

**EFFECTS OF INSULIN GENE DOSAGE ON BODY WEIGHT AND GLUCOSE  
HOMEOSTASIS**

by

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## Abstract

Obesity is one of the biggest health concerns around the world and is closely associated with insulin hypersecretion. However, the causality relationship between these conditions remains enigmatic. We tested the hypothesis that fasting hyperinsulinemia is necessary for diet-induced obesity by varying the pancreatic-specific *Ins1* gene dosage in *Ins2*<sup>-/-</sup> mice. Male *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> mice did not exhibit high fat diet-induced fasting hyperinsulinemia, when compared with their *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> littermate controls. This genetic inability to become hyperinsulinemic prevented the expected increase in pancreatic  $\beta$ -cell number, confirming a role for insulin in high fat diet-induced  $\beta$ -cell expansion. Male *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> mice were also protected from diet-induced obesity and hepatic steatosis when compared to high fat fed *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> littermate controls in the absence of sustained changes in glucose homeostasis. Genetic prevention of hyperinsulinemia increased energy expenditure while reducing adipose inflammation and fatty acid spillover. Female control *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> mice did not exhibit hyperinsulinemia or weight gain on the high fat diet we employed, so it was not possible to test the same hypothesis in the female mice. The effects of reducing *Ins2* gene dosage on the *Ins1* null background were also assessed. Male *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice had a phenotype that differed strongly between cohorts. In one cohort of the male mice, *Ins2* haploinsufficiency was associated with increased food intake of the high fat diet, relative to *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice fed the same diet, but no changes in circulating insulin levels. On the other hand, female *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice were partially protected from high fat diet-induced obesity relative to their littermate controls. The differences in the consequences of *Ins1* versus *Ins2* loss prompted analysis of the tissue expression of both insulin genes, focusing on the central nervous system. We demonstrated that, unlike *Ins1*, *Ins2* is expressed in the brain. High fat feeding reduced *Ins2* expression in the brain in a region- and sex-specific manner. Collectively, our data provide genetic

evidence that circulating hyperinsulinemia can drive obesity in mammals. These findings may be important for understanding the causes of obesity and eventually the development of approaches to prevent or treat it.

## Preface

I performed and analyzed all studies reported in this thesis, unless otherwise noted below. I was principally involved in all aspects of the research from design, data analysis, manuscript preparation, and submission for publication of all the data discussed in this thesis.

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## List of abbreviations

18S	18 S ribosomal RNA
Acacb	Acetyl coa carboxylase
Actb	$\beta$ -actin
Acyl	ATP citrate lyase
Adipoq	Adiponectin
Adipor	Adiponectin receptor
Adpn	Patatin-like phospholipase domain containing 3
AgRP	Agouti-related peptide
ANOVA	Analysis of variance
AOC	Area over the curve
ARC	Arcuate nucleus
Atf3	Activating transcription factor 3
Atgl	Phospholipase domain containing 2
ATP	Adenosine Triphosphate
AU	Arbitrary unit
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body mass index
Ca <sup>2+</sup>	Calcium
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and Amphetamine-regulated transcript

cDNA	Complementary DNA
Cebpa	CCAAT/enhancer binding protein
Chop	DNA-damage inducible transcript 3
Chrebp	Carbohydrate-responsive element-binding protein
CNS	Central nervous system
CSP	Cerebrospinal fluid
Ct	Threshold cycle
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
Egr1	Early growth response 1
ELISA	Enzyme-linked immunosorbent assay
Emr1	EGF-like module-containing mucin-like hormone receptor-like 1
ERK	Extracellular regulated kinases
Fasn	Fatty acid synthase
Fgf21	Fibroblast growth factor 21
Foxa2	Forkhead box a2
Foxc2	Forkhead box c2
Foxo1	Forkhead box o1
Fto	Alpha-ketoglutarate-dependent dioxygenase
GFP	Green fluorescent protein
GLUT	Glucose transporter protein
Glut4	Solute carrier family 2 member 4

Grb2	Receptor-bound protein-2
H&E	Hematoxylin and eosin
H3K4me1	Monomethylation of the 4th residue (lysine) from the start of the H3 protein
HDL	Low high-density lipoprotein
HNE	4-hydroxy-2-nonenal
Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1
HSD	Honest significant difference
Hsl	Hormone sensitive lipase
IGF	Insulin-like growth factor
Igfbp	Insulin-like growth factor binding protein
IKK $\beta$	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-6	Interleukin 6
<i>Ins1</i>	Insulin 1
<i>Ins2</i>	Insulin 2
Insr	Insulin receptor
IRS	Insulin receptor substrate
JNK1	JUN-N terminal Kinase 1
K <sub>ATP</sub>	ATP-sensitive potassium channel
Klf15	Kruppel-like factor 15
Krox20	Early growth response 2
LacZ	$\beta$ -galactosidase gene
Lep	Leptin

Lepr	Leptin receptor
LTD	Long-term depression
Lxr	Nuclear receptor subfamily 1
MAPK	Mitogen-activated protein kinase
MEK	MAPK kinase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
Neo	Neomycin
NeuN	Neuronal nuclei
NMR	Nuclear magnetic resonance
NPY	Neuropeptide Y
Nrip1	Nuclear receptor interacting protein 1
P	Probability of significance
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDK	Pyruvate dehydrogenase kinase
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B

PKC	Protein kinase C
Pomc	Pro-opiomelanocortin
Pnpla2	Adipose triglyceride lipase
Ppara	Peroxisome proliferator activated receptor alpha
Pparg	Peroxisome proliferator activated receptor gamma
Ppargc1a	Proxisome proliferative gamma coactivator 1 alpha
Ptpn1	Protein tyrosine phosphatase
qPCR	Quantitative polymerase chain reaction

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I believe that those in academia are more responsible than teaching a certain subject or studying it. It is our duty to set a good example for the rest of the society. We need to seek not just intelligence, but also wisdom. We must understand and explain science, literature, art, love, compassion, and simply how to be human and a good one at that. I only mention this to end this section by acknowledging one of the many quotes that have been a role model for me on my quest to achieve these, especially because of my endeavor to become a scientist and an educator:

“When we look at modern man, we have to face the fact that modern man suffers from a kind of poverty of the spirit, which stands in glaring contrast to his scientific and technological abundance; We've learned to fly the air like birds, we've learned to swim the seas like fish, and yet we haven't learned to walk the Earth as brothers and sisters.” Dr. Martin Luther King Jr.

## Dedication

*To my loving family and friends.*

# **Chapter 1: Introduction**

## **1.1 The hormone insulin**

### **1.1.1 Discovery of insulin**

One of the biggest discoveries in the history of medicine is the discovery of insulin as a glucose regulating hormone produced by the pancreatic islets. Several key observations led to today's understanding of this hormone. In 1869 a medical student from Germany, Paul Langerhans, discovered clumps of cells scattered throughout the pancreas. These clumps of cells became known as 'Islets of Langerhans' <sup>1</sup>. Then, Dr. Frederick Banting 92 years ago, with the help from a medical student Charles Best and support from John J. R. Macleod, began studies that led to the extraction, purification and discovery of insulin from these islets <sup>1,2</sup>. They observed that surgically preventing the flow of digestive pancreatic secretions from reaching the digestive system did not cause diabetes. However, if the pancreas were removed, dogs would begin drinking and urinating excessively, lose weight and become progressively weaker. These two pieces of evidence led them to believe that the anti-diabetic secretions of the pancreas were not in the digestive secretions. They ligated pancreatic ducts causing the pancreas to degenerate and lose the ability to secrete digestive secretions. They then removed the pancreata and sliced them in a mixture of salts and water. The ground slices were filtered, resulting in a substance named "isletin". They observed that infusion of isletin into diabetic dogs resulted in a sharp improvement in their health and that a few injections of this extract per day allowed the dogs to live longer. Later in 1921 they were joined by a biochemist, Bertram Collip, and continued their studies to extract and purify insulin for human use <sup>1,2</sup>.

### 1.1.2 Insulin and insulin signalling are evolutionarily conserved

Certain genes and pathways are evolutionarily conserved. This usually means that these genes are essential to the organism's survival. Insulin and insulin-like peptides, such as insulin-like growth factors (IGF), and their signalling pathways are among those that are highly conserved<sup>3,4</sup>. *Caenorhabditis elegans* have forty insulin-like genes and *Drosophila melanogaster* have seven. Humans and most mammals have eight insulin and insulin-like genes expressed that are closely related<sup>5-18</sup>. Almost all of these are involved in similar functions such as metabolism, energy balance, and growth<sup>10-13,19-32</sup>. For example, in *C. elegans*, mutation in *daf-28*, which encodes an insulin-like peptide, leads to dauer arrest and downregulation of DAF-2, an insulin receptor-like peptide, signalling<sup>33</sup>. In *Drosophila*, it has been shown that diet restriction increases lifespan and restoring the food intake to normal levels abolishes this effect<sup>11</sup>. However, partial ablation of median neurosecretory cells, which are responsible for production of three of the insulin-like peptides, protected against the decrease in lifespan when diet restriction was ended and food intake was returned to normal levels<sup>11</sup>. However, another study showed that ablating insulin producing cells that express four of the five genes that have significant similarity with mammalian insulin, *dilp1*, -2, -3, and -5, in flies leads to growth retardation and elevated circulating carbohydrate levels in hemolymph<sup>12</sup>. Collectively, these studies point to important conserved roles for insulin like peptides in longevity and energy homeostasis. However, to date, similar studies where insulin genes are manipulated in mammals have not been reported in the context of energy homeostasis and longevity.

### 1.1.3 Expression of insulin outside of pancreas

Insulin is best known for its role in glucose homeostasis. Indeed, the pancreatic islets of Langerhans are the sole site for the production of the insulin in the circulation that fulfills its endocrine function. Insulin expression is highly controlled at the transcriptional level in the 5' flanking region of its gene<sup>34</sup>. However, insulin production outside of the pancreas has also been reported<sup>35-115</sup>. Two major non-pancreatic sites of insulin production are the thymus and the central nervous system (CNS)<sup>35-115</sup>, the latter being more controversial and discussed later as a part of this research. However, insulin expression in thymus is well accepted and is thought to play a role in the pathogenesis of type 1 diabetes<sup>116</sup>. There are reports of other tissues, such as adipose tissue, spleen, bone marrow, and liver, producing minute amounts insulin, or proinsulin, under pathological circumstances<sup>108</sup>. Moreover, different isoforms of insulin can have different expression patterns within the same species. For example, unlike humans, mice have two insulin genes, *Ins1* and *Ins2*<sup>117</sup>. Most studies have shown that *Ins1* is restricted to pancreatic  $\beta$ -cells, where it contributes to approximately 1/3 of the expressed and secreted insulin<sup>117</sup>. The peptide product of the *Ins1* gene differs from that of the *Ins2* gene by two amino acids in the  $\beta$ -chain, at the B9 and B29 location, and is missing two amino acids in the connecting C-peptide<sup>118</sup>. *Ins1* also lacks an intron present in *Ins2*<sup>118</sup>. *Ins2* is the ancestral gene, with gene structure, parental imprinting, and a broad tissue distribution similar to human *INSULIN*<sup>47,117</sup>.

## 1.2 Insulin action

### 1.2.1 Insulin signalling

Insulin is an important regulator of cellular growth and survival, glucose homeostasis, energy metabolism, appetite and food seeking behavior and more<sup>23,24,119-127</sup>. Insulin receptors,

located on the plasma membrane, are activated upon binding with their cognate ligand. Upon binding of insulin, the insulin receptor's tyrosine kinase activity is initiated and leads to auto-phosphorylation at tyrosine residues. The resulting conformational change allows the activated insulin receptor to attract its downstream factors, insulin receptor substrate proteins (IRS1, IRS2, IRS3 and IRS4) and SH2-containing protein (Shc). IRS proteins, in turn, activate other proteins further downstream of insulin receptor<sup>23,128</sup>. There are two main pathways downstream of IRS proteins. In one pathway, phosphoinositide 3-kinase (PI3K), protein kinase B (PKB) are sequentially activated, resulting in the deactivation and exclusion of the forkhead family of transcription factors (FOXO) from the nucleus, thus changing gene transcription profiles among other effects<sup>24,129</sup>. In the other pathway, IRS proteins activate growth factor receptor-bound protein-2 (Grb-2), rat sarcoma (Ras), RAF proto-oncogene serine/threonine-protein kinase-1 (Raf-1), MAPK kinase (MEK), and ultimately lead to activation and translocation of extracellular regulated kinases (ERK1-2) into the nucleus where they can promote gene transcription<sup>23</sup>.

### **1.2.2 Autocrine insulin signalling**

Hormones are generally known for their ability to enter the bloodstream and reach other tissues. However, virtually all hormones are known to affect the same cell they are produced from as well as the surrounding cells<sup>130</sup>. Insulin is also believed to have such effects on insulin producing pancreatic  $\beta$ -cells<sup>23,27,131</sup>. In particular, insulin activates pro-survival pathways and inhibits pro-apoptotic pathways in insulin producing  $\beta$ -cells in face of cellular stress<sup>23,24,31,132</sup>. The endocrine pancreas continues to reshape and remodel itself throughout life in response to anti-apoptotic and pro-apoptotic signals<sup>133-135</sup>. Insulin can bind to its own receptor on the  $\beta$ -cells and affect  $\beta$ -cell growth and survival through the Raf/MEK/ERK pathway<sup>23,27,131</sup>. In fact, it has been

shown, by studies involving insulin receptor knockout mice, that insulin's local action on the  $\beta$ -cells is the key factor that allows for expansion of  $\beta$ -cell mass, particularly in response to peripheral insulin resistance<sup>27,136</sup>.

Insulin's autocrine actions are also important for pancreatic  $\beta$ -cell function<sup>23</sup>. Study of mice lacking insulin receptors specifically in  $\beta$ -cells ( $\beta$ IRKO) has shown that lack of insulin signalling in  $\beta$ -cells leads to reduced insulin secretion in response to glucose and progressive glucose homeostasis impairment<sup>27,131</sup>. These effects are thought to be in part due to the reduction in expression of key genes such as glucokinase and solute carrier family 2 member 2 (GLUT2)<sup>131</sup>. In  $\beta$ IRKO mice,  $\beta$ -cells also show reduced ability to increase their internal calcium ( $\text{Ca}^{2+}$ ) concentration that is required for insulin release<sup>131</sup>. Together, these studies show that insulin's autocrine actions are important for proper functioning of the pancreatic  $\beta$ -cells as well as their ability to respond to pathological conditions such as peripheral insulin resistance.

### **1.2.3 Insulin action in the periphery**

Insulin is a very important hormone with varied effects on many tissues. Insulin receptors are found throughout the body and one of their main functions is to promote uptake of glucose from the circulation into different cell types, such as the adipose tissue and muscle<sup>120</sup>. Once insulin binds to its receptor, the downstream signalling leads to translocation of glucose transporter proteins, mainly solute carrier family 2 member 4 (GLUT4), to the plasma membrane. This allows active transport of glucose from the bloodstream, across the plasma membrane and into the cytosol<sup>120,137-139</sup>. Although the adipose tissue is a target tissue for insulin stimulated glucose uptake, euglycemic and hyperglycemic insulin clamp studies have shown that the skeletal muscle is responsible for the majority of the insulin-dependent glucose uptake<sup>140-142</sup>. Glucose uptake from

the circulation is very important, not only because cells require glucose for their metabolism and survival, but also because hyperglycemia can have adverse health effects <sup>143-147</sup>. Although insulin is not the only factor, it is arguably the most important factor that allows different cell types to take the glucose from the circulation <sup>137-139,148-151</sup>.

In the white adipose tissue, where energy is stored as fat, insulin signalling allows for lipogenesis and prevents lipolysis <sup>152</sup>. This allows the excess energy to be safely stored as fat <sup>153</sup>. Fat is released from the adipocyte through the process of lipolysis <sup>152</sup>. One of the major pathways in lipolysis starts at the plasma membrane of the adipocyte. When adenylyl cyclase is activated, by such factors as catecholamine-activated  $\beta_{1,2}$  adrenergic receptors, and leads to increased cyclic-adenosine monophosphate (cAMP) levels within the adipocyte <sup>152,154</sup>. cAMP then activates protein kinase A (PKA) which in turn phosphorylates and activates lipolytic factors such as hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), monoglyceride lipase (MGL), and perilipin <sup>155-158</sup>. Insulin hinders lipolysis by activating phosphodiesterase-3B in an IRS1/PI3K/PKB manner, which hydrolyses cAMP into 5'AMP thus deactivating PKA <sup>159</sup>. Insulin induced activation of phosphodiesterase-3B has also been reported to stimulate GLUT4 translocation to the plasma membrane of adipocytes thus increasing glucose uptake and lipogenesis <sup>160,161</sup>.

In the liver, where energy can be safely stored in the form of glycogen, insulin works to inhibit glycogenolysis and gluconeogenesis <sup>162,163</sup>. Insulin increases the expression of genes, at the transcriptional level, involved in glycogen and lipid synthesis, such as glucokinase, phosphofructokinase, pyruvate kinase, acetyl-CoA carboxylase, and fatty-acid synthase <sup>164</sup>. In contrast, insulin decreases the expression of genes that are involved in gluconeogenesis, such as glucose-6-phosphatase, fructose-1,6-bisphosphatase, and phosphoenolpyruvate carboxykinase <sup>164</sup>.

Insulin also plays a role in phosphorylation and activation of enzymes that promote glycogen and lipid synthesis such as glycogen synthase and citrate lyase <sup>164</sup>. Thus, insulin works on multiple tissues to allow for clearance of glucose from the bloodstream and safe storage of energy in appropriate tissues <sup>52</sup>.

#### **1.2.4 Central actions of insulin**

The brain responds to many central and peripheral signals. There are specialized receptors on the blood brain barrier that actively transport only insulin, with the exception of minute amounts of IGF-1, from the circulation into the brain <sup>165,166</sup>. The amount of insulin crossing into the brain can change depending on the amount of circulating insulin <sup>167</sup>. However, there is a point at which these receptors can be saturated, which restricts the further increase of insulin crossing into the brain <sup>165,167,168</sup>. However, sites such as the median eminence are structured in such a way that the neuronal processes are in direct contact with the circulation <sup>169</sup>. The hypothalamus, one of the major sites of insulin action in the brain, is situated near this area <sup>169</sup>. Moreover, insulin receptors are expressed on many components of neurons, including neuronal processes and synapses <sup>165,170</sup>. Thus, increasing insulin in the circulation, beyond the saturation of the insulin transporters, could potentially affect the hypothalamus differently than the rest of the brain <sup>165,168</sup>.

Insulin that crosses from the circulation into the brain is not the only source of insulin within the brain. In some model systems, such as *C. elegans* and *Drosophila*, it is known that neurons are the cells responsible for the production of insulin-like peptides <sup>10,11,14,18,33,171,172</sup>. In mammalian systems, elegant studies have shown expression of insulin in neurons, especially those of hippocampus and olfactory bulb, *in vitro* <sup>44,48,60,173</sup>. In fact, in one study it was suggested that not only neurons are able to express one of the murine *Insulin* genes, but also that the lack of

expression of the murine *Ins1* gene, but not the *Ins2* gene, is because the *Ins1* gene is actively silenced<sup>173</sup>. However, one can argue that the *in vitro* expression of insulin can be an artifact of the cells having been removed from their natural setting and being cultured in an artificial environment. On the other hand, *in vivo*, it is hard to distinguish whether the insulin peptide in the brain has crossed into the central nervous system from the periphery or if it has been produced locally in the brain. In a recent study, a role for central insulin production was indirectly suggested by ablating neurons with ‘rat insulin promoter’, an *Ins2* specific promoter, activity<sup>174</sup>. It has long been debated whether insulin is produced locally in the CNS *in vivo*, but due to lack of proper controls (such as comparing mice lacking either the pancreas-specific *Ins1* or the multi-tissue-expressed *Ins2*), the debate continues<sup>38-115</sup>.

It is well accepted that within the brain, insulin plays key regulatory roles. Insulin can promote growth, survival, healthy function in neurons and memory<sup>175</sup>. There are strong links between neurodegenerative diseases, such as Alzheimer’s, and metabolic diseases such as obesity, insulin resistance, and diabetes. In fact, people with metabolic disorders have much higher risk for Alzheimer’s disease or other neurodegenerative pathologies<sup>176-214</sup>. Many trials have started, some with promising results so far, to test the effectiveness of treating dementia and memory loss by administration insulin nasally suggesting insulin has therapeutic potential for these disorders<sup>190,199,215-237</sup>.

Insulin also plays a key role in in the central nervous system in reward sensation, energy intake, storage and expenditure<sup>238-240</sup>. In the arcuate nucleus of hypothalamus (ARC) there are populations of neurons that produce orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) as well as anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART)<sup>241,242</sup>. In all of these neurons, insulin’s binding to its receptor leads

to phosphorylation of PKB and its translocation to the nucleus. Then PKB phosphorylates FOXO1 leading to its exclusion from the nucleus. FOXO1 inhibits expression of anorexigenic genes and promotes expression of orexigenic genes. PKB has the opposite effect to FOXO1 with respect to the expression of these genes. Thus, insulin works in these neurons by promoting satiety and decreasing food intake<sup>129,243-246</sup>. Leptin's action on these neurons with regards to gene expression is similar to that of insulin<sup>246,247</sup>. One puzzling fact about the effect of insulin on these neurons is that, unlike leptin, insulin decreases the firing rate of the anorexigenic neurons and increases the firing rate of the orexigenic neurons<sup>248,249</sup>. Potentially, a balance between them provides even a greater control on whole body energy control.

Insulin can also play a role in decreasing food intake by reducing the perceived reward from food intake. Insulin receptors are found on ventral tegmental area (VTA) neurons. The VTA is an important site for reward-seeking behavior that leads to increased food intake. Insulin can, through a retrograde endocannabinoid signalling, cause long-term depression (LTD) in excitatory neurons that synapse onto VTA, thus reducing food intake<sup>250,251</sup>.

Insulin in the brain is also important for whole body glucose sensing. Activation of insulin receptors in medial basal hypothalamus activates the ATP-sensitive potassium ( $K_{ATP}$ ) channels of steroidogenic factor-1 (SF-1) neurons in a PI3K, phosphatidylinositol (3,4,5)-trisphosphate (PIP3)-dependent manner<sup>252,253</sup>. This leads to inhibition of firing in these neurons. It then leads to a signal from the dorsovagal complex of the hindbrain, which is transmitted to the liver via the vagus nerve<sup>253</sup>. This then leads to inhibition of gluconeogenesis in the liver through an IL-6 and STAT3 dependent pathway<sup>248,249,252,254,255</sup>. Thus, insulin acts on the brain as a key signal for the preservation of energy balance and glucose homeostasis.

### **1.3 Metabolic syndrome**

Metabolic syndrome is a disorder of imbalance in energy storage and utilization. It is a collection of interrelated risk factors that promote the risk for obesity, heart disease, stroke, and type 2 diabetes <sup>256</sup>. For a person to be diagnosed with metabolic syndrome, they will have to meet three of the five following criteria: 1) abdominal obesity with waist circumference of more than 102 cm in men and 88 cm in women, 2) hypertriglyceridemia of equal or more than 150 mg/dL (1.69 mmol/L), 3) low high-density lipoprotein (HDL) cholesterol of less than 40 mg/dL (1.04 mmol/L) in men and 50 mg/dL (1.29 mmol/L) in women, 4) high blood pressure of equal to or more than 130/85 mm Hg, and 5) high fasting glucose of equal to or more than 110 mg/dL ( $\geq 6.1$  mmol/L) <sup>257</sup>. With abundance of high energy food and increasingly sedentary lifestyle, metabolic syndrome is increasing at an alarming rate causing a great load on health care and decreasing the quality of life for many <sup>256</sup>.

#### **1.3.1 Diabetes**

##### **1.3.1.1 Diabetes: A global epidemic**

It is well established that obesity is a strong risk factor for diabetes, especially type 2 diabetes <sup>258-261</sup>. The incidences of both major types of diabetes are also dramatically on the rise <sup>262,263</sup>. Moreover, what used to be considered mainly an adult and elderly disease, is now also a concern for children and adolescents <sup>264</sup>. In Canada, it is projected that the cost of dealing with diabetes and related complications will rise to nearly \$17 billion by 2020, putting a great burden on the Canadian economy and health care <sup>265</sup>. Diabetes mellitus is a complex disease and is divided into two main categories, type 1 and type 2. Both of these pathologies are mainly marked by

reduced functional  $\beta$ -cell mass; when  $\beta$ -cells can no longer efficiently produce, process, and release insulin efficiently, an inability to control glucose homeostasis is the result <sup>266</sup>.

### **1.3.1.2 Type 1 diabetes mellitus**

Type 1 diabetes is the less prevalent of the two main types of diabetes. Although the exact cause of it is unknown, it is known to be an autoimmune disorder <sup>116</sup>. Studies in non-obese diabetic mice have shown that, in contrast to the pancreas-specific *Ins1* gene, the *Ins2* gene expression in the thymus plays a protective role in a dose dependent manner in the pathology of type 1 diabetes mellitus <sup>36,267</sup>. The human *INSULIN* gene has also been reported to be expressed in the thymus and is associated with protection from type 1 diabetes <sup>268</sup>. Normally the thymus tolerizes the body's immune system towards insulin <sup>35,269</sup>, meaning that the immune system correctly recognizes insulin as a 'self' peptide and thus will not launch an immune reaction towards it. However, in some people, the immune system will incorrectly recognize insulin as foreign and will initiate an autoimmune response <sup>270</sup>. In this case, the immune cells attack insulin-producing  $\beta$ -cells and kill them. This destroys a majority of the body's insulin producing cells, leaving a person with type 1 diabetes almost completely insulin deficient and unable to control blood glucose homeostasis <sup>269,271</sup>. This is most commonly treated clinically by regular blood glucose level monitoring and insulin injections by the patient <sup>116,266,272</sup>, although other successful treatment avenues such as islet transplantation also exist <sup>273</sup>.

### **1.3.1.3 Type 2 diabetes mellitus**

Type 2 diabetes constitutes the majority of cases of diabetes mellitus <sup>262,263</sup>. Unlike type 1 diabetes, type 2 diabetes is generally characterized by resistance to actions of insulin and abnormal

and/or increased insulin secretion. Type 2 diabetic patients are initially not insulin-dependent<sup>119,120</sup>. In response to increasing insulin resistance,  $\beta$ -cells release more insulin in an effort to maintain glucose homeostasis and prevent hyperglycemia. This can lead to insulin depletion, release of immature insulin peptide, loss of insulin secretion pulsatility, increase in  $\beta$ -cell mass, and ultimately  $\beta$ -cell exhaustion and death<sup>27,136,274-281</sup>. Aside from lifestyle changes, such as diet and exercise, many patients are also treated with a variety of insulin sensitizing agents such as Metformin<sup>282</sup>. Though only in some cases, late stage type 2 diabetes with  $\beta$ -cell exhaustion and apoptosis, insulin therapy is required, some believe insulin therapy actually prevents  $\beta$ -cell exhaustion<sup>119,135,283-290</sup>.

## **1.4 Obesity**

### **1.4.1 Obesity: A poorly understood disease that is rapidly on the rise**

Obesity is a worldwide epidemic with increasing prevalence, and it is a major independent risk factor for heart disease, hypertension, stroke, type 2 diabetes, cancer, hepatic steatosis, and many other diseases<sup>291,292</sup>. The molecular causes of obesity remain largely unexplained, despite the identification of common gene variants that contribute modest risk<sup>293</sup>. Obesity is defined as having excess body fat and is frequently quantified using the body mass index (BMI); a BMI over 25 and below 30 is considered overweight and a BMI over 30 is considered obese<sup>294</sup>. The adipose tissue serves a very important role as the storage site for excess energy<sup>152,294-296</sup>. If the energy intake is greater than that of energy expenditure, the body must safely store these extra calories for when they are needed<sup>297</sup>. It is thought that this mechanism evolved to protect against starvation when food is scarce<sup>297-300</sup>. However, in many of today's societies, energy-dense foods are freely available and people's lifestyles are becoming increasingly sedentary. Thus, even a small

imbalance (a net positive balance) in energy intake and expenditure over time can lead to over-storage of energy as fat and ultimately to obesity<sup>294,300</sup>.

#### **1.4.2 Obesity and energy balance**

Energy intake is essential to the survival of any organism. When food is ingested it is transformed into a usable form for the body and used to fuel bodily functions and desired activity and excess energy is stored for later use<sup>130</sup>. Thus, carbohydrates, as well as amino acids, free fatty acids, and ketone bodies, are converted to adenosine triphosphate (ATP) through cellular respiration in the mitochondria within the cells<sup>130,301</sup>. The ATP produced can then be used to fuel all of our activities. Energy expenditure is measured by calorimetry and is generally looked at in two ways, direct and indirect calorimetry. Direct calorimetry refers to the amount of heat produced and indirect calorimetry is the amount of oxygen consumed<sup>301</sup>. Energy expenditure can be grouped in three main categories: 1) obligatory energy expenditure, 2) adaptive thermogenesis, and 3) physical activity<sup>130,301</sup>.

Obligatory energy expenditure is generally responsible for the majority of the total energy expenditure in a relatively sedentary adult<sup>130</sup>. Obligatory thermogenesis and basal metabolic rate (BMR) belong to this category. BMR defines the amount of energy required to perform the cellular and organ function of a resting organism at a thermo-neutral environment, which is approximately at 28°C for adult humans. BMR is also referred to as resting metabolic rate<sup>301,302</sup>. For mice, the thermo-neutral environment is approximately at 30°C<sup>303</sup>. Adaptive thermogenesis is dynamically controlled and can respond quickly to the environment and internal body state<sup>304-307</sup>. For example, exposure to cold can effectively and significantly increase adaptive thermogenesis, with a two to four fold increase in oxygen consumption<sup>304,305</sup>. The acute response to cold exposure can include

shivering<sup>306</sup>. However, with continued cold exposure the organism adapts and shivering is replaced by non-shivering thermogenesis<sup>304-306</sup>. Adaptive thermogenesis is the increased heat production from cellular respiration<sup>308,309</sup>. The heat produced is due to increase in ‘futile cycles’ or ‘mitochondrial uncoupling’ and the uncoupling protein 1 (UCP1) is a key factor in this process<sup>308,310</sup>. UCP1 is a protein on the inner mitochondrial membrane and allows for the leakage of protons from the mitochondrial matrix. Other variations of this protein, such as UCP2 and UCP3, also exist but some studies suggest that they do not contribute significantly to whole body adaptive thermogenesis and weight control in mammals<sup>311</sup>. Elimination of the proton gradient allows for heat production instead of ATP synthesis<sup>302</sup>. The main tissue responsible for adaptive thermogenesis is the brown adipose tissue (BAT), however, mitochondrial uncoupling does take place in other tissues such as skeletal muscle<sup>306</sup>.

Adaptive thermogenesis is highly regulated by the brain, specifically the sympathetic nervous system (SNS)<sup>312-316</sup>. Studies have shown that treatment with sympathomimetic agents, such as  $\beta$ -adrenergic receptor agonists increases adaptive thermogenesis in amounts comparable to that of cold exposure<sup>312,313,317</sup>. Conversely, administration of SNS blockers hindered the cold-induced increase in adaptive thermogenesis<sup>28</sup>. Similarly, knocking out the dopamine  $\beta$ -hydroxylase gene, responsible for expression adrenaline and noradrenaline, also prevented cold-induced thermogenesis<sup>317,318</sup>. This central control of adaptive thermogenesis could have very important implications in understanding the physiological basis of obesity. For example, hypothalamus is often studied for its role in control of food intake. However, studies have shown that hypothalamic lesions can also lead to obesity even if the food intake is restricted to equal that of control subjects<sup>319</sup>. It is believed that leptin, an adipocyte derived hormone, plays an important role in this process. Leptin is released in proportion to the amount of body fat. Thus, it serves as a

signal of the available energy stored in fat <sup>320</sup>. There also is a role played by the hypothalamic-pituitary-thyroid axis on adaptive thermogenesis. Changes in thyroid hormone levels parallel those in energy expenditure, which is partly mediated by changing leptin levels that mediate the expression of the hypothalamic thyrotropin-releasing hormone <sup>321-323</sup>. These studies show that there is a very important physiological link between obesity and adaptive thermogenesis.

