Metabolism (in press)*. **These authors contributed equally to this work.*

Studies in chapter 3 are published in the following article: D'souza AM, Kieffer TJ (2017). Restoration of *Lepr* in β cells of *Lepr* null mice does not prevent hyperinsulinemia and hyperglycemia. *Molecular Metabolism* 6(6): 585-593. All experiments were performed by AM D'souza with assistance from S. O'Dwyer. This publication was written by AM D'souza with editing by TJ Kieffer.

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Studies in chapter 5 and 6 were performed by AM D'souza. A Asadi performed the experiment in Figures 5.7B-C. Shannon O'Dwyer assisted with *in vivo* tolerance tests conducted in chapter 6.

Studies in chapter 7 were performed by AM D'souza with assistance from NV Krosigk. Monitoring of blood glucose and body weight in *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice were completed by NV Krosigk.

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The Animal Care Committee at the University of British Columbia examined and approved the use of animals for the studies in this thesis under the following Animal Care Certificates: A14-0081, A14-0063, A14-0162, A14-066.

TABLE OF CONTENTS

ABSTRACT ii
LAY SUMMARY iv
PREFACE v
TABLE OF CONTENTS vii
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF ABBREVIATIONS xiii
ACKNOWLEDGEMENTS xvi
CHAPTER 1: INTRODUCTION 1
1.1 Obesity & Type 2 Diabetes 1
1.2 Insulin resistance, hyperinsulinemia and obesity 3
1.3 Insulin 5
1.4 Leptin 8
1.5 Thesis Investigation 21
CHAPTER 2: MATERIALS & METHODS 23
2.1 Animals 23
2.2 Genotyping 28
2.3 Blood and Plasma Analytes 31
2.4 Analysis of Rodent Tissues 32
2.5 Body Composition & Metabolic Cages 33
2.6 Chemical Manipulations 34
2.7 Surgical Manipulations 35
2.8 In Vivo Assays 37
2.9 Histology 38
2.10 Statistical Analysis 40
CHAPTER 3: SUPPRESSING HYPERINSULINEMIA PREVENTS OBESITY BUT CAUSES RAPID ONSET OF DIABETES IN LEPTIN-DEFICIENT *OB/OB* MICE 42
3.1 Introduction 42
3.2 Results 43
3.3 Discussion 57
CHAPTER 4: RESTORATION OF *LEPR* IN Β CELLS OF *LEPR* NULL MICE DOES NOT PREVENT HYPERINSULINEMIA AND HYPERGLYCEMIA 61
4.1 Introduction 61
4.2 Results 63

4.3 Discussion 72
CHAPTER 5: LEPTIN DEFICIENCY IN RATS RESULTS IN HYPERINSULINEMIA AND IMPAIRED GLUCOSE HOMEOSTASIS 75
5.1 Introduction 75
5.2 Results 76
5.3 Discussion 93
CHAPTER 6: REDUCED INSULIN SENSITIVITY WITHOUT HYPERINSULINEMIA IS OBSERVED IN SUCKLING *LEP*^{KO} RATS 97
6.1 Introduction 97
6.2 Results 99
6.3 Discussion 108
CHAPTER 7: GENERATION AND CHARACTERIZATION OF MICE WITH INDUCIBLE KNOCKOUT OF LEPTIN IN ADULTHOOD 112
7.1 Introduction 112
7.2 Results 115
7.3 Discussion 126
CHAPTER 8: CONCLUSIONS AND FUTURE DIRECTIONS 130
8.1 General Conclusions 130
8.2 Contribution of Hyperinsulinemia to the Obese, Diabetic Phenotype of ob/ob Mice 131
8.3 Role of Lepr in β Cells 133
8.4 Hierarchy of Metabolic Defects that Arise as a Result of Leptin Deficiency in Rats 134
8.5 Loss of Leptin Signalling in Adulthood 137
8.6 Study Strengths and Limitations 138
8.7 Significance & Future Directions 140
REFERENCES 145
APPENDIX 186

LIST OF TABLES

Table 1.1: Summary of rodent models with impaired leptin signalling………………...20

Table 2.1: Primers sequences used for genotyping …………………………………….29

LIST OF FIGURES

Figure 1.1: Proposed chronology of obesity, insulin resistance and hyperinsulinemia ... 5

Figure 1.2: Glucoregulatory actions of leptin ... 17

Figure 2.1: Breeding scheme to generate *ob/ob* and *wt/wt* mice lacking 1, 2 or 3 insulin alleles.. ... 25

Figure 2.2: Schematic of genetic manipulation of Lepr gene in *Lepr*$^{loxTB/loxTB}$ mice ... 26

Figure 3.1: Reduction of insulin gene dosage suppresses hyperinsulinemia in *ob/ob* mice ... 44

Figure 3.2: Reduction of insulin gene dosage in ob/ob mice protects against obesity. . 46

Figure 3.3: Reduction of insulin attenuates adiposity in *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* mice ... 49

Figure 3.4: Reduction of insulin gene dosage in *ob/ob* mice is results in reduced survival ... 49

Figure 3.5: Adiposity is reduced in *ob/ob* mice lacking 2 insulin alleles independent of changes in food intake or energy expenditure... 50

Figure 3.6: Reduced body weight is not attributed to differences in VO_2 consumption or VCO_2 production... 51

Figure 3.7: Attenuation of hyperinsulinemia accelerates development of hyperglycemia in *ob/ob* mice ... 53

Figure 3.8: Attenuation of hyperinsulinemia does not improve glucose tolerance in *ob/ob* mice ... 55

Figure 3.9: Reduction of insulin gene dosage produces abnormal islet morphology in *ob/ob* mice. ... 56

Figure 4.1: *Lepr* restoration in β cells was validated by qPCR ... 64

Figure 4.2: Knock in of Cre into a single *Ins1* allele does not affect glucose homeostasis in *mT/mG* mice. ... 65

Figure 4.3: Restoration of *Lepr* in β cells does not improve islet morphology in *Lep*null mice. 67

Figure 4.4: Restoration of *Lepr* in β cells of *Lep*null mice does not prevent hyperinsulinemia 68

Figure 4.5: *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice are glucose intolerant and have impaired glucose stimulated insulin secretion 71

Figure 5.1: *Lep*^{KO} rats are obese and have increased lipid mass compared to *Lep*^{WT} littermates 78

Figure 5.2: *Lep*^{KO} rats have increased ectopic lipids compared to *Lep*^{WT} littermates 79

Figure 5.3: Leptin-deficient rats are hyperinsulinemic and males experience transient hyperglycemia. 82

Figure 5.4: Glucose intolerance and impaired glucose stimulated insulin secretion (GSIS) occurs in *Lep*^{KO} rats 83

Figure 5.5: *Lep*^{KO} rats have deficits in insulin clearance 84

Figure 5.6: Leptin deficiency in rats results in insulin resistance 90

Figure 5.7: Islets from *Lep*^{KO} rats are larger and secrete more insulin than *Lep*^{WT} islets 92

Figure 5.8: Leptin therapy reduces weight gain, food intake and fasting insulin levels in *Lep*^{KO} rats 92

Figure 5.9: Leptin therapy improves glucose tolerance and glucose stimulated insulin secretion (GSIS) in male *Lep*^{KO} rats 93

Figure 6.1: Increased fat mass is observed in suckling *Lep*^{KO} rats and significant increases in plasma insulin levels are observed by 3 weeks of age 100

Figure 6.2: Reduced insulin sensitivity is observed in suckling *Lep*^{KO} rat 102

Figure 6.3: Increased fat mass is not associated with elevated plasma triglycerides or ectopic lipid deposition in 2-week-old rats 104

Figure 6.4: Glucose-stimulated insulin secretion is higher in isolated islets from *Lep*^{KO} rats compared to *Lep*^{WT} littermates 106

Figure 6.5: Relative β and α cell area is comparable between suckling *Lep*^{KO} and *Lep*^{WT} rats 107

Figure 7.1: Plasma leptin levels are significantly lower in *Lep*$^{flox/flox}$ *ACTB*Cre mice compared to littermate controls 115

Figure 7.2: *Lep*$^{flox/flox}$ *ACTB*Cre mice are obese compared to littermate controls 117

Figure 7.3: Increased body weight is observed after weaning in *Lep*$^{flox/flox}$ *ACTB*Cre mice 118

Figure 7.4: Knockout of leptin in *Lep*$^{flox/flox}$ *ACTB*Cre mice results in impaired glucose homeostasis 120

Figure 7.5: Leptin therapy reverses weight gain and reduces blood glucose levels in *Lep*$^{flox/flox}$ *ACTB*Cre mice 122

Figure 7.6: Activation of Cre in *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice results in moderate and variable recombination of *ob* in gonadal adipose tissue and does not reduce immunoreactive leptin levels 123

Figure 7.7: Inducible activation of Cre in adult *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice does not result in obesity or impaired glucose homeostasis 125

Figure A1: *Lep*$^{flox/flox}$ *UBC*CreER^{T2} mice have minimal recombination in gWAT following tamoxifen injection 186

LIST OF ABBREVIATIONS

ACTB	beta-actin
AgRP	agouti-related peptide
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ARC	arcuate nucleus of the hypothalamus
ATP	adenosine triphosphate
AOC	area over the curve
AUC	area under the curve
BAT	brown adipose tissue
BMI	body mass index
CNS	central nervous system
CCK	cholecystokinin
Cre	Cre recombinase
DEXA	dual x-ray absorptiometry
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
GLP-1	glucagon-like peptide 1
GLUT	glucose transporter
gWAT	gonadal white adipose tissue
HBSS	Hank's balanced salt solution
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
h	hour(s)
ICV	intracerebroventricular

IGF	insulin-like growth factor
INS	human insulin gene
Ins1	mouse insulin 1 gene
Ins2	mouse insulin 2 gene
i.p.	intraperitoneal
Jak	janus kinase
JNK	c-Jun N-terminal kinsases
K_{ATP} channels	ATP-sensitive potassium channels
KO	knockout
Lep	leptin
Lepr	leptin receptor
NMR	nuclear magnetic resonance
NPY	neuropeptide Y
Ob	gene encoding leptin
OGTT	oral glucose tolerance test
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pdx-1	pancreatic/duodenal homeobox-1
PKC	phosphokinase C
POMC	pro-opiomelanocortin
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RER	respiratory exchange ratio
ROSA26	locus for constitutive, ubiquitous gene expression in mice

SEM	standard error of the mean
SOCS3	suppressor of cytokine signalling 3
STAT	signal transducers and activators of transcription
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes
TSH	thyroid stimulating hormone
Tyr	tyrosine
VMH	ventromedial nucleus of the hypothalamus
WAT	white adipose tissue
WT	wildtype

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To graduate students

struggling with mental illness in silence—

let your voice be heard, you are not alone

CHAPTER 1: INTRODUCTION

1.1 Obesity & Type 2 Diabetes

Obesity results from excessive accumulation of adipose tissue that impairs physical and psychosocial health and well being [1]. Clinically, obesity is defined based on body mass index (BMI), with overweight defined as a BMI between 25 kg/m^2 – 30 kg/m^2, while obesity is defined as a BMI greater than 30 kg/m^2 [2]. BMI is calculated by dividing the weight of an individual in kilograms by their height, in meters squared (kg/m^2). The number of overweight and obese individuals is predicted to rise to 57.8% of the world's adult population by 2030 [3]. In Canada approximately 1 in 7 children and 1 in 4 adults are obese [4]. The escalating prevalence of obesity in many developing and developed countries has resulted in an economic burden to the Canadian health care system estimated to be over $7 billion [5]. In addition, obesity is also highly correlated with a number of metabolic disorders including diabetes, heart disease, arthritis, and cancer [6].

Overweight and obesity are the most important predictors of type 2 diabetes (T2D) mellitus. As of 2015, approximately 435 million people have been diagnosed with some form of diabetes mellitus, and the annual number of deaths from diabetes rose from 1.2 million to 1.5 million over a 30-40 year period [7, 8]. Of those with diabetes worldwide, 90% of individuals have T2D. In 2010, it was estimated that roughly 2.7 million Canadians had T2D, and this number is projected to rise to 4.2 million people by 2020 [9]. Complications associated with diabetes accounted for one in ten deaths in Canadian adults in 2009 [10]. T2D is a polygenic disease that results from an insufficiency of insulin to meet the body's needs or an inability to respond properly to the actions of

insulin. Multiple theories have been postulated regarding the etiology of T2D. The most commonly held perspective is that insulin resistance, defined as a loss of sensitivity to insulin in peripheral tissues, results in a compensatory increase in β cell mass [11] and insulin secretory capacity [12, 13]. Eventually, the heightened demand for insulin cannot be met and β cell exhaustion occurs along with a loss of β cells and reduction of circulating insulin levels [14, 15]. T2D therefore develops as a result of reduced insulin secretion and reduced insulin sensitivity. Alternatively, another theory proposes that hyperinsulinemia, caused by consumption of environmental toxins contributes to development of insulin resistance, which may result in development of T2D [16].

There are many causes contributing to development of T2D, including genetic factors as well as environmental factors [17-19]. Genome wide association studies have identified polymorphisms in genes such as *TCF7L2, SLC30A8* and *CDKN2A/B* contributing to altered glucose induced insulin secretion [20-22]. Genetics may also result in certain ethnicities having an increased likelihood of developing obesity and diabetes [23, 24]. While genetics may predispose individuals to obesity and diabetes, the likelihood of developing these conditions is determined by environmental factors. These factors include: an increase of the aging population, an increase of sedentary lifestyles and access to calorie dense foods. While the molecular mechanisms linking diabetes and obesity remain unclear, recent studies have identified links between obesity and T2D involving an increase in ectopic fat deposition in the liver and skeletal muscle [25], cellular processes such as endoplasmic reticulum stress and mitochondrial dysfunction, as well as increased production of inflammatory cytokines [26-29]. Recent studies also suggest that impaired signalling of adipokine hormones such as leptin may mediate the link between obesity and diabetes [30].

1.2 Insulin resistance, hyperinsulinemia and obesity

Excessive nutrient intake or diminished energy output results in an obese phenotype that is often associated with insulin resistance and hyperinsulinemia. Insulin resistance is defined as the reduced responsiveness of target cells to insulin concentrations [31] while hyperinsulinemia refers to chronically elevated insulin levels in a fasted state. The association between obesity, insulin resistance and hyperinsulinemia is unclear. Over 50 years ago, Randle et al. hypothesized that obesity-associated insulin resistance was a result of increased circulating fatty acids (FAs) that competed with glucose for oxidative metabolism in insulin-responsive cells [32]. More recently, FAs have been implicated in the activation of kinases (e.g. JNK, NF-kB, PKC) that impair insulin signalling by increasing inhibitory serine phosphorylation of insulin receptor substrates (IRS) [33]. The chronically elevated circulating FAs lead to ectopic fat storage as triglycerides in muscle and liver [34], which in turn reduces insulin responsiveness in these tissues. This is primarily achieved by activation of deleterious pathways including those involved in generating mitochondrial dysfunction and endoplasmic reticulum stress [35]. Furthermore, the rise in FAs also facilitates increased activation of pro-inflammatory cytokines [36, 37]. The accumulation of lipids in non-adipose tissue, the rise in circulating FAs, and subsequent elevations of pro-inflammatory cytokines contributes to the development of insulin resistance, resulting in elevated blood glucose levels. This rise in blood glucose is thought to potentiate an increase in secretion of insulin [38]. Together, these studies provide compelling evidence that obesity is the primary metabolic defect contributing to dysregulation of insulin secretion and insulin action in peripheral tissues (Fig.1.1).

The compensatory rise of circulating insulin levels as a result of obesity-induced insulin resistance is a commonly accepted theory, but several clinical and *in vivo* studies have proposed that obesity and insulin resistance are outcomes of excess insulin. In mice made hyperinsulinemic by transfection of the human insulin gene, insulin resistance was observed post-transfection, and this was associated with reduced insulin receptor binding [39]. Similarly, rats administered with high levels of insulin exhibited down regulation of the insulin receptors [40]. Furthermore, it has been previously demonstrated that prevention of hyperinsulinemia can protect high-fat fed mice from diet-induced obesity [41, 42]. Clinical evidence indicates that hyperinsulinemia is a predictor of impaired glucose tolerance, T2D and hypertension [43]. Patients with insulin producing tumors exhibit elevated circulating insulin levels and concomitant insulin resistance as well as reduced insulin secretion in response to glucose [44, 45]. Furthermore, a recent report by Templeman et al. suggests that prevention of hyperinsulinemia in high fat fed mice can enhance insulin sensitivity [46]. Together, these studies indicate that hyperinsulinemia can produce insulin resistance. Despite the compelling evidence provided above, the chronology of obesity, insulin resistance and hyperinsulinemia remain unclear and warrant further investigation into the molecular mechanisms that promote these phenotypes.

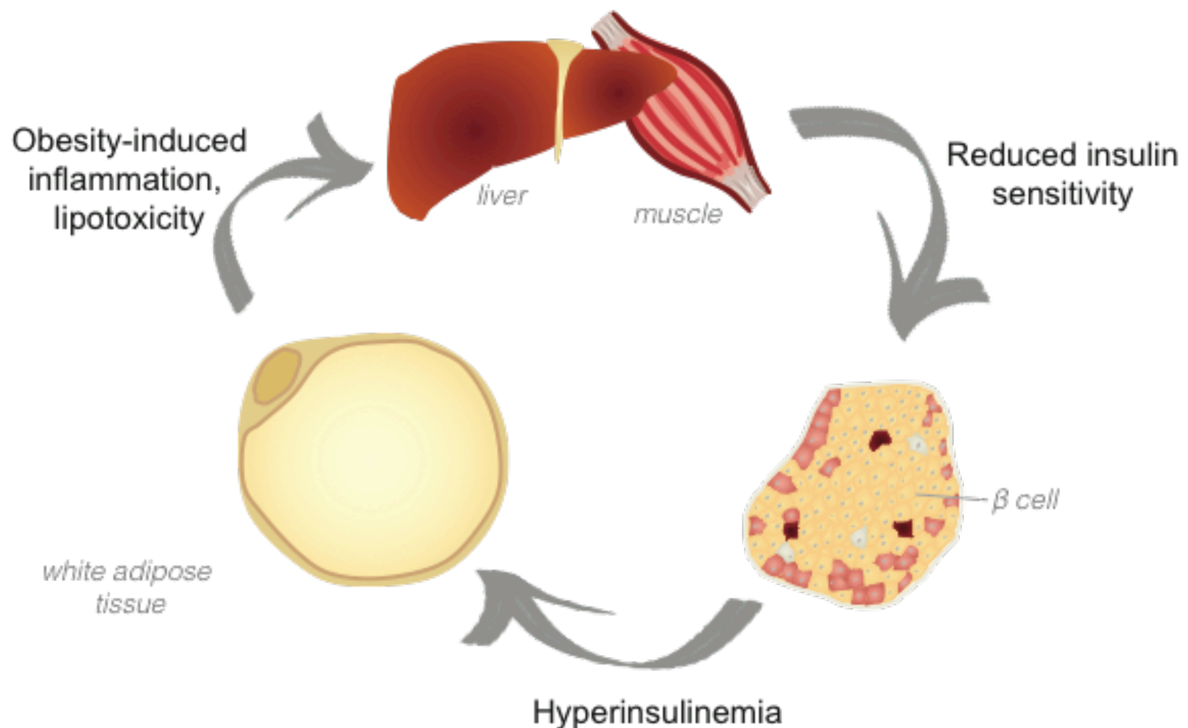


Figure 1.1: Proposed chronology of obesity, insulin resistance and hyperinsulinemia. Increased adiposity results in obesity-induced inflammation and lipotoxicity in peripheral tissues including liver and muscle. This in turn results in reduced insulin sensitivity in peripheral tissues. To compensate, pancreatic β cells secrete higher levels of insulin. Chronically elevated circulating insulin levels (hyperinsulinemia), promote increased lipogenesis of adipose tissue, contributing to increased fat mass.

1.3 Insulin

The outcome of diabetes was revolutionized through successful extraction and purification of a pancreatic substance over 100 years ago. This pancreatic extract effectively lowered blood glucose of diabetic individuals. Through the collaborative efforts of Banting, Best, Macleod and Collip, the substance secreted from the pancreas

into circulation in response to feeding was identified as insulin. Today, insulin remains a life-saving therapy for many individuals with Type 1 Diabetes (T1D), a disease that is caused by destruction of pancreatic β cells.

The insulin gene encodes a 110-amino acid precursor known as preproinsulin. Most animals only have one gene of insulin but in mice and rats, insulin is expressed as two distinct preproinsulin genes that differ in the number of introns and chromosomal locations [47]. In mice, the *Ins1* gene resides on chromosome 19 [48], while the *Ins2* gene resides on chromosome 7 [49]. Preproinsulin 2 (*Ins2*) is an ortholog to the insulin genes expressed in other mammals, while preproinsulin 1 (*Ins1*) is a rodent-specific retrogene and expresses one of the two introns found in *Ins2* [50]. Mammalian mature insulin is a 51-amino acid polypeptide hormone that is synthesized as a single precursor molecule, preproinsulin, which is post-translationally modified in the trans-Golgi to the mature hormone consisting of a 21 amino acid A-chain bound to a 30-amino acid B chain by disulfide bonds [50]. Cleavage of proinsulin in the Golgi yields mature insulin and C-peptide. Insulin along with C-peptide is stored in secretory granules within the β cell. Glucose plays a central role in insulin biosynthesis as it stimulates the transcription and mRNA translation of the insulin gene [51] .

In healthy subjects, pancreatic β cells release insulin in a precise manner to meet metabolic demand. In β cells, glucose sensors detect changes in plasma glucose concentrations due to the vast network of capillaries that are entangled in islets, and are able to respond accordingly to maintain blood glucose within a tight range. The ability of β cells to quickly respond to cellular signals is due to the availability of insulin granules. Insulin secretion from the pancreatic β cell is initiated by high glucose concentrations resulting in the closure of ATP-sensitive K+ channels (KATP). This triggers an increase

in intracellular [Ca^{2+}], resulting in calcium induced exocytosis of insulin [52]. Secretion of insulin is influenced by a number of circulating factors including: amino acids, fatty acids, growth hormones, glucose-dependent-insulinotropic polypeptide, glucagon-like peptide-1 and leptin.

Insulin exerts its major metabolic effects through insulin receptors (IR) located in the liver, white and brown adipose tissue, skeletal muscle, brain, α-cells and β-cells [53, 54]. The insulin receptor belongs to a subfamily of receptor tyrosine kinases that includes receptors for insulin-like growth factor I and II [55]. Insulin binding to the extracellular α-subunit of the IR induces a conformational change in the receptor molecule and brings the two β-subunits into close opposition. Insulin receptor activation initiates a cascade of phosphorylation events that regulates enzymes involved in growth and metabolism [56].

Insulin is a critical hormone involved in controlling energy and glucose homeostasis. Tight control of blood glucose is achieved by the opposing efforts of insulin, secreted from β cells, and glucagon, secreted from α cells. Once secreted, insulin promotes glucose uptake via Glut-4 receptors into skeletal muscle, and adipose tissue [57, 58]. In the liver, insulin action results in suppression of hepatic glucose production [59, 60]. Insulin has anabolic actions including storage of substrates as glycogen and fat, promotion of cell growth and differentiation, and inhibition of lipolysis, glycogenolysis and protein breakdown [61]. In addition to its role in glucose metabolism, insulin is also a potent growth factor. Insulin promotes uptake of amino acids and protein synthesis in muscle, liver and adipose tissue. In adipose tissue, insulin regulates lipid metabolism by decreasing the rate of lipolysis and stimulating the synthesis of

triglycerides [62-64]. Collectively, these data demonstrate that insulin is pivotal to glucose homeostasis and growth.

1.4 Leptin

The idea that a circulating factor could regulate body weight first came to light over 50 years ago with the discovery of two mutant mouse colonies exhibiting severe obesity at the Jackson Laboratories [65, 66]. These colonies were named *obese (ob)* and *diabetic (db)* due to dramatically increased body mass and the propensity to develop diabetes, respectively. Mice from both colonies are extremely obese, hyperinsulinemic and have elevated circulating blood glucose levels [67]. Parabiosis experiments revealed that *ob/ob* mice experienced weight loss, decreased feeding, and reduced blood glucose levels when they share continuous circulation with wild type mice, suggesting they are missing a circulating factor that regulates body weight and food intake [68]. In contrast, *db/db* mice did not experience weight loss when parabiosed to wild type mice, suggesting that they do not respond to the circulating factor [68]. More than 20 years later, it was discovered through positional cloning that the *ob* gene originally described by Coleman et al. encodes leptin [69], while the *db* gene encodes the leptin receptor [70]. Therefore *ob/ob* mice have a mutation in the gene encoding leptin resulting in leptin deficiency, while *db/db* mice have a mutation in the gene encoding the leptin receptor.

In humans and rodents leptin is a 167 amino acid protein secreted primarily from white adipose tissue [69] into the blood stream and is able to cross the blood brain barrier to enter the cerebrospinal fluid [71]. The *ob* gene consists of three exons spanning 3.5 kb in size. Expression of the *ob* gene is highest in the gonadal and

subcutaneous white adipose tissue [72]. Expression of leptin in adipose tissue is regulated by several hormones including insulin, epinephrine, growth hormone and dexamethasone [73], as well as by fasting or feeding [74, 75]. Leptin levels are reduced in states of low insulin, such as fasting and streptozotocin-induced diabetes [76, 77]. In contrast, acute or chronic elevations of insulin contribute to a rise in leptin levels [78, 79]. Similarly, mouse and rat adipocytes treated with insulin demonstrated enhanced leptin secretion [73, 80].

Leptin is also expressed in skeletal muscle, pancreas, stomach, hypothalamus and pituitary gland [81] however the relative contribution from these tissues to total circulating leptin levels is unclear. In muscle and pancreas, leptin expression is stimulated by nutrient excess, including conditions of hyperglycemia and hyperlipidemia [82, 83]. Similarly, nutrient availability is also believed to regulate leptin expression in the hypothalamus and pituitary gland [84, 85]. In the stomach, leptin expression is localized to the fundic epithelium. Leptin expression in the stomach is diminished by fasting as well as administration of the biologically active end of the peptide hormone cholecystokinin (CCK8) [86].

Human leptin protein is highly homologous to mouse leptin (84% homology) and rat leptin (83% homology) [69, 87]. In humans and rodents without mutations in the leptin gene, leptin levels generally correlate with total amount of body fat, except during fasting [88]. In normal healthy male mice, the half-life of leptin, based on injection of exogenous leptin, was determined to be 40.2 minutes [89]. Circulating leptin levels are similar between lean humans, rats, and mice typically ranging between ~5 – 15 ng/mL [74, 90, 91], ~0.5 – 5 ng/mL [92, 93], and ~1 – 10 ng/mL [94, 95] respectively. The majority of leptin in circulation is bound to the leptin soluble receptor protein [96, 97]. It

has been hypothesized that the free form of leptin is more physiologically active than the bound form [98]. Thus the bound form of leptin may function as a reservoir of inactive leptin to counteract a decline in active (free) hormone concentrations.

1.4.1 The leptin receptor

Following the discovery of the gene encoding leptin, Tartaglia et al. successfully identified the leptin receptor (*Lepr*) through expression cloning of the mouse choroid plexus [99]. Leptin exerts its effects on metabolism through binding to specific leptin receptors located throughout the central nervous system (CNS) as well as leptin receptors expressed in peripheral tissues The leptin receptor is a member of the interleukin-6-type cytokine receptor family and consists of an extracellular domain, a single membrane-spanning domain, and an intracellular domain [70, 100]. Leptin receptors do not contain intrinsic enzymatic activity but signal through Janus kinase 2 (JAK2) tyrosine kinase. JAK2 activation subsequently autophosphorylates multiple tyrosine kinase residues on the leptin receptor including Tyr^{985}, Tyr^{1077}, Tyr^{1138} in rodents. Activation of these tyrosine residues creates a binding site on the leptin receptor for the signal transduction and activators of transcription (STAT) molecules [101, 102]. Phosphorylation of STAT3 results in dimerization and translocation into the nucleus where it acts as a transcription factor for target genes such as suppressor of cytokine signalling 3 (SOCS3), resulting in suppression of leptin signalling [103-105].

Mutations of the specific tyrosine kinase binding sites on the leptin receptor were used to identify their downstream effects upon leptin binding. In mice, mutation of Tyr^{1107} of the leptin receptor resulted in modest increases in adiposity but had no effect on glucose tolerance or insulin levels [106]. Disruption of Tyr^{1138}, the binding site for

STAT3, resulted in obesity and hyperphagia in mice, although these mice remained 10-20% lighter than *db/db* controls [107]. In addition, these mice developed hyperglycemia, but blood glucose levels were ~50% lower than *db/db* controls [107], while glucose tolerance and insulin sensitivity were not altered [108]. However, when these mice were injected with streptozotocin (STZ) to induce insulin-deficient diabetes, the glucose lowering action of leptin was completely blocked [109]. These findings suggest that Tyr^{1138}-mediated STAT3 activation regulates body weight and food intake, but the degree to which it is involved in glucose homeostasis may differ between hyper- or hypo-insulinemic conditions. Deletion of STAT3 from the hypothalamus and β-cell using the Cre-*loxP* system resulted in impaired glucose tolerance but only mild increases in body weight and blood glucose levels [110]. Furthermore, injection of leptin and a STAT3 inhibitor into the brain prevented leptin-mediated reductions in body weight, food intake, and glucose production [101]. In contrast, mutation of Tyr^{985} of the leptin receptor, which recruits SOCS3, resulted in a lean phenotype in mice [111]. These mice also had a greater leptin-mediated reduction in body weight compared to controls, indicating enhanced leptin sensitivity. Similarly Howard et al. reported enhanced leptin sensitivity in SOCS3 haploinsufficient mice, suggesting that SOCS3 expression is a critical determinant of leptin sensitivity [112]. Collectively, these studies demonstrate that STAT3 mediates the effect of leptin on lowering body weight and normalizing glucose homeostasis, which is inhibited by activation of SOCS3.

In mice the leptin receptor gene is alternatively spliced to produce six isoforms, Lepr-a to Lepr-f. All isoforms, with the exception of Lepr-e, have identical extracellular and trans membrane domains but differ in length of the intracellular tail. The long form of the leptin receptor (Lepr-b) is the only isoform capable of JAK/STAT signalling and is the

major mediator of the metabolic effects of leptin in mice and humans [113, 114]. The Lepr-b isoform is distributed in both the central nervous system (CNS) [103, 115] and the periphery [116-119]. In addition to Lepr-b signalling, leptin action in the CNS is also dependent upon transport across the blood brain barrier via the Lepr-a isoform [120]. Lepr-a mRNA is highly expressed in cerebral microvessels suggesting that the Lepr-a isoform plays a role in the transport of leptin from the blood into the brain [121]. Deletion of exon 19a of the *Lepr* gene, which encodes the Lepr-a isoform, produced a mild enhancement of glucose tolerance beyond healthy controls and prevented weight loss in response to exogenous systemic leptin [122]. These findings suggest a potential role of Lepr-a in disrupting glucose regulation. Whether the other isoforms of the leptin receptor also affect glucose regulation is currently unknown and warrants further investigation.

The discovery of leptin generated considerable interest in its potential to cure obesity. However, it was soon discovered that leptin is an ineffective therapy to treat obese individuals [123]. Obese individuals and wildtype mice fed a high fat diet exhibit high levels of leptin expression in adipose tissue and have elevated circulating leptin levels [124, 125]. These high levels of leptin fail to maintain normal body weight, while exogenous leptin administration has little to no effect on body fat in obese mice and some obese subjects, indicating leptin resistance [123, 126]. Leptin insensitivity remains a barrier for the use of leptin to combat obesity. While the precise mechanisms contributing to leptin resistance are unclear, they may include increased SOCS3 activity [103, 112], reduced transport of leptin across the blood brain barrier [127, 128] as well as increased action of protein tyrosine phosphatase 1B (PTP1B), which dephosphorylates JAK2 thereby inhibiting leptin activity [129].

1.4.2 Leptin and energy homeostasis

The long (Lepr-b) isoform of the leptin receptor is highly expressed in the hypothalamus and other brain regions where it regulates energy expenditure and food intake. Leptin can modulate several aspects of feeding behaviour such as meal size [130], food reward [131, 132], and food preference [133]. In the arcuate nucleus of the hypothalamus, leptin activates the anorexigenic neurons that synthesize pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), and inhibits orexigenic neurons that synthesize agouti-related peptide (AgRP) and neuropeptide Y (NPY) [134]. With fasting, a decline in leptin levels results in expression of AgRP and NPY and reduction of POMC and CART, thereby increasing food intake and decreasing energy expenditure [135]. The ventromedial hypothalamus (VMH) appears to be another important site of leptin action. Loss of Lepr-b in high-fat diet fed mice results in more severe obesity and lower energy expenditure compared to mice expressing Lepr-b in the VMH [136]. Leptin levels are reduced with fasting and replacement of leptin during fasting can result in a blunting of the endocrine, behavioural and autonomic responses induced by fasting, suggesting that leptin levels are a key signal in the brain that regulates energy homeostasis [76]. The significance of leptin in energy homeostasis is most obvious in leptin deficient *ob/ob* mice. These mice rapidly become obese, and develop severe hyperphagia, low metabolic rate and high expression of NPY and MCH and low expression of POMC. Upon leptin treatment, these features are reversed and coincide with reduction of fasting glucose levels and insulin levels [137].

1.4.3 Leptin and glucose metabolism

In *ob/ob* mice, the improvements to fasting glucose levels following leptin therapy were initially attributed to reduced food intake and body weight, however several observations indicate that leptin can influence glucose homeostasis independent of its effects on body weight. Pair feeding *ob/ob* mice to consume the same amount of food as leptin-treated *ob/ob* mice did not improve glucose or plasma insulin to the same degree as leptin treatment [138]. Similarly, a low dose of leptin (1 mg/kg/day) was sufficient to normalize blood glucose and insulin levels without resulting in weight loss [137]. Furthermore, an acute disruption of leptin signalling using a leptin antagonist produced elevated plasma insulin and blood glucose levels prior to observed increases in body weight [139]. In addition, hyperinsulinemia precedes obesity in *ob/ob* and *db/db* mice, suggesting that impairments to glucose regulation occur distinctly from weight gain [140, 141]. Insulin deficient rodents, which had depleted white adipose tissue (WAT) depots, exhibited hyperglycemia, insulin resistance, and impaired glucose tolerance, all of which were normalized by leptin therapy [93, 94, 142-148]. Lastly, the hyperglycemia and insulin resistance that is present in lipodystrophic rodents and humans is corrected by leptin therapy [149-151]. Collectively, these findings demonstrate that leptin can regulate glucose homeostasis independent of its effects on body weight.

The central nervous system is proposed as an important site mediating the glucose-lowering effects of leptin. Intracerebroventricular (ICV) injection of leptin restores euglycemia and insulin sensitivity in *ob/ob* mice [152] and high fat fed mice [153], providing evidence that the CNS mediates the glucose-lowering actions of leptin. Within the CNS, the arcuate nucleus (ARC) plays a key role in regulation of blood

glucose via leptin. Restoration of leptin signalling in the ARC of leptin receptor null mice leads to marked improvements in hyperinsulinemia and normalized blood glucose [154]. Similarly, POMC and AgRP neurons of the hypothalamus also contribute to leptin-mediated lowering of blood glucose independent of changes in food intake and body weight [155]. While the mechanisms involved in this action have yet to be clearly elucidated, it has been suggested that leptin action in the ARC contributes to glucose homeostasis by improving insulin sensitivity [155, 156] and increasing glucose uptake in brown adipose tissue (BAT) [157].

In peripheral tissues, leptin acts both through indirect effects mediated by the CNS as well direct action through receptor expression within these tissues. The liver is a key organ involved in regulation of glucose homeostasis as it is a major site for glycogen synthesis and gluconeogenesis. In lean and *ob/ob* mice, ICV injection of leptin results in enhanced insulin-mediated suppression of hepatic glucose production [158, 159]. In mice rendered diabetic by streptozotocin, leptin therapy resulted in increased glycogen synthesis [145], resulting in an overall decrease in hepatic glucose output. In contrast to the indirect actions of leptin via the CNS, loss of direct leptin action via genetic ablation of leptin receptors that are expressed in rodent liver [114, 160] did not significantly alter glucose homeostasis [160]. Indeed loss of hepatic leptin receptors in high fat fed mice protected against glucose intolerance due to high fat diet and enhanced hepatic insulin sensitivity [160].

Leptin receptors have also been detected in white (WAT) and brown (BAT) adipose tissue [116, 161]. In cultured BAT leptin appears to counteract insulin action, resulting in inhibition of insulin-stimulated glucose uptake [162]. Similarly, in cultured WAT, leptin signalling results in inhibition of insulin signalling, leading to impaired

glycogen synthesis and phosphorylation of the insulin receptor (IR) [163, 164]. Interestingly, systemic leptin therapy has opposing effects in BAT and WAT. In BAT, leptin therapy in STZ-diabetic mice results in enhanced glucose uptake [165] while systemic leptin administration does not increase and may even suppress glucose uptake in WAT [158, 166-168]. The discrepancies between cultured and *in vivo* effects of leptin on BAT suggest that the CNS may mediate the enhanced glucose uptake observed *in vivo*. In WAT, it appears that leptin acts both directly and indirectly to offset insulin action.

In addition, leptin can also modulate secretion and expression of insulin in pancreatic β cells [169-171]. *In vitro*, leptin treatment of cultured islets or insulinoma cells results in suppression of insulin secretion and gene expression [172-174]. The proposed mechanism of the inhibitory role of leptin on insulin secretion occurs through controlling ATP-sensitive K+ channels in β cells [170]. A summary of the effects of leptin on glucose metabolism is outlined in Figure 1.2.

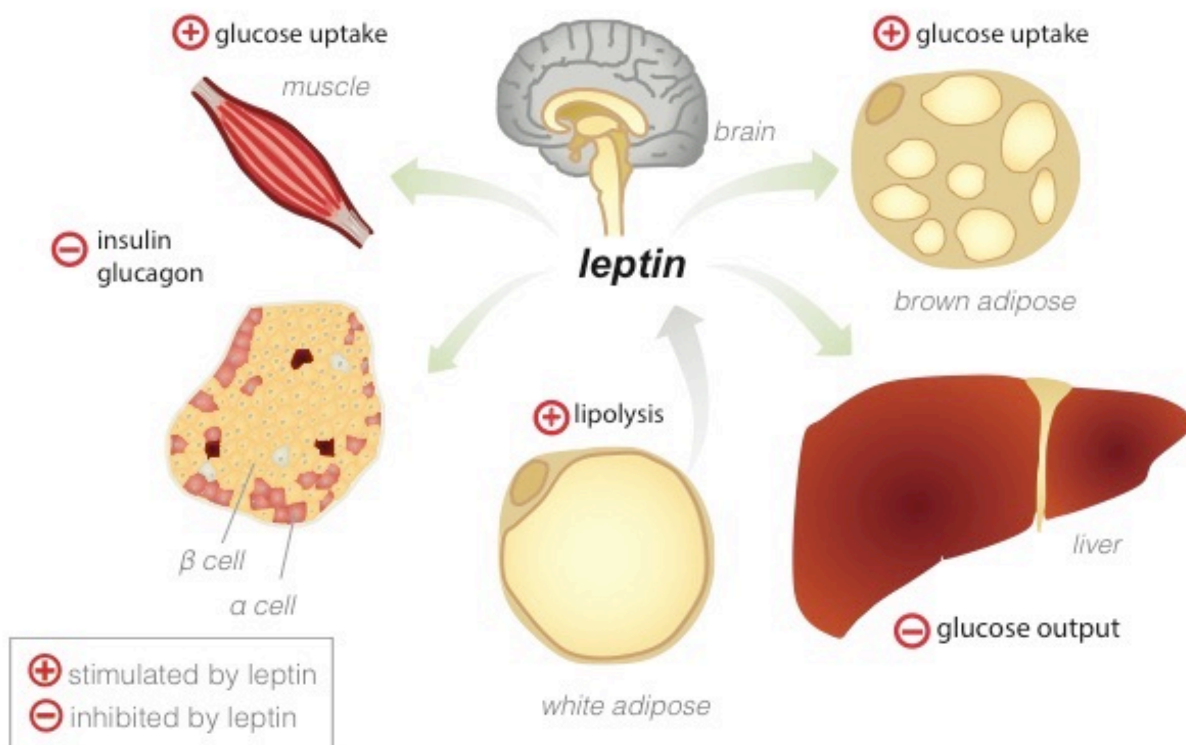


Figure 1.2: Glucoregulatory actions of leptin. Leptin is secreted primarily from white adipose tissue and can regulate blood glucose through direct action on peripheral tissues or indirectly through the CNS. Here we illustrate the main *in vivo* effects of leptin in lean or obese conditions. The effects of leptin may be inhibitory (red circle with minus sign) or stimulatory (red circle with plus sign). In lean or leptin deficient rodents, leptin stimulates glucose uptake in muscle and brown adipose tissue and suppresses release of insulin and glucagon secreted from β cells and α cells, respectively. In addition, leptin increases lipolysis in white adipose tissue, and causes an overall reduction in glucose output from the liver. (Figure adapted from D'souza et al. (2017) *Molecular Metabolism*, in press).