Physical movement, such as exercise, is another way of expending energy. Active muscles can efficiently take up fuels from their surroundings and convert them to the ATP that is required by the muscle fiber to produce movement <sup>130,324</sup>. Exercise increases the daily skeletal muscle-induced energy expenditure from 15% in sedentary subjects up to 40% of the total daily energy expenditure in athletes, reducing energy surplus and its storage as fat <sup>325</sup>. However, some studies have suggested that, unlike normal weight subjects, patients with obesity and the elderly show a compensatory reduction in non-exercise activity thermogenesis <sup>326,327</sup>. Non-exercise activity thermogenesis is the energy we expend for anything other than sleeping, eating, and exercise <sup>328</sup>. By doing so, exercise did not significantly contribute to weight management in obese patients and the elderly <sup>326,327</sup>. In summary, the three main categories of energy expenditure, obligatory energy expenditure, adaptive thermogenesis, and physical activity, constitute how energy is expended. If the energy intake exceeds its expenditure it will lead to increased internal energy storage and increased weight gain.

### **1.4.3 Diet and obesity**

While heritability can have a strong effect on obesity <sup>297,299,300,329-332</sup>, the type of food, such as diets high in long-chain saturated fatty acids, that is consumed can greatly affect weight gain <sup>333-336</sup>. Food consumption is also needed to provide the body with the essential macromolecules,

such as amino acids, that are needed for protein synthesis. To some extent, the body regulates the intake of different nutrients differently. For example, if a low-protein diet is consumed, the organism will increase its food intake to ingest enough protein to sustain protein synthesis, which will lead to increase in total energy intake<sup>337,338</sup>. Similarly, if a high-fat diet is consumed, the body cannot readily and acutely change its pattern of fuel oxidation and more food will be ingested until appropriate amount of energy is consumed<sup>339</sup>. Moreover, fat has a lower metabolic efficiency than carbohydrates; thus, a larger amount of a high-fat diet is needed to be consumed, compared to a calorie-matched diet with higher carbohydrate ratio, to produce the same amount of usable energy for the body<sup>340</sup>. Thus, to achieve energy homeostasis without over-consumption, the appropriate amounts of different necessary nutrients are needed in a healthy diet. In addition to the physiological need for different nutrients, there is also an evolutionarily conserved hedonic aspect to food consumption<sup>341</sup>.

#### **1.4.4 Molecular changes in obesity**

##### **1.4.4.1 Obesity and adipose tissue**

Obesity is defined by excessive fat accumulation in the body<sup>294</sup>. Fat can accumulate in a variety of organs, but it is ideally stored in the white adipose tissue. While the adipose tissue is known for its role in energy storage, it is also an important endocrine organ<sup>342</sup>. Adipose tissue is responsible for releasing different metabolites, hormones and other factors, whose production and release can change in obesity<sup>342-344</sup>.

Increased release of non-esterified fatty acids from adipocytes is thought to negatively affect insulin sensitivity and overall health<sup>345,346</sup>. It has been shown that insulin sensitivity can be greatly decreased within hours by acutely increasing circulating fatty acids<sup>347</sup>. Conversely, if anti-

lipolytic agents are administered and fatty acids levels are decrease acutely, enhanced insulin sensitivity results<sup>348</sup>. Increased delivery of fatty acids to different tissues such as the liver, muscle, and fat can cause insulin resistance<sup>349,350</sup>. Increased circulating fatty acids can increase intracellular fatty acid metabolites, such as diacyl glycerol, that activate protein kinase C (PKC)<sup>351,352</sup>. This leads to decreased insulin sensitivity by activating various serine/threonine kinases that can block activation of IRS-1 and IRS-2<sup>352-354</sup>. Activation of PKC also activates factors such as JUN-1 terminal Kinase 1 (JNK1), inhibitor of nuclear factor kappa-B kinase subunit beta (IKK $\beta$ ), and p38 mitogen-activated protein kinase (MAPK) that can both directly inhibit insulin signalling by phosphorylating IRS proteins at their inhibitory sites or by inducing expression of suppressor of cytokine signalling 3 (SOCS3) that also has the same inhibitory effect on IRS proteins<sup>343,355,356</sup>. Moreover, increased circulating fatty acids can activate toll-like receptor 4 (TLR-4) and elicit similar effects through JNK1, IKK $\beta$ , and MAPK<sup>357-360</sup>. Another factor that can activate TLR-4 pathway is the presence of the reactive oxygen species, which also reduces insulin signalling<sup>357-360</sup>. An increase in intracellular fatty acid concentrations leads to their usage in mitochondrial uncoupling and  $\beta$ -oxidation, thus producing increasing amounts of reactive oxygen species and ultimately activating aforementioned insulin desensitizing factors<sup>361-363</sup>.

The adipose tissue also secretes hormones such as leptin and adiponectin<sup>364-369</sup>. Leptin is released in proportion to the body's fat stores, and is thought to control energy balance by affecting the central nervous system<sup>246,247</sup>. As shown in models of diet-induced obesity, chronically high levels of circulating leptin can lead to central and peripheral leptin resistance, decreased activation of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway<sup>370-376</sup>. Leptin resistance is associated with increased SOCS3 activity<sup>370-376</sup>. Studies have shown that SOCS3 activity is a key factor in leptin resistance; for example, in the case of the Tyr-985 mutation

in the leptin receptor which is a SOCS3 binding site, leads to increased leptin sensitivity resistance to diet-induced obesity<sup>370,373,374,377-379</sup>.

Adiponectin is released in amounts that are inversely proportional to fat stores, and can have insulin-sensitizing effects<sup>380-382</sup>. A recent study has suggested that reduced adiponectin levels in obesity are related to the amount of bone marrow adipose tissue, which is inversely proportional to whole-body adiposity. Thus, the authors suggested that marrow adipose tissue has a higher influence on circulating adiponectin levels leading to decreased circulating adiponectin in obesity<sup>383</sup>.

#### **1.4.4.2 Adipose tissue subtypes**

The two biggest subcategories of adipose tissue are the white and brown adipose tissue. These different types of fat tissue have very distinct histological features, as well as partially distinct developmental origins<sup>384</sup>. Brown adipocytes generally are multilocular (have multiple fat droplets) and have a very high number of mitochondria, whereas the white adipocytes are unilocular<sup>384</sup>. In fact, it is reported that brown adipocytes, like skeletal muscles, arise from central dermomyotomes, since they express the transcription factor *Myf5*. This potential shared origin with muscle may be related to the propensity of brown adipose tissue to have high numbers of mitochondria, which leads to high energy consumption; features that are not shared with the white adipocytes<sup>385-387</sup>. Although this notion has been recently challenged that neither all brown adipocytes come from a *Myf5*-positive background nor do all white adipocytes come from a *Myf5*-negative background<sup>388</sup>. Moreover, other gender- or depot-specific factors may play a role in directing the fate of the adipocytes<sup>388</sup>. Nonetheless, whereas white adipocytes are generally responsible for the storage of energy, the UCP1-expressing brown adipocytes can play a major

role in producing heat and therefore consume energy<sup>384</sup>. A high-fat diet can stimulate the brown adipocytes to increase their energy consumption and diet-induced thermogenesis, to preserve energy balance<sup>389</sup>.

White adipocytes are derived from mesenchymal stem cells<sup>390,391</sup>. Two genes whose lack of expression in white adipocyte, either visceral or subcutaneous, is reported to differentiate them from brown adipocytes in mice are *HoxC4* and *HoxA1*<sup>390</sup>. Similar pattern of gene expression is also seen in human white and brown adipocytes<sup>390</sup>. In mature adipocytes, expression of *Leptin* is a hallmark of white adipocytes<sup>390</sup>. The leptin-producing white adipocytes generally play a predominant role in storing energy for later use<sup>384</sup>. The distribution of the white adipose tissue (WAT) within the body is also of great importance. Central adipose depots, mainly abdominal, have been established to have more deleterious effects on insulin sensitivity than peripherally distributed adipose depots<sup>392</sup>. Men are reported to have a more central distribution of fat, also known as “apple shaped” and women are reported to have more peripheral, or “pear shaped” fat distribution<sup>393</sup>. Intra-abdominal adipose depots are larger, are more lipolytic, and are less responsive to the anti-lipolytic effects of insulin compared to subcutaneous adipose depots<sup>394</sup>. Moreover, in intra-abdominal obesity, fatty acids released from centrally located white adipocytes will be directly delivered to the liver via the portal vein thus having a greater pathophysiological impact on this organ<sup>394,395</sup>.

There is a third type of adipocyte that is known as beige, bright, or brown-in-white adipocytes. These cells are more prominent within the subcutaneous tissue than in other WAT depots. These cells share some features with brown adipocytes, including being rich in mitochondria rich and a multilocular structure<sup>396-398</sup>. These cells can also express genes, such as *UCPI*, that are normally expressed virtually exclusively in brown adipocytes<sup>396</sup>. The emergence

of bright adipocytes are induced via various factors such as cold, peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) or  $\beta$ -adrenergic agonists, and factors that lead to the increase of cyclic-guanosine monophosphates like PDE5 inhibitors<sup>396-399</sup>. The beige adipocytes can either transdifferentiate from white adipocytes or differentiate from progenitor cells<sup>400</sup>. Moreover, it has been suggested that the browning of white adipocytes is an important factor that can contribute to the regulation of whole body metabolism and can ameliorate high fat diet-induced obesity<sup>387,401-404</sup>.

#### **1.4.4.3 Obesity, adipose tissue, and inflammation**

Adipose tissue is also responsible for the release of other factors, such as retinol binding protein-4, plasminogen activator inhibitor-1, resistin, interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), that are increased in obesity and counter insulin's actions in its target tissues<sup>355,405-412</sup>. The last two factors, IL-6 and TNF $\alpha$ , are pro-inflammatory cytokines that also induce systemic inflammation by inducing their own expression and increased release from both adipocytes as well as adipose tissue macrophages<sup>408-412</sup>. The obesity-induced inflammation is milder and more prolonged than the inflammatory response to infectious disease<sup>408-412</sup>. Though adipocytes are capable of producing pro-inflammatory cytokines, the majority of the cytokines are produced by the adipose tissue macrophages, whose recruitment to the adipose tissue is increased in obesity<sup>413,414</sup>. Thus, in obesity there is an increase in inflammation of the adipose tissue and increased infiltration of immune cells, which may ultimately promote insulin resistance<sup>413</sup>.

#### 1.4.4.4 Obesity and liver function

The liver plays a key role in glucose homeostasis, and liver function is affected in obesity<sup>350,106,125,126</sup>. Insulin plays an important role in the liver to promote glycogen synthesis and inhibit glucose production by reducing the activity of glycogen synthase kinase-3 and therefore increasing the activity of glycogen synthase, as well as decreasing the activity of the transcription factor FOXO1, which promotes expression of gluconeogenic genes<sup>120,415,416</sup>. Increased inflammation can lead to hepatic insulin resistance and thus dysregulation of glycogen synthesis and glucose production in the liver<sup>120,413,415</sup>. Moreover, as a result of large amounts of fatty acids delivered to the liver in obesity, lipid accumulation as well as oxidation is increased in this organ<sup>350</sup>. The increased mitochondrial lipid oxidation in liver results in the production of reactive oxygen species<sup>361-363</sup>. Increased levels of cellular reactive oxygen species can activate pathways involving JNK1, IKK $\beta$ , and p38 MAPK<sup>357-360</sup>. These then stimulate expression of genes, such as SOCS-3, that ultimately lead to phosphorylation of serine/threonine inhibitory sites on the IRS-1 and IRS-2 proteins leading to insulin resistance in hepatocytes<sup>361-363</sup>. This insulin resistance then results in an increase in expression of genes such as sterol regulatory element binding protein 1, a transcription factor that works to direct fatty acids away from the mitochondria, promotes the synthesis and accumulation of lipids in the liver, and ultimately leads to hepatic steatosis<sup>417-420</sup>. Moreover, increased accumulation of reactive oxygen species can lead to decreased cellular antioxidant defenses in the hepatocytes<sup>421</sup>. When the cellular defenses fail to reduce the level of reactive oxygen species, mitochondria are among the first organelles to be damaged which leads to peroxisomal fatty acid oxidation, thus increasing the amount of reactive oxygen species and exacerbating the situation<sup>421-423</sup>. This can potentially increase the cellular stress enough to activate apoptotic sequences in the hepatocytes<sup>424</sup>. This can lead activation of hepatic Kupffer cells, which

are responsible for production of pro-inflammatory factors in liver <sup>425</sup>, such as chitotriosidase <sup>426,427</sup>. Chitotriosidase in turn stimulates the hepatic stellate cells to produce collagen leading to hepatic cirrhosis <sup>428</sup>. Adipokines also play a role in hepatic metabolism. For example, leptin is involved in expression of hepatic lipogenic genes in liver, promote oxidation of fatty acids and increase resistance to hepatic steatosis <sup>429-432</sup>. Thus, in the state of metabolic stress, such as obesity, leptin resistance, proper lipid metabolism in liver could potentially be hindered <sup>433,434</sup>. Adiponectin, another important adipokine, works to reduce lipid accumulation in the liver and its reduced levels in obesity can contribute to hepatic steatosis <sup>435</sup>.

#### **1.4.4.5 Obesity and insulin resistance in skeletal muscle**

Approximately 70-90% of insulin-dependent glucose is taken up by the skeletal muscles <sup>142,436</sup>. Glucose uptake is promoted by insulin through translocation of glucose transporter proteins 4 (GLUT4) to the plasma membrane in a PI3K/PKB-dependent manner <sup>436</sup>. Obesity-induced inflammation has been reported to be one of the major contributors to insulin resistance in muscle, which is one of the earliest defects detected in those who may go on to progress to type 2 diabetes <sup>349,436,437</sup>. Since muscle is one of the major tissues responsible for insulin stimulated glucose uptake, it is logical to think that insulin resistance in muscle would have deleterious effects on whole body physiology. Using muscle-specific insulin receptor mice, it was shown that muscle insulin resistance leads to increased adiposity and circulating fatty acids and triglycerides <sup>438</sup>. This is probably due to carbohydrates being shunted away from muscles and ultimately taken up by the adipose tissue. Although reduced glucose uptake is one of the most studied changes in skeletal muscle in the context of obesity, other unfavourable changes do take place. For example, leptin promotes fatty acid oxidation and reduced lipid accumulation in the skeletal muscle through an

AMPK-dependent pathway<sup>430,439-441</sup>. Thus, peripheral leptin resistance, as seen in obesity, can adversely affect metabolism and lipid accumulation in skeletal muscle<sup>373,439</sup>.

#### **1.4.4.6 Obesity and pancreas function**

The endocrine pancreas is another organ that can be affected by obesity. In general, the pancreatic islets are able to respond to increased insulin demand from obesity-induced insulin resistance<sup>442-444</sup>. Pancreatic islets can effectively respond and increase in size through  $\beta$ -cell hyperplasia and hypertrophy. Increases in both cell number and cell size have been reported in obese humans, but an increase in beta-cell number is most often reported in rodent models<sup>445-447</sup>. Uptake of glucose and its metabolism is key in inducing insulin secretion in  $\beta$ -cells. In obesity and insulin resistance, glucose metabolism is increased to allow for increased insulin release through increased activity of glucokinase, the rate-limiting enzyme in glucose metabolism<sup>448,449</sup>.

Fatty acids potentiate insulin release from  $\beta$ -cells by either binding to the G-protein coupled receptor 40 or leading to increased intracellular acyl-CoA concentration, both of which result in increased intracellular  $\text{Ca}^{2+}$  concentration<sup>284,450,451</sup>. However, chronic exposure of  $\beta$ -cells to high levels of fatty acids can lead to both impaired glucose-stimulated insulin release as well as reduced insulin biosynthesis, thus impairing the function of the endocrine pancreas<sup>452,453</sup>. For example, with increased demand for insulin and chronic high release of insulin from  $\beta$ -cells, the usual pulsatility seen in insulin release is lost and a continuous pattern of insulin release could potentially contribute to further exacerbate insulin resistance<sup>274</sup>. Moreover, chronic increase in insulin release will also disproportionately increase the  $\beta$ -cell levels of immature proinsulin and also its release which can also contribute to reduced insulin action<sup>454</sup>. Our laboratory has previously shown that exposing primary human pancreatic  $\beta$ -cells and MIN6 cells to high levels of the fatty acid palmitate

leads to decreased protein levels of carboxypeptidase E, a key enzyme in the insulin processing pathway<sup>121</sup>. Our laboratory showed that palmitate-induced decrease in carboxypeptidase E leads to a disproportionate increase in proinsulin to insulin ratio as well as endoplasmic reticulum stress and  $\beta$ -cell apoptosis<sup>121</sup>. Loss-of-function mutations in carboxypeptidase E have been shown to lead to obesity,  $\beta$ -cell malfunction and failure, and type 2 diabetes in mice<sup>121,455-458</sup>. Obesity is a strong risk factor islet health and function as it can lead to impaired  $\beta$ -cells function and ultimately failure. In fact, the majority, ~90%, of those with type 2 diabetes are also either overweight or obese<sup>259-261</sup>.

#### **1.4.4.7 Obesity and the central nervous system**

Central nervous system structures, such as the hypothalamus, play key roles in energy balance, are also affected in diet-induced obesity. High-fat feeding is associated with increased CNS levels of pro-inflammatory genes, such as TNF $\alpha$ , Il-6, JNK, IKK, and SOCS3, in the hypothalamus. These pro-inflammatory cytokines then act to inhibit the energy-balancing effects of insulin and leptin in the brain<sup>247,459-464</sup>. Moreover, the hypothalamus also plays a role in glucose homeostasis through its effect on the control of gluconeogenesis in the liver. High fat diets have been shown to reduce the accumulation of long chain fatty acid-CoAs in the hypothalamus that is required for control of hepatic glucose production<sup>465,466</sup>. This is believed to be due to ability of the high fat diet to reduce the hypothalamic expression of malonyl-CoA which leads to increase in hypothalamic carnitine palmitoyltransferase I and ultimately reduction in hypothalamic long chain fatty acid-CoAs<sup>465,466</sup>. Thus, multiple organs, peripheral or central, can be impaired due to high fat diet or obesity and exacerbate its associated pathophysiological state.

## 1.5 Hyperinsulinemia

### 1.5.1 Hyperinsulinemia can precede obesity or insulin resistance

Obesity research has benefited from animal models, where genetic and environmental factors can be manipulated in ways that are impossible with humans. The *ob/ob* and *db/db* mice are such examples. The underlying cause of their phenotype is lack of leptin or its receptor, respectively<sup>365,467</sup>. There are also reports of humans with mutations in their leptin gene, who are also obese and hyperinsulinemic and their symptoms are normalized with leptin treatment<sup>468-472</sup>. Both the *ob/ob* and the *db/db* mice also show notable hyperphagia, insulin resistance, and hyperinsulinemia<sup>467,473-475</sup>. Since obesity can promote insulin resistance, it is logical to think that these mice become hyperinsulinemic to compensate for the obesity-induced insulin resistance. However, hyperinsulinemia in these mouse models is observed prior to any deviation from expected normal weight or glycemia compared to littermate controls<sup>476</sup>. This existence of hyperinsulinemia prior to obesity, insulin resistance, or hyperglycemia has been documented in other animal models of obesity, such as Zucker fatty rats<sup>477</sup>. Similarly, results from numerous human studies have also suggested that an increased insulin level is correlated with risk of later development of obesity<sup>25,478-487</sup>. Moreover, some studies suggested that humans with class I allele VNTR in the *INSULIN* gene produce and release more insulin from the pancreatic islets and are also more susceptible to obesity<sup>488-490</sup>, though this observation remains controversial<sup>491</sup>. On the other hand, studies of invertebrates with reduced insulin or insulin signalling have reported leaner, smaller bodies, along with increased lifespan<sup>33,171</sup>. Similarly, studies in mammalian models, such as the Zucker fatty rats, have also shown that treatment with diazoxide results in decreased insulin secretion, reduced weight and improved glucose intolerance<sup>492-498</sup>. Similarly, treatment of obese patients with diazoxide, a compound that reduces insulin secretion, is associated with weight loss

in some small clinical trials <sup>499,500</sup>. Lustig and his group found similar results using Octreotide, a somatostatin agonist that binds the sst5 somatostatin receptor, found on  $\beta$ -cells, which inhibits insulin release <sup>501-503</sup>. Therefore, such observations have raised the question of whether hyperinsulinemia itself is a primary defect in obesity.

## 1.6 Thesis investigation

Insulin and insulin-like peptides are some of the most studied hormones across species and have pivotal physiological roles, such as glucose homeostasis, control of adipose tissue form and function, energy balance, and control of food intake <sup>35,119,120,238-240,269</sup>. In mice, two insulin genes exist, and studies have suggested that they are partially redundant and capable of compensating for the loss of the other <sup>504</sup>. Other studies, such as those comparing the effect of the expression of the *Ins1* versus *Ins2* in the thymus in the context of type 1 diabetes have shown that the two genes are not entirely redundant <sup>184,430</sup>. Studies in other model systems, such as *C. elegans* and *Drosophila melanogaster*, have pointed to a role for insulin-like peptides in promoting weight gain and reducing longevity <sup>171,505</sup>. However, the effects of *Ins* gene dosage, and ultimately insulin levels, on glucose homeostasis and obesity have not been tested in mammals in a normal physiological context.

Studies in mammals have pointed to cell type-specific roles for the insulin receptor in diet-induced obesity. For example, fat-specific insulin receptor knockout mice are protected from diet-induced obesity and have extended longevity <sup>29,506</sup>, while eliminating insulin receptors from the brain has the opposite effect <sup>507</sup>. The interpretation of studies involving insulin receptor ablation is confounded by compensation between tissues and disruption of insulin-like growth factor signalling involving hybrid receptors <sup>25,29</sup>. Moreover, knockout of the insulin receptor, by

definition, induces tissue-specific insulin resistance, which alone can promote hyperglycemia and insulin hypersecretion<sup>25,85</sup>. Studies of fat-specific insulin receptor knockout mice, though elegant, were unable to specifically determine the role of hyperinsulinemia on obesity in the absence of confounding insulin resistance. We aimed to study both the peripheral and central role of insulin by genetically manipulating insulin gene dosage and also importantly without causing any global or tissue specific insulin resistance. We took advantage of the pancreas-specific expression of the murine *Ins1* gene to demonstrate that diet-induced hypersecretion of pancreatic insulin promotes obesity and its associated complications.

## Chapter 2: Materials and methods

### 2.1 Experimental animals

*Ins1*<sup>-/-</sup> and *Ins2*<sup>-/-</sup> mice were previously generated and described by the group of Jacques Jami (INSERM) in 1997<sup>504</sup>. *Ins1* was disrupted and most of its sequence was replaced by a neo cassette. Most of the *Ins2* sequence was replaced by a LacZ/neo cassette as described<sup>504</sup>. For genotyping, tail samples were collected and the DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Details of the genotyping primers can be found in Table 2.1.

For the studies described herein, groups of mice were divided into two diet groups at weaning (3 weeks), one group was kept on the control diet (total calories = 3.81 kcal/g; 25.3% calories from fat, 19.8% calories from protein, 54.9% calories from carbohydrate; Catalog #5015 Lab Diets, Richmond, IN) and the other group was put on a high-fat diet (total calories = 5.56 kcal/g; 58.0% calories from fat, 16.4% calories from protein, 25.5% calories from carbohydrate; Catalog # D12330 Open Source Diets/Research Diets, New Brunswick, NJ). Diets are compared in more detail in Table 2.2. After 52 weeks of age, mice were scanned for whole body fat to lean mass ratio using NMR Spectroscopy at the 7T MRI Research Center at the University of British Columbia (Vancouver, BC).

### 2.2 Glucose tolerance, insulin tolerance, and hormone secretion

Body weight and fasting glucose (OneTouch glucometer, LifeScan Canada, Burnaby, BC) were examined weekly after a 4-hour fast. Mice were fasted at approximately 8 am (one hour after the start of light cycle). Glucose tolerance was examined after intraperitoneal injection with 11.1  $\mu$ L per gram of body weight of 18% glucose in 0.9% NaCl saline. Insulin tolerance tests were assessed after injecting 0.75 U of insulin (Lispro Humalog VL-7510 in 0.9% NaCl solution) per gram of body weight. Serum insulin levels were measured using ultrasensitive mouse insulin ELISA kit (80-INSMSU-E01; ALPCO Diagnostics,

Salem, NH) and leptin levels were measured using mouse leptin ELISA kit (90030) from CrystalChem Inc. (Downers Grove, IL). Blood samples were collected from tail vein.

### **2.3 Metabolic cage analysis**

At 8 and 24 weeks of age, mice (n = 3-5 per genotype) were individually housed in PhenoMaster metabolic cages (TSE Systems Inc., Chesterfield MO) for indirect calorimetry. The cages were also equipped with food, drink and body weight monitors and an infrared beam grid to monitor activity in the x, y and z axes. Cages were placed in an environmental chamber to maintain constant temperature (21°C), with room lighting cycles (12 hr light, 7 am - 7 pm). Animals remained in the cages for 76 hours. Data from the first 4 hours were not used in analysis. Results from each of the 3 days were averaged and presented as a prototypical day for each genotype.

### **2.4 Tissue collection and analysis**

Mice were euthanized and tissues were collected and some samples were frozen instantly in liquid nitrogen and transferred to -80°C freezer for storage, while other samples were fixed in 4% paraformaldehyde (PFA) for tissue sectioning. Some brain samples were, post-PFA fixation, also dipped in optimal cutting temperature compound (OCT) and were frozen in an isopropanol/dry ice bath. Tissues collected were as follows: pancreas, epididymal fat pads, soleus muscle, liver, brain, kidney, spleen, heart, thymus and tibia. Tibias were placed in 2% KOH for removal of non-bone tissue for physical measurements. Serial paraffin sections were made at 5 µm thickness and serial frozen sections were made at 14 µm thickness. Tissue sections were prepared at Child and Family Research Institute Histology Core Facility (Vancouver, BC). Pancreatic islet morphology and hormone expression were approximated using the insulin positive area from three tissue sections 200 µm apart stained with guinea pig anti-insulin and rabbit anti glucagon (Linco/Millipore).

Alexa Fluor 488 and 594 raised in goat were used as secondary antibodies of choice (Life Technologies, Abtenau, Austria). Primary antibodies were diluted 1:100 and secondary antibodies were diluted 1:400. Incubations with primary antibodies were done overnight at 4 °C and secondary antibody incubations were done for one hour at room temperature. Sections were mounted in Vectashield solution with DAPI (Reactolab SA, Switzerland) and imaged through a Zeiss 200M inverted microscope equipped with a 10x (1.45 numerical aperture) objective, individual filter cubes for each color, and a CoolSnap HQ2 Camera (Roper Scientific). Images were analyzed using Slidebook software (Intelligent Imaging Innovations) as previously described<sup>121</sup>.

For the analysis of cultured hippocampal neurons, pregnant mice or rats were euthanized 1 day before timed birth and their hippocampi collected, cultured and fixed using paraformaldehyde. Slides were stained with rabbit anti-C-peptide 2 which would be expected to recognize C-peptide 2 as well as proinsulin 2 (Millipore catalog # 4020-01). It was confirmed independently, using islets from our knockout mice as negative controls, that this antibody specifically recognizes the C-peptide of insulin 2 but not insulin 1 (Fig. 3.6). In some studies, we also employed an antibody that only recognizes mature insulin (Biodesign, mAB1)<sup>508</sup>, and a guinea pig antibody expected to recognize mature and immature forms of insulin (Sigma). In order to determine whether insulin was being produced by neurons, we identified the endoplasmic reticulum using a calnexin antibody (Sigma). Neurons were marked with a mouse anti-NeuN antibody or identified morphologically after transfection with Green Fluorescent Protein (GFP).

## 2.5 Gene expression analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, Biosciences). 100 µg of RNA was used and cDNA was synthesized using qScript cDNA synthesis kit (Quanta, Biosciences). cDNA was subjected to Taqman real-time PCR (StepOnePlus, Applied Biosystems). PerfeCta qPCR supermix (Quanta, Biosciences) was used to perform the PCR in StepOnePlus real-time PCR system (Applied Biosystems). Primers and probes are listed in Table 2.3. PCR conditions were 2 min at 50 °C, 10 min at 95 °C and followed by 40 cycles of 15 sec at 95 °C, 1 min at 60 °C. *Actb* was used as the internal control. Reverse log of the raw values were used for analysis. The mouse brain cDNA panel (MDRT101) and human brain cDNA panel (HBRT101) were purchased from Origene (Rockville, MD). The human *INSULIN* probe set (Hs00355773-m1) was from Applied Biosystems.

Analysis of gene expression patterns in WAT, liver, and skeletal muscle was performed using custom Taqman mini-arrays. Each 96-well PCR plate was divided in half with 45 genes of interest and 3 internal controls (18S,  $\beta$ -actin/*Actb*, hypoxanthine guanine phosphoribosyl transferase 1/*Hprt1*). Genotypes, from the same diet group, were then compared on the same plate, side-by-side. We chose genes involved in lipid metabolism, insulin signalling, as well as several adipokines, inflammatory factors, and other genes believed to play a role in the pathogenesis of obesity. These genes are listed in Table 4.1.

## 2.6 Statistical analysis

All results are expressed as means  $\pm$  SEM. For glucose tolerance, insulin tolerance and insulin secretion tests statistical analysis was performed on values obtained from measuring area under the curve (AUC). The AUC is the area between the x-axis and line formed by connecting

the y-values. For insulin tolerance studies area over the curve (AOC) was used. Since the insulin tolerance studies are presented as percentage change from the basal four-hour fasted values, the AOC is the area between the y-values and a line drawn at  $y = 100$ . Statistical analysis was performed using Prism 5 (Graphpad). Two tailed t-test or two way ANOVA or one-way ANOVA followed by Tukey's HSD was used where appropriate. For all experiments  $p < 0.05$  was considered to be significant.

## 2.7 Tables

**Table 2.1 Primers used for genotyping the presence or absence of *Ins1* or *Ins2* alleles.**

Gene	Sequence 5'-3'	Annealing Temperature °C	PCR Product (bp)
<i>Ins1</i>	CCAGATACTTGAATTATTCCTGGTGTTTTATCAC	60	273 <i>Ins1</i> & 550 Neo
&	GCT GCA CCA GCA TCT GCT CCC TCT ACC		
Neo	TTC TCG GCA GGA GCA AGG TGA GAT GAC		
<i>Ins2</i>	TGC TCA GCT ACT CCT GAC TG	54	193
	GTG CAG CAC TGA TCT ACA AT		
LacZ	ACG GCA CGC TGA TTG AAG CA	59	420
	CCA GCG ACC AGA TGA TCA CA		

**Table 2.2 Comparison of the control medium fat and the high fat diets.**

<b>Diet</b>	<b>5015 (CD)</b>	<b>D12330 (HFD)</b>
Protein	19.805 kCal%	16.4 kCal%
Carbohydrate	54.858 kCal%	25.5 kCal%
Fat	25.337 kCal%	58.0 kCal%
Total Calories	3.81 kCal/g	5.55 kCal/g
Type of Fat	Lard	Hydrogenated Coconut Oil

**Table 2.3 Quantitative PCR Taqman primers used to measure the levels of *Ins1* and *Ins2* gene expression.**

<b>Gene</b>	<b>Description</b>	<b>Sequence</b>
<i>Ins1</i>	Forward	GAA GTG GAG GAC CCA CAA GTG
	Reverse	ATC CAC AAT GCC ACG CTT CT
	Probe	/56-FAM/CCC GGG GCT TCC TCC CAG CT/3IABlk_FQ/
<i>Ins2</i>	Forward	GAA GTG GAG GAC CCA CAA GTG
	Reverse	GAT CTA CAA TGC CAC GCT TCT G
	Probe	/56-FAM/CCT GCT CCC GGG CCT CCA /3IABlk_FQ/
<i>Actb</i>	Forward	CAG TAA CAG TCC GCC TAG AA
	Reverse	CTA GCA CCA TGA AGA TCA AGA
	Probe	/56-FAM/ACA CAG AGT ACT TGC GCT CAG GA/3IABlk_FQ/

### Chapter 3: Expression patterns of murine *Ins1* and *Ins2*

Rodents have become a very useful research tool for understanding mammalian biology as well as the biology of human disease. For example, the study of diabetes and obesity has benefited vastly from the use of mice and rats. There are some differences in between humans and rodent models. For example, mice and rats differ from humans, with respect to insulin. Mice have two insulin genes, *Ins1* and *Ins2*, whereas humans and virtually all other mammals have only a single *INSULIN* gene<sup>117</sup>. As a first step to understanding the *in vivo* biology of *Ins1* and *Ins2* in the mouse, we assessed the expression pattern of both genes and their corresponding proteins in selected tissues. While it is clear that pancreatic  $\beta$ -cells are the only significant source of circulating hormonal insulin<sup>34,127</sup>, it is known that some cells outside the pancreas express smaller amounts of *Ins2/INS*. For example, *Ins2* production in the thymus is required for immune tolerance<sup>35,37</sup>. Over 80 reports have suggested that insulin can be produced in the brain (e.g.<sup>38,39,42,47,50,51,509</sup>). Our collaborator, Dr. Brad Hoffman, provided us with chromatin immunoprecipitation data with antibodies to methylated histones (H3K4me1, marking regions of active transcription at protein-coding promoters) followed by direct sequencing that pointed to the presence of active transcription around the *Ins2* gene, but not the *Ins1* gene, in cerebral cortex and cerebellum<sup>127</sup>. In contrast, both genes had activity marks in pancreatic islets<sup>127</sup>. Interestingly, the active chromatin regions near the *Ins2* locus in brain were distinct from those in islets<sup>127</sup>. In contrast, both genes had activity marks in pancreatic islets<sup>127</sup>.

The observations of central *Ins2* gene activity and expression prompted us to employ Taqman qPCR primers, validated in islets from *Ins2*<sup>-/-</sup> or *Ins1*<sup>-/-</sup> mice (Fig. 3.1), to further elucidate the expression pattern of insulin in the brain. We observed brain expression of *Ins2* mRNA, significantly above the background seen in *Ins2*<sup>-/-</sup> brain tissue (Fig. 3.2). *Ins1* mRNA was absent

according to these criteria. Using an array with cDNA from multiple mouse brain regions, we found *Ins2* expression was highest in the hippocampus and *Ins1* was virtually absent (Fig. 3.3). Quantitative analysis (Fig. 3.4) confirmed that *Ins2* expression in hippocampal neurons is much lower than in pancreatic islets, which must deliver insulin to the entire body. Taqman RT-qPCR analysis of human brain cDNA array confirmed *INS* expression in several regions, including hippocampus (Fig. 3.5). Public gene expression databases also show expression of *Ins2/INS*, albeit at relatively low levels, in the rodent and human central nervous system ([www.gate2biotech.com/genenote](http://www.gate2biotech.com/genenote)), including expression the hippocampal formation ([www.brain-map.org](http://www.brain-map.org)). Interestingly, the active regions near the *Ins2* locus in brain were distinct from those in islets. Collectively, these observations are consistent with many reports that *Ins2* promoters, but not *Ins1* promoters, have robust activity in the brain<sup>509,510</sup>.

Next, we studied the localization of insulin protein throughout the brain, using antibodies validated in *Ins2*<sup>-/-</sup> or *Ins1*<sup>-/-</sup> islets, as well as *Ins1*<sup>-/-</sup>:*Ins2*<sup>-/-</sup> neurons (Fig. 3.6). Specific insulin immunoreactivity and C-peptide immunoreactivity were observed in the hippocampus, anterior olfactory nucleus, cerebral cortex, cerebellar Purkinje neurons, and several other discrete nuclei (Fig. 3.7 A-D). Next, we took advantage of the fact that the *Ins2*<sup>-/-</sup> mice used in our studies possess a LacZ knock-in in their endogenous *Ins2* locus (Fig. 3.8). We found that the same adult neurons positive for  $\beta$ -Gal also stained robustly for insulin in *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup>( $\beta$ gal) mice (Fig. 3.7 C). To rule out the possibility that insulin immunoreactivity arose from insulin taken up into these neurons via endocytosis, we cultured hippocampal neurons in defined insulin-free media. Indeed, using an antibody that recognizes mouse C-peptide 2, but not mouse C-peptide 1 (Fig. 3.6 A), we identified robust endoplasmic reticulum staining in cultured hippocampal neurons (Fig. 3.7 D). These observations strongly suggest that insulin is actively produced in these neurons. The diffuse

staining pattern of insulin distribution, also observed with an antibody to fully processed mature insulin, suggests a constitutive release expected for a local trophic factor (rather than being concentrated and stored in granules for triggered release, as in pancreatic  $\beta$ -cells). Together, these data demonstrate that *Ins2* is produced in the brain by a subset of neurons. Previous reports of insulin produced in the brain were highly controversial because it was difficult to distinguish low levels of insulin from background signal in the absence of ideal negative controls (i.e. *Ins2*<sup>-/-</sup> and/or *Ins1*<sup>-/-</sup> mice) and positive controls (i.e. *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup>( $\beta$ gal) mice). Our data confirm that the expression of insulin in neurons is evolutionarily conserved; neurons were the original insulin-producing cells<sup>12</sup>. The differential expression of two murine insulin genes and the availability of *Ins1* or *Ins2* knockout mice presents a unique opportunity to specifically dissect the roles of circulating *Ins1* derived from the pancreas and *Ins2* found in the brain.

### **3.1 High fat feeding reduces *Ins2* expression in the brains of female mice**

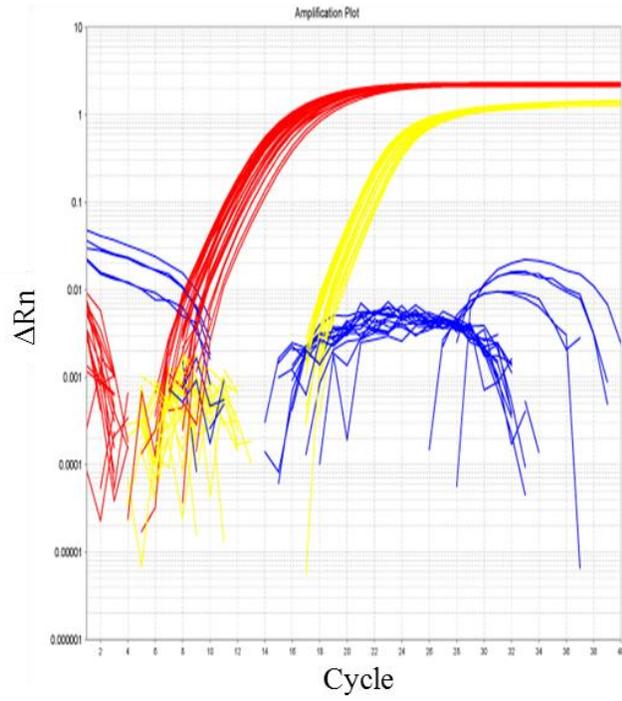
It is well established that insulin plays an important role in the brain with respect to balancing energy homeostasis, among other roles<sup>129,238-240,243-246</sup>. It is also widely accepted that diabetes and obesity are associated with insulin resistance in multiple tissues including the central nervous system<sup>168,191,199,225,511-525</sup>. We tested if environmental factors, specifically dietary differences, can affect insulin expression in the brain. To do this, we quantified the expression of *Ins2* in multiple brain regions of control and high fat fed wildtype male and female mice. In female mice high fat diet significantly reduced *Ins2* expression in hypothalamus compared to female mice on control diet (Fig. 3.9A). Though the difference was not significant, a similar trend was observed in the cortex of these female mice (Fig. 3.9B). Interestingly, high fat feeding did not alter central *Ins2* expression in the male mice we examined (Fig. 3.9C and D).

We also tested the effect of high fat diet in more brain regions in the *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice. Similar to the wildtype mice, *Ins2* expression was reduced in the hippocampus of high fat fed female mice (Fig. 3.10A). *Ins2* expression was also significantly reduced in the olfactory bulb of high fat fed female mice (Fig. 3.10A). High fat diet did not significantly alter *Ins2* expression in hypothalamus, cortex or cerebellum of the female mice (Fig. 3.10A). Also similar to the wildtype mice, high fat feeding did not alter *Ins2* expression in any of the tested brain regions in the male mice (Fig. 3.10B). Together these data suggest that *Ins2* expression is affected in certain regions of the brain in response to a high fat diet challenge.

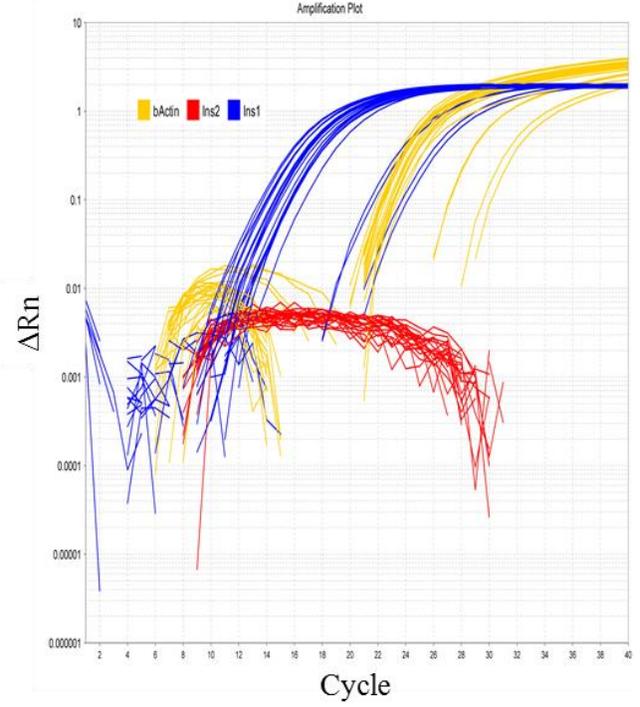
It has been reported in numerous studies that intake of high calorie and high fat diets as well as obesity are risk factors that contribute to central insulin resistance <sup>329,348,356,382,483-497</sup>. Moreover, these types of diets have been reported to be risk factors that promote neurodegenerative diseases <sup>176-178</sup>. Lastly, in early stages of Alzheimer's disease decrease in cerebrospinal fluid has been reported <sup>205,207,520</sup>. Our data help provide insight, with regards to changes in central insulin levels in response to high fat diet, that can potentially be related to increased risk of neurodegenerative disease such as Alzheimer's disease due to high fat diet intake <sup>526-529</sup>.

### 3.2 Figures

*Ins1*<sup>-/-</sup> islets

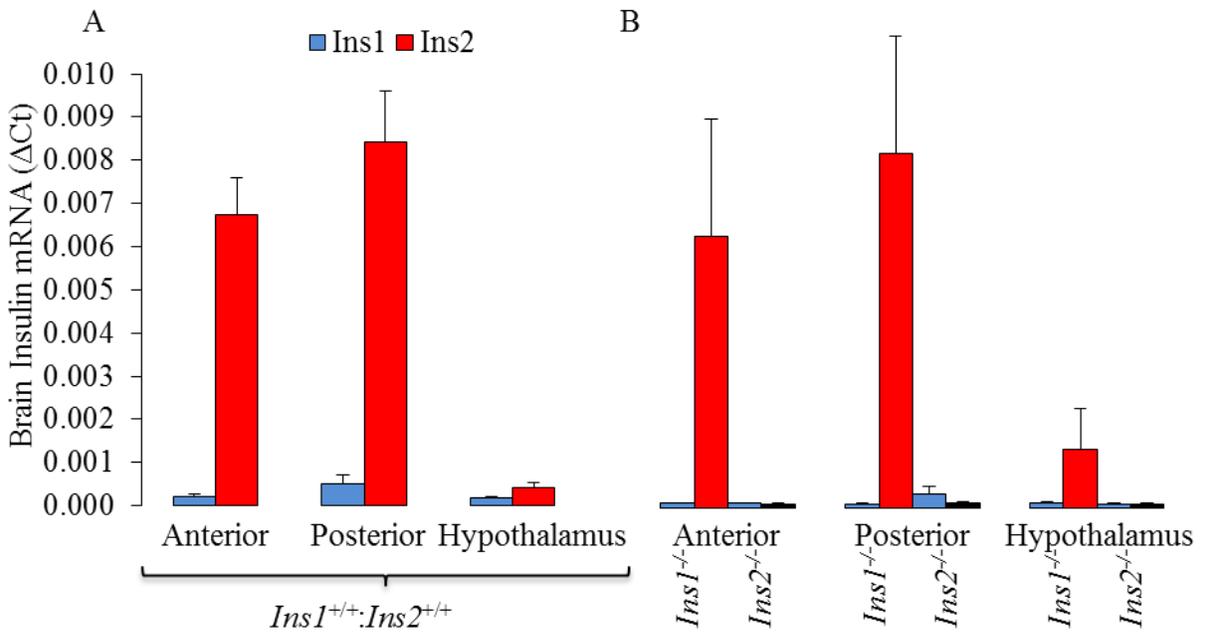


*Ins2*<sup>-/-</sup> islets



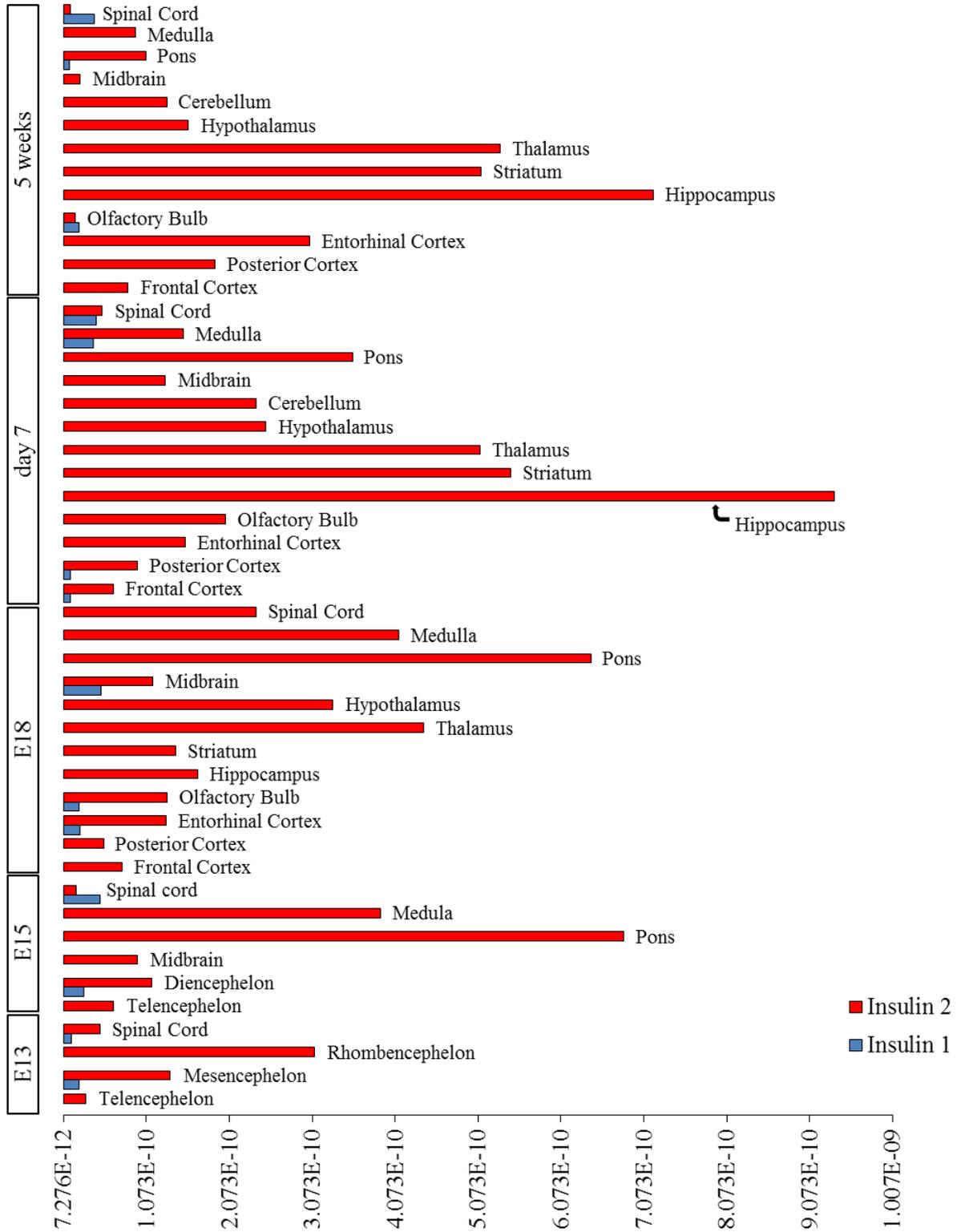
**Figure 3.1 Validation of Taqman real-time RT-qPCR primer/probe sets specific to each rodent *Insulin* gene.**

Raw Taqman real-time RT-qPCR data showing specific amplification *Ins1* (blue traces; absent in *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> mice) and *Ins2* (red traces; absent in *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> mice) in isolated islets. Beta-actin (yellow traces) is shown as a control.



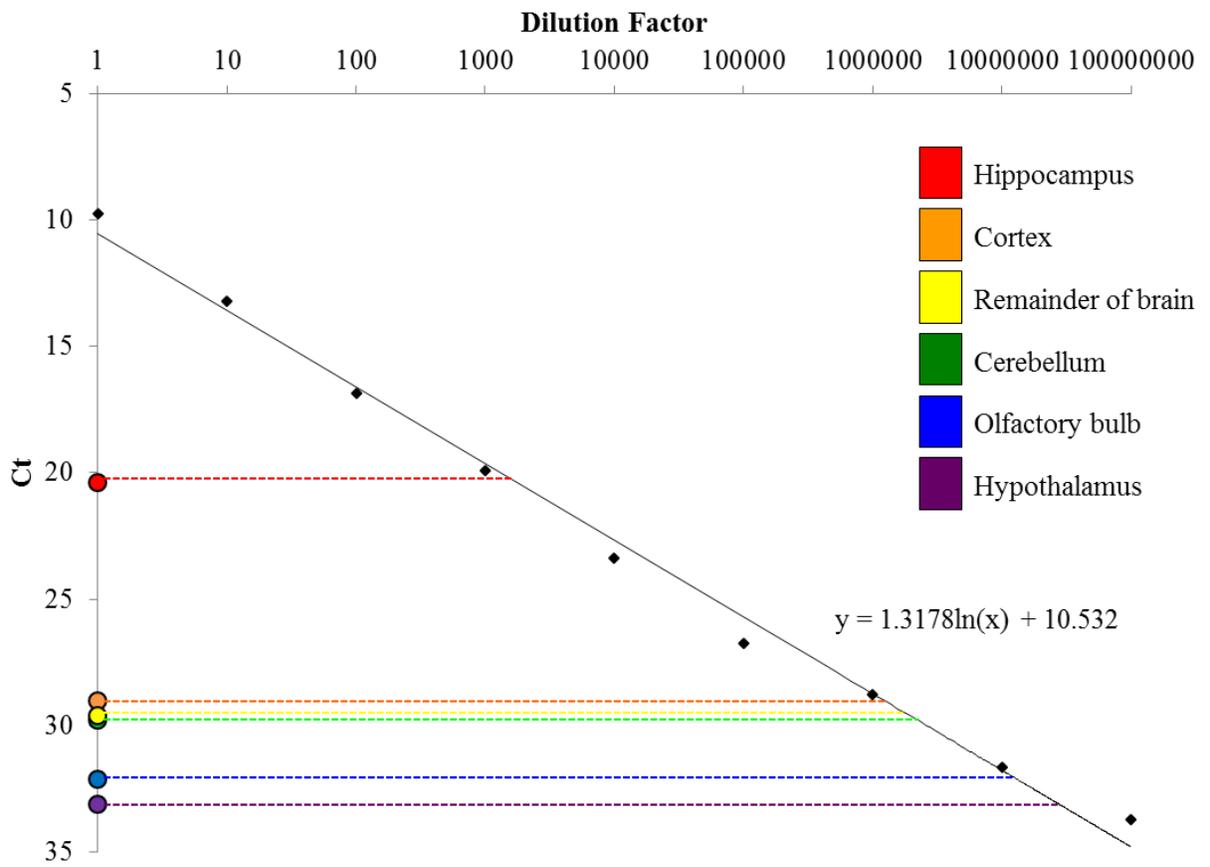
**Figure 3.2 Central nervous system expression of *Ins2*, but not *Ins1*.**

(A) Analysis of *Ins2* and *Ins1* mRNA expression (relative to the *Actb* control gene) in the hypothalamus, posterior brain and anterior brain of 6 month-old wildtype C57Bl6/J mice. (B) No significant compensatory up-regulation of either insulin gene in *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> or *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> mice (these mice also provide ideal negative controls for the RT-qPCR).



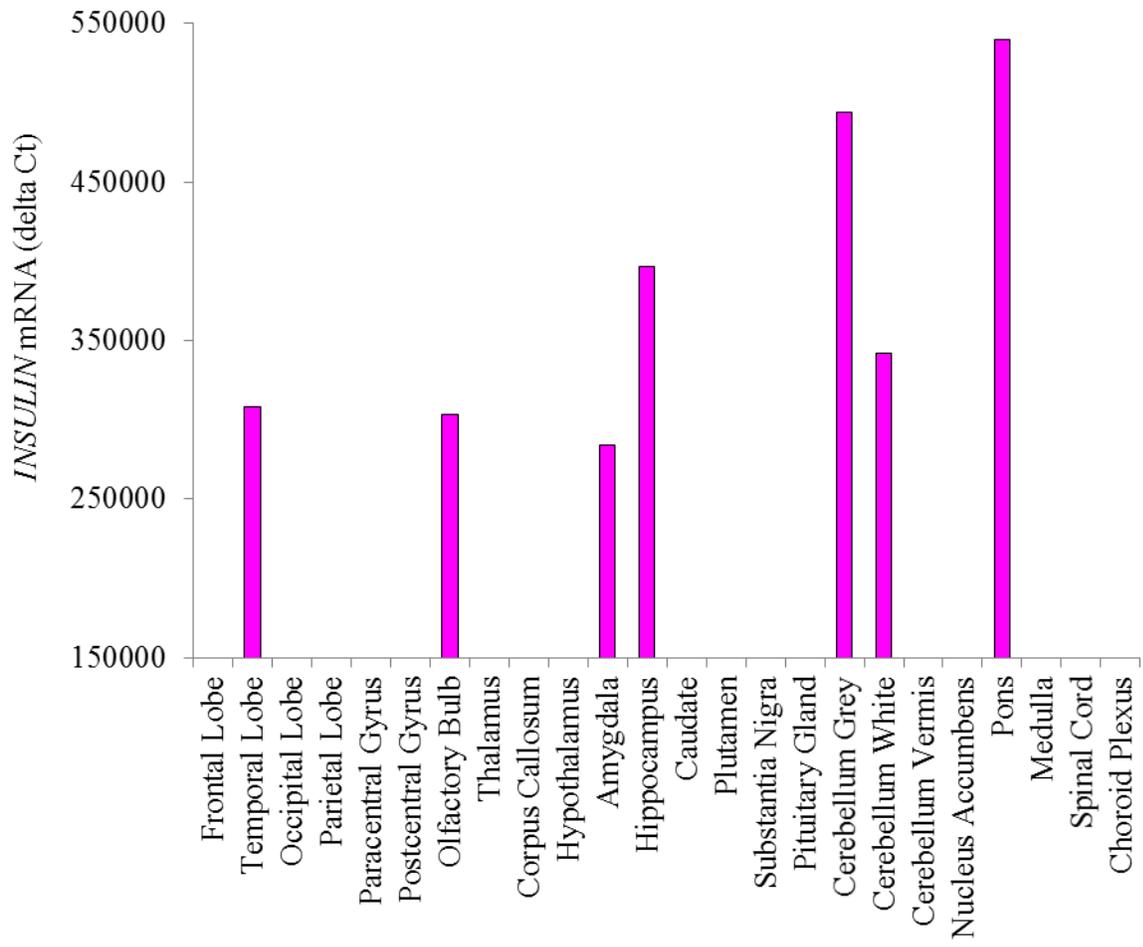
**Figure 3.3 *Ins1* and *Ins2* expression at different stages of development in mice.**

*Ins1* and *Ins2* gene expression assessed by Taqman real-time qPCR in arrayed cDNA from multiple murine brain regions at multiple stages of developing and post-natal brain.



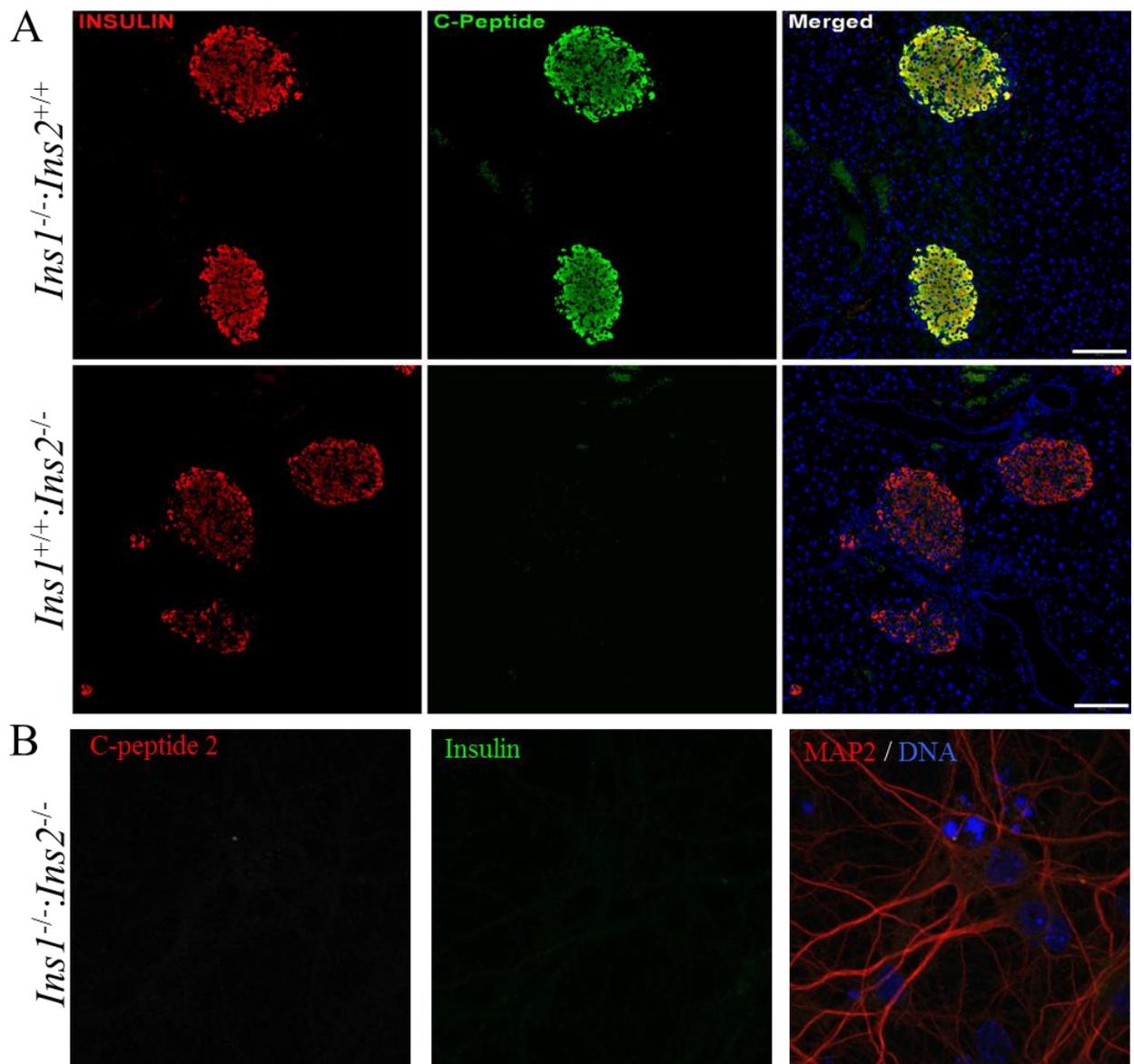
### **Figure 3.4 Comparison of *Ins2* mRNA levels in islets and brain.**

Comparison of *Ins2* mRNA levels in islets and brain regions using Taqman RT-qPCR (red, hippocampus; orange, cortex; yellow, remainder of brain; green, cerebellum; blue, olfactory bulb; purple, hypothalamus; in each case tissues were micro-dissected from a single male mouse brain). Data are presented as relative mRNA amounts per isolated tissue (i.e. not normalized for the amount of tissue/cells). *Ins2* mRNA was serially diluted from a sample of 236 hand-picked wildtype mouse islets to generate a standard curve. Thus, hippocampi from a single adult wildtype mouse has less than 1,000 times the amount of insulin mRNA when compared with 236 islets (with number of islets had roughly 8 times fewer cells than the hippocampi based on the nucleic acid recovered from the samples). Based on published estimations each islet would be composed of 1260  $\beta$ -cells<sup>530</sup> and each  $\beta$ -cell has ~50,000 copies of *Ins2* mRNA.



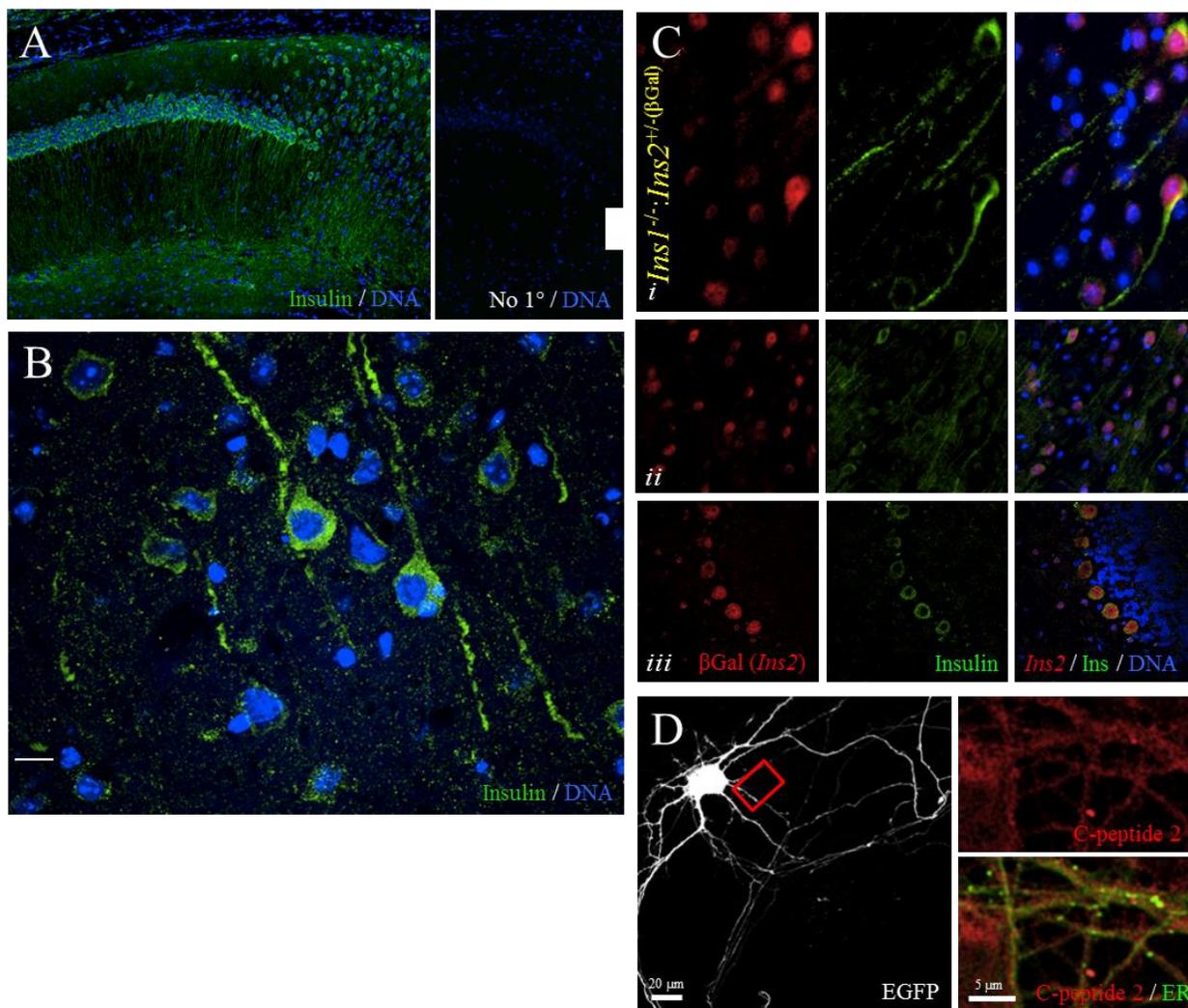
**Figure 3.5 Detection of *INSULIN* mRNA in brain.**

Taqman real-time RT-qPCR data showing amplification of human insulin from some brain regions (relative to the *Actb* control gene) in a commercial panel of brain cDNAs (OriGene, Rockville, MD, USA).