1.4.4 Leptin as a signal of energy deficiency

Accumulating evidence suggests that leptin serves as an indicator of energy deficiency rather than energy excess [175]. To protect the body in conditions of starvation, several physiological adaptations occur to divert energy away from the

processes not essential for survival. These include decreased reproductive function, decreased immune function and reduction of hormones involved in growth such as thyroid stimulating hormone (TSH). Interestingly, these changes are observed in leptin-deficient *ob/ob* mice [176-180]. Similarly, in patients with congenital leptin deficiency, several neuroendocrine disorders are present including hypogonadotrophic gonadism, [181]. Repletion of leptin in humans with leptin deficiency results in normal pubertal development [182], as well as increased IGF-1 and IGF-binding protein levels [183]. Energy deprivation also negatively affects immune function as individuals with congenital leptin deficiency have a higher incidence of infection than their peers due to defects in T-cell function [184]. In contrast, leptin replacement in leptin-deficient individuals improves cytokine levels [185] and results in increased inflammatory and platelet responses [186]. Together, these data provide compelling evidence that a decrease in leptin levels is an important signal conveying the need to conserve energy in times of starvation.

1.4.5 Phenotype of rodents and humans lacking functional leptin signalling

Our understanding of the role of leptin in metabolism has been greatly enhanced through assessment of rodents with spontaneous mutations of the gene encoding leptin (*ob/ob* mouse) or the leptin receptor (*db/db* mouse, Zucker fatty rat, obese Koletskey rat). These rodents have been used extensively in the study of the pathogenesis of obesity and diabetes [187, 188], and are also commonly used in drug discovery and testing of glucose lowering agents, insulin sensitizers, insulin secretagogues, and anti-obesity agents [189-191]. Table 1.1 summarizes the various rodent models along with the major phenotypes that arise from impaired leptin signalling. Common characteristics

that are observed in rodents with impaired leptin signalling include: obesity, hyperphagia, insulin resistance, glucose intolerance, dyslipidemia, and transient or overt hyperglycemia, depending on the strain and age of the animal [65, 67, 140].

Similar to rodents, humans with congenital leptin deficiency or mutations of their leptin receptor also develop obesity hyperphagia, dyslipidemia and hyperinsulinemia [192-194]. However, unlike rodents with impaired leptin receptor signalling, humans with mutations to their leptin receptors do not develop hyperglycemia. Furthermore, while *ob/ob* mice have non-linear growth, suppression of the growth hormone axis, and marked elevations of ACTH and corticosterone levels, humans with congenital leptin deficiency have normal linear growth and adrenal function [194, 195]. Understanding the discrepancies between rodent models of impaired leptin signalling and humans with spontaneous mutations to leptin or its receptor is essential in determining the metabolic actions of leptin.

Table 1.1: Summary of rodent models of impaired leptin signalling

Model name	Mutation	Hyperinsulinemia	Hyperglycemia	Insulin resistance	Obesity
ob/ob mouse	Nonsense mutation in codon 105 of *lep* resulting in truncated, biologically inactive leptin protein	Yes, early onset	age/strain dependent	Yes	Yes, early onset[1]
db/db mouse	Frame shift mutation of *Lepr* resulting in replacement of Lepr-b with Lepr-a	Yes, early onset	Yes strain dependent	Yes	Yes, early onset
Zucker fatty rat (*fa/fa*)	Missense mutation in *Lepr* gene resulting in glutamine to proline change in all isoforms of Lepr protein resulting in non-functional protein	Yes	No	Yes	Yes, early onset
Zucker diabetic fatty rat	Same mutation for Lepr but also carry an autosomal recessive defect in β cell transcription	Yes	Yes More severe in males than females	Yes	Yes, early onset
Koletsky corpulent rat	Autosomal recessive mutation of *Lepr* resulting in premature stop codon and truncation of extracellular domain of the leptin receptor.	Yes	Normal fasting glucose, post prandial hyperglycemia	Yes	Yes, early onset
Kilorat®	151 bp deletion in exon 1 of leptin gene resulting in leptin deficiency	Yes, early onset	Transiently fasting hyperglycemia observed in males	Yes, occurs early in life	Yes, early onset

[69, 70, 92, 113, 140, 141, 196-214]

[1] Early onset refers to onset prior to 6 weeks of age

1.5 Thesis Investigation

The aim of this thesis was to investigate the glucoregulatory actions of leptin. Prior studies have suggested that glucose homeostasis is achieved in part through the inhibitory actions of leptin on pancreatic β cells. Indeed, hyperinsulinemia is among the first metabolic disturbances that arise in leptin deficient *ob/ob* mice and is associated with obesity, insulin resistance and glucose intolerance [208].

In Chapter 3, we investigate whether suppression of hyperinsulinemia in leptin deficient *ob/ob* mice is sufficient to prevent impairments to glucose and energy homeostasis that have been observed in *ob/ob* mice. It has been suggested that hyperinsulinemia arises due to the absence of leptin signalling in β cells of mice. In Chapter 4, we sought to determine whether restoration of leptin receptors exclusively in β cells of mice lacking leptin receptors elsewhere is sufficient to prevent hyperinsulinemia.

We next wanted to investigate whether the metabolic phenotype that arises from leptin deficiency in *ob/ob* mice is conserved in rats, which are more metabolically similar to humans. To do so, in Chapter 5 we characterized glucose homeostasis in rats with a genetic mutation of the leptin gene resulting in leptin deficiency. In Chapter 6, we sought to determine whether the impairments to glucose homeostasis that arise in leptin deficient rats occur independent of obesity. To do so, we assessed glucose tolerance, insulin sensitivity and fast insulin levels in during the suckling period in rats, a period in development where body weight is comparable between rats with and without leptin.

Lastly, in the rodent lines used above to assess the effects of leptin on glucose homeostasis, the impairment to leptin signalling occurred through development, thus

making it difficult to delineate the direct effects of leptin on glucose homeostasis independent of its effects on development. In Chapter 7, we attempt to overcome this by generating and characterizing mice in which leptin expression was deleted by inducing Cre activation in adulthood.

CHAPTER 2: MATERIALS & METHODS

2.1 Animals

All procedures with animals were approved by the University of British Columbia (UBC) Animal Care Committee and carried out in accordance with the Canadian Council on Animal Care guidelines. Most *in vivo* studies involving the adult leptin knockout rats were performed at the Animal Research Unit (ARU), a conventional animal facility located on the UBC Point Grey campus. All studies involving *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice were performed in the barrier zone of the Modified Barrier Facility (MBF), while all studies on *ob/ob* mice with reduced insulin gene dosage, *Lep*$^{flox/flox}$*ROSA26*CreER^{T2} and *Lep*$^{flox/flox}$*ACTB*Cre mice were performed in the Center for Disease Modelling (CDM), a specific-pathogen free facility. In all facilities, rodents were housed at ambient temperature (21-25°C) on a 12 h light: 12 h dark cycle in filter-top cages containing EcoFRESH bedding (Absorption Corp, Ferndale, USA). Mice were group housed (3-5 mice per cage) except if separated due to fighting. Adult rats were housed in groups of 2-4 in filter top cages with Betachip bedding (Northeastern Products, Warrensburg, USA). All mice and rats housed in CDM and ARU had *ad libitum* access to irradiated standard chow (2918, Harlan laboratories, Indianapolis, USA) and water. Mice housed in MBF had *ad libitum* access to standard chow (5053, PicoLab Rodent Diet 20, LabDiet, St. Louis, USA) and water.

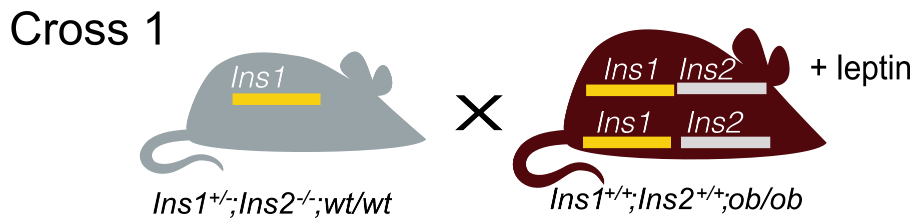
2.1.1 Leptin knockout rats

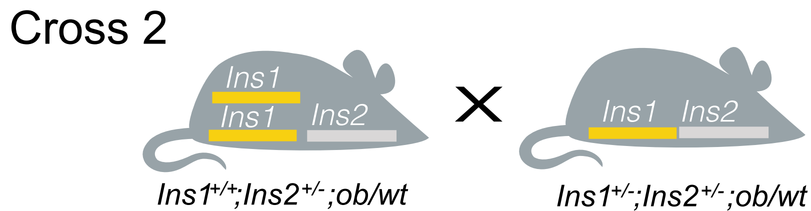
Leptin knockout rats, which have a 151 bp deletion in exon 1 of the leptin gene, were obtained from Sigma SAGE labs [213]. Male and female rats heterozygous for the mutant leptin gene were crossed together to generate homozygous knockout (*Lep*^{KO})

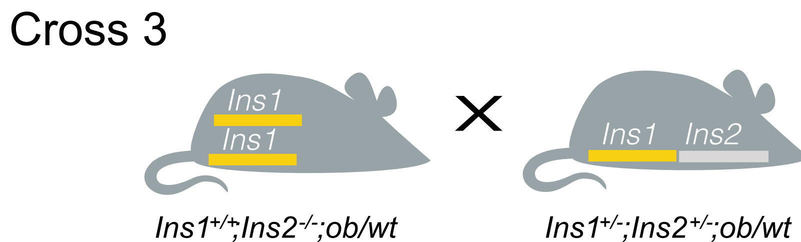
and homozygous wildtype (*Lep*^{WT}) littermates. For all experiments, male and female *Lep*^{KO} rats were compared to their *Lep*^{WT} littermate controls to minimize differences in genetic background, which can affect the phenotype of leptin deficiency [206].

2.1.2 Generation of ob/ob with reduced insulin gene dosage

Male C57Bl/6J mice homozygous for a mutation in the gene encoding leptin (*ob/ob*, JAX no. 000632, C57BL/6J) were implanted with 14-day mini osmotic pumps (0.5 μg/day murine leptin) to overcome sterility. These mice were then bred with female mice lacking 1 copy of *Ins1* and both copies of *Ins2* (*Ins1*$^{+/-}$;*Ins2*$^{-/-}$; 90% C57BJ6/J, 10% 129) kindly provided by Dr. James Johnson. The offspring from these mice (*Ins1*$^{+/-}$;*Ins2*$^{+/-}$;*ob/wt and Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/wt*) were bred to generate male and female *Ins1*$^{+/-}$;*Ins2*$^{+/-}$;*ob/wt and Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/wt* mice. These mice were then crossed together to generate mice lacking 2 (*Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob and Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*wt/wt*) or 3 insulin alleles (*Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob and Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*wt/wt*); refer to Fig. 2.1 for breeding scheme. Because it was not possible to efficiently generate *ob/ob* littermates with all four copies of insulin using the breeding strategy described above, littermates lacking 1 insulin allele (*Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* and *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*wt/wt*) were used as experimental controls. For all experiments, *ob/ob* mice of varying insulin dosage were compared with leptin expressing littermates (*wt/wt*) of matching insulin gene dosage.







Experimental Groups

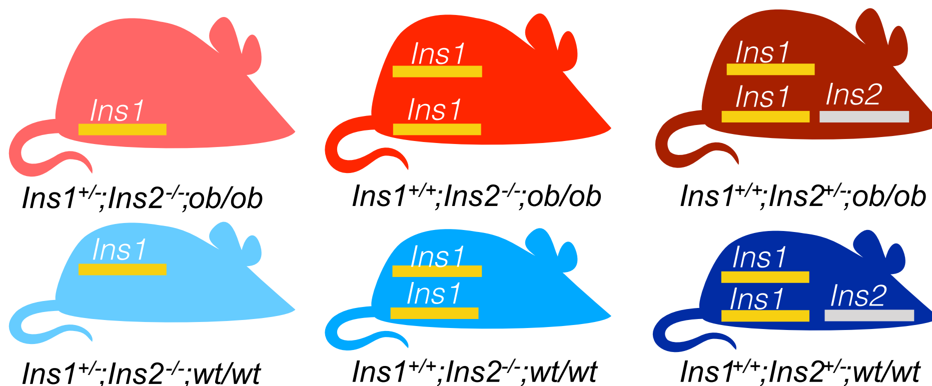


Figure 2.1 Breeding scheme to generate *ob/ob* and *wt/wt* mice lacking 1, 2 or 3 insulin alleles. A mini osmotic pump releasing 0.5 µg/day murine leptin was implanted in a male *ob/ob* mice to enable breeding with female *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*wt/wt* mice for the first of three crosses used to generate experimental groups. *Ob/ob* mice and *wt/wt* littermates lacking 1, 2 or 3 insulin alleles were generated by crossing *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/wt* mice with *Ins1*$^{+/-}$;*Ins2*$^{+/-}$;*ob/wt* mice.

2.1.3 Generation of $Lepr^{loxTB/loxTB}$ Ins1Cre mice

$Lepr^{loxTB/wt}$ mice (JAX no. 018989, 50% FVB, 50% C57BL/6J, <1% 129) and *Ins1*Cre mice (JAX no. 026801, C57BL/6J), in which Cre is knocked into a single *Ins1* allele, were obtained from Jackson Laboratory (Bar Harbour, ME, USA). *$Lepr^{loxTB/loxTB}$* mice contain a transcriptional blockade upstream of exon 17 of the leptin receptor gene, resulting in the *$Lepr^{loxTB}$* allele. Upon Cre-mediated recombination, the transcriptional blockade that resides upstream of exon 17 of the leptin receptor gene is excised, resulting in transcription of the full leptin receptor gene and production of *Lepr* (Fig. 2.2) [156]. The *Ins1*Cre was generated by knock-in of Cre into one allele of the *Ins1* gene, which is exclusively expressed in the β cells [215]. *$Lepr^{loxTB/wt}$* mice were bred with *Ins1*Cre mice for two generations to yield *$Lepr^{loxTB/loxTB}$ Ins1*Cre, *$Lepr^{loxTB/loxTB}$*, *$Lepr^{wt/wt}$ Ins1*cre, and *$Lepr^{wt/wt}$* mice on a B6.FVB hybrid background. Offspring were born at the expected Mendelian ratio. The result of the cre-mediated recombination is re-expression of the *Lepr* only in β cells of *$Lepr^{loxTB/loxTB}$ Ins1*Cre mice.

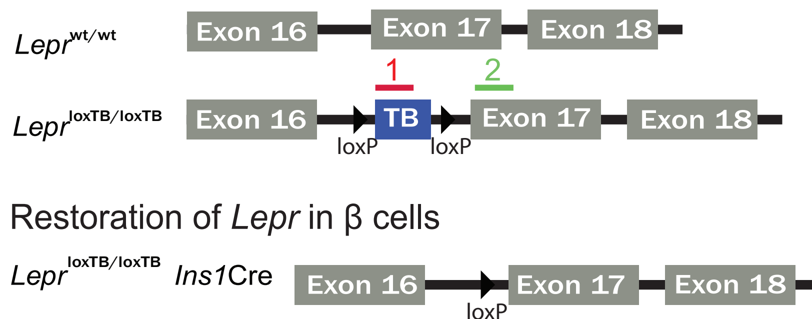


Figure 2.2: Schematic of genetic manipulation of Lepr gene in *$Lepr^{loxTB/loxTB}$* mice. Insertion of *loxP* sites flanking a transcriptional blockade (TB) upstream of Exon 17 results in impaired leptin receptor signalling. Cre-mediated excision of the TB results in restored *Lepr*. To determine the degree of Cre-mediated recombination, the ratio of PCR product between primers expressed within the TB (primer set '1') and downstream of the TB (primer set '2') was compared.

*2.1.4 mT/mG Ins1*Cre *mice*

To assess the specificity of *Ins1*Cre mediated recombination, *mT/mG* reporter mice (B6.129(Cg)-*Gt(ROSA)26Sor*$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J JAX no. 007676, B6.129, Jackson Laboratory) which possess a membrane-bound Tomato fluorescent protein (mT) flanked by two unidirectional *loxP* sites, followed by the open reading frame for membrane bound enhanced green fluorescent protein (EGFP, mG) driven by the chicken β actin promoter [216], were crossed with *Ins1*Cre mice for 2 generations to create homozygous *mT/mG* mice with or without 1 allele for *Ins1*Cre. Homozygous *mT/mG* mice were crossed with heterozygous Ins1Cre mice to generate offspring, 50% mT/mG Ins1Cre and 50% *mT/mG* only (no Cre controls). Cells that were marked by EGFP fluorescence indicated *Ins1*Cre activity in mice homozygous for *mT/mG*.

2.1.5 Lep$^{flox/flox}$*ACTBCre mice, Lep*$^{flox/flox}$*ROSA26CreER*^{T2} *mice*

Lep$^{flox/flox}$ mice (tm1a-EUCOMM-Hmgu) on a C56BL/6N were obtained from Dr. Richard Seavitt at the Baylor College of Medicine (Houston, Texas). The *Lep*$^{flox/flox}$ allele contains 2 unidirectional *loxP* sites flanking exon 3 of the leptin gene. Upon Cre-mediated recombination, the UTR region of exon 3 is excised, resulting in a frame-shift mutation and truncated, leptin protein.

Mice harbouring the human β actin transgene to ubiquitously express Cre [217] (*ACTB*Cre mice;) on a C57BL/6N background were obtained from a breeding colony at the Center for Molecular Medicine and Therapeutics (Vancouver, BC) and were mated with *Lep*$^{flox/flox}$ mice to generate *Lep*$^{flox/wt}$ mice with or without the *ACTB*Cre transgene. The offspring from this cross (50% *Lep*$^{flox/wt}$ *ACTB*Cre, 50% *Lep*$^{flox/wt}$ were mated to produced *Lep*$^{flox/flox}$ *ACTB*Cre, *Lep*$^{flox/flox}$, *Lep*$^{wt/wt}$ and *Lep*$^{wt/wt}$ *ACTB*Cre littermate

controls. Mice expressing the CreER^{T2} transgene driven by the tamoxifen inducible, ubiquitously expressed *ROSA26* promoter [218] (B6.129-Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J; #008463 Jackson Laboratories) were crossed with *Lep*$^{flox/flox}$ mice. Offspring from this cross (50% *Lep*$^{flox/wt}$ *ROSA26*CreER^{T2}, 50% *Lep*$^{flox/wt}$ were mated together to produce *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2}, as well as *Lep*$^{flox/flox}$, *Lep*$^{wt/wt}$ *ROSA26*CreER^{T2} and *Lep*$^{wt/wt}$ littermate controls.

2.2 Genotyping

Genomic DNA (gDNA) was extracted from mouse ear biopsies using a Chelex digest solution. Ear notches were incubated in 112.5 mg/mL Chelex 100 (BioRad, Mississauga, Canada), 0.1% Tween-20 (Fisher Scientific, Ottawa, Canada) and 0.1 mg/mL proteinase K (Fisher Scientific) at 55°C for 50 minutes followed by 10 minutes of heat inactivation at 95°C. Supernatant (2 μL) was used as a template for PCR based genotyping. Primers used to genotype the various mouse and rat strains used in this thesis investigation are outlined in Table 2.1.

2.2.1 Verification of cre-mediated recombination

To verify Cre-mediated recombination in *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice, gDNA was extracted from isolated islets using a salt homogenization buffer. Prior to gDNA extraction, the salt buffer (0.4 N NaCl, 10 mM Tris-HCl (pH 8.0) and 2 mM EDTA) was combined with 10% SDS and 10 mg/ml proteinase K. Islets were then incubated in 400 μl complete salt homogenization buffer at 55°C for 1.5 h.

Table 2.1: Sequence of primers used for PCR and quantitative PCR

Gene name	Forward primer (5’-3’)	Reverse Primer (5’-3’)	Probe sequence (5’-3’)
Lep (rat)	AAGAAGAAGAAGACCCCAGCGAGGAAA	CTTATGGTTTCATCATGGGAGCATAGGACAAC	n/a
Ob (mouse)[1]	TGTCCAAGATGGACCAGACTC	ACTGGTCTGAGGCAGGGAGCA	
Ins1	TCAGTGCTGCACCAGCATCT	TCCAGATACTTGAATTATTCCTGGTGTTTTATCAC	n/a
Ins1 (neo)	ACAACGTCGAGCACAGCTGC	CAGGAAGCAGAATTCCAGATACTTGAATTATTCCT	n/a
Ins2	TGCTCAGCTACTCCTGACTGGATTTTC	GTGCAGCACTGATCTACAATGCCA	n/a
Ins2 (LacZ)	CTGTATGAACGGTCTGGTCTTT	CGCTATGACGGAACAGGTATT	
Lepr	GTGAGATCATGAGACCCTAAA	GGAACTCAAGACCATCTATCA	TTCTGAATTGGTGTCCCTGGAGCC
Ins1 Cre	TCCAGCGACTTTAGGGAGAATGTGG	GCATTGCTGTCACTTGGTCGTGG	n/a
mT/mG	CATATATGGAGTTCCGCGTTACA	GAAAGTCCCTATTGGCGTTACT	TAACTTACGGTAAATGGCCCGCCT
Lep$^{wt/wt}$	GGCAGCTAGGAGAAGTCAGATATTC	CTAGTCATACTCTTCTGTGGTTCTCC	n/a
Lep$^{flox/flox}$	GCAGAATAAATAAATATGAACTGATGGCGAG	CTAGTCATACTCTTCTGTGGTTCTCC	n/a
β-actin Cre	CTTCCTTTGTCCCCAATCTG	TCTTGCGAACCTCATCAC	ATTTGCCTGCATTACCGGTCGATG
ROSA26	TGATCTGCAACTCCAGTCTTTC	GGAAGTCTTGTCCCTCCAATTT	TTTAAGCCTGCCCAGAAGACTCCC
*CreER*T2	TGATCTGCAACTCCAGTCTTTC	GGTGTACGGTCAGTAAATTGG	CGCAGTAGTCCAGGGTTTCCTTGA

[1]PCR product is digested by *Dde*I to distinguish between *wt/wt*, *wt/ob* and *ob/ob* products.

Following addition of 6N salt and centrifugation, the supernatant was transferred to a new tube, washed in isopropanol and combined with glycogen to reveal the DNA pellet. The DNA pellet was then stored in isopropanol overnight followed by 3 washes with 70% ethanol. The purified gDNA was then re-suspended in buffer TE and stored at 4°C. Percent recombination of *Lepr* was measured using qPCR primers that flanked a region of intron 17 of *Lepr* (Forward: 5'CCT TTC CAG ATA ATG CCT GAT AGA-3', Reverse: 5'-GCA CCA CAC TTA GCT CCA ATA-3', Probe: 5'-TAG GGC GGA TGA ACC AGC AAA TGT-3') and primers that flanked a portion of the transcriptional blockade sequence inserted upstream of exon 17 of *Lepr* (Forward: 5'-CTG CAT TCT AGT TGT GGT TTG-3', Reverse: 5'-GTC TCA TGA GCG GAT ACA TAT T-3' Probe: 5'-TCA TGT CTG GAT CGC TTA GGT GGC A-3').

To verify Cre-mediated recombination of the *Lep*$^{flox/flox}$ allele, genomic DNA was extracted as described above from gonadal white adipose of *Lep*$^{flox/flox}$ *ACTB*Cre, and *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice. The degree of recombination was assessed by a multiplex qPCR assay comparing the ratio of fluorescent dye produced by amplified gDNA detected from the floxed region of the *ob* gene (Forward: 5'-AAG GCG CAT AAC GAT ACC AC-3', Reverse: 5'-GCT GAT GGC TTG CTT CAG AT-3', Probe (FAM): 5'-GCG CCG GAA CCC GTC GAG ATA A-3') to fluorescence generated by amplification of a sequence from exon 17 of the *Lepr* gene, a sequence not expected to change in response to Cre activity (Forward: 5'- GTG AGA TCA TGA GAC CCT AAA-3', Reverse: 5'-GGA ACT CAA GAC CAT CTA TCA-3', Probe (HEX): 5'-TTC TGA ATT GGT GTC CCT GGA GCC-3').

2.3 Blood and Plasma Analytes

All blood samples were collected from the saphenous vein from conscious, restrained rodents, unless otherwise specified. Blood was collected in capillary tubes containing heparin to achieve a final concentration of 4.0 U heparin per mL of whole blood. Plasma was separated by centrifugation at 4600 RCF for 9 min at 4°C. Blood glucose was measured via a One Touch Ultra Glucometer (Life Scan Inc., Burnaby, Canada) from the saphenous vein. Cardiac puncture was used to collect blood from deeply anesthetised rodents under isoflourane.

Plasma insulin levels were determined using the Rat Insulin Enzyme Linked Immunosorbent Assay (ELISA) Kit, the Mouse Ultrasensitive ELISA Kit or the Rodent Chemiluminescent Insulin ELISA Kit (ALPCO Diagnostics, Salem, USA). Leptin (Mouse Leptin ELISA, Crystal Chem Inc., Dowers Grove, USA), C-peptide (Rat C-peptide ELISA, ALPCO), adiponectin (Total Adiponectin ELISA, ALPCO), glucagon (Glucagon ELISA, Mercodia, Uppsala, Sweden), growth hormone (Rat/Mouse Growth Hormone ELISA, Millipore), and glucagon like peptide-1 (GLP-1) (Total GLP-1 kit, Meso Scale Discovery, Rockville, USA) were measured from plasma according to manufacturer instructions. Plasma free fatty acids were measured by the HR Series NEFA-HR (2) Kit (Wako Chemicals, Richmond, USA) and plasma triglycerides were measured by Serum Triglyceride Determination kit (Sigma-Aldrich St. Louis, USA). Both assays were scaled down to fit a 96-well plate format. If samples were above the linear range of detection, they were diluted and re-measured.

2.4 Analysis of Rodent Tissues

2.4.1 Hepatic lipid content

Liver tissue (100 mg) was collected from adult rats following a 4 h fast and immediately flash frozen in liquid nitrogen. Hepatic triglycerides and cholesterol were quantified by a modified protocol from Briaud et al [219]. Liver was homogenized using an Ultra-Turrax in 3 mL of chloroform:methanol (2:1) followed by addition of 1.5 ml of cold H_2O. The homogenized solution was centrifuged and the lipid portion was extracted. A portion of the organic layer (500 μL) was dried under N_2 followed by combination with 30 μL of Thesit (Sigma-Aldrich, Oakville, Canada) mixed with N_2. Following reconstitution of dried contents with water, samples were incubated for 30 minutes at 37°C with intermittent vortexing. Triglycerides and cholesterol were measured according to manufacturer instructions using the Serum Triglyceride Determination kit (Sigma-Aldrich, Oakville, Canada) and Cholesterol E kit (Wako Diagnostics, Richmond, USA), respectively.

2.4.2. Western blot analyses

Liver tissue (100 mg) was harvested from adult rats and immediately frozen in liquid nitrogen. Frozen liver tissue was homogenized in 1 mL lysis buffer (0.5 M Tris-HCl pH 8, 5 M NaCl, 0.5 M NaF, 0.5 M EDTA, 1% w/v NP-40 (Sigma-Aldrich, St. Louis, USA), protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA) and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, USA) and centrifuged for 30 minutes at 12,000 g. Protein concentration was determined using the bicinchoninic acid (BCA) assay method on the collected supernatant. Protein extracts were subject to SDS page (10% acrylamide gel, A3353 Sigma-Aldrich) separation and were subsequently

transferred onto a PVDF membrane. Membranes were blocked for 1 h at room temperature in 5% BSA followed by overnight incubation at 4°C with the following primary antibodies combined with 5% BSA: phosphorylated AKT at Ser-473 (#4060s, Cell Signaling, 1:1000 dilution, Danvers, USA), AKT (#7076S, Cell Signaling, 1:1000 dilution, Danvers USA), phosphorylated ERK (#9101, Cell Signaling, 1:1000 dilution, Danvers, USA), total ERK (#9102, Cell Signaling, 1:1000, Danvers, USA) and β-actin (#NB600-503, Novus Biologicals, 1:5,000 dilution). Secondary antibodies used were donkey-anti rabbit HRP (GE Life Sciences, #NA934, Little Chalfont, United Kingdom). Bands were detected using Amersham ECL Detection Reagent (GE Life Sciences, #RPN3004, Litte Chalfont, United Kingdom) and film exposure. Band density was quantified using AlphaView analysis software (ProteinSimple, Santa Clara, USA).

2.5 Body Composition & Metabolic Cages

2.5.1 Nuclear magnetic resonance

Total body fat and lean mass was measured in *Lep*^{KO} and *Lep*^{WT} rats using nuclear magnetic resonance (NMR) in collaboration with the NMR and magnetic resonance imaging (MRI) core in the Life Science Centre at the University of British Columbia (Vancouver, Canada). Lean to lipid mass was measured in anesthetized 18 week old male rats using a Bruker Biospec 70/30 7 Telsa MRI scanner (Bruker Biospin, Ettlingen, Germany). NMR signal from the body was acquired using a quadrature volume RF coil tune to 300 MHz. The lean and fat mass was calculated from the NMR data as previously described [220]. Images of fat distribution in 18-week-old male and

female rats were captured from the thoracic and abdominal region and acquired in 1 mm longitudinal sections using MRI.

2.5.2 Dual x-ray absorptiometry

Body fat and lean percentage were measured in mice and young (<3 weeks old) rats using dual x-ray absorptiometry (DEXA) (Lunar PIXImus densitometer, InsideOutside Sales, Madison, WI, USA) in collaboration with the laboratory of Dr. S. Clee. Mice were anaesthetized under isofluorane immediately prior to assessment.

2.5.3 Indirect calorimetry and metabolic cage analysis

PhenoMaster metabolic cages (TSE Systems, Chesterfield MO, USA) were used for measure of activity, food intake, respiratory exchange ratio (RER) and energy expenditure (indirect calorimetry) in male mice. At 5 weeks of age, male mice were housed individually and allowed to acclimate in training chambers for 4 days prior to placement in metabolic cages. The cages were placed in environmental control chambers at 21°C for measurement of oxygen and carbon dioxide gas exchange, physical activity (beam breaks) and food intake over 72 h; data from the first 24 h were discarded. Data from two full light and dark cycles were averaged.

2.6 Chemical Manipulations

2.6.1 Tamoxifen injections

To induce Cre-activation, mice received a daily i.p. (intraperitoneal) injection of tamoxifen (2 mg/day or 4 mg/day; Sigma-Aldrich) prepared in corn oil (Sigma-Aldrich) at a concentration of either 10 or 20 mg/mL. Tamoxifen was dissolved in corn oil to

produce a 20 mg/ml solution that was filter sterilized and aliquoted into 1.5 ml eppendorf tubes prior to injection in *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice. Studies on *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice used a dose of 4 mg/day tamoxifen injected daily over 5 days. Male mice receiving daily injections of tamoxifen were susceptible to enlarged testes, which upon dissection of euthanized mice was revealed to be an increase in adipose storage within the testes. Additional groups (*Lep*$^{flox/flox}$ and *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice) were used to control for the effects of injection by receiving the same volume (200 μL) as tamoxifen-injected mice of corn oil via i.p. injection.

2.6.2 Leptin injections

To determine if leptin therapy would reverse the metabolic phenotype observed in *Lep*$^{flox/flox}$ *ACTB*Cre mice, 13 week old female in *Lep*$^{flox/flox}$ *ACTB*Cre mice received daily i.p. injections of peglyated mouse leptin (PEGLEP1; Protein Laboratories Rehovot) at a dose of 0.5 μg per day (0.1 ml injection of a 0.05 mg/ml solution) for six days. Leptin was prepared by dissolving 1 mg of pegylated leptin into 1 ml of ddH_2O. Littermate *Lep*$^{flox/flox}$ mice received i.p. injections of 0.1 mL of saline to control for the effects of injection.

2.7 Surgical Manipulations

2.7.1 Mini osmotic pump implantation

Recombinant rat leptin (100 μg/day, Peprotech, Rocky Hill, USA) was reconstituted with sterile ddH_2O and delivered via mini-osmotic pumps (28-day, 2ML4; Alzet, Cuperino, USA). Following equilibration overnight at 37°C in sterile PBS, mini-osmotic

pumps were implanted subcutaneously in anaesthetized male *Lep*^{WT} and *Lep*^{KO} rats at 16 weeks of age. Rats were given subcutaneous injections of ketoprofen (5 mg/kg; Merial; Baie d'Urfe, Canada) and bupivacaine (6 mg/kg; Hospira Healthcare Corporation, Montreal, Canada) prior to surgery. *Lep*^{WT} rats were implanted with mini osmotic pumps containing ddH_2O instead of leptin.

A similar procedure was performed in two 9 week old male *ob/ob* mice, but recombinant murine leptin (100 µg/day, Peprotech) and a 14-day mini osmotic pump was used instead to reverse sterility of *ob/ob* mice and enable breeding with *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*wt/wt* female mice.

2.7.2 Islet isolation

Rats were euthanized and pancreatic duct injections were performed with the assistance of Shannon O'Dwyer. Rat pancreata were distended by duct injection of 15 ml of collagenase (0.19 mg/mL Liberase TL grade, Roche Diagnostics, Mississauga, Canada) in Hank's balanced salt solution (HBSS – 137 mM NaCl, 5.4 mM KCl, 4.2 mM NaH_2PO_4, 4.1 mM KH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$ and 5 mM glucose, pH 7.2), and then digested in collagenase solution for 25 minutes in a 37°C water bath. The digested pancreata were then filtered three times and washed in Hank's solution (with $CaCl_2$), re-suspended in Hams F10 media (Sigma-Aldrich) and poured onto a 100 µM mesh cell strainer (BD Biosciences, San Jose, USA). Rat islets were then hand picked three times and cultured overnight at 37°C.

Mouse islets were isolated using a similar procedure as described with the following exceptions: 1000 U/mL collagenase type XI (Sigma-Aldrich) was prepared in filter-sterilized HBSS, pancreatic were distended with 3 mls of collagenase solution and

digestion at 37°C was performed for 11 minutes. Murine islets were purified from remaining exocrine tissue and pancreatic lymph nodes using a 70 µm cell strainer and were cultured in RPMI 1640 (Life Technologies, Burlington, Canada) supplemented with 0.5% BSA (Life Technologies), 1% penicillin-streptomycin (Life Technologies).

2.8 In Vivo Assays

2.8.1 Insulin tolerance test (ITT)

Rodents were fasted for 4 h and then given an intraperitoneal injection of 0.65-0.75 U/kg synthetic human insulin (Novolin GE Toronto, Novo Nordisk, Mississauga, Canada). Blood glucose was measured using a One Touch Ultra glucometer (Lifescan Canada) in samples taken from the saphenous vein at 0, 15, 30, 45, 60, 90 and 120 minutes after the insulin injection. Dose of insulin for each experiment is indicated in the figure legend.

2.8.2 Oral glucose tolerance test (OGTT) and glucose stimulated insulin secretion

Rodents were fasted for 6 h and glucose tolerance tests were performed by oral gavage of glucose (1.5 mg/kg body weight of a 40% solution). Glucose measurements were performed using a One Touch Ultra glucometer (LifeScan, Canada) on blood samples taken from the saphenous vein at 0, 15, 30, 45, 90, and 120 minutes following oral gavage. Blood was collected from the saphenous vein at 0, 15, 30, 60, 90 and 120 minutes following gavage of glucose for measurement of insulin from separated plasma. Plasma insulin levels were measured as described above.

2.8.3 Body temperature analysis and cold tolerance test

Seventeen week old rats were anaesthetized using isofluorane were implanted subcutaneously with sterile temperature transponders (IPTT-300; Bio Medic Data Systems, Seaford, USA) through a ~5 mm incision in the intrascapular region. Transponders were implanted parallel to the spine so as not to interfere with the animal's movement and the incision was closed with biodegradable sutures. Body temperature was assessed using a hand-held Pocket Scanner (DAS-5007; Bio Medic Data Systems, Seaford USA) to read the temperature transponders in rats. For the cold tolerance test, rats were singly housed with no bedding and transported to a cold room with room temperature of 4°C. Body temperature was measured in rats every 15 minutes for a total of 90 minutes using the hand-held Pocket Scanner.

2.9 Histology

2.9.1 Tissue processing

Mouse or rat pancreas tissue were collected and submerged in 4% paraformaldehyde (1:10 vol/vol ratio of tissue to solution made in PBS, pH 7.2), post-fixed overnight at 4°C and transferred to 70% ethanol for long-term storage. Pancreata were then embedded in paraffin and processed for sectioning by Wax-it Histology Services (Vancouver, Canada). Prior to immunostaining, paraffin sections were deparaffinized and rehydrated using a series of 5-min washes in xylene (x3), and graded alcohol solutions (70-100% ethanol). Heat-induced epitope retrieval was performed on slides for 10 minutes at 95°C in 10 mM sodium citrate buffer followed by addition of serum free protein block (DakoCytomation Inc., Carpinteria, USA).

2.9.2 Quantification of β-cell area, α-cell area and islet size

Three pancreas sections separated by 200 μm per mouse were incubated overnight in primary antibodies for insulin (rabbit anti insulin, Cat# C27C9, 1:1000, Cell Signaling), and glucagon (mouse anti glucagon, Cat# G2654, 1:1000 Cell Signaling, Danvers, USA) in a humid chamber at 4°C. On the following day, sections were washed in PBS and then incubated with AlexaFluor-conjugated secondary antibodies and mounted using VectaShield Hard Set Mounting Medium for Fluorescence with DAPI (Vector Laboratories, Burlingame, USA). All antibodies were diluted in Dako Antibody Diluent. Whole sections were scanned at 10x magnification and individual images were stitched together to recreate the entire pancreas. The insulin and glucagon positive areas were determined by setting a minimum threshold of pixel intensity and expressed relative to the whole pancreas tissue area. Total insulin- and glucagon-positive area relative to total pancreas area was measured using Meta Xpress High-Content Image Acquisition and Analysis software (Molecular Devices Corporation; Sunnyvale, USA). Quantification from three pancreas sections per mouse was averaged to obtain one value per animal (n=3-5 per group). Islet sizes were approximated by measuring insulin positive area of each islet in three pancreas sections per mouse. Islet sizes were then binned into ranges and the histogram analysis function in Microsoft Excel 2016 was used to determine the frequency of islets per size range. Frequency was represented as a percentage of total number of islets assessed per animal.

2.9.3 Glut-2 and EGFP immunostaining

To determine whether Glut-2 was present in the pancreas of rodents with impaired leptin signalling, one pancreas section from each animal was immunostained

for insulin (rabbit anti insulin, Cat# C27C9, 1:1000, Cell Signaling, Danvers, USA) and Glut-2 (Glut-2 (rabbit anti-Glut-2 antibody, Cat# 07-1402, 1:500, Millipore) overnight at 4°C. Slides were then incubated with AlexaFluor-conjugated secondary antibodies (Life Technologies, Burlington, Canada) for one h at room temperature. Slides were then washed three times in PBS and mounted with coverslips using Vectashield Hard Set Mounting Medium for Fluoresence with DAPI (Vector Laboratories, Burlingame, USA).

To determine whether recombination occurred in the presence of *Ins1*cre in pancreatic islets, one pancreas section from each *mT/mG Ins1*Cre and *mT/mG* control mice was immunostained for EGFP (rabbit anti-GFP antibody, Cat#A-11122, 1:1000, Invitrogen) and insulin (rabbit anti insulin, Cat# C27C9, 1:1000, Cell Signaling). Slides were incubated overnight in primary antibody at 4°C. Slides were then incubated for 1 h at room temperature with AlexaFluor-conjugated secondary antibodies (Life Technologies). Slides were then washed three times in PBS and mounted with coverslips using Vectashield Hard Set Mounting Medium for Fluorescence with DAPI (Vector Laboratories).