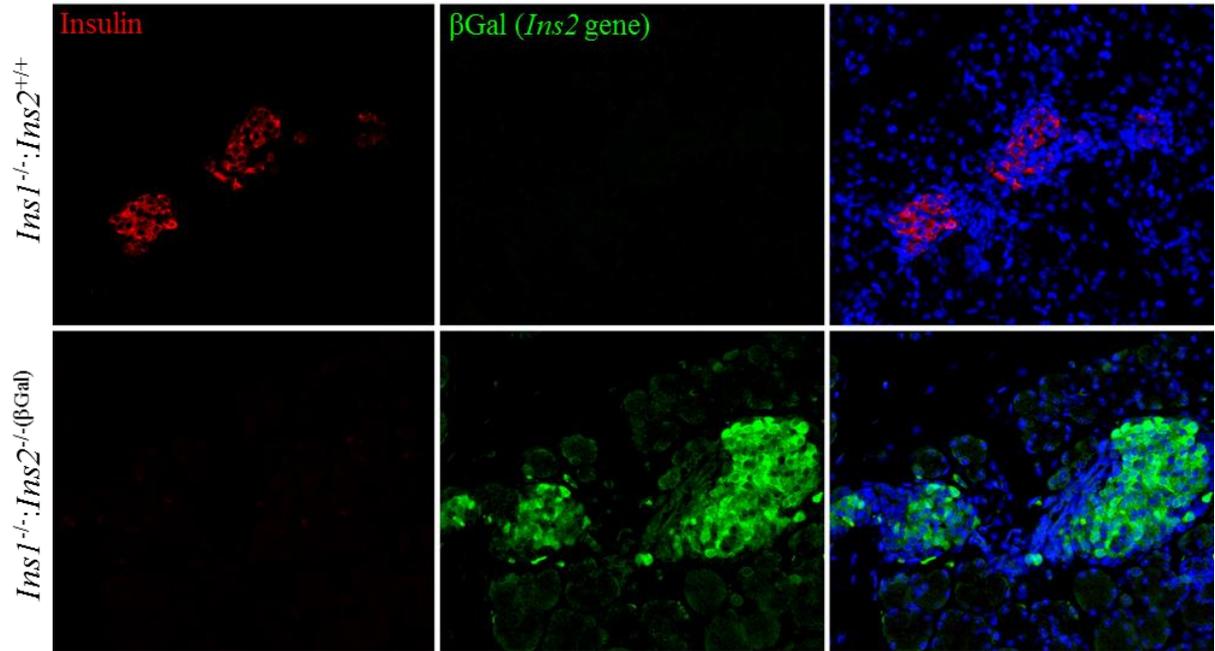
**Figure 3.6 Validation of specific *Ins1* and *Ins2* protein detection tools.**

(A) Millipore anti-C-peptide (# 4020-01) fails to stain *Ins2* knockout islets, demonstrating its specificity for the *Ins2* gene product. (B) Confocal imaging of cultured hippocampal neurons from *Ins1<sup>-/-</sup>:Ins2<sup>-/-</sup>* mice show no C-peptide 2 or (pan) Insulin immunoreactivity. MAP2 staining (far red) and DAPI (blue) are used to delineate the neurons.



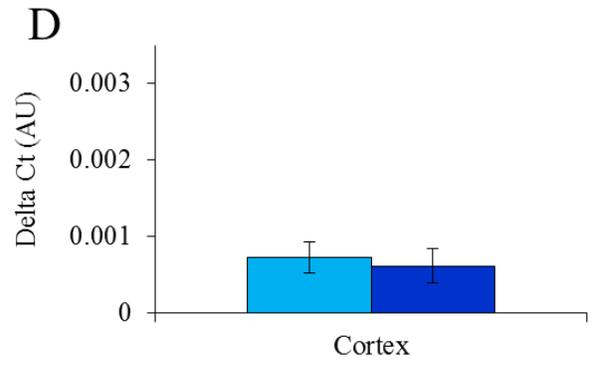
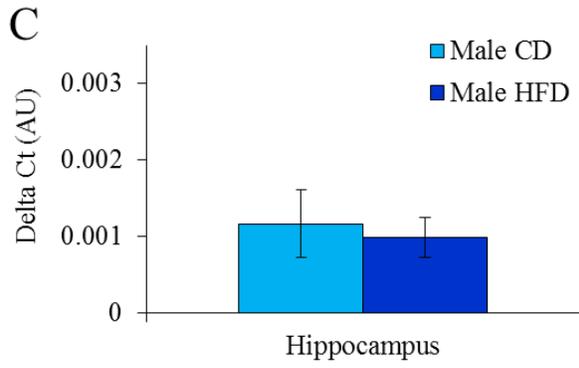
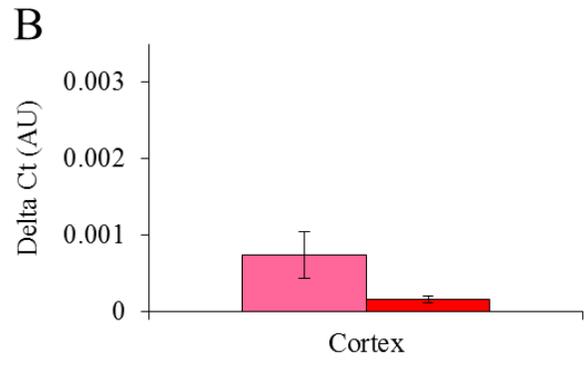
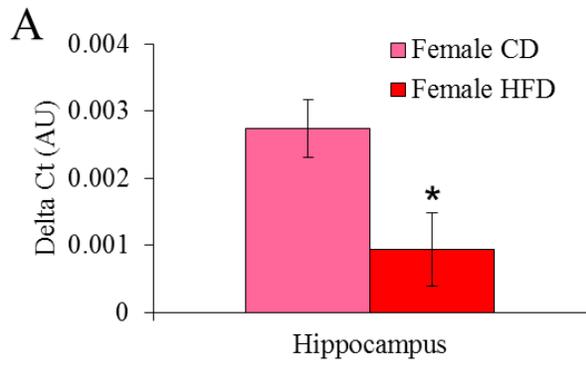
**Figure 3.7 Central nervous system expression of *Ins2*, but not *Ins1*.**

(A) Distribution of neurons expressing insulin protein in the hippocampus of 12 week-old mice. The inset shows a hippocampal image from the identical staining except that primary antibody was omitted. The antibodies were validated using *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> and *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> mice. (B) Close-up view of individual insulin neurons in the rostral cortex. (C) Colocalization of insulin (green-cell bodies and axons) and β-galactosidase (red-nuclear; knocked into the endogenous *Ins2* gene locus) in cortical neurons (i), anterior olfactory nucleus neurons (ii), and cerebellar Purkinje neurons (iii) from 12 week-old *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup>(βGal) mice. (D) Confocal imaging of a morphologically identified neuron showing punctate and reticular C-peptide 2 immunoreactivity (red) within calnexin-positive endoplasmic reticulum (ER; green) and in the somas of cultured rat hippocampal neurons (approximately half of the cells were positive). EGFP transfection was used to outline the boundaries and morphological health of individual neurons.



**Figure 3.8  $\beta$ -Gal expression in  $Ins2^{-/-}$  islets.**

Pancreatic islets from  $Ins1^{-/-}:Ins2^{+/+}$  mice express insulin but are negative for  $\beta$  Gal, while  $Ins1^{-/-}:Ins2^{-/-}$  mice are negative for insulin but shown immunoreactivity for  $\beta$ -Gal. In  $Ins2$  gene was replaced with the *LacZ* gene in our  $Ins2^{-/-}$  mice. In other words, the  $Ins1^{-/-}:Ins2^{-/-}$  mice are also  $Ins1^{-/-}:Ins2^{betaGal/betaGal}$  mice. The  $Ins1^{-/-}:Ins2^{-/-}$  mice were generated by breeding  $Ins1^{-/-}:Ins2^{+/+}$  mice together. The fully knockout mice were kept alive with daily (twice per day) insulin injections and these sections were collected at 4 weeks of age.



**Figure 3.9 High fat diet reduces *Ins2* expression in wildtype female mice.**

Levels of *Ins2* messenger RNA in one year-old wildtype female (A) Hippocampus, and (B) Cortex.

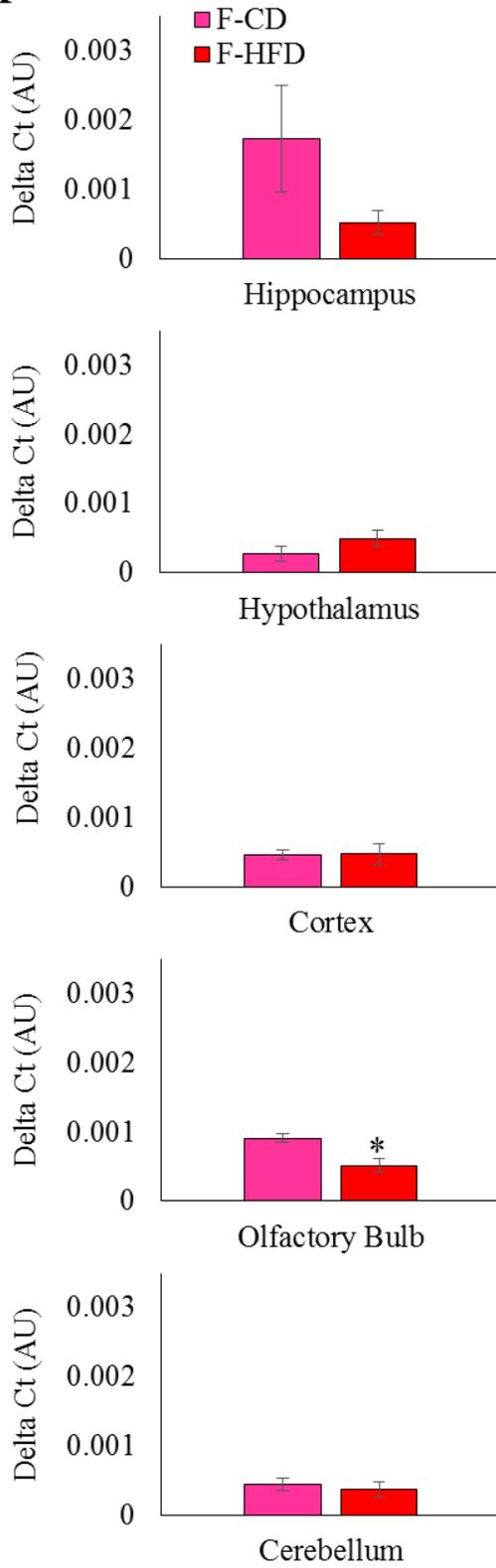
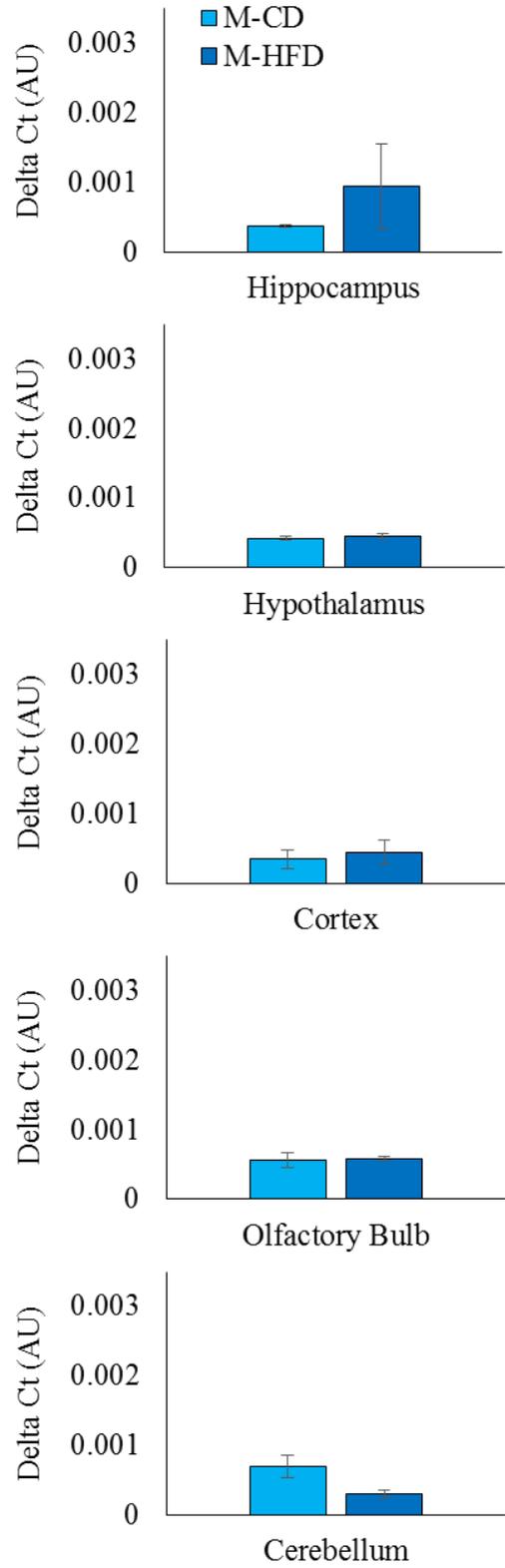
Levels of *Ins2* messenger RNA in one year old wildtype male (C) Hippocampus, and (D) Cortex.

Measured by Taqman real-time RT-qPCR assay. n = 7 for female mice and n = 8 for male mice at

1 year of age. \* denotes  $p < 0.05$ . The wildtype mice were derived from crossing the *Ins1*<sup>+/+</sup>:*Ins2*<sup>-</sup>

<sup>-</sup> mice and *Ins1*<sup>-</sup>:*Ins2*<sup>+/+</sup> mice and their progeny with the needed genotypes thereafter. CD refers

to our control diet with 25% fat and HFD refers to our high fat diet with 58% fat.

**A****B**

**Figure 3.10 High fat diet reduces *Ins2* expression in *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> female mice.**

Expression of *Ins2* messenger RNA in different brain regions of one year-old *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> (A) female, and (B) male mice. n = 8 or 9. Measured by Taqman real-time RT-qPCR assay. All samples were collected from 1 year-old mice. \* denotes  $p < 0.05$ . CD refers to our control diet with 25% fat and HFD refers to our high fat diet with 58% fat.

## Chapter 4: Systemic hyperinsulinemia is a causal factor in diet-induced obesity

### 4.1 Reduced *Ins1* prevents diet-induced $\beta$ -cell growth and fasting hyperinsulinemia

It is well accepted that hyperinsulinemia and obesity are strongly linked and many studies, both in humans and animal models, have reported hyperinsulinemia before any signs of obesity, insulin resistance, or glucose intolerance<sup>476,477,488,490,531</sup>. To test the hypothesis that peripheral, pancreas-derived insulin drives diet-induced obesity, we endeavored to selectively lower circulating insulin by generating mice null for the brain-expressed *Ins2* gene and modulating the gene dosage of pancreas-specific *Ins1* (Fig. 4.1). It was critical to first test whether removing one *Ins1* allele in *Ins2*<sup>-/-</sup> mice would result in a proportional reduction in *Ins1* mRNA and circulating insulin. Indeed, *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> mice exhibited a ~50% reduction in islet *Ins1* mRNA compared to *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> mice (Fig. 4.2A). Analysis of insulin content in islets at 8 weeks revealed some post-transcriptional compensation (Fig. 4.2B). By one year, insulin immunostaining (Fig. 4.2C), islet area (Fig. 4.3A) and fasting circulating insulin (Fig. 4.4), and glucose-stimulated insulin secretion (Fig. 4.5) were markedly decreased in mice with reduced *Ins1* gene dosage. Because *Ins2* is the primary contributor to islet insulin synthesis<sup>117</sup>, fasting insulin was near or below the limit of ELISA detection in young *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> and *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> mice, making detailed analysis of glucose-stimulated insulin secretion in these mice unfeasible.

Although the *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> control mice used in these experiments have been reported to be indistinguishable from wildtype on a control diet<sup>504,532</sup>, it was essential to determine if they would respond to a high fat diet with a similar hyperinsulinemia and  $\beta$ -cell mass increase seen in other control strains<sup>136</sup>. Relative to chow-fed controls, *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> mice fed a 58% fat diet since weaning developed age-dependent, high fat diet-induced fasting hyperinsulinemia (Fig. 4.4),

similar to what is observed in other strains of mice and in humans. Critically, persistent hyperinsulinemia was dependent on the full expression of both *Ins1* alleles.

The fasting hyperinsulinemia in high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice was associated with a ~2 fold increase in fractional  $\beta$ -cell area, compared with mice fed a control diet (Fig. 4.3A and E). However,  $\beta$ -cell area was equivalent to control diet levels in high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice, pointing to a critical role for basal circulating insulin in this process (Fig. 4.3A and E). Analysis of programmed cell death and proliferation in  $\beta$ -cells from *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice suggested that circulating insulin had gene dosage-dependent anti-apoptotic and pro-proliferative autocrine effects in the context of a high fat diet (Fig. 4.3B-D). These observations are consistent with studies showing that mice lacking insulin receptors on their  $\beta$ -cells fail to increase  $\beta$ -cell mass due to a defect in proliferation and increased apoptosis<sup>136</sup>. Thus, the long-term diet-induced fasting hyperinsulinemia was completely prevented in *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice (Fig. 4.4), which is associated with a complete lack of high fat diet-induced compensatory  $\beta$ -cell expansion (Fig. 4.3A and E). In other words, we generated mice that are genetically incapable of high fat diet-induced hyperinsulinemia.

## 4.2 Glucose homeostasis in mice with reduced insulin gene dosage

Insulin is an essential hormone for the maintenance of glucose homeostasis. Surprisingly, despite the observation that glucose-stimulated insulin release was reduced in *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice (Fig. 4.5), we observed only transient differences in fasting glucose between *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* and *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice (Fig. 4.6A). Significant worsening of glucose homeostasis in *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice was only observed in a small subset of high fat fed mice (2 mice) during the period of rapid somatic growth around 11 weeks of age and was not observed later in life or on the chow diet (Fig. 4.6B).

We did not observe any immune system activity near the pancreatic islets of the diabetic mice by immuno-staining for immune cell markers (data not shown). Insulin sensitivity was generally similar between the genotypes and diets (Fig. 4.6C). Therefore, comparing *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice and *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice enabled us to test the effects of genetically reduced pancreatic insulin secretion on obesity, in the absence of hyperglycemia or insulin resistance. To date, this is the only such model where the effect of a sustained reduction in circulating fasting insulin can be investigated in the absence of chronic differences in glucose homeostasis.

### **4.3 Reduced circulating pancreatic *Ins1* gene dosage prevents diet-induced obesity**

Insulin is a lipogenic hormone and its increased secretion has been associated with obesity<sup>353,354,365,367,393</sup>. We directly tested the hypothesis that pancreatic insulin hyper-secretion is required for diet-induced obesity by tracking body weight of *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice and *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice, fed a control diet or an obesity-inducing high fat diet (Fig. 4.1). We observed robust adult-onset obesity in high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* control mice with all the expected hallmarks, including increased fat-to-lean ratio measured by NMR, increased fat pad weight, increased size of individual adipocytes, as well as increased inflammation markers in white adipose tissue (Fig. 4.7). In striking contrast, *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice were completely protected from diet-induced adult-onset weight gain for the duration of these 1-year long experiments (Fig. 4.7A). The high fat diet-induced increase in epididymal fat pad weight was completely prevented (Fig. 4.7B), resulting in reduced circulating leptin (Fig. 4.7C). Whole body growth, measured by tibial length, was independent of *Ins1* gene dosage (Fig. 4.7D). Weights of other organs were not statistically different between any of the groups (Fig. 4.7E). Together with the results above, these data suggest that circulating *Ins1* is an adipocyte-specific and  $\beta$ -cell-specific growth factor.

Next, we sought to determine the physiological mechanisms accounting for the lack of high fat diet-induced adiposity (Fig. 4.7F and G) in the non-hyperinsulinemic *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice. Using indirect calorimetry, we found increased energy expenditure preceding the onset of differences in body weight (Fig. 4.8A). The observation of energy expenditure differences prior to differences in body mass was important because the normalization of energy expenditure between mice with different levels of adiposity is complicated<sup>533</sup>. Notably, we did not observe significant differences in food intake, physical activity, or respiratory quotient (Fig. 4.8B-E). Collectively, these *in vivo* data suggest that high fat diet-induced hyperinsulinemia promotes adult adipocyte growth and nutrient storage in the context of a high fat diet. The genetic loss of one *Ins1* allele, in *Ins2<sup>-/-</sup>* mice, was sufficient to promote reprogramming, as suggested by the resulting changes in expression of thermogenic genes, of white adipose tissue to increase energy expenditure and prevent adiposity.

#### **4.4 *Ins1* is a negative regulator of uncoupling, lipolytic, and inflammatory genes in white adipose tissue**

One of the factors that can play a role in weight gain is increased adaptive energy expenditure as discussed earlier. We expected changes in energy expenditure and fat cell growth over this long time scale to be associated with stable alterations in gene expression patterns and cellular reprogramming, rather than acute signalling events. Indeed, while hyperinsulinemic *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice exhibited marked adipocyte hypertrophy, white adipose tissue from high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice appeared similar to mice fed a control diet (Fig. 4.7H). To determine the molecular mechanisms underlying the protective effects of reduced fasting insulin, we designed a Taqman real-time PCR mini-array of 45 key metabolic, inflammatory, and insulin target genes (Table 4.1). In white adipose tissue, there were remarkably few differences between the two groups

of mice on the low fat diet, although insulin receptor mRNA was robustly lowered in mice with reduced *Ins1* (Fig. 4.9). In keeping with the lean phenotype and increase in energy expenditure, we observed a gene expression pattern associated with energy expenditure and lipid mobilization, including increased expression of uncoupling proteins and *Pnpla2* (adipose triglyceride lipase), in white adipose tissue from high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice versus high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice (Fig. 4.9). Perhaps the most notable observation was an increase in *Ucp1* gene expression in white adipose tissue isolated from high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice, suggesting brown adipose tissue-like features similar to what has been observed in other lean models, including mice lacking *Nrip1* (RIP140), a corepressor that suppresses *Ucp1* expression in white adipose tissue<sup>534,535</sup>. *Pparg1a* (*Pgc1α*) and *Pparγ*, positive regulators of energy expenditure and *Ucp1*, were also up-regulated in white adipose tissue from high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice. Furthermore, we observed down-regulation of factors previously shown to promote obesity and adipocyte differentiation in the white adipose of high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice, including the insulin target genes, *Egr2* (Krox20) and *Nrip1* (Fig. 4.9). Interestingly, none of the uncoupling proteins were differentially expressed between brown adipose tissue of high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice and high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice (Fig. 4.10). However, we did not measure the weight for BAT and thus cannot be sure if there were any differences in the size of BAT depots that could contribute to overall energy expenditure in these mice. We observed a tendency for a gene expression profile promoting energy expenditure in skeletal muscle from high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice, although these effects were not statistically significant (Fig. 4.11).

In collaboration with Ms. Templeman, we selected several differentially expressed genes with known roles in insulin signalling and/or adipocyte differentiation for additional analysis at the protein level. Immunocytochemistry suggested enhanced *Ucp1* protein in white adipose tissue

from high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice compared with high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* littermates <sup>127</sup>. A significant increase in Ucp1 levels in high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice was confirmed and quantified by immunoblot of white adipose tissue <sup>127</sup>. We also observed elevated protein levels of Pparg and Srebf1/SREBP-1c <sup>127</sup>. Immunoblot also confirmed a significant decrease in Egr2 protein in high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice <sup>127</sup>, mirroring the effects on its mRNA and suggesting a possible de-differentiation of white adipocytes. These quantitative data clearly demonstrate that the pancreas-specific *Ins1* gene, and by extension the circulating insulin hormone, controls the expression of a large number of key metabolic genes in white adipose tissue, including those responsible for energy expenditure.

It is well established that obesity is associated with chronic low-grade inflammation of multiple tissues, including white fat <sup>408-412</sup>. However, the cause and effect relationship between this phenomenon and hyperinsulinemia has not been fully established. Our Taqman RT-qPCR analysis revealed a broad reduction in inflammatory markers, including *Tnfα* and *Emr1* (F4/80; macrophages) in white adipose tissue from high fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice compared with high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* littermates. These data suggest that insulin regulates adipose inflammation either directly, or indirectly via fat cell size.

#### **4.5 Reduced pancreatic *Ins1* protects mice from lipid spillover and fatty liver**

*Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice were protected from elevated circulating free fatty acids when compared to their high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* littermate controls (Fig. 4.12A). In accordance with the lack of lipid spillover, livers of high-fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice were completely protected from diet-induced hepatic steatosis (Fig. 4.12B). Quantitative PCR analysis of liver showed that most of the differences were related to diet (reduced *Ddit3*, *Pparg*, *Ptpn1*, *Il6*, *Igfbp1*, *Egr1*, *Egr2*; increased

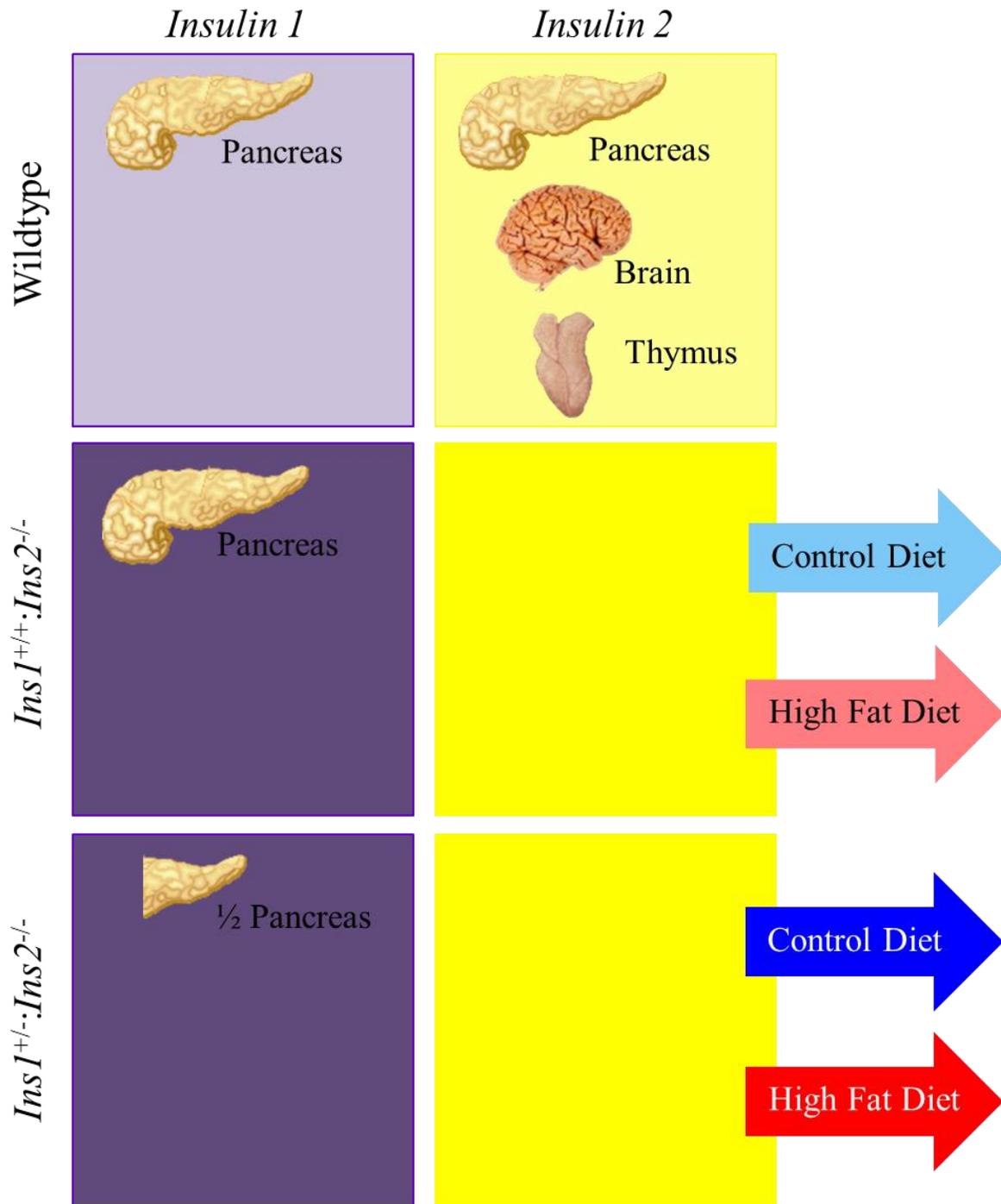
*Glut4*, *Srebf1*, *Ppargc1a*, *Pck1*), rather than between the two genotypes on the high fat diet (Fig. 4.12C). Some notable differences in inflammation markers prompted us to examine the gene expression of IL1 $\beta$ , TLR4, IL4 and interferon $\gamma$  in bulk liver tissue (Fig. 4.12C). The reduction in the stress marker Atf3 (Fig. 4.12C) prompted us to evaluate markers of oxidative stress and lipid peroxidation in this tissue, but the levels of genes coding for proteins carbonyl and 4-hydroxy-2-nonenal (HNE) were not different between high fat-fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice and their high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* littermates (Fig. 4.12C). Our data support a paradigm whereby high fat consumption leads to chronic basal insulin hyper-secretion (perhaps via direct insulinotropic effects of fatty acids), which then increases adipocyte size and lipid accumulation in adipose tissue. The spillover of free fatty acids subsequently leads to steatosis and ER-stress in the liver, which may eventually result in insulin resistance when combined with additional factors.

#### **4.6 Circulating insulin levels in female mice *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice**

This experiment was also conducted on female control *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice and experimental *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice. In contrast to male mice, reduction in *Ins1* gene dosage did not lead to a reduction in circulating insulin (Fig. 4.13A). There was no difference between the two genotypes in the female mice with respect to body weight (Fig. 4.13B). The only slight difference was between the different diets irrespective of their genotype. Mice on high fat diet had slightly higher circulating insulin and were also slightly heavier, but none of these differences were statistically significant in this study (Fig. 4.13B). Intraperitoneal glucose challenge showed that female mice in all four groups were able to equally control their blood glucose. However, at 12 and 26 weeks of age, female mice on high fat diet showed reduced responses to an intraperitoneal insulin challenge (Fig. 4.13C). This, however, was absent at either six or 52 weeks of age (Fig.