2.10 Statistical Analysis

Data were graphed and underwent statistical analysis using GraphPad Prism (GraphPad Software, La Jolla, USA). Metabolic cage data in chapter 3 were analyzed using Systat 13 (Systat Software Inc., San Jose, USA). Analysis of covariance (ANCOVA) was employed to assess energy expenditure with covariates for lean and fat mass in chapter 3. When data sets were small (n=<4) and/or highly variable, a normality transformation was performed prior to statistical analysis. Data are presented as mean ± standard error of the mean (SEM) or as a median or mean with individual data points

and bars indicating the range of values. The critical α-level was set at $P \leq 0.05$. A Repeated measures One or Two Way ANOVA was used to assess tracking data (e.g. fasting body weight, blood glucose), depending on number of experimental groups being compared, and multiple comparisons were made using Sidak or Tukey post hoc tests, indicated in each figure legend. A Two Way Repeated Measures ANOVA was used to assess acute *in vivo* measurements involving 2 groups (e.g. glucose tolerance test, insulin tolerance test). The Kruskal-Wallis test for non-parametric analyses was used instead of the One Way ANOVA to account for the large variability of the number of animals per experimental group. The method of statistical analysis is indicated in each figure legend.

CHAPTER 3: SUPPRESSING HYPERINSULINEMIA PREVENTS OBESITY BUT CAUSES RAPID ONSET OF DIABETES IN LEPTIN-DEFICIENT *ob/ob* MICE

3.1 Introduction

Obesity has a significant impact on human health yet the mechanisms underlying it are poorly understood. Mutation of the gene encoding leptin in mice (*ob/ob*) results in a deficiency of leptin, and increases in hyperphagia and adiposity [67]. In addition, loss of leptin also impairs glucose homeostasis manifesting as glucose intolerance, and at times fasting hyperglycemia in mice greater than 8 weeks of age [221]. In both *ob/ob* mice and mice lacking leptin receptors (*db/db*), hyperinsulinemia occurs prior to increases in body weight and fasting glucose levels [140, 208, 221], possibly as a result of removal of leptin's inhibitory effects on insulin gene expression and secretion [170, 172]. Administration of exogenous leptin to *ob/ob* mice results in attenuation of circulating insulin levels and improved fasting glycemia prior to reductions in body weight [137], conversely insulin upregulates the production of leptin [222]. The opposing actions of insulin and leptin results in an adipoinsular feedback loop that plays an important role in regulating fat deposition.

The sequence of events resulting in increased adiposity, hyperinsulinemia and insulin resistance that occur in obesity are not fully understood. In human studies, elevated insulin levels predict obesity later in life [223], suggesting that hyperinsulinemia contributes to excess adiposity. In obese rodents, suppression of insulin secretion using diazoxide lowers body weight [224, 225]. However, diazoxide can act independently of reducing insulin levels to attenuate adiposity and food intake [226]. Thus while these

studies suggest that lowering insulin pharmacologically may be a successful strategy to reduce body weight, the confounding effects of diazoxide [224, 227] make it difficult to delineate the direct effects of insulin on adiposity. To understand the direct implications of circulating insulin levels on obesity, an alternative strategy involves direct modification of insulin gene expression. In rodents, two insulin genes exist, *Ins1* and *Ins2*. Previous studies demonstrated that reduction of plasma insulin levels by lowering insulin gene dosage to either a single *Ins1* or *Ins2* allele protected mice against high fat diet-induced obesity [41, 42].

In this chapter, we examined the effects of reduced insulin gene dosage in *ob/ob* mice. The effects of reduced insulin gene dosage in the context of leptin deficiency had not been reported in prior studies modifying insulin gene dosage. We performed a thorough characterization of the metabolic effects of reduced insulin gene dosage in the context of leptin deficiency. This work has been published in the journal *Molecular Metabolism* [228].

3.2 Results

To determine if reduced insulin gene dosage is sufficient to protect *ob/ob* mice from developing obesity and impaired glucose homeostasis, we generated *ob/ob* mice lacking 1 (*Ins1*$^{+/+}$*;Ins2*$^{+/-}$*;ob/ob*), 2 (*Ins1*$^{+/+}$*;Ins2*$^{-/-}$*;ob/ob*) or 3 (*Ins1*$^{+/-}$*;Ins2*$^{-/-}$*;ob/ob*) insulin alleles, as well as leptin-expressing mice lacking 1, 2 or 3 insulin alleles (*Ins1*$^{+/+}$*;Ins2*$^{+/-}$*;wt/wt, Ins1*$^{+/+}$*;Ins2*$^{-/-}$*;wt/wt*, and *Ins1*$^{+/-}$*;Ins2*$^{-/-}$*;wt/wt*, (Fig. 2.1). We first determined the effects of reduced insulin gene dosage on plasma insulin levels. In male *wt/wt* mice, insulin levels were similarly low in mice lacking 1, 2 or 3 insulin alleles at both 5 and 7

weeks of age (Fig. 3.1A-B). In contrast, male *Ins1*$^{+/+}$*;Ins2*$^{+/-}$*;ob/ob* mice were hyperinsulinemic. Loss of 2 or 3 insulin alleles in male *ob/ob* mice resulted in plasma insulin levels that were comparable to *wt/wt* littermates (Fig. 3.1A-B). Female *ob/ob* mice lacking 3 insulin alleles also had comparable plasma insulin levels to *wt/wt* littermates, whereas plasma insulin levels of female *ob/ob* mice lacking 2 insulin alleles were higher than *wt/wt* littermates (Fig. 3.1C-D).

Ins1$^{+/-}$;*Ins2*$^{-/-}$;*wt/wt* *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*wt/wt* *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*wt/wt*
Ins1$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob*

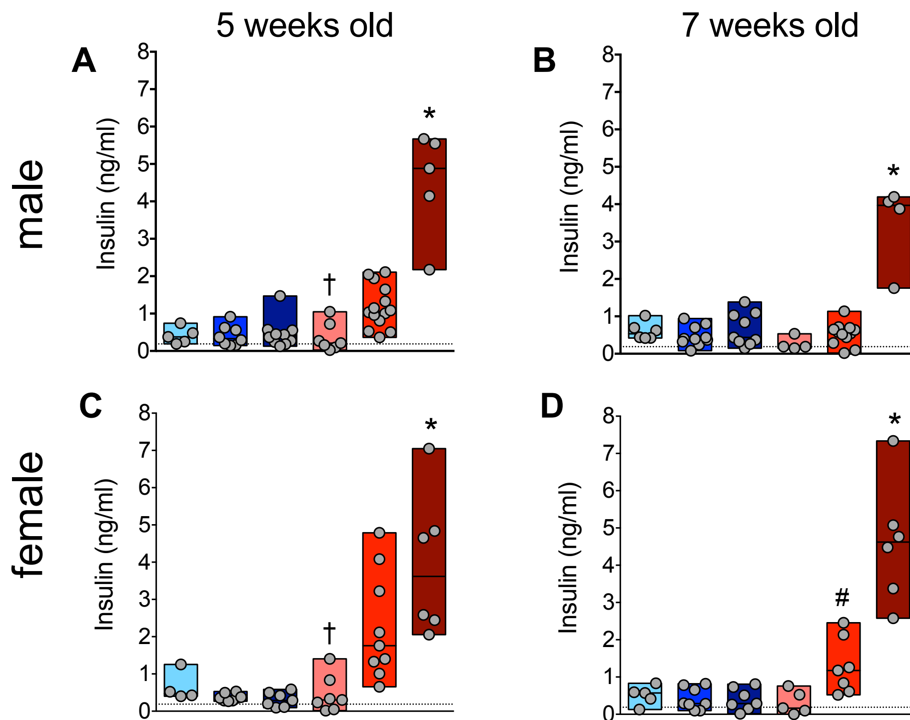


Figure 3.1: Reduction of insulin gene dosage suppresses hyperinsulinemia in ob/ob mice. Insulin levels were measured following a 4 h fast in male (A, B) and female (C, D) mice at 5 and 7 weeks of age. Values are presented individually with bars indicating the range of values and lines indicating the median value. A One Way ANOVA was used to assess significance. *P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* vs. all other

groups. †P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* vs. *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob;* #P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* vs. all other groups. Dotted lines on graph indicate detection limit of assay.

Despite a significant decrease of circulating insulin levels in female *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* mice compared to *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice, insulin levels remained significantly higher than *wt/wt* littermates by 7 weeks of age.

Since insulin is a potent anabolic hormone, we tested whether reductions of insulin gene dosage in the absence of leptin would protect against obesity. Body weight of male and female *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice doubled between 4-8 weeks of age, and these mice were significantly heavier than all other groups (Fig. 3.2A, B; P<0.05). Body weight of *wt/wt* mice did not differ between insulin gene dosage groups in both males and females. In *ob/ob* mice lacking 2 or 3 insulin alleles, differences in body weight were evident as early as 5 weeks of age. Reductions in body weight reflected a similar pattern as circulating insulin levels, with lower insulin gene dosage corresponding to a greater attenuation of body weight gain. By 8 weeks of age, body weight of male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* mice was comparable to *Ins1*$^{+/+}$*;Ins2*$^{-/-}$*;wt/wt* littermates, while body weight of *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* mice was significantly lower than *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*wt/wt* littermates. In contrast, body weight of female *Ins1*$^{+/+}$*;Ins2*$^{-/-}$*;ob/ob* mice was significantly higher than *Ins1*$^{+/+}$*;Ins2*$^{-/-}$*;wt/wt* littermates, but body weight of *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* mice was comparable to *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*wt/wt* mice. The lower body weight of male *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* mice compared to *wt/wt* mice was suggestive of growth impairments. To determine if reduced insulin gene dosage impacted growth, we assessed nasal-anus length at 8 weeks of age. We observed reduced body length of male *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* mice compared to male *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice (Fig. 3.2C, D).

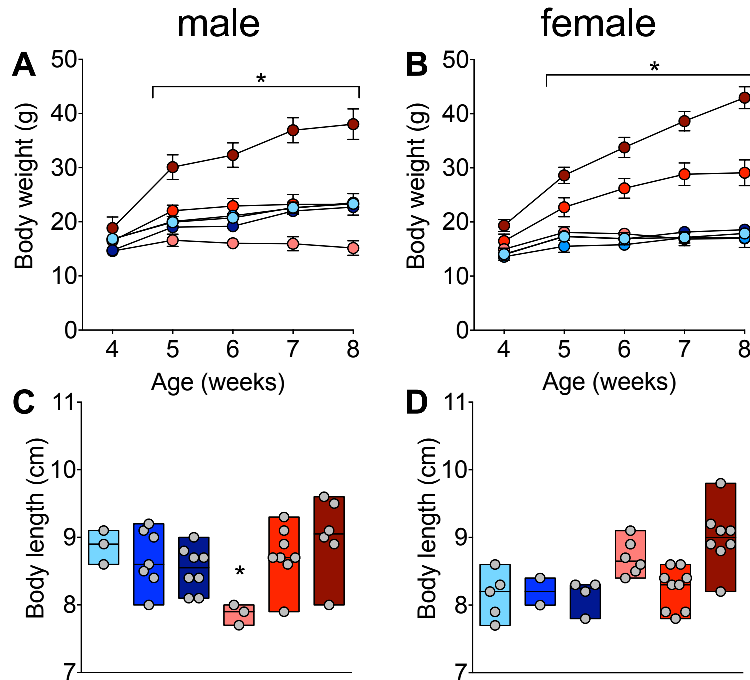


Figure 3.2: Reduction of insulin gene dosage in ob/ob mice protects against obesity. Body weight was measured weekly following a 4 h fast in male (A) and female (B) mice. Body length (naso-anus length) was measured at 8 weeks of age (C-D). Values are presented as mean ± SEM in A-B (n=5-11/group), and as individual data points with line indicating the median and bar indicating the range of values in C-D. *P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* vs. all other groups in A-B using a One Way Repeated Measures ANOVA and *P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* vs. *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* in C-D using a Kruskal Wallis non parametric test.

To further examine the relationship between hyperinsulinemia and obesity in the context of leptin deficiency, we next examined body composition and gonadal fat pad mass. Through DEXA analysis at 5 weeks of age, the total lean and lipid mass were measured and expressed as a fat/lean ratio. Fat/lean mass of *wt/wt* mice were

comparable among groups lacking 1, 2 or 3 insulin alleles. As expected, fat/lean mass was highest in *ob/ob* mice lacking 1 insulin allele. Reducing insulin gene dosage in male *ob/ob* mice resulted in corresponding reductions of fat/lean mass (Fig. 3.3A). Loss of 2 or 3 insulin alleles resulted in a similar reduction of fat/lean mass in female *ob/ob* mice compared to *ob/ob* female mice lacking 1 insulin allele (Fig. 3.3B). As reductions of fat mass are often associated with changes to lipid metabolism, we assessed plasma triglyceride and free fatty acid levels. Plasma triglyceride levels were highest in female *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* mice (Fig. 3.3F), and these mice also demonstrated a trend for the highest plasma free fatty acid levels (Fig. 3.3D). To assess ectopic lipid deposition, hepatic triglyceride content was measured at 8 weeks of age. *Ob/ob* males and females lacking 1 or 2 insulin alleles had highly variable hepatic triglyceride content as compared to *wt/wt* littermates (Fig. 3.3G-H). In male *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* mice, hepatic triglyceride levels were comparable to *wt/wt* littermates while no significant differences in hepatic triglyceride content were observed between female *ob/ob* and *wt/wt* mice.

The reduced weight and size of *ob/ob* mice lacking 3 insulin alleles was suggestive of growth impairments. To determine if growth impairments altered survival, *wt/wt* and *ob/ob* mice were monitored up to 8 weeks of age. Kaplan-meyer survival curves indicate that reduced survival is observed beyond 8 weeks in *ob/ob* mice lacking 2 or 3 insulin alleles compared to *wt/wt* littermates (Fig. 3.4).

Ins1$^{+/-}$;*Ins2*$^{-/-}$;*wt/wt* *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*wt/wt* *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*wt/wt*

Ins1$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob*

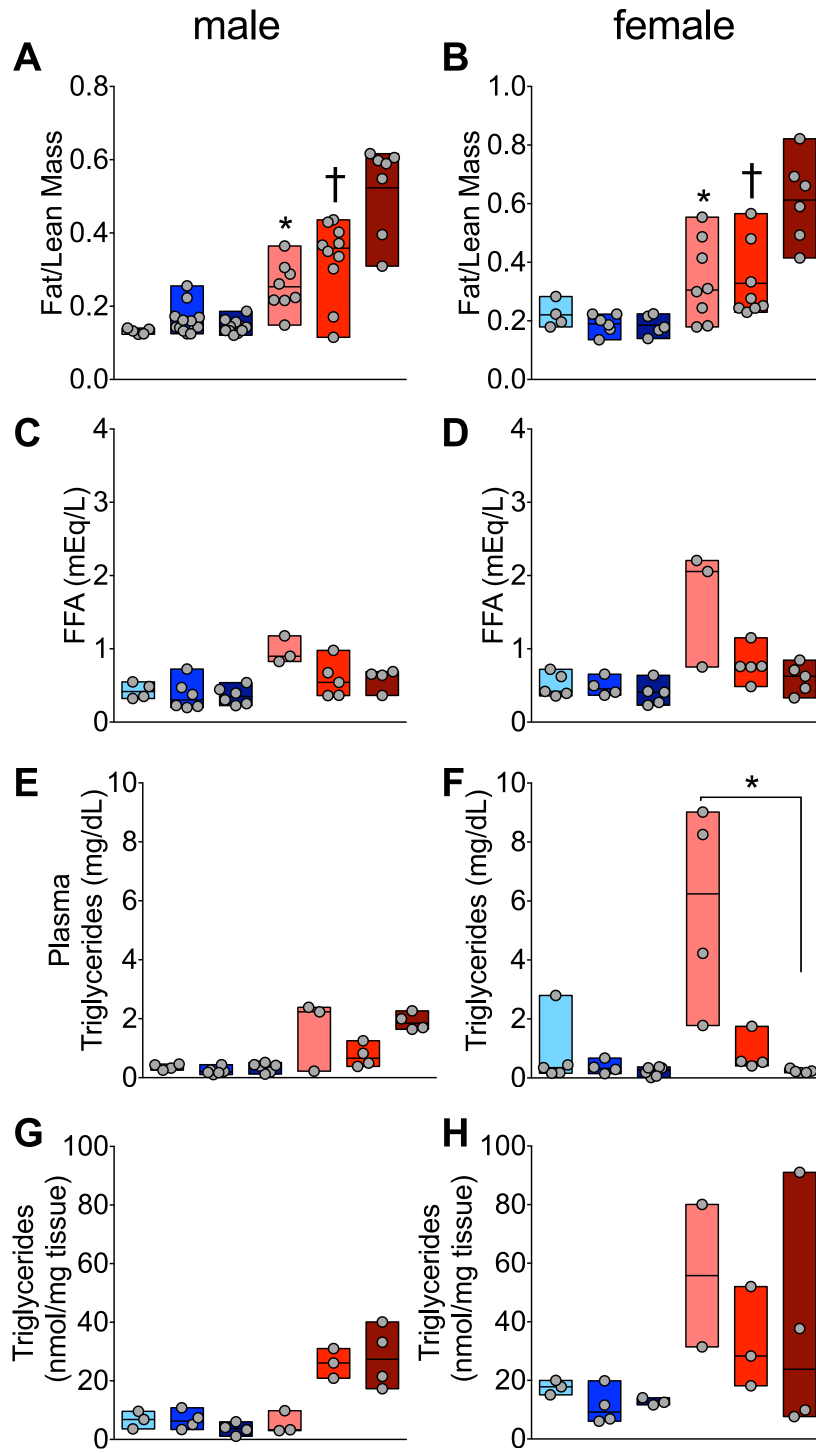


Figure 3.3: Reduction of insulin attenuates adiposity in *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* mice. At 5 weeks of age, body composition was assessed using DEXA analysis. Total body fat to lean ratio was assessed in 5-week-old male (A) and female (B) mice. At 8 weeks of age, cardiac plasma was collected following euthanization for assessment of plasma free fatty acids (C-D) and triglycerides (E-F). Hepatic triglycerides were measured from livers flash frozen at 8 weeks of age (G-H). Individual values are presented with bars indicating the range of values and a line indicating the median. A One Way ANOVA was used to assess significance in A-B. A Kruskal-Wallis non-parametric test was used to assess significance in C-H. *P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* vs. *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob*; †P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* vs. *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob*.

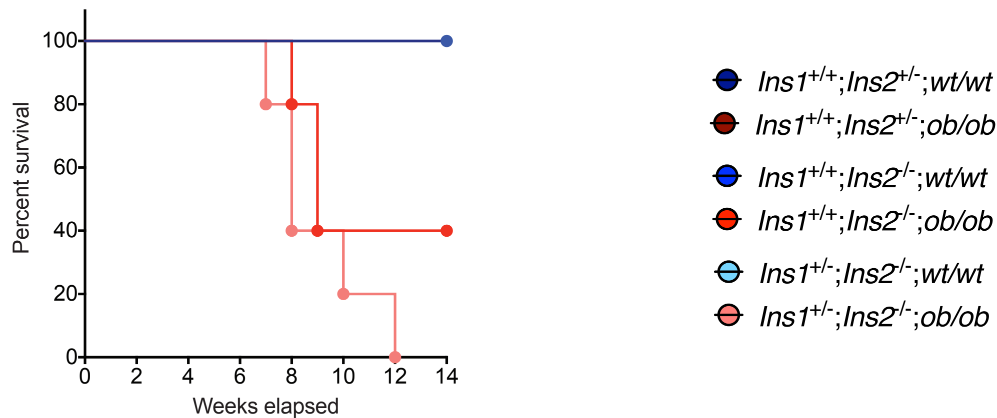


Figure 3.4: Reduction of insulin gene dosage in *ob/ob* mice is results in reduced survival. Kaplan-Meir survival curve of male and female *ob/ob* or *wt/wt* mice lacking 1, 2 or 3 insulin alleles monitored up to 14 weeks of age.

To examine the physiological mechanisms of improved body composition in *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* mice, we used indirect calorimetry to assess energy expenditure and food intake at 5 weeks of age. Due to the severe hyperglycemia of male *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* mice, comparisons of energy expenditure and food intake were only made between *ob/ob* mice lacking 1 or 2 insulin alleles and their respective *wt/wt* littermate controls. *Ob/ob* mice lacking 1 or 2 insulin alleles had significantly higher food intake and reduced physical activity compared to *wt/wt* mice (Fig. 3.5A, B). No differences in

respiratory quotient, physical activity, or food intake occurred between male *ob/ob* mice lacking 1 or 2 insulin alleles (Fig. 3.5A-C). Energy expenditure was highest in male *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice (heat, adjusted for lean body mass and 0.3x fat mass [229]; Fig. 3.5D). No differences in VO_2 or VCO_2 adjusted for lean mass were observed between groups (Fig. 3.6A, B). These data suggest that the improvements to body weight in *ob/ob* mice with reduced insulin gene dosage occur independent of increases in energy expenditure at the time of these measurements.

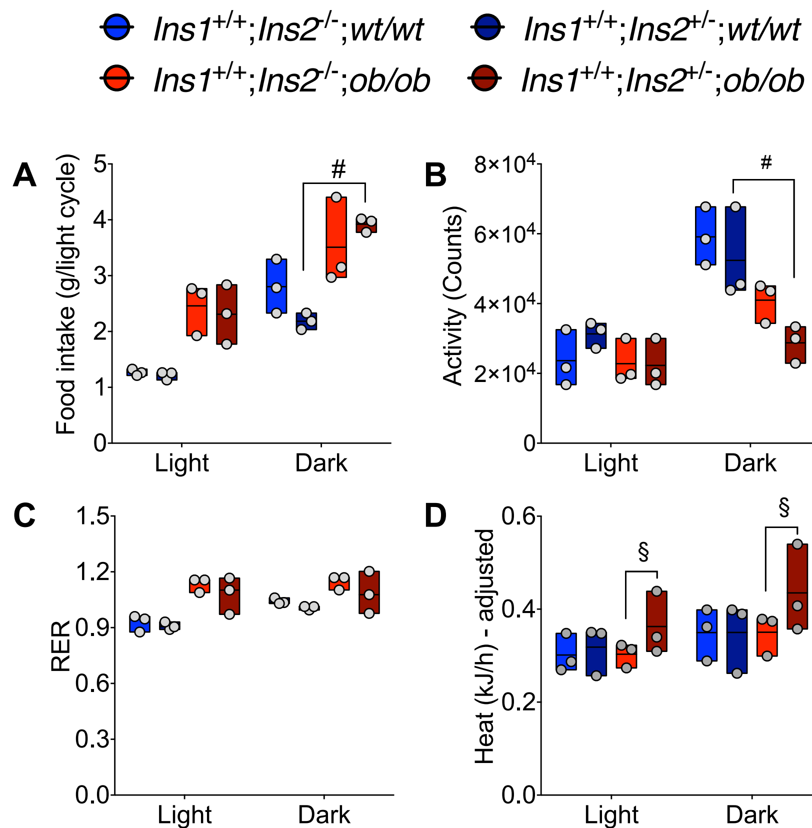


Figure 3.5: Adiposity is reduced in *ob/ob* mice lacking 2 insulin alleles independent of changes in food intake or energy expenditure. Food intake (A), activity (B), RER (C) and energy expenditure (D) was measured by indirect calorimetry in male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* and *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice and male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*wt/wt* and *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*wt/wt* mice at 5-6 weeks of age. To assess statistical differences, activity, heat and RER measures were adjusted for lean mass + 0.3x fat mass by

ANCOVA followed by Tukey post hoc analysis. Data are presented as unadjusted values of individual data points with bars indicating range of values and line indicating the mean. #P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* vs. *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*wt/wt* mice; §P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* vs. *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice.

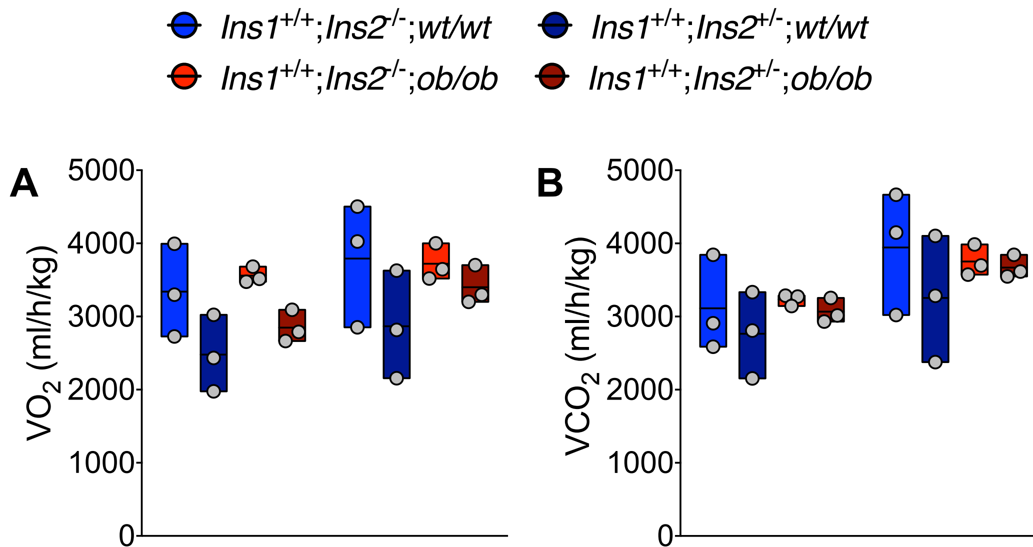


Figure 3.6: Reduced body weight is not attributed to differences in VO_2 consumption or VCO_2 production. VO_2 (A) and VCO_2 (B) were measured by indirect calorimetry in male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* and *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice at 5-6 weeks of age. Data are presented as unadjusted values. To assess statistical differences, all measures were adjusted for lean mass + 0.3x fat mass by ANCOVA followed by Tukey post hoc analysis. Individual data points are presented with lines indicating the mean and bars indicating the range of values.

As insulin is essential to glucose homeostasis, we next assessed whether reduction of insulin gene dosage affects fasting blood glucose levels in *ob/ob* mice between 4-8 weeks of age. *wt/wt* mice lacking 1, 2 or 3 insulin alleles had fasting glucose levels ranging from 4-12 mM that did not significantly differ between insulin gene dose groups (Fig. 3.7A, B). Blood glucose of male *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice were comparable to *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*wt/wt* mice between 4-7 weeks of age, however by 8 weeks of age,

$Ins1^{+/+}$;$Ins2^{+/-}$;*ob/ob* mice were hyperglycemic (blood glucose = 24.0±0.35 mM). In comparison, $Ins1^{+/+}$;$Ins2^{-/-}$;*ob/ob* and $Ins1^{+/-}$;$Ins2^{-/-}$;*ob/ob* male mice had become hyperglycemic (16.2±5.6 mM and 21.0±5.12 mM) by 4 weeks of age. $Ins1^{+/+}$;$Ins2^{-/-}$;*ob/ob* mice experienced a gradual rise in fasting glucose levels over time to 27.4±2.2 mM by 8 weeks of age, while fasting glucose rose above 25 mM by 5 weeks of age in $Ins1^{+/-}$;$Ins2^{-/-}$;*ob/ob* mice and remained elevated. In contrast to the mild hyperglycemia of 4 week old male $Ins1^{+/+}$;$Ins2^{-/-}$;*ob/ob* mice, fasting glucose levels of female $Ins1^{+/+}$;$Ins2^{-/-}$;*ob/ob* mice were comparable to *wt/wt* littermates at 4 weeks of age (Fig. 3.7B). By 8 weeks of age, blood glucose levels of $Ins1^{+/+}$;$Ins2^{-/-}$;*ob/ob* mice were elevated to >26 mM. By comparison, severe hyperglycemia (>29 mM) was evident in female $Ins1^{+/-}$;$Ins2^{-/-}$;*ob/ob* at 4 weeks of age and remained above 25 mM for the duration of tracking.

Since hyperglycemia may also be a result of impaired α cell function, we next assessed plasma glucagon levels in 8-week-old male and female mice. Fasting glucagon levels were highly variable in *ob/ob* mice and did not correlate with plasma glucose levels (Fig. 3.7C, D). Male $Ins1^{+/+}$;$Ins2^{-/-}$;*ob/ob* mice had significantly lower plasma glucagon levels despite having no differences in fasting glucose levels compared to *ob/ob* mice lacking 1 or 3 insulin alleles.

The development of hyperglycemia by 4 weeks of age in *ob/ob* mice with reduced insulin gene dosage indicates that reduction of insulin in *ob/ob* mice is detrimental to glucose homeostasis. To further examine the effects of reducing insulin gene dosage on glucose tolerance, we conducted an oral glucose tolerance test (GTT) and measured plasma insulin levels at selected time intervals in 7-week-old mice (Fig. 3.8A, D). Blood glucose levels of *wt/wt* littermates lacking 1, 2 or 3 insulin alleles peaked at ~16 mM but returned to baseline levels 2 h after glucose gavage. While fasting and 2 h post gavage

glucose levels of male *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice were comparable to *wt/wt* littermates, male *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice experienced a higher peak of blood glucose levels than *wt/wt* mice. In comparison, male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* and *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* mice were hyperglycemic at baseline, and blood glucose rose to levels above the detection limit of the glucose meter for the duration of the GTT.

Ins1$^{+/-}$;*Ins2*$^{-/-}$;*wt/wt* *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*wt/wt* *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*wt/wt*
Ins1$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob*

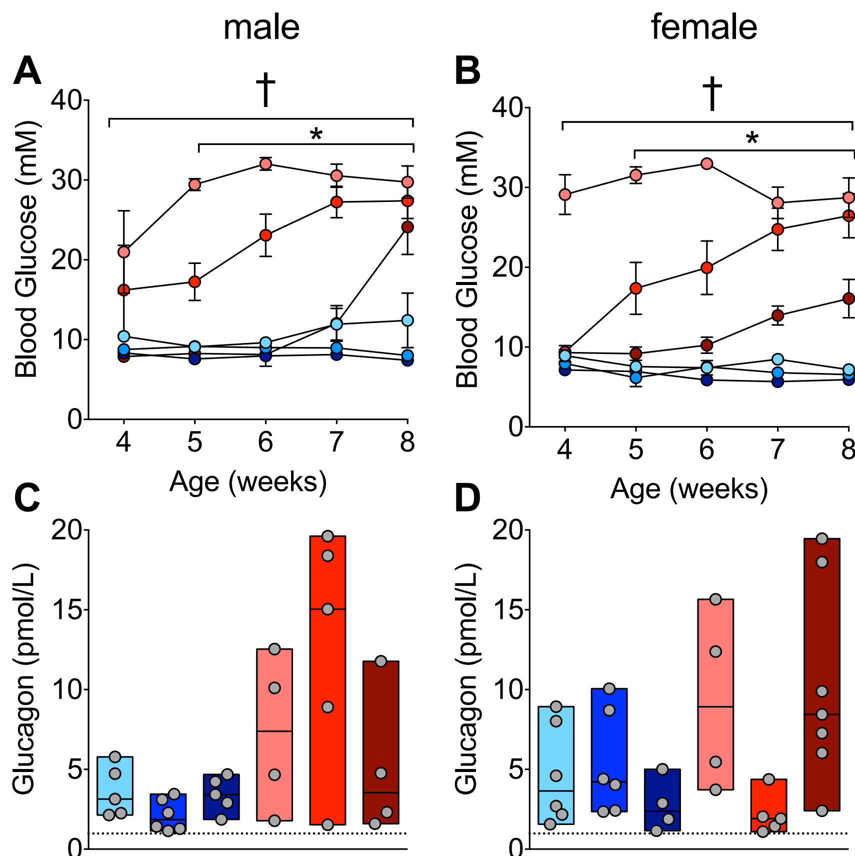


Figure 3.7: Attenuation of hyperinsulinemia accelerates development of hyperglycemia in *ob/ob* mice. Blood glucose was measured weekly following a 4 h fast in male (A) and female (B) mice. Fasting glucagon levels were measured from plasma collected at 8 weeks of age in male (C) and female (D) mice. Values are

presented as mean ± SEM in A-B (n=4-11/group), and as individual data points with lines indicating the median and bars indicating the range of values in C-D. A One Way Repeated Measures ANOVA was used to assess significance in A-B and a One Way ANOVA was used to assess significance in C-D. †P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* vs. *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob*. *P<0.05 *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* vs. *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob.*

In contrast to males, female *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice had elevated blood glucose following a 6 h fast (blood glucose = 11.6±1.02 mM), but trends in glucose tolerance were otherwise comparable between male and female mice. Area under the curve (AUC) analysis of glucose excursion reflected similar exacerbation of glucose intolerance among *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* and *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* male and female mice compared to *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice (Fig. 3.8B, E). To determine if glucose intolerance was due to altered insulin kinetics, plasma samples collected at 0, 15, 30 and 60 minutes during the OGTT were assayed for insulin. Male and female *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* and *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* mice maintained hyperinsulinemia relative to *wt/wt* littermates for the duration of the OGTT. *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* male and female mice failed to increase insulin secretion in response to glucose and had insulin levels equal to or below *wt/wt* (Fig. 3.8C, F).

Expansion of β cell area is a defining feature of *ob/ob* mice. To determine whether reduced insulin gene dosage alters β cell area, we co-stained pancreas sections taken from 3 different regions of the pancreas for insulin and glucagon. Islets from *ob/ob* mice lacking 2 or 3 insulin alleles appeared distorted in shape (Fig. 3.9A). Quantification of relative β and α cell areas revealed a trend for reduced insulin positive area and increased glucagon positive area in *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* mice (Fig. 3.9B, C). In contrast, islet morphology and relative β and α cell area were comparable between *wt/wt* mice lacking 2 or 3 insulin alleles.

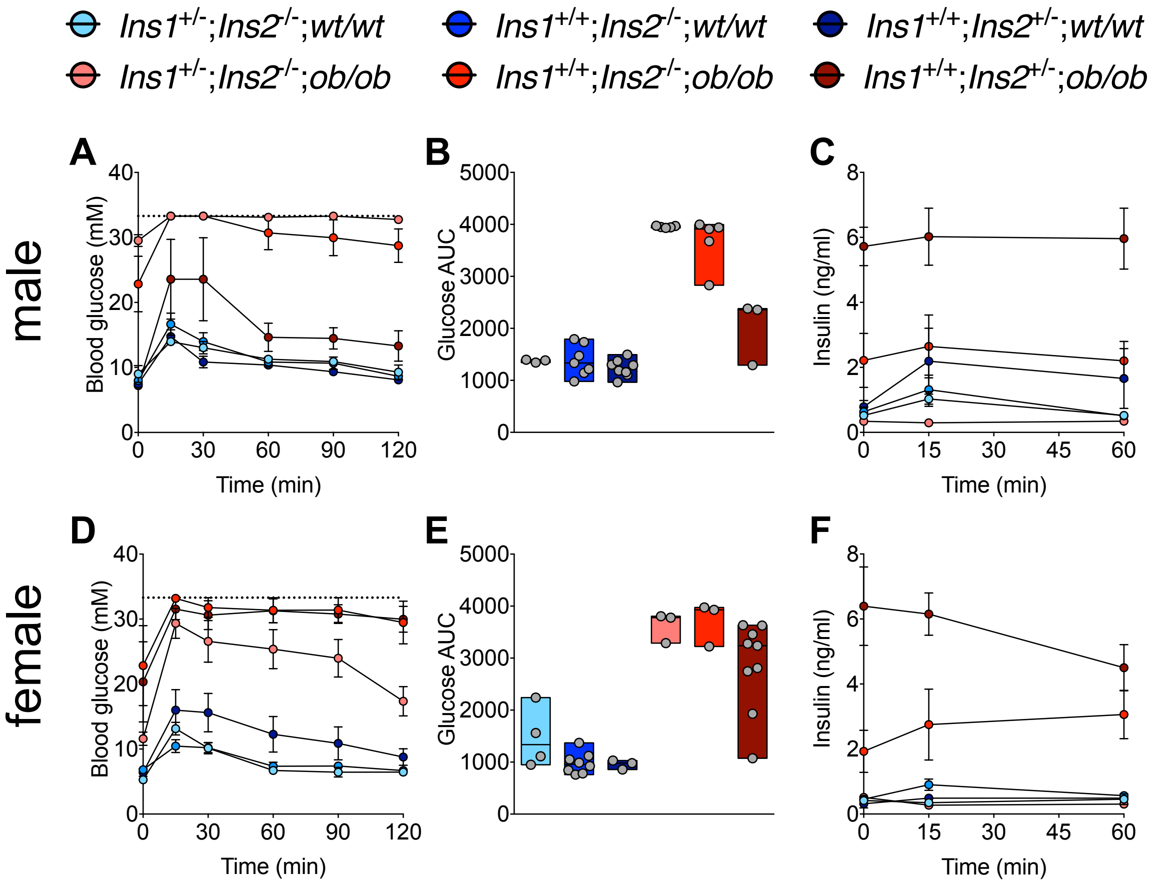


Figure 3.8: Attenuation of hyperinsulinemia does not improve glucose tolerance in *ob/ob* mice. At 7 weeks of age, an oral glucose tolerance test was performed following a 6 h fast (A, D). Plasma was collected to measure insulin during the OGTT (B, E). Values are presented as mean ± SEM in A, D, C and F (n=3-8/group). Individual values are presented in B, E with line indicating the median and bar indicating the range of values. A One Way Repeated Measures ANOVA was used to assess statistical significance in C, F. No statistical tests were performed on A, B, D and E due to values exceeding the limit of detection.

Ins1$^{+/-}$;*Ins2*$^{-/-}$;*wt/wt* *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*wt/wt* *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*wt/wt*

Ins1$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob*

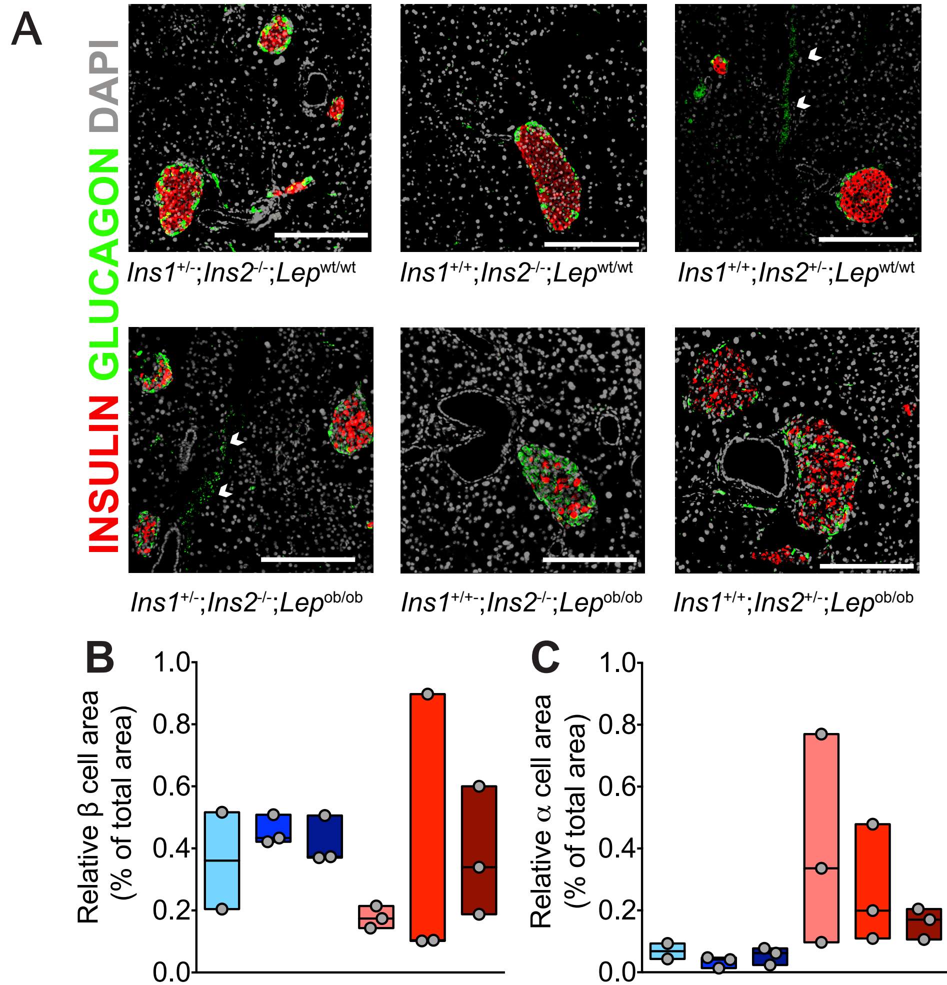


Figure 3.9: Reduction of insulin gene dosage produces abnormal islet morphology in *ob/ob* mice. Representative pancreas sections (A) stained with insulin (red) and glucagon (green) from 8-week-old male mice, scale bar corresponds to 200 µm. White arrowheads indicate autofluoresence of red blood cells. β (B) and α (C) cell area, based on insulin and glucagon staining relative to total pancreas area were quantified from three pancreas sections and averaged to provide one value per animal. Individual values are presented with line indicating the median and bars indicating the

range of values in B-C. Statistical tests were not performed due to low number of samples per group.