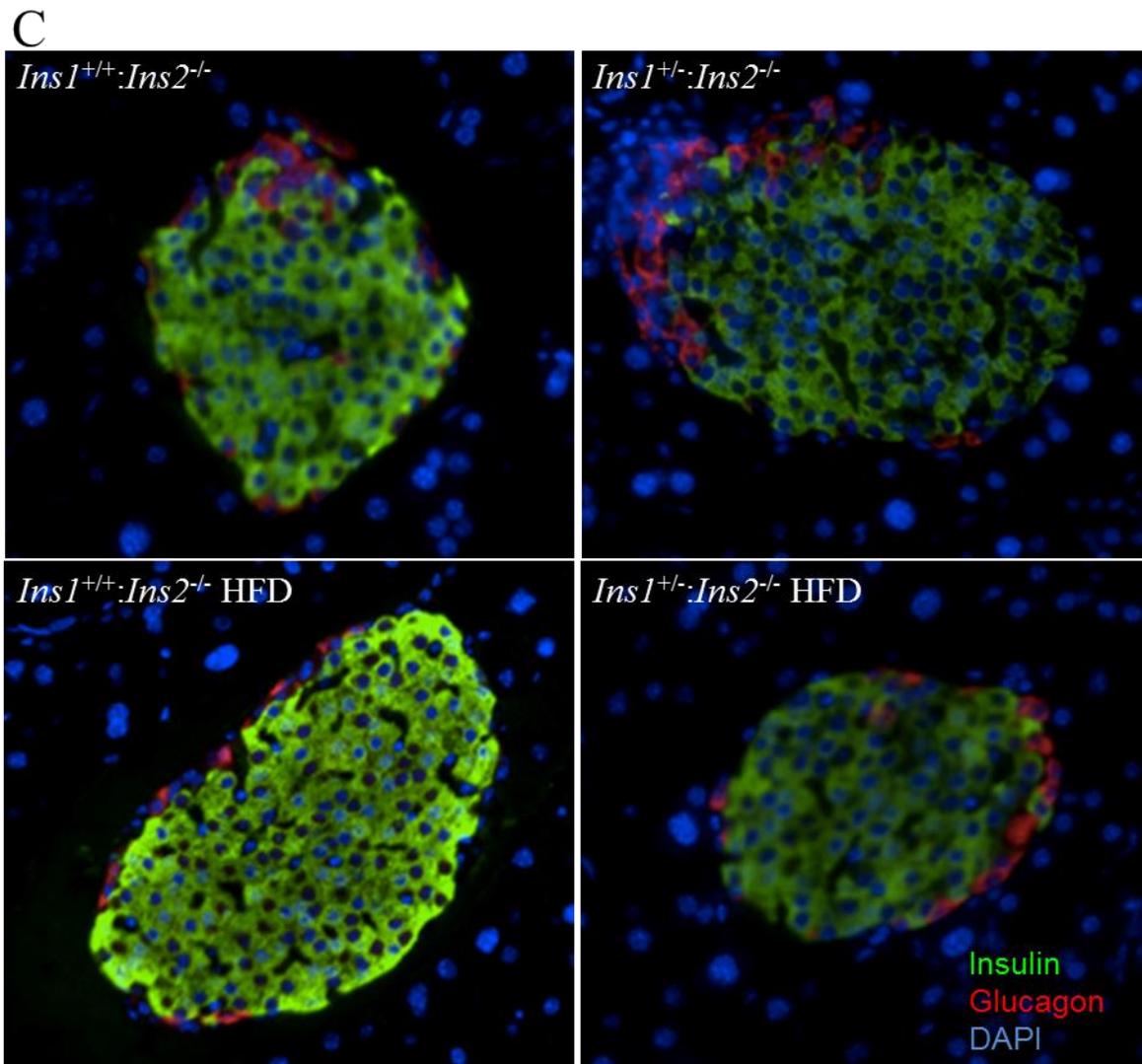
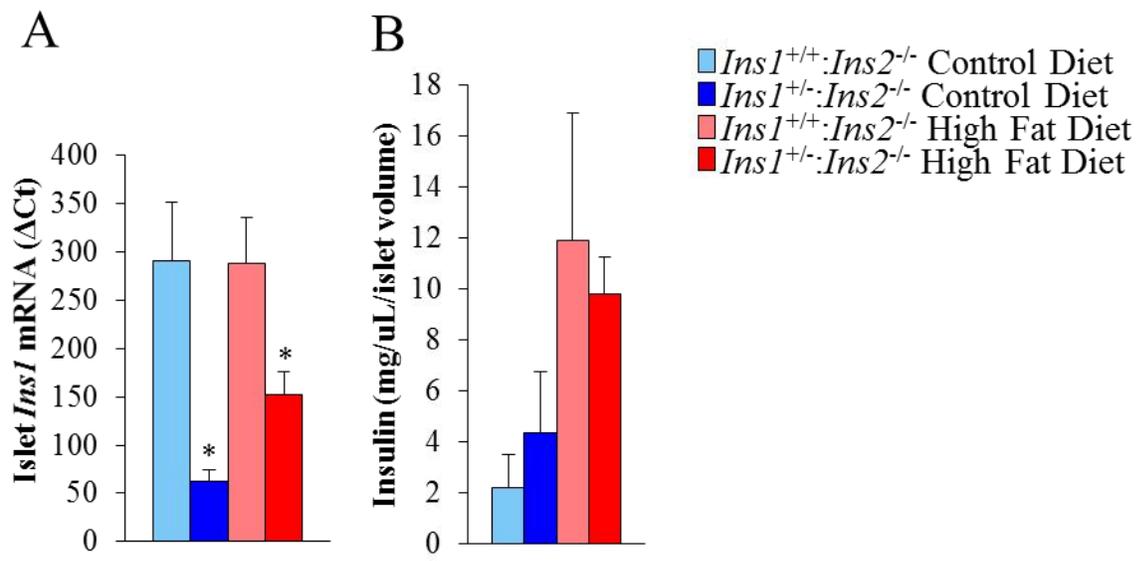
4.13D). These data are still consistent with our hypothesis that diet-induced hyperinsulinemia is upstream of obesity.

4.7 Figures



**Figure 4.1 Experimental design for mice with varying *Ins1* gene dosage on an *Ins2* null background.**

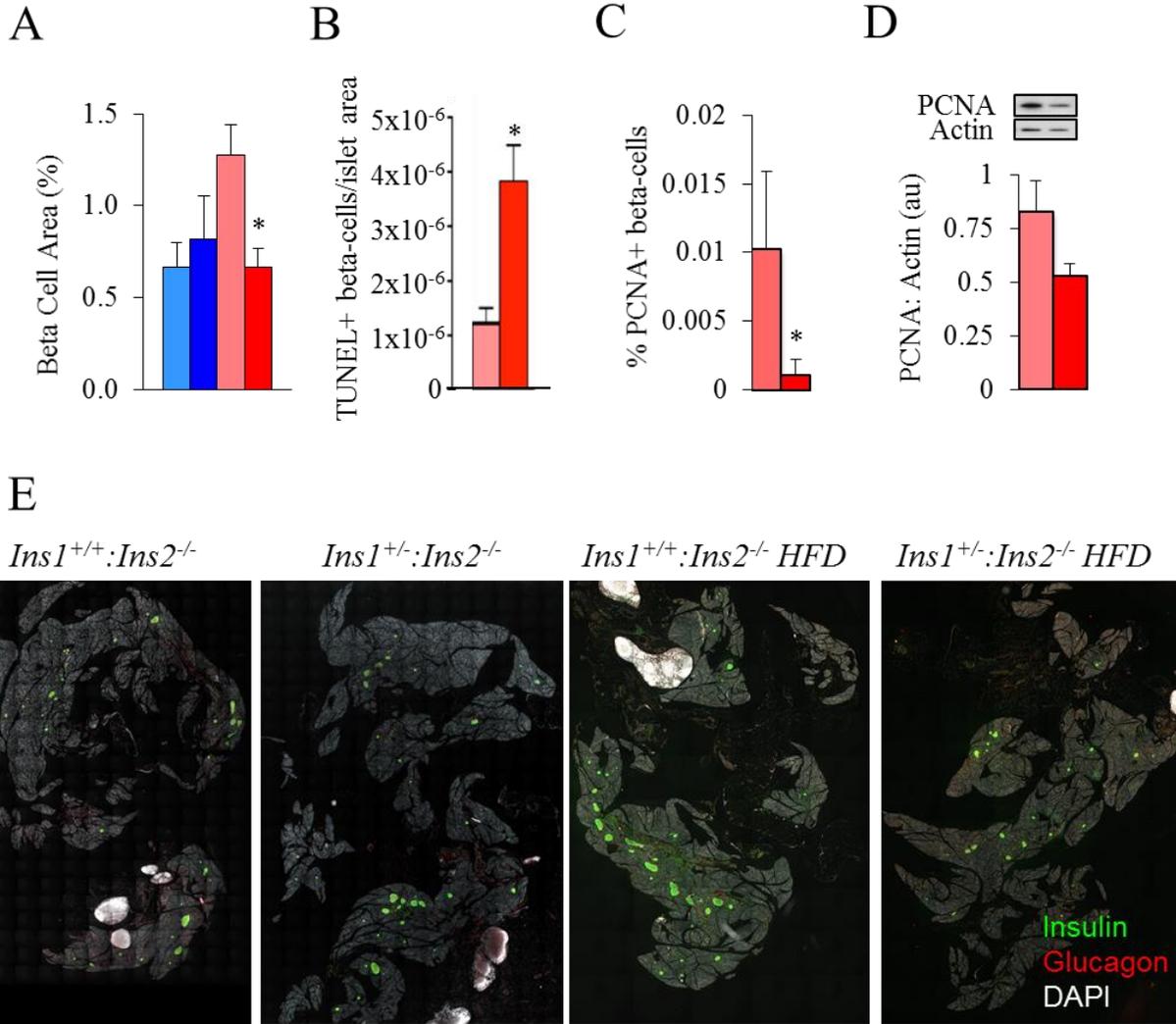
Experimental design to test the role of circulating insulin on diet-induced obesity in the absence of *Ins2*, which can be expressed outside of the pancreas, including in the brain.



**Figure 4.2 Reduced *Insulin* gene dosage results in reduced islet insulin mRNA and peptide.**

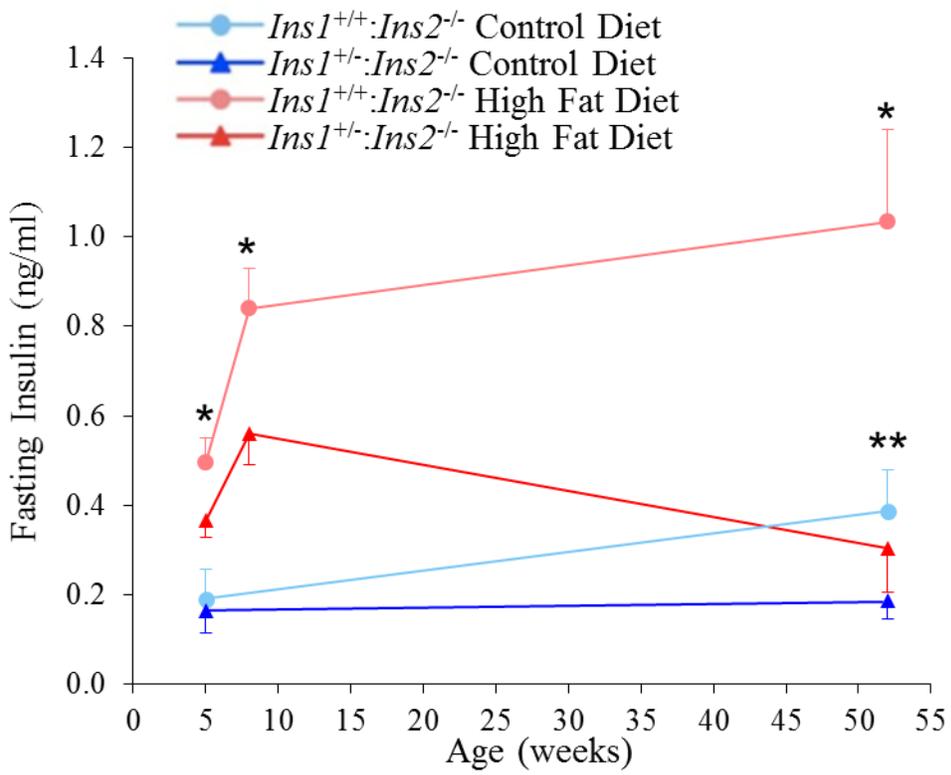
(A) Taqman RT-qPCR for *Ins1* mRNA in islets from 12-week old mice (n = 3-5). (B) Islet insulin content from 30 handpicked islets from 8 week-old mice (n = 3). (C) Representative staining using anti-insulin (green) and anti-glucagon (red) antibodies in pancreas tissue sections from 12 month-old mice. \* denotes significant difference between HFD mice.

■ *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> Control Diet  
■ *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> Control Diet  
■ *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> High Fat Diet  
■ *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> High Fat Diet



**Figure 4.3 Reduced *Insulin* gene dosage prevents the compensatory expansion of  $\beta$ -cell numbers in the context of a high fat diet.**

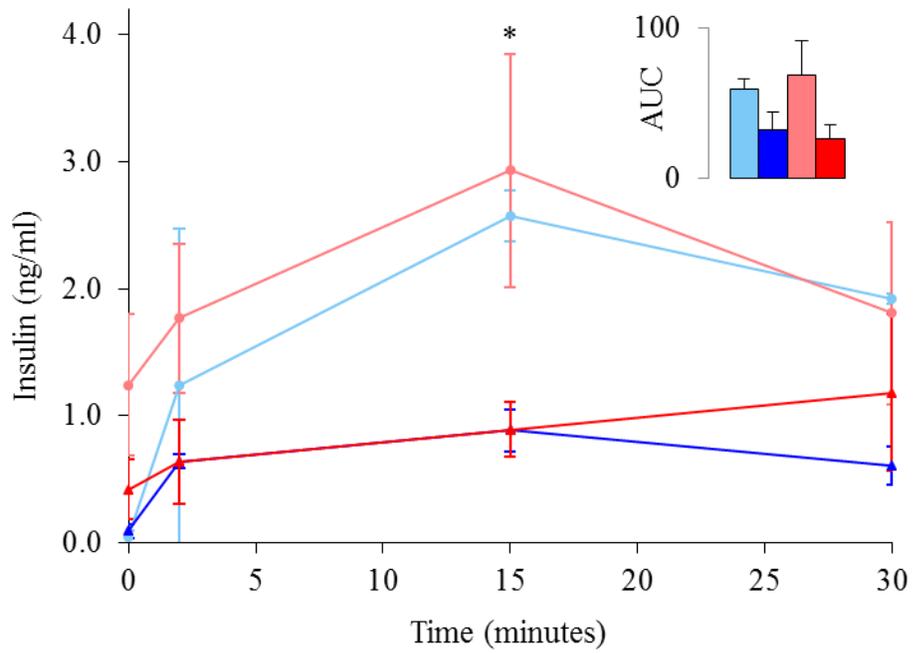
(A) *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice failed to increase  $\beta$ -cell mass in response to a high fat diet (n = 3). (B) Programmed cell death was assessed by counting the number of TUNEL positive  $\beta$ -cells in pancreatic sections from 8 week-old mice (n = 3-5). (C,D) Proliferation was estimated by quantifying the percentage of PCNA-positive  $\beta$ -cells in pancreata from 8 week-old mice (n = 3) and by performing PCNA immunoblot in islets isolated from 12 week-old mice (n = 4-6). (E) Representative staining using anti-insulin (green), anti-glucagon (red) and DAPI (white) antibodies in pancreas tissue sections from 12 month-old mice \* denotes significant difference between HFD mice. \* denotes  $p < 0.05$ .



**Figure 4.4 Reduced *Insulin* gene dosage prevents sustained hyperinsulinemia on a high fat diet.**

Age-dependent high fat diet-induced fasting hyperinsulinemia was prevented in *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice (n = 6-16). \* denotes significant difference between HFD mice. \*\* denotes significant difference between CD mice.

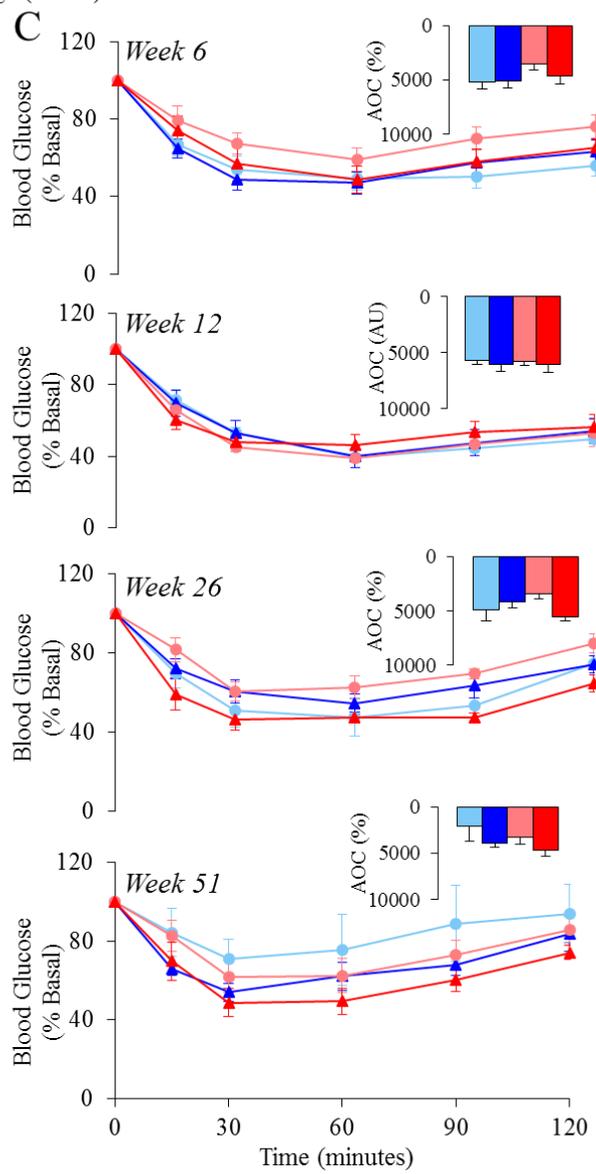
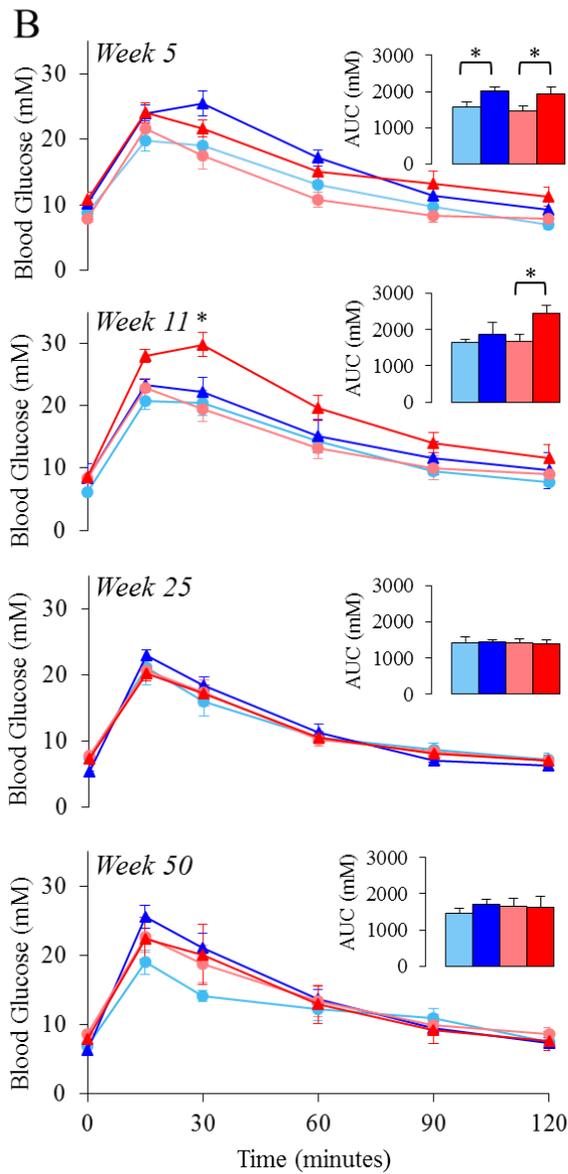
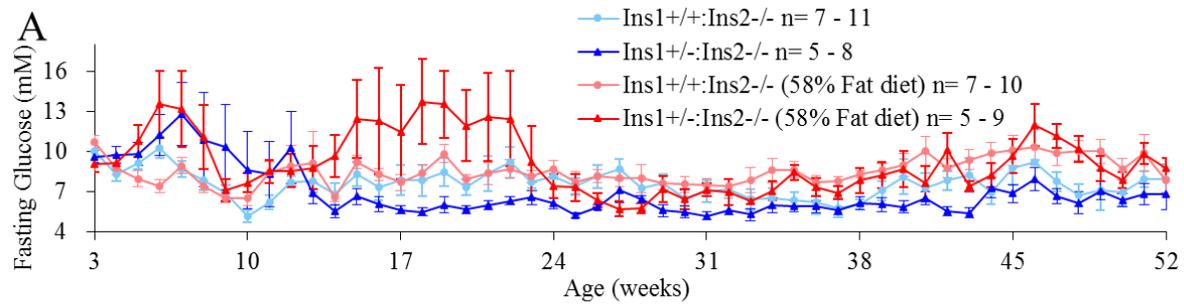
- *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* Control Diet
- ▲ *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* Control Diet
- *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* High Fat Diet
- ▲ *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* High Fat Diet



**Figure 4.5 Glucose stimulated insulin release in mice with reduced *Insulin* gene dosage.**

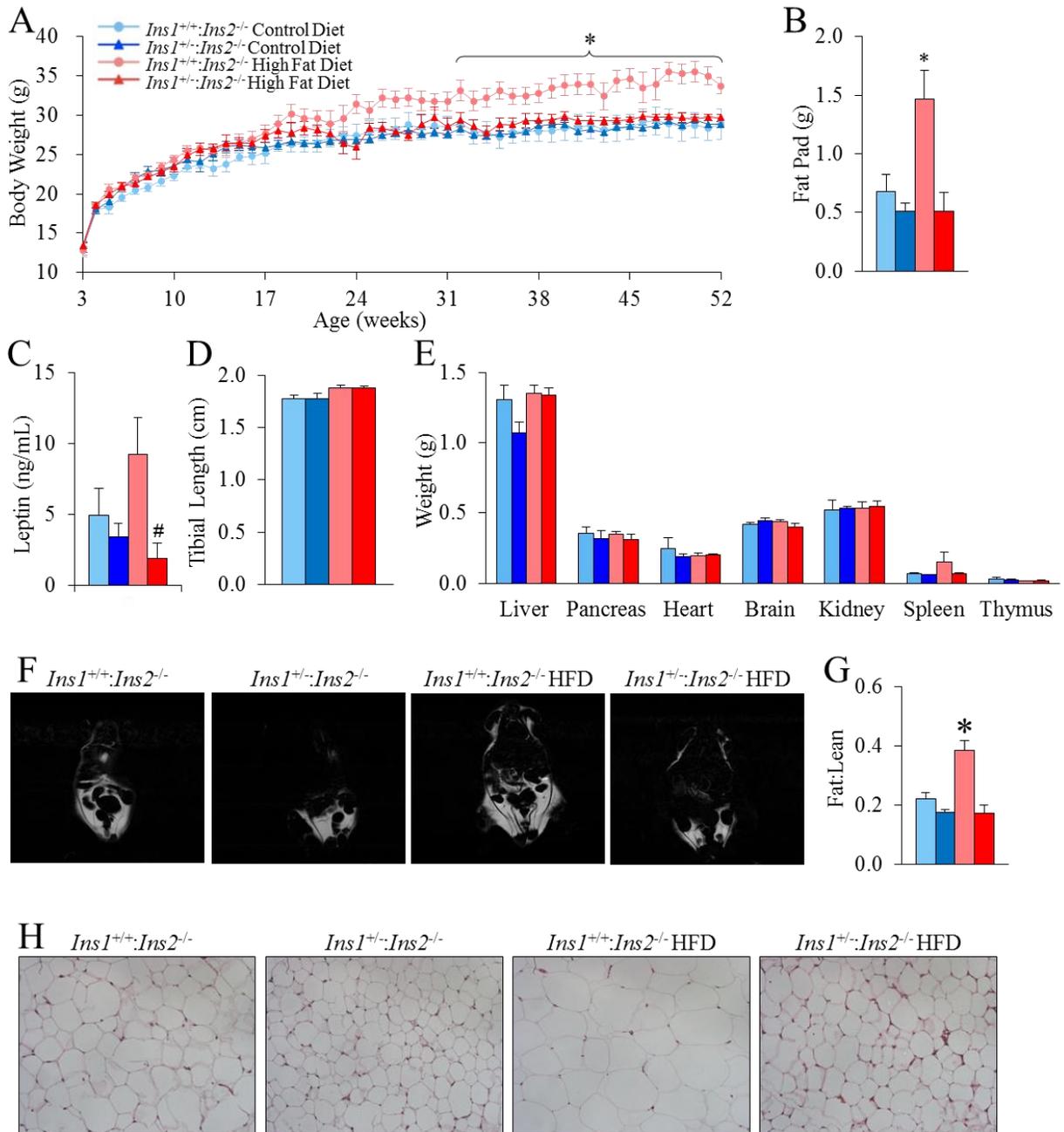
Insulin release in response to intraperitoneal injection of 18% glucose in 53 week-old mice (n =

6). \* denotes  $p < 0.05$ .



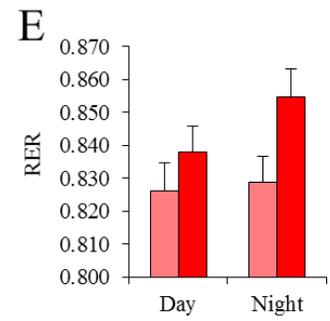
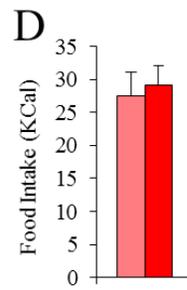
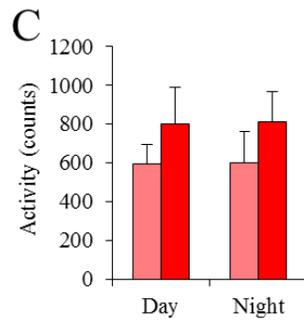
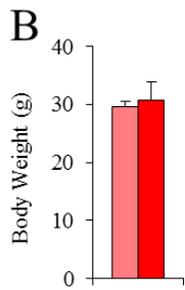
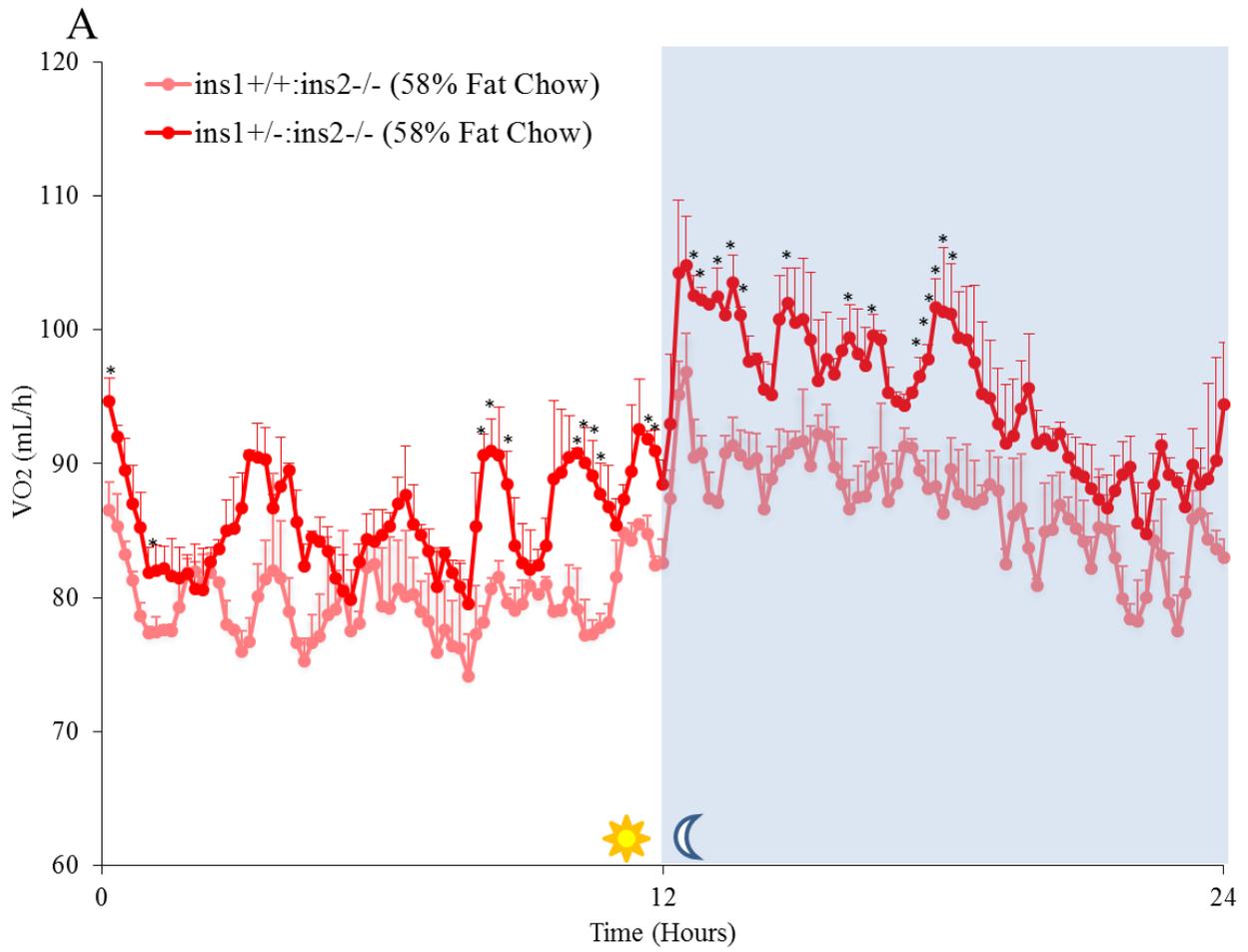
**Figure 4.6 Glucose homeostasis and insulin sensitivity in mice with reduced *Insulin* gene dosage.**

(A) Four-hour fasted glucose levels measured weekly over the 1st year of life. (B) Intraperitoneal glucose tolerance is impaired in young, but not old *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice. Insets show area under the curve (AUC). (C) Insulin tolerance is statistically similar between all groups at all ages studied. Insets show area over the curve (AOC). All data are represented as means of at least 5 male mice in each group at all time points. \* denotes statistical significance ( $p < 0.05$ ).



**Figure 4.7 Mice with reduced fasting insulin are protected from high fat-induced weight gain.**

(A) Body weight tracked weekly over 1 year, in multiple independent cohorts (assessed over several years; n = 5-11). (B) Epididymal fat pad weight in 1 year-old mice (n = 5-11). (C) Circulating leptin levels (n = 3). (D,E) Tibial length and organ weight were measured at 1 year as indexes of somatic growth (n = 5-11). (F,G) NMR spectroscopy (n = 3). (H) Hematoxylin and eosin-stained epididymal fat pad (n = 5-11).



**Figure 4.8 Mice with reduced fasting insulin have increased energy expenditure.**

(A, B) Energy expenditure was measured by indirect calorimetry in high fat fed mice at 20 weeks of age, prior to significant differences in weight gain (n = 5). (C-E) We did not note significant differences in activity, food intake, or respiratory quotient (n = 3-5).

	LF CD +/+	LF CD +/-	HFD +/+	HFD +/-
# $\$$ Acacb	100 ± 27	137 ± 52	113 ± 25	431 ± 89
#Pck1	100 ± 38	101 ± 39	84 ± 21	245 ± 60
ppargc1a	100 ± 42	139 ± 73	177 ± 40	310 ± 46
Ucp1	100 ± 37	68 ± 21	103 ± 33	234 ± 147
#Ucp3	100 ± 31	118 ± 33	70 ± 20	191 ± 26
#Pnpla2	100 ± 18	121 ± 24	109 ± 19	197 ± 30
pparg	100 ± 14	130 ± 38	89 ± 18	137 ± 20
Ppara	100 ± 15	126 ± 26	117 ± 9	165 ± 40
Cebpa	100 ± 10	169 ± 35	125 ± 22	172 ± 40
Fabp4	100 ± 15	124 ± 21	91 ± 15	127 ± 19
Adipor2	100 ± 24	112 ± 35	71 ± 14	104 ± 22
$\$$ Scd1	100 ± 26	112 ± 16	221 ± 40	251 ± 38
Foxa2	100 ± 27	83 ± 18	86 ± 21	110 ± 25
Nr1h3	100 ± 12	113 ± 21	83 ± 13	108 ± 14
Lepr	100 ± 12	70 ± 14	59 ± 9	83 ± 18
Ucp2	100 ± 13	105 ± 9	101 ± 10	121 ± 10
adipoq	100 ± 24	87 ± 17	65 ± 12	84 ± 10
Lipe	100 ± 18	105 ± 9	86 ± 12	104 ± 11
Klf15	100 ± 8	87 ± 19	77 ± 13	93 ± 11
Cd36	100 ± 19	118 ± 17	110 ± 18	127 ± 10
ptpn1	100 ± 16	99 ± 16	93 ± 12	109 ± 16
Slc2a4	100 ± 17	133 ± 10	110 ± 15	123 ± 21
Sirt1	100 ± 11	127 ± 18	117 ± 12	126 ± 10
Fto	100 ± 20	118 ± 14	104 ± 9	112 ± 12
Foxo1	100 ± 14	101 ± 16	76 ± 5	84 ± 10
& $\$$ Mlxipl	100 ± 8	115 ± 10	54 ± 1	61 ± 9
Foxc2	100 ± 23	104 ± 27	62 ± 10	67 ± 21
Rbp4	100 ± 18	120 ± 19	110 ± 21	113 ± 12
Adipor1	100 ± 12	82 ± 5	82 ± 4	81 ± 1
Ikbb	100 ± 17	107 ± 18	90 ± 15	86 ± 19
*& $\$$ Insr	100 ± 18	57 ± 3	51 ± 3	47 ± 1
Fasn	100 ± 24	131 ± 28	119 ± 20	106 ± 23
Ddit3	100 ± 11	91 ± 9	135 ± 16	117 ± 15
$\$$ Nrip1	100 ± 17	112 ± 12	91 ± 8	63 ± 9
Srebf1	100 ± 28	97 ± 11	111 ± 17	83 ± 24
#Fgf21	100 ± 18	59 ± 9	75 ± 9	40 ± 3
$\$$ Acly	100 ± 19	209 ± 47	114 ± 24	70 ± 15
Pnpla3	100 ± 22	167 ± 40	134 ± 22	88 ± 21
Atf3	100 ± 11	80 ± 25	140 ± 23	82 ± 24
#Tnf	100 ± 25	57 ± 8	143 ± 27	59 ± 11
Egr1	100 ± 17	103 ± 35	209 ± 108	113 ± 41
Emr1	100 ± 14	102 ± 19	248 ± 63	149 ± 32
IGFbp1	100 ± 36	86 ± 12	157 ± 60	52 ± 1
Il6	100 ± 24	81 ± 17	148 ± 57	35 ± 10
#&Egr2	100 ± 15	185 ± 88	380 ± 113	55 ± 17

#### **Figure 4.9 Gene expression profile of white adipose tissue.**

Taqman real-time qPCR quantification of 45 genes in epididymal white adipose tissue from 1 year-old mice. Results are sorted according to the magnitude of the difference between high-fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice and high-fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* littermates (# denotes  $p < 0.05$ ). Genes with a large negative relative expression are colored red; genes with increased expression are green. \$ denotes significant difference between diets in the *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice. & denotes significant differences between *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice compared with *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice on the control diet. Data are from 1-year old mice (n = 5-7).

	HFD +/+	HFD +/-
Klf15	100 ± 22	188 ± 38
Ikkkb	100 ± 92	141 ± 136
Nr1h3	100 ± 13	136 ± 31
Rbp4	100 ± 8	136 ± 16
Slc2a4	100 ± 24	124 ± 18
Foxo1	100 ± 24	122 ± 16
Insr	100 ± 8	121 ± 14
Adipoq	100 ± 12	115 ± 21
Cd36	100 ± 29	115 ± 16
Acacb	100 ± 26	115 ± 15
Adipor2	100 ± 0	111 ± 13
Fabp4	100 ± 0	109 ± 9
Ppargc1a	100 ± 13	107 ± 36
Pnpla2	100 ± 23	107 ± 15
Pck1	100 ± 31	104 ± 16
Lepr	100 ± 32	103 ± 33
Ucp1	100 ± 7	102 ± 5
Nripl	100 ± 16	101 ± 20
fto	100 ± 10	99 ± 1
Ucp3	100 ± 33	98 ± 26
Ucp2	100 ± 35	95 ± 38
Ppara	100 ± 28	93 ± 15
Adipor1	100 ± 13	90 ± 18
Srebf1	100 ± 24	90 ± 16
Ptpn1	100 ± 9	88 ± 3
Mlxipl	100 ± 19	87 ± 11
Lipe	100 ± 22	85 ± 18
Pnpla3	100 ± 20	82 ± 18
Fasn	100 ± 30	76 ± 12
Pparg	100 ± 1	73 ± 9
Cebpa	100 ± 31	72 ± 14
Acly	100 ± 29	70 ± 20
Il6	100 ± 31	69 ± 20
Emr1	100 ± 50	64 ± 11
Sirt1	100 ± 26	61 ± 7
Foxa2	100 ± 50	59 ± 12
Foxc2	100 ± 50	59 ± 12
Ddit3	100 ± 25	52 ± 10
Scd1	100 ± 2	52 ± 7
Egr1	100 ± 35	51 ± 9
Fgf21	100 ± 34	50 ± 2
Atf3	100 ± 25	40 ± 15
Tnf	100 ± 19	40 ± 8
Igfbp1	100 ± 34	39 ± 8
Egr2	100 ± 88	9 ± 1

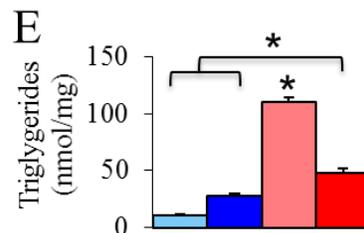
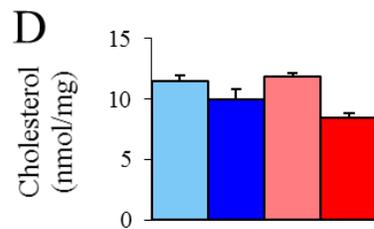
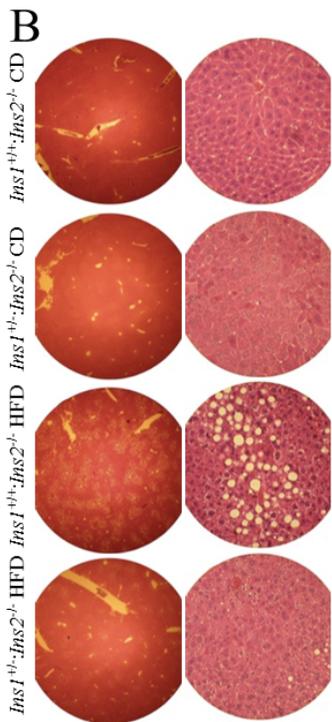
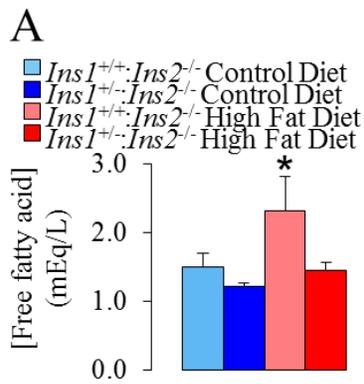
**Figure 4.10 Gene expression profile of brown adipose tissue.**

Taqman qPCR of intra-scapular brown adipose tissue in high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* and *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice. Data are ranked by numerical differences between conditions. No statistical significance was detected (n = 5-7).

	HFD +/+	HFD +/-
Tnf	100 ± 28	393 ± 76
Emr1	100 ± 30	239 ± 44
Pck1	100 ± 61	204 ± 61
Adipor1	100 ± 38	196 ± 62
Ucp2	100 ± 20	195 ± 39
Nr1h3	100 ± 14	194 ± 32
Ptpn1	100 ± 20	180 ± 26
Ucp3	100 ± 28	163 ± 27
Il6	100 ± 21	161 ± 36
Acly	100 ± 16	157 ± 17
Ucp1	100 ± 62	152 ± 51
Foxo1	100 ± 24	151 ± 15
Srebf1	100 ± 29	148 ± 32
Sirt1	100 ± 16	138 ± 14
Nripl	100 ± 27	136 ± 34
fto	100 ± 18	135 ± 20
Klf15	100 ± 35	134 ± 14
Acacb	100 ± 35	126 ± 22
Pnpla2	100 ± 19	125 ± 25
Lipe	100 ± 40	124 ± 47
Adipor2	100 ± 17	124 ± 22
Slc2a4	100 ± 15	122 ± 17
Insr	100 ± 30	119 ± 25
Cebpa	100 ± 35	118 ± 38
Rbp4	100 ± 42	110 ± 26
Cd36	100 ± 15	108 ± 22
Pparg	100 ± 34	106 ± 29
Lepr	100 ± 32	98 ± 33
Ikkkb	100 ± 34	97 ± 25
Egr2	100 ± 25	96 ± 27
Ddit3	100 ± 14	94 ± 24
Fabp4	100 ± 32	86 ± 25
Mlxipl	100 ± 22	80 ± 20
Scd1	100 ± 48	70 ± 49
Ppara	100 ± 47	65 ± 39
Fasn	100 ± 50	65 ± 36
Egr1	100 ± 46	64 ± 59
Pnpla3	100 ± 42	60 ± 24
Adipoq	100 ± 55	50 ± 42
Ppargc1a	100 ± 56	47 ± 19
Atf3	100 ± 48	23 ± 48

**Figure 4.11 Gene expression profile of skeletal muscle.**

Taqman qPCR of gastrocnemius skeletal muscle in high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* and *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice. Data are ranked by numerical differences between conditions. No statistical significances were detected (n = 7,5).

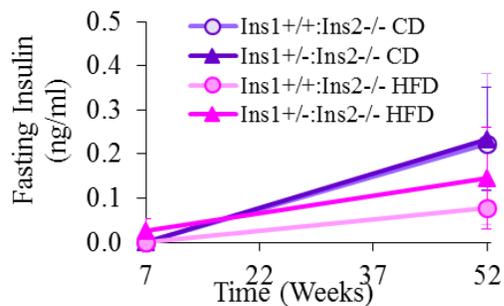
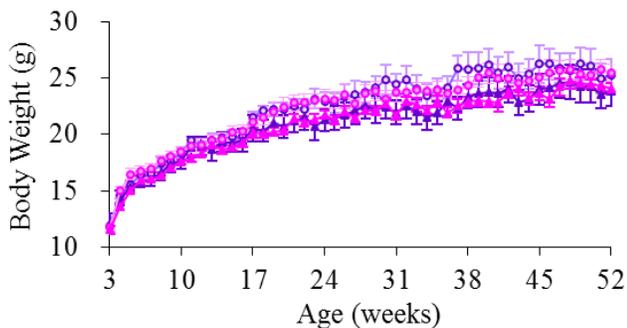
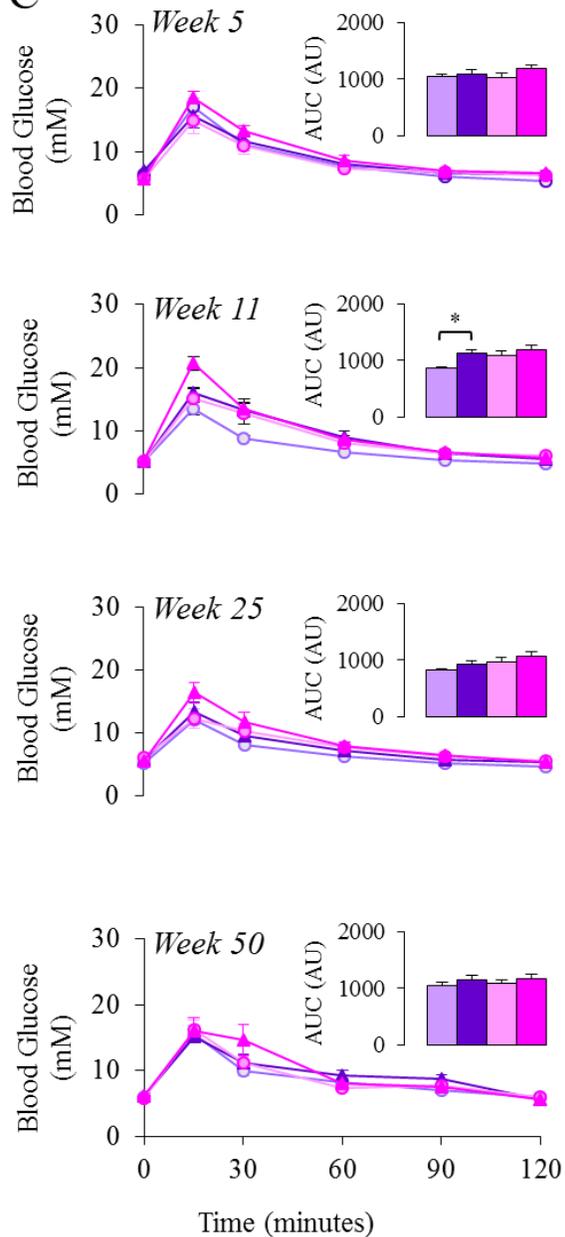
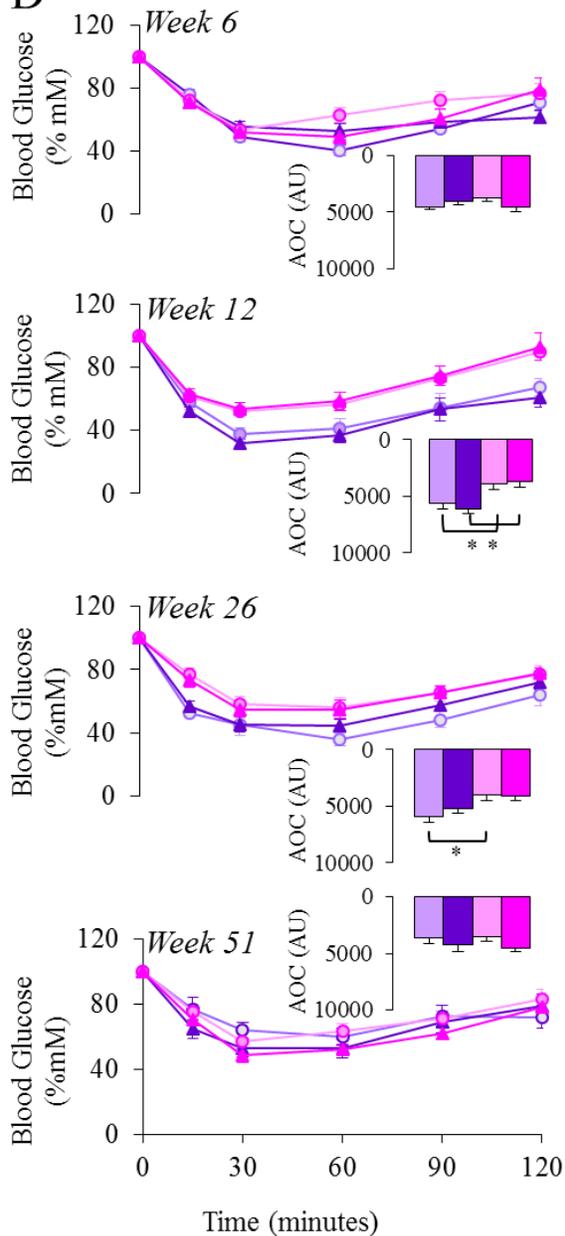


**C**

	LF CD +/+	LF CD +/-	HFD +/+	HFD +/-
IL-1b	100 ± 7	3459 ± 2922	28904 ± 16282	7270 ± 6428
&Il4	100 ± 23	589 ± 321	1056 ± 365	896 ± 553
Inf-y	100 ± 27	1946 ± 1202	1892 ± 838	11203 ± 8225
TLR-4	100 ± 19	2708 ± 2587	29780 ± 16571	13895 ± 8819
Slc2a4	100 ± 24	99 ± 25	278 ± 104	457 ± 251
Emr1	100 ± 19	239 ± 92	146 ± 31	278 ± 115
ppargc1a	100 ± 5	367 ± 100	478 ± 135	563 ± 109
Acacb	100 ± 27	92 ± 28	133 ± 22	213 ± 60
Fgf21	100 ± 11	121 ± 24	175 ± 41	217 ± 63
Pck1	100 ± 34	291 ± 71	324 ± 106	366 ± 114
Lipe	100 ± 27	92 ± 15	137 ± 38	165 ± 52
Tnf	100 ± 15	300 ± 120	118 ± 22	146 ± 63
Ucp3	100 ± 19	94 ± 14	88 ± 10	110 ± 42
Fasn	100 ± 18	94 ± 13	107 ± 23	125 ± 36
Pnpla2	100 ± 16	86 ± 11	107 ± 22	121 ± 35
Adipor2	100 ± 25	87 ± 14	116 ± 23	128 ± 26
Cebpa	100 ± 31	67 ± 16	84 ± 12	94 ± 33
Acly	100 ± 16	142 ± 31	89 ± 15	97 ± 19
Il6	100 ± 50	82 ± 54	33 ± 3	35 ± 10
Pnpla3	100 ± 39	74 ± 15	117 ± 19	117 ± 45
IGFbp1	100 ± 46	123 ± 67	24 ± 9	24 ± 9
Ucp2	100 ± 17	107 ± 18	83 ± 11	83 ± 5
Nr1h3	100 ± 31	79 ± 10	118 ± 24	117 ± 27
Ikbkb	100 ± 13	110 ± 14	85 ± 12	83 ± 12
Egr2	100 ± 46	89 ± 25	26 ± 4	23 ± 7
Insr	100 ± 26	69 ± 7	107 ± 18	104 ± 23
Rbp4	100 ± 25	83 ± 14	107 ± 20	103 ± 24
Fto	100 ± 16	92 ± 12	84 ± 10	80 ± 12
Sirt1	100 ± 17	85 ± 11	105 ± 13	98 ± 11
\$pparg	100 ± 28	93 ± 11	59 ± 11	49 ± 12
Fabp4	100 ± 25	66 ± 16	60 ± 15	49 ± 12
Egr1	100 ± 36	134 ± 65	26 ± 3	13 ± 7
Lepr	100 ± 47	97 ± 57	64 ± 25	52 ± 15
Nrip1	100 ± 15	98 ± 11	87 ± 13	74 ± 12
Foxc2	100 ± 18	128 ± 24	126 ± 27	113 ± 25
Ppara	100 ± 23	73 ± 16	118 ± 27	104 ± 20
Srebf1	100 ± 16	136 ± 18	216 ± 52	200 ± 37
Mlxipl	100 ± 23	119 ± 21	133 ± 30	117 ± 19
Klf15	100 ± 40	86 ± 24	104 ± 29	87 ± 16
Adipor1	100 ± 17	83 ± 9	92 ± 15	75 ± 13
Ucp1	100 ± 16	142 ± 45	93 ± 15	76 ± 11
Cd36	100 ± 20	88 ± 15	160 ± 25	142 ± 40
&\$Ddit3	100 ± 17	72 ± 8	60 ± 8	40 ± 7
ptpn1	100 ± 23	100 ± 46	55 ± 14	34 ± 5
Foxa2	100 ± 29	86 ± 15	112 ± 23	89 ± 34
Scd1	100 ± 26	77 ± 23	123 ± 22	94 ± 26
Foxo1	100 ± 14	115 ± 24	144 ± 33	114 ± 26
adipoq	100 ± 8	185 ± 91	119 ± 29	76 ± 21
Atf3	100 ± 29	105 ± 36	90 ± 30	35 ± 8

**Figure 4.12 *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice are protected from lipid spill over, fatty liver and ER-stress.**

(A) Circulating free fatty acids levels at one year of age. \* denotes  $p < 0.05$ . (B) Low and high magnification of H&E-stained liver. (C) Taqman real-time qPCR quantification of 49 genes in liver from 1 year-old mice (n = 3-7 individual mice). Results are sorted as in Figure 4.9. # denotes  $p < 0.05$  t-test between high-fat fed *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice versus high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice. \$ denotes significant difference between diets within the *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice. & denotes differences between *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice versus *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice on the control diet. (D, E) Liver cholesterol and triglyceride measurements (n = 3-5). \* denotes  $p < 0.05$ .

**A****B****C****D**

**Figure 4.13 Lack of fasting hyperinsulinemia and weight gain in high fat fed female *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> or *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> mice.**

(A) 4-hour fasting insulin was measured in female mice at the ages indicated (n = 3). (B) Body weight tracked weekly (n = 7-8 per group). (C) Glucose tolerance tests, and area under the curve insets (n = 7-8). (D) Insulin tolerance tests, and area over the curve insets (n = 7-8).

## 4.8 Tables

**Table 4.1 List of the genes studied in the mini-array.**

<b>Symbol</b>	<b>Name</b>
18S	18S ribosomal subunit
Acacb	Acetyl-CoA carboxylase
Acly	ATP citrate lyase
Actb	Actin, beta
Adipoq	Adiponectin, C1Q and collagen domain containing
Adipor1	Adiponectin receptor 1
Adipor2	Adiponectin receptor 2
Atf3	Activating transcription factor 3
Cd36	Cluster of differentiation 36
Cebpa	CCAAT/enhancer-binding protein alpha
Ddit3	DNA damage-inducible transcript 3
Egr1	Early growth response 1
Egr2	Early growth response 2
Emr1	EGF-like module-containing mucin-like hormone receptor-like 1
Fabp4	Fatty acid binding protein 4
Fasn	Fatty acid synthase
Foxo1	Forkhead box protein O1
Fto	Fat mass and obesity-associated

<b>Symbol</b>	<b>Name</b>
Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1
Ikbkb	Inhibitor of nuclear factor kappa-B kinase subunit beta
Il6	Interleukin-6
Insr	Insulin receptor
Lepr	Leptin receptor
Lipe	Hormone sensitive lipase
Klf15	Krüppel-like factor 15
Mlxipl	MLX interacting protein-like
Nr1h3	Nuclear receptor subfamily 1
Nrip1	Nuclear receptor-interacting protein 1
Pck1	Phosphoenolpyruvate carboxykinase 1
Pnpla2	Patatin-like phospholipase domain containing 2
Pnpla3	Patatin-like phospholipase domain containing 3
Ppara	Proliferator-activated receptor alpha
Pparg	Proliferator-activated receptor gamma
Ppargc1a	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
Ptpn1	Protein tyrosine phosphatase non-receptor type 1
Rbp4	Retinol binding protein 4
Scd1	Stearoyl-CoA desaturase-1

<b>Symbol</b>	<b>Name</b>
Sirt1	Sirtuin 1
Slc2a4	Solute carrier family 2 member 4
Srebf1	Sterol regulatory element binding transcription factor 1
Tnf	Tumor necrosis factor
Ucp1	Uncoupling protein 1
Ucp2	Uncoupling protein 2
Ucp3	Uncoupling protein 3

## Chapter 5: Effects of reduced *Ins2* gene dosage in *Ins1*<sup>-/-</sup> mice

### 5.1 Glucose homeostasis, insulin tolerance, and insulin levels in *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice

The studies in the preceding chapter demonstrate that the pancreas-specific *Ins1* gene is adipogenic (Figures from chapter 4), but they do not address the specific functions of the *Ins2* gene, which is more widely expressed (including in the brain), and more closely related to the human *INSULIN* gene. To assess the role of *Ins2* in obesity, we varied the *Ins2* gene dosage in mice lacking both alleles of *Ins1* (Fig. 5.1). Deleting one of the two alleles of the *Ins2* gene allowed us to successfully reduce the *Ins2* mRNA levels in the *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice (Fig. 5.2A). However, we were not able to detect significant differences in the circulating insulin levels in *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice compared to control *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> mice (Fig. 5.2B). Surprisingly, immunofluorescent staining of the islets showed only decreased signal in *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> male mice on control diet (Fig. 5.2C). In contrast to the previously discussed *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> male mice, we were unable to measure significant differences in pancreatic  $\beta$ -cell area or fasting insulin (Fig. 5.3). There were also no significant differences in fasting blood glucose levels throughout the entire experiment in the cohort of mice we tested (Fig. 5.4A). The glucose-stimulated insulin secretion was lower in the control diet fed *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice compared to *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> littermate controls on the same diet at seven weeks of age but not later (Fig. 5.4B). However, high fat fed *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice showed similar glucose-stimulated insulin secretion when compared to high fat fed *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> littermate controls (Fig. 5.4B). This indicates that a single allele of the *Ins2* gene is sufficient to generate enough insulin to mount an appropriate response to glucose in the context of a high fat diet (unlike the *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> mice described in the previous chapter). Mice in all groups, regardless of diet or genotype, showed normal glucose tolerance (Fig. 5.4C). On a control diet, 7 week-old mice with reduced *Ins2* gene dosage were hypersensitive to exogenous insulin, although this was not

observed in older mice (Fig. 5.4D). This group of high fat fed *Ins1*<sup>-/-</sup> mice (regardless of *Ins2* gene dose) were paradoxically insulin hypersensitive at all of the time points beyond 12 weeks (Fig. 5.4D). Collectively, these data suggests that the *Ins1* gene may be required for the deleterious effects of high fat diet on insulin resistance, although it would be important to replicate these findings in additional cohorts. Since there were no significant differences in peripheral circulating insulin, this model could potentially allow the effects of brain insulin (*Ins2*) on obesity to be evaluated in the absence of potentially confounding effects of hyperglycemia or insulin resistance.

## 5.2 Cohort dependent effects of *Ins2* gene dosage on diet-induced obesity

Comparing the effects of a control diet and high fat feeding of *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice and control *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> mice revealed a complex, cohort-dependent gene-environment interaction. In the first cohort, the control diet fed *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice were lighter throughout life than their *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> littermate controls (Fig. 5.5A and C), suggesting the potential for general growth factor roles for the conserved *Ins2* gene in the context of moderate fat intake. We also found that *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> mice did not gain weight on a high fat diet in this cohort (Fig. 5.5A and C), leading us to speculate that the hypersecretion of the *Ins1* gene product may be required for the proper storage of lipids in adipose tissue. Remarkably, in the first cohort a majority of *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice exhibited striking weight gain on the high fat diet (Fig. 5.5 A and C). Surprisingly, these somewhat paradoxical effects were not observed in a second cohort (Fig. 5.5 B and D). Weight gain the first cohort in high fat fed *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice relative to the other groups was due to a combination of increased adiposity (Fig. 5.6A and B) and increased somatic growth, assessed by tibial length and organ measurement (Fig. 5.6C and F). An unusual heterogeneity in adipocyte size was observed in all groups of *Ins1*<sup>-/-</sup> mice (Fig. 5.6A), reminiscent of mice lacking adipocyte insulin receptors

<sup>29,506</sup>. Circulating leptin was proportional to fat-pad weight and fat-to-lean ratio (Fig. 5.6D). We also did not detect differences in the circulating free fatty acids between groups in this cohort (Fig. 5.6E). Together, these data suggest that, in some currently un-defined conditions, *Ins2* may suppress body weight via a mechanism that is active specifically in the context of a high fat diet. Given the similarity in circulating insulin levels, these data suggest the possibility of altered local effects of *Ins2* in the brain. However, these data have to be looked at with caution as the differences observed were only found in the first cohort. The animal facility where this work was done no longer exists, so it is impossible to formally repeat these studies in the same environment. More studies, beyond the scope of this thesis, are in progress by another graduate student, in a different facility, to assess a potential role of *Ins2* gene dosage in obesity. In these studies, similar cohort-dependent variability has been observed (personal communication, Nicole Templeman). Collectively, the experience of our laboratory is that modulation of the *Ins2* gene, in the absence of *Ins1*, leads to striking diet-dependent variability in body weight. The source of this variability will be investigated by our collaborators specializing in epigenetics.

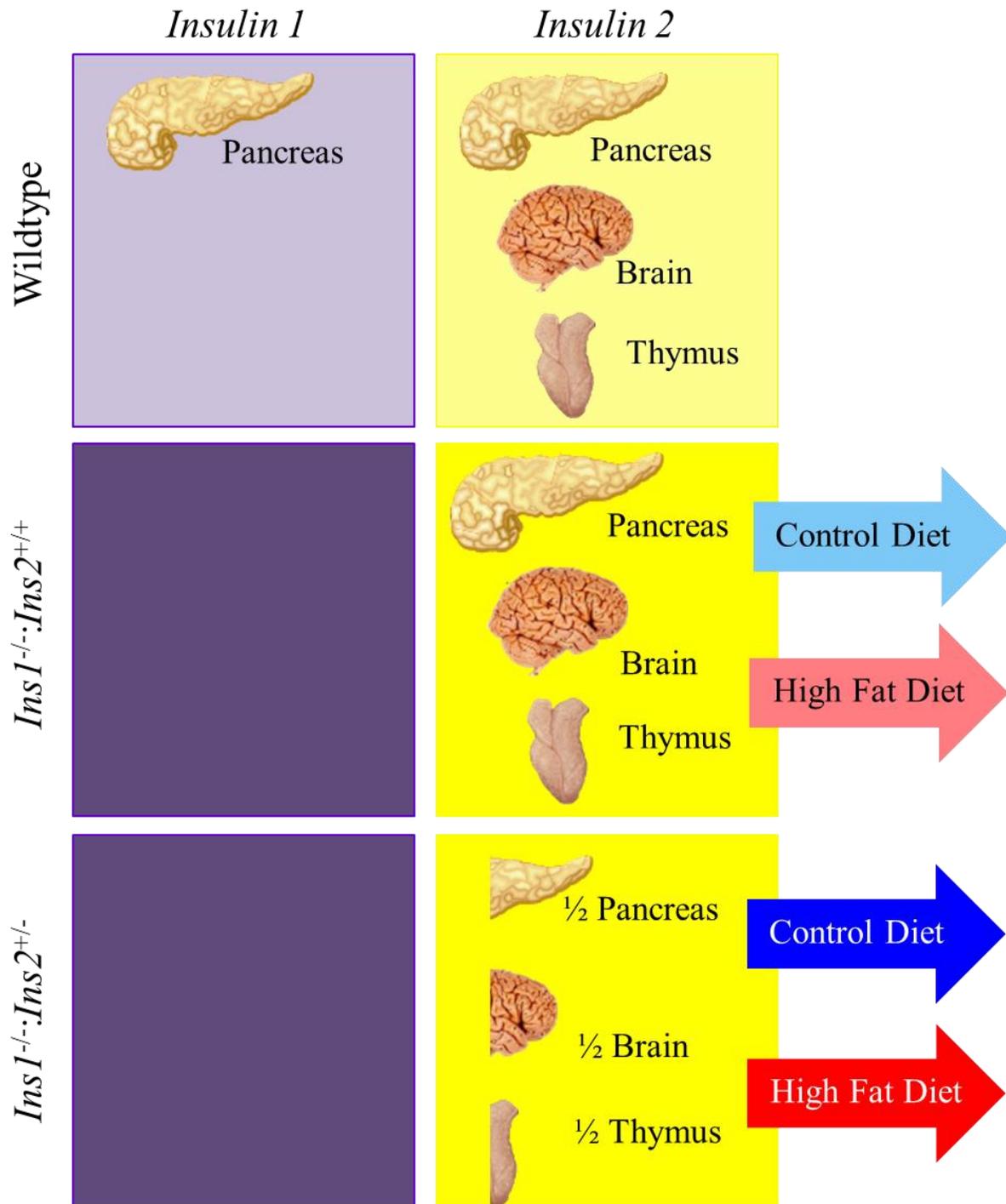
It is well established that insulin can act in the brain as a satiety factor <sup>215,536</sup>. We have confirmed that *Ins2* is expressed in multiple regions of the brain that can potentially control and project to feeding, reward and memory centers, raising the possibility that central *Ins2* gene expression may regulate food intake. We only conducted food intake and metabolic cage studies in our first cohort. In this cohort, reducing *Ins2* gene dosage changed body weight by significantly decreasing the ability to reduce food intake in response to high amounts of fat in diet (Fig. 5.7A). Importantly, the intake of the control diet was not different between *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice and *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* littermate controls. Other parameters such as activity and energy expenditure were also not different between any of the *Ins1<sup>-/-</sup>* groups (Fig. 5.7B-E).