3.3 Discussion

In rodents lacking leptin or its receptor, hyperinsulinemia has been observed prior to onset of obesity [221]. Given recent studies in mice implicating hyperinsulinemia as a cause for obesity [41], in this chapter we investigated whether the early and persistent hyperinsulinemia in *ob/ob* mice is a major contributor to their obesity. Unlike models of high fat feeding in which leptin levels rise with increases in adiposity, the present study sought to determine the direct contribution of insulin to body weight changes in leptin deficient mice, a model of extreme obesity. Here, we report that genetic reduction of 2 or 3 insulin alleles in leptin-deficient mice prevents the onset of hyperinsulinemia, and corresponds with a decrease in body weight and fat/lean mass.

In *ob/ob* mice lacking 2 or 3 insulin alleles, reduced weight gain is not observed until 5 weeks of age, and is consistent with a previous study reporting no differences in body weight of pre-weaning mice lacking 2 or 3 insulin alleles [42]. Since lipogenesis is driven by insulin [230] and adipose tissue expansion occurs after weaning in rodents [231], we speculate that the attenuated weight gain observed after 4 weeks of age in *ob/ob* mice lacking 2 or 3 insulin alleles is in part due to reduced insulin-stimulated adipogenesis and lipogenesis, and not due to developmental abnormalities.

The degree of hyperglycemia reported here in *ob/ob* mice lacking 2 or 3 insulin alleles is greater than previously reported in high fat diet-fed *Ins1*$^{+/-}$;*Ins2*$^{-/-}$ mice [41]. Though we did not assess urine glucose output in our study, it is likely that energy lost via this route contributed to the reduced body weight and size, and diminished the availability of glucose uptake into peripheral tissue such as muscle, adipose and liver in

male *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*ob/ob* [232]. Given the increases in energy expenditure that occurred in high fat fed mice with reduced insulin gene dosage [41], we hypothesized that reducing insulin levels may increase energy expenditure in *ob/ob* mice. However, heat production of *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob* mice was decreased compared to *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* animals, suggesting that increased energy expenditure does not mediate the decrease in body weight between male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*ob/ob and Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*ob/ob* mice. Thus in addition to changes in adipose metabolism, it is likely that increased urinary glucose output also contributes to the reduced body mass observed in *ob/ob* mice lacking 2 or 3 insulin alleles, while increased energy expenditure does not appear to be a major contributor.

Interestingly, sexual dimorphism was observed in *ob/ob* mice lacking 2 or 3 insulin alleles. In male *ob/ob* mice, loss of 2 insulin alleles reduced insulin levels and body weight to levels comparable to *wt/wt* littermates, and hyperglycemia was observed at 4 weeks of age. In contrast, plasma insulin levels and body weight of female *ob/ob* mice lacking 2 insulin alleles remained higher than *wt/wt* littermates, and onset of hyperglycemia did not occur until 5 weeks of age. In male *ob/ob* mice, loss of 3 insulin alleles resulted in significantly lower body weight and length than *wt/wt* mice. This reduction in body weight and size was not observed in female *ob/ob* mice lacking 3 insulin alleles, despite having similar fasting glucose and insulin levels. Sexual dimorphism was also observed upon assessment of plasma lipid levels and ectopic lipid accumulation. Hepatic triglyceride levels of male *ob/ob* mice lacking 3 insulin alleles were comparable to *wt/wt* mice. In contrast, female *ob/ob* mice lacking 2 or 3 insulin alleles had higher plasma lipid and hepatic triglyceride levels than their *wt/wt* littermates. Though we could not elucidate the mechanism for these sex-specific differences, it is

clear from our results that male *ob/ob* mice are more severely impacted by lower circulating insulin levels than females. These findings are in line with the increased inflammation and reduced insulin sensitivity observed in adipose tissue of high fat-fed male as compared to female mice [233]. Furthermore, it has been previously demonstrated that female sex steroids protect against development of diabetes in mice [234, 235]. Together, our results suggest that in the absence of leptin, reduction of insulin levels has a greater impact on body weight, ectopic lipid accumulation and body size of male as compared to female *ob/ob* mice.

In leptin deficient mice, reduced insulin sensitivity is observed [236, 237], and hyperinsulinemia precedes the onset of hyperglycemia [221, 238, 239]. We therefore examined the effects of reducing insulin alleles on fasting glucose, glucose tolerance and insulin secretion. Reduction of insulin to circulating levels similar to *wt/wt* levels resulted in rapid onset of hyperglycemia that was much more severe than that typically observed in *ob/ob* mice [137, 206, 221]. These results suggest that with reduced insulin gene dosage hyperinsulinemia is curtailed, but leptin deficient mice are unable to compensate for increased insulin demand, resulting in hyperglycemia. Though euglycemia was maintained in $Ins1^{+/+}$;$Ins2^{+/-}$;*ob/ob* mice up to 7 weeks of age, male $Ins1^{+/+}$;$Ins2^{+/-}$;*ob/ob* mice eventually developed hyperglycemia by 8 weeks of age. Thus hyperinsulinemia is able to transiently prevent hyperglycemia in this extreme model of obesity, but aging *ob/ob* mice with reduced insulin gene dosage are unable to keep up with higher insulin demands, resulting in hyperglycemia. Despite loss of 2 or 3 insulin alleles, *wt/wt* mice are euglycemic, suggesting that leptin plays a significant role in maintaining normal glucose levels. These results are consistent with studies in which leptin therapy is able to restore euglycemia in STZ-treated rodents [94, 145, 240], or

rodent models of T2D [147, 241]. Given the glucose-lowering effects of leptin [93, 242] and its role in regulation of insulin [170, 243] and insulin sensitivity [139, 160, 244], the lack of leptin in *ob/ob* mice may increase insulin demand as a compensatory mechanism to maintain euglycemia.

Despite reduced insulin gene dosage, glucose tolerance was normal, and relative α and β cell area were similar across all *wt/wt* groups. We observed a trend for decreased relative β cell and increased α cell area in *ob/ob* mice lacking 3 insulin alleles relative to *wt/wt* mice lacking 1 insulin allele. Hyperglucagonemia has been implicated as a contributor to hyperglycemia in mice lacking leptin signalling [240, 245], thus we hypothesized that it may contribute to the elevated glucose levels observed in *ob/ob* mice. However, glucagon levels of *ob/ob* mice were not significantly higher than *wt/wt* mice, and did not correlate with the degree of glycemia observed in *ob/ob* mice, suggesting that elevated glucagon levels were not the major cause of hyperglycemia.

In summary, our findings highlight the important role of hyperinsulinemia in regulation of adiposity and maintenance of euglycemia in the absence of leptin action. Though incidence of complete leptin deficiency is rare in humans, the present study uses a model of extreme obesity to demonstrate the compensatory role required by insulin to achieve euglycemia in the absence of leptin. Our findings suggest that with reduced insulin gene dosage, *ob/ob* mice have reduced ability to store excess glucose into fat and are unable to compensate for reduced insulin sensitivity. This results in decreases in fat mass and simultaneous development of hyperglycemia. Together, these findings suggest that in the absence of leptin, hyperinsulinemia promotes obesity, and is also a necessary response used to achieve euglycemia in young *ob/ob* mice.

CHAPTER 4: RESTORATION OF *LEPR* IN β CELLS OF *LEPR* NULL MICE DOES NOT PREVENT HYPERINSULINEMIA AND HYPERGLYCEMIA

4.1 Introduction

Obesity is often associated with elevated circulating insulin levels (hyperinsulinemia), glucose intolerance, and reduced insulin sensitivity [223, 246]. The molecular mechanisms underlying the increased insulin secretion and islet hyperplasia that are often concomitant with obesity remain unclear, but the adipose-derived hormone leptin may be a potential link between adipose tissue and β cells. Leptin has an established role in regulating food intake, adipose metabolism, and glucose homeostasis through signalling in the central nervous system (CNS) [247, 248]. In addition, leptin receptors are distributed in several peripheral tissues involved in glucose regulation including the liver, adipose and pancreas [116, 249]. Within these tissues, the long isoform of the leptin receptor (Lepr-b) is believed to mediate the glucoregulatory actions of leptin [114].

In vitro studies have demonstrated that *Lepr* is expressed in murine and human pancreatic β cells, as well as in β cell lines [172, 250-252]. Previous studies have attempted to assess whether leptin has a direct action on β cell function, but the results are contradictory. While some studies have found that incubation of human or rodent islets with leptin did not alter the expression or secretion of insulin [253-255], other studies demonstrated reduced insulin expression and secretion from islets treated with leptin [171-174, 253, 256]. The inhibitory actions of leptin on β cell function are supported by *in vivo* characterization of glucose homeostasis and circulating insulin levels in mice lacking leptin or its receptor. In leptin deficient mice (*ob/ob*) and mice with

mutated *Lepr* (*db/db*), hyperinsulinemia develops prior to increases in body weight, and islet hyperplasia is evident in these mice [67, 140, 207]. When *ob/ob* mice are treated with leptin, there is a reduction of insulin gene expression and secretion and a reduction of circulating insulin levels within hours, [257]. Moreover, doses of leptin insufficient to reduce body weight are sufficient to reverse hyperinsulinemia, indicating that the insulin-lowering effects of leptin can occur independent of weight loss [137].

To elucidate the mechanism by which leptin can regulate insulin secretion and expression, previous studies have employed Cre-*loxP* technology. Disruption of *Lepr* in β cells was achieved by Covey et al. using a rat insulin promoter driving Cre expression (*RIP*Cre) [238]. *Lepr*$^{flox/flox}$ *RIP*Cre mice were obese, hyperinsulinemic, glucose intolerant, and experienced islet hyperplasia relative to their *Lepr*$^{flox/flox}$ littermates. Interestingly, a similar approach by Morioka and colleagues to disrupt *Lepr* using a *Pdx-1* (pancreatic duodenal homeobox 1) promoter to drive Cre expression resulted in mice with mildly elevated fasting insulin levels, but the absence of obesity [258]. Furthermore, Morioka et al. reported improved glucose tolerance and enhanced glucose stimulated insulin secretion in chow-fed *Lepr*$^{flox/flox}$ *Pdx-1*Cre mice compared to *Lepr*$^{flox/flox}$ littermates [258]. Though intended to target β cells, the Cre recombinase used in both of these studies was present in other tissues including the hypothalamus [238] and brain stem, as well as the exocrine pancreas [258-260], confounding interpretation of the direct actions of leptin in pancreatic β cells. More recently, Soedling et al. crossed mice in which Cre was knocked into the insulin I gene (*Ins1*Cre*)* with *Lepr*$^{flox/flox}$ mice to selectively delete *Lepr* in pancreatic β cells [261]. Unlike previous studies which reported elevated plasma insulin levels following knockout of *Lepr* [238, 258], knockout of *Lepr* in β cells using *Ins1*Cre did not induce elevated plasma insulin levels. Moreover,

knockout of *Lepr* using *Ins1*Cre did not result in glucose intolerance or obesity. While the studies described above used very similar approaches to assess the role of *Lepr* in β cells, differences in genetic background and tissue specificity of Cre lines may have contributed to the different phenotypes observed upon deletion of *Lepr*. Furthermore, these knockout studies are limited by the potential compensation by *Lepr* expressed either in other peripheral tissues or the central nervous system (CNS) to make up for the life-long loss of direct action of leptin in β cells.

In this chapter, we exploited a murine model in which a transcriptional blockade flanked by two *loxP* sites is placed between exon 16 and exon 17 of *Lepr* (*Lepr*$^{loxTB/loxTB}$), resulting in impaired leptin signalling [156] to determine the actions of leptin in β cells. To assess the direct actions of leptin in β cells, we crossed these mice with *Ins1*Cre mice to selectively restore *Lepr* in pancreatic β cells, while functional leptin receptors were absent in other tissues. This provided a unique model with which to assess whether the direct actions of leptin in β cells are sufficient to prevent hyperinsulinemia. All data in this chapter are published in the journal *Molecular Metabolism* [262].

4.2 Results

To assess recombination of the *Lepr*$^{loxTB/loxTB}$ site in the presence of *Ins1*Cre, islets were isolated from 11-13 week old *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice, and gDNA was extracted for analysis by qPCR. A ~75% rate of recombination of *Lepr* was observed in islets of *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice, while gDNA of *Lepr* in islets of *Lepr*$^{loxTB/loxTB}$ mice was less than 10% (Fig. 4.1A). Recombination of *Lepr* in islets was compared to recombination of *Lepr* in the hypothalamus to assess the tissue specificity

of *Ins1*Cre. The percent of recombination in the hypothalamus was minimal and was comparable between *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre and *Lepr*$^{loxTB/loxTB}$ mice (Fig.4.1B).

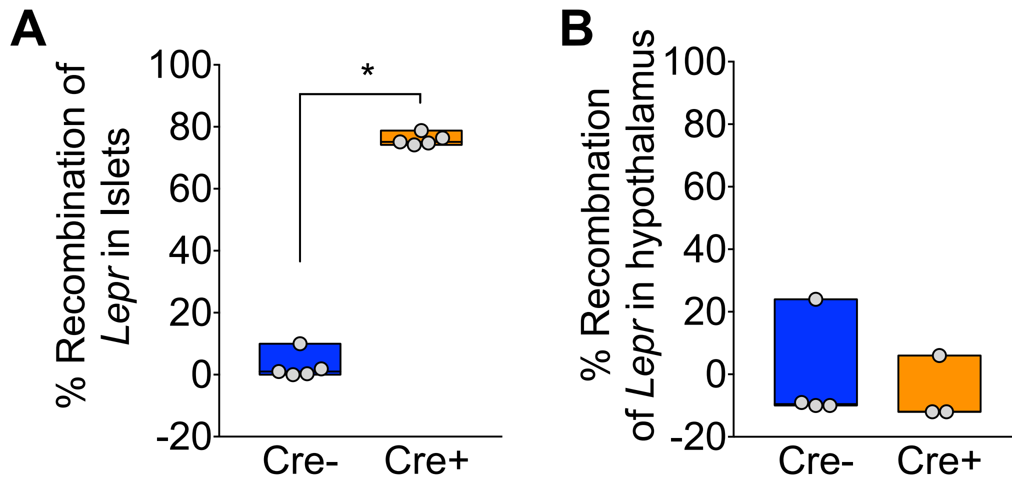


Figure 4.1: *Lepr* restoration in β cells was validated by qPCR. Percent recombination was assessed by measuring the ratio of qPCR product obtained using primers within and outside of the loxTB region (primer set 1 and 2, respectively in A) of gDNA extracted from islets (A) and hypothalamus (B). Individual values are presented with bars indicating the range of values and lines indicating the median value. *P<0.05 using a student's t-test.

Next, we crossed *Ins1*Cre mice with *mT/mG* reporter mice to confirm *Ins1*Cre produced recombination in β cells. These double fluorescent reporter mice express membrane-targeted dimer Tomato prior to Cre-mediated excision and membrane-targeted enhanced green fluorescent protein (EGFP) after excision [216]. Immunostaining of EGFP in fixed pancreas from *mT/mG Ins1*Cre and *mT/mG* control mice revealed EGFP expression co-localized in β cells of *mT/mG Ins1*Cre mice (Fig. 4.2A), indicating that Cre-induced recombination occurred in β cells. To determine whether replacing 1 insulin allele with Cre influenced glucose homeostasis and

metabolism, we also assessed body weight, fasting glucose, glucose tolerance and insulin sensitivity in male *mT/mG Ins1*Cre mice between the ages of 4-12 weeks.

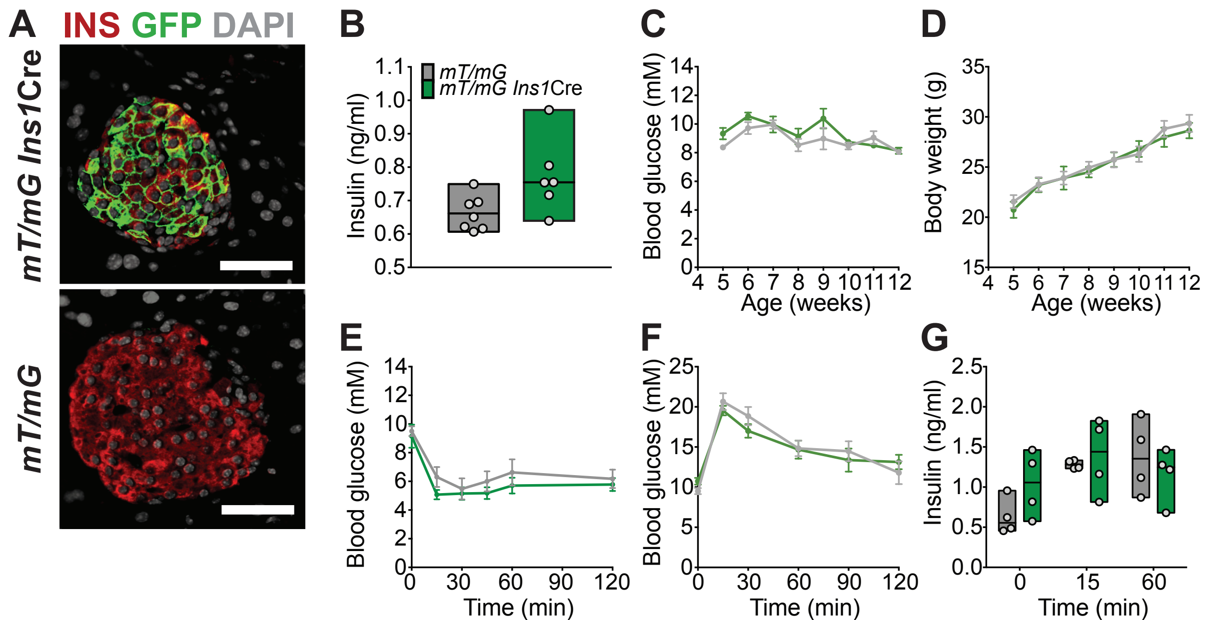


Figure 4.2: Knock in of Cre into a single *Ins1* allele does not affect glucose homeostasis in *mT/mG* mice. Representative images were collected from pancreata of *mT/mG Ins1*Cre and *mT/mG* mice co-stained for insulin (red) and EGFP (green) and DAPI (grey) (A). Expression of GFP indicates Cre-mediated recombination. Scale bar represents 100 µm. Fasted insulin levels were measured at 5 weeks of age (B), along with fasting glucose levels (C) and body weight (D) between 5-12 weeks of age in male *mT/mG Ins1*Cre and *mT/mG* mice. Blood glucose was measured following an i.p. injection of insulin (0.75 U/kg body weight) to assess insulin sensitivity in 6-week old mice (E). Plasma insulin levels were measured in 8-week old mice following a gavage of 40% glucose (1.5 g/kg body weight) to assess glucose tolerance and glucose stimulated insulin secretion (F, G). Values are presented as individual data points in B, and G with a line indicating the mean and bars indicating the range of values. Values in C-F are presented as mean ± SEM (n=4-6/group). A student's t-test with $P<0.05$ was used to determine statistical significance in B, a Repeated One Way ANOVA with Tukey post-hoc test was used to assess statistical significance in C-G.

Fasting insulin, blood glucose and body weight levels were similar between *mT/mG Ins1*Cre mice and *mT/mG* mice (Fig. 4.2B-D). Similarly, no differences in insulin tolerance, glucose tolerance or glucose stimulated insulin secretion were observed (Fig. 4.2E-G). Therefore, our characterization of the *Ins1*Cre line confirms and extends previously published data [215] and demonstrates that knock-in of Cre into one allele of *Ins1* does not appear to significantly affect glucose homeostasis.

It has been previously reported that whole body mutation of *Lepr* [140] or knockout of *Lepr* in the pancreas of chow-fed mice [258] results in increased β cell area. To determine if restoration of *Lepr* in β cells is sufficient to prevent the increase in β cell area, we immunostained pancreas sections collected from 12-14 week old *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre, *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{wt/wt}$ *Ins1*Cre mice with insulin, glucagon, and DAPI (Fig. 4.3A). Quantification revealed that *Lepr*$^{loxTB/loxTB}$ insulin positive area was ~10 fold higher compared to *Lepr*$^{wt/wt}$ Ins1Cre mice (Fig. 4.3C; p<0.01). Restoration of *Lepr* in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice resulted in a trend for reduced insulin positive area compared to *Lepr*$^{loxTB/loxTB}$ mice (p=0.06). In contrast, no differences in α cell area, as determined by glucagon positive immunostaining, were observed between experimental groups (Fig. 4.3D). Analysis of islet size revealed a significantly lower proportion of small islets (<500 μm^2) in *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice compared to *Lepr*$^{wt/wt}$ mice, while *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice had a greater number of large (>10,000 μm^2) islets than *Lepr*$^{wt/wt}$ littermates (Fig. 4.3E). To determine if restoration of *Lepr* in β cells is able to prevent the loss of Glut-2 expression in *Lepr*$^{loxTB/loxTB}$ mice, pancreas sections were co-immunostained for Glut-2, insulin and DAPI (Fig.4.3B). Glut-2 immunoreactivity was clearly present in *Lepr*$^{wt/wt}$ *Ins1*Cre mice, but generally lacking in *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. Together, these

findings suggest that direct action of leptin in β cells may not be sufficient to modify islet size or rescue Glut-2 expression in mice lacking leptin receptors elsewhere.

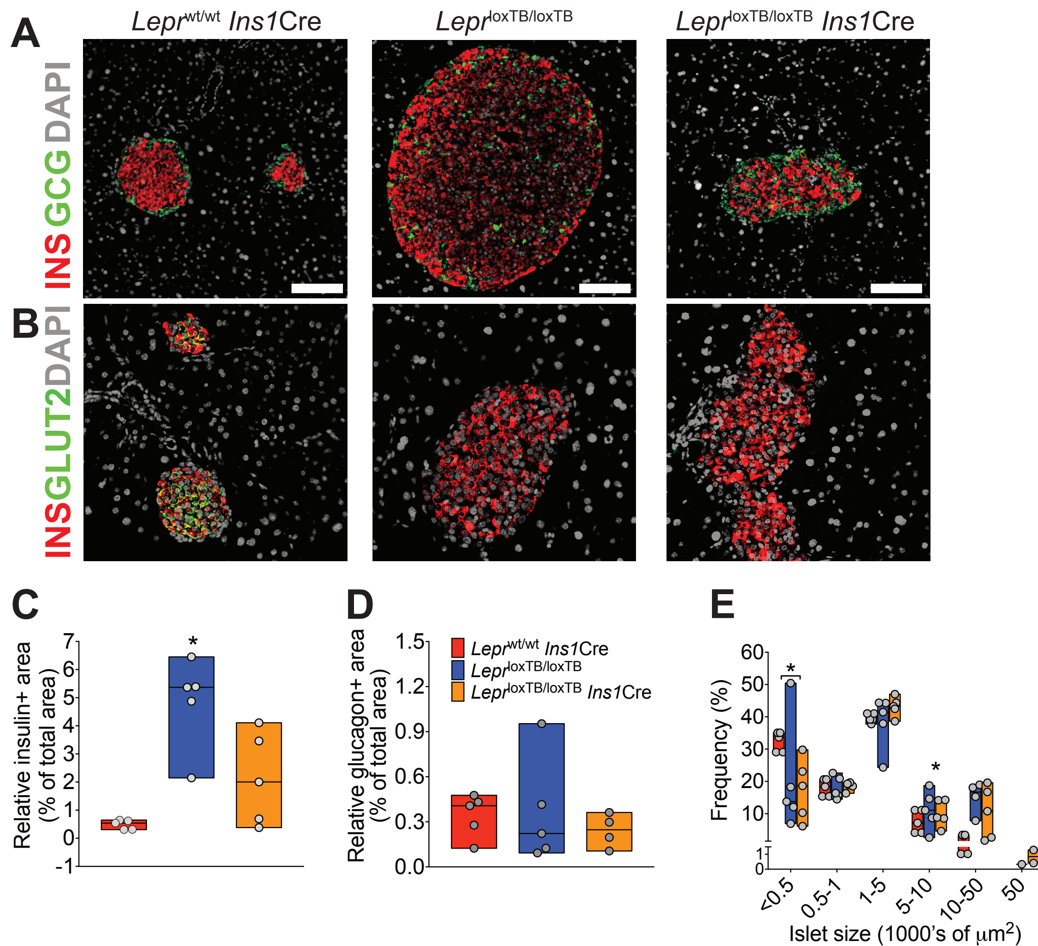


Figure 4.3: Restoration of *Lepr* in β cells does not improve islet morphology in *Lepr*null mice. Representative images were taken from pancreas sections of *Lepr*$^{wt/wt}$, *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice immunostained for insulin (INS; red), glucagon (GCG; green) and DAPI (grey) (A). Presence of Glut-2 in β cells was determined by co-staining pancreas sections for insulin (red) Glut-2 (green) and DAPI (white) in *Lepr*$^{wt/wt}$, *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice (B). Scale bar represents 100 μm. Relative β and α cell area were calculated by measuring total insulin and glucagon positive areas respectively, relative to total pancreas area (C, D). Islet size was approximated by measuring insulin positive area per islet. Islet size was divided into 6 categories and frequency of islets in each category was determined relative to total number of islets measured per animal (E). Values are presented as

individual data points with a line representing the median and the shaded area indicating the range of values. *P<0.05 compared to *Lepr*$^{wt/wt}$ *Ins1*Cre. A non-parametric One Way ANOVA with Kruskal-Wallis post hoc analysis was used to determine statistical significance.

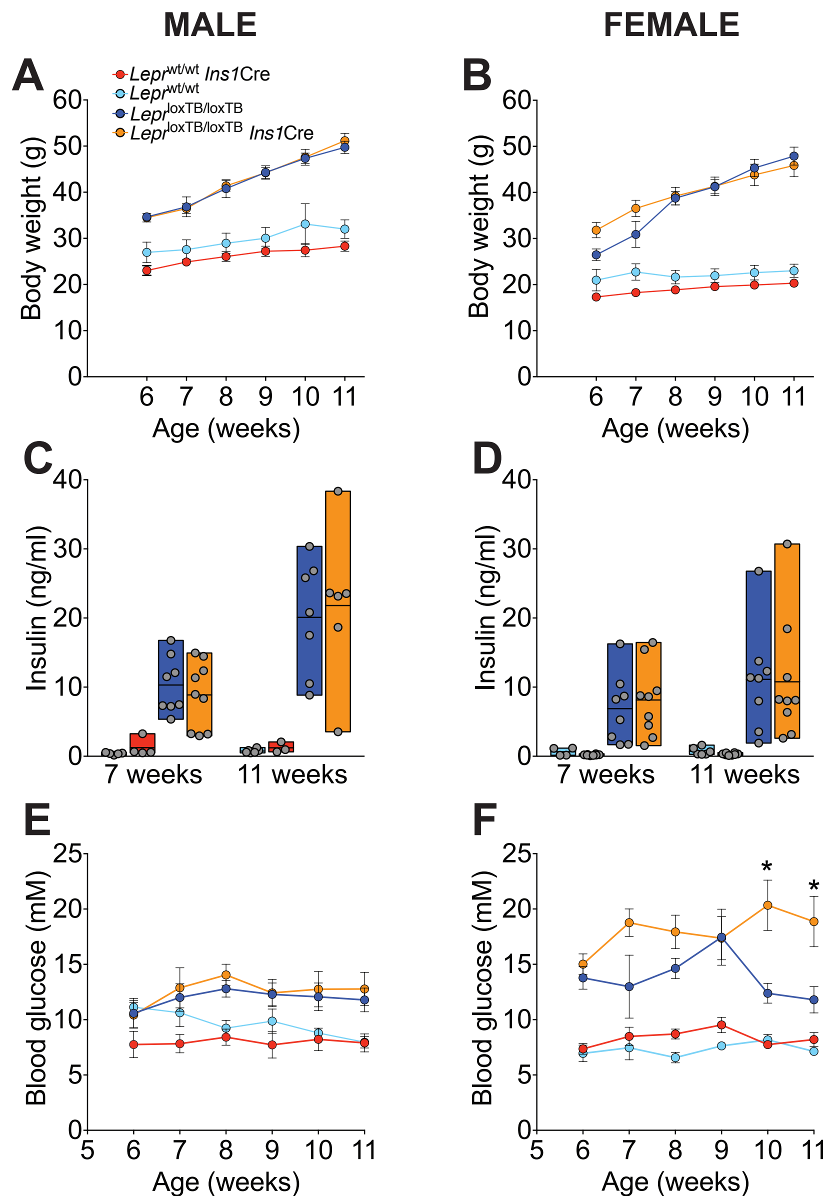


Figure 4.4: Restoration of *Lepr* in β cells of *Lepr*null mice does not prevent hyperinsulinemia. Body weight was measured weekly between 6-11 weeks of age in male (A) and female (B) *Lepr*$^{wt/wt}$, *Lepr*$^{wt/wt}$ *Ins1*Cre, *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre, mice. Fasting plasma insulin levels at 7 and 11 weeks of age in male (C) and

female mice (D). Values are presented as individual data points with a line indicating the median and the shaded region spanning the range of values. Fasting blood glucose levels were monitored comparing *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre and *Lepr*$^{loxTB/loxTB}$ using a One Way Repeated measures ANOVA with Tukey post hoc analysis.in male (E) and female (F) mice. Values in A, B, E, & F are presented as mean ± SEM (n=4-11/group). *P<0.05.

Along with increased insulin levels, obesity has been reported in *Lepr*$^{flox/flox}$ *RIP*Cre mice [238]. To examine the effects of restoration of *Lepr* in β cells, we measured body weight weekly between 6-11 weeks of age and collected plasma at 13 weeks of age to measure leptin levels. Body weight of male and female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice was comparable to *Lepr*$^{loxTB/loxTB}$ mice. Male *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice weighed 50% more than *Lepr*$^{wt/wt}$ littermate mice, while female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice weighed 80% more than their respective control littermates (Fig.4.4A, B). Similar to prior studies with *Lepr*$^{loxTB/loxTB}$ mice [156], we observed hyperleptinemia in *Lepr*$^{loxTB/loxTB}$ male and female mice, with leptin levels that were ~150 times higher compared to *Lepr*$^{wt/wt}$ *Ins1*Cre littermates (*Lepr*$^{loxTB/loxTB}$ = 252.9±26.7 ng/ml, *Lepr*$^{wt/wt}$ *Ins1*Cre = 1.65±0.20 ng/ml). Despite restoring *Lepr* in β cells, fasting leptin levels of *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice (279.8±11.67 ng/ml) were comparable to that of *Lepr*$^{loxTB/loxTB}$ mice. To assess the effects of β cell-specific signalling of leptin on insulin secretion and glucose homeostasis, we monitored blood glucose weekly between 6-11 weeks of age and collected plasma from male and female mice at 7 and 11 weeks of age to measure insulin levels. Hyperinsulinemia was evident in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice and plasma insulin levels were comparable to that of *Lepr*$^{loxTB/loxTB}$ mice (Fig. 4.4C, D). In contrast, plasma insulin levels of *Lepr*$^{wt/wt}$ and *Lepr*$^{wt/wt}$ *Ins1*Cre mice were ~20 fold lower than *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. Similar to fasting insulin levels, fasting

glucose levels were elevated in *Lepr*$^{loxTB/loxTB}$ mice and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice compared to *Lepr*$^{wt/wt}$ and *Lepr*$^{wt/wt}$ *Ins1*Cre mice (Fig. 4.4E, F). However, by 10-11 weeks of age, female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice exhibited significantly higher glucose levels than their *Lepr*$^{loxTB/loxTB}$ littermates. Together these findings suggest that selective restoration of *Lepr* in β cells does not prevent obesity and hyperinsulinemia in mice lacking leptin receptors elsewhere. Moreover, restoration of *Lepr* in β cells does not improve fasting glucose levels of male or female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice relative to their respective littermate *Lepr*$^{loxTB/loxTB}$ mice.

We next assessed the effects of restoring *Lepr* in β cells on insulin secretion in both male and female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. Following a 6 h fast, *Lepr*$^{wt/wt}$ mice were euglycemic, while *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice were hyperglycemic (blood glucose >11 mM; Fig. 4.5A, D). Fifteen minutes after glucose gavage, blood glucose levels of male and female *Lepr*$^{wt/wt}$ and *Lepr*$^{wt/wt}$ *Ins1*Cre mice peaked between 15-18 mM. In contrast, glucose levels of *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice peaked above the limit of detection, (33.3 mM, represented as a dashed line on the graph) indicating they were severely glucose intolerant. Analysis of area under the curve (AUC) revealed that *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice were significantly glucose intolerant compared to control littermate mice (Fig. 4.5B, E).

In response to glucose gavage, male and female *Lepr*$^{wt/wt}$ and *Lepr*$^{wt/wt}$ *Ins1*Cre mice had fasting plasma insulin levels between 0.1-0.7 ng/ml, and insulin levels peaked at 0.3-1.1 ng/ml at the 15-minute time point (Fig. 4.5C, F). In contrast, *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice had fasting insulin levels that were ~10-fold higher than *Lepr*$^{wt/wt}$ mice, and peak glucose stimulated insulin secretion levels that were between 15 to 30-fold higher than *Lepr*$^{wt/wt}$ mice. Fasting as well as glucose stimulated plasma

insulin levels were comparable between *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. Despite similar glucose levels in response to oral glucose gavage, basal plasma insulin levels and glucose stimulated insulin secretion was different between male and female mice. Female *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice had lower basal insulin levels compared to males (Fig. 4.4C-D, 4.5C,F), and insulin secretion in response to glucose was much higher in female *Lepr*$^{loxTB/loxTB}$ as compared to male mice of the same genotype (Fig. 4.5C, F).

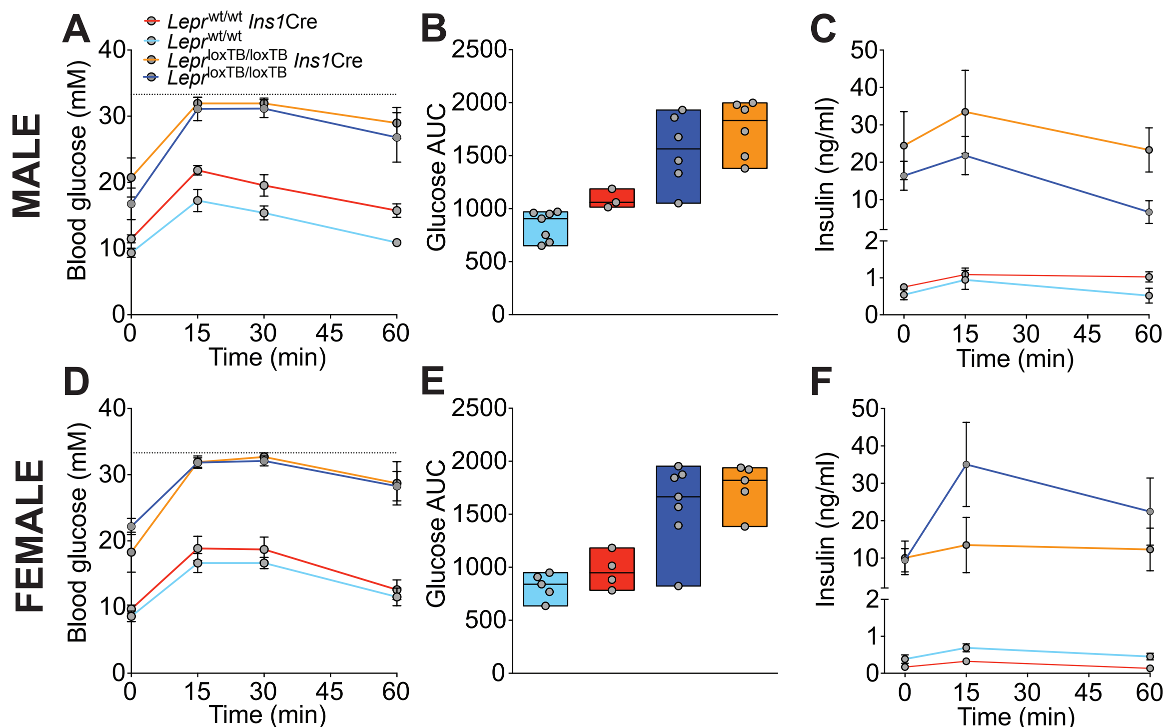


Figure 4.5: *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice are glucose intolerant and have impaired glucose stimulated insulin secretion. Male (A) and female (D) *Lepr*$^{wt/wt}$, *Lepr*$^{wt/wt}$ *Ins1*Cre, *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice between 7-12 weeks of age were fasted for 6 h to assess glucose tolerance by gavage of 40% glucose at a dose of 1.5 g glucose/kg body weight. Blood glucose was measured every 15 minutes and area under the curve was analysed (B, E). Blood collected at 0, 15 and 60 minutes of the glucose tolerance test was used to measure plasma insulin levels (C, F). Values are presented

as mean ± SEM in panels A, C, D, and F (n=4-11), while values in B and E are presented individually with a line indicating the median. *P<0.05 using a Repeated Measures One Way ANOVA with Tukey post hoc analysis in C and F. No statistical analysis was performed on A, B, D & E due to data points exceeding the limit of detection.

4.3 Discussion

To examine the effects of restoring *Lepr* in β cells on insulin secretion and morphology, we crossed mice expressing Cre knocked into the *Ins1* gene with *Lepr*$^{loxTB/loxTB}$ mice, which have a transcriptional blockade inserted between exon 16 and exon 17 of *Lepr,* resulting in impaired leptin signalling. Disruption of the leptin receptor signalling domain in *Lepr*$^{loxTB/loxTB}$ mice results in a phenotype similar to *db/db* mice, characterized by obesity, hyperinsulinemia, hyperglycemia, insulin resistance, and islet hyperplasia [65, 205]. Cre-mediated removal of the transcriptional blockade and restoration of *Lepr* can be achieved in *Lepr*$^{loxTB/loxTB}$ mice in a cell-type selective manner, enabling the assessment of the physiological impact of *Lepr* in specific tissues. Restoration of *Lepr* in β cells of *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice did not alter the development of hyperinsulinemia, hyperglycemia, glucose intolerance and obesity, suggesting that leptin signalling in β cells does not suppress insulin secretion. Moreover, islet hyperplasia was still present in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice, similarly to what is reported in *db/db* mice [205]. However, as we did not follow the mice for an extended period of time, our studies cannot rule out the possibility that the restoration of *Lepr* in β cells may have prevented age related β cell failure that eventually results in overt severe diabetes, like in *db/db* mice [263, 264].

A distinct difference between the present study and previous studies in which *Lepr* was selectively deleted [238, 258, 261] is the inability of compensation to occur from

non-β cells in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. In prior studies that examined the role of leptin signalling in β cells by deletion of *Lepr*, it is unclear whether *Lepr* acting in other tissues influenced pancreas morphology and β cell function. The ability of non-β cell leptin receptor action to influence β cell function is evident in mice with restored *Lepr* in brains of *db/db* mice. Overexpression of *Lepr-b* driven by synapsin 1 and neuron specific enolase promoters in *db/db* mice resulted in normalized islet morphology and partial correction of body weight [11]. These authors concluded that *Lepr* in the CNS is able to rescue the diabetic and obese phenotype of *db/db* mice. To prevent *Lepr* in non-β cells from influencing β cell function, we selectively re-expressed *Lepr* in β cells of mice lacking *Lepr* elsewhere. Our results suggest that *Lepr* in β cells is not sufficient to prevent hyperinsulinemia and glucose intolerance from occurring. It is likely that *Lepr* in the CNS and other islet cell populations are involved in inhibiting insulin secretion and preventing hyperplasia of β cells.

Previous studies have reported *Lepr* expression in β cells of human and rodent islets and cell lines [169, 170, 250, 265, 266], however, in another study, non-detectable levels of *Lepr* were reported in β cells from dispersed islets using RT-qPCR [261]. More recently, studies using single-cell RNA-seq to determine the transcriptome profiles of human and murine islets revealed that the expression of *Lepr* was higher in δ cells than β cells [117, 267]. Though insulin secretion and expression was reduced in isolated murine and human islets treated with leptin [172, 251, 253], this effect may have been a result of leptin acting on δ cells. Collectively, this suggests that despite restoration of *Lepr* in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice, the relatively low levels of *Lepr* in β cells may not be sufficient to provoke improvements in β cell function in the absence of leptin receptor signalling in other pancreatic islet populations.

The presence of hyperinsulinemia and islet hyperplasia despite restoration of *Lepr* in β cells of *Lepr*$^{loxTB/loxTB}$ mice may be due to hyperleptinemia that was present in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. Plasma leptin levels were ~150-fold higher in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice compared to control mice. Hyperleptinemia has been previously observed in *Lepr*$^{loxTB/loxTB}$ [156], and in mice with disruption of *Lepr* in peripheral tissues [268]. This increase of plasma leptin levels was attributed to the disruption of *Lepr* in white adipose tissue (WAT), which led to inactivation of a negative feedback loop regulating leptin synthesis in WAT [268]. Indeed the presence of hyperleptinemia can cause leptin resistance [269]. Thus, despite restoration of *Lepr* in β cells, the chronically elevated leptin levels in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice may have resulted in a state of leptin resistance in β cells of *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. It should also be noted that the Pfaffl method used to determine the degree of recombination of Lepr based on the qPCR assay resulted in values below zero for hypothalamic samples, indicating a certain degree of noise and variablity between samples in the assay.

In summary, selective restoration of *Lepr* in pancreatic β cells of *Lepr* knockout mice does not prevent the development of hyperinsulinemia and β cell expansion. These findings suggest that leptin receptor signalling directly in β cells does not regulate β cell function. We propose that actions of leptin on β cells are secondary to action of leptin signalling in other tissues. Whether this involves a combination of leptin signalling in other islet cell populations and in the CNS warrants further study.

CHAPTER 5: LEPTIN DEFICIENCY IN RATS RESULTS IN HYPERINSULINEMIA AND IMPAIRED GLUCOSE HOMEOSTASIS

5.1 Introduction

Leptin deficiency in *ob/ob* mice results in hyperglycemia and hyperinsulinemia [208]. This is also evident in *db/db* mice and Zucker Diabetic Fatty (ZDF) rats, which both lack functional leptin receptors as a result of a spontaneous mutation in the gene encoding the leptin receptor [70, 270]. Islets from *ob/ob* mice, *db/db* mice and ZDF rats have increased β cell mass compared to wildtype littermates but also have impaired glucose stimulated insulin secretion [207, 264, 271, 272]. These aberrations in glucose metabolism were initially thought to be a consequence of obesity; however leptin therapy normalized fasting glucose and reduced plasma insulin levels by 80% without significant reductions in body weight in leptin-deficient *ob/ob* mice [137, 273]. Similarly, pair feeding *ob/ob* mice to match food intake of wildtype littermates did not effectively improve glucose metabolism, while leptin therapy was able to normalize glucose tolerance, suggesting the effects of leptin on glucose homeostasis are independent of food intake [152]. Furthermore, glucose stimulated insulin secretion in *ob/ob* islets treated with leptin was increased by 15% at 5.5 mM glucose and 85% at 16.7 mM glucose [274]. The beneficial effects of leptin therapy on glucose homeostasis are also evident in animal models without leptin deficiency or genetic defects in leptin signalling. Leptin-treating the UCD-T2DM rat, a model of polygenic type-2 diabetes resulted in increased insulin sensitivity and reduced fasting glucose without any changes in food intake [241]. Together, these results reveal that leptin is able to alter glucose homeostasis independent of energy balance.

The phenotype resulting from leptin deficiency differs between *ob/ob* mice and humans. In humans with congenital leptin deficiency, severe obesity occurs but fasting glucose levels are typically in the normal range [193, 194]. In contrast, adult *ob/ob* mice are obese, and may have transient have fasting glucose levels >12 mM in early adulthood [138, 208, 273]. Thus, though the *ob/ob* mouse reflects some aspects of leptin deficiency observed in humans, it does not accurately portray all aspects of this condition. Rats have an extensive history of use in medical research as they are more physiological similar to humans when compared to mice. Genetic modifications were previously exclusive to mice, however, now, through the use of zinc finger nuclease technology a leptin knockout rat has been generated via a 151 bp deletion of exon 1. Initial characterization of these leptin-deficient rats revealed obesity, hyperphagia and elevated insulin levels [213]. However, other aspects of glucose metabolism were not reported.

In this chapter, we characterized the metabolic phenotype of leptin-deficient rats to determine whether perturbations to glucose and energy homeostasis previously observed in *ob/ob* mice also occur in rats. All data in this chapter are published in *Endocrinology* [92].

5.2 Results

Leptin has well-established effects on energy balance, so we characterized the effects of leptin deficiency in rats by measuring metabolic parameters including body weight and adiposity. Deletion of 151 bp within exon 1 of the gene encoding leptin (*Lep)* in Sprague Dawely rats resulted in male *Lep*^{KO} rats that were significantly heavier than

wildtype (*Lep*^{WT}) littermates by 7 weeks of age, and were 50% heavier by 12 weeks of age (Fig. 5.1A; p<0.01). Similarly, body mass was significantly higher in female *Lep*^{KO} rats by 6 weeks of age, and was 60% greater than *Lep*^{WT} littermates by 12 weeks of age (Fig. 5.1B; p<0.01). MRI imaging confirmed that the increase in body mass was attributed to increased visceral and subcutaneous adiposity in both male and female *Lep*^{KO} rats compared to *Lep*^{WT} littermates (Fig. 5.1C). Whole-body fat composition was determined by NMR analysis in 18-week-old rats. Total fat mass was 2-fold higher in *Lep*^{KO} males and 2.8-fold higher in *Lep*^{KO} females compared to their respective *Lep*^{WT} littermates (Fig. 5.1D, p<0.05). Lean mass did not differ between *Lep*^{KO} rats and *Lep*^{WT} littermates (p>0.05). Levels of adiponectin, an adipose-derived hormone that is inversely related to obesity and insulin resistance [206, 225], were significantly higher in male and female *Lep*^{KO} rats at 9 weeks of age compared to *Lep*^{WT} littermates while only female *Lep*^{KO} rats maintained higher adiponectin levels compared to *Lep*^{WT} at 34 weeks of age (Fig. 5.1E, p<0.05).

Obesity can be a consequence of both increased energy intake and reduced energy expenditure. Previous studies have suggested that leptin regulates energy expenditure in part through brown adipose tissue thermogenesis [275]; indeed, leptin deficiency in mice has been shown to diminish their capacity for cold thermoregulation [276]. Thus, we next sought to characterize thermoregulatory capacity in male *Lep*^{KO} rats using a cold tolerance test. After thirty minutes of cold exposure, body temperature of *Lep*^{WT} rats was not significantly different compared to baseline (Fig. 5.1F, p>0.05). In contrast, body temperature of *Lep*^{KO} rats was significantly reduced from baseline and remained lower for the duration of the cold exposure (Fig. 5.1F, p<0.05).

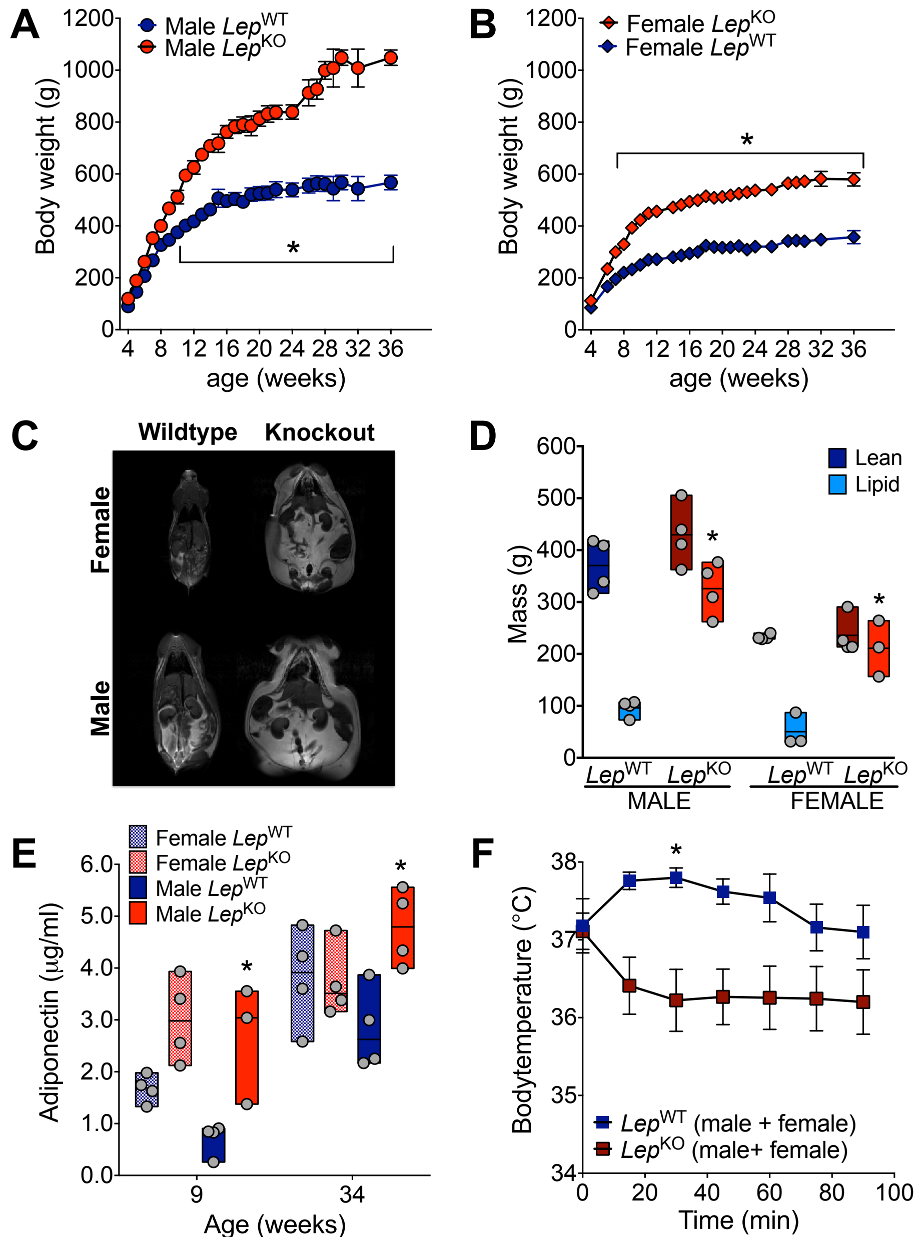


Figure 5.1: Lep^{KO} rats are obese and have increased lipid mass compared to Lep^{WT} littermates. Body weight was tracked in male (A) and female (B) $Lep^{WT}$and Lep^{KO} rats. MRI and NMR (C) were used to determine distribution of adiposity and lipid mass in male and female rats at 18 weeks of age. Plasma leptin levels were assessed following a 4-hour fast in 34-week-old male rats; dashed line indicates the limit of detection of the leptin assay (D). Plasma adiponectin levels were measured after a 4 h fast at 9 and 34 weeks of age in male and female rats (E). Cold tolerance was measured in 17 week old male and female rats via exposure to 4°C over 90 minutes (F).

Data are expressed as mean ± SEM in A-B and F (n=6-11), and are expressed as individual values with a bar indicating the range of values and line indicating the mean in D-E *P<0.05 by One Way Repeated measures ANOVA and Tukey post hoc analysis (A-B), student's t-test (D) or repeated measures Two Way ANOVA and Sidak's post hoc analysis (E-F).

Ectopic lipid accumulation has been previously observed in *ob/ob* mice [277] and ZDF rats, which lack functional leptin receptors [278]. To determine whether ectopic lipid accumulation was present in leptin-deficient rats, we quantified and compared muscle and hepatic lipid content of 36-week-old *Lep*^{KO} rats and *Lep*^{WT} littermates. Muscle triglyceride content in *Lep*^{KO} male and female rats was 4.6- and 4.2-fold greater compared to their respective *Lep*^{WT} littermates (Fig. 5.2A). Similarly, hepatic triglyceride content in *Lep*^{KO} male and female rats was 1.8- and 3.3-fold higher, compared to their respective *Lep*^{WT} littermates (Fig. 5.2B).

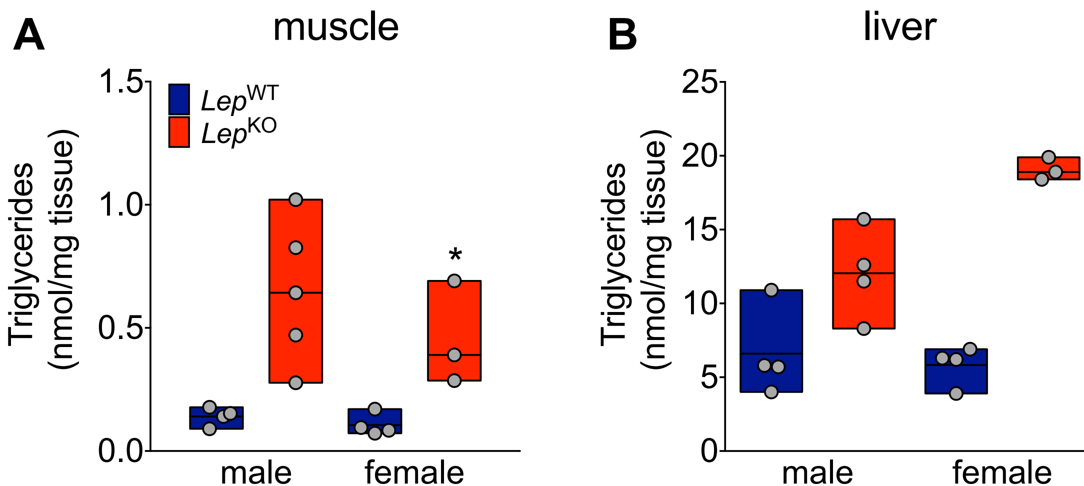


Figure 5.2: *Lep*^{KO} rats have increased ectopic lipids compared to *Lep*^{WT} littermates. Lipids were extracted and measured from muscle and liver of 36-week-old wildtype (*Lep*^{WT}) and leptin knockout (*Lep*^{KO}) rats. Data are presented as individually with a line indicating the median and bars depicting the range of data points. *P<0.05; by non-parametric Mann Whitney t-test.

To investigate whether leptin deficiency in rats affects glucose levels, fasting blood glucose and plasma insulin were measured in *Lep*^{KO} rats at multiple ages. Fasting glucose levels were initially comparable to *Lep*^{WT}, but by 8 weeks of age fasting glucose levels were typically 1.5-fold higher in male *Lep*^{KO} rats compared to *Lep*^{WT}, and were significantly elevated between 14-25 weeks of age (Fig. 5.3A; $p<0.05$ by one-way ANOVA). In contrast, female *Lep*^{KO} rats maintained fasting glucose levels similar to *Lep*^{WT} littermates, except at 12 weeks of age (Fig. 5.3B). These findings are in line with previous reports that indicate that female rats tend to be protected against increases in fasting glucose levels compared to males, an effect that may be mediated by estrogen [279, 280].

Previously, Vaira et al. reported that *Lep*^{KO} rats have elevated fasting plasma insulin levels compared to *Lep*^{WT} littermates at 4 weeks of age [213]. Here we report sustained fasting hyperinsulinemia in male and female *Lep*^{KO} rats up to 36 weeks of age. At 6 weeks of age, plasma insulin levels were 22-fold higher in male *Lep*^{KO} rats compared to *Lep*^{WT} (12.58 ± 1.82 ng/ml *vs.* 0.57 ± 0.12 ng/ml, $p<0.05$, Fig. 5.3C), while plasma insulin levels of female *Lep*^{KO} rats were 54-fold higher than *Lep*^{WT} (37.38 ± 3.84 ng/ml *vs.* 0.70 ± 0.02 ng/ml, Fig. 5.3D).

In addition to insulin, glucagon and growth hormone (GH) also contribute to regulation of glucose homeostasis. Fasted plasma glucagon levels were elevated in *Lep*^{KO} males (215.20 ± 16.44 ng/ml *vs*. 136.39 ± 8.92 ng/ml; $p<0.01$) and *Lep*^{KO} females (190.10 ± 13.82 ng/ml *vs.* 114.70 ± 8.73 ng/ml; $p<0.01$) compared to their respective littermate controls (Fig. 5.3E). A trend for reduced GH levels in male and female *Lep*^{KO} rats compared to their respective *Lep*^{WT} littermates between the ages of 10-14 weeks was observed (Fig 5.3F; $p=0.05$ and N.S., respectively). Despite a similar trend of

concomitant hyperinsulinemia, hyperglucagonemia and reduced GH levels, female *Lep*^{KO} rats do not develop a transient elevation in fasting glucose levels while male *Lep*^{KO} rats do.

To further characterize the effects of leptin deficiency on glucose handling, glucose tolerance and insulin sensitivity were assessed using an oral glucose tolerance test (GTT). Following an overnight fast, blood glucose levels were comparable between 7-week-old male and female *Lep*^{KO} and *Lep*^{WT} rats. However, by 20 minutes post- gavage, blood glucose levels were higher in both male (Fig. 5.4A) and female (Fig.5.4E) *Lep*^{KO} rats compared to *Lep*^{WT} controls. Area under the curve (AUC) analysis of glucose response revealed glucose intolerance in male *Lep*^{KO} rats (1274.50 ± 100.58 *vs.* 978.00 ± 24.39; $p<0.05$), but differences were not significant in female *Lep*^{KO} rats (1177.75 ± 112.43 *vs.* 1001.42 ± 30.35; $p>0.05$). In both *Lep*^{KO} and *Lep*^{WT} groups, glucose levels returned to baseline values by 2 h after glucose administration. Since glucose intolerance may arise from impaired glucose stimulated insulin secretion, we assessed plasma insulin levels during the GTT (Fig. 5.4C, G).

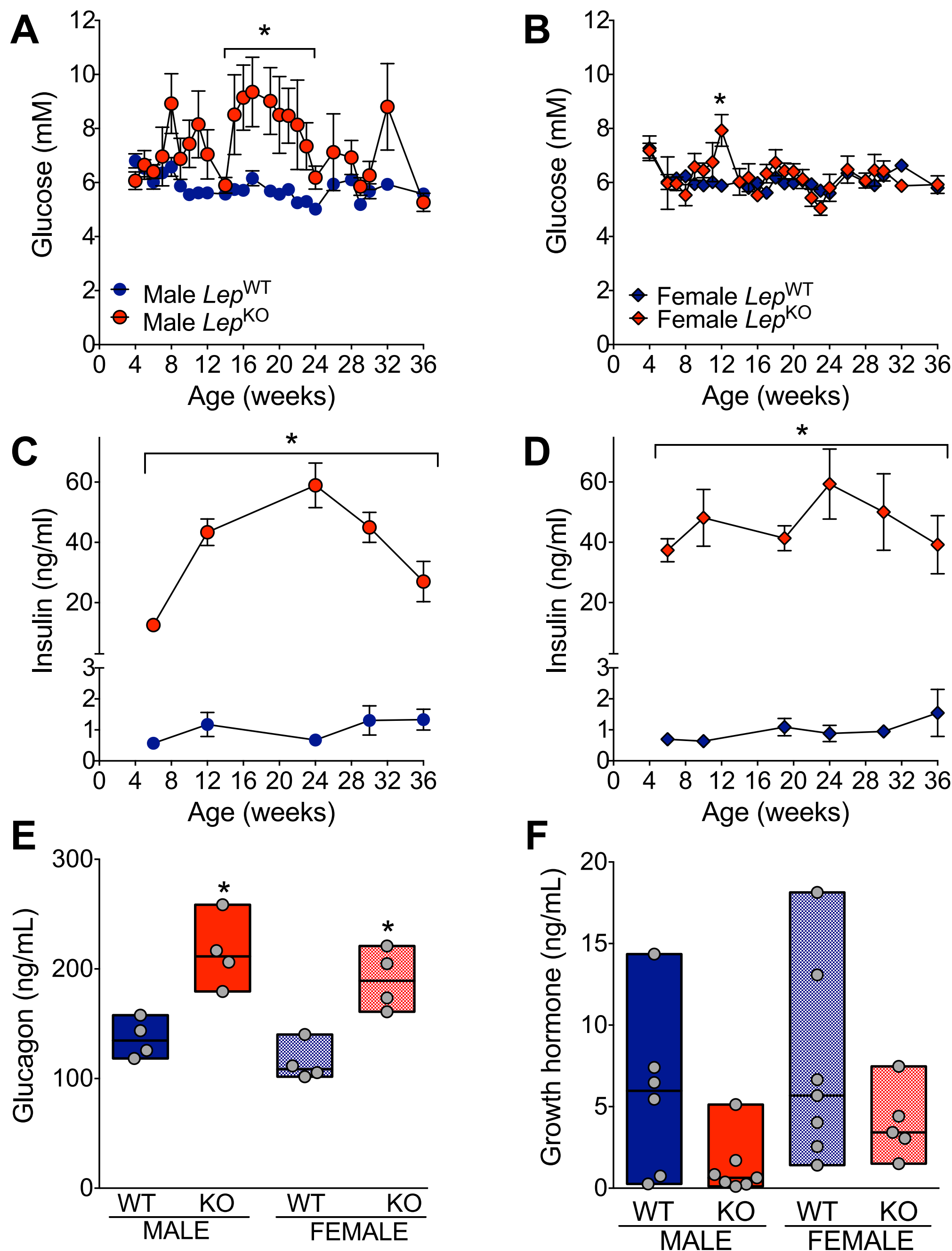


Figure 5.3 Leptin-deficient rats are hyperinsulinemic and males experience transient hyperglycemia. Fasting blood glucose levels (A-B) and plasma insulin (C-D) were measured in male and female rats. Fasted glucagon levels (E) and growth hormone levels (F) were measured in male and female rats at 26 weeks of age and 10-14 weeks of age, respectively. Data are expressed as mean ± SEM in A-D (n=6-11/group for A-B and n=4/group for C-D), and as individual data points with bars

indicating the range of values and a line indicating the mean in E-F; *P<0.05 by Two Way Repeated Measures ANOVA and Tukey post hoc analysis (A-D) and student's t-test within each sex (E-F).

In response to glucose gavage, male and female *Lep*^{WT} rats exhibited a 22- and 4-fold increase of plasma insulin levels 10 minutes after gavage (p<0.05 by two-way repeated measures ANOVA). In contrast, *Lep*^{KO} rats were hyperinsulinemic at baseline and throughout the GTT, but did not elicit a significant increase in plasma insulin levels 10 minutes post-gavage (p>0.05 by two-way repeated measures ANOVA).

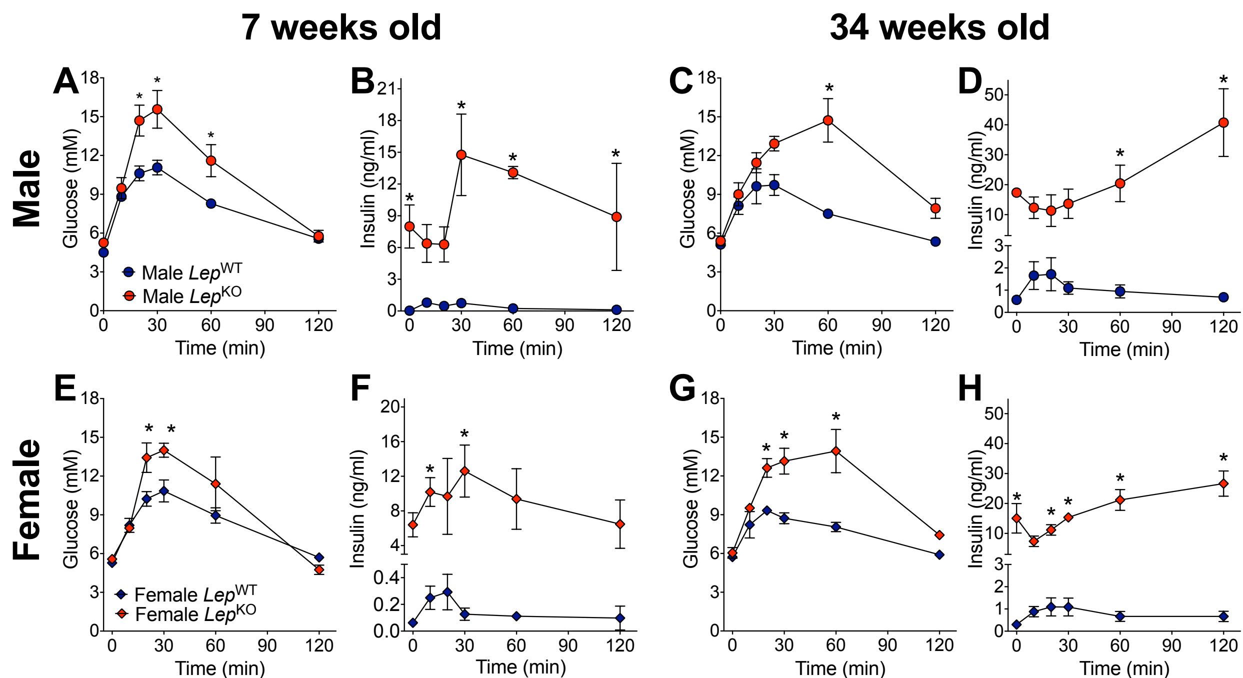


Figure 5.4: Glucose intolerance and impaired glucose stimulated insulin secretion (GSIS) occurs in *Lep*^{KO} rats. An oral glucose tolerance test (GTT) was performed on 7-week-old male (A) and female (E) rats following an overnight fast (1.5 g/kg glucose). Plasma was collected at each time point to measure GSIS in male (C) and female (F) rats. A GTT was repeated at 34 weeks of age in aging male (C) and female (G) rats, with corresponding measurement of plasma insulin levels (D, males; H, females). Data are expressed as mean ± SEM (n=5-6/group). *P<0.05 by Two Way Repeated measures ANOVA and Tukey post hoc analysis.

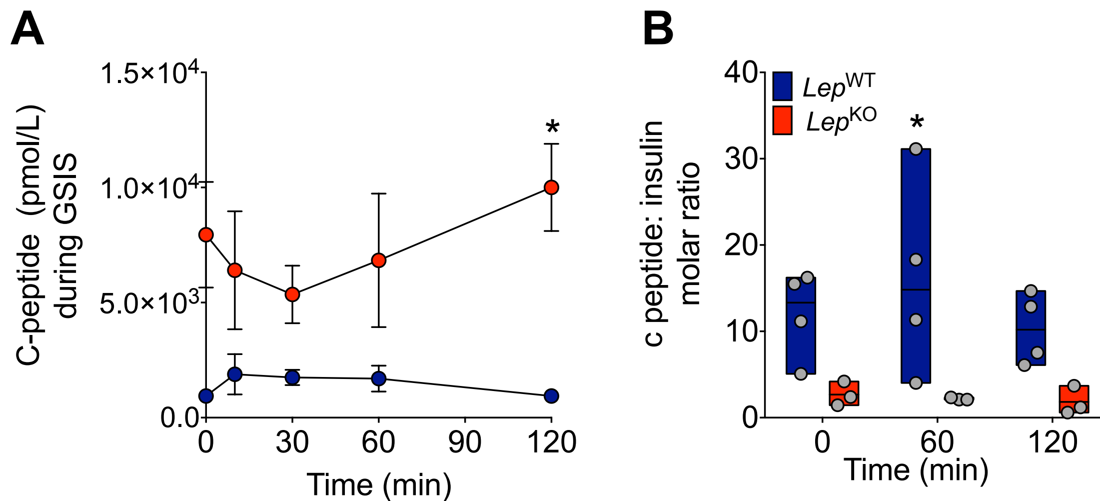


Figure 5.5: *Lep*^{KO} rats have deficits in insulin clearance. Plasma was collected during an OGTT performed in 34-week-old male rats to measure glucose stimulated c-peptide levels (A). C-peptide levels were divided by insulin levels to determine the molar ratio (B). Data are expressed as mean ± SEM in A (n=4/group) and as individual data points with a line indicating the median and bars indicating the range of values in B. *P<.05 by Repeated Measures Two Way Anova and Tukey multiple comparison analysis.

To assess glucose tolerance in older rats, we performed a GTT on 34-week-old rats (Fig. 5.4C-D, G-H). Glucose tolerance of *Lep*^{WT}rats at 34 weeks of age was comparable to results at 7 weeks of age, while glucose tolerance deteriorated with age in *Lep*^{KO} rats (Fig. 5.4C, G). Glycemic excursion was 1.4-fold greater in 34-week-old male *Lep*^{KO} rats (1390.50 ± 111.36 *vs*. 895.63 ± 38.67 $p<0.05$), and 1.5-fold greater in 34-week-old female *Lep*^{KO} rats (1364.50 ± 102.02 *vs.* 917.88 ± 19.54; $p<0.05$) compared to *Lep*^{WT} littermates of the same age. Blood glucose levels peaked at 60 minutes in *Lep*^{KO} rats at 34 weeks of age, in comparison to 30 minutes at 7 weeks of age. Though *Lep*^{KO} rats had pronounced hyperinsulinemia relative to *Lep*^{WT}, older

female Lep^{KO} rats failed to increase insulin levels until 60 after glucose administration and increases in insulin levels were evident in male Lep^{KO} rats only after 120 min ($p<0.05$ by two-way ANOVA). Fasting insulin levels are regulated by insulin output from the pancreas as well as clearance by the liver and kidney. In a leptin-deficient state, *ob/ob* mice have reduced insulin clearance [281]. To assess insulin clearance, plasma C-peptide levels were measured in male rats during the glucose challenge at 34 weeks of age and compared to insulin levels. A reduced C-peptide to insulin ratio indicates reduced clearance. Despite having 10-15 fold higher C-peptide levels (Fig. 5.5A), male Lep^{KO} rats had a lower C-peptide to insulin ratio compared to male Lep^{WT} littermates, indicating reduced insulin clearance (Fig. 5.5B, $p<0.05$). These findings suggest that deficits in insulin clearance in addition to increased basal secretion may contribute to hyperinsulinemia in this model.

Since hyperinsulinemia is often associated with insulin resistance [147], we next tested whether insulin resistance might contribute to the diminished glucose tolerance observed in Lep^{KO} rats. Sixty minutes after i.p. injection of insulin, glucose levels in 11-week-old male and female Lep^{WT} rats were reduced by 52% and 48%, respectively, compared to baseline (Fig. 5.6A-B). In contrast blood glucose levels were unchanged in Lep^{KO} rats following insulin injection, indicating significant insulin resistance. The integrated change in blood glucose levels (represented as area over the curve or AOC) remained positive in Lep^{WT} males and females (270.17 ± 76.32 and 363.03 ± 101.44, respectively), but was negative for Lep^{KO} males (-18.61 ± 79.51) and females (-7.33 ± 111.68). At 34 weeks of age, both male and female Lep^{KO} rats remained insulin resistant compared to Lep^{WT} (Fig. 5.6C; AOC = 168.33 ± 66.21 in male Lep^{WT} rats *vs.* 451.15 ± 17.42 in Lep^{KO} rats; Fig. 5.6D; AOC = 513.03 ± 21.46 in female Lep^{WT} rats *vs.*

-44.64 ± 85.68 in *Lep*^{KO} rats). Hepatic phosphorylation of Akt (S473) was reduced by 1.7 fold (Fig. 5.6E, p=0.05) while phosphorylation of Erk 1 (T202/Y204) and 2 (T185/Y187) was reduced by 2.4 fold relative to *Lep*^{WT} animals (Fig. 5.6F, p<0.05). Thus, *Lep*^{KO} rat had significant impairments in hepatic insulin sensitivity concomitant with hyperinsulinemia, which was sustained with aging.

To investigate the effects of leptin deficiency on postprandial glucose metabolism, 13-week-old rats were subject to an overnight fast followed by 1 h of free feeding of their normal diet. Prolonged fasting resulted in similar blood glucose levels in both male and female *Lep*^{KO} rats compared to *Lep*^{WT} littermate controls. After one h of feeding, blood glucose levels of male *Lep*^{KO} rats were 1.7-fold higher than *Lep*^{WT} littermates (Fig 5.6G, 10.32 ± 0.93 mM *vs.* 6.06 ± 0.20 mM; p<0.05). Similarly, female *Lep*^{KO} rats exhibited a 1.5-fold increase in blood glucose levels compared to *Lep*^{WT} following 1 h of free feeding (12.00 ± 1.30 mM *vs.* 7.92 ± 0.69 mM; p<0.05). Interestingly, these elevations in postprandial blood glucose levels occurred in *Lep*^{KO} rats despite hyperinsulinemia in the fasted and fed state (Fig. 5.6H; 12.68 ± 1.71 ng/ml to 74.75 ± 3.25 ng/ml in males; 29.64 ± 6.54 ng/ml to 160.72 ± 46.60 ng/ml in females; p<0.05 *Lep*^{WT} *vs.* *Lep*^{KO}, by two-way ANOVA). Feeding also increased insulin levels in *Lep*^{WT}rats (0.20 ± 0.07 ng/ml to 0.55 ± 0.14 ng/ml in males; 0.20 ± 0.03 ng/ml to 0.59 ± 0.13 ng/ml in females), but to a lesser extent.

A defining feature of *ob/ob* mice is their large pancreatic islets consisting predominantly of β cells [207]. To determine whether this is also the case in *Lep*^{KO} rats, we co-stained pancreas sections taken from 3 different regions for insulin and glucagon. Islets from 36-week-old male *Lep*^{KO} rats appear larger (Fig. 5.7A), and β cell area relative to total pancreas area, as determined by immunostaining, was 3-fold higher

compared to *Lep*^{WT} (Fig. 5.7B, 1.74 ± 0.46 % vs. 0.54 ± 0.05 %, *Lep*^{KO}; $p<0.05$). No differences in relative α cell area were observed between *Lep*^{WT} and *Lep*^{KO} (Fig. 5.7C, 0.014 ± 0.002 % vs. 0.016 ± 0.003 %; $p>0.05$). Despite having greater β cell area, insulin levels did not significantly increase in *Lep*^{KO} rats until 120 minutes after the glucose gavage (Fig. 5.4C-D, G-H), indicating a potential deficit in stimulus secretion coupling. GLUT-2 is believed to be a key component of β cell glucose sensing in rodents [282]. While GLUT-2 staining was clearly evident in *Lep*^{WT} rats, GLUT-2 immunoreactivity within *Lep*^{KO} islets appeared to be reduced (Fig. 5.7A). Though there was a delay in glucose stimulated insulin secretion, reduced GLUT-2 does not seem to result in reduced insulin output in *Lep*^{KO} islets.

We next determined whether changes in islet morphology were associated with alterations to islet function *ex vivo* by subjecting islets isolated from 12-week-old rats to a perifusion. Basal (3 mM glucose) insulin secretion was mildly higher from *Lep*^{KO} islets compared to *Lep*^{WT} (Fig. 5.7D; 6.10 ± 0.61 ng/ml *vs.* 3.07 ± 0.43 ng/ml, $p<0.05$). In response to 20 mM glucose, islets from *Lep*^{KO} rats secreted, on average, 2.5-fold more insulin compared to *Lep*^{WT} islets (16.4 ± 5.03 ng/ml *vs.* 5.66 ± 0.93 ng/ml; $p=0.058$). Insulin secretion (averaged over the duration of exposure to stimuli) was higher compared to *Lep*^{WT} islets in response to 30 mM KCl (31.8 ± 9.31 ng/ml *vs*. 8.61 ± 0.47 ng/ml; $p<0.05$) and 15 mM arginine (45.7 ± 14.3 ng/ml *vs.*19.3 ± 3.58 ng/ml; $p=0.12$). These data are consistent with results obtained *in vivo* in which *Lep*^{KO} rats had a higher peak of insulin following oral gavage of glucose compared to *Lep*^{WT} littermates (Fig. 5.4B).

Next we determined whether a reversal in phenotype could be induced by introducing recombinant leptin to 15-week-old leptin-deficient male rats via

subcutaneous mini osmotic pump. Prior to leptin therapy, plasma leptin values were undetectable (lower limit of detection = 0.15 ng/ml) in *Lep*^{KO} rats while fasting *Lep*^{WT} levels were 2.72 ± 0.41 ng/ml. Seven days after commencing leptin therapy, plasma leptin levels in leptin-treated *Lep*^{KO} rats were 5-fold higher than *Lep*^{WT} rats with vehicle (water) treatment (Fig. 5.8A, circulating leptin = 27.5 ± 2.59 ng/ml in *Lep*^{KO} rats *vs.* 5.11 ± 0.61 ng/ml in *Lep*^{WT}; p<0.001). Prior to leptin therapy, *Lep*^{WT} and *Lep*^{KO} rats had a similar rate of weight gain. However, *Lep*^{KO} rats experienced an attenuation of weight gain following leptin pump implantation, while vehicle-treated *Lep*^{WT} rats continued to gain weight (Fig. 5.8B, p<0.05). The attenuated weight gain observed in leptin-treated *Lep*^{KO} rats was partly attributed to decreased food intake. Prior to leptin therapy, leptin-deficient rats were consuming 1.6-fold more food than *Lep*^{WT} littermates (43.3 g/day *vs.* 26.7 g/day; p<0.001). Vehicle-treated *Lep*^{WT} littermates continued to consume between 25-35 g of food per day, however, 7 days after initiation of leptin therapy, *Lep*^{KO} rats were consuming an average of 13.33 ± 0.93 g of food per day, a 70% decrease from pre-leptin values (Fig. 5.8C, p<0.05).

It is well established that leptin can suppress insulin release via the bidirectional adipoinsular axis [283]. We therefore sought to determine whether leptin therapy was able to reduce plasma insulin levels in *Lep*^{KO} rats. Prior to leptin treatment, *Lep*^{KO} rats had circulating insulin levels averaging 12.74 ± 3.05 ng/ml (Fig. 5.8D). After 2 weeks of leptin therapy, *Lep*^{KO} rats remained hyperinsulinemic relative to *Lep*^{WT} rats, but insulin levels were reduced by 62% (Fig. 5.8D, 4.83 ng/ml ± 1.32 ng/ml, p<0.05). The reductions of insulin levels were not associated with attenuated GLP-1 levels as overnight fasted and 1 h fed GLP-1 levels were similar between *Lep*^{WT} and *Lep*^{KO} rats (Fig. 5.8E). In addition to reductions in body weight and plasma insulin, leptin therapy

also resulted in reduced circulating plasma triglyceride levels. After 3 weeks of leptin therapy, plasma triglyceride levels were reduced from pre-leptin values of 3.19 ± 0.47 mg/ml to 1.68 ± 0.46 mg/ml (Fig 5.8F, $p<0.05$). Leptin has been previously demonstrated to stimulate release of the gut peptide glucagon-like peptide-1 (GLP-1) [283], a hormone that has many glucose lowering actions [284]. However, leptin-treated *Lep*^{KO} rats did not have significantly elevated GLP-1 levels (Fig. 5.8E), suggesting that this may not be the mechanism mediating improvements to glucose homeostasis in this model.