Quantitative PCR analysis of the liver (Fig. 5.8) showed a significant decrease in the expression of the *Fto*, *Fasn*, and *Insr* genes in the *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice compared to the *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* littermate controls on the high fat diet. A variant of the *Fto* gene has been reported to be associated with the risk of being predisposed to obesity<sup>293,537</sup>. Furthermore, inactivation of the *Fto* gene has been reported to protect against obesity due to increased energy expenditure<sup>538</sup>. Fatty acid synthase, the peptide product of the *Fasn* gene, is a key component in lipogenesis that plays a role in the maximal rate of fatty acid synthesis, particularly in the liver<sup>539</sup>. However, it is reported that knocking out the *Fasn* gene leads to hepatic steatosis<sup>540</sup>. It is possible that in our case, and combined with changes in other genes, reduced, but not completely lost, *Fasn* expression has played a protective role in the context of high fat diet-induced hepatic steatosis. Lastly, we observed a reduction in the expression of the insulin receptor, *Insr* gene, in the liver. As we showed previously in chapter 4 (Fig. 4.12B), reduced circulating insulin level is associated with protection from high fat diet-induced hepatic steatosis<sup>127</sup>. Therefore, reduced insulin action in the liver could have also contributed to the reduced hepatic steatosis in the *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice compared to the *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* littermate controls on the high fat diet. Moreover, we observed up-regulation, though not significant, in the expression of genes such as *Fgf21* and *Pnpla3* in the high fat fed *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice compared to the *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* littermate controls. Collectively the aforementioned changes in the gene expression profile in the liver can potentially explain the lack of fat accumulation in the liver of the *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice compared to the *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice as observed in histological analysis of the liver (data not shown). However, the fat accumulation seen in the high fat fed *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice was much less than that of observed in the high fat fed *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice explained in the previous section.

### 5.3 Diet-induced weight gain in female *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice

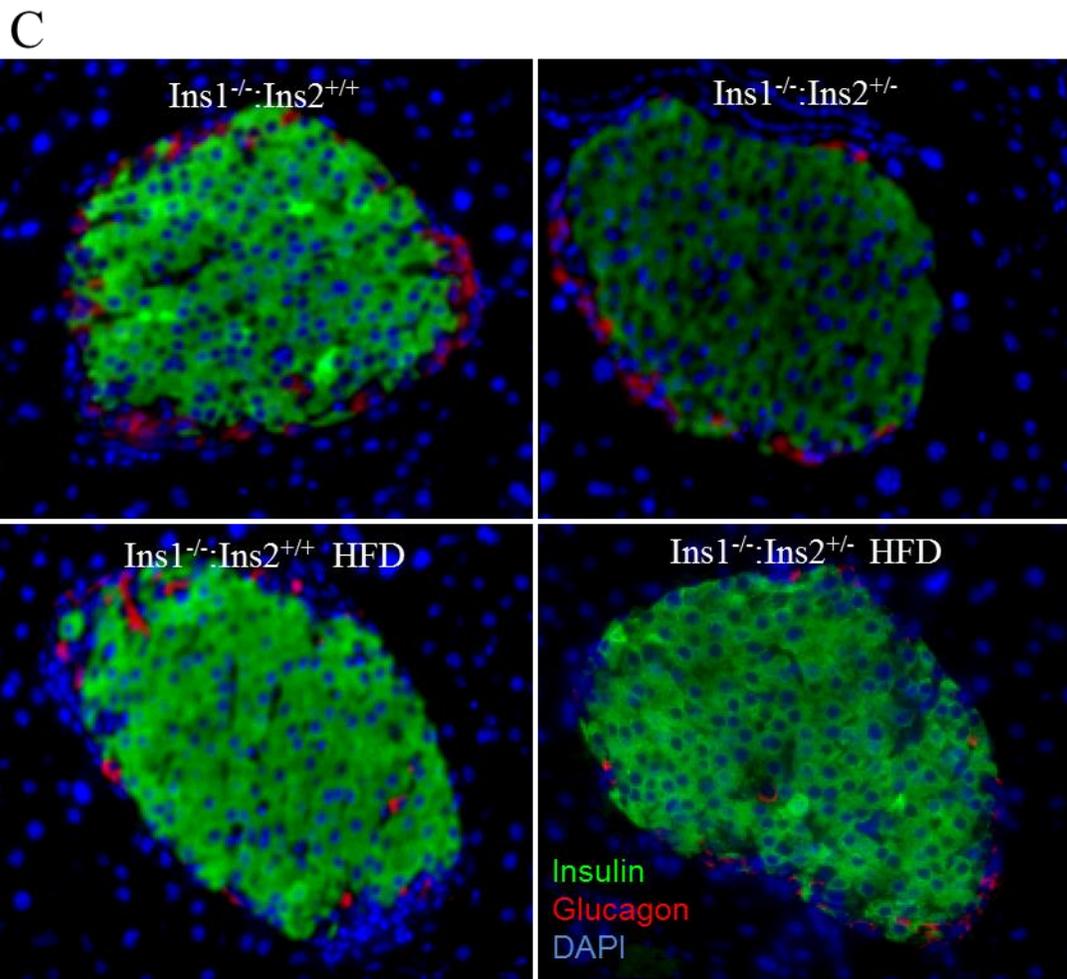
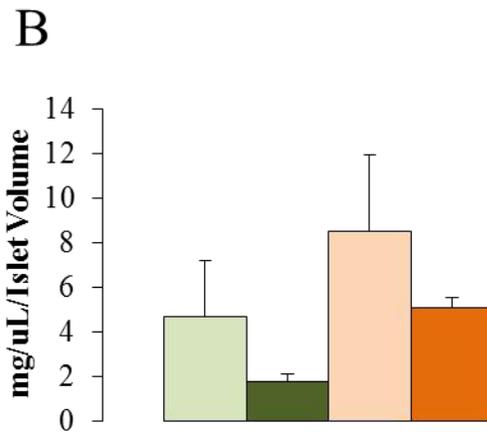
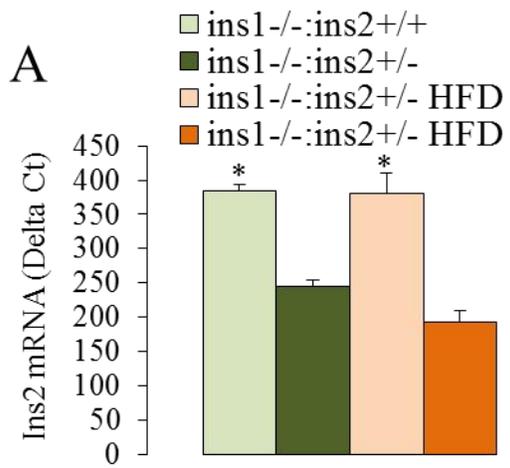
We also conducted an identical study on female control *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice and experimental *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice. Similar to mice in the previous chapter, reduction in *Ins2* gene dosage did not lead to a significant reduction in basal circulating insulin (Fig. 5.9A). Similarly, there were no significant differences between the two genotypes in the female mice with respect to their body weight (Fig. 5.9B). However, in this group of female mice, the high fat diet led to a significant increase in circulating insulin levels 15 minutes after an intraperitoneal glucose challenge on either genotype (Fig. 5.9A). Control *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice gained the most weight on a high fat diet when compared to the mice on control diet of the identical genotype (Fig. 5.9B). There was a tendency for *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice to gain less weight over the ~1 year period, although the study was underpowered to detect small differences (Fig. 5.9B). Another student has repeated this study with more mice, though in different facility, and her mice were protected to the level of statistical significance (personal communication, Nicole Templeman). Intraperitoneal glucose challenge showed that female mice in all four groups were able to equally control their blood glucose (Fig 5.9D). There were also no significant differences in these mice with respect to intraperitoneal insulin challenge (Fig. 5.9E). These data are also consistent with the notion that increased circulating insulin levels promote weight gain. High fat fed mice only transiently showed trends towards increased fasting blood glucose levels close to one year of age (Fig. 5.9F).

5.4 Figures



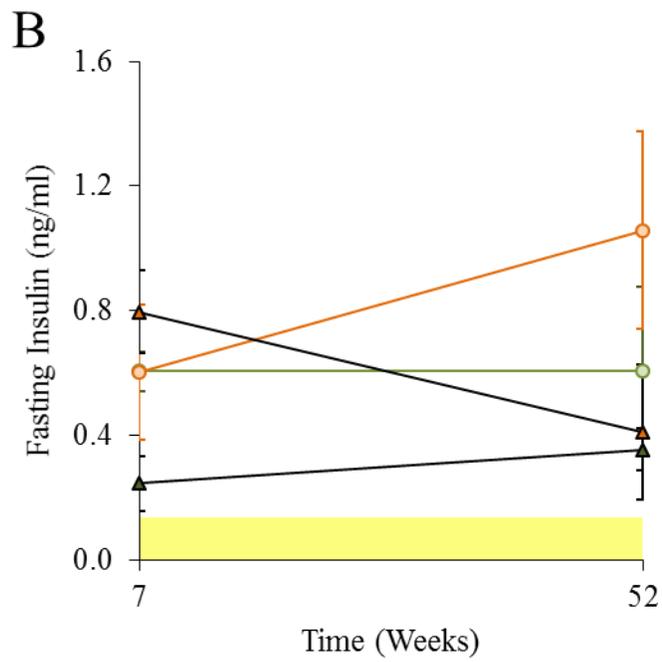
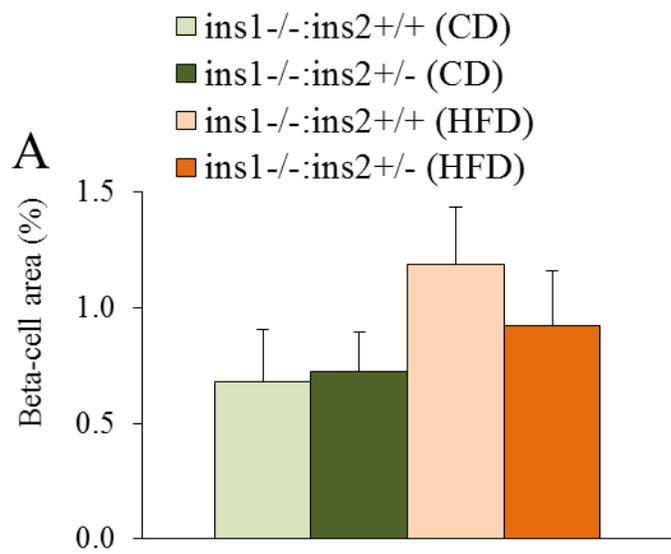
**Figure 5.1 Experimental design for mice with varying *Ins2* gene dosage on an *Ins1* null background.**

Experimental design for study testing the role of insulin 2 on diet-induced obesity.



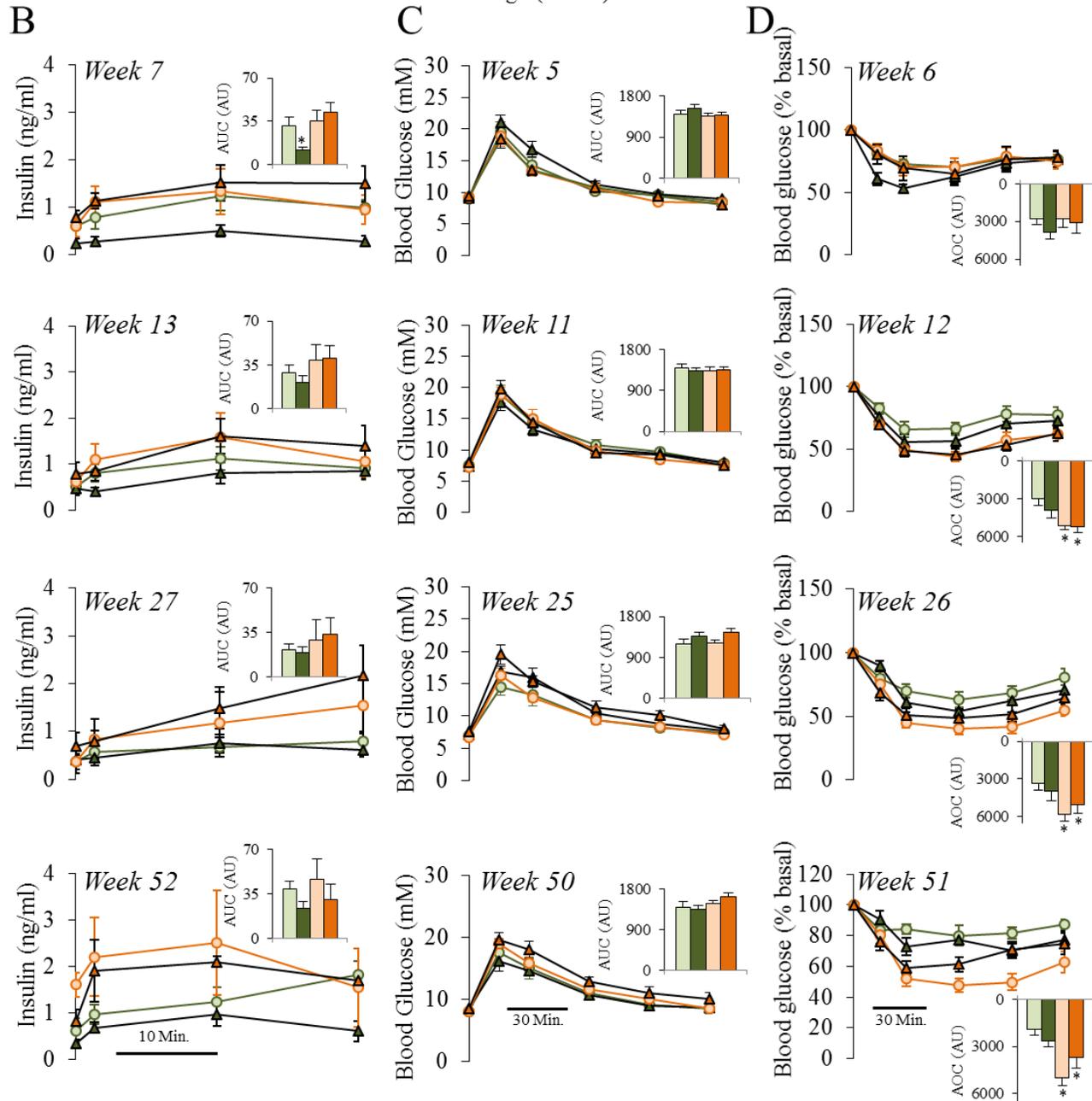
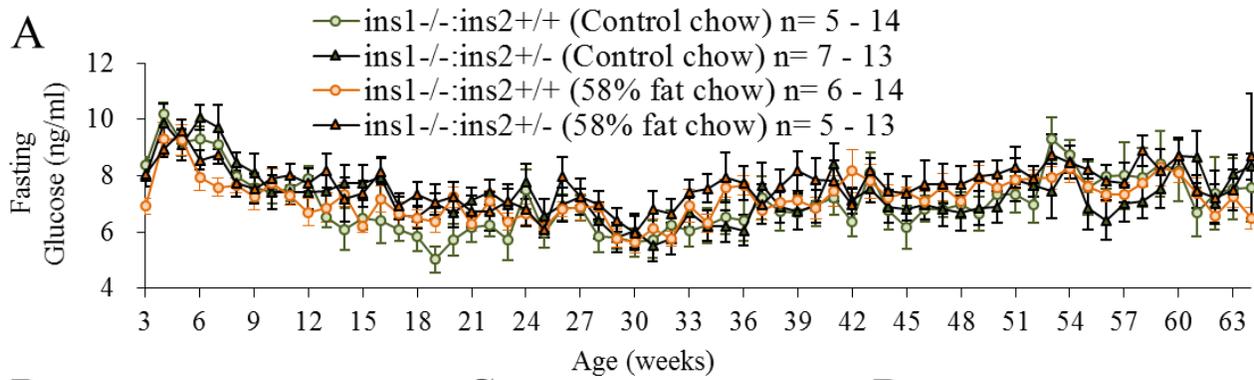
**Figure 5.2 Reduced *Ins2* expression in *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice.**

(A) Proportionally reduced *Ins2* mRNA in *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice regardless of diet (n = 3-4; 8 week old mice). As expected, *Ins1* mRNA was not found in these samples (not shown). (B) Insulin protein content in 30 size-matched islets isolated from 8 week old mice (n = 3-4). (C) Reduced insulin immunoreactivity in control diet fed *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice, but compensatory insulin production in *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice fed a high fat diet. Data are collected from cohort 1.



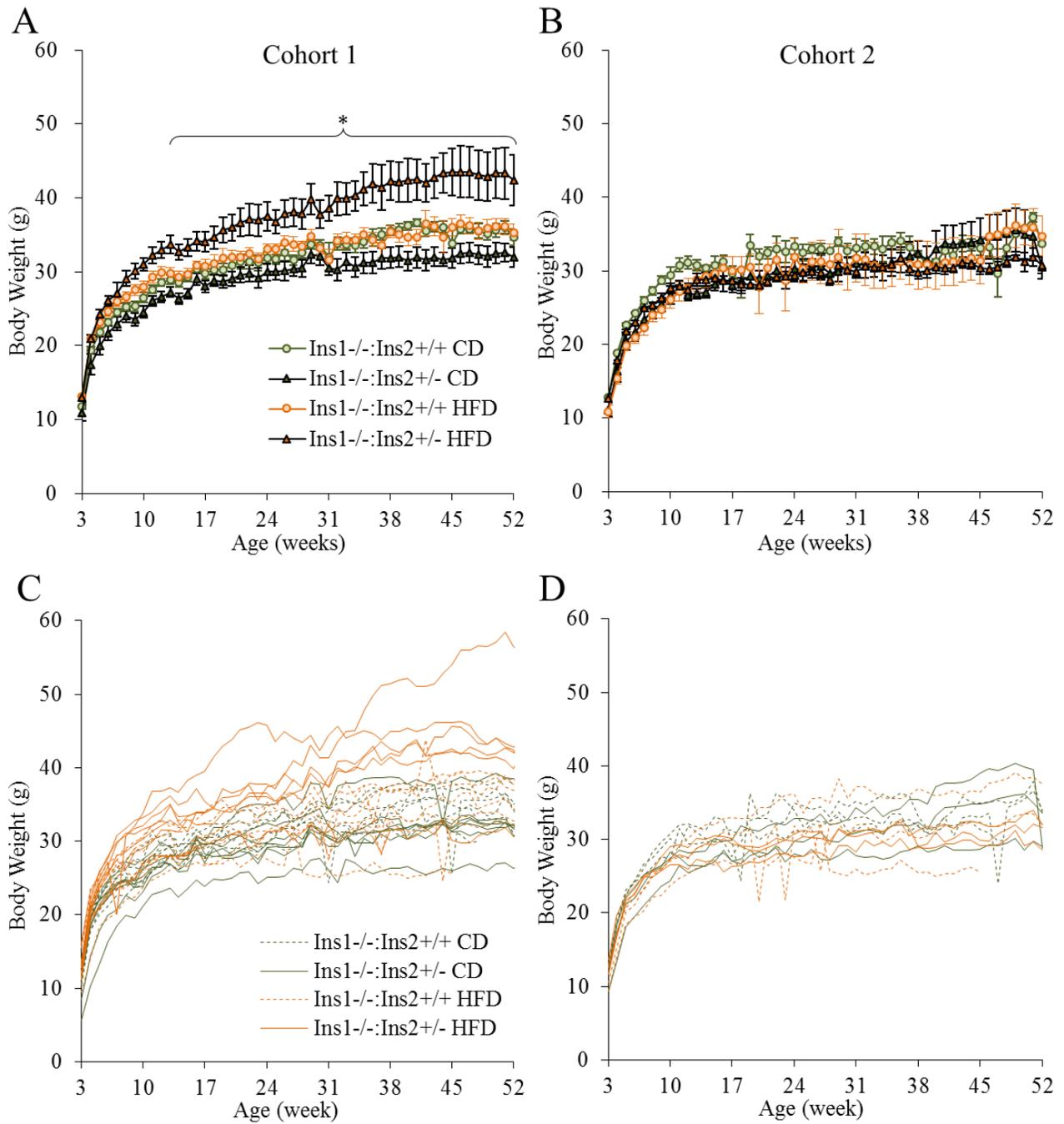
**Figure 5.3 Circulating insulin is not reduced in *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice.**

(A) We were unable to find differences in  $\beta$ -cell area (n = 3). (B) Fasting insulin was not significantly different between groups; data is from a combination of both cohorts (n = 6). The yellow area shows the detection limit for the ELISA kit. Data are collected from the first of the two cohorts, unless otherwise specified.



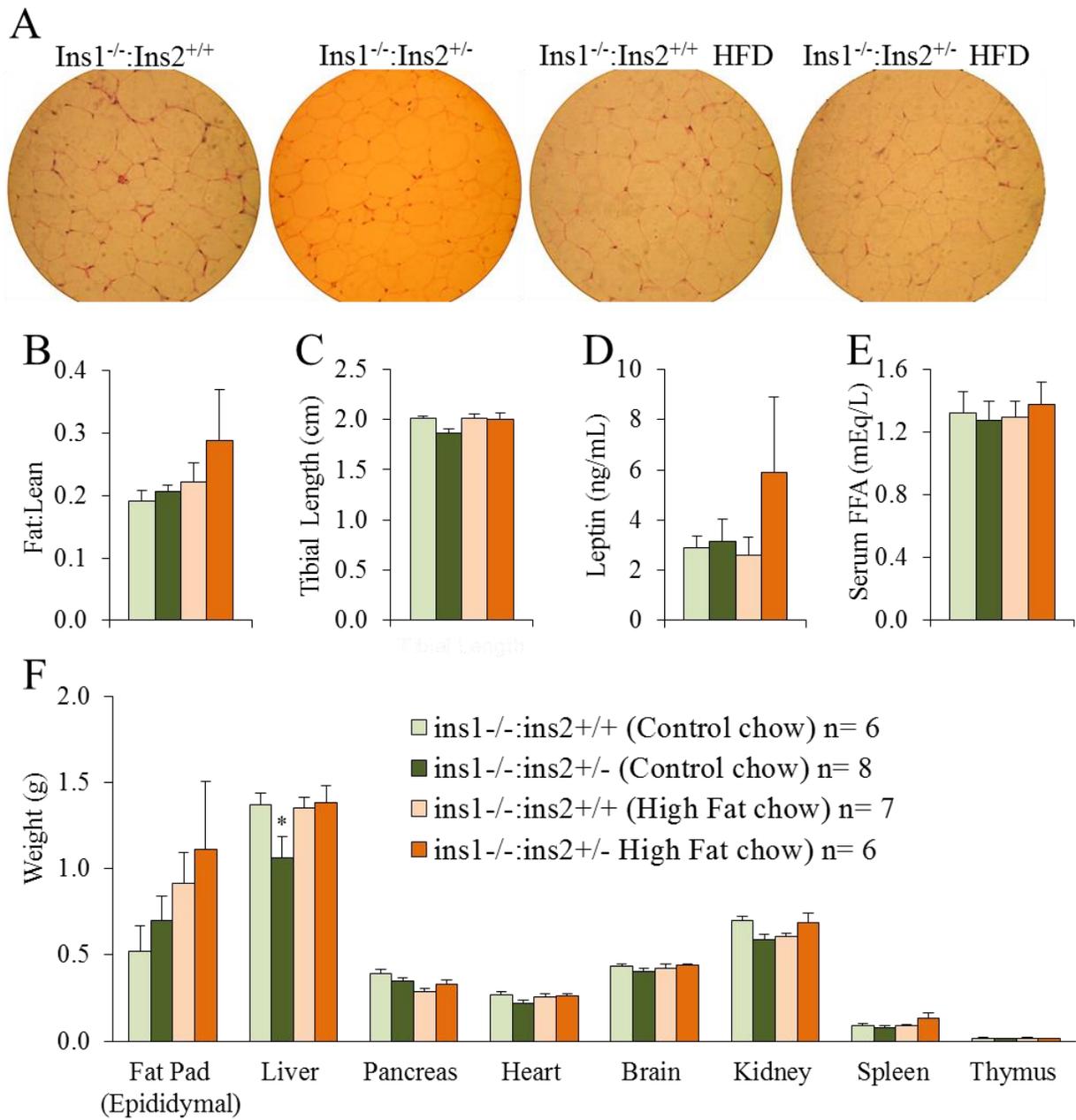
**Figure 5.4 Insulin secretion and glucose homeostasis in high fat fed *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice.**

(A) Weekly blood glucose after a 4-hour fast. (B) Glucose-stimulated insulin release. Insets show area under the curve (AUC). (C) Intraperitoneal glucose tolerance. (D) Insulin tolerance (0.75 U/g) after four hours of fasting. Insets show area over the curve (AOC). n = 6-14 unless otherwise specified. All data are from the average of results from both cohorts.



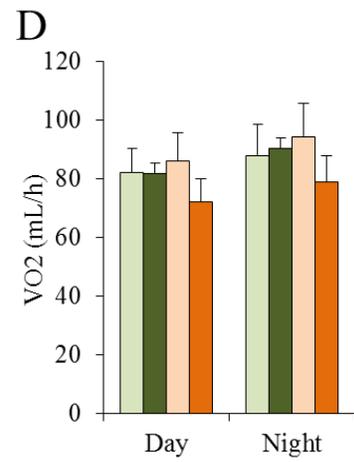
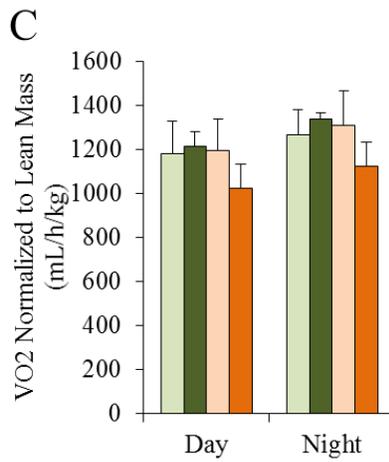
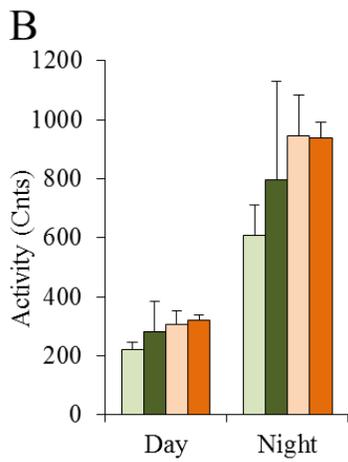
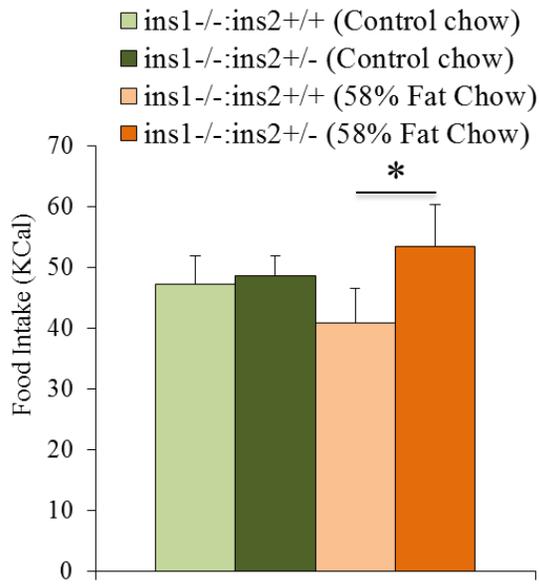
**Figure 5.5 Cohort-dependent and diet-dependent effects on body weight in *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice on high fat diet.**

Pooled body weight tracked weekly for 1 year in cohort 1 (A) and cohort 2 (B). Individual body weight tracked weekly for 1 year in cohort 1 (C) and cohort 2 (D). In cohort 1 (n = 6-8) and in cohort 2 (n = 3). \* denotes  $p < 0.05$  (ANOVA).



**Figure 5.6. A cohort of *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice exhibit high fat diet-dependent adiposity.**

(A) H&E-stained epididymal fat revealed heterogeneity in adipocyte size. (B) Whole body fat to lean ratio measure with NMR. (C) Tibial length was measured as an indicator somatic growth. (D) Circulating leptin levels (n = 3). (E) Serum free fatty acids. (F) Weights of indicated tissues. Data are from 1 year-old mice (n = 6-8). \*denotes p<0.05 (ANOVA). Data are collected from cohort 1.



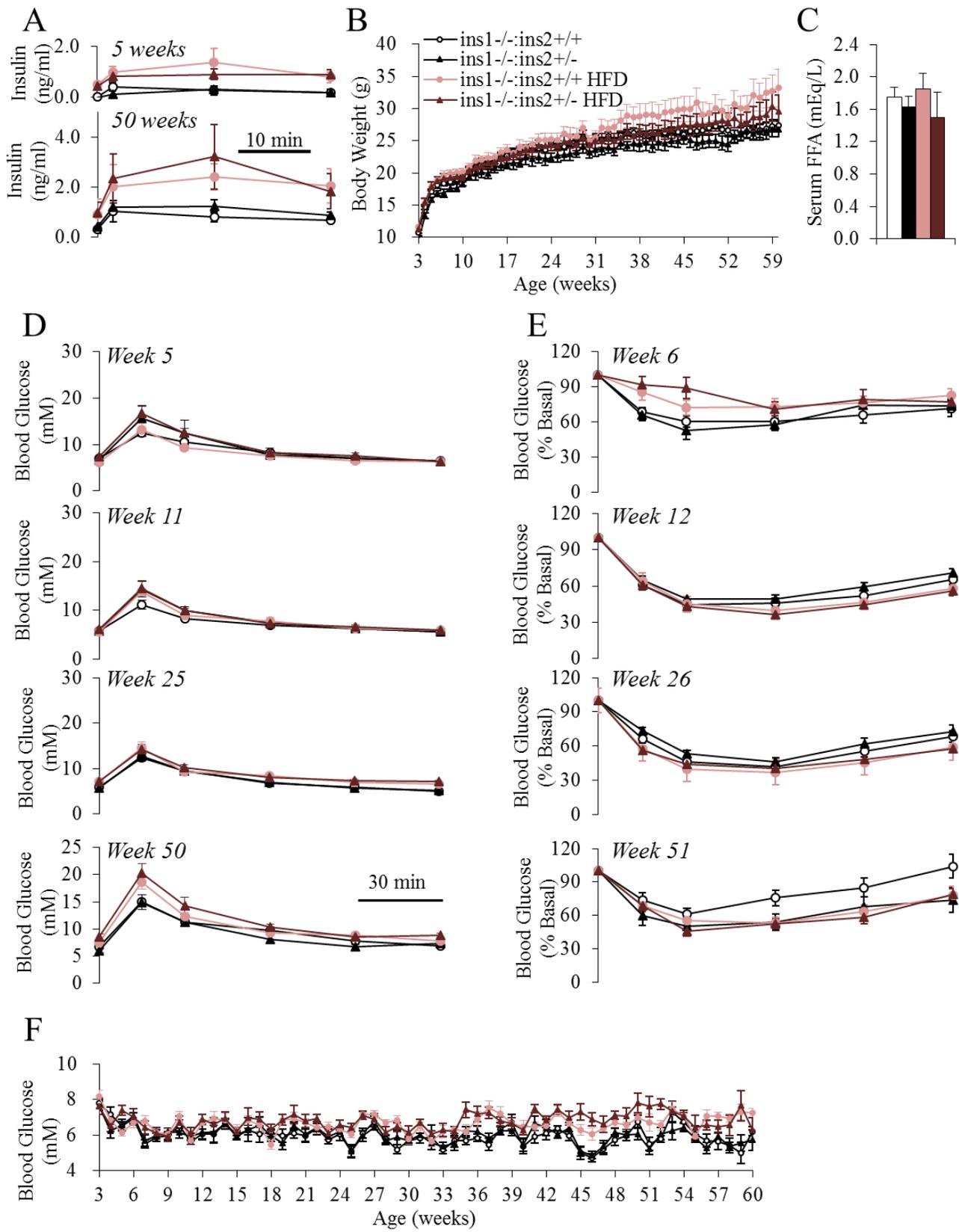
**Figure 5.7 Food intake and metabolic parameters in a cohort of *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice.**

(A) Food intake, (B) activity, and energy expenditure was measured by indirect calorimetry (C) oxygen consumption normalized to body weight and (D) total oxygen consumption in 8 week old male mice. n = 5-14 and \* denotes  $p < 0.05$ . Data are collected from cohort 1.