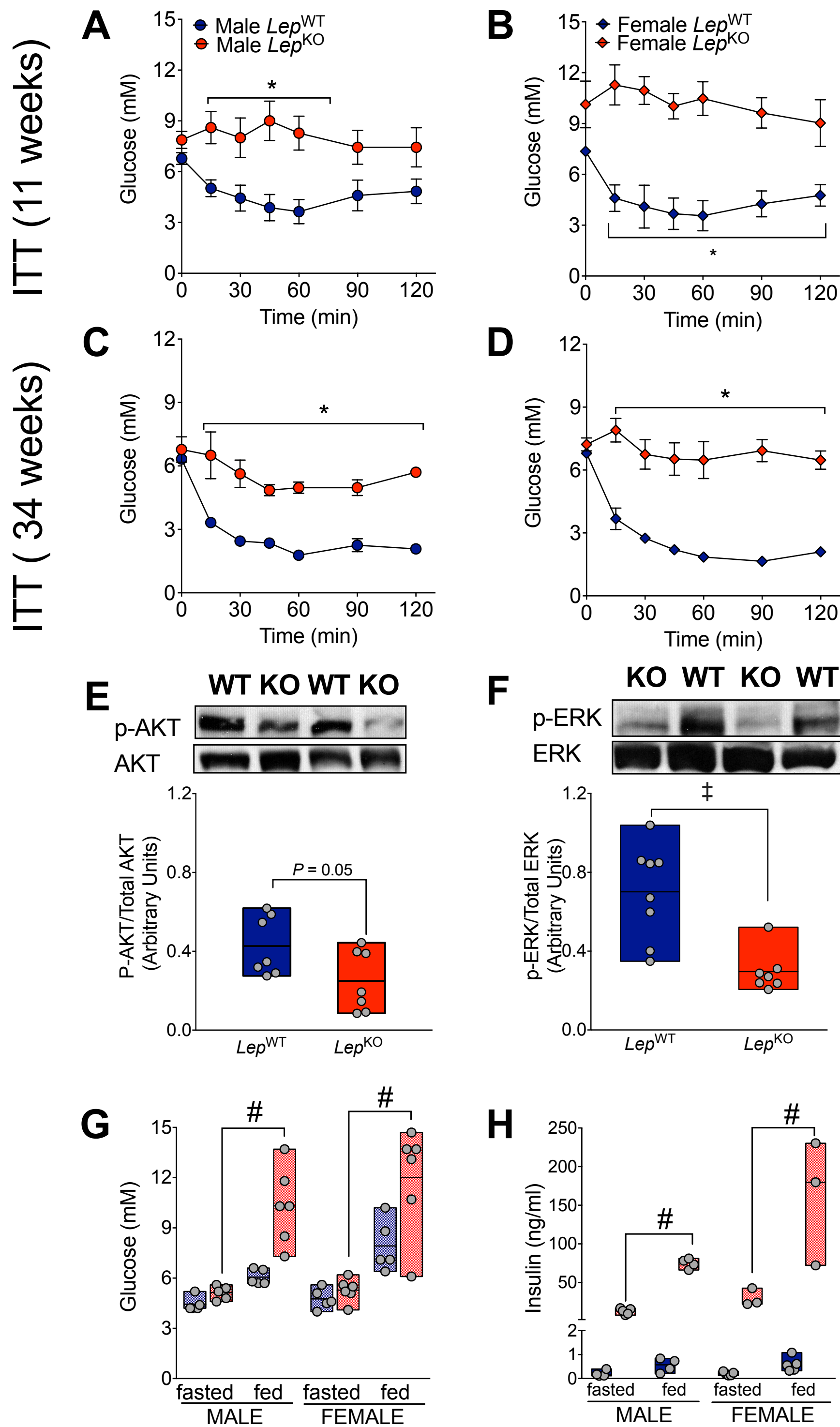


Figure 5.6: Leptin deficiency in rats results in insulin resistance. Insulin sensitivity was measured following i.p. injection of insulin (0.5 U/kg body weight) in male (A) and female (B) rats at 11 weeks of age and again at 34 weeks of age (C, male; D, female).

Phosphorylation of AKT (E; ser473) and Erk1/2 (F; Thr202/Tyr204 of Erk1, Thr185/Tyr187 of Erk2) was assessed in liver lysates of 36-week-old male and female rats. Blood glucose (E) and plasma insulin levels (F) were measured in 13-week-old rats after an overnight fast and 1 h of free feeding. Data are expressed as mean ± SEM in A-D (n=4-5/group) and as individual data points with bars indicating the range of values and a line indicating the mean in E-H. *P<0.05 by Two Way repeated measures ANOVA and Tukey post hoc analysis, #P<0.05 by Two Way ANOVA and Tukey post hoc analysis, ‡P<0.05 *Lep*^{KO} vs. *Lep*^{WT} by student's t-test.

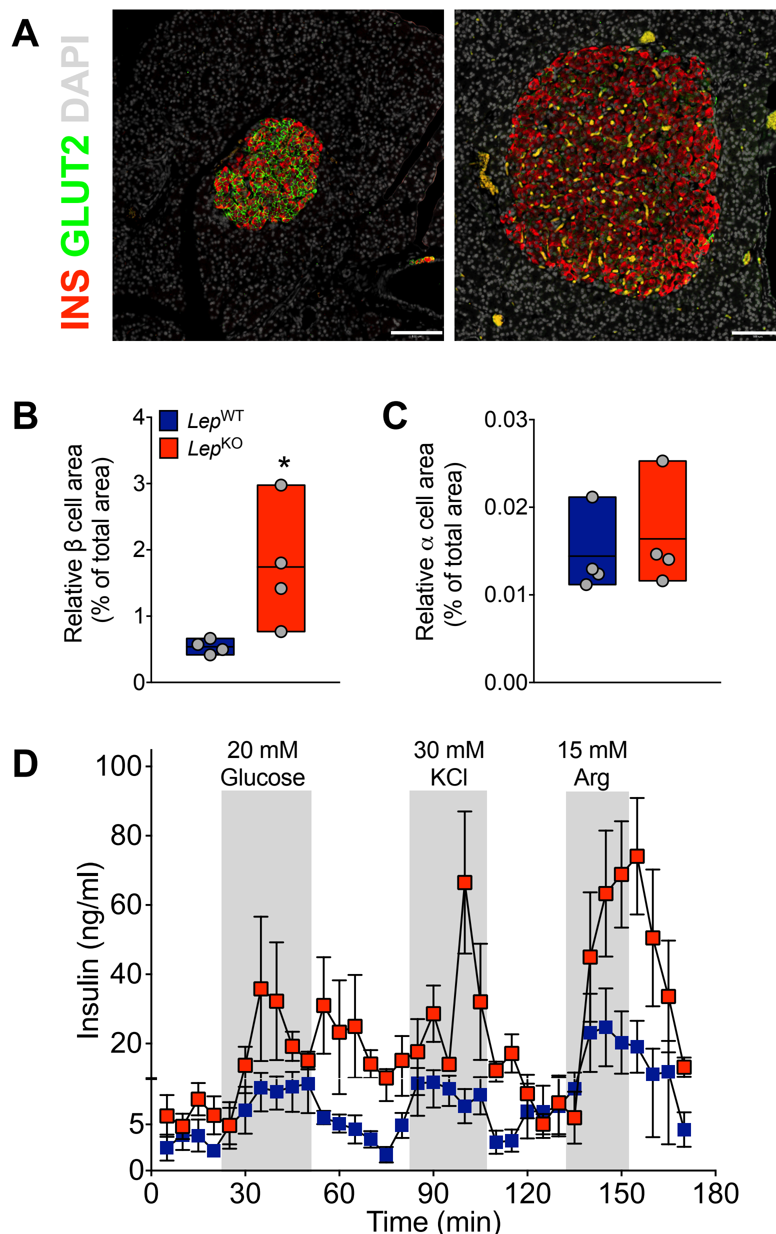


Figure 5.7: Islets from *Lep*^{KO} rats are larger and secrete more insulin than *Lep*^{WT} islets. Pancreas sections from 36-week-old male rats were stained for insulin (red) and GLUT-2 (green) (A; scale bar = 100 μm). Yellow indicates autoflouresence generated by red blood cells in fixed pancreas sections. β cell (B) and α cell (C) area relative to total pancreas area were quantified from pancreas sections stained with either insulin or glucagon and DAPI. Islets from 12-week-old male and female rats (100 per animal) were isolated, cultured overnight, and loaded into perifusion chambers to assess insulin secretion (D). Grey areas on graph indicate incubation of islets with glucose, KCl or arginine, basal glucose concentration = 3 mM. Values are presented as individual values with a line indicating the mean and with bars indicating range of values in B-C. In D, values are presented as mean ± SEM (n=3/group); *p<0.05 by student's t-test.

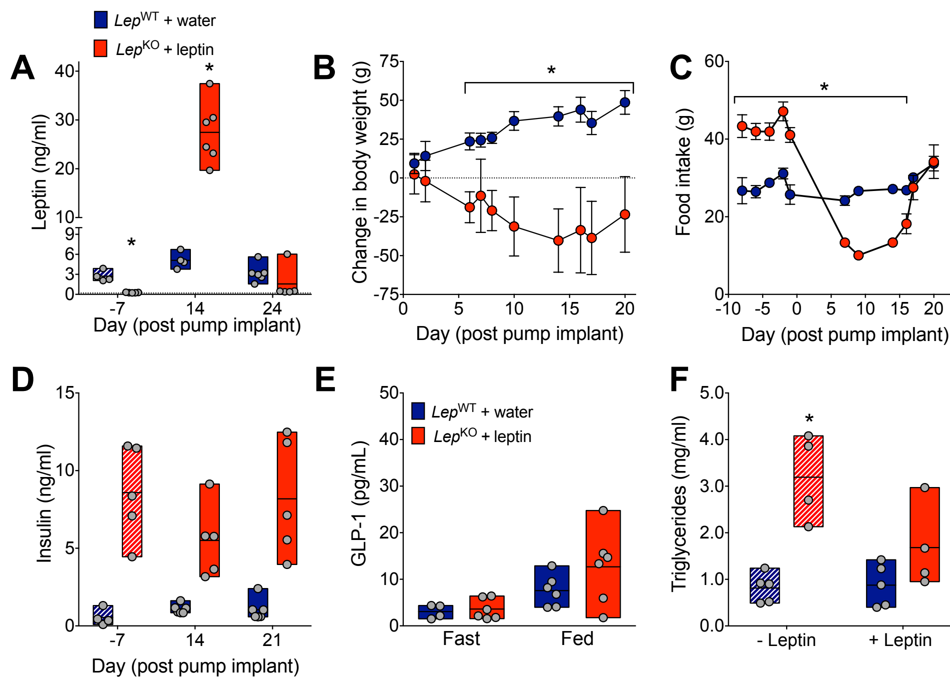


Figure 5.8: Leptin therapy reduces weight gain, food intake and fasting insulin levels in *Lep*^{KO} rats. Male *Lep*^{KO} rats (15 weeks old) were treated with continuous leptin infusion via mini osmotic pump (100 μg/day); *Lep*^{WT} littermate controls received pumps infusing vehicle (water). Plasma leptin levels (A), body weight (B), food intake (C), fasted plasma insulin levels (D), and plasma triglyceride levels (F) were measured prior to and following pump implantation. Two weeks after pump implantation, plasma GLP-1 levels were measured in rats following an overnight fast and 1 h of free feeding (E). Data are expressed as mean ± SEM in B-C (n=6/group), and presented as

individual values with line indicating the mean in A, D-F. Hatched bars in A, D and F represent pre-pump values. *P<0.05 *Lep*^{WT} vs. *Lep*^{KO} by Two Way ANOVA and Tukey post hoc analysis in B-C and multiple t-test in A, D-F.

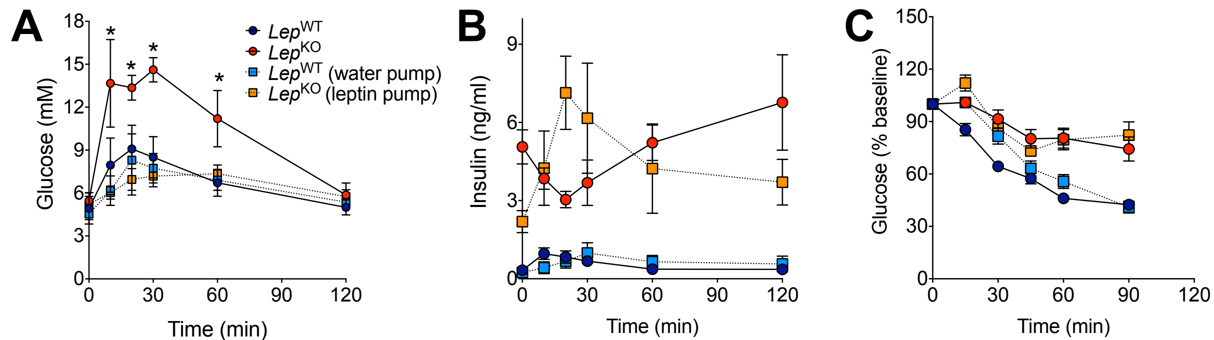


Figure 5.9: Leptin therapy improves glucose tolerance and glucose stimulated insulin secretion (GSIS) in male *Lep*^{KO} rats. Glucose tolerance (A), glucose stimulated insulin secretion (B) and insulin sensitivity (C) were assessed prior to and following leptin therapy in *Lep*^{WT} and *Lep*^{KO}. Data are expressed as mean ± SEM (n=6/group); *P<0.05 by Two Way repeated measures ANOVA and Tukey post hoc comparison, *P<0.05 before *vs.* after leptin in *Lep*^{KO} rats.

5.3 Discussion

The *ob/ob* mouse, which has a mutation in the *ob* gene resulting in leptin deficiency, has been the favored model for studying the effects of leptin deficiency on energy balance and glucose homeostasis. However, utilization of zinc finger nuclease technology has now enabled generation of a *Lep*^{KO} rat. Vaira and colleagues reported that *Lep*^{KO} rats, much like their murine equivalents, have impaired energy balance, impaired glucose tolerance and elevated insulin levels at 4 weeks of age [213]. In this chapter, we expanded upon these findings to more thoroughly characterize energy balance and glucose handling in both male and female rats at different ages.

Comparable to *ob/ob* mice [67], *Lep*^{KO} rats double their body weight between 4 and 8 weeks of age. In line with previous reports of elevated hepatic triglycerides observed in adult ZDF rats [284] and *ob/ob* mice [285], we observed increased ectopic triglyceride content in liver and muscle of *Lep*^{KO} rats. The altered lipid metabolism observed in *Lep*^{KO} rats is comparable to the increased plasma triglycerides and fatty liver recently reported in leptin-deficient F344/NS1c rat generated by ENU mutagenesis [286]. Consistent with previous reports of hyperglucagonemia and hyperinsulinemia in *ob/ob* mice [67, 68], glucagon levels were 1.6 fold higher in *Lep*^{KO} rats compared to *Lep*^{WT} at 26 weeks of age, and insulin levels were at times ~50-fold higher in *Lep*^{KO} rats compared to *Lep*^{WT}. Analysis of C-peptide levels demonstrated that the dramatic hyperinsulinemia in *Lep*^{KO} rats may be partly attributed to reduced insulin clearance. Insulin resistance and decreased hepatic insulin signalling in *Lep*^{KO} rats suggested that there might be reduced sensitivity of the α cell to paracrine insulin signalling [287]. This, combined with the absence of leptin signalling, which is known to suppress glucagon secretion in α cells [288], could contribute to the elevated glucagon levels observed. With further aging, or additional β cell insults such as feeding a high fat diet, β cell failure might occur, resulting in diabetes, as has been observed in ZDF rats, which have impaired leptin receptor signalling on a Wistar background strain [203].

Despite the similarities outlined above, some phenotypic inconsistencies were observed between the *Lep*^{KO} rat and *ob/ob* mouse. The transiently elevated fasting glucose levels in male *Lep*^{KO} rats have also been observed in *ob/ob* mice [68], however the degree of hyperglycemia was substantially lower than the fasting blood glucose levels reported in adult *ob/ob* mice [206, 273, 289]. In addition, adiponectin was increased in male and female *Lep*^{KO} rats compared to *Lep*^{WT} littermates at 9 weeks of

age, while it was reduced in *ob/ob* C57BL/6J mice [206]. Furthermore, we found that body temperature of *Lep*^{KO} rats was only mildly reduced (by 1°C) from their baseline values after 90 minutes of cold exposure, which was in contrast to the hypothermia at ambient temperature [290, 291], and death as a result of cold exposure [290, 292, 293] observed in *ob/ob* mice. Thus the leptin-deficient rat has a milder phenotype with respect to fasting glucose and thermoregulatory capacity compared to the *ob/ob* mouse.

In light of previous reports that have shown that leptin improves metabolic phenotype and glucose handling, we evaluated whether recombinant leptin administration could reverse the obese, glucose intolerant phenotype in *Lep*^{KO} rats. Similar to previous reports in mice [152, 273, 276, 294], administration of leptin to *Lep*^{KO} rats dramatically reduced weight gain compared to pre-leptin values. In addition, leptin therapy reduced circulating insulin levels in *Lep*^{KO} rats by 2.6-fold within 7 days. Leptin therapy also resulted in improved glucose tolerance, which is consistent with previous studies in both mice and rats that underwent leptin therapy [295, 296]. Though food intake initially decreased in leptin-treated *Lep*^{KO} rats, there was a gradual recovery in food intake over the duration of leptin therapy. Analysis of plasma leptin levels following pump implantation demonstrated a reduction in circulating levels of immunoreactive leptin in *Lep*^{KO} rats after 24 days. We speculate this could be a result of altered pump function in the *Lep*^{KO} rats, as the mini osmotic pump was predicted to work for 28 days or may be a result of altered leptin clearance *in vivo*.

Taken together, our findings demonstrate that despite some differences in phenotype, the function of leptin in glucose homeostasis is largely conserved between rats, mice and humans. Compared to murine models, rats more closely mimic responsiveness to environmental influences such as diet and stress [297]. For instance,

the mild perturbations to fasting glucose levels that we observed in *Lep*^{KO} rats are more consistent with the normal fasting glucose levels of leptin-deficient humans [193, 194]. The larger size of rats compared to mice provides additional benefits of greater blood volume and increased sequential blood sampling, allowing for easier measurements of physiological parameters. Though the glucose handling of other rat models of impaired leptin action (e.g. Zucker Diabetic Fatty rat and Koletzsky rat) has been well established, the absence of functional leptin receptors makes it challenging to assess the effects of leptin therapy on glucose homeostasis. Here we clearly show that leptin replacement in *Lep*^{KO} rats rapidly and dramatically improved glucose homeostasis and energy metabolism. Thus the *Lep*^{KO} rat provides an alternative model that may be used to study the metabolic and hormonal modifications that occur when leptin function is disrupted.

CHAPTER 6: REDUCED INSULIN SENSITIVITY WITHOUT HYPERINSULINEMIA IS OBSERVED IN SUCKLING *LEP*^{KO} RATS

6.1 Introduction

The adipose-derived hormone leptin is well known for its effects on energy balance, particularly through its actions in the CNS where it suppresses food intake [135]. In the absence of leptin signalling in *ob/ob* mice and *leptin knockout rats (Lep*^{KO}*)*, obesity and hyperphagia are observed [92, 188]. In addition, several defects to glucose homeostasis occur including the development of hyperinsulinemia, and glucose intolerance [70, 92, 298]. Several studies suggest that the glucoregulatory actions of leptin occur independent of its effects on body weight. In *ob/ob* mice, hyperinsulinemia is observed prior to obesity [208]. Second, in *ob/ob* mice, low dose leptin therapy is able to reduce hyperinsulinemia without lowering body weight [137]. In addition, acute antagonism of leptin by injection of a leptin antagonist peptide for 3 days in C57BL/6J mice raised plasma insulin levels without significant increases in body weight [139]. Together, these findings demonstrate that some effects of leptin on glucose homeostasis occur independent of its effects on energy balance.

The mechanisms underlying the glucoregulatory effects of leptin are poorly understood. In chapter 4, we report the potential for leptin to regulate insulin levels, as it has been previously reported that leptin can suppress insulin secretion from pancreatic β cells [172, 274, 283]. Leptin may also be involved in regulating insulin sensitivity. In the periphery, leptin signalling in insulin sensitive tissues such as skeletal muscle prevents lipotoxicity by promoting fat oxidation [299, 300], and leptin also promotes glucose uptake in BAT [301]. Both regulation of insulin sensitivity and insulin secretion are integral to glucose homeostasis, yet it remains unclear which is the primary method

by which leptin facilitates glucose homeostasis. Understanding the mechanisms by which leptin facilitates glucose homoeostasis provides a promising avenue for development of therapies aimed to treat diabetes.

In *ob/ob* mice hyperinsulinemia is observed prior to weaning and occurs independent of increases in body weight [208]. These findings suggest that dysregulation of glucose stimulated insulin secretion may be a primary defect that arises due to leptin deficiency. To further explore the relationship between leptin signalling, insulin secretion and insulin sensitivity, we assessed insulin sensitivity, β cell function and morphology of pancreatic islets in suckling Lep^{KO} rats. In this chapter, we build upon the characterization of Lep^{KO} rats done in chapter 5 to further elucidate the primary metabolic defects that arise from leptin deficiency in rats. The larger size of the rat compared to mice provides an opportunity to assess the development of these metabolic defects at an earlier age, without the confounding effects of obesity that develops after weaning [208]. Thus, assessment of glucose homeostasis in young Lep^{KO} rats will enable us to determine the hierarchy of impairments to glucose homeostasis that arise as a result of leptin deficiency.

Here we report development of insulin resistance and hyperinsulinemia prior to significant differences in body weight between Lep^{KO} rats and Lep^{WT} littermates. Furthermore, we show that hyperinsulinemia in Lep^{KO} rats does not result from increased pancreas content or relative β cell area, but rather may be a result of increased secretion of insulin as a result of stimulation by glucose.

6.2 Results

As reported previously, knockout of leptin in rats results in obesity and hyperinsulinemia [92]. To determine the age at which obesity develops in *Lep*^{KO} rats, we assessed body weight in *Lep*^{KO} and *Lep*^{WT} littermates from 1-3 weeks of age. Similar to *ob/ob* mice [208], no differences in body weight were observed between *Lep*^{KO} rats and *Lep*^{WT} littermates between 1-3 weeks of age (Fig. 6.1A). We next investigated the body composition of *Lep*^{WT} and *Lep*^{KO} rats using DEXA analysis. Despite similar body weight, *Lep*^{KO} rats demonstrated a significant increase in fat mass, assessed at 2 and 3 weeks of age (Fig. 6.1B; $p<0.05$). Prior reports have suggested that elevated insulin levels contribute to increased fat mass observed in obese rodents and humans [41, 223]. To determine if this would also be true of *Lep*^{KO} rats, we subjected 2- and 3-week-old rats to a 4 h fast and then collected blood to assess plasma insulin levels. No differences in plasma insulin levels were observed between *Lep*^{WT} and *Lep*^{KO} rats at 1 and 2 weeks of age. However, by 3 weeks of age, *Lep*^{KO} rats had significantly higher plasma insulin levels compared to *Lep*^{WT} littermates (Fig. 6.1C). Unlike previous observations in *ob/ob* mice [221] hyperinsulinemia was not accompanied by decreased blood glucose levels, and fasting glucose levels were comparable between *Lep*^{KO} rats and *Lep*^{WT} littermates between 1-3 weeks of age (Fig. 6.1D). Together these data suggest that elevated insulin levels and increased fat mass are among the first metabolic disturbances to occur in *Lep*^{KO} rats.

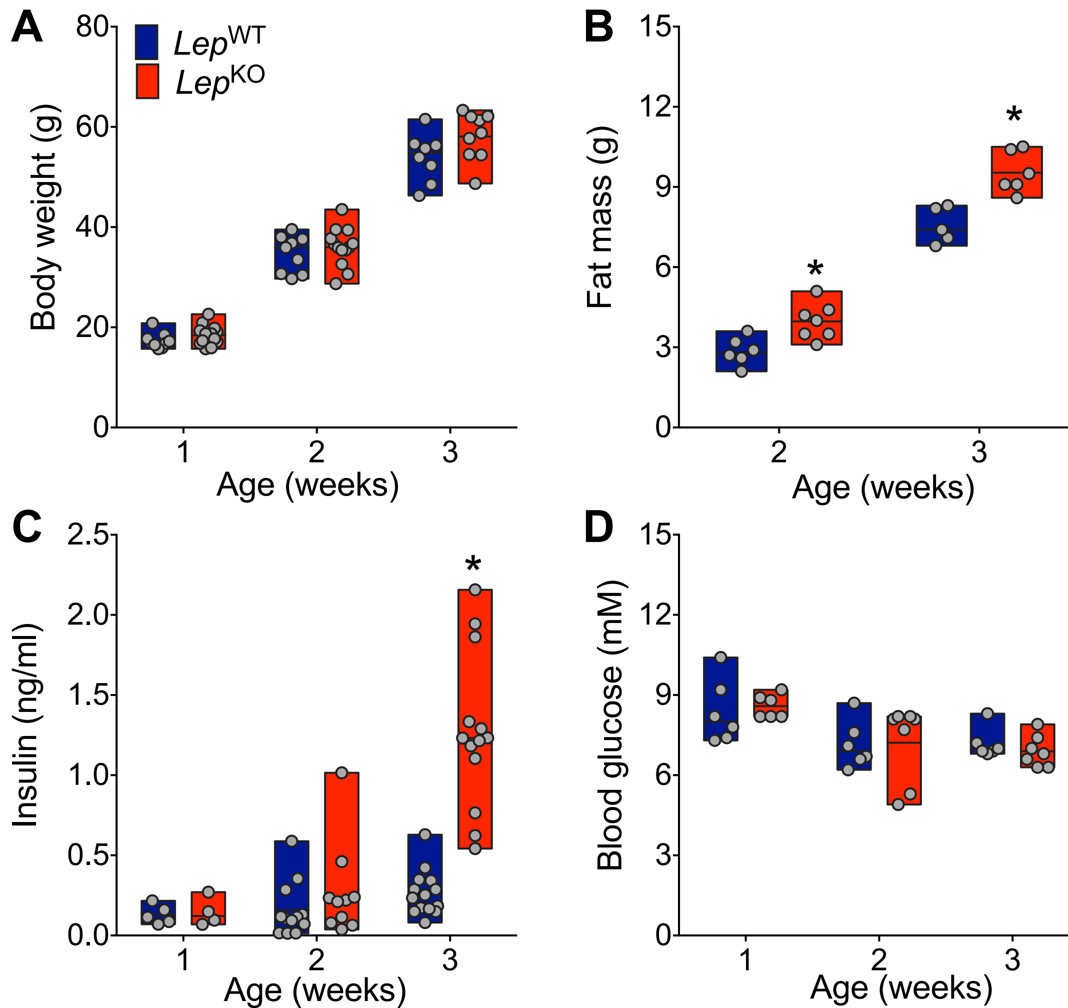


Figure 6.1: Increased fat mass is observed in suckling *Lep*^{KO} rats and significant increases in plasma insulin levels are observed by 3 weeks of age. Body weight (A) was measured in *Lep*^{KO} rats and *Lep*^{WT} littermates between 1-3 weeks of age. At 2 and 3 weeks of age, a subset of *Lep*^{KO} and *Lep*^{WT} male rats underwent DEXA analysis to measure total fat mass (B). Plasma insulin levels (C) and blood glucose (D) were measured in random-fed rats ranging from 1-3 weeks of age. Individual data points are presented with a line indicating the median and bars indicating the range of values. *P<0.05 using Mann-Whitney non-parametric t-test for each time point.

Because elevated circulating insulin levels are often associated with decreases in insulin sensitivity [209], we evaluated insulin sensitivity in pre-weaning *Lep*^{KO} rats compared to their *Lep*^{WT} littermates. At 2 weeks of age, animals were fasted for 4 h followed by an i.p. injection of insulin (0.7 U/kg of body weight). Blood glucose levels were then assessed at 15-minute intervals after injection up to 120 minutes after injection. While both *Lep*^{WT} and *Lep*^{KO} rats demonstrated a reduction in blood glucose in response to insulin injection, blood glucose of *Lep*^{KO} rats was significantly less responsive to insulin injection (Fig. 6.2A). Analysis of area over the curve, which measures the area between blood glucose at each time point compared to baseline blood glucose levels (100%), revealed a trend for decreased insulin sensitivity in 2 week old *Lep*^{KO} compared to *Lep*^{WT} rats (Fig. 6.2C). At 3 weeks of age, blood glucose levels of *Lep*^{KO} rats did not decrease in response to i.p. injection of insulin, while as much as a ~40% decrease in blood glucose was observed in *Lep*^{WT} rats compared to baseline values (Fig. 6.2B). This corresponded with a trend for reduced area over the curve in *Lep*^{KO} rats compared to *Lep*^{WT} rats (Fig. 6.2D).

Previous studies have suggested that increased fat mass is associated with reduced insulin sensitivity [302, 303]. To determine if increased fat mass predicted reductions in insulin sensitivity, a correlation was plotted between the two variables. No significant correlation was observed between percent body fat and area over the curve of the insulin tolerance test in 2-week-old rats, but a moderate correlation was observed at 3 weeks of age (Fig. 6.2E, F). Together, these data suggest that the modest reduction of insulin sensitivity observed at 2 and 3 weeks of age in Lep^{KO} rats is not likely a result of increased fat mass observed, as a correlation between insulin sensitivity and fat mass was only observed at 3 weeks of age.

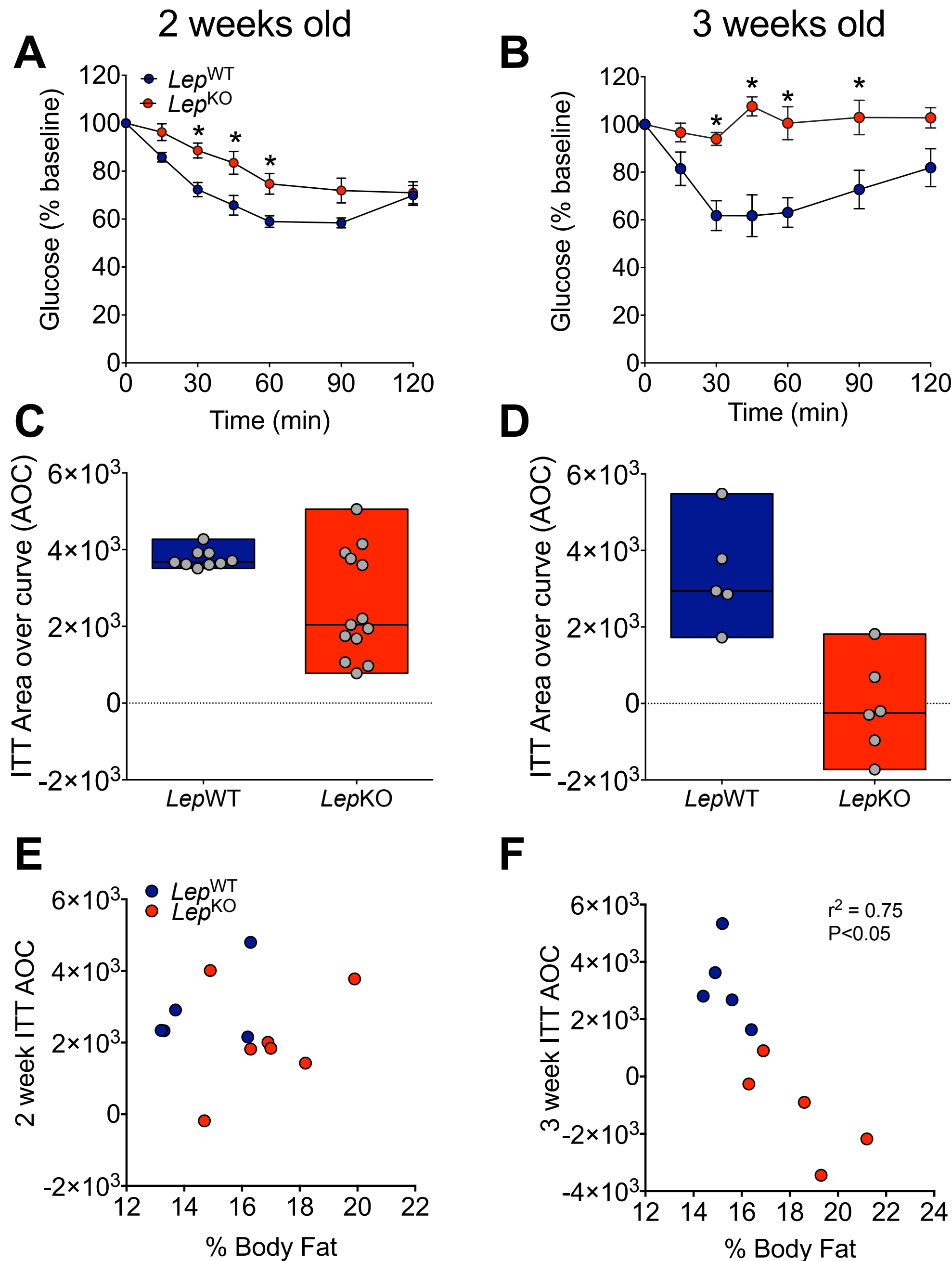


Figure 6.2: Reduced insulin sensitivity is observed in suckling *Lep*^{KO} rat. Two-week-old *Lep*^{KO} rats and *Lep*^{WT} littermates were subject to 4 h of fasting followed by an i.p. injection of insulin (0.7 U/kg body weight). Blood glucose levels were measured at 15-minute intervals over a 2 h period post injection (A). The same procedure was performed in 3-week-old rats (B). Percent change in blood glucose during the ITT was plotted against time and area over the curve (AOC) was analyzed (C-D). AOC was

plotted against body fat mass (indicated in Fig. 6.1B) of 2 and 3 week old rats (E-F). In A-B, values are presented as mean ± SEM (n=9-14/group in A and n=5-6/group in B) and *P<0.05 representing statistical significance based on a Two Way Repeated measures ANOVA and Tukey post hoc analysis. In C-D individual data points are presented with a line indicating the median and bars indicating the range of values. Multiple t-tests on normalized data were used to determine significance in C-D. A correlation analysis was performed in E-F.

The development of insulin resistance is highly related to the accumulation of neutral lipids in non adipose tissue such as liver and muscle [304]. To determine if the reduction of insulin sensitivity in 2-week-old Lep^{KO} rats was attributed to increased ectopic lipid deposition, we assessed plasma triglyceride levels as well as hepatic lipid content in 2-week-old rats. Analysis of plasma triglyceride levels revealed no significant different between Lep^{WT} and Lep^{KO} rats (Fig. 6.3A). Furthermore, extraction and quantification of hepatic triglyceride and cholesterol levels (Fig. 6.3B-C) revealed no significant differences between 2-week-old Lep^{WT} and Lep^{KO} rats. Together, these findings suggest that the reduction of insulin sensitivity observed in Lep^{KO} rats compared to Lep^{WT} rats at 2 weeks of age (Fig. 6.2A) is not attributed to increased ectopic lipid deposition.

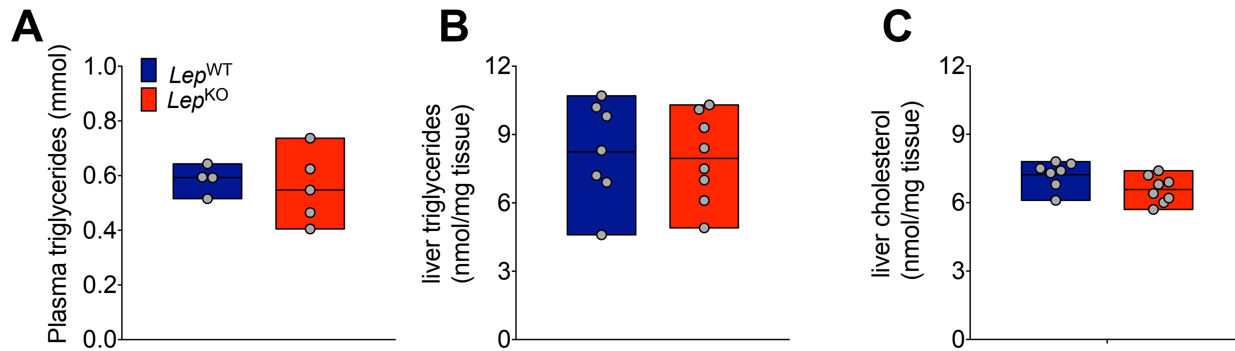


Figure 6.3: Increased fat mass is not associated with elevated plasma triglycerides or ectopic lipid deposition in 2-week-old rats. Plasma triglyceride levels were measured in 4 h fasted 2-week-old rats (A). Triglyceride (B) and cholesterol (C) levels were also measured from lipid extracts from livers of 2-week-old rats. Values are presented as individual data points with a line indicating the mean and bars indicating the range of values. Student's t-test were performed and revealed no significant differences between *Lep*^{WT} and *Lep*^{KO} rats.

To further characterize the effects of leptin deficiency on glucose handling, glucose tolerance was assessed in *Lep*^{KO} rats and *Lep*^{WT} littermates. At 3 weeks of age, rats were fasted for 4 h followed by an oral gavage of glucose (1.5 g/kg body weight; 40% glucose solution). Blood glucose levels were comparable following oral gavage of glucose in 3-week-old *Lep*^{KO} rats and *Lep*^{WT} rats, though *Lep*^{KO} rats did have an trend for elevated glucose levels at 7 and 15 minutes post glucose gavage (Fig. 6.4A). During the glucose tolerance test, blood was collected to assess glucose stimulated insulin secretion. *Lep*^{KO} rats experienced a trend for higher insulin secretion in response to glucose 7 minutes after glucose gavage compared to *Lep*^{WT} littermates (Fig. 6.4B; p=0.05). To further assess the function of β cells, islets were isolated from 3 week old *Lep*^{KO} and *Lep*^{WT} rats and subjected to a perifusion. Basal (3 mM glucose) insulin secretion from *Lep*^{KO} islets was comparable to *Lep*^{WT} islets, though a trend in elevated insulin levels was observed in *Lep*^{KO} islets (Fig. 6.4C, p=0.05), and insulin secretion

from two of the three *Lep*^{KO} islets samples was almost double that of *Lep*^{WT} islets. After incubation in 3 mM glucose, islets were then exposed to 20 mM of glucose for 40 minutes. Secretion of insulin from *Lep*^{KO} islets was significantly higher than *Lep*^{WT} islets (Fig. 6.4D, $p<0.05$). Following exposure to high glucose, islets were then subjected to perifusion of 30 mM KCl. A trend for higher insulin secretion from *Lep*^{KO} islets compared to *Lep*^{WT} islets was observed, but was not significantly different (Fig. 6.4D; $p=0.08$). To determine whether the higher insulin secretion observed in response to 20 mM glucose was due to higher insulin content, we assessed total pancreatic content in *Lep*^{WT} and *Lep*^{KO} rats at 2 and 3 weeks of age. No differences in pancreatic insulin content were observed at 2 weeks (Fig. 6.4E) or 3 weeks of age (Fig. 6.4F). Together these data suggest that even prior to weaning, insulin secretion from *Lep*^{KO} rats is higher in response to glucose stimulation, but is not due to higher pancreatic insulin content.

We next determined whether changes in islet function were associated with alterations to islet morphology by collecting pancreata from 2- and 3- week old rats and co-staining pancreas sections taken from three different regions of the pancreas for insulin and glucagon. Immunostained images revealed similar islet morphology between *Lep*^{WT} and *Lep*^{KO} rats at 2 weeks and 3 weeks of age (Fig. 6.5A). Quantification of relative cell area revealed no differences in β cell area (Fig. 6.5B) or α cell area (Fig. 6.5D) between *Lep*^{WT} and *Lep*^{KO} rats at 2 weeks of age. Similarly, analysis of relative β cell (Fig. 6.5C) and α cell (Fig. 6.5E) area in pancreata collected from 3-week-old rats revealed no significant differences between *Lep*^{WT} and *Lep*^{KO} rats. Together, these findings suggest that increased glucose stimulated insulin secretion observed from islets collected at 3 weeks of age (Fig. 6.4D) may not be due to increased β cell area. However, due to the limited number of animals assessed and large variability within

groups in Fig. 6.5D-E, further studies of β cell area analysis at these time points are required to confirm this finding.

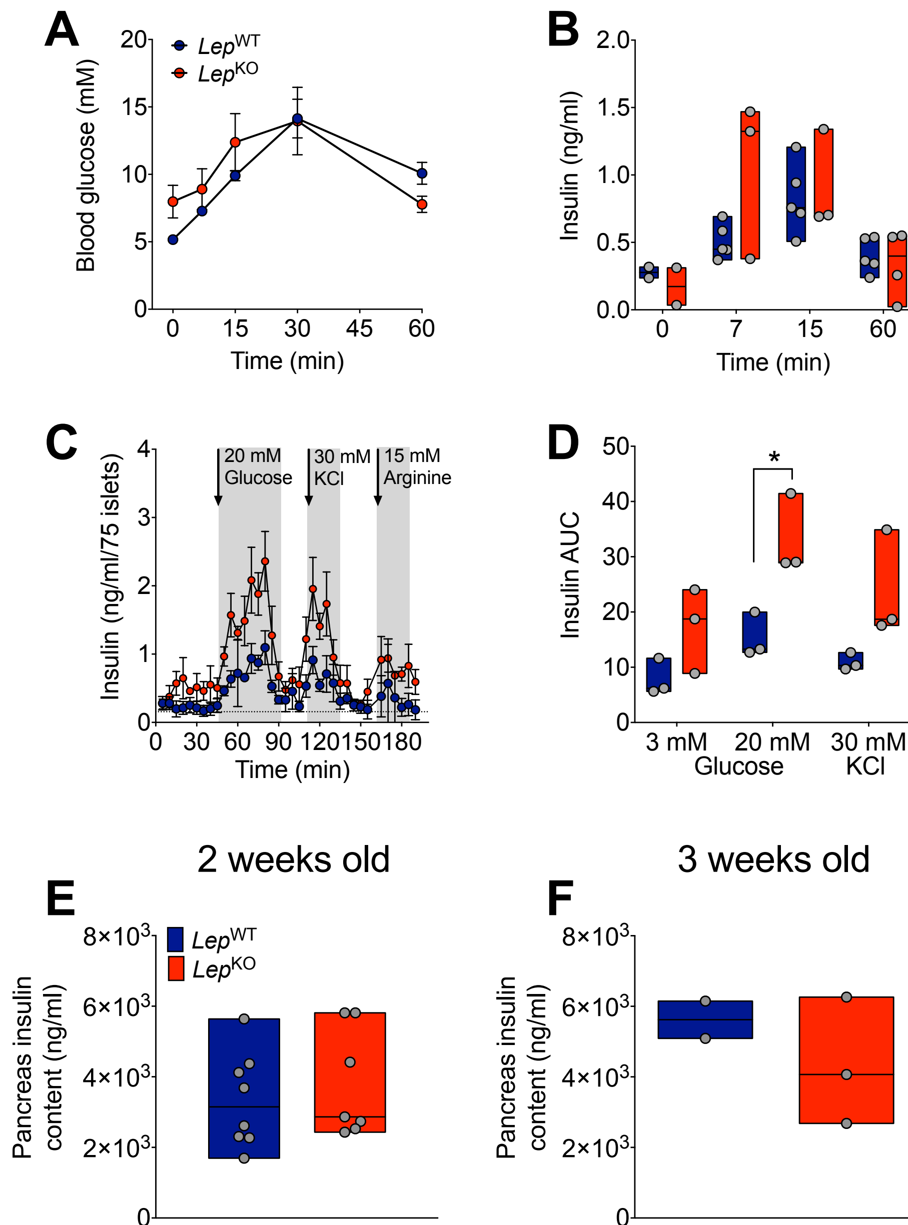


Figure 6.4: Glucose-stimulated insulin secretion is higher in isolated islets from *Lep*^{KO} rats compared to *Lep*^{WT} littermates. At 3 weeks of age, *Lep*^{WT} and *Lep*^{KO} rats were subject to a 4 h fast followed by an oral gavage of 1.5 g/kg glucose. Blood glucose was measured at 15-minute intervals for 1 h following injection (A). In addition, blood

samples were collected to assess plasma insulin levels (B). Islets were isolated from 3-week-old rats and subject to a perifusion. Samples were collected from the perifusion every 5 minutes to assess insulin secretion of islets in response to stimulation by 3 mM glucose, 20 mM glucose, 30 mM KCl or 15 mM arginine (C). Area under the curve of insulin response to each condition of the perifusion was plotted in (D). Total pancreas insulin content was measured in whole pancreas from 2-week-old rats (E) and 3-week-old rats (F). Individual data points are presented in B and D, E and F with lines indicating the median and bars indicating the range of values. *P<0.05 *Lep*^{WT} vs. *Lep*^{KO} analyzed by student's t test. In A and C, values are presented as mean ± SEM (n=2-4/group).

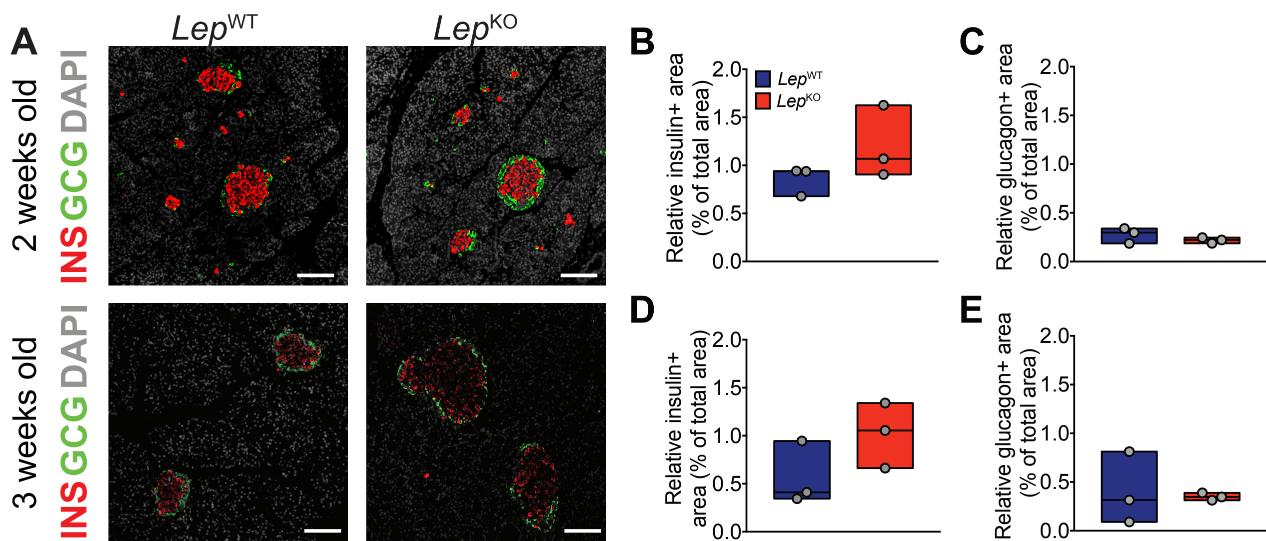


Figure 6.5: Relative β and α cell area is comparable between suckling *Lep*^{KO} and *Lep*^{WT} rats. Pancreas sections from 2- and 3-week-old rats were immunostained for insulin (red) and glucagon (green; A). Scale corresponds to 100 μm. β cell (B, D) and α-cell (C, E) area relative to total pancreas area were quantified from pancreas sections collected at 2 (B-C) and 3 (D-E) weeks of age. Individual values are presented with a line indicating median and bars indicating the range of values in B-E. A student's t-test was used to assess statistical significance.

6.3 Discussion

In this chapter, we have attempted to delineate the primary disturbances to glucose homeostasis that develop as a result of leptin deficiency. To do so, we assessed blood glucose, fasting insulin, insulin sensitivity, glucose tolerance and islet function in suckling (1-3 weeks of age) Lep^{KO} rats and Lep^{WT} littermates. We report reduced insulin sensitivity in Lep^{KO} rats at 2 weeks of age, and this occurred prior to significant differences in body weight or fasting insulin levels between Lep^{KO} and Lep^{WT} rats. Furthermore, we report an exaggerated insulin response is islets isolated from 3-week-old Lep^{KO} rats compared to islets from Lep^{WT} littermates. This exaggerated insulin response does not appear to be a result of increased relative β cell area or increased pancreatic insulin content.

Similar to *ob/ob* mice [208], we observed hyperinsulinemia around the age of weaning (3 weeks old) in Lep^{KO} rats, and prior to significant increases in body weight compared to Lep^{WT} littermates. Despite our efforts to assess glucose homeostasis without the confounding effects of obesity, we observed an increase in fat mass in suckling Lep^{KO} rats. These findings are in line with previous observations by Dubuc et al., who reported significantly elevated carcass fat percentage in suckling *ob/ob* mice compared to lean littermates [208]. In addition to increased carcass fat percentage, Dubuc et al. also reported increased insulin levels in 17-day-old and 21-day-old *ob/ob* mice compared to lean littermates. However, unlike the findings by Dubuc et al, we report comparable insulin levels in 2-week-old Lep^{WT} and Lep^{KO} rat and elevated fat mass in Lep^{KO} rats at this age. Thus, it appears that hyperinsulinemia is not required to drive the increase in fat mass observed in suckling leptin deficient rats. The increase in adipose mass in suckling leptin deficient rats could be due to a number of reasons.

Leptin inhibits accumulation of lipid in adipocytes by inhibition of de novo lipogenesis and stimulation of lipolysis [161, 163, 305]. *In vivo* studies using antisense RNA to down regulate leptin receptor expression in adipose tissue of wildtype mice resulted in a doubling of body fat mass, implying that leptin signalling in adipose is necessary to regulate body fat [306]. Thus the absence of leptin in suckling *Lep*^{KO} rats may result in reduced lipolysis and increased lipogenesis, contributing to increased adipose mass. Interestingly the increased fat mass observed in 2-week-old rats was not associated with elevated plasma triglyceride levels or elevated hepatic lipid content, suggesting that the reduction of insulin sensitivity is not attributed to ectopic lipotoxicity. However, the increased adiposity may be associated with increased secretion of inflammatory cytokines including TNF-α, which can impede insulin-facilitated glucose uptake thus contributing to reduced insulin sensitivity [307, 308]. Further analysis of circulating inflammatory cytokine levels are required to determine whether the reduced insulin sensitivity observed in 2-week-old *Lep*^{KO} rats is secondary to increases in adiposity and associated increase of cytokine release, or is a direct consequence of leptin deficiency.

Our lab has previously reported that correction of hyperinsulinemia prevents insulin resistance in *Lepr*$^{flox/flox}$ *RIP*Cre mice, suggesting that hyperinsulinemia is the primary defect resulting in insulin resistance [239]. To assess insulin sensitivity in suckling *Lep*^{KO} rats compared to their *Lep*^{WT} littermates, we subjected these rats to a 4 h fast, followed by an injection of insulin. We observed higher blood glucose levels in 2-week-old *Lep*^{KO} rats compared to *Lep*^{WT} littermates at multiple timepoints following insulin injection, indicating reduced insulin sensitivity. This reduction of insulin sensitivity in *Lep*^{KO} was observed despite comparable fasting insulin levels between 2-week-old *Lep*^{KO} and *Lep*^{WT} rats. These findings indicate that in the context of leptin deficiency in rats,

reduced insulin sensitivity is not a result of fasting hyperinsulinemia. This is in line with previous observations in rodents with uncontrolled diabetes mellitus induced by STZ who experience a loss of insulin that results in depletion of fat mass and in turn, depletion of leptin levels. Replacement of physiological levels of leptin in these rodents is sufficient to reverse insulin resistance independent of changes to plasma insulin levels, food intake or body weight [147], suggesting that leptin can directly influence insulin sensitivity. Together, these data collectively suggest that leptin regulating of insulin sensitivity plays a key role in regulating glucose homeostasis.

A defining feature of leptin deficient rodents is exaggerated insulin secretion in response to glucose [92, 309]. To determine whether defects to glucose-stimulated insulin secretion occur independent of increased body weight, we assessed glucose stimulated insulin secretion *in vivo* and in isolated islets from Lep^{KO} rats at 3 weeks of age. In line with previous observations in leptin deficient rodents, we report a trend for increased insulin secretion *in vivo* and exaggerated insulin secretion from islets of 3-week-old Lep^{KO} rats in response to 20 mM glucose. While we were unable to assess insulin response at a younger time point due to insufficient number of isolated islets, Chen et al. reported no differences in responsiveness of islets isolated from 2-week-old *ob/ob* compared to lean littermates following perfusion of 20 mM of glucose [310]. Collectively, these studies suggest that defects to insulin secretion may be associated with impaired insulin sensitivity. Whether the exaggerated insulin response observed in Lep^{KO} rats is a result of reduced insulin sensitivity remains to be elucidated and warrants further investigation.

Taken together, our findings reveal that reduced insulin sensitivity is among the first metabolic defects to arise in the absence of leptin signalling in rats, along with

elevated insulin levels. It remains to be determined how reductions in insulin sensitivity affect insulin secretory capacity of β cells.

CHAPTER 7: GENERATION AND CHARACTERIZATION OF MICE WITH INDUCIBLE KNOCKOUT OF LEPTIN IN ADULTHOOD

7.1 Introduction

Our understanding of the physiological actions of leptin is largely a result of studies performed in mice with spontaneous mutations of the genes encoding leptin (*ob*) or the leptin receptor (*db*). Over forty years ago, parabiosis experiments demonstrated that a missing circulating factor in *ob/ob* mice produced their obese phenotype, as they were able to lower food intake and weight, as well as improve glycemic control when joined to lean mice [68]. Many decades later, the gene encoding this circulating factor was identified as leptin in 1994 [69]. The *ob/ob* mouse, lacks leptin due to a spontaneous mutation in the *ob* gene, and is characterized by obesity, hyperphagia, transient hyperglycemia (on a BL/6 background) [207], and elevated plasma insulin concentrations (10-50 times normal) [67]. In addition, these mice have enlarged pancreatic islets due to an increase in the number and size of β cells [214].

Rodents with spontaneous mutations in the gene encoding leptin or its receptor have been used extensively to assess the pathophysiology of obesity and T2D. Rodents with spontaneous mutations of leptin or the leptin receptor have deficient leptin signalling throughout development, resulting in dysregulated brain development [311], dysregulation of thermogenesis [293], and obesity along with defects to insulin secretion that arise early in life [214, 310], among other metabolic defects. The chronic disruption to energy balance and development of obesity and hyperinsulinemia early in life make it difficult to distinguish the direct effects of leptin on glucose homeostasis from the secondary effects that arise due to the development of obesity.

In recent years, the growing interest in the role of leptin in the pathophysiology of metabolic diseases has resulted in the generation of multiple experimental models to assess the effects of disrupted leptin action. Previously, our lab has utilized a leptin antagonist administered via i.p. injection or osmotic pump over a short period of time into lean, healthy mice. After 3 days of antagonist administration, mice developed hyperinsulinemia and reduced insulin sensitivity without significant perturbations to body weight [139]. These results suggest that leptin plays a significant role in regulation of glucose homeostasis. However, due to the significant expense associated with chronic administration of antagonist peptides, studies on chronic leptin inhibition in adult mice could not be assessed. An alternative approach to assess the chronic effects of leptin inhibition in a tissue specific or temporal manner is the use of Cre-*loxP* technology. Many studies have been published on the effects of tissue-specific deletion of leptin receptors [160, 238, 312]. However, similar to the *ob/ob* and *db/db* mouse lines, the Cre recombinases used in these studies are constitutively active, making it difficult to assess the effects of leptin on metabolism independent of the effects chronic obesity or the effects of leptin on neuronal development.

To circumvent these confounding variables, genetic techniques have been developed that allow for introduction of a mutation at a chosen time and in a specific cell type [313]. These include the development of CreER^{T2} recombinase, consisting of a Cre recombinase protein fused to the human estrogen receptor ligand binding domain with a G400V/M543A/L554A triple mutation [314]. The CreER^{T2} recombinases are inactive until exposure to the synthetic estrogen receptor ligand 4-hydroxytamoxifen (OHT), enabling temporal control of Cre activity. Previous studies have shown that loss of leptin signalling in adulthood can have variable effects on glucose homeostasis. Guo et al.

utilized mice expressing an inducible Cre driven by the globally expressed ROSA26 promoter (*ROSA26*CreER^{T2}) to induce peripheral loss of *Lepr* signalling in adult mice. They report no significant alterations in body weight or blood glucose levels, despite resulting in significant hyperleptinemia [268]. In contrast, inducible loss of leptin receptors using mice expressing the human ubiquitin c promoter to drive inducible Cre expression (*UBC*CreER^{T2}) by Cox et al. resulted in significant increases in body weight, transient hyperglycemia and glucose intolerance observed three weeks after tamoxifen administration [315]. The discrepancy between these studies suggests that further research is required to understand how inducible loss of leptin signalling at various stages of development alters metabolic function.

In this chapter, we utilize a mouse line in which exon 3 of the *ob* gene was flanked by *loxP* sites (*Lep*$^{flox/flox}$), and crossed these mice with *ROSA26*CreER^{T2} mice. Upon tamoxifen injection, Cre-mediated recombination of the floxed region of the *ob* gene is predicted to produce a truncated, non-functional leptin protein, thus resulting in impaired leptin signalling. Thus, the effects of leptin on metabolism can be assessed in tissue-specific and temporal manner, depending on the Cre recombinase used. We hypothesized that recombination of the floxed leptin gene in adult *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice will produce a metabolic phenotype similar to *ob/ob* mice. We report partial and variable recombination in adult *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice following administration of tamoxifen. The data presented in this chapter are preliminary findings and warrant further investigation to delineate the significance of loss of leptin signalling in adulthood.

7.2 Results

To determine the effects of selective deletion of exon 3 of the *ob* gene, we first crossed *Lep*$^{flox/flox}$ mice with mice expressing the constitutively active, ubiquitously expressed *ACTB*Cre, which is driven by the human β actin promoter and is expressed in all tissues [217]. The use of this Cre line enables assessment of the metabolic phenotype that occurs as a result of complete recombination of *Lep*$^{flox/flox}$, without the effects of tamoxifen dose and heterogeneity of inducible Cre expression confounding the results. *Lep*$^{flox/flox}$ *ACTB*Cre mice were compared to *Lep*$^{flox/flox}$, *Lep*$^{wt/wt}$ *ACTB*Cre and *Lep*$^{wt/wt}$ littermates. To verify deletion of leptin following Cre-mediated recombination, we measured circulating leptin levels in 4 h fasted 5 week old mice. Plasma leptin levels were significantly lower in male (Fig. 7.1A) and female (Fig. 7.1B) *Lep*$^{flox/flox}$ *ACTB*Cre mice compared to *Lep*$^{flox/flox}$, *Lep*$^{wt/wt}$ and *Lep*$^{wt/wt}$ *ACTB*Cre littermates. Leptin levels of *Lep*$^{flox/flox}$ *ACTB*Cre mice fell at or below the limit of detection of the ELISA, indicating that *ACTB*Cre mediated recombination of *ob* results in a leptin protein that is not detected by the leptin ELISA.

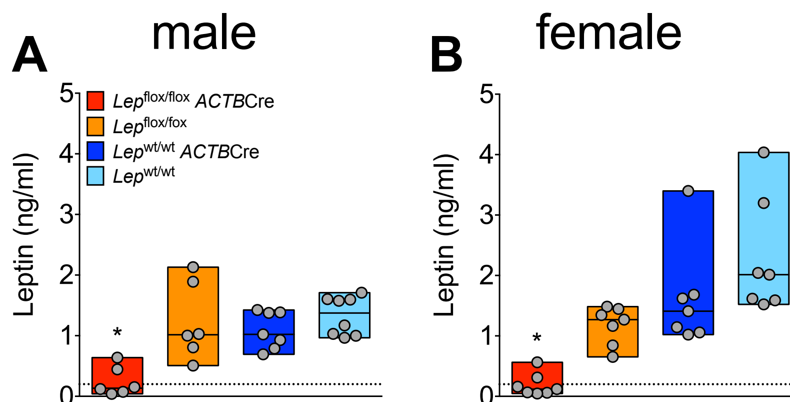


Figure 7.1: Plasma leptin levels are significantly lower in *Lep*$^{flox/flox}$ *ACTB*Cre mice compared to littermate controls. Plasma leptin levels were measured in 5-week-old male (A) and female (B) mice; dashed line indicates the limit of detection of the leptin assay. Individual data points are presented with a line indicating the mean and bars

indicating the range of values. A One Way ANOVA and Tukey's multiple comparison analysis were used to determine statistical significance. *P<0.05 *Lep*$^{flox/flox}$ *ACTB*Cre mice vs. all other groups.

To characterize the physiological effects of deletion of part of exon 3, we first assessed body weight and composition. Weekly measurements of body weight were conducted in male and female mice between 7-13 weeks of age. Body weight of male *Lep*$^{flox/flox}$ *ACTB*Cre mice was approximately 1.6-fold higher than *Lep*$^{flox/flox}$, *Lep*$^{wt/wt}$ and *Lep*$^{wt/wt}$ *ACTB*Cre littermates (Fig. 7.2A). Similarly, body weight of female *Lep*$^{flox/flox}$ *ACTB*Cre mice was approximately 2-fold (7.2B) higher than their respective littermates. To assess body composition, DEXA analysis was performed on 5-week-old *Lep*$^{flox/flox}$ *ACTB*Cre mice and littermate controls. Lean mass was comparable between *Lep*$^{flox/flox}$ *ACTB*Cre mice and littermate controls. In contrast, fat mass was approximately 7-fold higher in male (Fig. 7.2C) and female (Fig. 7.2D) *Lep*$^{flox/flox}$ *ACTB*Cre mice. Together, these findings indicate that recombination of the floxed ob allele results in increased fat mass and increased body weight compared to wildtype and Cre negative littermates.

In *ob/ob* mice and *Lep*^{KO} rats, weight gain is observed after weaning ([208] and Fig. 6.1). To determine whether differences in body weight are observed in *Lep*$^{flox/flox}$ *ACTB*Cre mice compared to control mice post weaning, body weight was measured between 3-6 weeks of age. Body weight was comparable between mice in the control groups (*Lep*$^{flox/flox}$, *Lep*$^{wt/wt}$ and *Lep*$^{wt/wt}$ *ACTB*Cre littermates), between 3-6 weeks of age. In contrast, significant increases in body weight were observed by 5 weeks of age in male (Fig. 7.3A) and female (Fig. 7.3B) *Lep*$^{flox/flox}$ *ACTB*Cre mice. Thus, deletion of part of exon 3 of *ob* using the constitutively expressed *ACTB*Cre results in an obese phenotype resembling *ob/ob* mice.

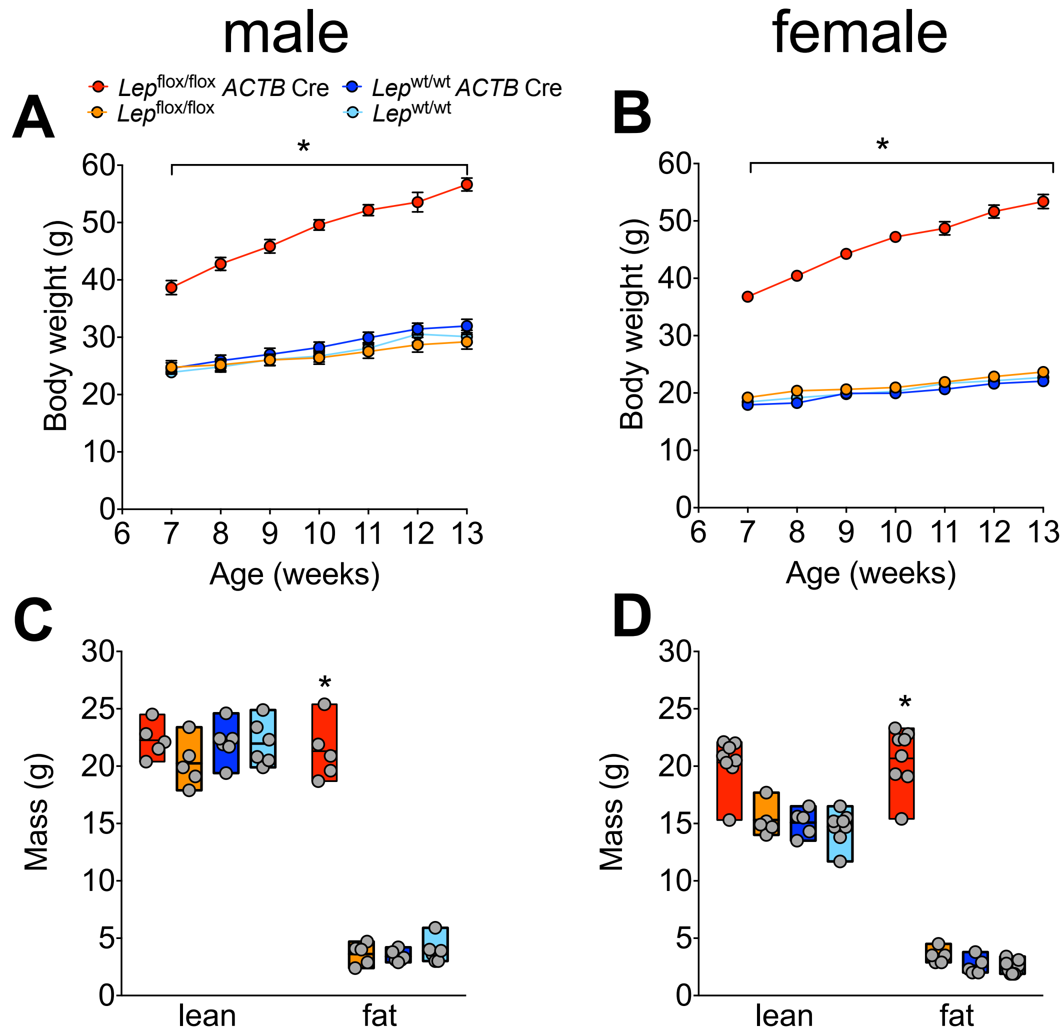


Figure 7.2: *Lep*$^{flox/flox}$ *ACTB*Cre mice are obese compared to littermate controls. Body weight was measured weekly in male (A) and female (B) *Lep*$^{flox/flox}$ *ACTB*Cre mice and *Lep*$^{flox/flox}$, *Lep*$^{wt/wt}$ and *Lep*$^{wt/wt}$ *ACTB*Cre littermates between 7-13 weeks of age. DEXA analysis was used to determine lean and fat mass in male (C) and female (D) mice. Values are presented as mean ± SEM in A-B (n=6-10/group) and as individual data points with bars indicating the range of values in C-D. *P<0.05 by Repeated Measures ANOVA (A-B) and Two Way Anova (C-D) and Tukey's multiple comparison analysis.

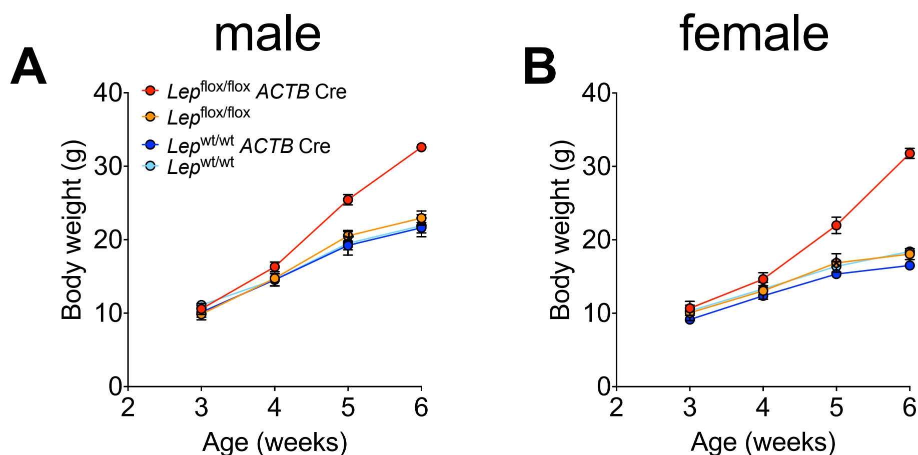


Figure 7.3: Increased body weight is observed after weaning in *Lep*$^{flox/flox}$ *ACTB*Cre mice. Body weight was measured between 3-6 weeks of age in male (A) and female (B) mice. Values are presented as mean ± SEM (n=2-5/group). Statistics were not done due to limited number of samples per group.

To explore the effects of leptin knockout on glucose homeostasis, fasting blood glucose, fasting insulin levels, glucose tolerance tests and insulin tolerance tests were performed in adult *Lep*$^{flox/flox}$ *ACTB*Cre mice and control littermate mice. Fasting glucose levels were transiently higher in male *Lep*$^{flox/flox}$ *ACTB*Cre mice compared to all other groups between 8-10 weeks of age (Fig. 7.4A). Similarly, in female *Lep*$^{flox/flox}$ *ACTB*Cre mice, fasting glucose levels were significantly elevated at 7, 11 and 12 weeks of age compared to all other groups (Fig. 7.4B). As leptin deficiency is commonly associated with hyperinsulinemia, we next assessed fasting insulin levels in 10-week-old mice. Male *Lep*$^{flox/flox}$ *ACTB*Cre mice exhibited significant hyperinsulinemia (10.4±1.35 ng/ml, Fig. 7.4C), which was also observed in female *Lep*$^{flox/flox}$ *ACTB*Cre mice (4.51±0.74 ng/ml, Fig. 7.4D), while littermate control mice had fasting insulin levels ranging from 1.15-1.70 ng/ml. The transient elevations in blood glucose levels despite

hyperinsulinemia suggested that *Lep*$^{flox/flox}$ *ACTB*Cre mice might have impairments to glucose tolerance.

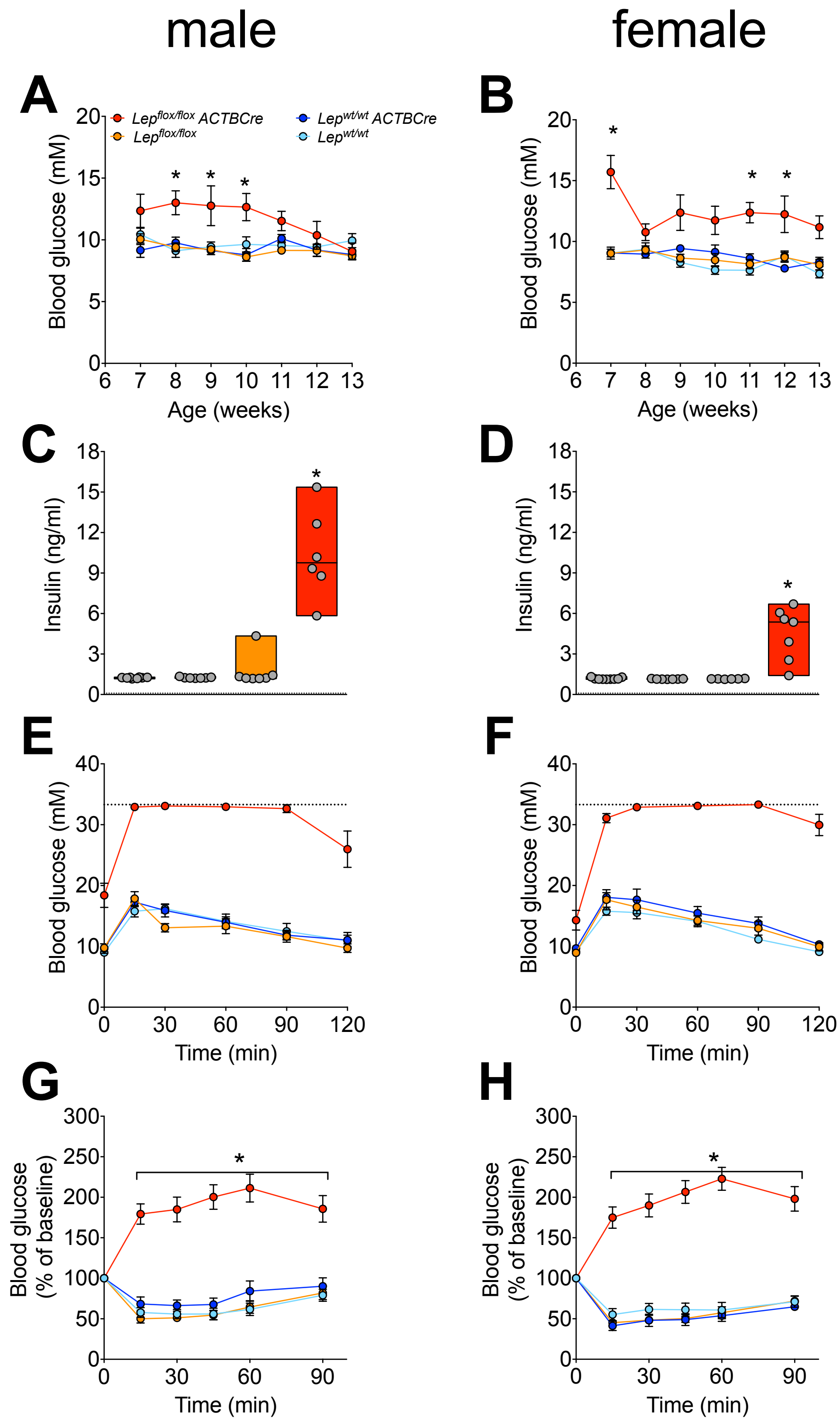


Figure 7.4: Knockout of leptin in *Lep*$^{flox/flox}$ *ACTB*Cre mice results in impaired glucose homeostasis. Male (A) and female (B**)** *Lep*$^{flox/flox}$ *ACTB*Cre mice and control littermates were subject to a 4 h fast followed by measurement of blood glucose levels between 7-13 week of age. At 10 weeks of age, plasma was collected to measure plasma insulin levels in male (C) and female (D) mice. Glucose tolerance was assessed in male (E) and female (F) mice at 9 weeks of age following a 6 h fast; dashed line indicates limit of detection by the glucose meter (33.3 mM). At 12 weeks of age, insulin sensitivity was assessed in male (G) and female (H) mice and presented as percentage of baseline blood glucose levels. Values in A-B and E-H are presented as mean ± SEM (n=8-11/group), while values in C-D are presented as individual data points with bars representing the range of values. *$P<0.05$ *Lep*$^{flox/flox}$ *ACTB* Cre mice vs. all other groups by Two Way Repeated Measures ANOVA and Tukey's multiple comparison analysis in A-B and G-H, and Two Way Anova in C-D. No statistical analyses were performed on E-F due to data points exceeding the limit of detection.

To assess glucose tolerance, mice were subjected to a 6 h fast followed by oral gavage of glucose and subsequent measurement of blood glucose levels. Male and female *Lep*$^{flox/flox}$, *Lep*$^{wt/wt}$ and *Lep*$^{flox/flox}$ *ACTB*Cre mice were euglycemic at baseline and peak blood glucose levels 15 minutes following glucose gavage did not exceed 20 mM (Fig. 7.4E, F). In contrast, *Lep*$^{flox/flox}$ *ACTB*Cre mice were hyperglycemic at baseline, and peak blood glucose levels exceeded the limit of detection (33.3 mM, represented as a dashed line on the graph), indicating severe glucose intolerance. Following an i.p. injection of insulin, a similar reduction of blood glucose levels was observed in *Lep*$^{flox/flox}$, *Lep*$^{wt/wt}$ and *Lep*$^{flox/flox}$ *ACTB*Cre mice (Fig. 7.4G, H). In contrast, blood glucose levels of *Lep*$^{flox/flox}$ *ACTB*Cre mice increased and remained higher following i.p. injection of insulin, indicating severe insulin resistance. Thus, *Lep*$^{flox/flox}$ *ACTB*Cre mice develop transient hyperglycemia, hyperinsulinemia, glucose intolerance and insulin resistance that is comparable to *ob/ob* mice [208].

Next we sought to determine whether the obese, glucose intolerant phenotype observed in *Lep*$^{flox/flox}$ *ACTB*Cre mice could be reversed by restoring leptin levels. To do so, we injected female *Lep*$^{flox/flox}$ *ACTB*Cre mice daily with pegylated recombinant mouse leptin for six days. Body weight and fasting blood glucose levels were measured daily up to 15 days after initiating leptin injections. Nine days after initial leptin injections, we observed an 11.4±0.85% decrease of body weight in *Lep*$^{flox/flox}$ *ACTB*Cre mice, which was reversed following cessation of leptin therapy (Fig. 7.5A). In contrast, no differences in body weight were observed in saline-injected *Lep*$^{flox/flox}$ littermate mice. In *ob/ob* mice, leptin therapy is able to significantly reduce blood glucose levels [137]. To determine whether daily i.p. leptin injections in *Lep*$^{flox/flox}$ *ACTB*Cre mice would affect blood glucose levels, we measured blood glucose via the saphenous vein before and after leptin therapy. Injection of leptin resulted in a 35% reduction of blood glucose levels in *Lep*$^{flox/flox}$ *ACTB*Cre mice, and blood glucose levels were lower than saline-injected *Lep*$^{flox/flox}$ littermates by day +7 of leptin therapy (Fig. 7.5B). Cessation of leptin therapy resulted in a gradual elevation of blood glucose levels in *Lep*$^{flox/flox}$ *ACTB*Cre mice, such that by day +14, blood glucose levels (10.32±0.41 mM) were similar to baseline levels (10.86±0.61 mM). These data suggest that leptin deficiency is the primary defect resulting in obesity and impaired glucose homeostasis in *Lep*$^{flox/flox}$ *ACTB*Cre mice.

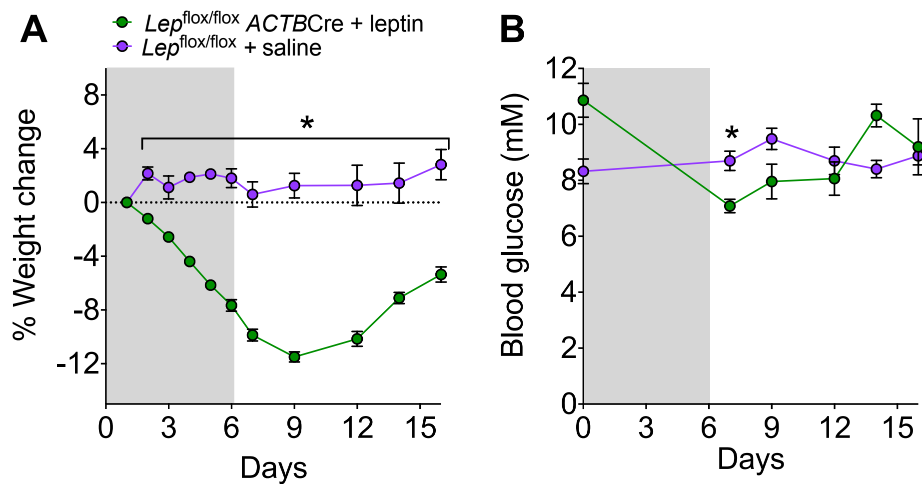


Figure 7.5: Leptin therapy reverses weight gain and reduces blood glucose levels in *Lep*$^{flox/flox}$ *ACTB*Cre mice. Thirteen-week-old female *Lep*$^{flox/flox}$ *ACTB*Cre mice were injected daily with pegylated mouse leptin for 6 days while *Lep*$^{flox/flox}$ received daily injections of saline for the same duration. Body weight (A) and blood glucose (B) was measured prior to and following cessation of leptin therapy. Grey shading represents the period of leptin injections. Values presented as mean ± SEM (n=5/group). *$P<0.05$ by Two Way repeated measures ANOVA and Tukey's multiple comparisons.

To examine the impact of inducible knockout of leptin on body weight and glucose homeostasis, we crossed *Lep*$^{flox/flox}$ mice with *ROSA26*CreER^{T2} mice, resulting in tamoxifen-induced Cre recombinase activation. The Cre variant CreER^{T2} has a mutated human estrogen receptor that has greater affinity for tamoxifen than estrogen [316]. Activation of Cre is dependent on binding by an estrogen receptor agonist, which results in translocation of the Cre protein into the nucleus where it excises DNA that are flanked by *loxP* sites. At 6-9 weeks of age, male mice were injected daily for a period of 5 days with 4 mg of tamoxifen via i.p. injection. To assess recombination of the floxed *Lep* in the presence of tamoxifen-activated *ROSA26*CreER^{T2}, we collected gonadal adipose tissue and extracted gDNA for analysis by qPCR. A highly variable rate of recombination was observed in *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice; with one mouse

exhibiting 52.0% recombination while another exhibited 31.3% recombination (Fig. 7.6A). Next, we measured fasting leptin levels to determine if inducible knockout of leptin via tamoxifen-activated Cre would result in reduce immunoreactive leptin levels, similar to our observations in *Lep*$^{flox/flox}$ *ACTB*Cre mice. We observed no significant differences in plasma leptin levels in *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice compared to littermate controls following tamoxifen injections (Fig. 7.6B). Together, these data suggest that tamoxifen induced activation of Cre resulted in moderate and variable genetic recombination in *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice, and was not sufficient to alter immunoreactive leptin levels of these mice.