	CD +/+	CD +/-	HFD +/+	HFD +/-
Pnpla3	100 ± 45	89 ± 27	83 ± 27	298 ± 88
Igfbp1	100 ± 50	126 ± 37	105 ± 23	288 ± 45
\$ Fgf21	100 ± 38	118 ± 22	177 ± 23	318 ± 23
\$ Lepr	100 ± 36	101 ± 24	202 ± 30	272 ± 22
Fabp4	100 ± 23	73 ± 13	140 ± 32	201 ± 49
Cd36	100 ± 17	77 ± 20	130 ± 16	168 ± 52
Foxo1	100 ± 12	108 ± 10	124 ± 14	149 ± 27
Pnpla2	100 ± 10	71 ± 14	107 ± 27	124 ± 24
Cebpa	100 ± 17	92 ± 16	79 ± 11	95 ± 45
Pparg	100 ± 18	82 ± 20	60 ± 14	74 ± 64
Ppargc1a	100 ± 31	97 ± 11	109 ± 26	121 ± 28
\$ Sirt1	100 ± 9	113 ± 2	140 ± 14	147 ± 9
Ucp2	100 ± 32	62 ± 14	58 ± 30	52 ± 35
Pck1	100 ± 20	110 ± 18	104 ± 20	98 ± 31
Adipor2	100 ± 23	79 ± 17	80 ± 14	71 ± 6
Acly	100 ± 22	112 ± 29	78 ± 31	67 ± 36
* Atf3	100 ± 22	43 ± 17	64 ± 17	53 ± 29
Adipor1	100 ± 20	74 ± 15	109 ± 20	96 ± 20
* Ddit3	100 ± 9	51 ± 18	102 ± 40	85 ± 61
Foxa2	100 ± 21	84 ± 20	105 ± 15	85 ± 23
Nripl	100 ± 12	87 ± 8	103 ± 10	81 ± 23
Ppara	100 ± 20	85 ± 13	114 ± 19	90 ± 18
Lif15	100 ± 16	94 ± 8	102 ± 19	74 ± 13
Rbp4	100 ± 11	88 ± 11	128 ± 22	98 ± 24
Ptpn1	100 ± 17	82 ± 14	106 ± 21	75 ± 17
# \$ Fto	100 ± 10	115 ± 5	107 ± 9	74 ± 8
Tnf	100 ± 69	24 ± 14	77 ± 41	34 ± 55
# Insr	100 ± 13	90 ± 14	123 ± 10	76 ± 15
Nr1h3	100 ± 10	78 ± 11	120 ± 18	73 ± 21
\$ Lipe	100 ± 6	78 ± 12	91 ± 27	43 ± 18
Srebf1	100 ± 11	168 ± 29	137 ± 19	78 ± 33
Mlxipl	100 ± 23	91 ± 12	145 ± 18	77 ± 22
Acacb	100 ± 11	149 ± 15	247 ± 25	177 ± 28
Emr1	100 ± 34	67 ± 28	107 ± 45	32 ± 28
# \$ Fasn	100 ± 18	157 ± 20	146 ± 13	62 ± 12
& Scd1	100 ± 23	164 ± 22	636 ± 18	420 ± 34

**Figure 5.8 Gene expression profile of liver of *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* and *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice.**

Taqman real-time qPCR quantification of 45 genes in epididymal white adipose tissue from 1 year-old mice. Results are sorted according to the magnitude of the difference between high-fat fed *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice and high-fat fed *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* littermates (# denotes  $p < 0.05$ ). Genes with a large negative relative expression are colored red; genes with increased expression are green. \$ denotes significant difference between diets in the *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice. & denotes significant difference between diets in the *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice. \* denotes significant differences between *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice compared with *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice on the control diet. Data are from 1-year old mice (n = 5-11). Data are collected from cohort 1.



**Figure 5.9 Female *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice show trends towards increased fasting hyperinsulinemia and weight gain due to high fat diet intake.**

(A) Intraperitoneal glucose stimulated insulin release at 5 and 50 weeks of age. (B) Weekly body weight. (C) Serum free fatty acids at one year of age. (D) IPGTT after a four-hour fast. (E) ITT after a four-hour fast. (F) Four-hour fasting glucose. For all data in this figure (n = 8-11) except for (A) and (C) for which (n = 6).

## Chapter 6: Discussion

### 6.1 Pancreatic *Ins1* hyper-secretion promotes fat storage and reduces fat burning

A canon of diabetes and obesity research is that obesity-associated insulin resistance causes hyperinsulinemia as a compensatory mechanism because the pancreatic  $\beta$ -cells are hyperstimulated to release more insulin<sup>442,443</sup>. However, the possible physiological mechanisms for this hyperstimulation remain unclear, since it often occurs prior to hyperglycemia<sup>476,477</sup>. Our data demonstrate for the first time that prevention of high fat diet-induced hyperinsulinemia through partial ablation of the pancreas-specific *Ins1* gene protects mice from diet-induced obesity and associated complications<sup>127</sup>. Genes that mobilize lipids were up-regulated and increased mitochondrial uncoupling producing heat from calories stored in white adipose tissue, and down-regulated genes required for adipocyte differentiation. Specifically, we proposed a model for diet-induced obesity whereby hyperinsulinemia negatively regulates white adipose tissue *Ucp1* expression, via a gene network involving *Ppar $\gamma$*  and *Nrip1*, to suppress energy expenditure<sup>127</sup>. This is consistent with a well-established general anabolic role for insulin and the observation that reducing circulating insulin with diazoxide promotes weight loss in obese mice via an increase in basal metabolic rate<sup>541</sup>. Our *in vivo* data place the pancreas-specific *Ins1* gene ‘functionally upstream’ of obesity, and establish the causality of circulating hyperinsulinemia in adult fat growth in the absence of insulin resistance or hyperglycemia (Fig. 7.1). In our study, increased basal insulin secretion occurred without hyperglycemia or hypoglycemia. Indeed, both insulin resistance and insulin hypersecretion can occur in normoglycemic humans (including at and before birth), preceding obesity and insulin resistance<sup>25,478,480,484,485,487,490</sup>. It is notable that early hyperinsulinemia was the strongest predictor of type 2 diabetes in a 24-year study<sup>542</sup>. Insulin resistance and obesity can also be uncoupled in human lipodystrophies<sup>543</sup> and in many knockout

mouse models<sup>70,544-547</sup>. Further evidence for the concept that hyperinsulinemia can be a primary factor in the metabolic syndrome in humans can be found in the increased risk of childhood weight gain observed in individuals with elevated pancreatic insulin production and release caused by inheritance of class I alleles of the human *INSULIN* gene<sup>490</sup>. Genetics and epigenetics likely combine with environmental and dietary factors that stimulate insulin hypersecretion early in life, including *in utero*, promoting fat growth and eventually complications including type 2 diabetes. The therapeutic potential of drugs that inhibit circulating insulin has previously been demonstrated<sup>499,541,548</sup>, but here we provide insight into the molecular mechanism of these clinical observations. Our study provides additional rationale for efforts to therapeutically block some peripheral insulin, as tested here by the changes in the pancreatic specific *Ins1* gene dosage, action to combat obesity.

## **6.2 Diet-induced $\beta$ -cell expansion is regulated by insulin *in vivo***

Previous studies have suggested that insulin receptor signalling modulates post-natal  $\beta$ -cell mass expansion in response to a high fat diet<sup>136</sup>, but this concept has remained controversial. The lack of  $\beta$ -cell mass ‘compensation’ in *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice provides the first direct *in vivo* evidence that insulin, in this case the pancreas-specific *Ins1* gene, plays a role in  $\beta$ -cell growth and survival under stressed conditions. Importantly, our results demonstrate that  $\beta$ -cell mass can increase in the absence of sustained hyperglycemia. Moreover, our results from chapter 5 demonstrated that in *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice, which also only had insulin expression from only one allele, no significant changes in circulating insulin levels or islet mass was observed. In the past, *in vitro* cultures of dispersed primary islet cells, a system where insulin and glucose can be clamped independently of each other, have been used to demonstrate that insulin but not glucose stimulates  $\beta$ -cell proliferation<sup>24,132</sup>. We have also shown that glucose-stimulated ERK activation is proportionally

reduced in islets with reduced insulin gene dosage<sup>31</sup>, strongly suggesting that glucose acts mainly via local autocrine/paracrine insulin signalling. Indeed, >80% of the effects of glucose on  $\beta$ -cell gene expression are lost in cultured  $\beta$ -cells with insulin receptor knockdown<sup>549</sup>. Together, these lines of evidence from multiple groups demonstrate conclusively that insulin acts via insulin receptors to mediate the compensatory increase in  $\beta$ -cell mass and basal insulin release in the context of high fat diet. This has implications for efforts to increase  $\beta$ -cell mass in both type 1 and type 2 diabetes.

### **6.3 Tissue-specific roles for *Ins1* and *Ins2*: Relevance to human *INSULIN***

Insulin is the most studied hormone in biology, yet the present study provides new integrated insight into its localization and physiological functions. Clinically, insulin insufficiency resulting from a loss of greater than ~80% of  $\beta$ -cell mass results in diabetes, promoting the prevailing mindset that insulin's roles are predominately positive<sup>550,551</sup>. However, elegant studies in flies and worms have demonstrated that deleting insulin genes, or blocking elements of the insulin signalling pathway, dramatically increases lifespan and prevents diseases associated with adiposity<sup>33,171</sup>. These model systems also clearly indicate that individual insulin-like peptide genes have distinct physiological functions despite signalling through a single receptor<sup>33,171</sup>. It has been previously shown that the mouse *Ins1* and *Ins2* genes have opposing effects on type 1 diabetes incidence in the NOD mouse due to the induction of thymic tolerance by *Ins2*,<sup>44,49</sup>. Specifically, *Ins2* expression was associated with reduced incidences of type 1 diabetes and the reverse was true for the expression of the *Ins1* gene<sup>44,49</sup>. Our observation that the pancreatic-specific *Ins1* is dose-dependently required for diet-induced obesity, defines the first specific role for *Ins1* outside the context of type 1 diabetes. It is noteworthy that these *Ins1*<sup>+/+</sup>:*Ins2*<sup>-/-</sup> and *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> mice are

missing the *Ins2* gene and by definition are hypoinsulinemic compared to a wildtype *Ins1<sup>+/+</sup>:Ins2<sup>+/+</sup>* mouse. However, the relative systemic hyperinsulinemia observed in the, *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* mice compared to the *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* allowed us to understand the causal role for high fat diet-induced increase in circulating insulin in the pathogenesis of obesity.

We elucidated the tissue-specific expression patterns of *Ins1* and *Ins2*. Using multiple approaches, we found that *Ins2* is clearly present in neurons of the CNS, at both the mRNA and protein level. Importantly, we present both negative and positive controls arguing against qPCR or staining artifacts. Through work with our collaborators, we also provided information on distinct active chromatin marks on the *Ins2* gene in the CNS<sup>127</sup>. Given the conservation between the rodent *Ins2* gene and the human *INS* gene, we were not surprised to find preliminary evidence for insulin production in the human brain. In both human and rodent brains, we found robust insulin expression in the hippocampus, pointing to potential roles in memory and cognition<sup>351,360,376-398</sup>. It is interesting to note the recent interest in clinical trials using nasal insulin to deliver this putative central neurotrophic factor for the treatment of Alzheimer's disease<sup>351,360,376-398</sup>. A complete understanding of the role of *Ins2* in the CNS will require conditional *Ins2* knockout mice.

#### **6.4 Changes in *Ins2* gene dosage may potentially affect food intake**

The central nervous system oversees many physiological functions in the periphery, one of which is energy homeostasis<sup>238-240</sup>. Insulin, regardless of its origin, is thought to play a key regulatory role in central control of energy intake and expenditure<sup>238-240</sup>. Brüning *et al.* showed that knocking out the insulin receptor specifically in the central nervous system leads to hyperphagia and obesity<sup>507</sup>. The presence of small amounts of insulin protein and mRNA in the mammalian brain has long been reported<sup>552</sup>. However, we showed, unlike previous studies that

lacked high fidelity molecular methods and ideal negative controls such as the insulin knockout mice, that the *Ins2* gene is expressed in multiple regions of the brain, most notably in hippocampal neurons. Furthermore, we also provided the first evidence that *Ins2* mRNA in the brain is regulated by high fat feeding. Since insulin has been proposed to be a satiety factor <sup>553</sup>, downregulation of insulin expression and/or action may be expected to increase food intake. Consistent with this notion, *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice in our first cohort had increased high fat food intake and were obese when compared to the control high-fat fed *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice. Although our experimental treatment was expected to reduce *Ins2* gene dosage in multiple tissues, including the pancreas, thymus and brain, several lines of evidence could potentially hint that a partial reduction of brain *Ins2* was associated with the diet-dependent hyperphagia and obesity, at least in some specific conditions. In our studies, circulating insulin levels were not different between *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice and *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice on the high fat diet. Other investigators have shown that *Ins2* knockout in the thymus had no effect on body weight <sup>61</sup>. On the other hand, it has been reported that insulin receptor knockout in the brain leads to increased high fat food intake and obesity <sup>507</sup>, suggesting the possible presence of a local signalling network. Our work should open new avenues for investigating the biology of insulin in neurons and their connections. However, it is imperative that these data are interpreted with caution as we observed conflicting results in a second cohort. This observed variability in response to changes in *Ins2* gene dosage is currently being studied by another graduate student in our laboratory in a new animal housing facility and is beyond the scope of this thesis (personal communications, Nicole Templeman).

## 6.5 High fat feeding affects central insulin expression

Neurodegenerative diseases are on the rise, similar to the ascendance of obesity and diabetes. In particular Alzheimer's disease and dementia are strongly correlated with obesity and insulin resistance<sup>526-529</sup>. Thus, the rising numbers of patients with obesity and insulin resistance are also at risk for these neurodegenerative diseases, which reduce cognitive function and the ability to form memories<sup>176-178</sup>. It is established that obesity and a high fat diet regimen can lead to central insulin resistance<sup>168,191,199,225,511-525</sup>. High calorie diets with excess fat and carbohydrates are also noted as important environmental factors that can increase the risk of these neurodegenerative diseases<sup>176-178</sup>. Animal studies have also shown that such dietary regimes can have adverse cognitive health outcomes<sup>176,180-185</sup>. Moreover, it is reported that during the early stages of the Alzheimer's disease there is a notable decrease in the levels of insulin in the cerebrospinal fluid<sup>68,70,554</sup>. We have been able to show, for the first time and through reliable and precise molecular methodology, that high fat diet can reduce the expression of central murine *Ins2* gene. Remarkably, this reduction is apparent in both the hippocampi and the olfactory bulbs (Fig. 3.9 and 3.10). The hippocampus is responsible for memory formation and neuronal death in this region is a key factor in Alzheimer's disease<sup>526-529</sup>. Both the olfactory bulb and the hippocampus are similar in that they both receive new cells from neuronal stem cell depots, dentate gyrus in case of hippocampus and the subventricular zone in case of the olfactory bulb<sup>555-559</sup>. Being a pro-survival/anti-apoptotic factor<sup>23</sup>, insulin fits as an important factor to promote differentiation of these stem cells and protection of the existing neurons. This notion is also in agreement with the result of the studies of intranasal insulin therapy that is currently being tested for treating Alzheimer's disease with promising results<sup>190,199,215-237</sup>. Our data adds to our understanding of these neurodegenerative diseases by showing that insulin is being produced at these brain regions.

Thus, for fully understanding these diseases, and inventing new therapeutic means, it is crucial to consider the role of the centrally expressed insulin.

## 6.6 Summary

In the present studies, we have used genetic partial reduction of pancreatic insulin to demonstrate a causal role for insulin in obesity. Studies have shown that the loss of the *Ins2* gene, compared to the *Ins1* gene, has a more pronounced effect on islet function, insulin production and glucose homeostasis, which is expected since *Ins2* constitutes the major insulin isoform in the pancreas<sup>504,560-562</sup>. Moreover, it has been shown that there is a striking increase in the incidences of early-onset diabetes, due to depletion of insulin in the islets as opposed to autoimmunity towards insulin, in the male *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>*, but not in *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>*, *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>*, or *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>*, mice on a non-obese diabetic background<sup>561</sup>. Our results are also in line with these findings that although there is compensation in expression from the remaining *Ins* gene when one of the two murine *Ins* genes is ablated, expression from only one allele of the *Ins1* gene may not be able to adequately compensate for the missing alleles. However, we and others<sup>561</sup> observed that once past the early stage of life, after approximately ten weeks of age, *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* male mice are able to control their glucose homeostasis despite reduced insulin levels. The fact that the adult male *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice are able to maintain their glucose homeostasis despite reduced insulin levels was a key factor in the interpretation of our data. Because of this, we were able to study the causal role of systemic hyperinsulinemia in the pathogenesis of diet-induced obesity by comparing male *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* and *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>*, but not *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* and *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>*, mice; as expression from even one allele of the *Ins2* gene proved capable of preventing changes in circulating insulin levels. Using the male *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* and *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>*, we were able to conclusively show that hyperinsulinemia is causal

to the changes in adiposity, independent of glucose tolerance, as previously suggested by numerous other studies<sup>102,104,476,480,492,494-499,541</sup>. Our studies are also in agreement with previous studies showing that reduced systemic insulin is associated with increased metabolic rate and reduced adiposity<sup>495,496,498</sup>. However, our studies do not allow us to confirm or rule out that there also may be functional and isoform-specific effect difference(s) between the two murine insulin peptides.

In contrast to the male mice, we were unable to detect any significant changes in insulin levels in response to reduction in *Ins* gene dosage in female mice. Notably, we did not encounter any cases of diabetes in the female mice. The interpretations of these data are more complex and some of its aspects, such as the variability observed in male *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* and *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice, are being studied by another student in our laboratory (personal communication, Nicole Templeman). However, generally, there are differences between males and females that can affect adiposity, fat distribution, inflammation, and insulin sensitivity. For example, higher levels of estrogen in females have been associated with a healthier physiological state compared to males such as lower visceral and higher peripheral adiposity, higher adiponectin levels, increased lipid oxidation, reduced production of reactive oxygen species, better insulin sensitivity and protection of  $\beta$ -cell health and function during metabolic stress<sup>393,563-570</sup>. These factors could potentially contribute to the lack of differences in the circulating insulin levels due to changes in *Ins* gene dosage in our studies. The only non-significant trends observed in the female mice, with respect to changes in circulating insulin levels and weight gain, was observed in *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* and *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice. Both of these groups of female mice showed higher circulating levels and trends towards higher weight that was high fat diet-dependent regardless of their genotype. In agreement with this observation, it has been previously shown that women are more affected by the type of food they eat compared to men<sup>571</sup>.

Interestingly, the phenotypes presented in chapter 5 appear to be variable, sex-dependent, and context-dependent with respect to reduction of the *Ins2* gene. We also provide evidence that insulin is produced locally in the brain, where it may have potential effects on satiety<sup>553</sup>. Moreover, *Ins2* expression was reduced in certain parts of the high fat fed female, but not male, brain and that could potentially be associated with the aforementioned diet-dependent differences observed in the *Ins1* null female mice. Our data provide strong rationale to investigate approaches to limit peripheral hyperinsulinemia and augment the central effects of insulin for not only the prevention and treatment of obesity, but also neurodegenerative disease in humans.

## **6.7 Limitation of the studies and future directions**

Although our studies provided some insights into the effects of changes in insulin gene dosage in mice, there are improvements that can be made. These studies were conducted using diets that, though almost calorically matched, contained different sources for the type of fat. The high fat diet contained 58% coconut oil whereas the control diet contained 25% lard (Table 2.3). Coconut oil is believed to be less deleterious than lard and it can also increase the amount of beneficiary HDL cholesterol<sup>572</sup>. Thus this may have introduced confounding effects in the results that may have not been accounted for. However, usage of a high fat diet that contains 60% lard produces dramatic glucose intolerance (Appendix A) compared to the 58% coconut oil where glucose tolerance was unaffected (Figs. 4.6B and 5.4C). Thus, the diet-induced glucose resistance would have prevented us from studying the role of reduced circulating insulin in the absence of glycemic abnormalities. To avoid this issue, it is best that in future studies a control diet is chosen with lower amounts of coconut oil.

Although the studies above hinted at a role for insulin expression in the brain for controlling food intake, the reduction in *Ins2* gene dosage was made globally throughout the body. Thus, it is imperative to isolate the *Ins2* gene deletion to the central nervous system, leaving it intact elsewhere, especially in the pancreas and thymus. To test the role of local insulin expression in food intake we can utilize the Cre-Lox recombination system. This system is derived from bacteriophage P1<sup>573</sup>. Since this system does not naturally exist in mammals, it can be used to efficiently remove specific genes from mammalian models<sup>573</sup>. The enzyme Cre recombinase finds and excises any floxed, flanked by two LoxP sequences, DNA sequence<sup>573</sup>. We can use mice with a floxed *Ins2* gene on an *Ins1* null background, which are available in our laboratory. Our earlier experiments show that there is no compensatory expression from the *Ins1* allele in the absence of *Ins2* in the brain. However, to prevent any potential compensation from the other murine *Ins* gene, we can use mice on an *Ins1* null background. This ensures that when *Ins2* is removed, compensatory *Ins1* expression will not confound the results. Keeping in mind that the *Ins2* gene is capable of sufficiently compensating for the lack of the pancreatic-specific *Ins1* gene<sup>560</sup>. To knock out *Ins2* specifically in the neurons we can use a mouse line that expresses Cre recombinase under the control of a CNS-specific promoter. Normally the *Nestin* gene is used for this purpose. Nestin is expressed in a plethora of cell types, such as neurons, cartilage, bone, skin, skeletal muscle, pancreas, kidney and heart, both during embryonic development and adult life<sup>574-576</sup>. However, a specific NestinCre line, developed by R. Klein, has increased specificity to central and peripheral neurons<sup>577</sup>. This specificity is due to the fact that this mouse line has transgene that carries the neuron specific enhancer element of the intron 2 of the *Nestin* gene<sup>577,578</sup>. We did attempt this method, with a similar but not identical NestinCre deleter mouse from Ruth Slack at the University of Ottawa, but unfortunately we did not observe changes in central *Ins2* expression between *Ins1*

$Ins1^{-/-};Ins2^{fl/fl};Cre^{Nes}$  and control  $Ins1^{-/-};Ins2^{fl/fl}$  or  $Ins1^{-/-};Ins2^{+/+};Cre^{Nes}$  mice. There are reports, through lineage-tracing studies, that in the pancreas *Nestin* and *Insulin* are not expressed in the same cells<sup>579</sup>. This could potentially explain the negative results we have in our attempt, as *Nestin* may also not be expressed in the same neurons that *Ins2* is expressed. A different Cre-expressing mouse line that could be used to conduct a similar experiment would be mice that produce Cre under the control of the *Synapsin* promoter which is expressed in neurons of the brain and spinal cord<sup>580-582</sup>. Moreover, we are not aware of any study indicating that *Synapsin* and *Insulin* are not expressed in the same cells. Future studies, including those using *Ins2*:GFP knock-in mice, are required to clearly map the distribution of brain insulin relative to the domains of the various Cre lines available.

Insulin's effect on satiety largely depends on the population of neurons in the arcuate nucleus of the hypothalamus that express orexigenic and anorexigenic peptides such as POMC and AgRP/NPY, respectively. However, it has been shown that if the genes that express these peptides are knocked out from the embryonic stages, the brain can completely compensate for their absence<sup>583</sup>. Thus, to study the role of local brain insulin expression on food intake, insulin must be knocked out acutely and postnatally. This can be achieved by injecting, through stereotactic surgery, the  $Ins1^{-/-};Ins2^{fl/fl}$  mice with a Cre expressing virus. We also tried this approach. We were able to successfully inject the mice in desired locations as tested by injecting a dye into the third ventricle. We injected both a virus that expressed Cre and GFP (Vector Biolabs catalog # 7088, Philadelphia, USA), and another virus that expressed only GFP (Vector Biolabs catalog # 7006, Philadelphia, USA). However, the Cre-expressing virus used failed to infect as we were unable to detect any significant GFP expression in the brain using fluorescent microscopy (data not shown). We tested the virus *in vitro* as well and found no signs of successful infection (i.e. GFP expression) using

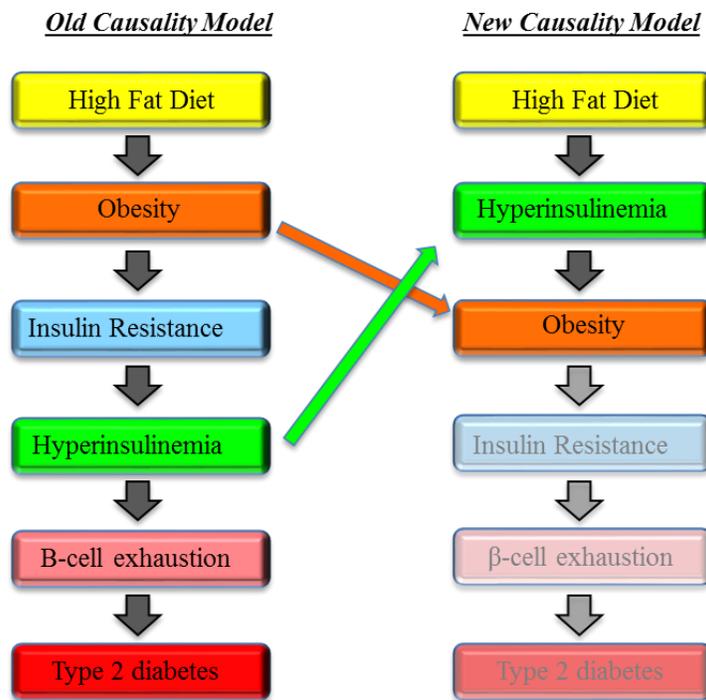
fluorescent microscopy (data not shown). It was recently also reported that acutely killing hypothalamic cells with *Ins2* promoter activity can alter food intake and weight gain<sup>174</sup>. However, their approach is confounded by the fact that the cells with *Ins2* promoter activity could have other non-insulin dependent functions. Thus, the specific *Ins2* gene knockout approaches we have suggested here will allow studying only the effects of removing central *Ins2* gene. Moreover, a similar method can be used by studying the role of local insulin expression in the hippocampus and its effect on memory and learning by bilaterally injecting the Cre-expressing virus in the hippocampi.

There are numerous reports indicating that there is a link between insulin and longevity<sup>4,13,584,585</sup>. In our studies, we allowed one cohort of *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>*, and littermate control *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>*, mice to age. We noticed that *Ins2<sup>-/-</sup>* mice with reduced *Ins1* gene dosage and on a control diet had about 30 percent higher survival rate at 2 years (data not shown). Additional studies are underway in the laboratory to more thoroughly evaluate the relationship between insulin and longevity. The tools we have at our disposal, the insulin knockout mice, allow us to study the relation between insulin and longevity without adding any confounding factor such as caloric restriction or inducing insulin resistance.

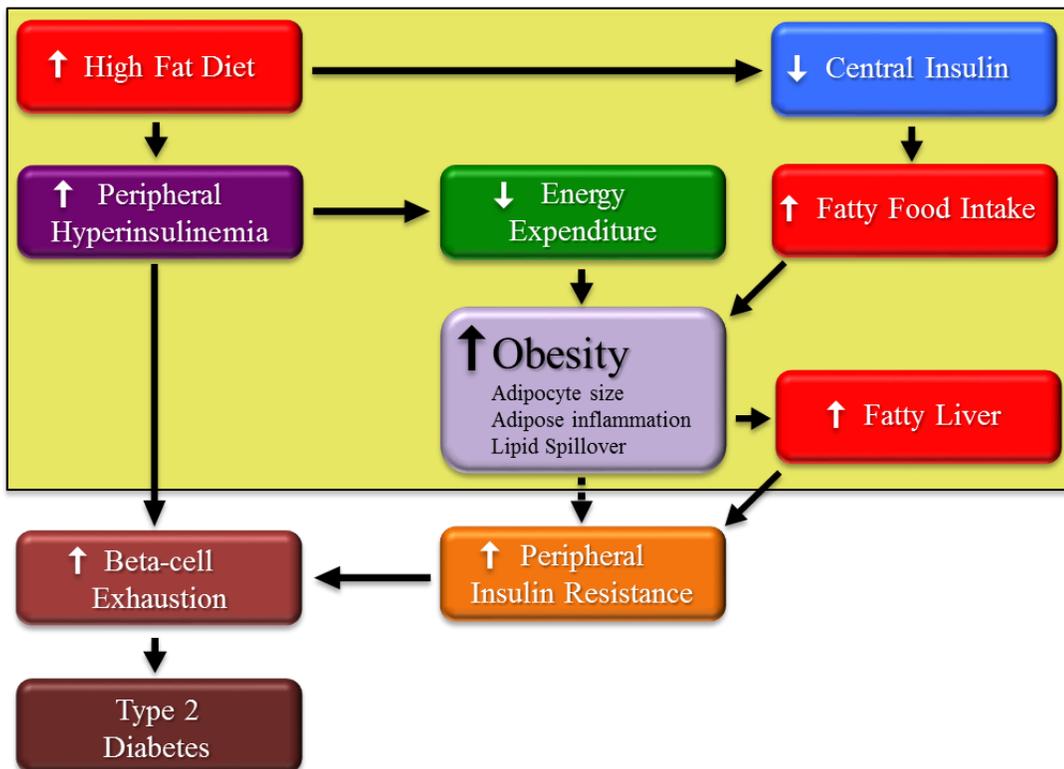
Despite these caveats, we have been able to show that high fat diet-induced hyperinsulinemia is a causal factor in the pathogenesis of high fat diet-induced obesity and that prevention of hyperinsulinemia is an effective way to prevent high-fat diet induced obesity. Moreover, using knockout controls we were able to show that in mice, unlike the *Ins1* gene, the *Ins2* gene is expressed in the brain like that of the human *INSULIN* gene; this can potentially help for further research for treatment of cognitive disorders that are related to reduced central insulin levels or action.

## 6.8 Summary Figure

A



B



**Figure 6.1 Revisiting the central model of obesity and type 2 diabetes.**

(A) The most widely accepted model of the causality relationships in the pathogenesis of obesity and type 2 diabetes dictates that a high fat diet leads to obesity, which via multiple factors including a chronic state of inflammation, which results in insulin resistance. Insulin resistance leads to hyperinsulinemia then  $\beta$ -cell exhaustion then type 2 diabetes. The accepted model is incompatible with our results that put the hypersecretion of pancreatic insulin 1 genetically upstream of obesity.

(B) Specifically, our data suggest a model whereby low levels of insulin are required to maintain energy expenditure in white adipose tissue via the expression of *Ucp1* (perhaps in a Nrip1-dependent manner). Our data also suggest that central insulin 2 may play an anti-obesity role by suppressing the intake of high fat food. This satiety signal could be alleviated by the reduction of brain insulin 2 in the context of a high fat diet, suggesting an additional vicious cycle. Our data do not address the order of subsequent events after obesity (outside the yellow box), such as insulin resistance and/or type 2 diabetes, since they were not observed in our studies.

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## Appendices

### Appendix A Effect of Diets with Lard as The Source of Fat on Glucose Tolerance

#### A.1 High Fat Diet Induced Glucose Intolerance