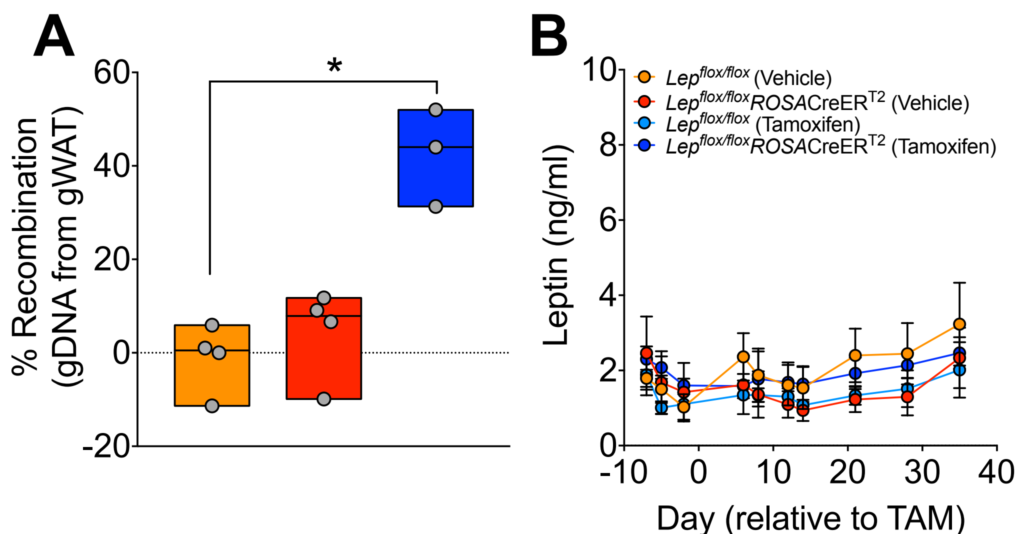


Figure 7.6: Activation of Cre in *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice results in moderate and variable recombination of *ob* in gonadal adipose tissue and does not reduce immunoreactive leptin levels. Gonadal adipose tissue was collected 8 weeks after initial tamoxifen injections in *Lep*$^{flox/flox}$ *ROSA*26CreER^{T2} mice and qPCR was used to assess degree of recombination in *Lep*$^{flox/flox}$ *ROSA*26CreER^{T2} mice and littermate controls (A). Plasma leptin levels were measured prior to and following tamoxifen or vehicle (corn oil) injections in mice. Individual values are presented in A, with bars indicating the range of values and line indicating the median value. Data are presented as mean ± SEM in B (n=3-5/group). *P<0.05 by Kruskal-Wallis non parametric multiple comparison test in A and Two Way Repeated Measures Anova in B.

Next, we characterized the metabolic phenotype of *Lep*$^{flox/flox}$ *ROS*A26CreER^{T2} mice and littermate control mice. Assessment of weight gain, based on calculating increase in weight from the average weight from Day -2 to -9 prior to tamoxifen injections indicated an increase in weight gain at multiple time points post tamoxifen injection in *Lep*$^{flox/flox}$ *ROSA*26CreER^{T2} mice compared to *Lep*$^{flox/flox}$ mice (Fig. 7.7A). DEXA analysis was performed 34 days after initiating tamoxifen injections to determine the proportion of lean and fat mass in these mice. DEXA analysis revealed no significant differences in proportion of lean and fat mass in tamoxifen-treated *Lep*$^{flox/flox}$ *ROS*A26CreER^{T2} mice compared to control littermate mice (Fig. 7.7B). Plasma insulin levels were also measured at select days after initiation of tamoxifen injection to determine if Cre activation to induce recombination of the *ob* gene in *Lep*$^{flox/flox}$ *ROS*A26CreER^{T2} mice would result in hyperinsulinemia. Following tamoxifen injections, no significant increases in plasma insulin levels were found in *Lep*$^{flox/flox}$ *ROSA*26CreER^{T2} mice compared to littermate controls (Fig. 7.7C), though a trend for elevated insulin levels was observed on Day 21, 28 and 35 following tamoxifen injections. No significant differences of fasting glucose levels were observed between *Lep*$^{flox/flox}$ *ROSA*26CreER^{T2} mice compared to littermate controls (Fig. 7.7D). To thoroughly examine glucose homeostasis, glucose tolerance tests and insulin tolerance tests were also conducted 42 and 19 days after the first tamoxifen injection. No significant differences in glucose tolerance were observed in *Lep*$^{flox/flox}$ *ROSA*26CreER^{T2} mice compared to littermate controls (Fig. 7.7E). Similarly, blood glucose levels following an i.p. injection of insulin (0.75U/kg body weight) were comparable between all groups (Fig. 7.7F), indicating no significant differences in insulin sensitivity.

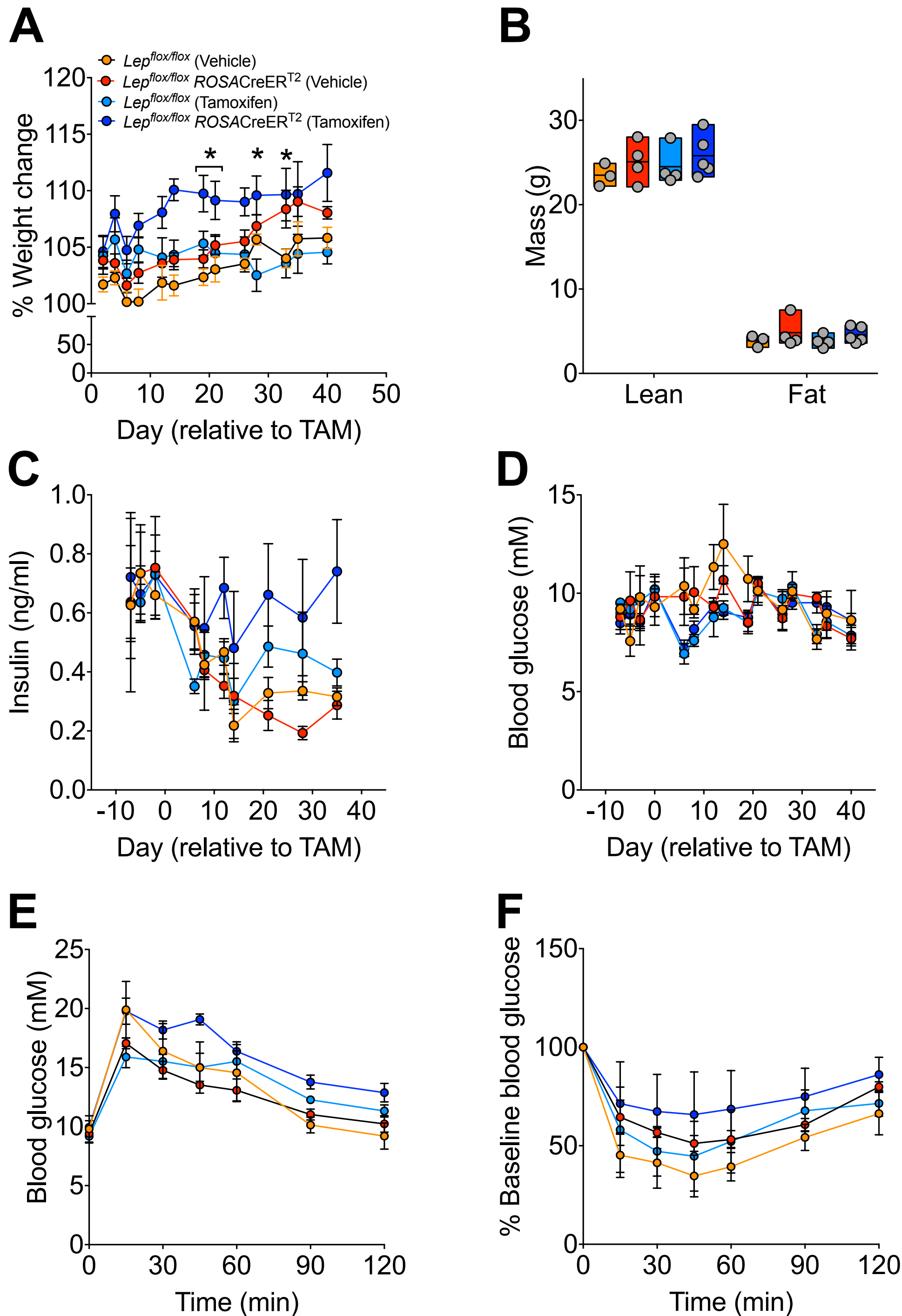


Figure 7.7: Inducible activation of Cre in adult *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice does not result in obesity or impaired glucose homeostasis. Body weight gain was normalized to average body weight between Day -2 to -9 (A). Fat and lean mass was measured by DEXA analysis 34 Days after initial tamoxifen or vehicle injections (B). Mice were fasted for 4 h to collect blood for measurement of plasma insulin (C) and

blood glucose (D). On day 42, mice were fasted for 6 h followed by oral gavage of glucose (1.5 g/kg body weight) and subsequent measurement of blood glucose levels during an OGTT (E). On day 19, mice were fasted for 4 h followed by an i.p. injection of insulin (0.75 U/kg body weight) to assess insulin sensitivity. Data are presented as mean ± SEM (n=3-5/group). A Two Way Repeated Measures ANOVA and Tukey post hoc analysis was used to assess statistical significance. *P<0.05 vs. *Lep*$^{flox/flox}$ (tamoxifen) group.

Together, these findings suggest that moderate recombination of *ob* following activation of Cre by tamoxifen does not alter body weight nor disrupt glucose homeostasis.

7.3 Discussion

In *ob/ob* mice, a point mutation in the gene encoding leptin results in undetectable plasma leptin levels [69]. It is well established that leptin deficiency in *ob/ob* mice results in significant disruption of glucose and energy homeostasis, but whether these effects would also be observed in mice with disruption of leptin in adulthood is unclear. A previous study by our lab has attempted to assess the effects of disrupted leptin action using leptin antagonism [139]. However, the antagonism of leptin was acute, and it is unclear whether loss of leptin action in adult mice produces a similar metabolic phenotype as has been observed in *ob/ob* mice. In this chapter, we aimed to identify whether knockdown of leptin in adulthood effects glucose homeostasis and energy homeostasis. To do so, we utilized mice that have *loxP* sites flanking part of the open reading frame of the leptin gene located within exon 3 along with mice expressing an inducible Cre. Cre-mediated recombination was predicted to produce a truncated, non-functional leptin protein.

To determine if Cre-induced recombination in *Lep*$^{flox/flox}$ mice would recapitulate the phenotype of *ob/ob* mice, we used the *ACTB*Cre mouse line, which constitutively

expresses Cre in all organs [217]. Deletion of the open reading frame in exon 3 of *Lep*$^{flox/flox}$ *ACTB*Cre mice resulted in plasma leptin immunoreactivity below the limit of detection in male and female mice. Furthermore a metabolic phenotype similar to *ob/ob* mice and *Lep*^{KO} rats, characterized by obesity, transient hyperglycemia, hyperinsulinemia, glucose intolerance and insulin resistance was observed [92, 141, 207]. Our observations of obesity in *Lep*$^{flox/flox}$ *ACTB*Cre mice are consistent with results from Odle et al. who used a similar strategy to disrupt leptin signalling by inserting *loxP* sites flanking exon 3 of leptin. These floxed mice were then crossed with mice expressing EIIa Cre, to create a whole-body knockout of leptin [317]. EIIa Cre-mediated recombination of the floxed ob gene resulted in obesity in addition, Odle et al also observed increased food intake and decreased energy expenditure in *ob* floxed mice expressing EIIa Cre compared to Cre negative control mice [317, 318]. Collectively, these results suggest that deletion of the floxed region of exon 3 of the leptin gene results in disruptions to glucose and energy homeostasis.

Compared to the robust reduction of plasma leptin levels observed in *Lep*$^{flox/flox}$ *ACTB*Cre mice, no significant differences in plasma leptin levels were observed in tamoxifen treated *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice compared to control littermates. While plasma leptin levels of *Lep*$^{flox/flox}$ *ACTB*Cre mice were below the detection limit of the leptin assay, plasma levels of *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice ranged between 1.5-2.5 ng/ml, and were comparable to control littermates. Furthermore, a moderate recombination of leptin gDNA (approximately 42%) was observed in gonadal adipose tissue following tamoxifen administration to activate Cre. This corresponded with no significant differences in body weight, fasting insulin, fasting blood glucose or insulin

sensitivity between *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice and control littermates following delivery of 20 mg total of tamoxifen.

Previous studies have employed inducible Cre mouse lines to selectively knockout leptin receptors in adult mice [268, 315]. Guo et al. used *ROSA26*CreER^{T2} to selectively delete exon 17 of *Lepr* in adult mice. Guo et al. reported 85% recombination of *Lepr* in perigonadal fat of *Lepr*$^{flox/flox}$ *ROSA26*CreER^{T2} after delivery of 5 mg of tamoxifen via i.p. injection [268]. A similar degree of recombination of targeted alleles was reported in WAT of mice expressing CreER^{T2} under the control of the ROSA26 promoter following tamoxifen injection [319, 320]. In contrast, we observed variable recombination of *ob* in gonadal adipose tissue, ranging between 31-51% in *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2}, despite injecting more than double the amount of tamoxifen as Guo et al. It is unclear why our studies achieved relatively lower levels of recombination in adipose tissue compared to the studies described above as important details regarding tamoxifen administration including dose of tamoxifen, route of delivery or solution used to dissolve tamoxifen were not described in the published studies mentioned above.

The comparable levels of plasma leptin levels between *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice and littermate controls despite the moderate (31-51%) knockdown of leptin gDNA in gonadal adipose tissue suggests that more than a 50% decrease of leptin gene expression is required to produce significant decreases in plasma leptin levels. These findings are in line with the phenotype of *ob/wt* mice, who are phenotypically similar to *wt/wt* mice [208], and have comparable circulating plasma leptin levels unadjusted for fat mass [321].

The moderate recombination of leptin observed in gonadal adipose tissue may not accurately reflect plasma leptin levels as multiple adipose depots contribute to

circulating leptin levels [87]. In particular, subcutaneous adipose depots express more leptin than omental depots [322, 323]. Furthermore, variable Cre recombination rates have been reported in *ROSA26*CreER^{T2} mice, with small intestine and liver experiencing the greatest Cre-mediated recombination (approximately 85-90%), while Cre expression in muscle, BAT and kidney resulted in moderate recombination (approximately 20-40%) [268]. Thus alternative adipose depots including the subcutaneous inguinal depot may need to be assessed in future studies to determine the effects of Cre-mediated knockdown of leptin.

Although our attempt to characterize the effects of inducible knockdown of leptin in *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice did not result in complete deletion of leptin following tamoxifen administration, future directions to achieve complete inducible knockout of the leptin gene are proposed in chapter 8 that can be investigated using a similar strain of mice.

CHAPTER 8: CONCLUSIONS AND FUTURE DIRECTIONS

8.1 General Conclusions

The discovery of leptin over 20 years ago generated significant excitement in the hopes that it would resolve obesity. Subsequent clinical trials revealed that leptin alone is ineffective in treating obesity [123]. However, leptin therapy following weight loss was beneficial in changing neural activity, such as increased hunger that occurs with weight loss [324]. These studies suggest that rather than preventing weight gain, leptin levels are a reflection of long term energy stores.

Leptin therapy in patients with congenital mutations resulting in leptin deficiency or patients with generalized lipodystrophy has revealed an important role of leptin in glucose homeostasis. These patients are often characterized by significant perturbations to glucose homeostasis including hyperinsulinemia, insulin resistance and glucose intolerance. Leptin therapy is able to reverse these impairments to glucose homeostasis by lowering fasting insulin levels, improving insulin sensitivity and glucose tolerance [325-327], suggesting that it may have a significant role in regulating glucose homeostasis independent of its effects on energy metabolism. However, the mechanism by which leptin acts to improve glycemia and the hierarchy of metabolic defects that arise when leptin action is disrupted is unclear.

To elucidate the role of leptin in maintaining glucose homeostasis, this thesis investigation focused on understanding the effects of leptin on β cell function. Furthermore, we aimed to investigate the primary metabolic defects that arise with loss of leptin signalling. Through characterization of mouse and rat strains with disrupted leptin signalling, we obtained a better understanding of the role of leptin in regulating

insulin and glucose to achieve glucose homeostasis, along with observations that now raise new questions of the hierarchy of metabolic defects that arise as a result of impaired leptin signalling.

8.2 Contribution of Hyperinsulinemia to the Obese, Diabetic Phenotype of ob/ob Mice

A fundamental focus of this thesis was to understand the effects of leptin on pancreatic β cells. In mice and humans with impaired leptin signalling due to spontaneous mutations in the genes encoding for leptin or the leptin receptor, hyperinsulinemia is commonly observed [140, 193, 194, 208]. Development of hyperinsulinemia is among the first metabolic defects to arise in *ob/ob* mice and is concomitant with obesity, insulin resistance, glucose intolerance and transient or mild hyperglycemia [67, 137, 208]. Previous research in diet-induced obese mice has suggested that reduction of insulin gene dosage is able to prevent hyperinsulinemia and concomitant obesity [41], suggesting that hyperinsulinemia can drive other metabolic defects including obesity and insulin resistance that occur as a result of leptin deficiency.

The first part of this thesis focuses on understanding the significance of hyperinsulinemia in the absence of leptin signalling, as well as the role of leptin in regulating β cell function. Our approach to attenuate hyperinsulinemia involved reduction of insulin gene dosage in *ob/ob* mice. We found that loss of 2 or 3 insulin alleles in *ob/ob* mice produced significant reductions of body weight and fat mass. However, loss of body weight was also accompanied by hyperglycemia and worsened

glucose intolerance. We found that hyperinsulinemia in *ob/ob* mice, while detrimental to weight gain is required to prevent severe perturbations to glucose homeostasis. Without the elevated insulin levels, insulin resistant *ob/ob* mice quickly developed extremely high levels of blood glucose. The reduction in body weight observed in *ob/ob* mice is much greater than previous reports in high fat fed C57BL/6J mice [41], but the severe hyperglycemia that was observed was unique to *ob/ob* mice, and suggests that in the absence of leptin, and perhaps its role in facilitating insulin sensitivity, much higher levels of insulin are required to achieve lower blood glucose levels. Thus, this thesis provides evidence that hyperinsulinemia is a compensatory mechanism that helps to manage blood glucose levels in the absence of leptin.

Sexual dimorphisms were observed as a result of reduced insulin gene dosage in male and female *ob/ob* mice. Male mice experienced greater weight loss and had higher blood glucose levels with loss of 2 or 3 insulin alleles. The findings reported in this thesis are in line with previous observations indicating that males and females respond differently to reductions of insulin gene dosage [41, 42]. Furthermore, these results are consistent with previous reports suggesting that males are more susceptible to glucose intolerance and insulin resistance compared to females [328, 329]. The discrepancy between the sexes may be related to the influence of sex hormones, as estrogen is thought to enhance insulin sensitivity, decrease hepatic glucose production and enhance glucose transport into muscle [330, 331]. Whether sex steroids can also contribute to regulation of insulin secretion is unclear and warrants further investigation.

8.3 Role of Lepr in β Cells

Previously it has been suggested that leptin regulates glucose homeostasis by acting in pancreatic β cells to suppress insulin secretion and insulin gene expression [170, 238, 256, 257]. Deletion of *Lepr* in β cells of mice produced contrasting results with one study reporting hyperinsulinemia, obesity and glucose intolerance [238], while another reported mildly elevated insulin levels without significant increases in body weight or glucose intolerance [258]. It was later revealed that promiscuity of *RIP*Cre and *PDX-1*Cre, which were also expressed in the hypothalamus and exocrine pancreas respectively, may have confounded the interpretation of these results [260]. In contrast to these studies, we assessed the function of *Lepr* in β cells by selectively restoring *Lepr* only in β cells using the *Ins1*Cre, resulting in mice with disruption to leptin signalling in all tissues except the pancreatic β cells. In these experiments, we found that restoration of *Lepr* did not improve plasma insulin levels, glucose tolerance or normalize islet mass and β cell area in male and female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice.

These data differ from previous reports suggesting that leptin action in the β cell is a key site for mediating the effects of leptin on glucose homeostasis [238, 258]. However, these data are consistent with a recent study using the *Ins1*Cre to knockout *Lepr* in β cells [261]. Our results suggest that *Lepr* in β cells are not sufficient to regulate glucose homeostasis. Additional sites of leptin signalling are needed to achieve glucose homeostasis may include the hypothalamus, which is an integral site of regulating insulin secretion and blood glucose levels [312]. Indeed, mice with selective restoration of *Lepr* in the CNS are reported to have attenuated plasma insulin levels and restoration of euglycemia [154, 332] providing further evidence that the CNS is a primary site through which leptin-mediated regulation of insulin secretion occurs.

8.4 Hierarchy of Metabolic Defects that Arise as a Result of Leptin Deficiency in Rats

Most of our knowledge of the physiological mechanisms underlying the metabolic actions of leptin is based upon data collected from *in vivo* studies using mice possessing spontaneous mutations to leptin or its receptor, mice with manipulated genetics to target deletion of leptin receptors in specific tissues, or wildtype mice that have undergone chemical or dietary manipulations (e.g. STZ treatment, high fat diet) provided with exogenous leptin therapy. These studies have enhanced our knowledge of the physiological role of leptin, especially in relation to energy and glucose metabolism. However, mouse models are an approximation of human disease, and may not fully recapitulate the role of leptin in human physiology. The difference in leptin physiology between humans and mice is illustrated in humans with congenital leptin deficiency or mutations causing lack of leptin receptors. Humans with impaired leptin signalling due to mutations have normal functioning hypothalamic-pituitary-adrenal axis function and normal body temperature whereas *ob/ob* mice have a hyperactive HPA axis and an inability to handle cold temperatures [293]. Furthermore, humans lacking leptin have relatively normal linear growth and mild impairments in the hypothalamic-pituitary-thyroid axis [192-194] whereas *ob/ob* mice have stunted growth and marked hypothalamic hypothyroidism [333, 334]. Of utmost relevance to this thesis is the difference in glycemic response to leptin deficiency between humans and mice. Humans with mutations to leptin or the leptin receptor maintain relatively normal blood glucose levels [193, 194], while *db/db* mice and young *ob/ob* mice on a C57BL/BKs background are hyperglycemic [206, 207]. The mechanism underlying these differences is unclear,

but suggests that caution should be taken when interpreting the results of murine studies and applying them to human physiology.

An alternative species to mice that is frequently used in pharmacological studies is rats. For over 150 years, the rat has been used in numerous areas of research ranging from drug discovery, psychiatric disorders, cardiovascular disease and diabetes. In many areas of research, rats are an ideal model due to their physiological similarities to humans. Additionally, many human genes associated with diseases have counterparts in the rat genome [335]. Finally, rats are often preferred to mice due to their larger size, making handling, sampling and performing procedures easier.

For the reasons mentioned above, another aim in this thesis was to characterize the effects of leptin deficiency on glucose homeostasis in rats. Our findings revealed that loss of leptin in rats results in a similar metabolic phenotype as has been previously observed in *ob/ob* mice including development of obesity, hyperinsulinemia, insulin resistance, glucose intolerance and islet hyperplasia. However, several differences in metabolic phenotype exist. First, Lep^{KO} rats appear to be less sensitive to reduced environmental temperature, with only minimal decreases in body temperature, suggesting that thermogenic capacity in greater in rats than mice, and may be more similar to human thermogenic capacity. Second, fasting glucose levels were mildly and transiently elevated in male rats, while in *ob/ob* mice on a C57BL/Ks background, significant hyperglycemia has been reported [207]. Finally, glucose response is near normal 2 h post glucose gavage in Lep^{KO} rats while *ob/ob* mice demonstrate higher blood glucose levels 2 h post glucose challenge [95]. The milder perturbations to glucose homeostasis observed in leptin deficient rats compared to leptin deficient mice suggests that rats are more similar to humans lacking leptin signalling. The thorough

characterization of glucose homeostasis in the *Lep*^{KO} rat provides a resource for researchers to use this model to better understand the effects of various drugs involved in glucose regulation with the opportunity to control leptin levels.

To follow up on our characterization of glucose homeostasis in *Lep*^{KO} rats, we assessed rats in the early suckling period of life, well before the development of overt differences in body weight between *Lep*^{KO} and *Lep*^{WT} rats. This was done to determine the primary defects to glucose homeostasis that arise as a result of leptin deficiency and independent of the effects of obesity. Interestingly, we found that reduced insulin sensitivity was observed prior to significant increases in plasma insulin levels of *Lep*^{KO} rats. Depletion of leptin levels as a result of uncontrolled diabetes mellitus have been associated with development of insulin resistance, and replacement of leptin in rats with uncontrolled diabetes mellitus have resulted in reversal of insulin resistance independent of effects on food intake or body weight [147]. Thus, our results are in line with previous findings indicating the significance of insulin sensitizing effects of leptin to achieve glucose homeostasis, independent of the effects of leptin on insulin. However, questions still remain regarding the hierarchy of metabolic effects that develop as a result of impaired leptin signalling. First, it is unclear whether insulin resistance drives hyperinsulinemia, or if dysregulation of glucose stimulated insulin secretion observed in rodents lacking leptin signalling occurs independently of this. Secondly, as we did not assess plasma insulin levels or insulin sensitivity continuously, it is unclear whether there are incidences in which elevated plasma insulin levels were observed prior to reductions in insulin sensitivity. In addition, the measurement of plasma insulin levels and insulin sensitivity were not performed on the same rodents, thus preventing us from associating plasma insulin levels with measures of insulin sensitivity.

8.5 Loss of Leptin Signalling in Adulthood

The study of metabolism in rodents with spontaneous mutations resulting in impaired leptin signalling has been an invaluable resource used to better understand the role of leptin in energy and glucose homeostasis. Similarly, development of transgenic models ranging from tissue-specific knockout of the leptin receptor [160, 238, 258, 261], to whole body knockout of the leptin receptor [156, 312, 336] have provided us with a better understanding of the tissue specific actions of leptin and metabolic defects that can arise when leptin signalling is disrupted. However, many questions regarding the effects of leptin on metabolism still remain.

The *in vivo* models described above are limited by their constitutive expression of mutated leptin or leptin receptors, An aim of this thesis was to develop an animal model in which leptin expression could be manipulated at different stages of development. An inducible transgenic model that can be used to manipulate leptin expression at various time points during development may provide insight into the effects of reduced leptin action in adulthood. In most cases of T2D, defects to glucose homeostasis are not observed until later in adulthood [337]. The poor efficacy of rising leptin levels to prevent weight gain and impairments to glucose homeostasis is suggestive of the development of leptin resistance in adulthood [338]. Understanding the effects of loss of leptin signalling later in life as well as the mechanisms of leptin resistance is crucial to development of therapies to overcome obesity and diabetes.

The findings in this thesis suggest that targeted deletion of leptin by floxing exon 3 of the leptin gene is an effective strategy to disrupt leptin expression. Use of the constitutively expressed *ACTB*Cre line confirms that complete Cre recombination of the loxP sites in *Lep*$^{flox/flox}$ mice results in depletion of leptin levels below detectable limits

and results in a metabolic phenotype that resembles *ob/ob* mice. However, our preliminary results using the inducible *ROSA26*CreER^{T2} resulted in moderate recombination (approximately 42%) of leptin in gonadal white adipose tissue, with no discernable effects on metabolic phenotype. These findings indicate that a higher dose of tamoxifen or an alternative method of tamoxifen delivery is required to induce Cre activation.

8.6 Study Strengths and Limitations

The fundamental focus of this thesis was to assess the role of leptin in regulating blood glucose levels. Work from this thesis has helped to define the significance of hyperinsulinemia in the context of leptin deficiency, as well as the implications of direct leptin action on pancreatic β cells. Furthermore, we were able to demonstrate the consistency of the glucoregulatory actions of leptin by assessing the effects of leptin deficiency in rats as well as mice.

The applicability of our findings was increased by including appropriate control groups and including both sexes whenever possible. Indeed, studies in chapters 3, 4, 5, and parts of chapter 7 show that there are subtle differences in the phenotype that develops when leptin signalling is disrupted in males and females. A few instances of sexual dimorphisms were observed. For example, in chapter 3, female *ob/ob* mice develop a milder phenotype resulting from reduction of insulin gene dosage than *ob/ob* males, while in chapter 5 euglycemia is observed in female but not male leptin-deficient rats. These studies should be followed up by more in-depth analyses to determine the

mechanisms by which females are able to regulate glucose homeostasis better than males.

In spite of our efforts to design studies that limited confounding variables, there are a number of caveats presented in these studies. Firstly, the variable number of animals per group limited the power of our statistical analysis. For example, in chapters 4 and 7, there is a significant discrepancy in the number of animals in the control groups versus the experimental groups. This variability is partly attributed to the fact that most of the strains used in this thesis were bred in our own animal facility, and the availability of mice from each experimental group was variable for each round of breeding. To prevent this from occurring, measures such as generating a large cohort of breeders and timed breeding could have been employed. Secondly, some of our studies included a mixed background of strains, and were not backcrossed multiple generations to generate mice on a pure B6 background. While mixed background strain may be more reflective of the complex genetics of humans, it provides another variable to consider when interpreting results. In addition, studies in chapter 4 and parts of chapter 5 were performed in a different facility (Modified Barrier Facility and Animal Resource Unit) compared to all other studies (performed in the Center for Disease Modelling). Recent studies suggest that background strain and environment can influence the gut microbiota and metabolic phenotype of mice [339], and thus caution should be taken in interpreting the results of our studies. However, wherever possible, we used littermate controls to limit the significance of mixed background strains from confounding our results. The studies presented in this thesis have several limitations in study design. In chapter 3, we were limited by the complicated breeding scheme to generate *ob/ob* mice with loss of 2 or 3 insulin alleles and were unable to compare these mice to littermate control *ob/ob* or

wt/wt mice expressing all 4 insulin alleles. In chapter 4, the hyperleptinemia observed in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice may have prevented us from exploring the effects of *Lepr* restoration exclusively in β cells as it has been previously observed that hyperleptinemia is sufficient to induce leptin resistance [269]. In chapter 6, we were unable to assess glucose tolerance and islet function in 2-week-old rats, and thus the majority of our conclusions about islet function are made from islets at 3 weeks of age, when hyperinsulinemia is already present. Thus, we are unable to ascertain whether impaired islet function is a source of elevated fasting insulin levels. Similarly, the low sample sizes of pancreatic insulin content and relative β and α cell area warrant an increase in sample size to enhance confidence in our findings. Finally, the preliminary data in chapter 7 is limited by small samples sizes and our limited analysis of tissue-specific Cre recombination. It is likely that analysis of tamoxifen mediated-recombination in alternate adipose depots may yield a different degree of recombination, given the heterogeneity of *ROSA26*CreER^{T2} expression [268]. Regardless of this, it is clear that the level of knockdown achieved by tamoxifen injection was not sufficient to significantly alter circulating leptin levels, and thus an alterative strategy to enhance the efficiency of knockdown is required in order to determine the effects of inducible loss of leptin in adult mice.

8.7 Significance & Future Directions

The application of leptin in the clinic has been successful among patients with congenital leptin deficiency and those suffering from lipodystrophy [150, 326]. Whether or not leptin can be used to treat obese and diabetic individuals is unclear. Recent

clinical trials have revealed no significant effects of leptin therapy for type 1 and type 2 diabetes, though mild improvements in hbA1C levels were observed [340-342]. Improved understanding of the metabolic effects of leptin may help to develop therapies that target leptin therapies in a tissue specific manner.

The findings reported in this thesis have provided insight into a number of potential mechanisms by which leptin may or may not be acting to regulate glucose homeostasis. We reveal that regulation of glucose homeostasis by leptin in rats may be primarily mediated through the insulin sensitizing effects of leptin [296]. Our findings demonstrate that loss of leptin signalling results in insulin resistance in both adult rats and mice. Furthermore, reduced insulin sensitivity is among the first metabolic defects that is observed in pre-obese rats. Though we initially believed that resolution of hyperinsulinemia would be beneficial to *ob/ob* mice, it appears that hyperinsulinemia in *ob/ob* mice is necessary to protect these mice from severe hyperglycemia that arises as a result of reduced insulin sensitivity associated with leptin deficiency. Furthermore, we show that direct leptin signalling in β cells does not improve glycemia nor does it resolve glucose intolerance in mice lacking *Lepr* elsewhere. These findings collectively suggest that in the hierarchy of the glucoregulatory actions of leptin, its effects on insulin sensitivity are more significant than its influence on β cell function.

To further investigate the significance of the insulin-sensitizing effects of leptin, future studies can employ the inducible leptin knockout model and combine it with an insulin sensitizing therapy such as metformin. This would help to determine whether the loss of insulin sensitivity is a primary defect contributing to hyperinsulinemia and obesity that are typically observed in rodents lacking leptin signalling.

The data in this thesis regarding the direct actions of *Lepr* in the β cell could also be followed up on. Recent single cell transcriptomics data of islet cell types reveals that *Lepr* expression is relatively low in β cells but much higher in δ cells [117]. However, little is known about the role of *Lepr* in δ cells on regulation of insulin secretion. Studies utilizing the *Lepr*$^{loxTB/loxTB}$ mouse line crossed with a δ-cell specific Cre such as the somatostatin Cre, could be done to determine the influence of *Lepr* signalling in the δ cell on glucose homeostasis. However, care must be taken to ensure that recombination in neurons also expressing somatostatin do not confound the interpretation of these results.

Finally, the development of the inducible leptin knockout mouse represents a valuable tool that can be used to elucidate the effects of tissue-specific sources of leptin. Though we were unable to induce significant knockdown of leptin in adult *Lep*$^{flox/flox}$ *ROSA26*CreER^{T2} mice, the development of a phenotype resembling the *ob/ob* mouse in *Lep*$^{flox/flox}$ *ACTB*Cre mice suggests that knockdown of leptin gene expression is sufficient to produce undetectable leptin levels can be achieved by using the right Cre line. Alternative strategies to achieve temporal knockdown of leptin may include use of tamoxifen metabolites to induce Cre activation, in addition to use of alternative ubiquitously expressed inducible Cre lines. Tamoxifen is commonly used to induce activity, but can often result in toxic side effects including cardiomyocyte apoptosis and gastric toxicity, depending on the dose used [343, 344]. In addition, scrotal swelling has been previously observed as a result of 24 mg of tamoxifen [345], and this phenotype was similar to our own observations using 20 mg of tamoxifen (data not shown). To avoid these toxic effects and increase the efficiency of Cre recombination, future studies utilizing inducible Cre lines may benefit from the administration of the tamoxifen

metabolite, 4-hydroxytamoxifen (4-OHT). Compared to tamoxifen, 4-OHT is less toxic, and has 100 times higher affinity for the mutated estrogen receptor than tamoxifen [346, 347]. Additional tamoxifen metabolites that may be effective in inducing Cre activity also include Endoxifen, which, similar to 4-OHT, is generated from metabolism of tamoxifen by cytochrome p450. Endoxifen is reported to be more stable in storage than 4-OHT, while retaining strong potency to induce CreER^{T2} activation [348].

The *ROSA26*CreER^{T2} line used in our preliminary studies to achieve leptin knockout in adult mice failed to produce a complete knockdown of leptin. This may be a result of the heterogeneous and inconsistent efficiency of Cre that has been previously reported. An alternative strategy to generate a higher efficiency of recombination is the use of alternative ubiquitously expressed and inducible Cre lines such as the *UBC*CreER^{T2}. This inducible Cre is driven by the human ubiquitin c promoter [349], and has been previously shown to result in efficient and consistent knockdown across several tissues including visceral adipose tissue [349]. Furthermore, the use of this inducible Cre line has resulted in obesity and impairments to glucose tolerance following disruption of *Lepr* [315], suggesting that Cre induced recombination was widespread. However, pilot studies involving *Lep*$^{flox/flox}$ *UBC*CreER^{T2} mice have reported miniscule levels of recombination (averaging approximately 9% in gonadal adipose tissue following i.p. injection of 10 mg of tamoxifen, Appendix Fig. A1), suggesting that higher doses of ligand are required to induce CreER^{T2} activity.

In addition to assessing the effects of temporal knockdown of leptin, the *Lep*$^{flox/flox}$ line may be used to understand the role of tissue-specific sources of leptin. It is well established that the primary source of leptin is derived from adipose tissue [69], but it remains unclear whether leptin derived from alternative tissue sources including the

gastric epithelium [86], pancreas and muscle [82, 83], or pituitary [84] influence glucose regulation. The *Lep*$^{flox/flox}$ mice that are already available in the lab can be crossed with tissue specific Cres such as the Adiponectin Cre [350] to determine the effects of adipose-derived leptin on glucose homeostasis.

In summary, the studies from this thesis suggest that leptin signalling has a profound influence on regulation of glucose homeostasis, and these effects are, for the most part, similar in both mice and rats. Further exploration of the impact of modulating leptin action in adulthood and the impact of leptin signalling on insulin sensitivity may provide new insight to fuel therapeutic applications aimed at treating obesity and diabetes.

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APPENDIX

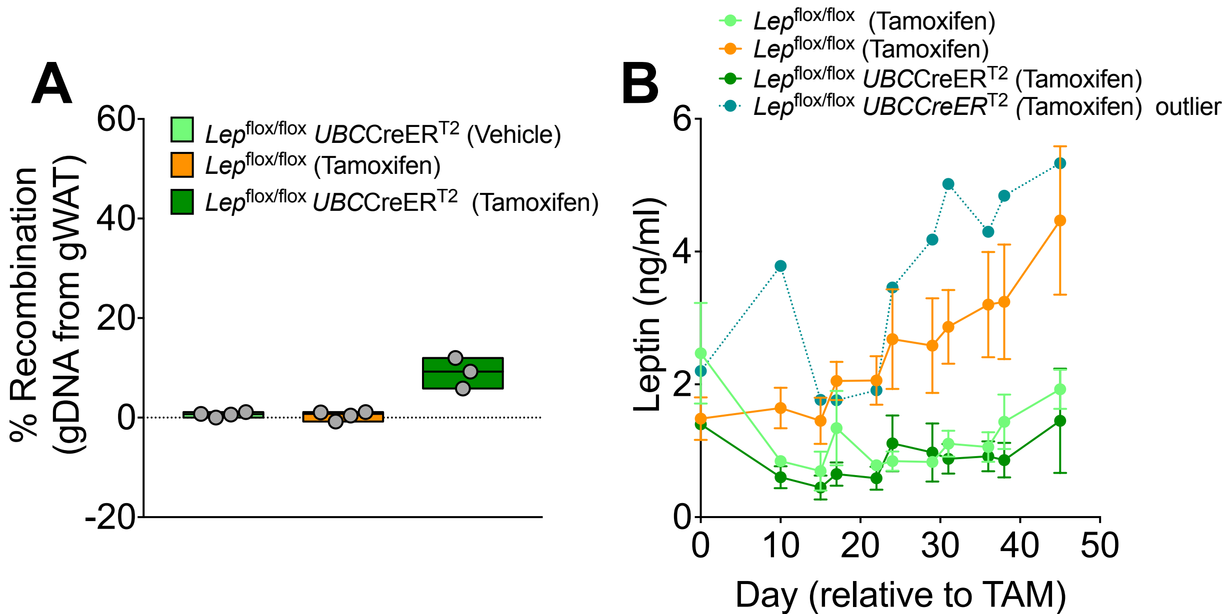

Figure A1: *Lep*$^{flox/flox}$ *UBC*CreER^{T2} mice have minimal recombination in gWAT following tamoxifen injection. Leptin gDNA was measured in gonadal white adipose tissue (gWAT) 50 days after i.p. injection of tamoxifen (10 mg total dose) (A). Plasma leptin levels were not significantly different between *Lep*$^{flox/flox}$ *UBC*CreER^{T2} mice and *Lep*$^{flox/flox}$ littermates treated with tamoxifen. One outlier expressing significantly higher plasma leptin levels was observed in the *Lep*$^{flox/flox}$ *UBC*CreER^{T2} tamoxifen treated group (B). Individual data are presented with a bar indicating the range of values and line indicating the median value in A, while values are presented as mean ± SEM (except for outlier) in B.